Identification of Microtubule-associated Proteins in The Centrosome, Spindle, and Kinetochore of The Early Drosophila Embryo

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Abstract. We have developed affinity chromatography methods for the isolation of microtubule-associated proteins (MAPs) from soluble cytoplasmic extracts and have used them to analyze the cytoskeleton of the early Drosophila embryo. More than 50 Drosophila embryo proteins bind to microtubule affinity columns. To begin to characterize these proteins, we have generated individual mouse polyclonal antibodies that specifically recognize 24 of them. As judged by immunofluorescence, some of the antigens localize to the mitotic spindle in the early Drosophila embryo, while others are present in centrosomes, kinetochores, subsets of microtubules, or a combination of these structures. Since 20 of the 24 antibodies stain microtubule structures, it is likely that most of the proteins that bind to our columns are associated with microtubules in vivo. Very few MAPs seem to be identically localized in the cell, indicating that the microtubule cytoskeleton is remarkably complex.

MICROTUBULE-ASSOCIATED proteins (MAPs)1 are thought to play a central role in determining the structure and function of microtubule networks in eukaryotic cells. MAPs were originally identified in mammalian brain tissue extracts as abundant proteins that cosediment with microtubules (for reviews, see Vallee et al., 1984; Olmstead, 1986). More recently, similar criteria have been used to identify MAPs in a number of nonneuronal systems (Vallee and Collins, 1986; Goldstein et al., 1986; Lye et al., 1987).

None of the MAPs that cosediment with microtubules have been found to localize to the centrosome or the kinetochore, two extensively studied microtubule-organizing centers in the cell. Most MAPs that have been identified thus far are relatively abundant proteins that bind along the entire length of microtubules (Vallee and Bloom, 1983; Bloom et al., 1984; Huber and Matus, 1984; Binder et al., 1985). MAPs that function as components of the centrosome or the kinetochore would be expected to be present only near the ends of microtubules, and are therefore likely to be much less abundant. These low abundance MAPs may be difficult to detect by current methods.

We have developed procedures for the isolation of MAPs by microtubule affinity chromatography, with the hope that even minor proteins that bind to microtubules can be identified and characterized. Previous work has shown that affinity chromatography is a powerful technique for the identification, purification, and characterization of interacting proteins (for examples, see Formosa and Alberts, 1984; Dedhar et al., 1987). We have used our technique to isolate a large number of previously uncharacterized MAPs from the early Drosophila embryo, which seems to provide an especially good model system for studying cytoskeletal functions.

The Drosophila embryo begins development as a giant syncytial cell. The earliest nuclear divisions occur in the interior of the embryo, but, after nine nuclear divisions, the majority of the nuclei have migrated to the cortex where they form an evenly spaced monolayer. The nuclei in this monolayer divide four more times and then become synchronously cellularized by invaginations of the plasma membrane to form the cellular blastoderm (Rabinowitz, 1941; Zalokar and Erk, 1976; Foe and Alberts, 1983). The nuclear divisions that precede this cellular blastoderm stage take place at intervals of 8–20 min, and the dynamic rearrangements of the microtubule arrays during each nuclear cycle can be observed in living embryos after injection of fluorescently-labeled tubulin subunits (Kellogg et al., 1988). Large quantities of early embryos are readily available for biochemical analyses, and these embryos are also amenable to immunofluorescence, microinjection, and genetic studies. These factors combine to make the early Drosophila embryo an attractive system in which to study the morphogenesis of microtubule arrays and their role in embryonic development.

To begin a characterization of the affinity-purified Drosophila embryo MAPs, we have made a library of mouse polyclonal antibodies that specifically recognize 24 of them. These antibodies have allowed us to determine the subcellular localizations of the affinity-purified proteins in embryos.

1. Abbreviations used in this paper: MAP, microtubule-associated protein; TAME, N-p-tosyl arginine methyl ester.

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The results suggest that the majority of the many proteins that bind to microtubule affinity columns are associated with microtubules in the cell, and they have allowed us to identify MAPs that localize to centrosomes and kinetochores.

Materials and Methods

Materials

All chemicals used were reagent grade. N-p-tosyl arginine methyl ester (TAME), pepstatin A, leupeptin, aprotinin, and Freund's adjuvant were from Sigma Chemical Co. (St. Louis, MO). Rhodamine-conjugated goat anti–mouse antibody was from Cappel Laboratories (Malvern, PA), and alkaline phosphatase-conjugated goat anti–mouse antibody was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Monoclonal antibodies against alpha and beta tubulin were from Amersham Corp. (Arlington Heights, IL). Taxol was a generous gift of Dr. Matthew Saffness (National Institutes of Health).

Buffers and Stock Solutions

Protease inhibitor stock: 1 mM benzamidine-HCl, 0.1 mg/ml phenanthroline, 1 mg/ml each of aprotinin, leupeptin, and pepstatin A (this stock is used at dilutions of 1:100–1:1,000, as noted). PMI buffer: 0.1 M Pipes-KOH, pH 6.8, 2 mM Na3 EGTA, 1 mM MgCl2, 1 mM GTP, 1 mM TAME. PMIX buffer: PMI buffer plus 0.5 mM DTT and protease inhibitor stock (1:100). PMIX.1 buffer: as above, but with 1:1000 dilution of protease inhibitor stock. C buffer (column buffer): 50 mM Hepes-KOH, pH 7.6, 1 mM MgCl2, 1 mM Na3 EGTA. CX buffer: C buffer supplemented with 10% glycerol, 25 mM KCl, 0.5 mM DTT, and protease inhibitor stock (1:1000). BRB80 buffer (microtubule assembly buffer): 80 mM Pipes-KOH, pH 6.8, 1 mM MgCl2, 1 mM Na3 EGTA. BRB80X buffer: BRB80 supplemented with 0.5 mM DTT, 1 mM GTP, 5 mM taxol, and protease inhibitor stock (1:100). PBS: 10 mM sodium phosphate, pH 7.3, 0.15 M NaCl. TBS: 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl. Polyacrylamide gel sample buffer: 63 mM Tris-HCl, pH 6.8, 3% sodium dodecylsulfate (SDS), 5% β-mercaptoethanol, 10% glycerol.

Purification of Drosophila Tubulin

Tubulin is purified from early Drosophila embryos by procedures modified from those used by Detrich and Wilson (1983) to purify tubulin from sea urchin embryos. As starting material, we use 0.3–0.5 g of embryos that have been frozen in liquid nitrogen and stored at −70°C. Frozen chunks of embryos are placed between several layers of aluminum foil, crushed with a hammer, and added to 2 ml of PMI buffer at 15°C containing protease inhibitor stock (1:100). After stirring briefly, PMF5 is added to 1 mg/ml, and the embryos are homogenized by several passes of a motor-driven teflon pestle, and added to 2 ml of PMI buffer at 15°C containing protease inhibitor stock (1:100). The homogenate is centrifuged at 10,000 g for 70 min, the supernatant is brought to 0.5 mM DTT and mixed with 0.5 vol of packed phosphocellulose (Whatman Inc., Clinlon, NJ), previously equilibrated with PMIX buffer. After 30 min of gentle mixing on a rotator, the phosphocellulose resin is pelleted by centrifugation for several min at 10,000 g, and the supernatant is saved. The phosphocellulose is washed twice with 0.5 vol of PMIX buffer, and these supernatants are combined with the first supernatant.

The tubulin in the above supernatants is bound to DEAE cellulose by adding the supernatant to 0.33 vol of packed DEAE cellulose (DE52; Whatman Inc.) previously equilibrated with PMIX buffer. After gentle mixing the DEAE cellulose and the extract on a rotator for 30 min, the DEAE cellulose is pelleted and washed twice with 0.5 vol of PMIX buffer. A slurry of the resin is poured into a column (3.5-cm-diam for 150 ml of DEAE cellulose) and washed with 2 column vol of PMIX.1 buffer containing 0.15 M NaCl. Tubulin is then eluted from the DEAE cellulose column with PMIX.1 buffer containing 0.4 M NaCl. Fractions of 2–3 ml are collected at a flow rate of 2 column vol/h. The eluted fractions are assayed for protein (Bradford, 1976), and peak fractions are pooled and dialyzed for 40 min against 10 vol of BRB80 buffer containing 0.5 mM DTT, followed by another 40 min against fresh buffer. If the protein concentration is below 2 mg/ml at this point, the solution is concentrated using an ultrafiltration device (Amicon Corp., Danvers, MA). Tubulin is polymerized into microtubules by addition of GTP to 10 mM, MgCl2 to 4 mM, and DMSO to 10%, followed by incubation at 25°C for 40 min. The microtubules are collected by cen-
PMSF is added to 1 mM, the embryos are homogenized at 4°C, and the Microtubule Affinity Chromatography ter loading, the columns are washed with 3-4 column vol of CX buffer, and then eluted in succession with this buffer plus either 1 mM MgATP, 0.1 M vol/h, and all chromatography steps are at 4°C.

Microtubule Affinity Chromatography

Embryo extracts are loaded onto affinity columns at 0.5-1 column vol/h. After loading, the columns are washed with 3-4 column vol of CX buffer, and then eluted in succession with this buffer plus either 1 mM MgATP, 0.1 M KCl, or 0.5 M KCl. The wash and elution steps are carried out at 4°C.

After the protein concentration in each fraction is determined (Bradford, 1976), the peak fractions are pooled. The protein in each pool is precipitated with 10% TCA as described by Miller and Alberts (1989), resuspended in gel sample buffer (0.5 ml for the eluate from a 15-ml column), and neutralized with the vapor from a Q-tip soaked in ammonium hydroxide. The pellets are solubilized by incubation at 50°C for 30 min, followed by 100°C for 3 min; each is then analyzed by SDS-PAGE (Laemmli, 1970), using Coomassie blue staining to visualize protein bands.

Cosedimentation of MAPs with Taxol-stabilized Microtubules

As an alternative to microtubule affinity chromatography, MAPs were iso- lated by virtue of their ability to cosediment with taxol-stabilized microtubules, using the procedures described by Vallee and Collins (1986). Briefly, 0-3-h embryos (dechorionated and washed as described above) are added to 2 vol of BRB80 buffer containing protease inhibitor stock (1:100). After PMSF is added to 1 mM, the embryos are homogenized at 4°C, and the extract is clarified as described earlier. The supernatant is supplemented with 0.5 mM DTT, 1 mM GTP, 20 μM taxol, warmed to 25°C for 5 min, and then centrifuged at 100,000 g for 45 min at 4°C to allow sedimentation of the endoge- nous tubulin. The taxol-stabilized microtubules and their associated protein- teins are collected by centrifugation through a sucrose cushion (BRB80X containing 10% sucrose) at 48,000 g for 30 min at 4°C. The pellet is washed twice at 4°C by resuspension in 0.2 times the original extract volume of BRB80X buffer, followed by a second centrifugation through a sucrose cushion. MAPs are dissociated from the microtubules by resuspending the fraction in BRB80X buffer containing 1 mM ATP and 0.5 M KCl (0.07 times the original extract volume) at 25°C. The microtubules are removed by centrifugation at 45,000 g for 15 min at 20°C, and the MAP-containing supernatant is saved. Release of the MAPs at 25°C prevents dissociation of tubulin subunits (see Vallee and Collins, 1986).

Isolation of MAPs in a pH 7.6 Hepes buffer (see text) is carried out in the same manner, except that the BRB80X buffer is replaced by C buffer, BRB80X buffer is replaced by CX buffer, and the release of the MAPs from microtubules is carried out at 4°C, since taxol-stabilized microtubules are more stable when exposed to ATP and KCl in the pH 7.6 Hepes buffer.

Generation of Mouse Polyclonal Antibodies to Gel-Purified Proteins

We have produced mouse polyclonal antibodies using procedures modified from those of Amero et al. (1987). A total of ~1.6 mg of affinity-purified MAPs are separated on two preparative 7-12%, 14.5 cm × 22 cm, SDS- containing polyacrylamide gradient gels (0.82-mm thick). The gels are stained with Coomassie blue, destained for 0.5 h, and treated with a solution of 2% glutaraldehyde in water for 1 h at 25°C. The gels are then transferred to 7% acetic acid to complete the destaining procedure. After soaking in water to remove the acetic acid, bands of interest are excised with a razor blade and homogenized with a motor-driven teflon dounce homogenizer in the presence of a small amount of water. The homogenized gel bands are then lyophilized and resuspended in 1.5 ml of sterile PBS.

Each gel slice is used for a total of four immunizations given infra- tionally with a 22-gauge needle and spaced at 2 wk intervals. The first immunization is with 0.45 ml of homogenized gel slice slurry and 0.1 ml of complete Freund's adjuvant, while the remaining three immunizations use 0.34 ml of the slurry and 0.1 ml of incomplete Freund's adjuvant. The adjuvants are warmed to 37 °C and vortexed before being loaded into the syringe. Beginning 1 wk after the final immunization, the mice are bled intraorbitally every 7-10 d, and the sera are tested for the presence of specific antibodies. Mice are anesthetized with ether before all immuniza- tions and bleeds.

Western Blotting

Proteins were detected by western blotting according to standard procedures (Towbin, 1979). Approximately 0.15 mg of crude extract protein or affinity- purified MAPs are electrophoresed on a preparative minigel (8 × 6 × 0.5 mm) and then transferred from the gel to a sheet of 0.45 μm porosity nitrocellulose (Schleicher & Schuell Inc., Keene, NH) in the presence of 25% methanol, 0.15 M glycine, 0.02% SDS (transfer for 90 min at 300 mA in a Hoeffer electroblotting apparatus). The nitrocellulose sheet is incubated for 45 min in PBS containing 5% BSA and 0.02% sodium azide and then placed in a miniblottor apparatus (Immunetics, Cambridge, MA). Each lane of this miniblottor is loaded with 55 μl of antibody diluted into PBS contain- ing 0.05% Tween 20 detergent (Bio-Rad), 5% BSA, and 0.02% sodium azide, and the miniblottor is then placed on a rocker platform for 2 h at room temperature. Alkaline phosphatase-conjugated goat anti-mouse secondary antibody is used to visualize protein bands. To reduce nonspecific back- ground staining, this secondary antibody was predesorbed to methanol-fixed embryos by diluting the antibody into ~1 ml of PBS containing 0.5 ml (set- tled volume) of methanol-fixed embryos. After mixing on a rotating wheel (60 min, 25°C), the soluble antibody is diluted to its final working concentration and used immediately.

Fixation and Immunofluorescent Staining of Drosophila Embryos

Methanol is the fixative that best preserves microtubule structures in the Drosophila embryo (Warn and Warn, 1986; Kellogg et al., 1988). For im- munofluorescence staining, the methanol-fixed embryos are incubated in PBS containing 5% BSA, 0.05% Tween 20, and 0.02% sodium azide for 20 min. The embryos are then incubated overnight at 4°C in a 1:400 dilution of immune mouse serum in PBS containing 5% albumin, 0.1% Tween, and 0.02% sodium azide. These embryos are washed and treated with rhodo- minamine-conjugated secondary antibody as described by Karr and Alberts (1986).

Affinity Purification of Antibodies

Affinity purification of antibodies using nitrocellulose-bound antigen was carried out according to procedures described by Smith and Fisher (1984), with modifications. Affinity-purified MAPs are resolved on two preparative minigels and transferred to nitrocellulose sheets. The location of the desired protein band is determined by using a miniblottor to treat the lanes on both edges of the nitrocellulose sheet with primary antibody. After staining with alkaline phosphatase-conjugated secondary antibody, the central strip of un- stained nitrocellulose that corresponds to the desired protein band is excised and cut into small pieces, which are placed into a 1.5-ml tube. We used the 190-kD band to affinity purify the SI-24 antibody, the 105- and 89-kD bands for the S5-47 antibody, and the 190-kD band for the SI-24 antibody, the 105- and 89-kD bands of complete Freund's adjuvant, while the remaining three immunizations use 0.34 ml of the slurry and 0.1 ml of incomplete Freund's adjuvant. (The adjuvants are warmed to 37 °C and vortexed before being loaded into the syringe. Beginning 1 wk after the final immunization, the mice are bled intraorbitally every 7-10 d, and the sera are tested for the presence of specific antibodies. Mice are anesthetized with ether before all immuniza- tions and bleeds.

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Table I. Purification of Tubulin from Drosophila Embryos*

| Fraction       | Volume (ml) | Total protein (mg) | Tubulin (mg) | Percentage of yield | Relative purification |
|----------------|-------------|--------------------|--------------|--------------------|-----------------------|
| Cleared lysate | 330         | 5,115              | ~100         | (100)              | (1.0)                 |
| Phosphocellulose| 450         | 4,275              | ~100         | ~100               | 1.15                  |
| DEAE cellulose | 47          | 183                | 103          | ~100               | 28                    |
| Cycled tubulin | 15          | 56                 | 56           | 56                 | 50                    |

* The starting material was 160 g of frozen 0-4 h Drosophila embryos. The amount of tubulin in each fraction was determined according to Bradford (1976) (cycled tubulin) or by Western blotting, using purified tubulin as a standard. For the procedures used, see Materials and Methods.

Results

Microtubule-Affinity Columns

We have constructed microtubule-affinity columns using methods modeled on those used to construct actin filament affinity columns (Miller and Alberts, 1989). The columns are constructed by covalently linking microtubules to an inert agarose support matrix, using tubulin purified from bovine brain (Mitchinson and Kirshner, 1984) or from early Drosophila embryos (Table I). Fig. 1 shows the result of polyacrylamide gel electrophoresis of the purified tubulins used for column construction. The more slowly migrating second band seen in the purified Drosophila tubulin is a variant form of the α-tubulin subunit, as revealed by Western blotting (Fig. 1 B). This band may correspond to the highly divergent α-4 tubulin identified by Theurkauf et al. (1986).

Microtubules are highly labile structures, and they must be stabilized to survive the conditions required for an affinity chromatography experiment (for example, low temperatures and high salt concentrations). Taxol, a low molecular mass plant toxin, binds tightly to microtubules and confers suitable stability (Horwitz et al., 1982). We routinely construct microtubule-affinity columns with bed volumes ranging from 2 to 20 ml that contain approximately 1 mg/ml of bound microtubules (see Materials and Methods). The columns can be reused at least five times over a 1-mo period without detectable changes in their properties. A control column is constructed by coupling BSA to the same agarose matrix.

Affinity Purification of MAPs from the Early Drosophila Embryo

A cytoplasmic extract is made from early Drosophila embryos under conditions that solubilize >90% of the endogenous tubulin, making it likely that most of the proteins bound to microtubules (MAPs) are solubilized by the extraction procedure. The affinity columns are loaded with the clarified extract, washed extensively with buffer, and then eluted in three steps with CX buffer containing 1.0 mM MgATP, 0.1 M KCl, and 0.5 M KCl, respectively. Fig. 2 is an elution profile comparing the protein obtained from a bovine microtubule-affinity column and an albumin control column. Whereas 1.2% of the total extract protein binds to the microtubule to carry out about five immunofluorescent stainings, whereas the amount of the SI-4 antibody that we obtained allowed only one staining before it was exhausted. Controls that used the apparent background bands at 210-230 kD in Fig. 6 A (0.1 M KCl elution) for affinity purification failed to select any antibody that stained centrosomes from the SI-4 or SI-24 antisera.

Figure 1. SDS polyacrylamide gel and immunoblot analysis of the tubulins used for construction of microtubule-affinity columns. Purified tubulins were resolved on SDS-containing 8.5% polyacrylamide gels (Laemmli, 1970) and visualized by Coomassie blue staining (A). Immunoblot analysis was carried out by transferring the resolved Drosophila tubulin bands to nitrocellulose and probing with antibodies against either alpha or beta tubulin (B). Alkaline phosphatase-conjugated secondary antibody was used to visualize antibody staining. We did not use a high pH running buffer to resolve the alpha and beta subunits of tubulin (Gard and Kirshner, 1985) because these conditions gave poor resolution of the slow-migrating Drosophila α-tubulin band.

Figure 2. An elution profile comparing the amount of protein eluted from a microtubule-affinity column (bovine) and an albumin control column. In this experiment, 15-ml columns were used and 30 ml of extract was loaded onto each column. The columns were eluted with ATP, 0.1 M KCl, and 0.5 M KCl. The amount of protein in each fraction was determined (Bradford, 1976) by using BSA as a standard. The volume of each fraction is 1.5 ml; the protein concentration in the peak fractions from the salt elutions of the microtubule-affinity columns range from 40 to 80 μg/ml.
column, <0.1% binds to the control column, indicating that specific interactions with microtubules are observed.

To examine the proteins that elute from each of the columns, the column fractions were concentrated by TCA precipitation. The proteins were then separated by electrophoresis through an SDS-containing polyacrylamide gel and visualized by Coomassie blue staining (Fig. 3). A large number of different proteins bind specifically to Drosophila and bovine microtubule affinity columns, whereas very little binding is observed to an albumin control column. Moreover, the eluates are not contaminated with any of the major proteins in the extract applied to the columns. Greater than 90% of the total protein that elutes from the microtubule affinity columns, including all of the major species, will rebind to the columns after dialysis back into a low salt buffer, suggesting that the majority of these proteins bind directly to microtubules or are components of tightly associated multiprotein complexes that bind to microtubules (data not shown). Because the proteins eluting from the columns constructed with bovine and Drosophila microtubules are largely identical, it appears that most of the binding sites for MAPs on tubulin are conserved between these two species.

For comparison, we have also isolated MAPs from Drosophila embryo extracts by a procedure based on cosedimentation of MAPs with taxol-stabilized microtubules (Vallee and Collins, 1986). In this procedure, taxol is added to a concentrated cytoplasmic extract to polymerize the endogenous tubulin. Microtubules and their associated proteins are then collected in a pellet by centrifugation. After washing this pellet, MAPs are dissociated from the microtubules with a buffer containing 0.5 M KCl and 1.0 mM MgATP. Since the microtubule protein pellet forms clumps that are difficult to homogenize except in the presence of high salt, we were unable to elute MAPs from it with MgATP alone or with 0.1 M KCl to mimic more closely the microtubule affinity column elutions.

The proteins obtained when the cosedimentation procedure is used to isolate MAPs from a Drosophila embryo extract are analyzed by PAGE in Fig. 4. Comparison of these proteins (lane B) with the total proteins that are retained on a microtubule affinity column (lane D) reveals that a greater number of different MAPs are isolated by the affinity chromatography procedure. Moreover, the proteins isolated by the cosedimentation procedure constitute only 0.5% of the total extract protein, compared with 1.3% of the total protein isolated by affinity chromatography. It is possible that the yield of MAPs obtained by the cosedimentation procedure could be improved by the addition of more tubulin to the extract.

The published cosedimentation procedures for isolating MAPs use a pH 6.8 Pipes buffer. When affinity chromatography experiments are carried out in this buffer, significant amounts of tubulin are lost from the columns in the presence of high salt (data not shown). Our affinity chromatography experiments are therefore carried out at pH 7.6 in Hepes buffer (see Materials and Methods). When the cosedimentation experiment is repeated in the same buffers used for the chromatography experiments, the results more closely resemble those obtained by affinity chromatography. In both cases, the MAPs obtained constitute ~1.2% of the total extract protein, and the pattern of proteins obtained by each procedure is more similar (compare lanes C and D in Fig. 4). Although most of the major MAPs are enriched by both procedures, there are large differences in the relative amounts of various proteins, and many of the minor bands are different. Each procedure has advantages: although the cosedimentation procedure is more rapid and convenient, affinity chromatography allows better control of the elution conditions.

Generation of a Library of Polyclonal Antibodies Against Drosophila MAPs

To begin a characterization of the many proteins that bind to
microtubule affinity columns, we have generated a library of mouse polyclonal antibodies that recognize a large number of them. By means of such antibodies, the proteins obtained in our chromatography procedure can be localized in Drosophila embryos by immunofluorescent staining, so that those associated with microtubules in vivo can be identified. Moreover, MAPs with particularly interesting localizations can be identified and selected for further study, using the antibodies as probes to aid in their further purification and characterization.

In several studies, it has been found that mice will generate an immune response against only the few most abundant and/or immunogenic of the proteins in a complex mixture, limiting the range of the monoclonal antibodies that can be prepared with impure antigens (Miller, K., D. R. Kellogg, and B. M. Alberts, unpublished results; Burke et al., 1982). We have therefore purified our MAPs to homogeneity by preparative PAGE, so as to be able to use each purified protein as an antigen. Mice will generate remarkably specific polyclonal antisera when immunized with a single protein (see below). Hence, the sera may be used directly for immunofluorescence and Western blotting. In addition, because mice can be immunized and bled with relative ease, large numbers of sera can be rapidly screened for the presence of antibodies that give particularly interesting immunofluorescence patterns.

Fig. 5 shows portions of the preparative gradient gels that we used to resolve the proteins that elute from microtubule affinity columns with ATP, with 0.1 M KCl, and with 0.5 M KCl. There are ~40 protein bands of molecular mass >25,000 that can be resolved in the ATP elution, and ~70 protein bands in each of the 0.1 M KCl and 0.5 M KCl elutions. Each of these proteins is assigned an identification code according to its elution behavior and relative molecular mass. For example, the protein designated ATP-1 is the protein of greatest apparent molecular mass that elutes from...
microtubule affinity columns with ATP, and the protein designated S5-1 is the largest protein that elutes with 0.5 M salt. Thus far we have immunized mice with eight of the ATP-eluting proteins, 24 of the 0.1 M KCl-eluting proteins, and 18 of the 0.5 M KCl-eluting proteins (see examples marked on Fig. 5). As a control, five mice were subjected to the same immunization protocol with polyacrylamide containing no protein.

Sera derived from these 50 immunized mice have been screened for the presence of specific antibodies by both Western blotting and immunofluorescence. Preimmune sera from 10 mice showed no response when tested by immunofluorescence. In addition, sera from the five mice subjected to a control immunization protocol showed no response when tested by Western blotting. Although several of these control sera produced a weak centrosomal staining when tested by immunofluorescence on whole fixed embryos, this staining persisted only for 2–3 wk after the final immunization. The experimental mice were therefore scored as reacting positively to an injected protein only if the serum from the mouse maintained a prolonged (>6-wk) titer of an antibody that produced a distinct immunofluorescence staining pattern and/or identified an affinity-purified protein by Western blotting. By these criteria, 24 of the 50 mice injected with a protein band generated an immune response against the injected protein (19 of which were positive by Western blotting). Several mice generated an immune response to proteins that were barely detectable by Coomassie blue staining. We estimate that in these cases we immunized the mice with a total of only 10–25 μg of protein.

Western blotting data for some of the antibodies are shown in Fig. 6. Serum from each mouse was diluted 1:400 and then tested against both microtubule affinity column fractions (Fig. 6 A) and against the crude extract that was loaded onto the affinity columns (Fig. 6 B). Some of the antibodies recognize more than one protein band; this could be because several proteins share the same epitope; more likely, however, it is attributable to proteolysis. We have therefore used arrow heads in Fig. 6 to indicate the band that is of the same molecular mass as the protein band originally injected into the mouse as antigen. In those few cases where only protein bands of different molecular mass than the injected band are detected, we assume that our initial immunization was carried out with a minor proteolytic fragment or that the antibody recognizes an epitope shared by more than one protein. In addition, some of the antibodies that work well for immunofluorescence react only weakly with a protein band on Western blots. This required us to allow the color development reaction to proceed for longer than usual, resulting in a substantial background. The background protein bands are those that are found across all of the lanes in each group (see legend to Fig. 6).

The weak signal observed for some antibodies is not because of a low antibody concentration, since the same signal is obtained over a wide range of dilutions, and the antibodies work well for immunofluorescence. We suspect that in some cases the signal is weak because of poor transfer of proteins to nitrocellulose. This may also explain why some of the antibodies that work well for immunofluorescence do not identify proteins by Western blotting. Antibodies that gave no reaction on Western blots are not shown in Fig. 6; 5 of the 24 antibodies in our library are in this category.

The Western blotting results in Fig. 6 demonstrate that the majority of the sera recognize proteins that are greatly enriched in the microtubule affinity column fractions, as compared to the starting extract (compare A with B). These blots also demonstrate the specificity of mouse polyclonal antibodies, since there is little or no background staining of bands in the extract. The specificity of these antibodies makes them suitable for immunofluorescence localization of their cognate proteins in fixed preparations of *Drosophila* embryos.

**The Subcellular Distribution of MAPs in the Early *Drosophila* Embryo**

We have used immunofluorescent staining of whole *Drosophila* embryos to study the subcellular distribution of the proteins recognized by the 24 antibodies just described. As examples of the results obtained, photomicrographs showing the immunofluorescent localization of the MAPs recognized by three of these antibodies are presented in Fig. 7, 8, and 9. These micrographs display a small area of the surface of an embryo at nuclear cycle 10, the first cycle after the nuclei reach the embryo cortex. In addition to the antibody staining, the DNA has been stained with a second fluorochrome to reveal the location of the nuclei and their stage in the nuclear cycle. The distribution of microtubules in the early *Drosophila* embryo has been discussed in other studies (Karr and Alberts, 1986; Warn and Warn, 1986; Kellogg et al., 1988).

The SI-4 antibody most strongly recognizes a 190-kD protein that elutes from microtubules with 0.1 M KCl. It stains each centrosome as a bright dot throughout the nuclear cycle. Examples of the staining at metaphase (Fig. 7 A) and telophase (Fig. 7 B) are shown. The staining is brightest during anaphase and telophase, and is weakest during interphase.

The S5-39 antibody recognizes a 59-kD protein that elutes from microtubules with 0.5 M KCl. It stains a diffuse and irregular region around each centrosome at prophase (not shown). This staining persists through mitosis, but, in addition, a punctate staining appears at the metaphase plate that suggests kinetochore staining (Fig. 8 A). The staining at anaphase confirms the kinetochore localization. At this stage, one can see localization to the centrosomal region, as well as clear localization to the kinetochore region of the separating chromosomes (Fig. 8 C). There is little or no staining at interphase.

Finally, the distribution of the SI-8 protein (a 175-kD protein that elutes from microtubules with 0.1 M KCl) is illustrated at prometaphase, metaphase, and telophase in Fig. 9. At prometaphase, the antibody appears to stain a series of small dots that are either on the nuclear envelope or the outer edge of the nucleus (Fig. 9 A). The antibody also stains the centrosome at prometaphase, but this is not visible in the focal plane shown in Fig. 9 A. The antibody continues to stain the centrosome at metaphase, but in addition shows a diffuse localization around the spindle (Fig. 9 C). At telophase, the antibody stains both the centrosomal region (arrows, Fig. 9 E) and a region in the middle of the interzonal microtubules (arrowhead). During interphase there is little or no visible staining. Similar data have been obtained for all of the antibodies in our library. The results are summarized in Table II, which lists the molecular mass and relative abundance of the pro-

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Figure 6. Analysis by Western blotting of the antibodies raised against affinity purified MAPs. Procedures are discussed in Materials and Methods. Each antibody has been blotted against both the appropriate affinity column fractions (A) and against the crude extract (B). The blots that compare the affinity column fractions versus the crude extract were allowed to develop in the alkaline phosphatase substrate solution for equal amounts of time, so that the intensities of bands could be compared directly. We tested a total of 23 different mouse sera by Western blotting against the 0.1 M KCl-eluting proteins. All of these sera stained the group of bands in the 210–230 kD region (0.1 M KCl elution), even though 13 of the sera showed no reactivity by immunofluorescence and no specific reactivity by Western blotting. We conclude that these high molecular mass bands represent background staining, a conclusion supported by their failure to affinity purify any centrosome-staining antibodies from several antisera tested (see Materials and Methods). Since Fig. 6 represents a composite of several Western blots, the molecular masses indicated to the left of each set of lanes are approximate (within 10 kD); the exact molecular masses of the protein bands recognized by each of the antibodies are indicated in Table II. An arrowhead is used to indicate the band that is of the same molecular mass as the protein band originally injected into the mouse as antigen. Antibodies that gave no specific reaction on Western blots are not shown. See text for further discussion.
Figure 7. The distribution of the SI-4 antigen at metaphase (A) and telophase (C) of nuclear cycle 10, as revealed by immunofluorescence staining. The embryos have also been labeled with the fluorescent dye 4,6-diamidino-2-phenylindole to reveal the distribution of the DNA, and B and D show the distribution of the DNA in the region of each embryo corresponding to A and C, respectively. For clarity in A and B, the centrosomes corresponding to one mitotic spindle are indicated with arrows, and the position of the corresponding metaphase chromosomes is indicated by arrowhead. Bar, 10 μm.

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Figure 8. The distribution of the S5-39 antigen at metaphase (A) and telophase (C) of nuclear cycle 10, as revealed by immunofluorescence staining. B and D show the distribution of the DNA in the corresponding region of the embryos in A and B. For clarity, the location of the centrosomes and chromosomes corresponding to one mitotic spindle are indicated with arrows and arrowheads, respectively. Bar, 10 μm.

Figure 9. The distribution of the SI-8 antigen at prophase (A), metaphase (C), and telophase (E), as revealed by immunofluorescence staining. B, D, and F show the distribution of the DNA in the corresponding region of the embryos in A, C, and E, respectively. At telophase, the antibody stains not only the centrosomal region (Fig. 9 E arrows), but also a region in the middle of the interzonal microtubules (arrowhead). There is no detectable staining in interphase. Bar, 10 μm.

Discussion

We have used microtubule affinity chromatography to isolate a large number of MAPs from the early Drosophila embryo. This method supplements the more conventional MAP puri-
| Antibody | Molecular mass of antigen (kD) | Antigen abundance | Molecular mass on blots (kD) | Immunofluorescence distribution |
|----------|-------------------------------|-----------------|----------------------------|--------------------------------|
| ATP-2    | 205 + +                       | 205, 192        | Strongly localized to the centrosomal region throughout the nuclear cycle. Weakly localized to microtubule arrays. |
| ATP-3    | 192 + +                       | 205, 192 (205)  | Localized to most or all microtubules throughout the nuclear cycle. |
| ATP-4    | 184 +                         | 164, 147        | Similar to ATP-3, except preferentially localized to astral microtubules at anaphase. |
| ATP-5    | 147 +                         | 164, 147        | Localizes to the centrosomal region throughout the nuclear cycle during preblastoderm divisions. Localizes to microtubule arrays during late cycle 14. |
| ATP-10   | 90 + +                        | 142, 107, 75    | Cytoplasmic localization. Does not appear to be microtubule associated. |
| ATP-12   | 77 + +                        | 77 (77)         | Diffuse blotchy localization near the spindle pole. |
| S1-1     | 335 +                         | 157             | Centrosomal localization from metaphase through telophase. Appearance as a very fine dot. Very weak localization to the spindle. |
| S1-4     | 222 +                         | 222,* (190)     | Centrosomal localization throughout the nuclear cycle. Appears as a fine bright dot, weakest during late interphase and early prophase. See Fig. 7. |
| S1-6     | 194 + +                       |                 | Similar to S1-1. |
| S1-7     | 182 +                         |                 | Localized to spindle during early metaphase, to spindle and centrosomes during late metaphase. Localized to centrosomes and interzonal microtubules during anaphase and telophase. |
| S1-8     | 175 +                         | 175, 103, 93    | Localized to the centrosome at all stages except interphase. Also localized to the nuclear envelope region at prophase, the spindle, and to a region in the middle of the interzonal microtubules. See Fig. 9. |
| S1-14    | 136 + +                       | 160 (160)       | Similar to S1-7. |
| S1-18    | 110 +                         | 110, 96, 92 (110) | Similar to S1-8, but localizes over all interzonal microtubules rather than to a central band during telophase. |
| S1-20    | 102 + +                       |                 | Centrosomal localization from metaphase through telophase. Appearance as a cluster of fine dots. |
| S1-24    | 89 + +                        | 105, 89         | Centrosomal localization from anaphase through early interphase. Appears as a fine dot, somewhat weak. |
| S1-25    | 86 + + +                      |                 | Centrosomal localization from metaphase through telophase. Appears as a diffuse region, slightly punctuate. Very weakly localized to the spindle. |
| S1-27    | 84 + +                        | 148, 109        | Not detectable by immunofluorescence. |
| S1-28    | 79 + +                        |                 | Centrosomal localization throughout the nuclear cycle. Very weak localization to the spindle. |
| S5-32    | 68 + +                        | 82, 68 (82, 68) | Localized to centrosome at anaphase, telophase, and early cycle 14. Localized to DNA strongly during interphase and telophase, weakly during the remainder of the nuclear cycle. Neuronal? |
| S5-38    | 60 + +                        | 60 (60)         | Neuronal? |
| S5-39    | 59 + +                        | 60 (60)         | Neuronal? |
| S5-45    | 52 +                         | 93 (93)         | Centrosomal localization from prophase through telophase. See Fig. 8. |
| S5-47    | 50 +                         | 190             | Centrosomal localization throughout the nuclear cycle. Appears as a fine bright dot. |
| S5-50    | 46 + + +                      | 180, 80, 46 (180, 80, 46) | Centrosomal localization throughout nuclear cycle. Very bright at anaphase/telophase, weak at interphase. Localized weakly to the spindle. Neuronal? |

Regular numbers indicate the molecular masses of protein bands recognized by Western blotting in the enriched microtubule affinity column fractions, whereas numbers in parentheses indicate the molecular masses of protein bands recognized in the crude extract. A minus sign indicates that no protein bands are detectable under the conditions that we have used for Western blotting.

* The 222-kD band recognized by the S1-4 antibody is also recognized as a background band by other antibodies (see legend to Fig. 6). The S1-4 antibody most strongly recognizes the 190-kD band.
Figure 10. Immunofluorescent staining with affinity-purified S5-47 antibody. The antibody was affinity purified using a specific MAP gel band immobilized on nitrocellulose, as described in the text. Antibody staining is shown in Fig. 10 A, whereas Fig. 10 B shows the distribution of the DNA in the corresponding region of the embryo, as revealed by 4,6-diamidino-2-phenylindole staining. The embryo is in metaphase of nuclear cycle 10. Similar results have been obtained with antibodies SI-4 and SI-24. Bar, 10 μm.
larly interesting to us, since they represent the first biochemically
defined MAPs that localize uniquely to this important
microtubule organizing center (for reviews, see Karsenti and
Maro, 1986; Vorobjev and Nadezhdina, 1987). Previously,
the centrosome has been characterized only by functional or
morphological criteria (Mitchison and Kirschner, 1984;
Vorobjev and Chentsov, 1982; Rieder and Borisy, 1982), or
by its staining with monoclonal and autoimmune antibodies
(Calarco-Gilliam et al., 1983; Moroi et al., 1983; Gosti-
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