Centrosome Assembly In Vitro: Role of γ-Tubulin Recruitment in Xenopus Sperm Aster Formation

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Abstract. Centrioles organize microtubules in two ways: either microtubules elongate from the centriole cylinder itself, forming a flagellum or a cilium ("template elongation"), or pericentriolar material assembles and nucleates a microtubule aster ("astral nucleation"). During spermatogenesis in most species, a motile flagellum elongates from one of the sperm centrioles, whereas after fertilization a large aster of microtubules forms around the sperm centrioles in the egg cytoplasm. Using Xenopus egg extracts we have developed an in vitro system to study this change in microtubule-organizing activity. An aster of microtubules forms around the centrioles of permeabilized frog sperm in egg extracts, but not in pure tubulin. However, when the sperm heads are incubated in the egg extract in the presence of nocodazole, they are able to nucleate a microtubule aster after isolation and incubation with pure calf brain tubulin. This provides a two-step assay that distinguishes between centrosome assembly and subsequent microtubule nucleation. We have studied several centrosomal antigens during centrosome assembly. The CTR2611 antigen is present in the sperm head in the peri-centriolar region. γ-tubulin and certain phosphorylated epitopes appear in the centrosome only after incubation in the egg extract. γ-tubulin is recruited from the egg extract and associated with electron-dense patches dispersed in a wide area around the centrioles. Immunodepletion of γ-tubulin and associated molecules from the egg extract before sperm head incubation prevents the change in microtubule-organizing activity of the sperm heads. This suggests that γ-tubulin and/or associated molecules play a key role in centrosome formation and activity.

At fertilization the contents of two cells, the oocyte and the sperm, are mixed. The genetic material is equally contributed by both gametes, whereas the other cellular components that preside to early development are asymmetrically derived. Whereas most cytoplasmic components are stored in the oocyte, the centrioles are generally paternally contributed and are required for cleavage and development (Maller et al., 1976). In most species a large aster of microtubules forms around the sperm centriolar area after fertilization and extends into the egg cytoplasm. This aster is required for the migration of the male pronucleus towards the center of the egg, for the migration of the female pronucleus towards the male pronucleus and for the segregation of developmental determinants, leading for instance in Xenopus to dorso-ventral asymmetry (Chambers, 1939; Manes and Barbieri, 1977; Wilson, 1925).

Thus the sperm centrioles are at the locus of two different microtubule-organizing activities before and after fertilization. In the sperm flagellum the nine doublets of flagellar microtubules elongate directly from the centriolar microtubules themselves ("template" type of nucleation; Kuriyama and Kanatani, 1981). In the sperm aster the astral microtubules have no structural continuity with the centriolar microtubules and are anchored to pericentriolar (centrosomal) material ("astral" type of nucleation). These astral microtubules are nucleated from the pericentriolar material, not merely elongated. The association between the centrioles and the material around them is very dynamic, leading to extensive variations in the naming of the centrioles and their associated material. We define a centriole according to its overall structure, irrespective of its activity. A centriole may also be called a basal body when it forms the base of a flagellum or a cilium. We define a centrosome according to its activity: throughout the paper we call the centrioles and their associated material a centrosome only when they can nucleate an aster of microtubules (Bornens, 1992). Therefore we consider the change in microtubule organization at the sperm centrioles at fertilization as the appearance of an active centrosome, as well as a change in centriolar activity (from basal body to centrosome organizer).

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Little is known about the mechanism of microtubule nucleation and the molecular composition of the centrosome. Several lines of research are now aiming at the molecular characterization of centrosomal factors involved in microtubule nucleation (for review see Kalt and Schliwa, 1993). Genetic approaches have led to the discovery of γ-tubulin, a new subtype of tubulin. The γ-tubulin gene has been first found in the fungus Aspergillus nidulans as an extragenic suppressor of a mutation in the β-tubulin gene (Oakley and Oakley, 1989). It has since been found almost ubiquitously in eukaryotic cells and is found in small quantities, when compared to α and β-tubulins (Horio et al., 1991; Liu et al., 1993; Stearns et al., 1991; Zheng et al., 1991). Gene disruption is lethal both in Aspergillus (Oakley et al., 1990) and in fission yeast (Horio et al., 1991). In Aspergillus, mixed heterokaryons are viable but those spores that contain the transformed nuclei and therefore lack the γ-tubulin gene contain only few cytoplasmic microtubules and do not divide. In vertebrate cells, injection of a polyclonal antibody directed against a synthetic peptide of the γ-tubulin sequence prevents microtubule regrowth after nocodazole or cold-induced depolymerization, and mitotic spindle formation (Joshi et al., 1992).

In this report we devise an in vitro assay with which the role of γ-tubulin, and other centrosomal components, can be assayed by other means than function-blocking antibodies. We can manipulate centrosome formation in vitro by mixing Xenopus egg cytoplasmic extracts and permeabilized Xenopus sperm. Xenopus eggs are laid arrested in the metaphase of second meiotic division. This block is rapidly released (within 5 min) after sperm-egg contact (Gerhart et al., 1984). We therefore chose to use interphase extracts prepared from parthenogenically activated eggs. This in vitro system proved to be useful to demonstrate the origin and requirement of some centrosomal molecules (like γ-tubulin) for centrosome assembly.

Materials and Methods

Preparation of Xenopus Egg Extracts

Interphase low-speed extracts were prepared from Xenopus activated eggs incubated for 90 min with 200 μg/ml of cycloheximide, as described in Félix et al. (1989, 1993). High-speed supernatants were obtained by centrifugation twice at 80,000 rpm for 30 min in the TL-100.1 rotor in a Beckman table-top ultracentrifuge (Leiss et al., 1992). ATP-depleted extracts were prepared by addition of apyrase (Sigma Chem. Co., St. Louis, MO) at a concentration of 10 U/ml (Buendia et al., 1992), to an extract prepared without the ATP-regenerating system. DMAP (6-dimethylaminopurine) was purchased from Sigma Chem. Co.

Preparation of Permeabilized Xenopus Sperm

Permeabilized frog sperm was prepared basically according to Blow and Laskey (1986). One male frog was injected one week before the preparation with 100 U human chorionic gonadotropin (HCG, Sigma). The testes were homogenized in Eppendorf tubes in 2 ml cold SuNaSp: 50 mM Hepes pH 7.0, 250 mM sucrose, 75 mM NaCl, 0.5 mM spermidine (Sigma S-2501), 0.15 mM spermine (Sigma S-2876), with protease inhibitors (1 mM PMSF, 1 μg/ml leupeptin, 2 μg/ml pepstatin and 2 μg/ml aprotinin). The debris were removed by centrifugation at 20 *g* for 30 s. The sperm was then pelleted at 3,000 rpm at 4°C for 10 min in the Sorvall HB-4 rotor. The red blood cells were lysed by resuspending the pellet in 10 mM Tris, pH 7.5, 1 mM EDTA. The sperm was pelleted again, washed twice in SuNaSp, and permeabilized by addition of 5 μg/ml of lysolecithin (L α-lysophosphatidylcholine, Sigma L-4129, freshly prepared at 1 mg/ml in H_2O, at room temperature). The reaction was stopped by washing twice with 3% BSA in SuNaSp. The sperm was finally resuspended at a concentration of $10^4/\mu l$ (counted in a Mallassez cell counting chamber) in SuNaSp containing 25% glycerol, and stored at −80°C.

Purification of Bovine Brain Tubulin

Tubulin was purified from bovine brain as in Bré and Karsenti (1990), by two cycles of polymerization-depolymerization (Shelanski et al., 1973) followed by phosphocellulose chromatography (Weingarten et al., 1975). After addition of GTP (1 mM), the tubulin was frozen in liquid nitrogen at a concentration of 5 μg/ml (determined by the Bradford procedure, using BSA as a standard). This concentration was below the critical concentration for free nucleation in the absence of centrosomes, as observed in Bré and Karsenti (1990). Microtubule-associated proteins (MAPs)[1] were eluted from the phosphocellulose column with 0.8 M KCl and stored frozen in liquid nitrogen.

Preparation of Rhodamine-labeled Tubulin

Tubulin was coupled to rhodamine using tetramethylrhodamine succinimidyl ester (Molecular Probes, Eugene, OR; C-1171), according to Hyman et al. (1991). The stoichiometry of the labeling was 0.75 mole of rhodamine per mole of tubulin dimer and the concentration of tubulin in the preparation was 40 μg/ml. It was diluted 100-fold for use in the egg extract or in the unlabeled brain tubulin preparation.

Sperm Aster Formation in the Egg Extract

Permeabilized sperm was added to the egg extract at a concentration of 5×10^2 sperm nuclei/μl of extract (unless otherwise specified), in a volume that never exceeded 1/10 of the extract volume. After incubation at room temperature, the extract was fixed and centrifuged onto a coverslip as in Verde et al. (1990). If desired, the coverslips were immediately postfixed in methanol at −20°C for 5 min.

Centrosome Assembly in the Egg Extract and Assay of its Microtubule Nucleation Activity with Pure Bovine Brain Tubulin

The sperm heads were incubated in the extract (typically 20 μl) in the presence of 100 μM of nocodazole (Sigma, M-2759; from a 10-mM stock in DMSO). The extract was then diluted fourfold in SuNaSp and layered onto a 50% glycerol cushion in SuNaSp and centrifuged at 18,000 *g* for 2 min. (It was found necessary to lower the concentration of glycerol for the pelleting of nuclei that did not swell and decondense properly, for instance when they were incubated in diluted, or high-speed, extracts.) The sperm pellet was resuspended in 6 μl of SuNaSp and either used immediately, or frozen in SuNaSp containing 25% glycerol. The microtubule nucleation activity of the centrosomes was tested by incubating 2 μl of the resuspended pellet with 10 μl of pure calf brain tubulin (40 μM final) for 10 min at 37°C. The asters were fixed and centrifuged as in Mitchison and Kirschner (1984). The coverslips were postfixed immediately in methanol at −20°C for 5 min.

Antibodies and Immunofluorescence

Microtubules were labeled with anti-α or anti-β-tubulin antibodies (Amersham Corp., Arlington Heights, IL; diluted 1:500). The CTR2611 monoclonal antibody (IgM) was obtained by immunization of mice with human centrosomes purified from the KE37 cell line (Bornens et al., 1987). The 125 monoclonal antibody (IgM) was raised by injection of mice with rat brain MAPs (Ulloa et al., 1993*a*, *b*). The MPM2 mouse monoclonal antibody was raised against mitotic cells and recognizes certain phosphorylated epitopes (Davis et al., 1983).

Several anti γ-tubulin antisera were raised in rabbits against specific peptides of the γ-tubulin sequence. The immunization procedure was as described in Julian et al. (1993). Serum '38' was raised against the EEFATEGGDRKDV peptide, serum '70' against NIIQGEADPTDVHKSL and serum '75' against EYHAATRPDYISWGTEQ. The guinea pig serum C3 was raised against the same γ-tubulin peptide as serum 75. The two first sequences are integrally found in the Xenopus γ-tubulin sequence. The last sequence is the carboxyterminal end of the human γ-tubulin and is found in the Xenopus γ-tubulin, with the exception of the two last amino-acids

1. *Abbreviation used in this paper*: MAPs, microtubule-associated proteins.

(Stearns et al., 1991). These peptides are not found in the α or β-tubulin sequences. The crude sera were used at a dilution between 1:500 and 1:1,000. Sera 70 and 75 were purified by affinity for their antigenic peptide coupled to CNBr-activated Sepharose 4B beads (Pharmacia LKB Biotechnology, Piscataway, NJ). The bound antibodies were eluted from the column with 0.1 M glycine, pH 2.8 and immediately neutralized with a Tris buffer, pH 8.8. Serum 75 was purified by affinity for the reactive 50-kD band on immunoblots. Xenopus egg extracts were run on 2 minigels and the gels processed for immunoblotting as described below. After incubation of the blot with serum 75 and extensive washing, a 2 millimeter-wide strip was cut at the level of the reactive 50-kd band. The bound antibodies were then eluted with 0.2 ml of 0.2 M glycine, 0.2% fish skin gelatin, pH 2.8. The solution was immediately neutralized and used at a twofold dilution in further experiments.

The secondary antibodies were FITC-labeled goat anti-mouse (KPL; 1/50), Texas red-labeled sheep anti-mouse (Amersham; 1/40), FITC-labeled goat anti-rabbit (KPL; 1/50), and rhodamine-labeled goat anti-rabbit (Miles; 1/150). 0.5 μg/ml of HOECHST dye 33258 were added to the second antibody reaction in order to stain the DNA.

All antibodies other than the 125 antibody were used after methanol postfixation. The coverslips were rinsed in PBS containing 0.1% Triton X-100 at room temperature and treated with 0.1% sodium borohydride in PBS for 10 min. Immunoreactions were performed for 10 min at room temperature in PBS containing 3% BSA and 0.02% NaN_3. The coverslips were mounted in Mowiol and observed with a Leitz Diaplan epimicroscope, with x63 Planapo (1.4 NA) and x100 Fluotar (1.32 NA) objectives. Photographs were taken using a Leitz Orthomat camera and Kodak T-Max 400 films.

Microtubule Number Per Centrosome

The number of nucleated microtubules was estimated by focusing up and down in the aster under the microscope. Microtubule number was assigned to one of the five following classes: 0–9 (no aster), 10–19, 20–39, 40–59, or more than 60. It was not possible to count the number of microtubules when higher than 60 (for example in the aster in Fig. 2 *d*), but the microtubules were well resolved in less furnished asters.

Electron Microscopy

For electron microscopy, samples were fixed and centrifuged onto a coverslip as described above. The coverslips were rinsed in PBS + 0.1% TX-100 and treated with 0.1% sodium borohydride in PBS for 10 min. After incubation for 10 min with PBS containing 1% FCS and 0.1% fish skin gelatin (Sigma, G-7765), the coverslips were incubated for 45 min at room temperature with the primary antibody (fivefold more concentrated than for immunofluorescence), washed in PBS + 0.1% TX-100, and incubated for 45 min with the secondary antibody. The secondary antibodies were 15 nm gold-labeled goat anti-mouse (BioCell, 1/10), 10 nm gold-labeled goat anti-rabbit (Amersham, 1/10), and 5 nm gold-labeled goat anti-rabbit (Amersham, 1/10). After extensive washing, the coverslips were transferred into Sørensen buffer (phosphate buffer 0.1 M, pH 7.2). They were postfixed for 30 min in 2% glutaraldehyde, 0.4% tannic acid in Sørensen at room temperature, washed in Sørensen, and incubated on ice for 10 min with 0.5% OsO4 in Sørensen. They were then stained en bloc for 15 min at room temperature in 2% uranyl acetate in 50% ethanol, dehydrated in a 50–100% ethanol series and flat embedded in Epon/Araldite. Sections (around 120 nm) were cut on a Reichert ultramicrotome, contrasted with uranyl acetate and observed on a Philips EM 410 electron microscope at 80 kV.

For immunostaining with the γ-tubulin anti-peptide antibodies, it was found necessary to incubate the coverslips in methanol at −20°C for 3 min before starting this procedure, after the fixation with glutaraldehyde. It was difficult to immunostain the centrosomes in the center of very dense asters because of accessibility problems for the antibodies. Dilution of the extract by 30% (in the acetate buffer + ATP regenerating system) led to less dense asters and allowed a much better penetration of the antibodies (Fig. 6).

Electrophoresis and Immunoblotting

The egg extract was diluted 10-fold in SDS-gel sample buffer and 5 μl were loaded on a SDS-PAGE minigel. For immunoblotting the proteins were transferred to nitrocellulose filters (pore size 0.45 μm, Schleicher and Schuell, Keene, NH), using a semi-dry blotting system (Biolyon). The blots were incubated for 30 min in 3% milk (Gloria) in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.5) before incubation with the first antibody. They were then washed in TBST (4 × 5 min) and incubated either directly with protein A-peroxidase (Sigma P-8651; 10 μg/ml in TBST) if the primary antibody was raised in rabbit, or else first with a purified rabbit anti-mouse antibody (Nordic Immunology, RAM/Ig/75, 10 μg/ml in TBST), washed and then incubated with protein A-peroxidase. After extensive washing, the peroxidase activity was detected using the ECL Western Blotting Detection System (Amersham) according to the manufacturer's instructions.

Immunodepletion of the Egg Extract

Immunoprecipitin (Gibco, formalin-fixed Staphylococcus aureus cells) was processed according to the manufacturer's instructions and washed in PBS. Serum 75 (raised against the COOH-terminal peptide of γ-tubulin, see above) was preincubated for 60 min at room temperature in the presence or absence of the antigenic peptide (5 mg/ml). 20 μl of Staph A cells were then incubated for 30 min at room temperature with 20 μl of a fourfold dilution of the serum in PBS. The Staph A cells with the bound immunoglobulins were washed in PBS, PBS + 0.1% TX-100, PBS and acetate buffer (100 mM K-acetate, 2.5 mM Mg-acetate, pH 7.2). The frog egg extract was prepared without DTT in the buffer. Before incubation with the Staph A cells, it was diluted one to one in the acetate buffer + ATP-regenerating system + protease inhibitors and centrifuged twice at 6,000 rpm for 5 min in a Sigma 2K-15 table-top centrifuge to eliminate fast-sedimenting aggregates. The Staph A cells were then incubated for 60 min at 4°C with 30 μl of this diluted frog egg extract. The cells were finally pelleted by centrifugation at 6,000 rpm for 5 min. The supernatant was recovered and used in the sperm centrosome assembly assay described above. The sperm heads were incubated at a concentration of $3 \times 10^3/\mu$l for 30 min in the extracts.

Results

Sperm Aster Formation in Interphase Xenopus Egg Extracts

Permeabilized Xenopus sperm heads were incubated at 22°C in low-speed interphase extracts prepared from activated Xenopus eggs. After fixation and centrifugation onto a coverslip, the microtubules were revealed by anti-tubulin immunofluorescence and the nuclei by HOECHST 33258 staining. The incorporation of tubulin into microtubules during the incubation was revealed by addition of a small quantity of fluorescent tubulin to the egg extract. Comparison of the rhodamine-tubulin pattern with the FITC anti-tubulin immunofluorescence permitted the discrimination of microtubules assembled in the extract from tubulin polymerized already (centrioles, flagella).

The centrioles were seen as two dots by immunofluorescence (Fig. 1 *a*, *top part*). The flagella were ruptured during sperm preparation, but sometimes a small segment remained attached to the distal centriole (Fig. 1 *a*, *bottom part*). After 3 min of incubation of the sperm heads in the extract, no new microtubules were formed around the sperm centrioles (Fig. 1 *a'*, *top part*). Axoneme elongation sometimes took place when a segment of the flagellum was still connected to its associated centriole (Fig. 1 *a'*, *bottom part*). After 30 min of incubation, a dense aster of microtubules was formed around the centriolar region of all sperm heads (Fig. 1 *b*). Membrane vesicles accumulated in these asters, as seen by phase contrast (Fig. 1 *c*) and lipid dye staining (DHCC, not shown).

Aster formation took place after 10 min of incubation of the sperm heads in the egg extract (not shown). It was never observed after 3 min (Fig. 1 *a*). The slow kinetics of aster formation suggested that the sperm centrosome was not immediately active for microtubule nucleation in the extract. Moreover, after 10 min of incubation of the sperm heads in the egg extract at 22°C followed by 30 min at 4°C to

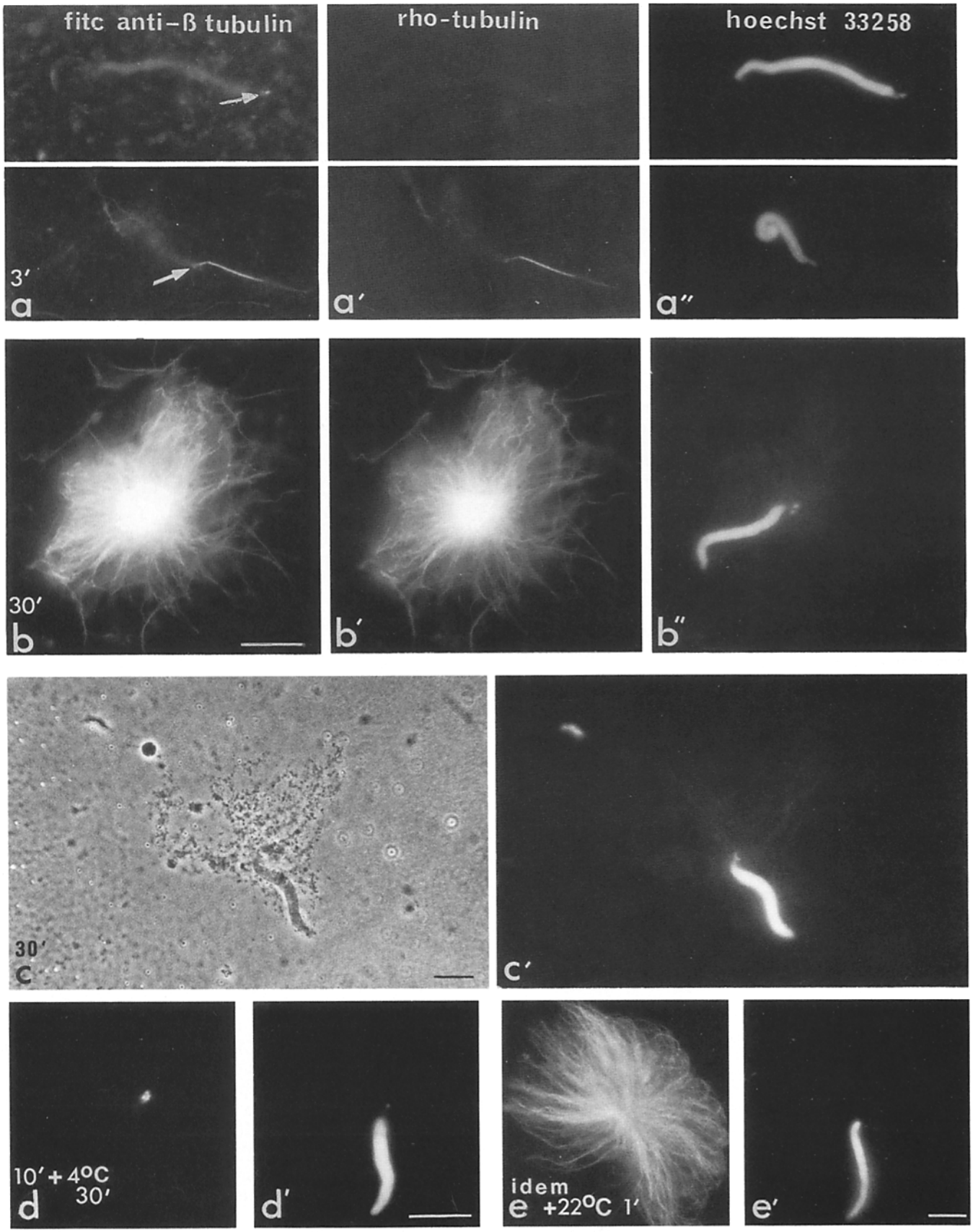

Figure 1. Time course of sperm aster formation in interphase frog egg extracts. Permeabilized Xenopus sperm was incubated in an interphase egg extract (500 nuclei/μl of extract). Exogenous rhodamine-labeled brain tubulin was added in *a* and *b*. The extract was incubated at 22°C for 3 min (*a*); 30 min (*b* and *c*); 10 min at 22°C then 30 minutes at 4°C (*d*); as in *d*, then 1 min at 22°C (*e*). The extracts were fixed, centrifuged onto a coverslip and processed for anti-tubulin immunofluorescence (*a* and *b*) and HOECHST 33258 staining for the

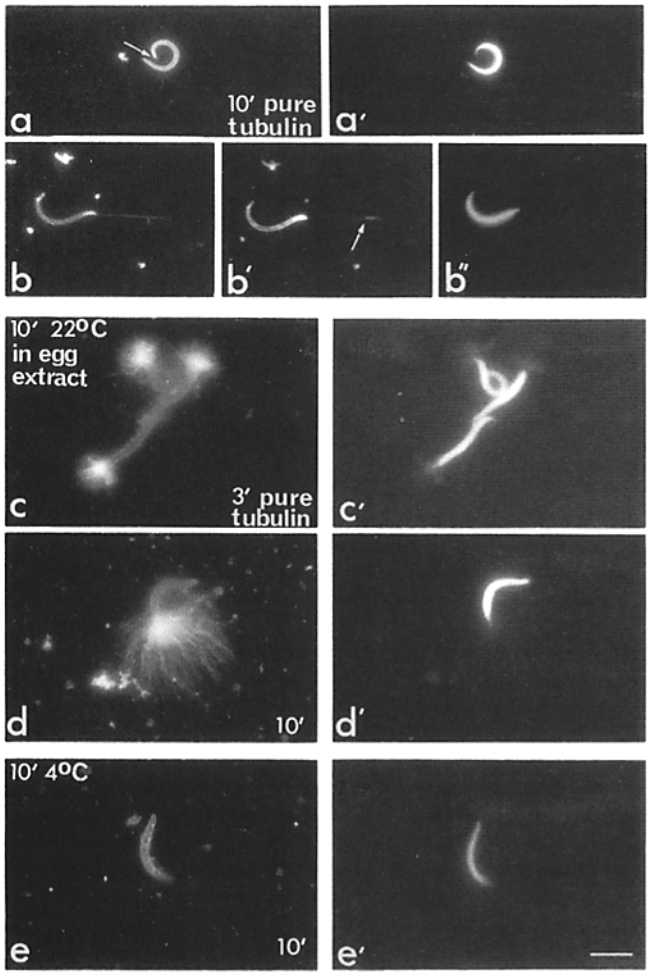

Figure 2. Nucleation of brain tubulin microtubules on sperm centrosomes assembled in an egg extract. (*a* and *b*) Permeabilized Xenopus sperm heads were incubated with pure bovine brain tubulin at 37°C for 10 min. (*c–e*) Sperm heads were first preincubated for 10 min in an interphase frog egg extract in the presence of 100 μM of nocodazole (500 nuclei/μl), at 22°C (*c* and *d*), and at 4°C (*e*), pelleted through a glycerol cushion and resuspended. They were then incubated with pure bovine brain tubulin at 37°C for 10 min (*d* and *e*) or 3 min (*c*). The material was fixed, centrifuged onto a coverslip and processed for anti β-tubulin immunofluorescence (*left panels*) and HOECHST 33258 staining (*right panels*). In *b*, rhodamine-labeled tubulin was included in the incubation with pure tubulin; the corresponding channel is shown in the middle panel (*b'*). No astral microtubules polymerized when the sperm heads were incubated with pure brain tubulin (*a*); in some cases the microtubules of the remaining part of the flagellum elongate (compare *b* and *b'*; the *arrow* designates the junction between the remaining piece of the flagellum and the newly elongated microtubules). An aster of microtubules forms when the sperm heads have been incubated in the egg extract at 22°C (*c* and *d*), but not at 4°C (*e*). (*Top left corner*) Incubation times in the egg extract. (*Bottom right corner*) Incubation times with pure tubulin. Bar, 10 μm.

depolymerize the microtubules (Fig. 1 *d*), 1 min of incubation at 22°C was sufficient to allow the reformation of a large aster around the sperm centrosome (Fig. 1 *e*). Therefore the delay in aster formation did not correspond to the time required for the extract to warm up and microtubules to polymerize. Free microtubules (not nucleated by a centrosome) were indeed observed after 3 min of incubation. Finally centrosomes purified from cell lines such as human lymphoblastic KE37 nucleated microtubules after only 30 s of incubation in the egg extract (Verde et al., 1990). The delay observed in our assay showed that the sperm heads were not competent immediately to nucleate an aster in the egg extract. To determine whether the egg extract actually triggered the formation of an active centrosome, we attempted to separate experimentally the 'complementation' of the sperm heads by the egg extract from microtubule nucleation and aster formation per se.

Centrosome Assembly on Sperm Centrioles in Egg Extracts in the Presence of Nocodazole

Permeabilized frog sperm incubated with purified calf brain tubulin did not nucleate astral microtubules (Fig. 2 *a*). Some microtubules elongated from the sperm axoneme (Fig. 2 *b*) as happened after 3 min of incubation in the egg extract (Fig. 1 *a*, *bottom part*). In contrast, KE37 centrosomes nucleated asters when incubated with pure tubulin (Bornens et al., 1987). Our data demonstrated that there were no active centrosomes around the sperm centrioles before their incubation in the egg extract.

The sperm heads were incubated in the egg extract for 10 min at 22°C in the presence of 100 μM nocodazole (that prevented totally microtubule formation in the extract, not shown). They were then isolated through a glycerol cushion and incubated at 37°C with purified calf brain tubulin. Under these conditions they were able to nucleate an aster (Fig. 2, *c* and *d*). Sperm heads that were incubated in the egg extract at 4°C (Fig. 2 *e*), or mixed with an extract that was incubated for 10 min at 22°C and subjected to the same centrifugation through the glycerol cushion (not shown), were unable to nucleate an aster.

It was possible to quantify the number of nucleated microtubules, at a given sperm concentration in the extract and at a given tubulin concentration. The data displayed in the first histogram of Fig. 3 (*top left*) show that 97% of the sperm centrosomes nucleated more than 10 microtubules in the conditions used (see figure legend). This allowed us to test the effect of diluting the egg extract. The extract was diluted 3, 10, 30, or 100-fold in the presence of an ATP-regenerating system and the activity assayed as above. In this experiment sperm head concentration was maintained at 2 × 10^3/μl, which is similar to the concentration of centrosomes at the midblastula transition in Xenopus (Newport and Kirschner, 1982). Sperm heads that failed to nucleate 10 or more astral microtubules appeared when the extract was

DNA (*a''*, *b''*, *c'*, *d'*, and *e'*), or observed for the rhodamine-labeling of the incorporated exogenous tubulin (*a'* and *b'*), or by phase contrast (*c*). The sperm centrioles were found at one end of the elongated sperm nucleus (*arrows* in *a*). After 3 min in the extract, on some sperm heads no microtubule nucleation/elongation has taken place (*a*, *top part*), while on others a bundle of microtubules has elongated from the remaining part of the flagellum (*a*, *bottom part*). After 30 min at 22°C, a dense microtubule aster is formed around the sperm centrosome (*b* and *c*). After 10 min at 22°C followed by 30 min at 4°C, microtubules can no longer be seen in the extract (*d*). However an additional 1 min at 22°C is sufficient for the growth of a large aster (*e*). Bars, 10 μm.

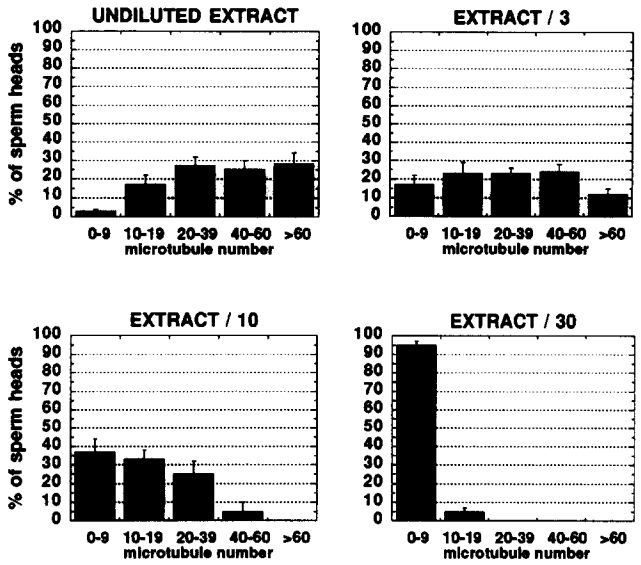

Figure 3. Distribution of the number of nucleated microtubules per sperm head after preincubation with various dilutions of egg extract. Permeabilized Xenopus sperm heads ($2 \times 10^3/\mu l$) were incubated in an interphase egg extract that was either undiluted or diluted 3, 10, or 30-fold in acetate buffer containing an ATP-regenerating system. Sperm heads were then pelleted through a glycerol cushion, resuspended and incubated with pure bovine brain tubulin at 37°C, for 10 min. Samples were fixed, centrifuged onto a coverslip and processed for anti-tubulin immunofluorescence. The number of microtubules per sperm head was assigned to one of five classes: 0–9 (no aster), 10–19, 20–39, 40–59, or more than 60. Histograms show the percentage of sperm heads belonging to each class. The data are the mean of three independent experiments. At least 50 centrosomes were examined in each experiment for each condition. Standard deviations are shown. 100% of sperm heads that had been incubated in a 100-fold dilution of the extract, or in buffer alone, nucleated less than 10 microtubules.

diluted threefold, but some centrosomal activity was still observed after a 10-fold dilution of the extract (Fig. 3). This was important in defining the system for further manipulation of the egg extract (see below the immunodepletion).

In summary, these experiments demonstrated that active centrosomes indeed formed around the sperm centrioles upon incubation in the egg extract. We could distinguish two steps: (i) centrosome formation in the egg extract and (ii) microtubule nucleation in pure brain tubulin.

Changes in Molecular Composition of the Sperm Centrosome: Recruitment of γ-Tubulin and Appearance of Phosphorylated Epitopes

We studied the molecular composition of the sperm centriolar area. Immunostaining was performed on untreated sperm heads or sperm heads incubated in the egg extract in the presence of nocodazole, using several antibodies known to recognize pericentriolar components. Anti β-tubulin antibodies stained the centrioles as two dots, both before and after incubation in the extract (Fig. 4, *a* and *f*). The monoclonal CTR2611 antibody raised against purified mammalian centrosomes (Bornens et al., 1987; Buendia et al., 1990) reacted also before and after incubation of the sperm heads in the extract. The staining was not restricted to the centrioles but covered a large peri-centriolar area, often displaying the shape of an elongated 'X' (Fig. 4, *b* and *g*).

Four anti-peptide sera were raised against three different specific and conserved sequences of the γ-tubulin molecule (rabbit sera 38, 70, and 75, and guinea pig serum C3; Julian et al., 1993). Sera 70 and 75 were affinity-purified on their respective antigenic peptides. Serum 75 was also affinity-purified on the 50-kD γ-tubulin band revealed by immunoblotting (see below Fig. 7). None of the sera and affinity-purified antibodies stained the peri-centriolar area of the untreated sperm heads. In all cases, a strong staining of the sperm centrosome was observed after incubation of the sperm heads in the egg extract (Fig. 4, *c* and *h* and 5, and not shown). The staining appeared as spots in the peri-centriolar area (the separation of these different spots was difficult in the light microscope). This staining was abolished when the anti γ-tubulin sera were incubated with their corresponding antigenic peptides (Fig. 5, *g* and *i*).

By electron microscopy the two centrioles (labeled by stars in Fig. 6) were seen oriented at a wide angle. Striated fibers linked the centrioles to both the nucleus and the proximal part of the flagellum (this was especially clear in a sperm preparation that had not been incubated in the extract; not shown and Bernardini et al., 1986). When the sperm heads were incubated in the egg extract, globular electron-dense aggregates of typically 50–100 nm diam accumulated around the two centrioles, suggesting that centrosomes were assembled de novo in the extract (Fig. 6). Microtubules seemed to originate from these structures. Serum 38 stained these electron-dense aggregates (Fig. 6, *a–b*). It did not label the striated fibers (*arrow* in Fig. 6 *c*), but labeled other centriolar appendages (*arrowheads*). The staining was greatly reduced in the presence of the antigenic peptide (Fig. 6 *d*). It was clearly peripheral with respect to the CTR2611 staining (Fig. 6 *e*), which appeared along the striated fibers in untreated sperm heads (not shown).

These results suggested strongly that γ-tubulin was recruited from the egg cytoplasm into these structures that accumulated around the sperm centrioles. An alternative explanation would be that γ-tubulin was already present in the sperm and that the three epitopes became unmasked in the extract. To clarify this point, we performed immunoblotting experiments (Fig. 7 *A*). An egg extract sample was loaded in lanes *1–4*. Serum 75 recognized a major band at 50-kD (lanes *1* and *3*), the expected molecular weight of the Xenopus γ-tubulin molecule (Stearns et al., 1991). In lanes *1* and *2*, we used a high serum concentration. Under these conditions several polypeptides reacted with the primary serum. Yet only the 50-kD band was abolished upon incubation of the serum with the specific peptide (lane *2*). Only this band was revealed when a high serum dilution was used (lane *3*). Moreover, when the serum was affinity-purified on the 50-kd band, only this band (and a faint lower molecular weight band) appeared in a second round of immunoblotting (lane *4*). The same centrosomal staining was observed with these affinity-purified antibodies (Fig. 4, *c* and *h*).

γ-tubulin was not present in high-speed extract supernatants, from which all insoluble material was pelleted (lane *5*). The presence of γ-tubulin was then monitored in the sperm heads. No staining could be seen when 5×10^4 sperm heads were loaded onto the gel (lane *7*). Lane *8* contained 5×10^4 sperm heads incubated for 30 min in the ex-

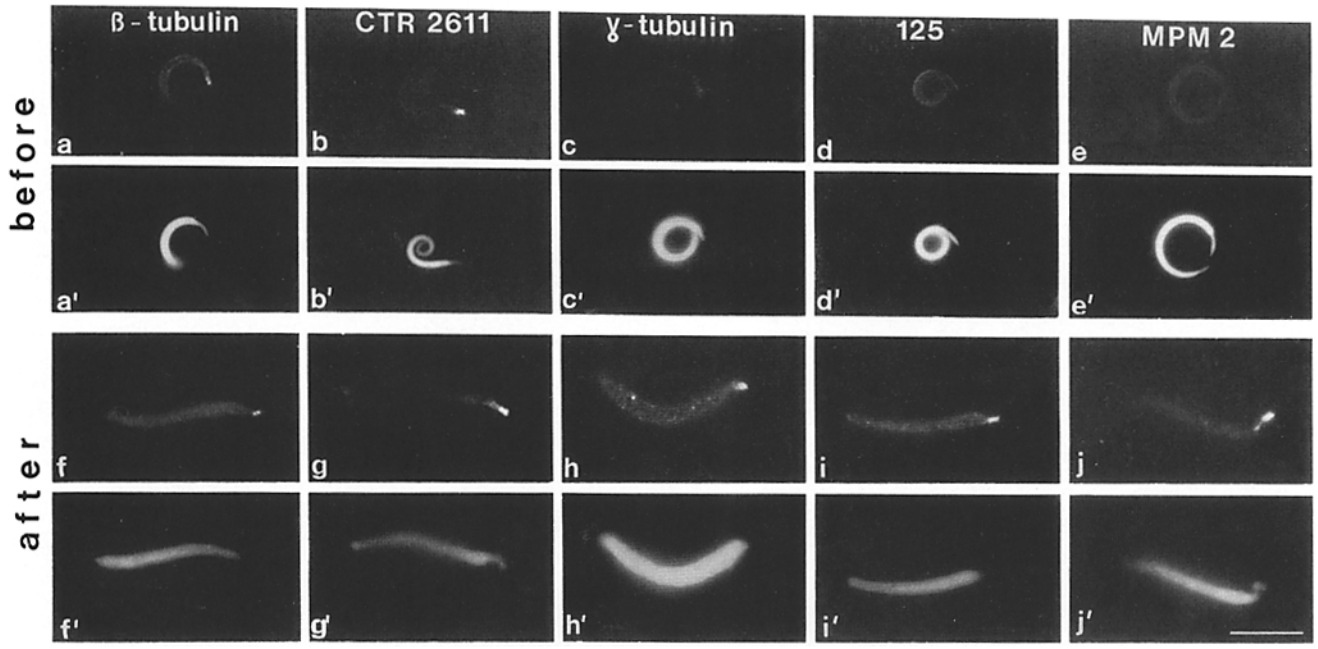

Figure 4. Immunofluorescence staining of sperm heads with specific anti-centrosome antibodies. Control sperm heads (*a–e*), or sperm heads preincubated in an interphase egg extract for 30 min in the presence of nocodazole (*f–j*), were stained with anti β-tubulin antibodies (*a* and *f*), the CTR2611 antibody (*b* and *g*), serum 75 directed against a specific peptide of the γ-tubulin sequence and affinity-purified on the 50-kD γ-tubulin band on an immunoblot (*c* and *h*), and the monoclonal antibodies 125 (*d* and *i*) and MPM2 (*e* and *j*), which recognize phosphorylated epitopes. Lower panels show the corresponding HOECHST 33258 staining (*a'–j'*). While β-tubulin and CTR2611 immunoreactivities were observed in control sperm heads, reactivity with the anti γ-tubulin, 125, and MPM2 antibodies only appears after incubation in the egg extract. Bar, 10 μm.

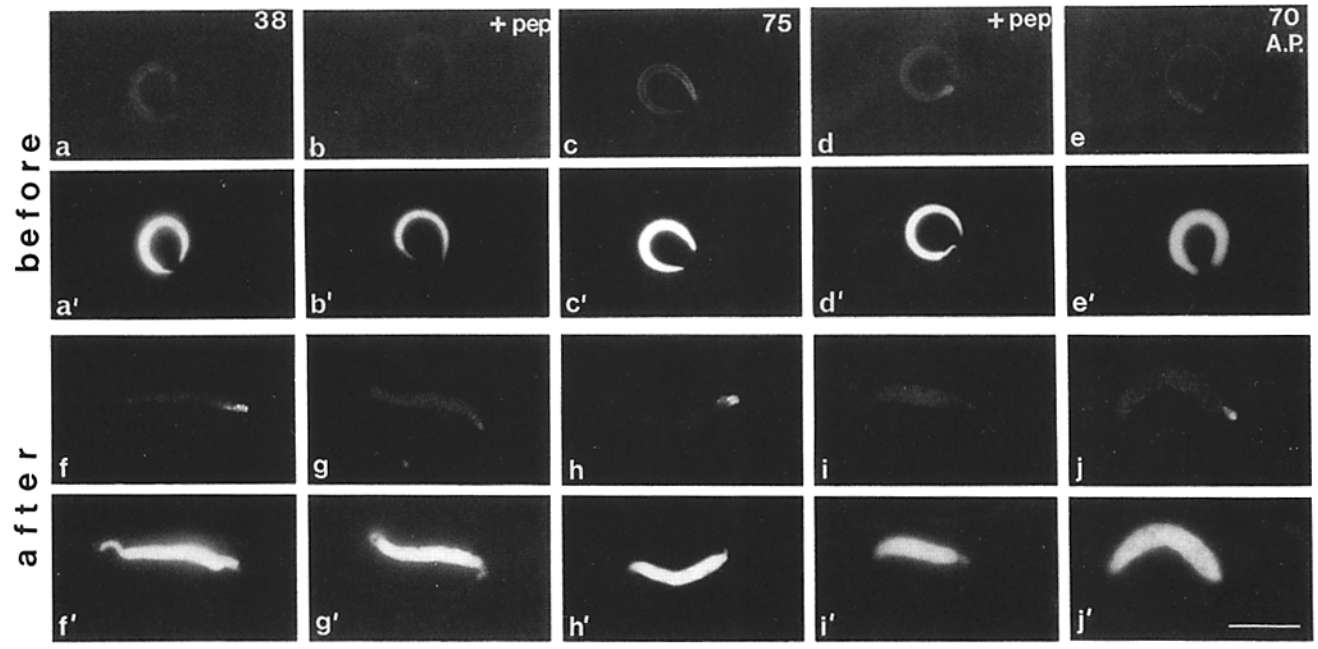

Figure 5. Immunofluorescence staining of sperm heads with antisera directed against different specific peptides of the γ-tubulin molecule. (*a–e*) Control sperm heads. (*f–j*) Sperm heads incubated for 30 min in the egg extract. In *a*, *b*, *f*, and *g* they were stained by immunofluorescence with serum 38, in *c*, *d*, *h*, and *i* with serum 75, in *e* and *j* with serum 70 affinity-purified on its antigenic peptide. In *b*, *d*, *g*, and *i* the immunological reaction was performed in the presence of an excess of the corresponding antigenic peptide. Lower panels show the HOECHST 33258 staining (*a'–j'*). With both crude sera (and sera 70 and C3 as well, not shown), the speckled centrosomal staining is blocked by the corresponding peptide. Note that the weak unspecific staining of the nucleus with serum 75 (and 70) is not blocked by incubation with the peptide. Bar, 10 μm.

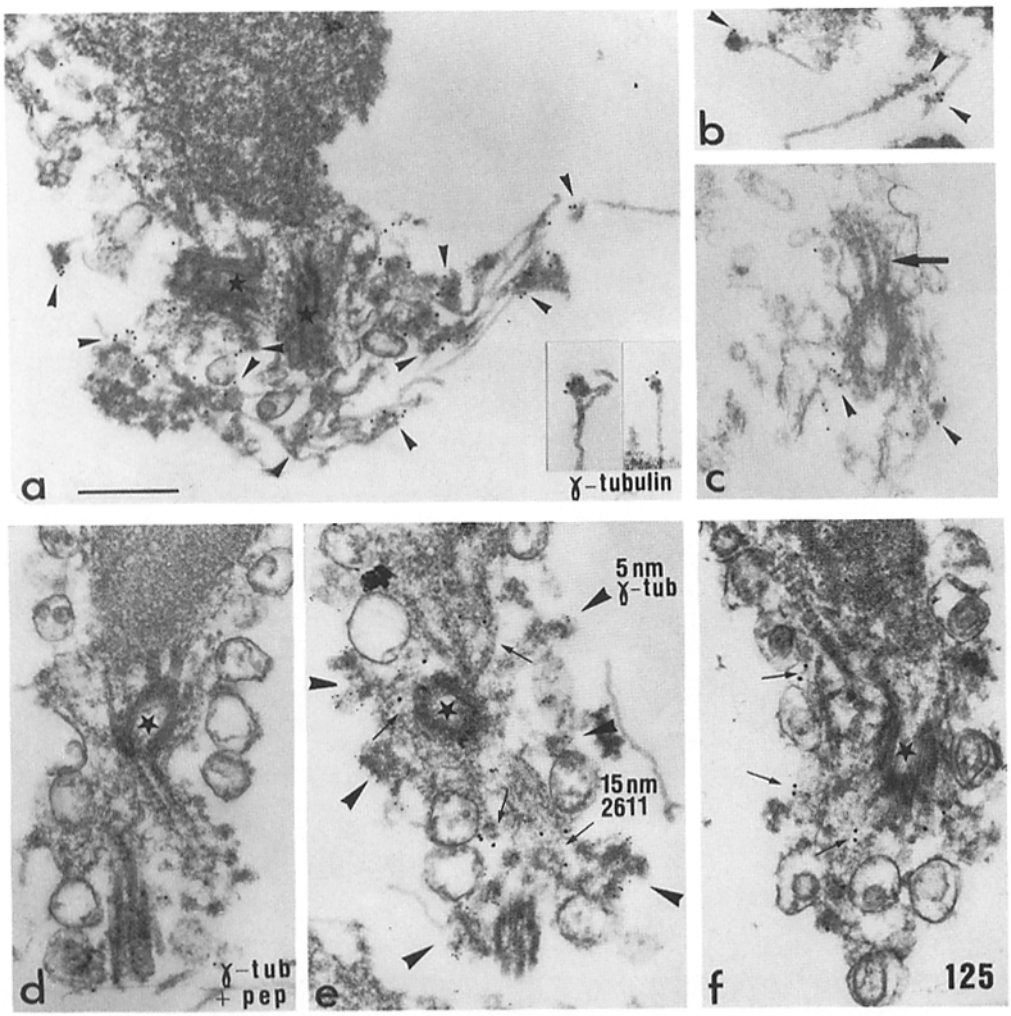

Figure 6. Immuno-electron microscopic localization of γ-tubulin, CTR2611, and 125 antigens in sperm heads incubated in the egg extract. Xenopus sperm heads were incubated in an interphase egg extract for 30 min, then processed for immunoelectron microscopy. (*a–d*) Immunostaining with the anti γ-tubulin serum 38 and 10-nm gold secondary antibodies, in the absence (*a–c*), or presence (*d*) of the antigenic peptide. (*e*) Double immunostaining with the 38 anti γ-tubulin serum (5-nm gold anti-rabbit antibodies) and the CTR2611 antibody (15-nm gold anti-mouse antibodies). (*f*) Immunostaining with the 125 monoclonal antibody. No staining was seen with the secondary antibody alone (not shown). Centrioles are marked by a star. Arrowheads point to the electron-dense aggregates that contain γ-tubulin and from which microtubules originate. Arrows in *e* point to the striated rootlets stained by the CTR2611 serum. Same magnification for all micrographs. Bar, 0.3 μm.

tract and reisolated through a glycerol cushion, and an extract incubated with the sperm storage buffer was run in parallel in lane *9*. The 50-kD band recognized by serum 75 appeared in the sample containing the reisolated sperm heads (lane *8*) and not in the control without sperm heads (lane *9*). This demonstrated that the γ-tubulin molecule was actually recruited from the egg extract onto the sperm centrosome during the incubation.

Two monoclonal antibodies shown previously to react with phosphorylated epitopes at the centrosome were also tested. Antibody 125 was originally raised against brain MAP1B (Ulloa et al., 1993*a*). The antibody is specific for a phosphorylated epitope that can be phosphorylated by casein kinase II (Ulloa et al., 1993*b*). It recognizes a protein related to MAP1B in human centrosomes (Domínguez, J., personal communication). The MPM2 antibody was raised against mitotic cells and recognizes certain phosphorylated epitopes (Davis et al., 1983), some of them at the centrosome, even during interphase (Buendia et al., 1992; Centonze and Borisy, 1990). Neither of these antibodies stained the peri-centriolar area of sperm heads that had not been incubated in the egg extract (Fig. 4, *d* and *e*). However, both antibodies stained it after incubation in the egg extract. The stained area appeared more like the X-shaped CTR2611 labeling than like the γ-tubulin spots (Fig. 4, *i* and *j*). Using electron microscopy the 125 antibody seemed not to stain the peripherical globular aggregates but rather more internal material (Fig. 6*f*). Double staining combined with γ-tubulin was not possible because of incompatibility of fixation conditions.

In the interphase frog egg extract, the 125 antibody recognized two polypeptides of 48 and 250 kD on SDS-PAGE gels (Fig. 7 *B*, lane *6*). No staining was observed when 5×10^4 untreated sperm heads were loaded (lane *7*). Two bands were observed specifically in the sample containing sperm heads preincubated with the egg extract and reisolated: at 120 kd and above 250 kd (lane *8*, *arrows*). The sperm heads in lane *8* were incubated in an extract volume of 25 μl whereas only 0.5 μl of extract were loaded in lane *6*. It is therefore possible that these epitopes already existed in the egg extract, albeit at undetectable levels, and were greatly enriched on sperm heads. Alternatively, they may have appeared on the sperm heads through a phosphorylation event in the extract. The 48 and 250-kD polypeptides were found in the control pellet of the egg extract centrifugation (lane *9*; fast-sedimenting proteins were found in this fraction), but they were not enriched in the presence of sperm heads (lane *8*). The MPM2 antibody recognized a similar pattern of polypeptides on gels (Fig. 7 *C*).

These results show that phosphorylated epitopes appeared

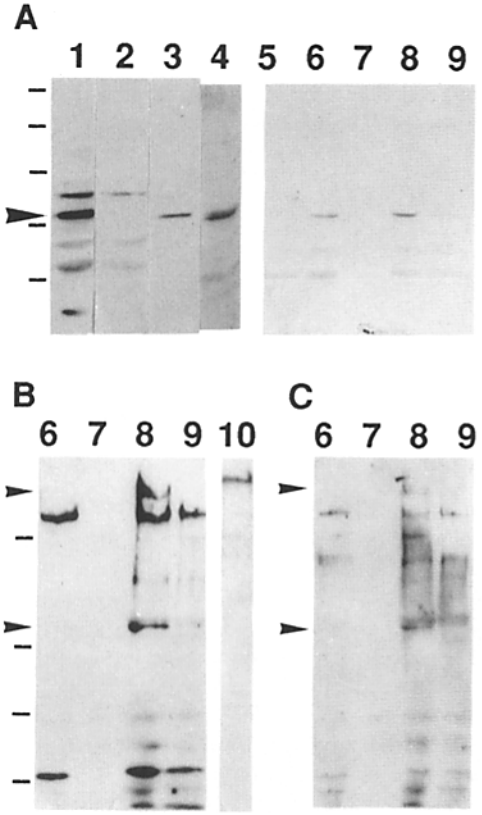

Figure 7. Immunoblotting of egg extract and sperm heads with serum 75 directed against γ-tubulin (*A*), and 125 (*B*), and MPM2 (*C*) monoclonal antibodies. (Lanes *1–4*) 0.5 μl of egg extract were run by SDS-PAGE, transferred to nitrocellulose, and immunoreacted with serum 75. (lane *1*) 1:1,000 dilution. (lane *2*) 1:1,000 dilution plus the antigenic peptide (4 μg/ml). (lane *3*) 1:10,000 dilution. (lane *4*) Serum 75 was first affinity-purified on the 50-kD γ-tubulin band and the purified antibodies used for a second round of immunoblotting. The reactive band at 50 kd is indicated by an arrowhead. (lane *5*) 0.5 μl of high-speed egg extract supernatant were loaded. (lane *6*) 0.5 μl of low-speed egg extract. (lane *7*) 5×10^4 sperm heads. (lane *8*) 5×10^4 sperm heads incubated for 30 min in the egg extract (2×10^3/ μl) and reisolated. (lane *9*) Egg extract incubated with the sperm head storage buffer and processed through the same centrifugation steps. (lane *10*) Brain MAPs. Arrowheads in *B* and *C* point to the polypeptides that become specifically stained by 125 and MPM2 antibodies when the sperm heads are incubated in the egg extract. (*A*) 10% SDS-PAGE. (*B* and *C*) 7% SDS-PAGE. The molecular weight markers indicated on the left correspond, from top to bottom, to 200, 97, 69, 46, and 30 kd, respectively.

on the sperm centrosome during incubation in the egg extract, either by recruitment of molecules from the extract, and/or by phosphorylation at the centrosome.

ATP Requirement for Centrosome Complementation

ATP-Mg^{2+} was depleted from the egg extract by addition of either 10 U/ml apyrase, a concentration that totally depletes ATP in the extract (Buendia et al., 1992), or 5 mM EDTA, a concentration that chelates all Mg^{2+} ions in the extract (Félix et al., 1989). Table I shows that centrosome formation (tested as in Fig. 2 for astral microtubule nucleation with pure tubulin) required ATP. No MPM2, 125, nor γ-tubulin staining appeared on the sperm heads in the absence of ATP. 1 mM DMAP (6-dimethylaminopurine, an ATP analog that inhibits phosphorylations in the extract; Félix et al., 1989), strongly inhibited the appearance of MPM2 and 125 epitopes. On those centrosomes that still stained weakly with these antibodies, γ-tubulin staining appeared as two dots slightly distal to the centrioles (apparently on opposite sides of the distal centriole). A similar γ-tubulin staining was observed when sperm heads that had been complemented already by incubation in an egg extract were added to a fresh extract in the presence of apyrase. MPM2 staining was then confined to the two centrioles (not shown). This suggested that the presence of γ-tubulin in a particularly wide area in the sperm centrosome (compared to other centrosomes) required constant energy supply and possibly phosphorylation events.

When the sperm heads were incubated in the extract with 1 mM DMAP, reisolated and incubated with an ATP-regenerating system, the phosphorylated epitopes appeared around the centrioles. Under the same experimental conditions, γ-tubulin was not recruited. Sperm heads treated in this way did not nucleate microtubules when incubated with pure tubulin. The appearance of phosphorylated epitopes did not occur if the ATP-regenerating system was omitted in the last step (Table I). A similar situation (presence of the phosphorylated epitopes, absence of γ-tubulin and of nucleation activity) was observed when the sperm heads were incubated in a high-speed extract supernatant that did not contain γ-tubulin (Fig. 7 *A*, lane *5*, and Table I). This suggested that γ-tubulin recruitment might be important for the formation of an active centrosome.

Immunodepletion of γ-Tubulin from the Egg Extract

To establish firmly the role of γ-tubulin, we immunodepleted it from the egg extract and assayed thereafter the activity of the depleted extract in the sperm centrosome complementation assay. γ-Tubulin was removed from the egg extract using immobilized immunoglobulins specific for the COOH terminus of γ-tubulin (serum 75). As seen by immunoblotting, the procedure depleted γ-tubulin from the extract (Fig. 8 *a*, lane *3*). The preimmune serum did not deplete γ-tubulin (lane *2*). Preincubation of the antibodies with the antigenic peptide prevented complete depletion of γ-tubulin from the extract (lane *4*).

Sperm heads were incubated in the different extracts, then examined using immunofluorescence for the presence of the γ-tubulin molecule, using serum 38 that was raised against another peptide of the γ-tubulin sequence. No γ-tubulin was detected around the centrioles of sperm heads incubated in the depleted extract (Fig. 8 *b, middle panel*). γ-Tubulin was detected on sperm heads incubated in the extracts processed with the preimmune serum or the immune serum plus the antigenic peptide (*left* and *right panels*). This confirmed the immunoblotting results and showed that, when present, γ-tubulin could still bind in the sperm centriolar area in the processed extracts.

The sperm heads incubated in the γ-tubulin depleted extracts were unable to nucleate astral microtubules after reisolation and incubation with pure tubulin. By contrast, the sperm heads incubated in the extracts treated with the preimmune serum or the immune serum in the presence of

Table I. Conditions for Centrosome Complementation and Appearance of γ-Tubulin and Phosphorylated Epitopes

Extract	Centrosome complementation	γ-Tubulin	MPM2	MAP1B IBO
Control	+	+	+	+
Apyrase 10 U/ml	−	−	−	−
EDTA 5 mM	−	−	−	−
DMAP 1 mM	−	− or 2 spots	− or weak	− or weak
DMAP, reisol., − ATP reg.	−	− or 2 spots	− or weak	N.D.
DMAP, reisol., + ATP reg.	−	−	+	+
High-speed supernatant	−	−	+	+
Buffer + ATP reg. sys.	−	−	−	−

The complementation conditions were as in Fig. 3. Centrosome complementation was either positive (presence of asters, '+') or totally negative (only axoneme elongation, '−'). The presence of a weak MPM2/MAP1B staining in the presence of DMAP on some centrosomes correlated with the presence of two spots of γ-tubulin. When the sperm heads were incubated in an egg extract, reisolated on a glycerol cushion and incubated in the same volume of acetate buffer, a very strong MPM2 and MAP1B-IBO staining appeared on the sperm centrosome provided an ATP-regenerating system was added to the acetate buffer ('DMAP, reisol., + ATP reg.'). See text for details.

the antigenic peptide were able to nucleate astral microtubules (Fig. 8 *c*). These data suggest strongly that the presence of γ-tubulin (and associated molecules) in the extract was an absolute requirement for the assembly of an active centrosome around the sperm centrioles.

Discussion

A Two-Step Assay for Centrosome Assembly and Activity

Only few in vitro systems are available for the study of centrosomes. Centrosomes have been purified and their nucleation activity studied by incubation with pure tubulin (Bornens et al., 1987; Bré and Karsenti, 1990; Kuriyama, 1984; Mitchison and Kirschner, 1984). A centrosome complementation assay has been designed by Buendia et al. based on the loss of nucleation activity after extraction of centrosomes purified from KE37 lymphoblastic cells by 2M urea. The urea-treated centrosomes incubated in Xenopus egg extracts in the presence of nocodazole can be reisolated by high-speed centrifugation on sucrose gradients and are able to nucleate microtubules (Buendia et al., 1992). Although this complementation assay could be useful to characterize some

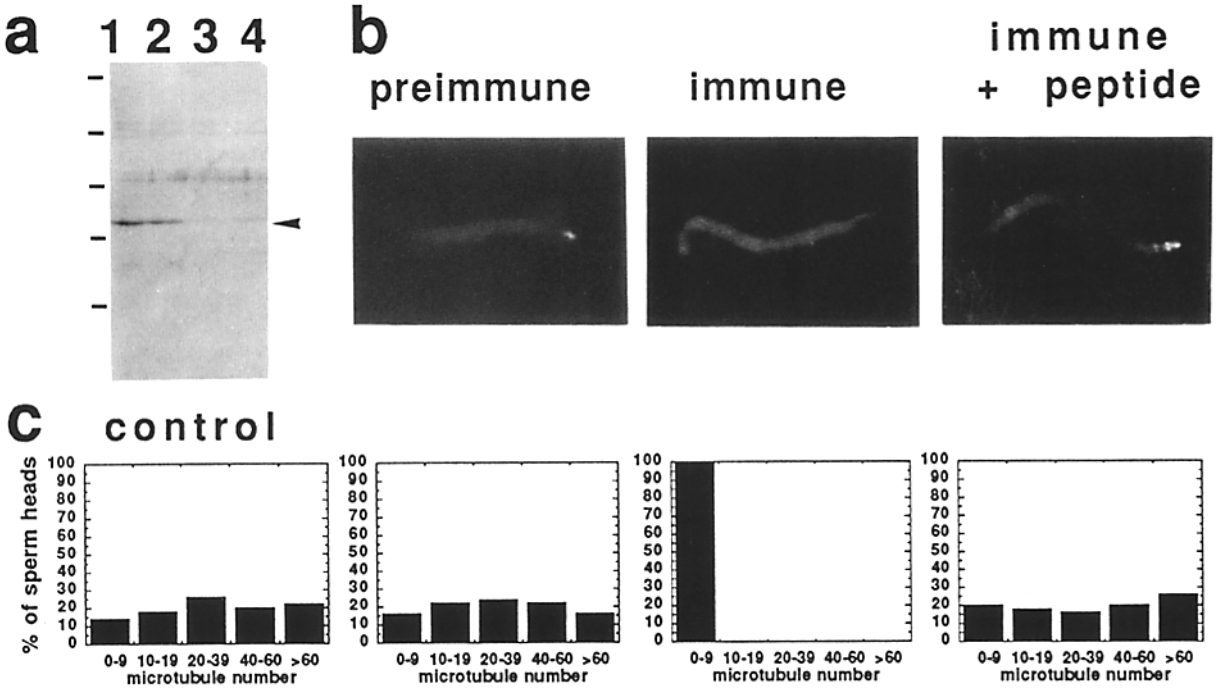

Figure 8. Immunodepletion of γ-tubulin from the egg extract. (*a*) Immunoblotting of the egg extract processed for immunodepletion with no antibody (lane *1*), preimmune serum 75 (lane *2*), immune serum 75 (lane *3*), and immune serum 75 plus the corresponding antigenic peptide (lane *4*). The blots were incubated with anti γ-tubulin serum C3, then protein A coupled to peroxidase. (*b*) Immunofluorescence of sperm heads incubated for 30 min in the egg extracts immunodepleted with the indicated sera. The fixed samples were incubated with anti γ-tubulin serum 38, then rhodamine-labeled secondary antibody. (*c*) Distribution of the number of microtubules nucleated per sperm head after preincubation with the egg extracts used above (treated, from left to right, without antibody, with preimmune serum, with immune serum, and with immune serum in the presence of the antigenic peptide). 50 centrosomes were observed in each case.

of the proteins involved directly or indirectly in microtubule nucleation, it makes use of biochemical extraction to obtain inactive centrosomes. In contrast, the sperm centrioles provide a more physiological system for centrosome assembly.

Sperm aster formation was first reconstituted in the egg extract. The Xenopus sperm centrioles and their associated material are not able by themselves to nucleate astral microtubules: microtubules only grow from the axoneme when the sperm heads are incubated with purified brain tubulin. However, they are able to organize asters after incubation in the egg extract in the presence of nocodazole, reisolation, and incubation with pure brain tubulin. Thus we distinguish two sequential steps in the formation of the sperm aster: (i) the formation in the egg extract of a functional centrosome onto the sperm centrioles and (ii) the nucleation of microtubules from pure tubulin on the mature centrosome. These two steps can be manipulated independently. In the first step, active centrosomes form in the absence of microtubules (nocodazole is added to the extract). Centrosome formation requires ATP and does not occur at 4°C. It is thus an energy-dependent process that does not rely only on a preestablished affinity of maternal and paternal components. In the second step of the assay (nucleation of microtubules), we select a tubulin concentration at which no extensive free tubulin polymerization takes place, in contrast to the egg extract where microtubules polymerize freely (see also Verde et al., 1990). The two-step procedure used shows that the sperm centrosome actually nucleates microtubules and does not merely capture them. Obviously it is also able to keep these nucleated microtubules anchored.

Our assay offers two practical advantages over the complementation of urea-treated centrosomes (Buendia et al., 1992), since the sperm centrioles are associated to the nucleus. Firstly, their reisolation does not require a high-speed centrifugation step. Secondly, they are easily localized under the microscope, since they are found at one end of the elongated frog sperm nucleus that can be readily visualized using fluorescent DNA dyes. The conditions of complementation of the urea-treated centrosomes and of formation of the sperm centrosomes in the egg extract are different: (i) complementation of the urea-treated centrosomes does not require ATP and (ii) it occurs in high-speed supernatants of the egg extract (Buendia et al., 1992), in contrast to the formation of the sperm centrosome; (iii) the urea-treated centrosomes still contain some γ-tubulin (Buendia, B., personal communication), whereas the γ-tubulin of the sperm centrosome is recruited from the egg extract. The egg extract thus contributes differently to the complementation of the urea-treated centrosomes and to the activation of the sperm centrosome.

Xenopus (mitotic) egg extracts have also been used to confer to fission yeast spindle pole bodies a microtubule-nucleating activity which they have only in mitosis in vivo. In this case the acquisition of the nucleation activity correlates with the appearance of MPM2 staining, and the presence of γ-tubulin in interphase spindle pole bodies is not sufficient for microtubule nucleation activity (Masuda et al., 1992). We find conversely that the presence of the MPM2 antigen around sperm centrioles in the absence of γ-tubulin is not sufficient for microtubule nucleation (Table I). The presence of MPM2 epitopes at the centrosome has been shown to correlate with microtubule nucleation activity (Centonze and Borisy, 1990), especially during the cell cycle: the increased centrosomal staining by MPM2 antibody at the onset of mitosis parallels the increased microtubule nucleation in prophase (Vandré et al., 1984). In our study, as in Buendia et al. (1992), the MPM2 antibody stains also centrosomes incubated in interphase extracts.

The phosphoepitope recognized by the 125 antibody is probably related to the phosphoepitope recognized by the MPM2 antibody (at least in interphase egg extracts), since both antibodies recognize a similar set of polypeptides by immunoblotting in the different samples. The 125 antibody gives clearer results by immunoblotting, which allows the characterization and purification of these phosphoproteins (Buendia, B., and J. Domínguez, personal communication). Whether the appearance of these phospho-epitopes on the sperm centrosome corresponds to the recruitment of a phosphoprotein or to a de novo phosphorylation remains to be determined. Kinases that have been localized to the centrosome, like the cdc2 kinase (Bailly et al., 1989), cAMP-dependent kinase (Keryer et al., 1993), and MAP kinase (Verlhac et al., 1993), may be involved in this phosphorylation.

Role of γ-Tubulin Recruitment in Astral Microtubule Nucleation

γ-Tubulin is present in the sperm centrosome around the centrioles in the electron-dense aggregates from which microtubules originate. From our biochemical study, it is clear that it is recruited from the egg extract. We made use of the two-step centrosome assembly assay to investigate the role of γ-tubulin. In contrast to antibody interference experiments, γ-tubulin can be first removed from the egg extract by immunoprecipitation and no free immunoglobulins are left in the extract (Fig. 8 *a*). The sperm heads incubated in the depleted extract do not nucleate astral microtubules. The specificity of the γ-tubulin immunodepletion is suggested by the recovery of the complementation activity after preincubation of the anti γ-tubulin serum with the antigenic peptide. It does not appear from the immunoblotting experiments in Fig. 7 that serum 75 recognizes another protein, the binding to which would be competed for by the γ-tubulin peptide. Moreover γ-tubulin seems to be the only polypeptide immunoprecipitated and subsequently recognized by immunoblotting by serum 75 (not shown). Our results thus suggest strongly that the recruitment from the egg extract of a complex that comprises γ-tubulin is required for the formation of an active centrosome, able to nucleate microtubules, and possibly for the process of microtubule nucleation itself.

γ-Tubulin, as a tubulin, may participate structurally in the base of the microtubule, establishing thereby microtubule polarity (Oakley, 1992). Four possible and not mutually exclusive mechanisms for the involvement of γ-tubulin in microtubule nucleation can be proposed, which we name 'localized tubulin', γ-tubulin 'miniseeds', increased affinity and capping. (i) γ-Tubulin could favor nucleation merely by being a localized tubulin, increasing the probability that a microtubule be formed in this region. (ii) Moreover, the recruitment of several γ-tubulin molecules in miniseeds could locally lower the apparent critical concentration of α/β dimers required for nucleation (Stearns et al., 1991). In this case γ-tubulin should be inactive in solution. (iii) γ-Tubulin may also favor nucleation by having an increased affinity in

lateral/axial interactions for itself, or for one, or both, of the other tubulins. Lateral interactions are weak and are a limiting step in nucleation before their enhancement by cooperative interactions (Carlier and Pantaloni, 1978; Erickson and Pantaloni, 1981; Voter and Erickson, 1984). Nucleation favored by γ-tubulin may constrain the nature of the lateral interactions (α-α, α-β, or β-β), for instance to those of the microtubule A-lattice with three-start helices. This dimer arrangement along the whole base of the microtubule would lead to a microtubule with 13 protofilaments (for review see Wade and Chrétien, 1993). This could explain how centrosomes nucleate microtubules that contain mostly 13 protofilaments (Evans et al., 1985). One mechanism for greater affinity would be through the regulation of GTP hydrolysis, which is thought to destabilize the tubulin interactions at the end of the microtubule (for review see Kirschner and Mitchison, 1986). There is a putative GTP-binding motif in the γ-tubulin sequence, but it is not known whether it is exchangeable, or even occupied (Burns, 1991). γ-Tubulin lateral binding to an α/β dimer could also inhibit GTP hydrolysis in this dimer, thereby stabilizing the microtubule. (iv) Such an increased affinity would also lead to an effective tubulin cap by preventing depolymerization of minus-ends located at the centrosome. Because minus-ends are difficult to visualize at the centrosome it is not known whether microtubules ever depolymerize completely, or whether microtubule seeds always remain at the centrosome. In this respect we observed that the microtubules nucleated in the sperm aster depolymerize completely in the presence of nocodazole (not shown), whereas a β-tubulin staining (and an α-tubulin staining, not shown) similar to the γ-tubulin staining remains after cold-induced depolymerization (Fig. 1 *d*). This could reflect a cold-insensitive, but nocodazole-sensitive interaction of the first α/β tubulin dimers with the nucleating/anchoring material, possibly γ-tubulin.

γ-Tubulin Recruitment and the Regulation of Centriolar Activity during Development

Changes in the molecular composition of the centriolar region have been demonstrated previously during the cell cycle (Bailly et al., 1989; Buendia et al., 1990; Price and Pettijohn, 1986; Sager et al., 1986). Here we show that γ-tubulin is recruited from the egg cytoplasm around the sperm centrioles at fertilization. The recruitment of γ-tubulin could be a general characteristic of specific stages in development, that would be involved in the conversion of centrioles from a basal body function to the nucleation of astral microtubules. In apparent contradiction with this hypothesis, γ-tubulin has been observed recently in the basal body region of monociliated bovine retinal photoreceptors and multiciliated cells from the bovine trachea and oviduct (Muresan et al., 1993). However, in these epithelial cells, cytoplasmic microtubules emanate from the basal body region. Thus, the centrioles have two activities in these cells, as basal bodies and as organizers of centrosomal material. It would be interesting to determine whether γ-tubulin is also present in the centriolar region of other epithelial cells where microtubule nucleation in the cytoplasm is uncoupled from the centrioles (Bacallao et al., 1989; Bré et al., 1990; Tucker et al., 1992).

In the fertilized egg the centrosome is assembled from both paternal components (the centriole itself, the CTR2611 antigen) and maternal components (for example γ-tubulin). The latter are stored in the oocyte cytoplasm during oogenesis in sufficient quantities to contribute to the formation of thousands of centrosomes until the onset of massive zygotic gene expression, at the midblastula transition (Gard et al., 1990; Newport and Kirschner, 1982) (see Fig. 3). Extracts prepared from embryos taken later in development actually cannot assemble centrosomes onto sperm centrioles (data not shown). In egg extracts, γ-tubulin sediments faster than would be expected for a monomer or a dimer (Fig. 7). This suggests that it is stored as a complex in the egg cytoplasm, either as a multimer or complexed with other molecules. A complex of γ-tubulin with other molecules has been described in Drosophila egg extracts (Raff et al., 1993). The role of the other molecules in this complex is unclear. They could either contribute to the recruitment of γ-tubulin around the sperm centrioles by binding to a 'receptor' site, or they could themselves be involved in microtubule nucleation. Alternatively, they could sequester γ-tubulin in the egg cytoplasm.

In some species, astral microtubule nucleation can take place in the absence of centrioles. In the mouse, centrosomal material can self-aggregate in the oocyte cytoplasm and at the poles of the cleavage spindles (Calarco-Gillam et al., 1983; Maro et al., 1985). γ-tubulin is present in these acentriolar structures (Gueth-Hallonet et al., 1993; Palacios and Joshi, 1993). In sea urchin, centrioles can appear de novo in artificially activated eggs (Kuriyama and Borisy, 1983). In Xenopus, a parent centriole is required both for centriole generation, in contrast to the sea urchin, and for organization of the cytoskeleton and cleavage, in contrast to the mouse (Bornens et al., 1987; Maller et al., 1976). Thus in Xenopus, the paternal centriole could be seen as a receptor site for maternal complementary components necessary for both microtubule nucleation and daughter centriole budding.

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References

Bacallao, R., C. Antony, C. Dotti, E. Karsenti, E. Stelzer, and K. Simons. 1989. The subcellular organization of MDCK cells during the formation of a polarized epithelium. *J. Cell Biol.* 109:2817–2832.

Bailly, E., M. Dorée, P. Nurse, and M. Bornens. 1989. $p34^{cdc2}$ is located in both nucleus and cytoplasm. Part is centrosomally associated at G2/M and enters vesicles at anaphase. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3985–3995.

Bernardini, G., R. Stipani, and G. Melone. 1986. The ultrastructure of Xenopus spermatozoon. *J. Ultrast. Mol. Struct. Res.* 94:188–194.

Blow, J. J., and R. A. Laskey. 1986. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of Xenopus eggs. *Cell.* 47:577–587.

Bornens, M. 1992. Structure and functions of isolated centrosomes. *In* The Centrosome. V. I. Kalnins, editor. Academic Press, San Diego. 1–43.

Bornens, M., M. Paintrand, J. Berges, M. C. Marty, and E. Karsenti. 1987. Structural and chemical characterization of isolated centrosomes. *Cell Motil.* 8:238–249.

Bré, M.-H., and E. Karsenti. 1990. Effects of brain microtubule-associated proteins on microtubule dynamics and the nucleating activity of centrosomes. *Cell Motil. Cytoskeleton.* 15:88–98.

Bré, M.-H., R. Pepperkok, A. M. Hill, N. Levilliers, W. Ansorge, E. H. K. Stelzer, and E. Karsenti. 1990. Regulation of microtubule dynamics and nucleation during polarization in MDCK II cells. *J. Cell Biol.* 111:3013–3021.

Buendia, B., C. Antony, F. Verde, M. Bornens, and E. Karsenti. 1990. A centrosomal antigen localized on intermediate filaments and mitotic spindle poles. *J. Cell Sci.* 97:259–271.

Buendia, B., G. Draetta, and E. Karsenti. 1992. Regulation of the microtubule nucleating activity of centrosomes in Xenopus egg extracts—role of cyclin-A-associated protein kinase. *J. Cell Biol.* 116:1431–1442.

Burns, R. G. 1991. Alpha-tubulin, beta-tubulin, and gamma-tubulins—sequence comparisons and structural constraints. *Cell Motil. Cytoskeleton.* 20:181–189.

Calarco-Gillam, P. D., M. C. Siebert, R. Hubble, T. Mitchison, and M. Kirschner. 1983. Centrosome development in early mouse embryos as defined by an autoantibody against pericentriolar material. *Cell.* 35:621–629.

Carlier, M.-F., and D. Pantaloni. 1978. Kinetic analysis of cooperativity in tubulin polymerization in the presence of Guanosine Di- or Triphosphate nucleotides. *Biochemistry.* 17:1908–1915.

Centonze, V. E., and G. G. Borisy. 1990. Nucleation of microtubules from mitotic centrosomes is modulated by a phosphorylated epitope. *J. Cell Sci.* 95:405–411.

Chambers, E. L. 1939. The movement of the egg nucleus in relation to the sperm aster in the echinoderm egg. *J. Exp. Biol.* 16:409–424.

Davis, F. M., T. Y. Tsao, S. K. Fowler, and P. N. Rao. 1983. Monoclonal antibodies to mitotic cells. *Proc. Natl. Acad. Sci. USA.* 80:2926–2930.

Erickson, H. P., and D. Pantaloni. 1981. The role of subunit entropy in cooperative assembly, nucleation of microtubules and other two-dimensional polymers. *Biophys. J.* 34:297–309.

Evans, L., T. Mitchison, and M. Kirschner. 1985. Influence of the centrosome on the structure of nucleated microtubules. *J. Cell Biol.* 100:1185–1191.

Félix, M.-A., P. R. Clarke, J. Coleman, F. Verde, and E. Karsenti. 1993. Frog egg extracts as a system to study mitosis. *In* The Cell Cycle, A Practical Approach. P. Fantes, editor. IRL Press, Oxford. In press.

Félix, M. A., J. Pines, T. Hunt, and E. Karsenti. 1989. A post-ribosomal supernatant from activated Xenopus eggs that displays post-translationally regulated oscillation of its cdc2+ mitotic kinase activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:3059–3069.

Gard, D. L., S. Hafezi, T. Zhang, and S. J. Doxsey. 1990. Centrosome duplication continues in cycloheximide-treated xenopus blastulae in the absence of a detectable cell cycle. *J. Cell Biol.* 110:2033–2042.

Gerhart, J., M. Wu, and M. Kirschner. 1984. Cell-cycle dynamics of an M-phase-specific cytoplasmic factor in Xenopus laevis oocytes. *J. Cell Biol.* 98:1247–1255.

Gueth-Hallonet, C., C. Antony, J. Aghion, A. Santa-Maria, I. Lajoie-Mazenc, M. Wright, and B. Maro. 1993. γ-tubulin is present in acentriolar MTOCs during mouse early development. *J. Cell Sci.* 105:157–166.

Horio, T., S. Uzawa, M. K. Jung, B. R. Oakley, K. Tanaka, and M. Yanagida. 1991. The fission yeast γ-tubulin is essential for mitosis and is localized at microtubule organizing centers. *J. Cell Sci.* 99:693–700.

Hyman, A., D. Drechsel, D. Kellogg, S. Salser, K. Sawin, P. Steffen, L. Wordeman, and T. Mitchison. 1991. Preparation of modified tubulins. *Methods Enzymol.* 196:478–485.

Joshi, H. C., M. J. Palacios, L. McNamara, and D. W. Cleveland. 1992. γ-tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature (Lond.).* 356:80–83.

Julian, M., Y. Tollon, I. Lajoie-Mazenc, A. Moisand, H. Mazarguil, A. Puget, and M. Wright. 1993. γ-tubulin participates in the formation of the mid-body during cytokinesis in mammalian cells. *J. Cell Sci.* 105:145–156.

Kalt, A., and M. Schliwa. 1993. Molecular components of the centrosome. *Trends Cell Biol.* 3:118–128.

Keryer, G., R. M. Rios, B. F. Landmark, B. Skalhegg, S. Lohmann, and M. Bornens. 1993. A high-affinity binding protein for the regulatory subunit of cAMP-dependent protein kinase II in the centrosome of human cells. *Exp. Cell Res.* 204:230–240.

Kirschner, M., and T. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell.* 45:329–342.

Kuriyama, R. 1984. Activity and stability of centrosomes in chinese hamster ovary cells in nucleation of microtubules in vitro. *J. Cell Sci.* 66:277–295.

Kuriyama, R., and H. Kanatani. 1981. The centriolar complex isolated from starfish spermatozoa. *J. Cell Sci.* 49:33–49.

Kuriyama, R., and G. G. Borisy. 1983. Cytasters induced within unfertilized sea-urchin eggs. *J. Cell Sci.* 61:175–189.

Leiss, D., M. A. Félix, and E. Karsenti. 1992. Association of cyclin-bound $p34^{cdc2}$ with subcellular structures in Xenopus eggs. *J. Cell Sci.* 102:285–297.

Liu, B., J. Marc, H. C. Joshi, and B. A. Palevitz. 1993. A γ-tubulin related protein associated with the microtubule arrays of higher plants in a cell cycle dependent manner. *J. Cell Sci.* 104:1217–1228.

Maller, J., D. Poccia, D. Nishioka, P. Kidd, J. Gerhart, and H. Hartman. 1976. Spindle formation and cleavage in Xenopus eggs injected with centriole-containing fractions from sperm. *Exp. Cell Res.* 99:285–294.

Manes, M. E., and F. D. Barbieri. 1977. On the possibility of sperm aster involvement in dorso-ventral polarization and pronuclear migration in the amphibian egg. *J. Embryol. Exp. Morphol.* 40:187–197.

Maro, B., S. K. Howlett, and M. Webb. 1985. Non-spindle microtubule organizing centers in metaphase II-arrested mouse oocytes. *J. Cell Biol.* 101:1665–1672.

Masuda, H., M. Sevik, and W. Z. Cande. 1992. In vitro microtubule-nucleating activity of spindle pole bodies in fission yeast Schizosaccharomyces pombe—cell cycle-dependent activation in Xenopus cell-free extracts. *J. Cell Biol.* 117:1055–1066.

Mitchison, T., and M. Kirschner. 1984. Microtubule assembly nucleated by isolated centrosomes. *Nature (Lond.).* 312:232–237.

Muresan, V., H. C. Joshi, and J. C. Besharse. 1993. γ-tubulin in differentiated cell types: localization in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia. *J. Cell Sci.* 104:1229–1237.

Newport, J., and M. Kirschner. 1982. A major developmental transition in early Xenopus embryos: 2. Control of the onset of transcription. *Cell.* 30:687–696.

Oakley, B. R. 1992. γ-tubulin: the microtubule organizer? *Trends Cell Biol.* 2:1–5.

Oakley, C. E., and B. R. Oakley. 1989. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mip*A gene of Aspergillus nidulans. *Nature (Lond.).* 338:662–663.

Oakley, B. R., C. E. Oakley, Y. S. Yoon, and M. K. Jung. 1990. γ-tubulin is a component of the spindle pole body that is essential for microtubule function in aspergillus-nidulans. *Cell.* 61:1289–1301.

Palacios, M. J., and H. C. Joshi. 1993. γ-tubulin reorganization during mouse fertilization and early development. *J. Cell Sci.* 104:383–389.

Price, C. M., and D. E. Pettijohn. 1986. Distribution of the nuclear mitotic apparatus protein (NuMA) during mitosis and nuclear assembly. *Exp. Cell Res.* 166:295–311.

Raff, J. W., D. R. Kellogg, and B. M. Alberts. 1993. Drosophila γ-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* 121:823–835.

Sager, P. R., N. L. Rothfield, J. M. Olicer, and R. D. Berlin. 1986. A novel mitotic spindle pole component that originates from the cytoplasm during prophase. *J. Cell Biol.* 103:1863–1872.

Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA.* 70:765–768.

Stearns, T., L. Evans, and M. Kirschner. 1991. γ-tubulin is a highly conserved component of the centrosome. *Cell.* 65:825–836.

Tucker, J. B., C. C. Paton, G. P. Richardson, M. M. Mogensen, and I. J. Russell. 1992. A cell surface-associated centrosomal layer of microtubule-organizing material in the inner pillar cell of the mouse cochlea. *J. Cell Sci.* 102:215–226.

Ulloa, L., J. Avila, and J. Díaz-Nido. 1993*a*. Heterogeneity in the phosphorylation of microtubule-associated protein MAP1B during rat brain development. *J. Neurochem.* 61:961–972.

Ulloa, L., J. Díaz-Nido, and J. Avila. 1993*b*. Depletion of casein kinase II by antisense oligonucleotide prevents neuritogenesis in neuroblastoma cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:1633–1640.

Vandré, D. D., F. M. Davis, P. N. Rao, and G. G. Borisy. 1984. Phosphoproteins are components of mitotic microtubule organizing centers. *Proc. Natl. Acad. Sci. USA.* 81:4439–4443.

Verde, F., J.-C. Labbé, M. Dorée, and E. Karsenti. 1990. Regulation of microtubule dynamics by cdc2 protein kinase in cell-free extracts of Xenopus eggs. *Nature (Lond.).* 343:233–238.

Verlhac, M. H., H. de Pennart, B. Maro, M. H. Cobb, and H. J. Clarke. 1993. MAP kinase becomes stably activated at metaphase and is associated with microtubule-organizing centers during meiotic maturation of mouse oocytes. *Dev. Biol.* 158:330–340.

Voter, W. A., and H. P. Erickson. 1984. The kinetics of microtubule assembly. *J. Biol. Chem.* 259:10430–10438.

Wade, R. H., and D. Chrétien. 1993. Cryoelectron microscopy of microtubules. *J. Struct. Biol.* 110:1–27.

Weingarten, M. D., A. H. Lockwood, S.-Y. Hwo, and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA.* 72:1858–1862.

Wilson, E. B. 1925. The Cell in Development and Heredity. MacMillan Publishing Co., New York. 687 pp.

Zheng, Y. X., M. K. Jung, and B. R. Oakley. 1991. γ-tubulin is present in *Drosophila-melanogaster* and *Homo-sapiens* and is associated with the centrosome. *Cell.* 65:817–823.