

# *Drosophila* $\gamma$ -Tubulin Is Part of a Complex Containing Two Previously Identified Centrosomal MAPs

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**Abstract.**  $\gamma$ -tubulin is a minor tubulin that is localized to the microtubule organizing center of many fungi and higher eucaryotic cells (Oakley, B. R., C. E. Oakley, Y. Yoon, and M. C. Jung. 1990. *Cell*. 61: 1289-1301; Stearns, T., L. Evans, and M. Kirschner. 1991. *Cell*. 65:825-836; Zheng, Y., M. K. Jung, and B. R. Oakley. 1991. *Cell*. 65:817-823). Here we show that  $\gamma$ -tubulin is a component of a previously isolated complex of *Drosophila* proteins that contains at least two centrosomal microtubule-associated proteins called DMAP190 and DMAP60. Like DMAP190 and DMAP60, the  $\gamma$ -tubulin in extracts of early *Drosophila* embryos binds to microtubules, although this

binding may be indirect. Unlike DMAP190 and DMAP60, however, only 10-50% of the  $\gamma$ -tubulin in the extract is able to bind to microtubules. We show that  $\gamma$ -tubulin binds to a microtubule column as part of a complex, and a substantial fraction of this  $\gamma$ -tubulin is tightly associated with DMAP60. As neither  $\alpha$ - nor  $\beta$ -tubulin bind to microtubule columns, the  $\gamma$ -tubulin cannot be binding to such columns in the form of an  $\alpha$ : $\gamma$  or  $\beta$ : $\gamma$  heterodimer. These observations suggest that  $\gamma$ -tubulin, DMAP60, and DMAP190 are components of a centrosomal complex that can interact with microtubules.

THE centrosome is the main microtubule organizing center (MTOC)<sup>1</sup> in animal cells (for reviews see Brinkley, 1985; Karsenti and Maro, 1986; Mazia, 1987; Cande, 1990; Huang, 1990; Johnson and Rosenbaum, 1992; Karsenti, 1992). In the 100 yr since the centrosome was first described, it has become apparent that this organelle plays a crucial role in many aspects of cellular organization (Wilson, 1928; McIntosh, 1983; Kreis, 1990; Schliwa, 1992) as well as in development (Wilson, 1928; Bornens et al., 1987; Raff and Glover, 1989). Despite its central importance, the centrosome has remained enigmatic: it is unclear how it nucleates microtubules, how it replicates, or how its functions are regulated during the cell cycle.

To understand how the centrosome functions, we will need to identify the proteins that constitute it. A number of such proteins have already been identified (for example, Kuriyama and Boris, 1985; Klotz et al., 1986; Baron and Salisbury, 1988; Huang et al., 1988b; Whitfield et al., 1988; Kellogg et al., 1989; Rout and Kilmartin, 1990), and cDNAs encoding several of them have been cloned and sequenced (Huang

et al., 1988a; Kuriyama et al., 1990; Ohta et al., 1990; Joswig et al., 1991). Moreover, antibodies raised against certain centrosomal proteins have been shown to inhibit aspects of centrosomal function when injected into cells (Moudjou et al., 1992; Joshi et al., 1992), and genetic studies in yeasts have identified genes whose products are involved in the function of the spindle pole body, the centrosome equivalent in yeasts (Byers, 1981; Baum et al., 1986, 1988; Rose and Fink, 1987; Uzawa et al., 1990; Winey et al., 1991; Vallen et al., 1992). It is still unclear, however, how any of these proteins function in the centrosome.

The recent discovery of  $\gamma$ -tubulin could be a breakthrough in the attempt to understand how centrosomes nucleate microtubules (Oakley et al., 1990; Oakley, 1992; Cande and Stearns, 1991).  $\gamma$ -tubulin was first identified as a suppressor of a  $\beta$ -tubulin mutation in the fungus *Aspergillus nidulans* (Oakley and Oakley, 1989): when the gene encoding the suppressor was sequenced it was found to encode a new member of the tubulin superfamily and was named  $\gamma$ -tubulin. Unexpectedly, antibodies raised against the protein did not stain microtubules, but instead stained the spindle pole body (Oakley et al., 1990).  $\gamma$ -tubulin has since been found in a wide variety of animal and plant cells (Stearns et al., 1991; Zheng et al., 1991; Horio et al., 1991; Cande and Stearns, 1991), and in all cases it has been found to be located exclusively in MTOCs. These findings led Oakley and his colleagues to suggest that  $\gamma$ -tubulin may act as a highly conserved site of microtubule nucleation in all MTOCs (Oakley et al., 1990; Oakley, 1992). Several observations support

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1. *Abbreviations used in this paper:* GST, glutathione-S-transferase; MAPs, microtubule-associated proteins; MBP, maltose-binding protein; MTOC, microtubule organizing.

this suggestion: (a) Inactivation of the  $\gamma$ -tubulin gene in *Aspergillus* produces a lethal phenotype that is consistent with a failure in microtubule function (Oakley et al., 1990). (b)  $\gamma$ -tubulin has been localized ultrastructurally to the pericentriolar material (PCM), the site of microtubule nucleation in the centrosome (Stearns et al., 1991). (c) An injection of anti- $\gamma$ -tubulin antibodies into cultured cells inhibits the nucleation of new microtubules from the centrosome but does not interfere with microtubules that are already formed (Joshi et al., 1992). A crucial prediction of this hypothesis, which has so far not been demonstrated, is that  $\gamma$ -tubulin can interact with microtubules composed of  $\alpha$ : $\beta$  tubulin heterodimers.

We have previously identified a complex of *Drosophila* proteins that contains a number of centrosomal microtubule-associated proteins (MAPs) (Kellogg and Alberts, 1992). This complex was originally isolated by passing *Drosophila* embryo extracts over an affinity column of antibodies raised against DMAP190 – a 190-kD centrosomal MAP whose cDNA has been cloned (Kellogg and Alberts, 1992; Whitfield et al., 1988, where DMAP190 is called the BX63 antigen). The affinity column quantitatively depleted DMAP190 from the extract, and, in addition, specifically retained a number of other proteins. The DMAP190 antibodies did not recognize any of these other proteins in Western blotting experiments, suggesting that they are retained on the antibody column via an interaction (either direct or indirect) with the DMAP190 protein. One of these proteins, called DMAP60, also binds to a microtubule affinity column and is localized to the centrosome, strongly suggesting that DMAP190 and DMAP60 form a complex in vivo (Kellogg and Alberts, 1992). We now show that  $\gamma$ -tubulin is a component of this complex, and like DMAP190 and DMAP60,  $\gamma$ -tubulin in *Drosophila* embryo extracts binds to microtubules, although the binding of these proteins to microtubules could be indirect. These observations suggest that these three proteins are components of a complex involved in the interaction of the centrosome with microtubules.

## Materials and Methods

### Preparation and Purification of Anti-*Drosophila*- $\gamma$ -Tubulin Antibodies

Oligonucleotides were synthesized to allow full length *Drosophila*- $\gamma$ -tubulin to be amplified by PCR using standard procedures (Sambrook et al., 1989) from an appropriate plasmid (kindly supplied by Trish Wilson, Yixian Zheng, and Berl Oakley). The oligonucleotides contained EcoRI sites that allowed the final PCR product to be ligated, in frame, into the vectors pGEX1 (Amrad Corp., Ltd., Australia) or pMALc (New England Biolabs Inc., Beverly, MA). DH5 $\alpha$  cells were transformed with the  $\gamma$ -tubulin-pMALc construct. Maltose-binding protein (MBP)- $\gamma$ -tubulin fusion protein was expressed in these cells according to the manufacturers instructions. The induced cells were isolated by centrifugation, resuspended in sample buffer (Laemmli, 1970), and boiled for 5 min. This whole cell extract was fractionated by preparative SDS-PAGE (Laemmli, 1970), and the full-length fusion protein was excised from the gel and used to inject two rabbits (injection and serum production was carried out by the Berkeley Antibody Co., Richmond, CA).

Cells transformed with the pGEX1- $\gamma$ -tubulin construct expressed a glutathione-S-transferase (GST)- $\gamma$ -tubulin fusion protein which was purified and used to affinity purify the antibodies produced from the rabbits described above. Because this fusion protein was otherwise highly insoluble, modifications were made to standard procedures (Smith and Johnson, 1988). Cells were grown in 6 liters of LB (Sambrook et al., 1989) at 37°C

until they reached an  $A_{600}$  of  $\sim 0.5$ . They were cooled to 20°C, induced with 100  $\mu$ M IPTG, and grown for an additional 3–4 h at room temperature. The cells were harvested by centrifugation and lysed by a freeze/thaw cycle followed by resuspension in 4 vol of PBS (140 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.2, 138 mM NaCl, 2.7 mM KCl) plus 1 mM Na<sub>3</sub>EDTA and 1 mM Na<sub>3</sub>EGTA. After sonication, the bacterial extract was spun at 100,000 g for 1 h to remove particulate matter and the fusion protein was purified by passing the extract over a glutathione-agarose column (Sigma Chem. Co., St. Louis, MO), followed by elution of the fusion protein in 50 mM Tris-Cl (pH 8.0), 5 mM glutathione. We routinely obtained 2–3 mg of soluble fusion protein in this way. The protein was dialyzed into 50 mM Hepes, pH 7.6, 25 mM KCl, and coupled to Affigel-10 (Bio-Rad Labs., Cambridge, MA) at 2 mg/ml as per manufacturers instructions. The rabbit antibodies were purified on this column using standard procedures (Harlow and Lane, 1988).

### Microtubule-Affinity Chromatography

The procedures for constructing microtubule columns, for making embryo extracts, for loading extracts over the microtubule column, and washing the column were described previously (Kellogg et al., 1989). In the experiments reported here, a sample of the proteins flowing through the column was taken as soon as the concentration of protein in the flow-through reached that of the protein in the extract. For this reason, we refer to the flow-through from a column as the “initial” flow-through. After loading all of the extract, the column was washed with 10 vol of CX buffer (50 mM Hepes, pH 7.6, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>EGTA, 10% glycerol, 1 mM DTT, 1  $\mu$ M each of leupeptin, pepstatin A, and aprotinin) plus 50 mM KCl, and the proteins retained on the microtubule column were eluted in a single step in CX buffer containing 1 mM ATP and 0.5 M KCl. TCA was added to the eluate to a final concentration of 10%, and this was left on ice for 20 min, and then centrifuged in a microfuge for 10 min. Pellets were resuspended in sample buffer, neutralized with the vapors from a cotton swab dipped in NH<sub>4</sub>OH, boiled for 5 min, and separated by SDS-PAGE followed by Western blotting.

### Microtubule Spin-Down Experiments

0–3-h embryos were homogenized with 1 vol of C buffer (50 mM Hepes, pH 7.6, 1 mM MgCl<sub>2</sub>, 1 mM Na<sub>3</sub>EGTA), plus 10  $\mu$ M each of leupeptin, pepstatin A, and aprotinin. The extract was centrifuged at 100,000 rpm for 1 h at 4°C in a TL100 rotor (Beckman Instrs. Inc., Fullerton, CA), and the supernatant was then taken through a second round of centrifugation at 100,000 rpm for 15 min. To polymerize the microtubules in the extract, GTP was added to 1 mM and taxol to 10  $\mu$ M. The extract was warmed to 25°C for 5 min and allowed to sit at 4°C for an additional 15 min. 100  $\mu$ l of the extract was loaded on top of a 150- $\mu$ l cushion of C buffer plus 50% glycerol and centrifuged for 10 min at 100,000 rpm in a TL100 rotor. 50  $\mu$ l of the supernatant was added to 50  $\mu$ l of 2 $\times$  sample buffer. The entire pellet was resuspended in 60  $\mu$ l of C buffer, and 20  $\mu$ l of 4 $\times$  sample buffer was added. The samples were boiled and separated by SDS-PAGE before Western blotting. In control experiments no GTP or taxol was added to the extract, and the extract was not warmed to 25°C. In experiments where extra tubulin was added to the extract, 100 or 200  $\mu$ g (5 or 10% by volume) of cyclic bovine or *Drosophila* tubulin was added to 120  $\mu$ l of extract before the addition of GTP and taxol. This is  $\sim 2$  and 4 $\times$  the amount of the endogenous tubulin present in this amount of extract (confirmed by the size of the microtubule pellet and by looking at the tubulin band in samples of the extracts before and after addition of the tubulin on Coomassie blue stained gels). To shear microtubules, the extract was pipetted vigorously for 30 s after GTP and taxol had been added to the extract, and the microtubules had been allowed to polymerize at 25°C for 5 min. The microtubules were then incubated for an additional 15 min at 4°C. The effectiveness of the shearing at reducing the viscosity of the extract was confirmed by low-shear viscometry.

### Sucrose Gradient Sedimentation

The proteins eluted from a microtubule column were concentrated to  $\sim 1$  mg/ml in a centricon microconcentrator (Amicon, Beverly, MA), and frozen in liquid nitrogen in 120  $\mu$ l aliquots. Sucrose gradients (5–25% sucrose in CX buffer plus 0.5 M KCl) were equilibrated at 4°C for 1 h. The MAP samples were thawed and clarified for 15 min at 100,000 rpm in a TL100 rotor, and 100  $\mu$ l was loaded per gradient. The gradients (2 ml) were spun for 15 h at 55,000 rpm in a TLS-55 rotor in the same centrifuge. After cen-

trifugation, 14 140- $\mu$ l fractions were collected from the top of the gradient, TCA-precipitated, and analyzed by Western blotting as described above. The concentrated high speed extract that was sedimented on a sucrose gradient was made in the same way as the normal high speed extract except that the embryos were diluted only 1:1 (wt/vol) in C buffer instead of the usual 1:10. Glycerol was added to 10% and KCl to 0.5 M before loading on the gradient to mimic the conditions in the MAP fractions.

### Preparation of Antibody Columns and Antibody-Affinity Chromatography

The procedures for constructing the DMAP190 antibody column, for making the embryo extract, for loading the extract over the column, for washing the column, and for eluting the proteins bound to the column were described previously (Kellogg and Alberts, 1992). DMAP60 antibody columns were constructed and treated in exactly the same way. In the experiments where isolated MAPs were run over a DMAP60 antibody column, 200  $\mu$ l of concentrated MAPs (at  $\sim$ 1 mg/ml—see above) were loaded onto a 0.5-ml antibody column (at 2–3 mg of antibody per ml) that had been preequilibrated with CX buffer plus 0.5 M KCl. The MAPs were allowed to sit on the column for 5 min, and the column was then washed with 200  $\mu$ l of CX buffer plus 0.5 M KCl. The MAPs flowing through the column were processed for Western blotting as described above.

### Western Blotting

Proteins were transferred to nitrocellulose membranes using standard procedures (Towbin et al., 1979). Blots were incubated with TTBS (20 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 50 mM KCl, 0.05% Tween-20) containing 3% dehydrated milk and 10% glycerol for 20 min, and then incubated in this buffer with the appropriate antibodies for 2 h at room temperature. The blots were washed three times in TTBS for 15 min, and then incubated with a 1:10,000 dilution of the appropriate peroxidase-conjugated secondary antibody (Amersham Intl., Buckinghamshire, U.K.) for 1 h, followed by three 15-min washes in TTBS. The antibody was detected using an enhanced chemiluminescence detection system (Amersham Intl., U.K.). As this method of detection is highly nonlinear, multiple exposures of all Western blots were obtained, and in many experiments control Western blots of known amounts of proteins were performed to allow accurate quantitation of the relative amounts of signal. Hence the relative amounts of the proteins in the different experimental fractions quoted in the text may appear at odds with what is shown in some of the figures.

### Antibodies

The following antibodies were used in Western blotting experiments: affinity-purified rabbit polyclonal antibodies against  $\gamma$ -tubulin, DMAP190, and DMAP60 at 1–2  $\mu$ g/ml; DM1 $\alpha$  (anti- $\alpha$ -tubulin—ICN Biomedicals, Inc., Costa Mesa, CA) and DM1 $\beta$  (anti- $\beta$ -tubulin—ICN Biomedicals Inc.) at 1:500 and 1:2,000 dilutions, respectively. The titer of the anti- $\gamma$ -tubulin, -DM1 $\alpha$ , and -DM1 $\beta$  antibodies was tested by incubating known concentrations of the antibodies with Western blots of serial dilutions of either purified *Drosophila* tubulin (to compare the titers of the DM1 $\alpha$  and DM1 $\beta$ ) or full-length GST- $\alpha$ -tubulin and full-length GST- $\gamma$ -tubulin (to compare the titers of the DM1 $\alpha$  and the anti- $\gamma$ -tubulin antibodies). We found that the titer of the DM1 $\beta$  and anti- $\gamma$ -tubulin antibodies were approximately the same, while the titer of the DM1 $\alpha$  antibody was  $\sim$  fivefold lower.

### Colchicine Treatment, Fixation, and Immunofluorescence Staining of Embryos

Embryos were collected, dechorionated, fixed, and stained according to Kellogg et al. (1988). The  $\gamma$ -tubulin, DMAP190, and DMAP60 affinity-purified antibodies were used at 1–2  $\mu$ g/ml. A mouse anti-histone mAb (Chemicon Intl., Inc., Temecula, CA) was used at a 1:500 dilution to allow the DNA to be visualized. Appropriate rhodamine- or fluorescein-coupled secondary antibodies were used at 1:500 dilutions.

To depolymerize microtubules, embryos were dechorionated in bleach, washed in water plus 0.05% Triton X-100, and then placed in heptane for 30 s. An equal volume of Grace's *Drosophila* medium containing 0.5 mg/ml colchicine (Sigma Chem. Co.) was added, and the embryos were allowed to sit at the aqueous/heptane interface for 20–30 min with gentle agitation. The aqueous phase was then removed, an equal volume of 37% formaldehyde was added, and the embryos were allowed to sit at the interface for an additional 4 min with gentle agitation. The formaldehyde was then re-

moved and the embryos were devitellinized by vigorous shaking with an equal volume of methanol, as described in Kellogg et al. (1988).

## Results

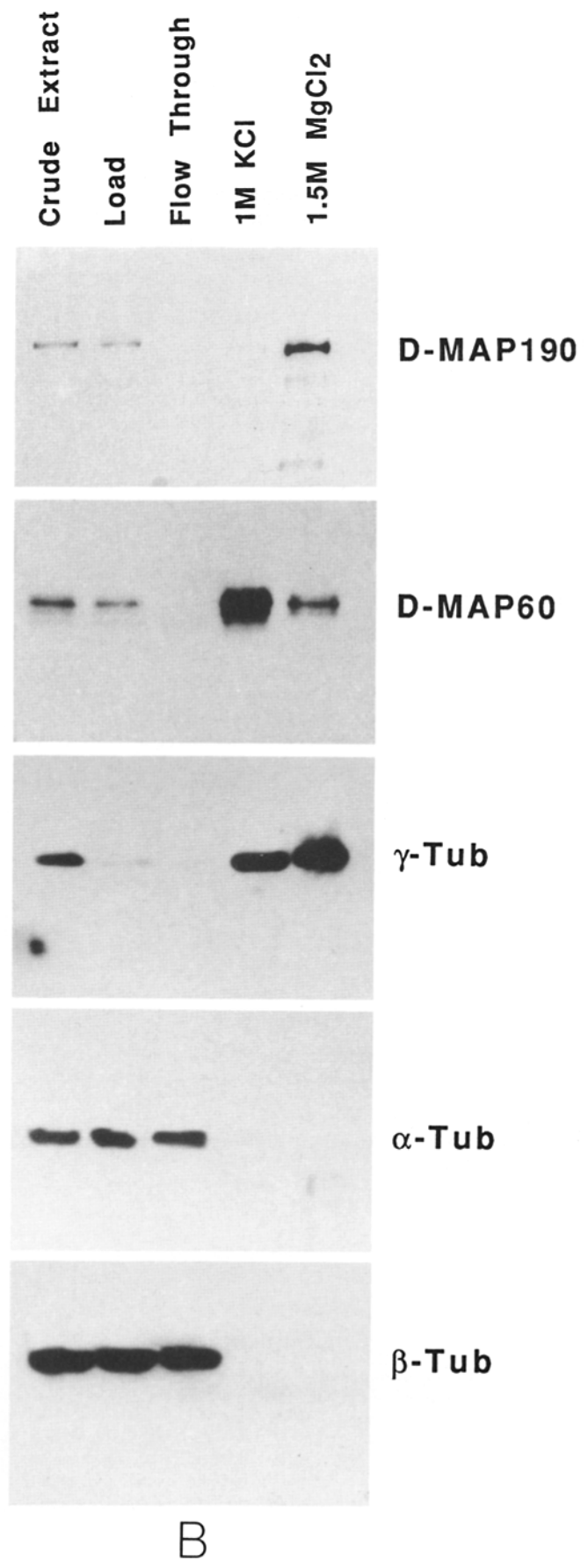
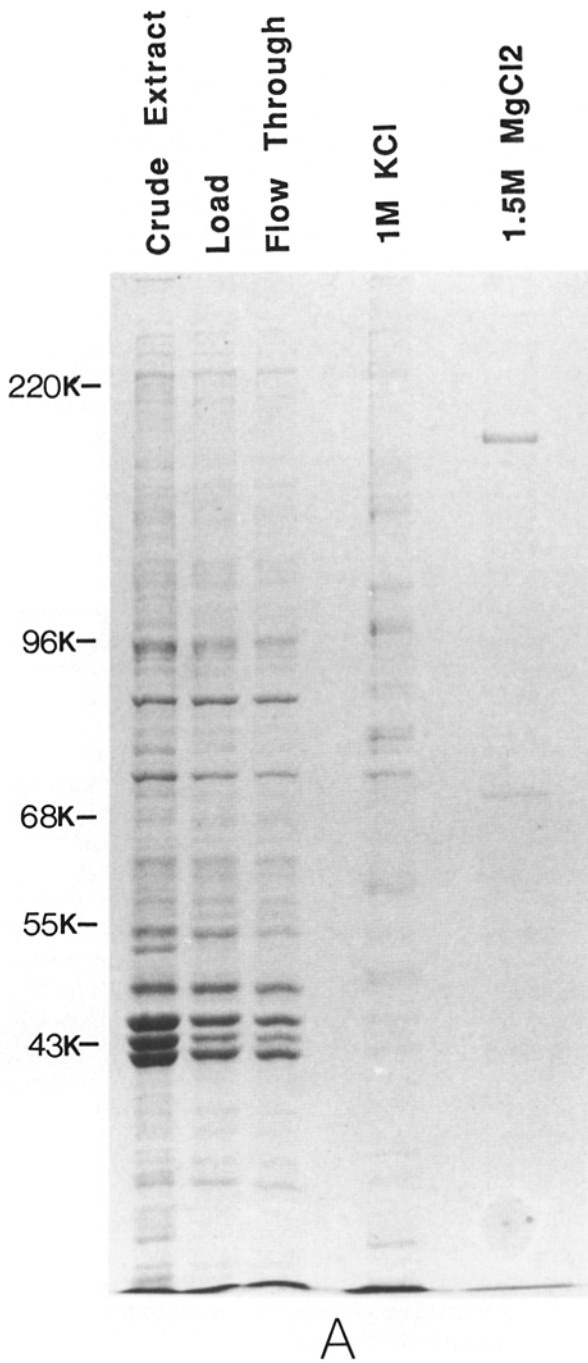
### $\gamma$ -Tubulin Is Part of a Complex that Contains DMAP190 and DMAP60

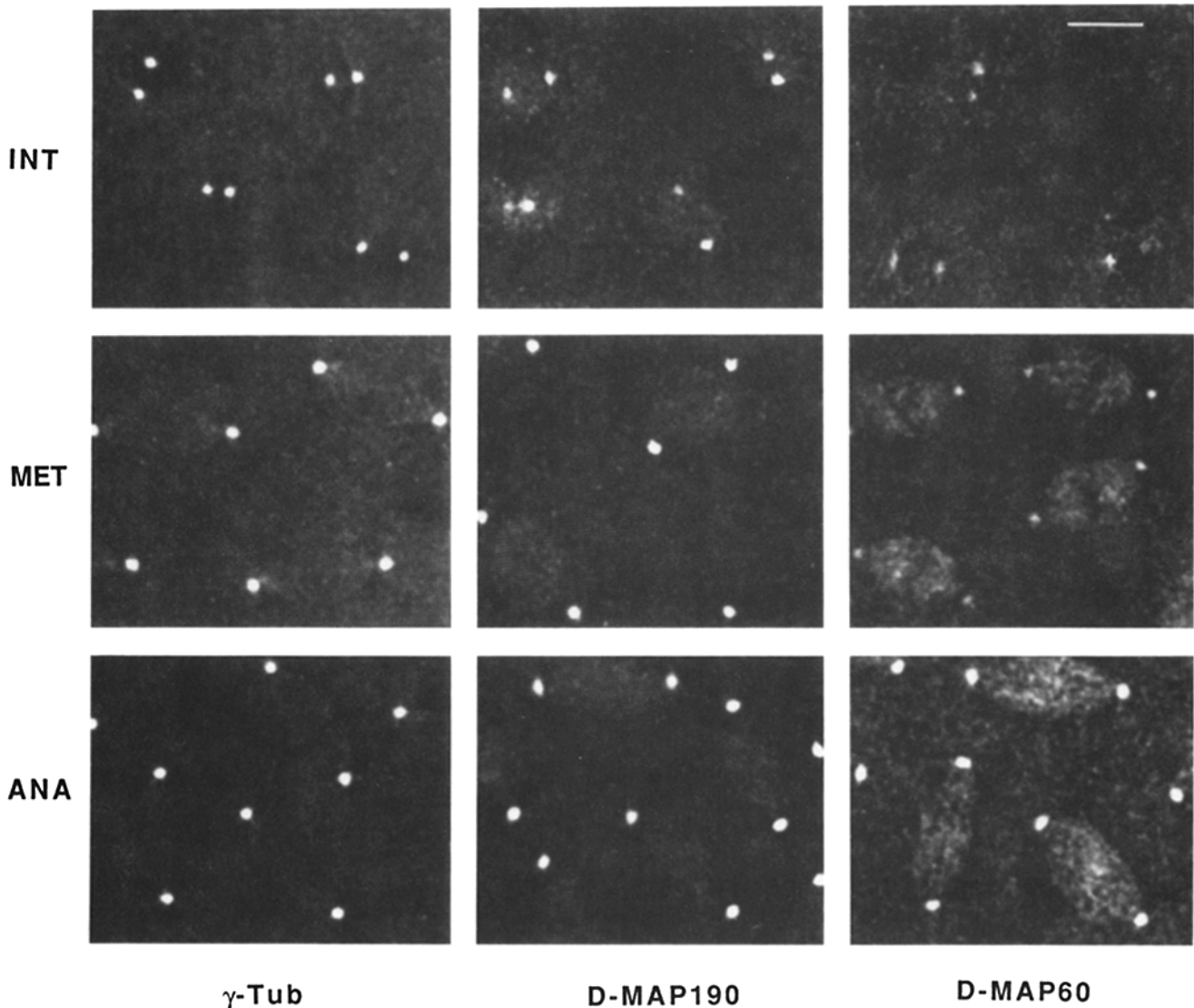
We previously cloned a cDNA encoding a 190-kD centrosomal MAP called DMAP190 (Kellogg and Alberts, 1992). We constructed immuno-affinity columns with low-affinity antibodies raised and purified against a bacterially expressed DMAP190 fusion protein. These columns were used to purify a number of proteins that bind to the columns through their association (either direct or indirect) with DMAP190 (Kellogg and Alberts, 1992). In these experiments, a high speed extract from 0–3-h old *Drosophila* embryos (containing no nuclei or assembled centrosomes) is passed over the anti-DMAP190 antibody column. After extensive washing, any proteins retained on the column via an association with DMAP190 are eluted with 1 M KCl. The DMAP190 is then eluted with 1.5 M MgCl<sub>2</sub>. This procedure yields highly purified DMAP190 in the 1.5 M MgCl<sub>2</sub> elution, and 8–10 major proteins in the 1 M KCl elution (Kellogg and Alberts, 1992; Fig. 1 A). None of these proteins are retained on control columns constructed with purified IgG from nonimmunized rabbits (Kellogg and Alberts, 1992). Antibodies raised against one of these proteins recognized a 60-kD protein, now called DMAP60, which is also a centrosomal MAP (Kellogg and Alberts, 1992), strongly suggesting that DMAP190 and DMAP60 are associated in vivo.

Because  $\gamma$ -tubulin is a centrosomal protein that is postulated to interact with microtubules (Oakley, 1992), we wished to determine whether it was a component of the complex of proteins retained on an anti-DMAP190 antibody column. We therefore made antibodies against a bacterially produced fusion between MBP and full-length *Drosophila*  $\gamma$ -tubulin (see Materials and Methods) and purified them by affinity chromatography. On Western blots, these antibodies recognized *Drosophila*  $\gamma$ -tubulin, but not *Drosophila* (or bovine)  $\alpha$ - or  $\beta$ -tubulin (see below), and in indirect immunofluorescence assays on whole-mount embryo preparations they stained only the centrosome (Fig. 2).

These antibodies were used to probe Western blots of the protein fractions shown in Fig. 1 A. We found that  $\gamma$ -tubulin was greatly enriched in the fraction of proteins that bound to the anti-DMAP190 antibody column (Fig. 1 B), although there was not always a detectable band (by Coomassie blue staining) at the expected molecular weight for  $\gamma$ -tubulin, suggesting that  $\gamma$ -tubulin may be a relatively minor component of the complex. The  $\gamma$ -tubulin that bound to the column, however, bound very tightly: only 30–50% of it eluted with 1 M KCl, while the rest eluted with the DMAP190 in 1.5 M MgCl<sub>2</sub>. In contrast, 80–90% of the DMAP60 eluted with 1 M KCl, and only 10–20% eluted with the DMAP190. Neither  $\alpha$ - nor  $\beta$ -tubulin bound significantly to the anti-DMAP190 antibody column (Fig. 1 B), and neither  $\gamma$ -tubulin nor DMAP60 bound significantly to an identical control column of IgG prepared from a nonimmunized rabbit (not shown).

Although the anti-DMAP190 antibodies showed no detectable cross-reactivity with  $\gamma$ -tubulin in Western blotting ex-



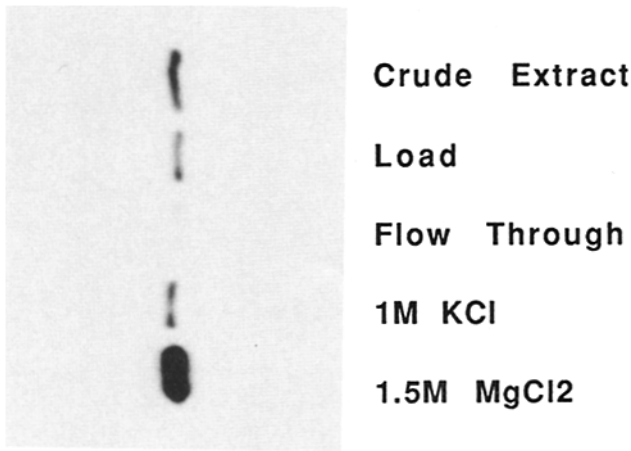


**Figure 2.** Immunohistochemical staining of  $\gamma$ -tubulin, DMAP190, and DMAP60 in the early *Drosophila* embryo. The figure shows the typical distribution of  $\gamma$ -tubulin, DMAP190, and DMAP60 at interphase (*INT*), metaphase (*MET*), and anaphase (*ANA*) visualized with a scanning confocal microscope in embryos at nuclear cycle 10 or 11. All three proteins are localized mainly to the centrosome during these early cycles. Anti-DMAP60 antibodies, and to a lesser extent anti-DMAP190 antibodies, however, also show a weak spindle-like staining during mitosis, and, while all of the antibodies stained the centrosome most strongly at mitosis, this was most marked for the anti-DMAP190 and anti-DMAP60 antibodies. In addition, after nuclear cycle 12, both anti-DMAP190 and anti-DMAP60 antibodies strongly stained interphase nuclei as well as centrosomes (not shown, see Whitfield et al., 1988), while anti- $\gamma$ -tubulin antibodies continued to stain only the centrosome. Bar, 5  $\mu$ m.

periments (not shown), we wished to exclude the possibility that the binding of  $\gamma$ -tubulin to the anti-DMAP190 antibody column was due to a cross-reactivity of these antibodies with native  $\gamma$ -tubulin. We therefore passed embryo extracts over an antibody column constructed from anti-DMAP60 antibodies. Like the anti-DMAP190 antibody columns (Kellogg

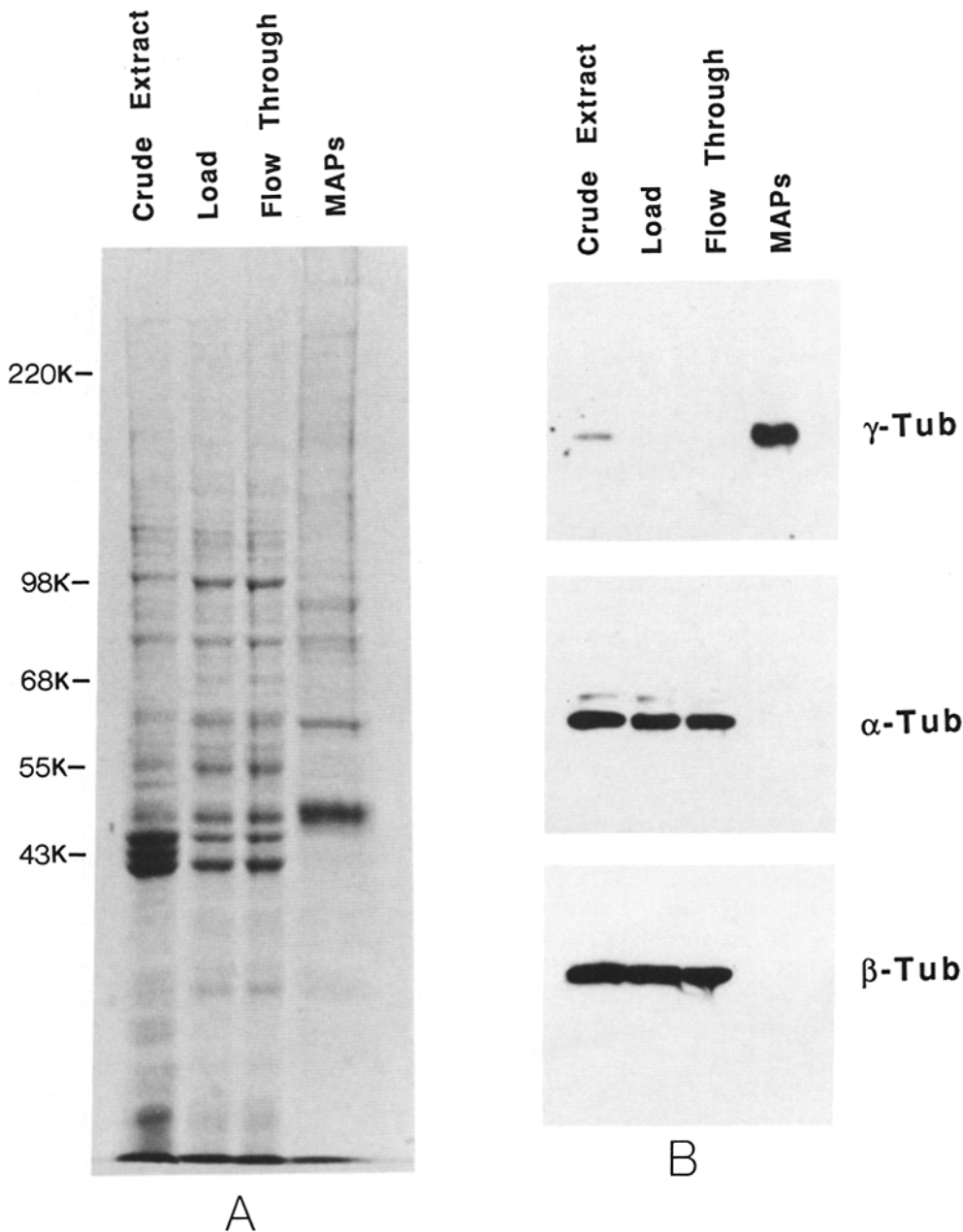
and Alberts, 1992), these columns quantitatively depleted both DMAP60 and DMAP190 from the extract initially flowing through the column (not shown; see Materials and Methods for the definition of initial flow-through).  $\gamma$ -tubulin bound to the DMAP60 antibody columns even more tightly than it did to the anti-DMAP190 antibody columns: only

**Figure 1.** The binding of  $\gamma$ -tubulin to an anti-DMAP190 antibody column. (A) Coomassie blue stained gel of an anti-DMAP190 antibody column experiment. (Lane 1) crude embryo extract; (lane 2) the same extract after centrifugation at 100,000 g for 1 h, as loaded onto the antibody column; (lane 3) the proteins that initially flowed through the column; (lane 4) the proteins eluted in 1 M KCl; and (lane 5) the proteins eluted in 1.5 M MgCl<sub>2</sub>. (B) Western blots of the protein gel shown in A probed with antibodies against DMAP190, DMAP60,  $\gamma$ -tubulin,  $\alpha$ -tubulin, and  $\beta$ -tubulin. 10–20  $\mu$ g of protein was loaded per lane with approximately equal amounts of protein in each lane.



10–20% of the  $\gamma$ -tubulin was eluted with 1 M KCl; the remaining 80–90% was eluted in the 1.5 M  $\text{MgCl}_2$  fraction (Fig. 3).  $\alpha$ - and  $\beta$ -tubulin did not bind significantly to anti-DMAP60 antibody columns (not shown). Thus, antibodies raised against either DMAP190 or DMAP60 can be used to

**Figure 3.** The binding of  $\gamma$ -tubulin to an anti-DMAP60 antibody column. A Western blot of the proteins retained on an anti-DMAP60 antibody column was probed with anti- $\gamma$ -tubulin antibodies. (Lane 1) crude embryo extract; (lane 2) the same extract after centrifugation for 1 h, as loaded onto the antibody column; (lane 3) the proteins that initially flowed through the column; (lane 4) the proteins eluted in 1 M KCl; and (lane 5) the proteins eluted in 1.5 M  $\text{MgCl}_2$ . 10–20  $\mu\text{g}$  of protein was loaded per lane with approximately equal amounts of protein in each lane.



**Figure 4.** The binding of  $\gamma$ -tubulin to a column of polymerized microtubules. (A) Coomassie blue stained gel of a MAP purification experiment. (Lane 1) crude *Drosophila* embryo extract; (lane 2) the same extract after centrifugation at 100,000  $g$  for 1 h, as loaded onto the microtubule column; (lane 3) the proteins that initially flowed through the microtubule column; and (lane 4) the proteins eluted from the microtubule column in 1 mM ATP plus 0.5 M KCl. (B) Western blots of the protein fractions shown in A, probed with anti- $\gamma$ -,  $\alpha$ -, or  $\beta$ -tubulin antibodies. 10–20  $\mu\text{g}$  of protein was loaded per lane with approximately equal amounts of protein in each lane.

purify a complex of proteins that contains DMAP190, DMAP60, and  $\gamma$ -tubulin, as well as a number of unidentified proteins.

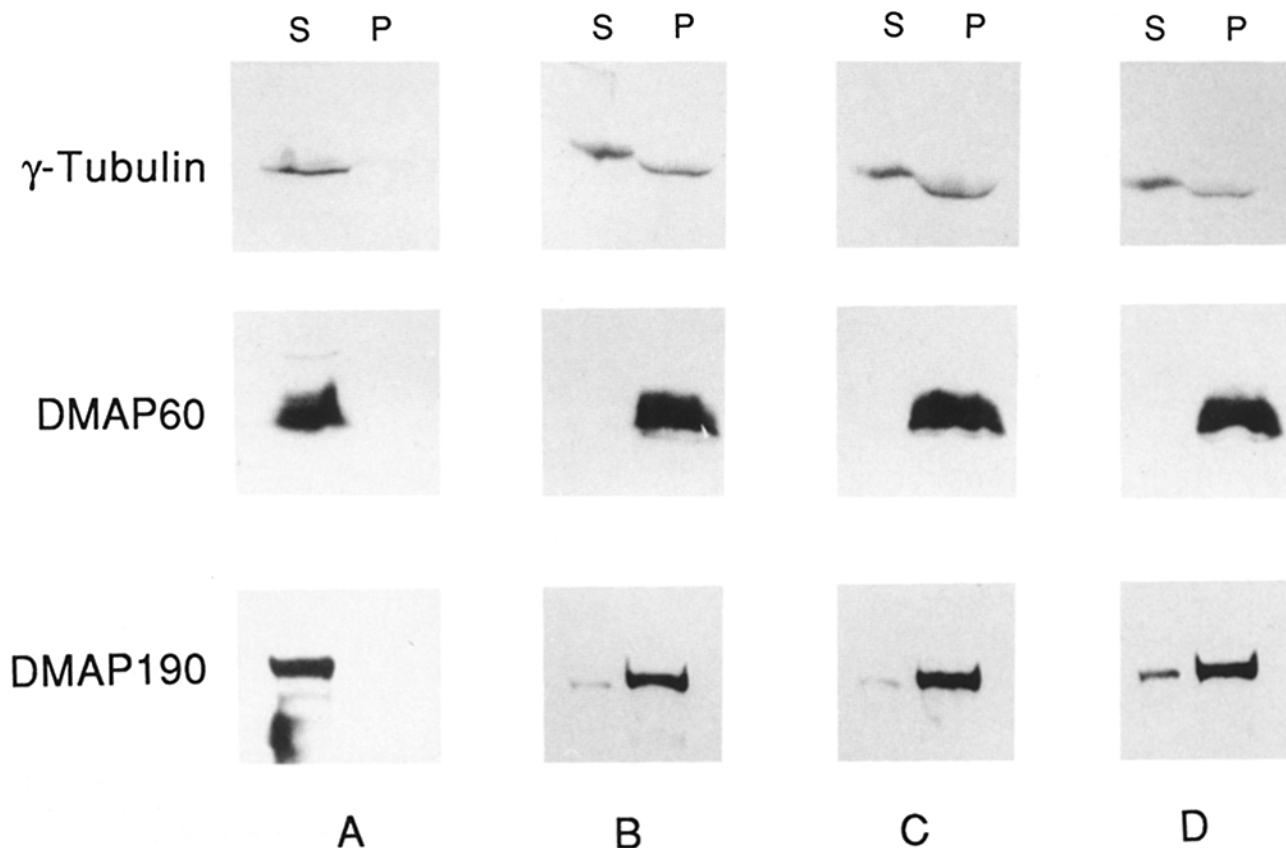
#### $\gamma$ -Tubulin in Embryo Extracts Binds to Microtubules

To test whether  $\gamma$ -tubulin in embryo extracts could bind to microtubules, clarified extracts of 0–3-h old embryos (containing no nuclei or assembled centrosomes) were passed over a column of taxol-stabilized microtubules, as described previously (Kellogg et al., 1989). After extensive washing, the proteins retained on the microtubule column were eluted in a single step with 1 mM ATP and 0.5 M KCl. An SDS-polyacrylamide gel analysis of the proteins obtained is shown in Fig. 4 A. We probed Western blots of the protein fractions shown in Fig. 4 A with anti- $\gamma$ -tubulin antibodies and found that  $\gamma$ -tubulin was greatly enriched in the MAP fraction (Fig. 4 B). In contrast,  $\alpha$ - and  $\beta$ -tubulin did not bind significantly to the microtubule column (Fig. 4 B); this result was expected because the extract was passed over the column under conditions that inhibit microtubule polymerization (Kellogg et al., 1989).

We compared the titers of the antibodies we used to detect  $\alpha$ -,  $\beta$ -, and  $\gamma$ -tubulin by quantitating their binding to known

amounts of purified *Drosophila* tubulin, or known amounts of bacterially expressed, full-length GST-*Drosophila*  $\alpha$ -tubulin or GST-*Drosophila*  $\gamma$ -tubulin (see Materials and Methods). From these measurements, we estimate that there is at least 10–30 times more  $\gamma$ -tubulin than either  $\alpha$ - or  $\beta$ -tubulin in the MAP fraction. Thus,  $\gamma$ -tubulin could not have eluted from the microtubule column as part of an  $\alpha$ : $\gamma$  or  $\beta$ : $\gamma$  heterodimer. Neither  $\alpha$ -,  $\beta$ -, nor  $\gamma$ -tubulin bound significantly to control columns of either matrix alone or matrix coupled to BSA (not shown).

To confirm that the  $\gamma$ -tubulin in embryo extracts could bind to microtubules, we performed more conventional microtubule spin-down experiments. We used taxol to polymerize the endogenous tubulin in a concentrated embryo extract and then pelleted the microtubules and associated proteins through a sucrose cushion. In the absence of taxol, no microtubules were formed and virtually all of the DMAP190, DMAP60, and  $\gamma$ -tubulin in the extract remained in the extract supernatant (Fig. 5 A). When taxol was added to the extract to promote the formation of microtubules, ~70–90% of the DMAP190 and >95% of the DMAP60 pelleted with the microtubules (Fig. 5 B), even though >95% of the total protein remained in the supernatant. Interestingly, only 10–50% (variable between experiments) of the  $\gamma$ -tubulin in the extract



**Figure 5.** The behavior of  $\gamma$ -tubulin, DMAP60, and DMAP190 in microtubule spin-down experiments. S, supernatant; P, Pellet. (A) No taxol control; (B) taxol was added to polymerize the endogenous microtubules; (C) purified tubulin was added to 4 $\times$  the level present in the endogenous extract before the addition of taxol; and (D) the endogenous microtubules were sheared to create more microtubule ends before pelleting the microtubules. In these Western blots, 1/10 of the total protein left in the supernatant and 1/3 of the protein in the pellet was loaded per lane, but because >95% of the total protein remained in the supernatant, there is ~10–20 $\times$  more total protein in the supernatant lane than in the pellet lane. Note that the  $\gamma$ -tubulin signal in the pellet lane is shifted down slightly because of the large amount of tubulin in the pellet that runs just above the  $\gamma$ -tubulin.

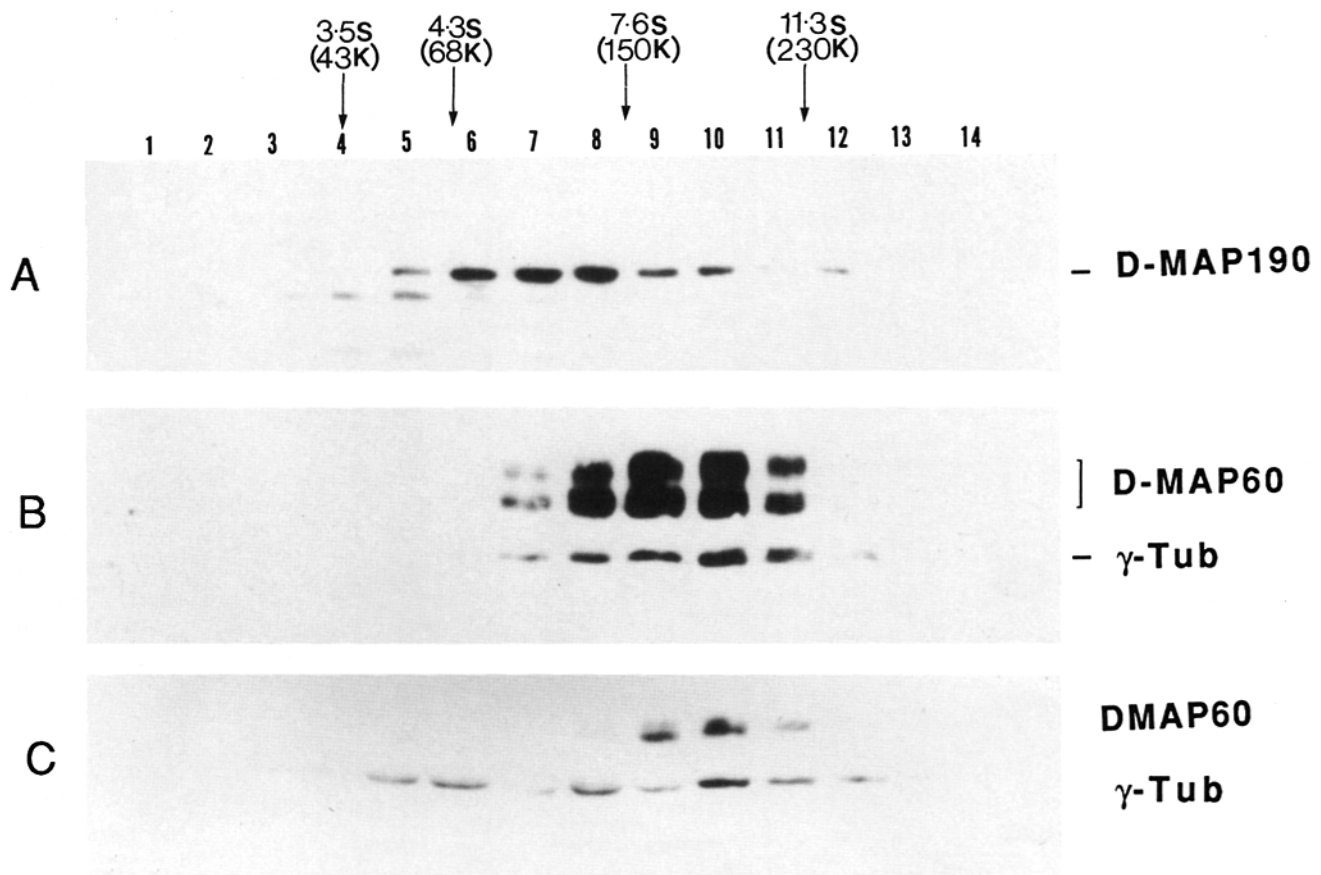
pelleted with the microtubules (Fig. 5 B). To test whether the reason that most of the  $\gamma$ -tubulin in the extract failed to bind to the microtubules was because the sites for binding  $\gamma$ -tubulin were saturated, we added an excess of purified tubulin to the extract before adding taxol. Even when the equivalent of at least four times the total amount of endogenous tubulin was added, no increase in the amount of  $\gamma$ -tubulin that pelleted with the microtubules was seen (Fig. 5 C). Vigorous pipetting of the extract (to shear the microtubules to create more microtubule ends) also had no effect on the amount of  $\gamma$ -tubulin that pelleted with the microtubules (Fig. 5 D).

To determine if microtubule columns also removed only a fraction of the  $\gamma$ -tubulin from embryo extracts, we compared the amounts of  $\gamma$ -tubulin in the load and flow-through from these columns. As was the case in the microtubule spin-down experiments, microtubule columns removed only ~10–50% (variable between experiments) of the  $\gamma$ -tubulin

initially loaded (not shown), whereas it quantitatively depleted the DMAP190 and DMAP60 (Kellogg and Alberts, 1992). Thus, in two different assays, only 10–50% of the  $\gamma$ -tubulin in the extract bound to microtubules.

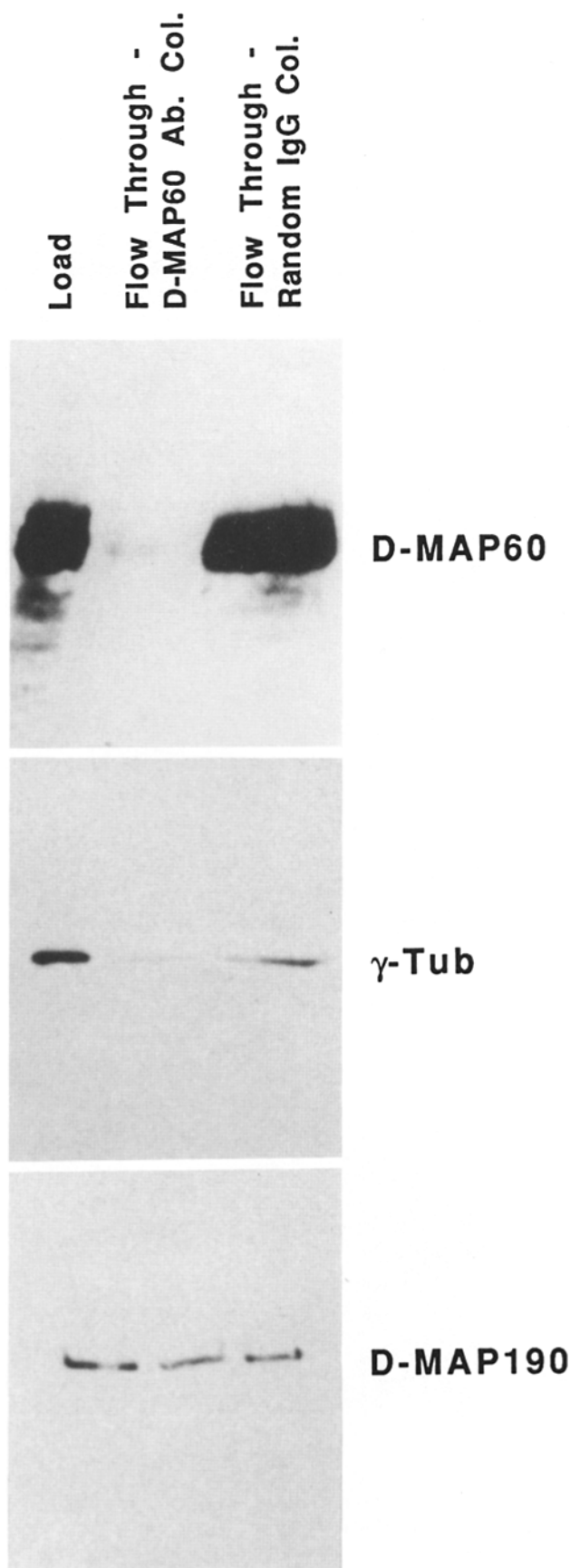
#### $\gamma$ -Tubulin Binds to Microtubules as Part of a Complex

We wished to know whether  $\gamma$ -tubulin bound to microtubules as a monomer or as part of a complex with DMAP190, DMAP60, and/or other proteins. Unfortunately, it was not possible to assay the state of these proteins while they remained bound to microtubules. We therefore tested whether these proteins behaved as monomers after elution from a microtubule column by subjecting the MAPs to velocity sedimentation through sucrose gradients. As the proteins were eluted from the microtubule column in 0.5 M KCl, we used this concentration of salt in the sedimentation experiments; therefore, only salt-stable interactions would be de-



**Figure 6.** Sedimentation behavior of  $\gamma$ -tubulin, DMAP60, and DMAP190 on sucrose gradients. After elution from a microtubule column, MAPs were separated on a sucrose gradient run in 0.5 M KCl. The resulting fractions were analyzed by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose sheet was cut in half, separating the proteins of 100 kD and higher molecular weight from those of lower molecular weight. The higher molecular weight half was probed with anti-DMAP190 antibodies (A), while the other half was probed with a mixture of anti- $\gamma$ -tubulin and anti-DMAP60 antibodies (B).  $\gamma$ -tubulin and DMAP60 both sediment at ~8 S, whereas DMAP190 sediments at ~6.5 S. This gel was run to maximize the resolution between the  $\gamma$ -tubulin and DMAP60 proteins so they could both be probed on the same Western blot, and the DMAP60 triplet (Kellogg and Alberts, 1992) has resolved into a doublet on this blot. (C) A concentrated embryo extract was subjected to sedimentation through a sucrose gradient under the same conditions as the MAP proteins. The DMAP60 is in the form of a higher molecular weight complex, while a fraction of the  $\gamma$ -tubulin now behaves as a smaller protein. Arrows at the top of the gel indicate the positions of the roughly spherical marker proteins that were sedimented on an identical gradient and run in parallel to the gradients shown in the figure. The markers were ovalbumin (43 kD; 3.5 s), BSA (68 kD; 4.3 s), alcohol dehydrogenase (150 kD, 7.6 s), and catalase (230 kD, 11.3 s).





**Figure 7.** An anti-DMAP60 antibody column depletes DMAP60 and partially depletes  $\gamma$ -tubulin from isolated MAPs. Isolated MAPs, in 0.5 M KCl, were passed over either an anti-DMAP60

ected in this experiment. As shown in Fig. 6 B, the  $\gamma$ -tubulin that eluted from a microtubule column ran as a peak sedimenting at  $\sim 8$  s, the size expected of a roughly spherical 150–200-kD protein ( $\gamma$ -tubulin has a molecular mass of 52 kD). The DMAP60 that eluted from the microtubule column migrated in a similar manner, sedimenting at  $\sim 8$  s (Fig. 6 B), whereas DMAP190 ran as a smaller protein with a peak at  $\sim 6.5$  s (Fig. 6 A). Thus, both  $\gamma$ -tubulin and DMAP60 eluted from a microtubule column as components of salt-stable complexes. From the predicted amino acid sequence of DMAP190 (Whitfield, W., personal communication), its sedimentation rate was about that expected for a DMAP190 monomer. It seems, therefore, that if DMAP190 is binding to microtubules in association with DMAP60 and/or  $\gamma$ -tubulin, this complex is not stable under the conditions used in this experiment.

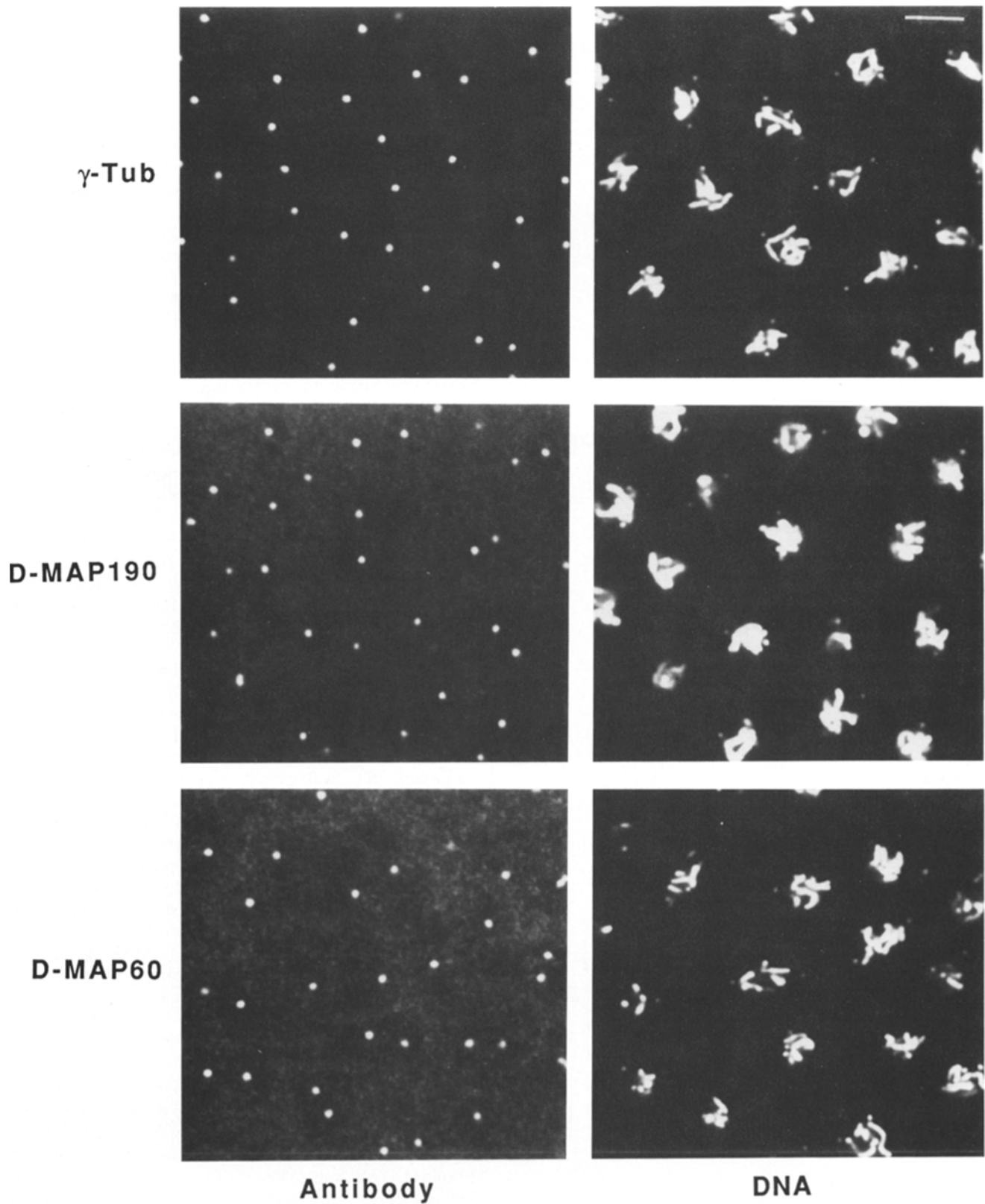
To test whether all of the  $\gamma$ -tubulin and DMAP60 in the extract could be in the form of these salt-stable complexes, we ran concentrated embryo extracts on sucrose gradients. We used concentrated extracts so that the behavior of the  $\gamma$ -tubulin, which is a very minor component, could be followed, and these experiments were performed in 0.5 M KCl to mimic the conditions under which the MAPs were assayed. We found that most of the DMAP60 in the extract migrated at  $\sim 8$  s (Fig. 6 C), which was similar to its migration when the MAP fraction was analyzed. In contrast, while some of the  $\gamma$ -tubulin migrated with a peak at 8 s, a significant portion ran at a lower molecular weight (Fig. 6 B), suggesting that not all of the  $\gamma$ -tubulin in the extract can be in the form of a salt-stable complex.

Because the  $\gamma$ -tubulin- and DMAP60-containing complexes that eluted from a microtubule column were similar in size, we tested whether these proteins were a part of the same complex. We passed isolated MAPs, in 0.5 M KCl, over a column of anti-DMAP60 antibodies, and then assayed the proteins initially flowing through the column to see if the  $\gamma$ -tubulin was depleted along with the DMAP60. In three experiments, we found that  $>95\%$  of the DMAP60 and 40–70% of the  $\gamma$ -tubulin were depleted from the initial flow-through when compared to the flow-through from a control column constructed from preimmune IgG (Fig. 7), strongly suggesting that a substantial proportion of the  $\gamma$ -tubulin in the MAP fraction is associated with DMAP60. In contrast, under these high salt conditions, the DMAP190 was not depleted, as expected from the sucrose gradient experiments.

#### *$\gamma$ -Tubulin, DMAP190, and DMAP60 Are All Integral Components of the Centrosome*

Because  $\gamma$ -tubulin, DMAP190, and DMAP60 are all centrosomal MAPs, we tested whether any of these proteins required intact microtubules to maintain their localization at the centrosome. We treated embryos with the microtubule depolymerizing drug colchicine for 20–30 min before fixation and then stained them with antibodies against each of these proteins. Although such treatment depolymerized vir-

antibody column or a nonimmune IgG column. The proteins that flowed through these columns were subjected to SDS-PAGE, blotted, and probed with antibodies against DMAP60,  $\gamma$ -tubulin, and DMAP190.



**Figure 8.** Immunohistochemical staining of  $\gamma$ -tubulin, DMAP190, and DMAP60 in colchicine-treated *Drosophila* embryos. The figure shows a field of nuclei at the cortex of embryos at nuclear cycle 11 or 12 that were treated for 30 min with colchicine before fixation and antibody staining. The embryos were also stained with an anti-histone antibody to visualize the chromatin (*right-hand panels*). Note the characteristic disorganized “metaphase arrest” configuration of the chromosomes, and the very bright staining of the centrosomes with anti- $\gamma$ -tubulin, anti-DMAP190, and anti-DMAP60 antibodies. Bar, 10  $\mu$ m.

tually all of the microtubules in the embryo (not shown), it had little effect on the staining of centrosomes by these antibodies (Fig. 8). Thus, although these centrosomal proteins can associate with microtubules, they behave as "integral" components of the centrosome whose presence in the centrosome does not require intact microtubules.

## Discussion

In an attempt to gain a better understanding of how the centrosome organizes microtubules, we have used immunaffinity chromatography to isolate a complex of *Drosophila* centrosomal proteins that can interact with microtubules. We have previously shown that antibodies raised against DMAP190, a centrosomal MAP, can be used to purify a complex of proteins that contains a number of other centrosomal MAPs including DMAP60 (Kellogg and Alberts, 1992), and a novel 85-kD protein (Raff, J. W., and B. M. Alberts, manuscript in preparation). In this report we show that  $\gamma$ -tubulin, a highly conserved component of probably all centrosomes, is also a component of this complex and that, like DMAP190 and DMAP60,  $\gamma$ -tubulin in *Drosophila* embryo extracts can bind to microtubules, although it is not clear whether any of these proteins bind to microtubules directly (see below). The finding that these centrosomal proteins associate with one another and with microtubules in vitro, strongly suggests that they are components of a centrosomal microtubule-binding complex.

We have used two approaches to demonstrate that  $\gamma$ -tubulin, DMAP60, and DMAP190 in embryo extracts can interact with microtubules. First, we passed embryo extracts over columns of taxol-stabilized microtubules and showed that these proteins bind to the column; this had been shown previously for DMAP60 and DMAP190 (Kellogg and Alberts, 1992). Second, we polymerized the endogenous tubulin in an embryo extract and showed that these proteins copellet with microtubules. We previously demonstrated that the vast majority of proteins that bind to a microtubule column are associated with microtubule structures in vivo and that the proteins that copellet with microtubules in vitro are a very similar set to those that bind to a microtubule column (Kellogg et al., 1989). These findings, together with our observation that  $\gamma$ -tubulin, DMAP190, and DMAP60 all colocalize to the centrosome in vivo, suggest that the association of these proteins with microtubules in vitro is likely to reflect their function in cells.

An intriguing finding in the present study is that in both the microtubule column and microtubule spin-down experiments, only 10–50% of the  $\gamma$ -tubulin in the extract binds to microtubules. These results are unlikely to be due to the presence of a limited number of  $\gamma$ -tubulin binding sites on the microtubules since adding an excess of tubulin to the extracts before polymerizing the tubulin into microtubules, or shearing the microtubules to create more free microtubule ends, does not increase the proportion of  $\gamma$ -tubulin that binds to microtubules. Moreover, in microtubule column experiments we find that  $\gamma$ -tubulin is not depleted from the extract flowing through the column in an initial burst, but is instead continuously partially depleted from the extract (unpublished observations—determined by collecting the flow-through from the column in small fractions and assaying the extent of  $\gamma$ -tubulin depletion in each fraction). It seems,

therefore, that a substantial fraction of the  $\gamma$ -tubulin in *Drosophila* embryo extracts is in a form that cannot bind to microtubules in vitro. There are at least two possible explanations for this result: (a)  $\gamma$ -tubulin in the extract may have an intrinsically low-affinity for microtubules, and it is only when it becomes associated with other proteins that it can bind to microtubules; (b)  $\gamma$ -tubulin in the extract is associated with other proteins that prevent it from interacting with microtubules with high affinity. Our finding that  $\gamma$ -tubulin elutes from a microtubule column as part of a complex with another centrosomal MAP (see below), and that not all of the  $\gamma$ -tubulin in the extract can be in the form of this complex, is consistent with the first possibility, but cannot exclude the second.

It is possible that the inability of all of the  $\gamma$ -tubulin to bind to microtubules reflects a special requirement of the early *Drosophila* embryo, in which enough  $\gamma$ -tubulin may have to be stored to make the large numbers of centrosomes needed during the first 2–3 h of development (Foe and Alberts, 1983). In this case, it could be important to prevent any stored  $\gamma$ -tubulin from binding to microtubules with high affinity until it has become incorporated into a functioning centrosome. The existence of a large maternal store probably explains why we are able to obtain  $\gamma$ -tubulin in a soluble form from extracts.

Although we have shown that  $\gamma$ -tubulin is part of a complex that contains DMAP190 and DMAP60, and that these three proteins associate with microtubules in vitro, we did not know whether these proteins were in a complex together while they were bound to microtubules. Unfortunately, it was not possible to directly analyze these proteins while they were bound to microtubules. We therefore analyzed the sedimentation behavior of these proteins after they were eluted from a microtubule column in high salt. We found that the  $\gamma$ -tubulin and DMAP60 behaved very similarly, both sedimenting at  $\sim 8$  s, whereas DMAP190 sediments as a smaller protein, with a peak at  $\sim 6.5$  s. Thus, both  $\gamma$ -tubulin and DMAP60 elute from a microtubule column as components of salt-stable complexes, while, under these conditions, the majority of the DMAP190 cannot be a component of these complexes.

To test if  $\gamma$ -tubulin and DMAP60 were components of the same complex, we passed the eluted MAPs, in 0.5 M KCl, over an anti-DMAP60 antibody column. The column depletes >95% of the DMAP60 and 40–70% of the  $\gamma$ -tubulin compared to control columns, strongly suggesting that at least fractions of the  $\gamma$ -tubulin and DMAP60 are in the same complex. It is puzzling, however, that not all of the  $\gamma$ -tubulin in the MAP fraction binds to the anti-DMAP60 antibody column because virtually all of the  $\gamma$ -tubulin in the MAP fraction appears to be in a complex that comigrates with the DMAP60 on sucrose gradients. We suspect, therefore, that after elution from a microtubule column, these two proteins may exist in a variety of complexes, all of which extensively comigrate in sucrose gradients. This is supported by our observation that the sedimentation behavior of the  $\gamma$ -tubulin and DMAP60 eluted from a microtubule column is very similar, but not identical (unpublished observations).

Although  $\gamma$ -tubulin binds to microtubules as part of a complex, neither  $\alpha$ - nor  $\beta$ -tubulin bind to microtubule columns, excluding the possibility that  $\gamma$ -tubulin binds to the columns as an  $\alpha:\gamma$  or  $\beta:\gamma$  heterodimer. While it is possible that

$\gamma$ -tubulin forms  $\gamma$ : $\gamma$  homodimers, its migration on sucrose gradients suggests that the complex is too big to be a homodimer alone (whereas  $\gamma$ -tubulin migrates at 8 s,  $\alpha$ : $\beta$  homodimers migrate at 6 s), and, as discussed above, at least a fraction of the  $\gamma$ -tubulin binding to a microtubule column is associated with DMAP60.

Although we have shown that  $\gamma$ -tubulin, DMAP190, and DMAP60 in embryo extracts can bind to microtubules, it is still uncertain whether any of these proteins can bind directly to microtubules, and, if they do, whether they bind exclusively to the ends of microtubules. Our observation that virtually all of the DMAP60 in an extract binds to microtubules suggests that DMAP60 must either bind directly to microtubules, or all of the DMAP60 in the extract is associated with proteins that bind to microtubules. It has recently been found that bacterially produced DMAP60 can cosediment with polymerized microtubules in vitro, suggesting that DMAP60 can bind directly to microtubules (Schneider, K., personal communication). Although  $\gamma$ -tubulin, DMAP60, and DMAP190 all associate with microtubules at some level, preventing microtubule polymerization does not affect their localization in centrosomes. Thus, these proteins appear to be integral components of the centrosome.

There are two important limitations to the approaches we have used to analyze how  $\gamma$ -tubulin, DMAP60, and DMAP190 are associated with one another and with microtubules in the cell. First, all of our studies are conducted with the soluble forms of these proteins, which may behave differently than they would in the environment of a functional centrosome. Second, we have only analyzed the behavior of these proteins either before loading on a column or after elution from a column in high salt. It is probable that these proteins assemble with yet other proteins into much larger complexes while they are bound to microtubules. This is certainly the case for proteins bound to an anti-DMAP190 antibody column, which retains at least 8–10 major proteins through their direct or indirect association with DMAP190 (Kellogg and Alberts, 1992). Thus,  $\gamma$ -tubulin and DMAP60 might bind to microtubules in a complex with DMAP190, but this larger complex is disrupted after the proteins are eluted from the column in high salt, and then diluted on a sucrose gradient. To understand how these proteins function in the centrosome, one will need to determine which of them bind directly to microtubules, to discover exactly how they interact with one another, and to identify the other components that interact with these proteins in the centrosome.

We thank Tim Stearns for supplying anti-*Xenopus*- $\gamma$ -tubulin antibodies used in the initial stages of this study and for much helpful discussion and advice. Trish Wilson, Yixian Zheng, and Berl Oakley supplied the *Drosophila*  $\gamma$ -tubulin construct used to amplify full-length  $\gamma$ -tubulin for cloning and fusion protein production. Bill Theurkauf supplied the *Drosophila*  $\alpha$ -tubulin clone used to amplify full-length  $\alpha$ -tubulin for cloning and fusion protein production. Will Whitfield and Ken Schneider generously communicated data before publication. We thank Chris Field, Becky Kellum, Bin Liu, Tim Mitchison, Michelle Moritz, Tim Stearns, and Ken Schneider for critical reading of the manuscript, and the two referees whose comments greatly improved the manuscript.

J. W. Raff is a Lucille Markey Visiting Fellow, and this work was supported by National Institutes of Health grant RO1 GM239828.

Received for publication 6 October 1992 and in revised form 25 February 1993.

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