Centrosomes Isolated from *Spisula solidissima* Oocytes Contain Rings and an Unusual Stoichiometric Ratio of α/β Tubulin

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Abstract. Centrosome-dependent microtubule nucleation involves the interaction of tubulin subunits with pericentriolar material. To study the biochemical and structural basis of centrosome-dependent microtubule nucleation, centrosomes capable of organizing microtubules into astral arrays were isolated from parthenogenetically activated *Spisula solidissima* oocytes. Intermediate voltage electron microscopy tomography revealed that each centrosome was composed of a single centriole surrounded by pericentriolar material that was studded with ring-shaped structures ∼25 nm in diameter and <25 nm in length. A number of proteins copurified with centrosomes including: (*a*) proteins that contained M-phase–specific phosphoepitopes (MPM-2), (*b*) α-, β-, and γ-tubulins, (*c*) actin, and (*d*) three low molecular weight proteins of <20 kD. γ-Tubulin was not an MPM-2 phosphoprotein and was the most abundant form of tubulin in centrosomes. Relatively little α- or β-tubulin copurified with centrosomes, and the ratio of α- to β-tubulin in centrosomes was not 1:1 as expected, but rather 1:4.6, suggesting that centrosomes contain β-tubulin that is not dimerized with α-tubulin.

I N most organisms, microtubules (Mts)[1] are not randomly distributed in cells but exist as highly ordered arrays that are essential for various functions (for review see Brinkley, 1985; Kellogg et al., 1994). The organization of these arrays is determined by Mt-organizing centers (Pickett-Heaps, 1969). In animal cells, the major Mt-organizing center is the centrosome (Brinkley et al., 1981). Despite the critical role of the centrosome in many cellular processes, surprisingly little is known about the organelle's molecular composition or how Mt nucleation is regulated biochemically. A major barrier has been the inability to isolate sufficient quantities of functional centrosomes for direct biochemical analysis (Brinkley, 1985). Recently, methods have been developed for the isolation of centrosomes from mammalian cell lines (Mitchison and Kirschner, 1984, 1986; Bornens et al., 1987), insects (Moritz et al., 1995*a*), and the major Mt-organizing center in fungal cells, the spindle pole body, from yeast (Rout

and Kilmartin, 1990). These procedures provide an important first step for the characterization of proteins required for centrosome function.

Early electron microscopy studies revealed that in most animal cells, centrosomes are composed of one or two centrioles surrounded by an amorphous cloud of electron-dense material, the pericentriolar material (PCM) (Porter, 1966; Gould and Borisy, 1977). The PCM is largely proteinaceous (Kuriyama, 1984; Klotz et al., 1990) and serves as the site of origin for Mts (Gould and Borisy, 1977; Soltys and Borisy, 1985; Moritz et al., 1995*a*). At this time, relatively few PCM proteins have been identified (Sellitto et al., 1992; Kellogg et al., 1994). An extensively characterized PCM protein required for Mt nucleation is γ-tubulin, a member of the tubulin superfamily first identified in *Aspergillus nidulans* (Oakley and Oakley, 1989). A large body of genetic and biochemical evidence indicates that γ-tubulin plays a crucial role in Mt nucleation. First, γ-tubulin is localized to centrosomes and spindle pole bodies and is required for spindle assembly and progression through mitosis (Oakley and Oakley, 1989; Oakley et al., 1990; Stearns et al., 1991; Zheng et al., 1991). Second, γ-tubulin antibodies inhibit centrosome-dependent Mt nucleation (Joshi et al., 1992). Third, recruitment of γ-tubulin to sperm basal bodies is necessary for the assembly of an Mt nucleation-competent paternal centrosome (Felix et al., 1994; Stearns and Kirschner, 1994). Fourth, γ-tubulin binds tightly to the minus ends of Mts with a stoichiometry that suggests that one

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1. *Abbreviations used in this paper*: IVEM, intermediate voltage transmission electron microscopy; IOD, integrated optical density; MPM, M-phase–specific phosphoepitope; Mts, microtubules; PCM, pericentriolar material.

γ-tubulin is bound per tubulin subunit exposed at the minus ends of Mts (Li and Joshi, 1995). Finally, γ-tubulin is a component of nucleation-competent complexes, 25-nm-diam "rings," recently isolated from *Xenopus* oocyte extracts (Zheng et al., 1995), and ring structures of similar diameter have been identified as components of isolated *Drosophila* centrosomes (Moritz et al., 1995*b*). Based on these studies, it is clear that γ-tubulin is a critical PCM component required for centrosome-dependent Mt nucleation. However, the role, if any, of conventional α- or β-tubulin in centrosome-dependent Mt nucleation has yet to be investigated.

Microtubule nucleation must involve the interaction of tubulin subunits (α- and/or β-tubulin) with centrosome components. Since γ-tubulin was originally discovered as a second-site suppressor of a β-tubulin mutation in *Aspergillus nidulans* (Oakley and Oakley, 1989), it was proposed that γ-tubulin interacts with Mts via a physical interaction with the β-tubulin subunit of the tubulin heterodimer (Mandelkow and Mandelkow, 1994; Oakley, 1994). In addition, evidence gained from the binding of GTP-analogs covalently attached to fluorescent beads suggests that β-tubulin is the terminal subunit at the plus end of the Mt; however, it was proposed that the minus end may have an alternative structure, perhaps consisting of β:γ heterodimer (Mitchison, 1993). Thus, it is important to investigate whether conventional (α, β) tubulins, and in particular β-tubulin, are important for centrosome-dependent Mt nucleation. In addition, numerous other questions remain regarding the cell cycle–dependent regulation of centrosome assembly and duplication and the regulation of Mt nucleation during meiosis and mitosis.

To address these and other questions, we have used the unique properties of *Spisula solidissima* (surf clam) oocytes to develop an in vitro system for the study of centrosome function. These oocytes can be obtained in 100-g quantities, thus facilitating preparative biochemistry. Furthermore, since they are arrested at prophase of meiosis I (Rebhun, 1959), they represent a pure synchronous culture of cells. Importantly, fertilization or parthenogenetic activation induces the synchronous assembly and maturation of functional centrosomes within minutes (Allen, 1953; Rebhun, 1959; Kuriyama, 1984; Palazzo et al., 1992), and extracts prepared from activated oocytes assemble asters (Weisenburg and Rosenfeld, 1975; Palazzo et al., 1988), offering the possibility of a biochemical approach to understanding the regulation of centrosome assembly and maturation. Previously, this system was used to study centriole assembly and maturation in vitro (Palazzo et al., 1992). Here we report methods for isolating homogeneous centrosomes from a specific time point in the meiotic cell cycle for biochemical and structural analysis, and the discovery that centrosomes contain an unexpected stoichiometric ratio of α/β tubulin.

Materials and Methods

All reagents were from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted.

Lysate Preparation

Adult *Spisula* were collected by the Marine Resources Department of the Marine Biological Laboratory (Woods Hole, MA) and maintained in flow-through sea water tanks at ~13°C. Oocytes were dissected from ripe ovaries, passed through cheese cloth, and washed in sea water by three cycles of suspension/sedimentation. Oocytes were activated by treatment with KCl for 4 min, and lysates were prepared as previously described (Palazzo et al., 1988). Aster formation in lysates was assessed with polarized light microscopy by adding 3% hexylene glycol to small aliquots and warming to 24°C (Palazzo et al., 1988). The remaining lysate was aliquoted, snap frozen, and stored at −80°C. Frozen lysates retain the ability to assemble asters after years of storage.

Tubulin Preparation

Sea urchin (*Strongylocentrotus purpuratus*) Mts were prepared by three cycles of polymerization/depolymerization as previously described (Suprenant and Marsh, 1987). Aliquots of ~2 mg/ml protein were stored in 100 mM potassium-Pipes, pH 6.9, 1 mM EGTA, 5 mM $MgSO_4$, 2 mM GTP (reassembly buffer) at −80°C. Three-cycled tubulin was diluted with reassembly buffer to 0.7 mg/ml for all functional assays unless otherwise stated.

Three-cycled tubulin was also prepared from *Spisula* oocyte lysates using a modification of the procedure described in Suprenant (1989). Lysates were thawed and diluted with 0.8 vol of dilution buffer (100 mM potassium-Pipes, pH 7.2, 4 mM EGTA, 1 mM $MgSO_4$, 1 mM DTT) containing 1 mM GTP, 10 mg/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride. Diluted lysate was gently resuspended on ice and centrifuged (39,000 *g*/2°C/30 min) using a rotor (model JA20; Beckman Instruments, Fullerton, CA) to clarify. Supernatants were collected and three-cycled tubulin prepared as previously described (Suprenant, 1989).

When necessary, *Spisula* tubulin was purified from three-cycled tubulin by polymerizing in the presence of Na-glutamate (Simon et al., 1992), followed by successive cycles of polymerization/depolymerization. The final five-cycled Mts were pelleted through a cushion of 30% glycerol/reassembly buffer. Pellets were aspirated dry, snap frozen in liquid nitrogen, and stored at −80°C.

Centrosome Isolation

Centrosomes were isolated from lysates by sucrose density-gradient centrifugation using a modification of the procedures described by Mitchison and Kirschner (1984, 1986) for isolation of mammalian centrosomes. Lysates were thawed on ice, and aliquots were removed and tested for aster formation using hexylene glycol and polarized light microscopy as previously described. For each preparative gradient, 2–3 ml of crude lysate (~9–18 × 10⁶ centrosomes) was diluted with 0.60 vol of aster buffer (Aster Buffer: 20 mM sodium-Pipes, pH 7.2, 100 mM NaCl, 5.0 mM $MgSO_4$), resuspended, and centrifuged (5,500 *g*/4°C/10 min) using a rotor (model JS 13.1; Beckman Instruments). Supernatants were collected, diluted to 50.2% sucrose by addition of a stock solution of 70% (wt/wt) sucrose in PEM (5 mM potassium-Pipes, pH 7.2, 1 mM EGTA, 1 mM $MgSO_4$), and loaded onto a two-step gradient consisting of 66% (3.0 ml) and 52.5% (5.0 ml) sucrose steps. Gradients were centrifuged at 70,000 *g* at 4°C for 90 min using a rotor (model SW-28; Beckman Instruments). Fractions (1.0 ml) were collected at 4°C by bottom puncture of centrifuge tubes, and sucrose density was determined with the use of a hand-held refractometer.

Fractions were tested for centrosome content based on their ability to organize Mts in the form of radial astral arrays when reconstituted with tubulin media. Thus, 10 μl aliquots from each fraction were diluted into 40 μl of tubulin media (0.5 mg/ml three-cycled sea urchin tubulin in RA buffer containing 2 mM GTP) and incubated for 10 min at ambient temperature, and asters were counted using a hemacytometer viewed with a polarized light microscope. The fraction containing the highest density of centrosomes (centrosome fraction) was used directly, or snap frozen in liquid nitrogen and stored at −80°C for further use.

Immunofluorescence

Centrosome proteins were localized by immunofluorescence as previously described (Mitchison and Kirschner, 1986; Palazzo et al., 1992) with the following modifications: Aliquots of the centrosome fraction were reconstituted with tubulin media and incubated for 30 min at room temperature. Samples were fixed for 5 min at room temperature by adding 100 vol of 1% glutaraldehyde (Ted Pella, Inc., Redding, CA) in PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl, pH 7.2). Fixed samples were layered onto a 30% glycerol/PBS cushion, and centrosomes were centrifuged onto polylysine-coated coverslips at 8,000 *g* using a rotor (model JS13.1; Beckman Instruments) (Mitchison and Kirschner, 1984).

The coverslips were incubated in ice-cold methanol for 5 min and then washed three times with PBS to rehydrate. All subsequent steps used PBS as buffer. Coverslips were blocked with 5% BSA for 30 min, followed by application of the primary antibody. Tubulins were detected using the following antibodies: DM1A (α-tubulin; Amersham Corp., Arlington Heights, IL), TU27 (β-tubulin; a gift of Dr. A. Frankfurter, University of Virginia, Charlottesville, VA), or EAD24 (γ-tubulin). Detection of antigen/antibody complexes was accomplished using either anti–mouse FITC or anti–rabbit rhodamine conjugates (Calbiochem, La Jolla, CA). Images were acquired with a laser scanning confocal microscope (model MRC-100; BioRad Labs, Hercules, CA).

Electron Microscopy Tomography

Centrosome fractions were diluted into 2 vol of 3% glutaraldehyde in PEM buffer and incubated on ice for 10 min. After fixation, centrosomes were concentrated into a loose pellet by centrifugation at 70,000 g for 30 min at 4°C in a rotor (model TLA 100.3; Beckman Instruments). Pellets were aspirated, postfixed with 1% OsO_4, dehydrated, and embedded as described previously for *Spisula* asters (Palazzo et al., 1988, 1992). Semithick (200–300 nm) serial sections were then cut from each preparation. Each section was subsequently mounted in the center of a formvar-coated slot grid on which 15-nm colloidal gold had been lightly deposited to facilitate subsequent micrograph alignment. Sections were next stained with uranyl acetate and lead citrate. Selected regions within a section, containing the area to be reconstructed, were irradiated at 400 kV and normal beam intensity in an intermediate voltage electron microscope (IVEM) (model JEM-4000 FX; JEOL U.S.A., Inc., Peabody, MA) until the initial mass loss was completed (~10 min). They were then photographed on SO 163 film, using a tilt-rotation stage, around two orthogonal (x and y) axes at increments of 2° over a ± 60° range. To limit specimen deformation during sequential photography, the specimen was further irradiated only during image recording. 122 tilt images were used for each reconstruction, with 61 images around the x-axis and 61 images around the y-axis. Images were then scanned so that each pixel was 2.6 nm and each image was 800 × 800 pixels. Three-dimensional tomographic reconstructions were then calculated by methods previously described in detail (Penczek et al., 1995; see also McEwen et al., 1993). Images were displayed in negative form to enhance the contrast.

Protein Analysis

To collect centrosomes, the centrosome fractions were diluted with equal volume of PEM buffer followed by centrifugation at 128,000 g/4°C/20 min using a rotor (model TLA 100.3; Beckman Instruments). For protein quantitation or SDS-PAGE analysis, centrosome pellets were first resuspended in 25 mM Tris, pH 7.2, containing 0.5% SDS (TS). For two-dimensional gel analysis, pellets were resuspended in urea sample buffer (O'Farrell, 1975). Protein content was determined with a bicinachromic acid protein assay according to manufacturer's instruction (Pierce, Rockford, IL).

Denaturing Electrophoresis

Centrosome pellets were solubilized in 25–50 μl of TS. Equal volume of 2× SDS-sample buffer was added (Laemmli, 1970), samples were boiled, and proteins were separated on either 10 or 4–20% gradient (BioRad Labs) polyacrylamide gels (Laemmlli, 1970). Proteins were visualized by staining with Coomassie G-250 (Neuhoff et al., 1988).

Two-dimensional Electrophoresis

The Millipore (Bedford, MA) high-resolution electrophoresis system (Patton et al., 1990) was used for two-dimensional gel electrophoresis. Centrosome pellets (containing 10–20 × 10^6 centrosomes) were solubilized in 50 μl of urea sample buffer (10 mM Tris, pH 8.8/0.06% SDS/0.1 M β-mercaptoethanol/100 mM DTT/9 M urea/4% NP-40/10 mM CHAPS). Approximately 5 μg of protein (~90,000 centrosomes) was loaded onto each isoelectric focusing gel and proteins focused for 18,000 volt h using 4.1% gels containing ampholytes with the pH ranges of 3–10, 5–7, and 4–8 (Millipore Corp.) at a ratio of 1:1:2, respectively. In the second dimension, proteins were separated on 10% SDS-PAGE gels (O'Farrell, 1975), and proteins were detected by either silver stain (Patton et al., 1990) or by immunoblot. Apparent molecular weights and isoelectric points were determined using external standards (mol wt; BioRad Labs) (pI; Pharmacia LKB Nuclear, Gaithersburg, MD).

Immunoblots

For immunoblot analysis, proteins resolved by SDS-PAGE were transferred to Immobilon-P membrane (Millipore Corp.) according to the methods of Towbin et al. (1979). Membranes were blocked with 5% BSA in TBS containing 0.2% Tween-20 (TBS/T) for 1 h, followed by incubation with primary antibodies DM1A (α-tubulin; Amersham Corp.), DM1B (β-tubulin; Amersham Corp.), C4 (actin; ICN Biomedicals, Costa Mesa, CA), or EAD24 (γ-tubulin) and HRP-IgG–conjugated secondary antibody (Promega Corp., Madison, WI). All antibodies were diluted in 0.5× blocking buffer. Blots were washed between hybridizations with TBS/T. Chemiluminescence (ECL; Amersham Corp.) was used to detect the antibody–antigen complexes according to product instructions.

Quantitation of Centrosome Tubulin

To quantify tubulin in centrosome samples, *Spisula* glutamate–purified tubulin was used as a standard. The *Spisula* tubulin stock sample was determined to be 97% tubulin, with one contaminant representing <3% of the protein. The stoichiometric ratio of α/β tubulin was determined by densitometric measurement of Coomassie-stained tubulin separated in 7.5% polyacrylamide gels and found to be 1:1 in these samples. Therefore, the concentration of either α- or β-tubulin for each standard was considered to be half of the protein concentration. Tubulin (2 mg/ml in reassembly buffer containing 2 mM GTP) was diluted to 1 mg/ml on ice with ultrapure H_2O, followed by serial dilution in SDS-sample buffer to give standards with the following concentrations: 0.1, 0.03, 0.01, 0.003, and 0.001 mg/ml.

Centrosome protein (5 μg) and 10 μl of each tubulin standard were separated on 10% polyacrylamide gels and transferred to Immobilon-P membrane (Millipore Corp.) as described in Towbin et al. (1979). After transfer, gels were stained as described in Neuhoff et al. (1988) to confirm that all the protein had been electroeluted to the membrane. Immunoblots were prepared and visualized by chemiluminescence as described previously.

Images of immunoblots were digitized using a BioImage analysis system (Milligen, Ann Arbor, MI) and analyzed using the one-dimensional module of the BioImage Visage software package. The integrated optical density (IOD) of each standard was determined and plotted relative to the tubulin concentration to determine the linear range. Tubulin standards within the linear range were used for first-order regression analysis. The IOD for the tubulin content of centrosome samples was obtained, and tubulin concentration was calculated using the equation $x = b - y/m$, where y is equal to the IOD value of the centrosome tubulin signal, b is equal to the y-intercept (integrated optical density), and m is equal to the slope. Since only half of the tubulin in each standard represented α- or β-tubulin (e.g., α-tubulin = $a/a + b = 0.5$), the tubulin concentration in centrosome samples obtained by this regression analysis was adjusted by multiplying the value by 0.5.

Results

Isolation of Centrosomes

Centrosomes were isolated from *Spisula* oocyte lysates by a discontinuous two-step sucrose gradient centrifugation system, consisting of 66 and 52.5% sucrose steps, similar to that used for isolation of mammalian centrosomes (Mitchison and Kirschner, 1984). The centrosome content of gradient fractions was determined using a functional reconstitution assay based on the ability of centrosomes to organize Mts into astral arrays (Mitchison and Kirschner, 1984; Palazzo et al., 1988, 1992). Thus, aliquots of gradient fractions were diluted into tubulin-containing media, and aster formation was visualized by polarized light microscopy (see Fig. 2 *A*).

In these gradients, the highest density of centrosomes was consistently found in a 59% sucrose fraction at the interface between the 52.5% step and the 66% cushion (centrosome fraction) (Fig. 1 *A*). Protein analysis revealed that the centrosome fraction contained relatively little protein compared to the rest of the gradient fractions collected
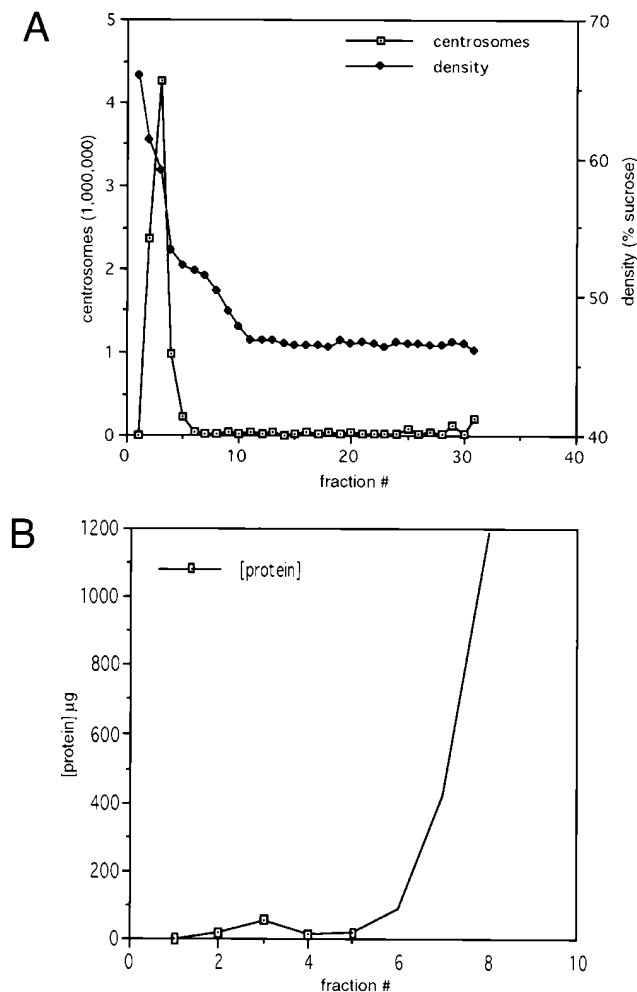
Figure 1. Sedimentation of centrosomes in preparative (30 ml) discontinuous sucrose gradients. Gradient fractions were analyzed for centrosome content using a functional reconstitution assay. Centrosomes were isolated from *Spisula* oocyte lysates as described in Materials and Methods. 5 μl of each fraction was combined with 20 μl of tubulin media containing 0.5 mg/ml sea urchin three-cycled tubulin and incubated at 22°C to allow aster formation. (*A*) Concentration of asters (centrosomes) /ml was determined using a hemacytometer and polarized light microscope and plotted against fraction density (% sucrose). The peak of aster forming activity, reflecting the number of centrosomes per fraction, corresponded to the 59–60% fraction (59.7 ± 0.628, n = 5). (*B*) To determine the protein concentration relative to centrosome content, fractions were dialyzed against PEM buffer and then against PEM/0.2% SDS for 2 h. Protein was precipitated with 20% TCA on ice and pelleted in a microfuge. Pellets were resuspended in 0.1 N NaOH and the protein concentration determined using a Coomassie assay. Aster-forming activity corresponded to a small peak of precipitable protein. The protein content of centrosome-containing fractions represented a small fraction of the total protein recovered from the gradient.

(Fig. 1 *B*). The number of centrosomes present in each fraction was determined by reconstituting gradient fractions with exogenous tubulin and counting the number of asters that formed with a hemocytometer viewed by polarized light microscopy (Fig. 2 *A*). Immunofluorescence analysis of asters using antibodies that recognize β-tubulin (TU27)
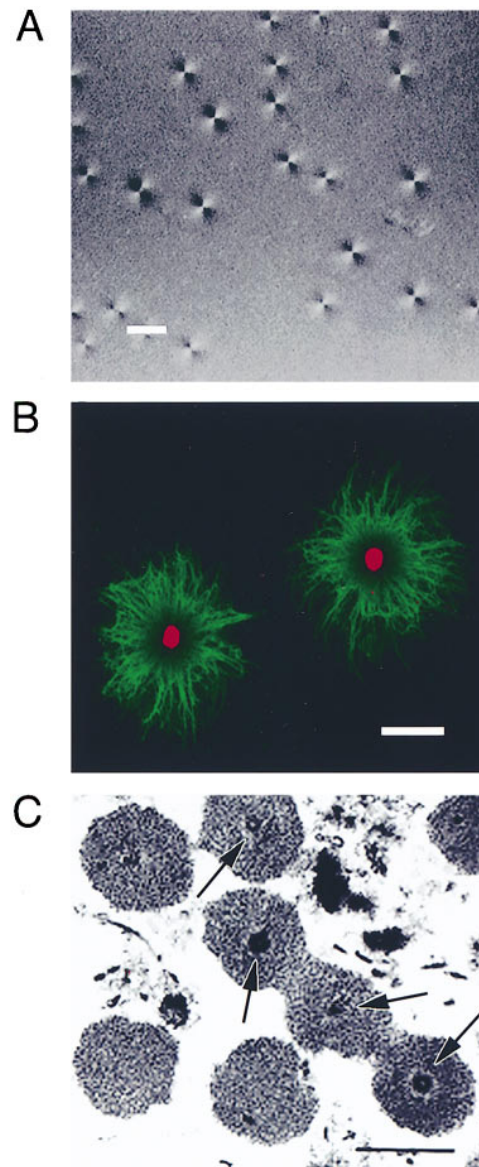
Figure 2. In vitro aster formation using centrosome-containing sucrose fractions. Aliquots of centrosome fractions were reconstituted with tubulin media to assemble asters. (*A*) Aster-forming activity was quantified using polarized light microscopy. (*B*) Asters were composed of microtubules (*green*) stained by β-tubulin antibody TU27, which project from discrete central foci (*red*) stained by γ-tubulin antibody EAD24. (*C*) EM analysis revealed that centrosome fractions contained bona fide centrosomes composed of centrioles (*arrows*) surrounded by a mass of pericentriolar material ∼2 μm in diameter. Bar, (*A* and *B*) 10 μm; (*C*) 2.0 μm.

or γ-tubulin (EAD24) revealed that these reconstituted asters contain Mts emanating from a discrete γ-tubulin staining center (Fig. 2 *B*). IVEM verified that centrosome fractions contained bona fide centrosomes (Fig. 2 *C*), which were easily identified based on their centriole content (Palazzo et al., 1992). As expected, each centrosome contained a single centriole surrounded by PCM (Fig. 2 *C*).

Using this isolation method, 4–10 × 10^6 centrosomes could be obtained from a single 30-ml gradient, representing a yield of 2–3 × 10^6 centrosomes per ml of original ly-

Table I. Isolation of Centrosomes from Spisula Oocyte Lysates

Sample	Protein	Aster-forming units $\times 10^6$	Percent yield	Fold-purification
	mg			
Lysate	209.2	9.7	–	–
Clarified lysate	170.4	9.8	101	1.2
Centrosome fraction*	0.053	6.6	68	2684

Total protein in lysate and clarified lysate (5,500 g supernatant) was determined by solubilizing an aliquot of each sample in TS buffer and determining protein content with a bicinochromic protein assay. Sucrose fractions with peak aster-forming activity were pooled,* and centrifuged at 70,000 g to pellet centrosomes. The centrosome pellet was resuspended in TBS/S, and protein content was determined. Aster-forming units (centrosomes) were quantitated in lysate and clarified lysate by warming for 10 min at 24°C in the presence of 3% hexylene glycol, diluting the sample with 4 vol of 20 mM MES/1 mM EGTA/1 mM $MgSO_4$, pH 6.5, and determining the number of asters/ml in sucrose fractions using the hemacytometer assay (see Methods and Materials).

sate. When centrosome fractions from multiple gradients were pooled and centrifuged, the final centrosome pellet represented an ~3,000-fold purification from lysate. Results from a typical preparation are shown in Table 1. The degree of purification was variable (~2,400–4,000 fold) depending on the protein concentration of the original lysates used, which ranged between ~75 mg/ml to ~150 mg/ml. Based on the capacity of the SW28 rotor (six gradients), these preparations yielded ~300 μg of protein per run, allowing the preparation and storage of more than 900 μg of centrosome protein per day.

Structural Analysis

IVEM analyses of serial semi-thick sections confirmed our original finding (Palazzo et al., 1992) that centrosomes isolated from *Spisula* oocytes shortly after activation contain a single centriole embedded in the middle of an extensive sphere of PCM (e.g., Fig. 3, *A* and *G*). When viewed by tomography, this PCM was seen to be studded throughout its volume with numerous, conspicuous ring-shaped structures (Fig. 3, *C–F, arrowheads*). The depth (z-axis dimension) of each ring was less than its 25–28-nm diameter, and most were found to be completely contained within the 150–200-nm-thick (after irradiation) volume of each tomogram. These rings were similar, if not identical, to those recently detected by IVEM tomography in the PCM of isolated *Drosophila* centrosomes (Moritz et al., 1995a). IVEM also revealed that the great majority of centrosomes isolated from *Spisula* oocytes shortly after activation contained an unusual vesicular structure at the base of the centriole (Fig. 3, *G* and *H*). It is possible that this structure is involved in assembling the second centriole, which we have previously shown to occur near this time point.

Protein Composition

Centrosome protein composition was analyzed using both SDS-PAGE and two-dimensional gel electrophoresis. In addition, immunoblot analysis was conducted to assay for the presence of proteins known to be components of mitotic centrosomes; the MPM-2 phosphoepitope proteins (Davis, et al., 1983; Vandre et al., 1986; Ohta et al., 1993), α-tubulin,
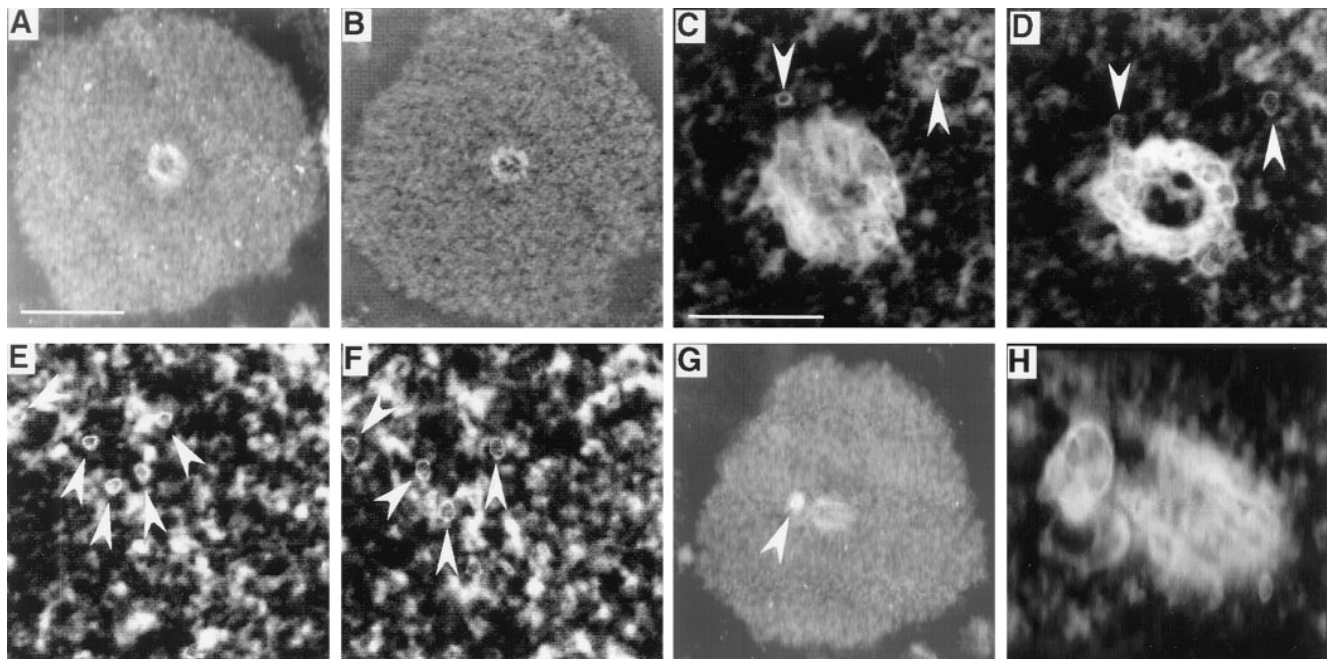
Figure 3. IVEM-tomography of centrosomes. (*A*) IVEM micrograph, (*B*) 2.6-nm-thick slice, and (*C–F*) rendered projections through the volume of a tomographic reconstruction from a 0.25-μm section through a *Spisula* centrosome isolated from a 4-min extract. *C* and *D* and *E* and *F* show highly magnified sections from this tomogram in untilted (*C* and *E*) and tilted (*D* and *F*) views. Numerous ring-shaped structures (e.g., *arrowheads*), completely contained within the section, are found throughout the centrosome, including regions near the centriole (*C* and *D*) and near its periphery (*E* and *F*). A 2.6-nm-thick slice from a second tomogram, obtained from a 0.25-μm-thick section cut from one end of the centriole in an isolated centrosome, is pictured in *G* and at higher magnification in *H*. Note that a conspicuous electron-opaque "vacuole" is associated with the centriolar end. Bars: (*A, B,* and *G*) 0.50 μm; (*C–F,* and *H*) 250 nm.
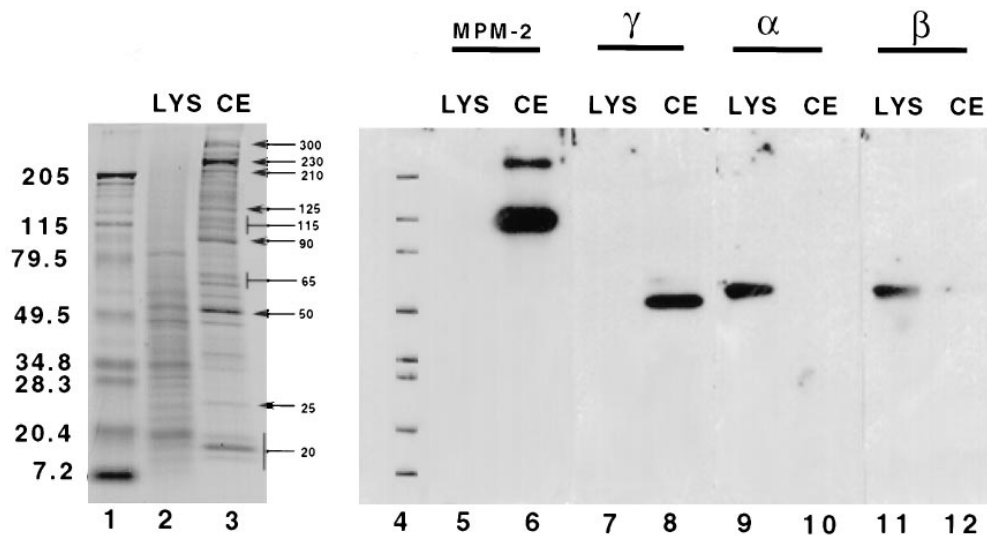
Figure 4. Protein composition of centrosomes. *Spisula* oocyte lysate and isolated centrosomes were compared. 5 μg of total protein was loaded per lane. Proteins were separated in 4–20% linear gradient minigels and stained with colloidal Coomassie G-250. Molecular mass standards are shown in lanes *1* and *4*. A number of proteins are enriched in centrosome fractions (lane *3*) over lysate (lane *2*). These include a triplet of 20 kD, and proteins of 25, 50, 65, 90, 115, 125, 210, 230, and 300 kD, respectively. Immunoblots were probed with antibodies specific for the following: MPM-2 phosphoepitope (lanes *5* and *6*), γ-tubulin, (lanes *7* and *8*), α-tubulin (lanes *9* and *10*), and β-tubulin (lanes *11* and *12*). Two MPM-2 phosphoepitope proteins of 115 and 230 kD (lanes *5* and *6*) and γ-tubulin (lanes *7* and *8*) are enriched in centrosome fractions over lysate. Conventional tubulins (α- and β-tubulin) are not detected in centrosome fractions under these conditions (lanes *9–12*). *LYS,* lysate; *CE,* isolated centrosome fraction.

β-tubulin (Bornens, 1987), and γ-tubulin (Stearns et al., 1991; Zheng et al., 1991).

SDS-PAGE revealed that the centrosome fraction contained a number of proteins, including a prominent triplet of proteins of ∼20 kD (Fig. 4, lane *3*). Comparison with the lysate protein profiles revealed that these proteins were highly enriched in the centrosome fraction (Fig. 4, lanes *2* and *3*). Thus, a number of specific proteins copurify with *Spisula* centrosomes. Immunoblot analysis revealed that relative to lysate, the centrosome fraction was enriched in two MPM-2 phosphoepitope proteins of 230 and 115 kD (Fig. 4, lanes *5* and *6*). Importantly, while γ-tubulin copurified with centrosomes as expected, it did not appear to be an MPM-2 protein (Fig. 4, lane *6*). Finally, relatively little α- or β-tubulin was found in the centrosome fraction (Fig. 4, lanes *9–12*).
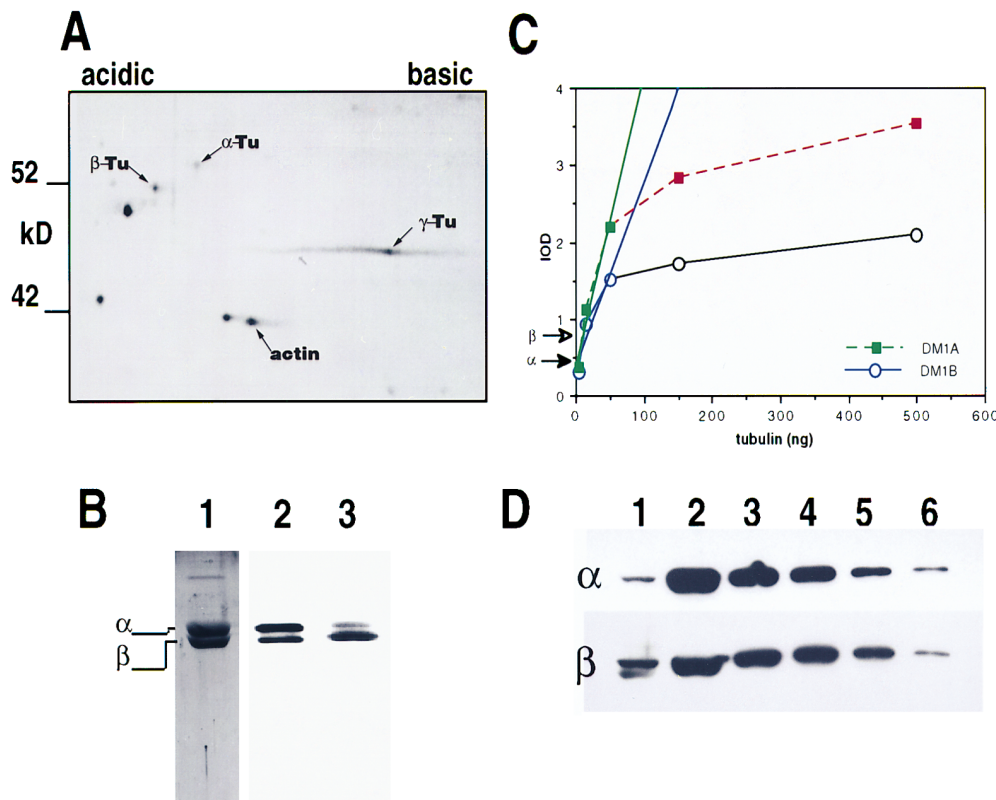
Quantitation of Centrosome Tubulins

Two-dimensional gel analysis of centrosome proteins visualized with silver stain revealed that the major form of tubulin to copurify with centrosomes was γ-tubulin (Fig. 5 *A*). The staining intensity of γ-tubulin was higher than that observed for either α- or β-tubulin (Fig. 5 *A*). Densitometric analysis of silver-stained gels revealed that centrosomes contain ∼10-fold more γ-tubulin than β-tubulin, consistent with the tubulin ratios reported for γ-tubulin ring complexes isolated from *Xenopus* oocytes (Zheng et al., 1995). Surprisingly, the results of two-dimensional gel analysis suggested that centrosomes contain more β- than α-tubulin (Fig. 5 *A*).

The intriguing possibility that the stoichiometric ratio of α/β tubulins was not 1:1 in centrosomes was tested further. To conduct this analysis, the ratio of α/β tubulin present in purified *Spisula* tubulin was first determined, and the staining intensity was assessed for α- (DM1A) and β- (DM1B) tubulin-specific antibodies to be used as probes for quanti-

tative immunoblot analyses. Comparison of the IOD values obtained from SDS-PAGE gels stained with Coomassie blue revealed that glutamate-purified *Spisula* tubulin contained stoichiometrically equivalent amounts of α- and β-tubulin as expected (Fig. 5 *B*, lane *1*). Furthermore, Western blot analysis revealed that the relative staining intensity of DM1A and DM1B for equimolar protein was similar (Fig. 5 *B*, lane *2*), although DM1A was found to be a slightly more sensitive probe than DM1B. However, Western blot analysis of centrosome proteins resulted in significantly higher staining intensity for DM1B than for DM1A (Fig. 5 *B*, lane *3*), confirming that centrosomes contain more β-tubulin than α-tubulin.

To extend this analysis further, the stoichiometric ratio of α/β tubulin in centrosomes was determined by quantitative immunoblot analysis. Because of the differences in the staining intensity observed for DM1A and DM1B when challenged with equivalent protein (Fig. 5 *B*, lanes *1* and *2*), identical immunoblots were prepared and probed with each antibody separately. Gels were loaded with *Spisula* glutamate–purified tubulin diluted in series (from 1.0 μg to 0.01 μg/lane) to provide standards, and centrosome proteins were loaded onto adjacent lanes on the same gels. Proteins were separated, transferred to immobilon membranes, probed with antibodies in parallel and blots visualized by chemiluminescence (Fig. 5 *D*), as described in Materials and Methods. The signal for α- or β-tubulin produced by chemiluminescence was digitized, and the staining intensity (IOD) of tubulin in standards and centrosome samples was measured by quantitative densitometry (Fig. 5 *C*). The IOD values obtained were plotted relative to tubulin concentration, and the linear range of DM1A and DM1B staining for tubulin was determined (Fig. 5 *C*). The standard curves of both DM1A and DM1B staining showed saturation at tubulin concentrations greater than 0.3 μg, but both standard curves were linear for tubulin concentrations between 0.1 and 0.03 μg (Fig. 5 *C*). In all

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Figure 5. Analysis of centrosome tubulins. Centrosome preparations were analyzed by two-dimensional gel electrophoresis (*A*) and quantitative immunoblot (*B–D*). Three tubulin types were found in centrosomes, α, β, and γ (*A*). γ-Tubulin has a major isoform with a pI of 5.52 and is the major form of tubulin found in centrosome fractions. SDS-gel analysis of glutamate-purified *Spisula* oocyte tubulin stained with Coomassie blue (*B*, lane *1*) provided an α- and β-tubulin standard (*B*, lane *2*). Stoichiometrically equivalent amounts of α- and β-tubulin (*B*, lane *1*) were probed simultaneously with antibodies specific to either α-tubulin (DM1A) or β-tubulin (DM1B) (*B*, lane *2*). In these samples, the α-tubulin–specific signal is more intense than the β-tubulin–specific signal, indicating that DM1A is a more sensitive probe than DM1B (*B*, lane *2*). In contrast, probing centrosome fractions resulted in a higher β-tubulin–specific (DM1B) signal than the α-tubulin–specific (DM1A) signal (*B*, lane *3*). The tubulin content of centrosomes was determined using quantitative immunoblot analysis. (*D*) 5 μg of centrosome protein (lane *1*) and series dilution of purified *Spisula* tubulin standards (lane *2*, 1 mg/ml; lane *3*, 0.3 mg/ml; lane *4*, 0.1 mg/ml; lane *5*, 0.03 mg/ml; lane *6*, 0.01 mg/ml) were separated in 10% polyacrylamide gels and processed for immunoblot analysis using DM1A and DM1B (see Materials and Methods for details). The IOD of tubulin stain for centrosome samples and each standard lane in *D* was determined by densitometry and plotted (*C*). The IOD of only the top band of the two stained by DM1B in *D*, lane *1*, was used in this analysis, the lower band being an artifact in this particular blot. The linear range (*C*, *solid lines*) for DM1A (*C*, *filled squares*) and DM1B (*C*, *open circles*) ranges from 0.01 to 0.1 mg. The IOD obtained for the tubulin signals in the centrosome sample for both DM1A and DM1B lay within the linear range of detection (*C*, *arrows*). First order regression analysis was used to calculate the tubulin content of centrosomes (results presented in text and Table II). The r^2 value for the regression for DM1A was 0.962, and for DM1B was 0.893.

cases, the IOD values obtained for centrosomal tubulin bands stained by DM1A or DM1B lay within the linear region of these curves (Fig. 5 *C*; note *arrows*). The linear range of each standard curve was used to derive a first order equation that was used to calculate the tubulin concentration in the centrosome protein sample as described in Materials and Methods. The results, summarized in Table II, show that α- and β-tubulin together represent 0.3% of the total centrosome protein. Calculations indicate that centrosomes contain 0.10 ± 0.021 pmol of α-tubulin (*n* = 5) and 0.48 ± 0.099 pmol of β-tubulin (*n* = 5) per centrosome. Most importantly, the stoichiometric ratio of α/β in centrosomes was not 1:1, but found to be an average of 1:4.6 in these experiments.

Discussion

Despite its critical role in a variety of cellular processes (Brinkley, 1985), the centrosome remains a relatively enigmatic organelle. A major barrier to understanding how it functions has been the difficulty in obtaining highly purified and homogeneous preparations for biochemical analyses (Brinkley, 1985). Recently, procedures have been de-

veloped for isolating centrosomes from mammalian cell lines (Mitchison and Kirschner, 1984, 1986; Bornens et al., 1987), *Drosophila* embryos (Moritz et al., 1995*a*), and spindle pole bodies from yeast (Rout and Kilmartin, 1990). As described here, surf clam (*Spisula solidissima*) oocytes possess a number of characteristics that make them uniquely suited as a model system for centrosome research. First, *Spisula* is commercially fished, and substantial quantities of pure oocytes (100 g) can be quickly and easily harvested. In addition, *Spisula* oocytes and embryos develop synchronously (Allen, 1953; Rebhun, 1959; Palazzo et al.,

Table II. Quantitation of α- and β-Tubulin in Centrosome Fractions

Tubulin	*n*	Percent protein/fraction	pmol (10^{-1})	β/α
α	5	0.07 ± 0.027	1.0 ± 0.21	
β	5	0.30 ± 0.098	4.8 ± 0.99	4.6 ± 0.62

The tubulin content of centrosome fractions relative to total fraction protein was determined by quantitative immunoblot as previously described. The stoichiometric ratio of α/β tubulin was determined by calculating the pmoles of α- and β-tubulin (mol wt = 50,000) in the sample. Values from five independent experiments were averaged, and the standard deviation was calculated using standard methods.

1988), allowing for the acquisition of large numbers of cells from specific time points in the meiotic and mitotic cell cycle. Importantly, *Spisula* oocytes are arrested naturally in prophase of meiosis I and enter M-phase within minutes of oocyte activation (Allen, 1953; Rebhun, 1959). Finally, parthenogenetic activation of oocytes induces centrosome maturation, which includes centriole duplication (Palazzo et al., 1992) and assembly of the meiosis I spindle within 15 min (Allen, 1953; Rebhun, 1959).

Crude homogenates (Weisenberg and Rosenfeld, 1975) and lysates (Palazzo et al., 1988) prepared from parthenogenetically activated *Spisula* oocytes spontaneously assemble robust asters, which are easily discernible by conventional polarized light microscopy. Moreover, treating lysates prepared from unactivated oocytes with high-speed supernatants prepared from parthenogenetically activated oocytes induces centriole duplication and centrosome maturation within lysates (Palazzo et al., 1992). The unique properties of *Spisula* oocytes, together with these advancements, suggested that this system could be developed as a powerful model for studying how centrosome function is regulated. To this end, it becomes important to develop methods for isolating centrosomes from various time points in the cell cycle for analyses. Here we have described a method for isolating centrosomes from one specific time point, 4 min after parthenogenetic activation.

Asters are clearly present in *Spisula* oocytes 4 min after activation (Allen, 1953; Rebhun, 1959) and in lysates prepared from oocytes at this stage in development (Weisenberg and Rosenfeld, 1975; Palazzo et al., 1992). Past analyses have shown that these asters contain bona fide centrosomes composed of a single centriole surrounded by PCM (Weisenberg and Rosenfeld, 1975; Palazzo et al., 1992). *Spisula* centrosomes were isolated from these lysates by sucrose-density gradient centrifugation using a procedure patterned after the methods of Mitchison and Kirschner (1986). Centrosomes in sucrose fractions were detected by simply diluting gradient fractions in tubulin-media under conditions that support Mt polymerization and observing aster formation by polarized light microscopy. Sucrose gradient fractions that contained aster-forming activity were analyzed to determine if they contained bona fide centrosomes by several methods. First, we assayed for centrosome-dependent Mt nucleation (aster formation) using a functional reconstitution assay. Furthermore, double immunofluorescence revealed that fractions capable of inducing aster formation contained discrete structures that stained for γ-tubulin, a known component of centrosomes (Stearns et al., 1991; Zheng et al., 1991). Finally, EM studies revealed that these fractions contained numerous centrioles surrounded by abundant PCM. Together, these observations indicate that we have successfully isolated centrosomes from activated *Spisula* oocytes.

Because the Mt nucleation potential of isolated *Spisula* centrosomes is so robust, centrosomes could be quantified at every step of the purification process by simply counting asters. Our isolation procedure yields an average of ~3.0 × 10^6 centrosomes/ml of lysate, with recovery as high as 70%. This recovery is comparable to that reported by Mitchison and Kirschner (1986) and Bornens et al. (1987), but our system yields significantly more protein for analyses. Indeed, as much as 53 μg of centrosome protein, repre-

senting a 2,500–4,000-fold purification from lysate, can be collected from a single preparative gradient. Since six gradients can be run simultaneously, and three preparations can be run in a 9-h period, milligram quantities of centrosomal protein can be obtained in a single day.

Our biochemical analyses of *Spisula* centrosomes were facilitated by our ability to obtain relatively large quantities of these organelles in a highly enriched state. We found vandre that a minimum of thirty proteins ranging in molecular mass from 20–300 kD copurify with *Spisula* centrosomes, including γ-tubulin and a prominent triplet of proteins of ~20 kD. Immunoblot analyses revealed that the 230- and 115-kD proteins appear to contain mitosis-specific, or in this case meiosis-specific, phosphoepitopes (Vandre et al., 1986). Also, the 230- and 115-kD proteins appear to contain mitosis-specific, or in this case meiosis-specific, phosphoepitopes (Vandre et al., 1986) since they cross-reacted with the MPM-2 monoclonal antibody (Davis et al., 1983). Actin was also relatively abundant in our preparations, suggesting that *Spisula* centrosomes contain one or more actin-binding proteins. Finally, we found that γ-tubulin was the major tubulin component of *Spisula* centrosomes, which also contained more minor amounts of α- and β-tubulin.

γ-Tubulin (Oakley and Oakley, 1989) is a highly conserved component of centrosomes (Stearns et al., 1991; Zheng et al., 1991; Sunkel et al., 1995), and it is required for centrosomal Mt nucleation in diverse organisms. However, the respective roles of the α- and β-tubulins in this nucleation process remain ambiguous. Based on extragenic suppression of a β-tubulin mutation by the mipA allele in *Aspergillus*, Oakley and Oakley (1989) proposed that γ- and β-tubulin interact physically to initiate Mt nucleation. Recently, an Mt nucleation-competent γ-tubulin complex, also containing α- and β-tubulins in stoichiometrically equivalent amounts, was identified in *Xenopus* cytoplasmic extracts (Zheng et al., 1995). Other multiprotein complexes containing α, β, and γ-tubulins have also been reported for other cell types (Marchesi and Ngo, 1993; Melki et al., 1993; Raff et al., 1993; Stearns and Kirschner, 1994), but the stoichiometry of the various tubulins in all of these complexes remains to be determined. Regardless, given the recent discovery in *Drosophila* of γ-tubulin–containing ring-structures, with the appropriate diameter of a 13-protofilament Mt (Moritz et al., 1995a,b), it is possible that α- and/or β-tubulin are also components of γ-tubulin–based Mt-nucleating sites.

We found that α- and β-tubulins were minor components of *Spisula* centrosomes, representing <0.3% of the total centrosome protein. This is dramatically lower than the α and β tubulin content reported for mammalian centrosomes (3–6%; Mitchison and Kirschner, 1984, 1986; Bornens et al., 1987) and yeast spindle pole bodies (Rout and Kilmartin, 1990). Two-dimensional gel analysis revealed that γ-tubulin was at least 10-fold more abundant in *Spisula* centrosomes than conventional tubulins. This finding is similar to that reported for the γ-tubulin–containing complexes isolated from *Xenopus* extracts (Zheng et al., 1995). However, we unexpectedly found that β-tubulin is significantly more abundant in *Spisula* centrosomes than α-tubulin. Since, to our knowledge, there are no known posttranslational modifications of the epitope recognized by the α-tubulin antibody (DM1A), it is unlikely that the amount of α-tubulin in the centrosome is underreported

by our analysis. It is also unlikely that this disparity arises from significant differences in the α/β tubulin ratios within the centriole in each centrosome, since the α/β tubulin ratio in these organelles is expected to be 1:1 (Kochanski and Borisy, 1990).

The results presented indicate that isolated *Spisula* centrosomes contain ring structures like those first reported for *Drosophila* (Moritz et al., 1995b). Since the diameter of these rings matches the diameter of microtubules and isolated ring complexes from *Xenopus* oocytes increase the rate of microtubule polymerization (Zheng et al., 1995), it is likely that these rings represent oligomeric protein complexes that serve as initiation sites for centrosome-dependent microtubule assembly. The data presented here indicates that while isolated *Spisula* centrosomes contain similar ring structures, they also contain an unusual ratio of α/β tubulin that is not 1:1, but rather 1:4 or 1:5. This indicates that in isolated centrosomes, at least some of the β-tubulin is not complexed in the form of conventional α/β heterodimers. This suggests the possibility that β-tubulin might be a component of the oligomeric ring structure, possibly associated with γ-tubulin. Thus, the data presented is consistent with the hypothesis that β-tubulin plays an important role in centrosome structure and/or microtubule nucleation, a role that may be independent of α-tubulin.

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