Molecular Biology of the Cell
Vol. 3, 1–11, January 1992

Purification of a Multiprotein Complex Containing Centrosomal Proteins from the *Drosophila* Embryo by Chromatography With Low-Affinity Polyclonal Antibodies

Douglas R. Kellogg* and Bruce M. Alberts†

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143-0448.

Submitted September 5, 1991; Accepted October 3, 1991

A 190-kDa centrosomal protein interacts with microtubules when *Drosophila* embryo extracts are passed over microtubule-affinity columns. We have obtained a partial cDNA clone that encodes this protein. Using a fusion protein produced from the clone, we have developed a novel immunoaffinity chromatography procedure that allows both the 190-kDa protein and a complex of proteins that associates with it to be isolated in a single step. For this procedure, the fusion protein is used as an antigen to prepare rabbit polyclonal antibodies, and those antibodies that recognize the 190-kDa protein with low affinity are selectively purified on a column containing immobilized antigen. These low-affinity antibodies are then used to construct an immunoaffinity column. When *Drosophila* embryo extracts are passed over this column, the 190-kDa protein is quantitatively retained and can be eluted in nearly pure form under nondenaturing conditions with 1.5 M $MgCl_2$, pH 7.6. The immunoaffinity column is washed with 1.0 M KCl just before the elution with 1.5 M $MgCl_2$. This wash elutes 10 major proteins, as well as a number of minor ones. We present evidence that these KCl-eluted proteins represent additional centrosomal components that interact with the 190-kDa protein to form a multiprotein complex within the cell.

INTRODUCTION

The centrosome is thought to play a central role in a variety of cellular events, including cell division, chromosome segregation, directed cell movements, and the overall organization of the cytoplasm (for reviews, see Brinkley, 1985; Karsenti and Maro, 1986; Vorobjev and Nadezhdina, 1987). Yet this important organelle remains poorly understood. For instance, little is known about the factors that control centrosomal duplication or the nucleation of microtubules by the centrosome, and most of the proteins that function as components of the centrosome have yet to be discovered. A biochemical characterization of the centrosome has been difficult, not only because centrosomes are present in small quantities but also because we lack good assays to aid in the purification of centrosomal proteins. Genetic approaches to understanding the centrosome have also gone slowly, perhaps because it is difficult to predict the phenotypes of mutations in centrosomal proteins.

In a recent study, we used a combination of microtubule-affinity chromatography and immunocytology to identify *Drosophila* microtubule-associated proteins (MAPs)[1] that are components of the centrosome (Kellogg *et al.*, 1989). One of the antibodies described in this

* Present address: Department of Physiology, University of California, San Francisco, San Francisco, CA 94143.
† American Cancer Society Research Professor.

[1] Abbreviations used: EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; GST, glutathione S-transferase; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MAP, microtubule-associated protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; Tris, tris(hydroxymethyl)-aminomethane.

study recognized a 190-kDa centrosomal protein (initially designated as S1-4). Until more is known about the function of this protein, we have chosen to call it DMAP 190, indicating that it is a 190-kDa microtubule-associated protein from *Drosophila melanogaster*. In the present report, we describe the cloning of a partial cDNA for DMAP 190. Starting with this clone, we have developed a procedure for immunoaffinity chromatography that has allowed us to purify native DMAP 190 and its associated proteins from *Drosophila* embryo extracts.

Immunoaffinity chromatography has not been generally useful for the purification of proteins in their native state, because the relatively harsh conditions required for the dissociation of antigens from antibodies will cause denaturation of most proteins. In some cases, however, it has been possible to obtain monoclonal antibodies that bind to their antigens with low affinity. The antigens may be dissociated from these low-affinity antibodies under relatively mild conditions, thus preserving their native function. Immunoaffinity columns constructed with such low-affinity monoclonal antibodies have been very effective in the purification of proteins (Chang *et al.*, 1984; Simanis and Lane, 1985; Chang and Bollum, 1986; Reyland and Loeb, 1987; Ishimi *et al.*, 1988).

We reasoned that similar low-affinity antibodies should exist in a typical polyclonal serum, and we designed a procedure that selects such antibodies. We report here the isolation of low-affinity polyclonal antibodies that recognize DMAP 190 and the use of these antibodies to construct an immunoaffinity chromatography column. Fractionation of *Drosophila* embryo extracts on this column allows convenient single-step purification of DMAP 190, as well as the purification of additional centrosomal proteins that appear to interact with DMAP 190 to form a multiprotein complex within the cell.

MATERIALS AND METHODS

Materials

Glycerol was spectrophotometric grade from Eastman Kodak (Rochester, NY). Affigel 10 was from Bio-Rad (Richmond, CA). Dimethylpimelimidate was from Sigma (St. Louis, MO). Protein A-Sepharose CL-4B and bovine serum albumin (Fraction V) were from Sigma. All other materials were as described in Kellogg *et al.* (1989).

Buffers

Extract buffer contained 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, 50 mM KCl, 1.0 mM Na_3 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1.0 mM Na_3EDTA, 0.05% NP-40. Protease inhibitor stock contained 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 1:100–1:1000, as noted). Column buffer contained 50 mM HEPES, pH 7.6, 50 mM KCl, 1.0 mM Na_3EGTA, 1.0 mM $MgCl_2$, 10% glycerol. Tris(hydroxymethyl)aminomethane (Tris)-buffered saline (TBS) contained 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl. Phosphate-buffered saline (PBS) contained 140 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2, 138 mM NaCl, 2.7 mM KCl. Polyacrylamide gel sample buffer contained 63 mM Tris-HCl, pH 6.8, 3% sodium dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol.

DNA Cloning

To clone DMAP 190, we screened a lambda gt11 cDNA expression library with mouse polyclonal antibodies (antibody S1-4 in Kellogg *et al.*, 1989). The expression library was constructed from *Drosophila* ovarian mRNA by Steinhauer *et al.* (1989) and was screened as described (Huynh *et al.*, 1985) with the following modifications. For plaque lifts, we leave the nitrocellulose filters on the plates for 4–8 h. At the end of this incubation period, the filters are lifted off of the plates and placed in a glass dish containing water that has just been brought to a boil. The filters are left in the dish for 5 min without further heating of the water and are then washed several times with water, stored in TBS, and probed with the antibody within 20 h. Treating the filters with boiling water in this way reduced the background and seemed to prolong the life of the diluted primary antibody solution that was used repeatedly to screen the library.

All antibodies used for screening the library were diluted into PBS containing 0.05% Tween-20 and 5% nonfat dry milk or bovine serum albumin. As the primary antibody, we used a mouse polyclonal antibody at a dilution of 1:500, and we monitored the quality of the diluted primary antibody throughout the screening procedure by using it at intervals for immunofluorescence staining of embryos. Additional primary antibody was added to refresh the original solution as needed. As the secondary antibody, we used alkaline phosphatase-conjugated goat anti-mouse antibody (Boehringer-Mannheim, Indianapolis, IN).

We screened ~600 000 phage plaques and obtained five potential positive clones. One of these phage clones carried an insert that yielded three fragments when cut with *Eco*R1. We subcloned the smallest of these fragments (831 bp) into the *Eco*R1 site of the vector pGEX.1 (Smith and Johnson, 1988). This vector directs expression of protein sequences fused to the enzyme glutathione S-transferase (GST). The subclones that carry the *Eco*R1 fragment in the correct orientation were identified by screening transformants for those that synthesize a protein recognized by the mouse polyclonal antibody. We have called the 73-kDa fusion protein synthesized by this construct GST/190-c. Rabbit polyclonal antibodies raised against this fusion protein recognize a *Drosophila* 190-kDa protein that is enriched by microtubule-affinity chromatography, and the antibodies stain the centrosome in a manner that is identical to the staining observed with the original mouse polyclonal serum. These results demonstrate the correct identity of the cDNA clone.

Construction of GST and GST/190-c Protein Affinity Columns

The purification of polyclonal antibodies that are specific for DMAP 190 requires affinity columns that have GST and GST/190-c bound to them (see below). To construct such columns, the GST protein and the GST/190-c fusion protein are purified by glutathione-affinity chromatography as described (Smith and Johnson, 1988), except that the glutathione resin is washed and eluted on a column rather than batchwise. This gives a purified fusion protein that is more concentrated and largely free of contaminating proteins. From 6 g of packed *Escherichia coli* cells, we obtain 125 mg of GST and 45 mg of GST/190-c. The purified proteins are dialyzed into 50 mM HEPES, pH 7.6, 25 mM KCl and are coupled to Affigel 10 agarose at 5–10 mg protein per ml of agarose, according to the manufacturer's instructions. All antigen and antibody columns used in these experiments are stored at −20°C in PBS containing 50% (wt/vol) glycerol.

Isolation of Low-Affinity Antibodies that Recognize DMAP 190

To generate antibodies against DMAP 190, a total of 6 mg of the GST/190-c fusion protein was used to immunize three rabbits. The

rabbits were bled periodically to obtain 300 ml of serum. The immunizations and bleeds were carried out by the Berkeley Antibody Company (Richmond, CA). Before antibodies can be isolated that are specific for the DMAP 190 portion of the fusion protein, it is necessary to remove the antibodies that recognize the GST portion of the protein. This is accomplished by passing the serum over a column containing 200 mg of purified GST at room temperature, using a flow rate of 3–5 column volumes/h. The serum is centrifuged at 100 000 × *g* for 30 min before passing it over the column. To regenerate the column, the anti-GST antibodies are eluted from the column with acid (0.5% acetic acid, 0.15 M NaCl) and then with base (100 mM triethylamine, pH 11.5).

The serum is passed over the GST column several times until no more antibody is bound. The antibodies in the serum that are specific for the DMAP 190 portion of the protein are then isolated by passing the depleted serum over a 4-ml column containing 25 mg of bound GST/190-c fusion protein. This column is loaded at room temperature with a flow rate of 4 column volumes/h and is washed with TBS until no protein can be detected in the flow-through (~25 column volumes). The low-affinity anti-DMAP 190 antibodies are eluted with buffer containing 1.4 M $MgCl_2$, 10% glycerol, and 50 mM HEPES, pH 7.6. The elution is carried out by pipetting 1.0-ml aliquots of the elution buffer directly onto the top of the column bed. The buffer is allowed to flow through the column by gravity and is collected in 1.5-ml plastic tubes. The column fractions are assayed for the presence of antibody by measuring absorbance at 280 nm, and the peak fractions are pooled. The yield of antibody is determined using an extinction coefficient of 1.35 mg ml^{-1}. Bovine serum albumin (Sigma, Fraction V) is added to these low-affinity antibodies as a carrier protein (5 mg albumin/1 mg antibody), and the antibodies are then dialyzed into PBS containing 0.02% sodium azide and 50% (wt/vol) glycerol. The low-affinity antibodies are stored at −20°C until use. Elution of antibodies from the column with buffer containing 1.0 M $MgCl_2$ instead of with 1.4 M $MgCl_2$ yields about one-half the antibody obtained with the 1.4 M $MgCl_2$ elution.

After elution of the low-affinity antibodies, the high-affinity anti-DMAP 190 antibodies are eluted from the column with 0.5% acetic acid, 0.15 M NaCl. Each column fraction from the acid elution is immediately neutralized by the addition of Na_2HPO_4 from a 1-M stock. The yield of low- and high-affinity antibodies from 300 ml of serum was 16 and 42 mg, respectively. A similar ratio of low- to high-affinity antibodies has been obtained from different rabbits injected with the same antigen and from rabbits injected with a different antigen. Additional high-affinity antibody can be obtained by eluting the column with 100 mM triethylamine (pH 11.5) after the acid elution. We use the low-affinity antibodies for immunoaffinity chromatography and the high-affinity antibodies for immunofluorescence and western blotting.

Immunoaffinity Chromatography

The low-affinity antibodies to DMAP 190 are coupled to protein A Sepharose using dimethylpimelimidate as described (Harlow and Lane, 1988). For our experiments, we coupled 3 mg of low-affinity antibody to 1 ml of protein A Sepharose. A control column was constructed by coupling 3 mg of IgG from preimmune sera to the same column matrix. The control IgG was purified by use of a protein A column (Harlow and Lane, 1988).

We used 15–20 g of *Drosophila* embryos (age 0–4 h) as the starting material for our immunoaffinity purification procedure. The embryos were harvested, dechorionated, and washed extensively with distilled water as previously described (Miller and Alberts, 1989). They were then suspended in 6 volumes of extract buffer containing protease inhibitor stock (1:100). Phenylmethylsulfonyl fluoride was added to 1 mM, and the embryos were immediately homogenized by several passes of a motor-driven Teflon dounce homogenizer. The crude extract was centrifuged for 10 min at 10 000 rpm in a SS34 rotor (Dupont Sorvall, Wilmington, DE), followed by 60 min at 40 000 rpm in a Beckman (Fullerton, CA) 50.2Ti rotor. The supernatant was collected with a pipette, taking care to avoid the loose pellet at the bottom of the tube. We also tried to avoid collecting the floating lipid layer, although we were unable to exclude this material completely.

The clarified extract was passed through three different columns connected in series. The first column was a 15-ml column that was constructed with Sepharose CL-4B (Pharmacia, Piscataway, NJ). This column served to filter out any protein aggregates that may form in the extract during the column loading procedure. Such aggregates would otherwise be filtered out by the immunoaffinity column, producing a contaminating background of nonspecific proteins in the final eluate due to solubilization by the elution buffers. After flowing through the Sepharose CL-4B, the extract flowed through a 1-ml control IgG column and then through a 1-ml anti-DMAP 190 immunoaffinity column. The columns were loaded in series to load the maximal amount of extract onto the anti-DMAP 190 immunoaffinity column. The columns were initially loaded at a flow rate of 12 ml/h, and a sample was taken from the first flow-through fraction that contained the same concentration of protein as the extract (this is the "flow-through" fraction in Figures 4 and 5). The flow rate was increased to 20–30 ml/h to load the remainder of the extract, and the three columns were then disconnected from each other. The control IgG column and the immunoaffinity column were washed with 75 column volumes of column buffer containing protease inhibitors (1:200) and 0.05% NP-40 detergent. This wash step was carried out overnight using gravity to produce a flow rate of 10–30 ml/h. All columns were run at 4°C.

Before elution, the columns were washed with several column volumes of column buffer to remove the NP-40 detergent present in the wash buffer. The columns were then eluted with column buffer containing 1.0 M KCl, followed by a buffer containing 50 mM HEPES, pH 7.6, 1.5 M $MgCl_2$, and 10% glycerol. The elutions were carried out by pipetting 0.3-ml aliquots of the elution buffer directly onto the surface of the column bed. The 0.3-ml aliquots were then allowed to flow through the column by gravity and were collected in 1.5-ml plastic tubes containing 3 μl of 0.1 M dithiothreitol. This reducing agent was excluded from the extract and wash buffers due to concerns that it could destabilize the antibodies on the column. The column fractions were assayed for protein by the method of Bradford (1976), using bovine gamma globulin as a standard. Small samples were removed from the peak fractions and mixed with four times gel sample buffer for analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).

The maximal yield of DMAP 190 from our column was 0.25 mg, which represents the capacity of the column. This was only ~5–10% of the theoretical maximum capacity. A large fraction of the antibody was apparently either inaccessible or inactivated by the procedure used to couple the antibody to the column. Alternatively, some of the polyclonal antibodies on the column may recognize sites on DMAP 190 that are masked by *Drosophila* embryo proteins that bind to DMAP 190. We carried out experiments to determine whether coupling antibody to hydra-azide activated column matrices (Bioprobe International, Tustin, CA) would yield immunoaffinity columns with higher capacities for binding of the antigen. Thus far, we have observed only moderate increases in the binding capacity, and we lost significant amounts of antibody in the steps used to prepare these columns (K. Oegema, unpublished results).

Generation of Mouse Polyclonal Antibodies Against DMAP 60

Mouse polyclonal antibodies that specifically recognize DMAP 60 were generated by immunizing mice with polyacrylamide gel purified protein as described (Kellogg *et al.*, 1989). Approximately 100 μg of DMAP 60 was resolved from the other proteins in the 1.0 M KCl-eluting fraction by preparative SDS-PAGE. After staining with Coomassie blue, three separate protein bands can be resolved in the 60-kD region on an 8.5% polyacrylamide gel. Each of these three bands was excised and used to immunize a separate mouse.

Western Blotting and Immunofluorescence

For western blotting experiments (Towbin *et al.*, 1979), proteins are separated by electrophoresis on 8.5% SDS-containing polyacrylamide gels, and are transferred to nitrocellulose (pore size 0.1 μm) in the presence of 25% methanol, 0.15 M glycine, and 0.02% SDS. The blots are preincubated for 15 min in PBS containing 5% nonfat dry milk, 10% glycerol, 0.05% Tween-20 detergent, and 0.02% sodium azide. The addition of glycerol to this solution helps prevent the milk from precipitating during prolonged storage at 4°C. The antibodies are diluted in the same buffer. For secondary antibodies, we use fresh dilutions of alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibodies. The immunofluorescence staining of methanol-fixed embryos is carried out as described (Kellogg *et al.*, 1988). For both western blotting and immunofluorescence, the affinity-purified anti-DMAP 190 rabbit polyclonal antibody was used at 1–2 μg/ml and the anti-DMAP 60 mouse serum was used at a dilution of 1:500.

RESULTS

Cloning of the Gene for DMAP 190

Partial cDNA clones encoding DMAP 190 were isolated by screening an ovarian lambda gt11 cDNA expression library with mouse polyclonal antibodies. The antibodies were raised against a *Drosophila* embryo MAP identified by microtubule-affinity chromatography (antibody S1-4 in Kellogg *et al.*, 1989). One phage clone identified in the screen carried an insert that yielded three fragments when cut with *Eco*R1. The smallest of these fragments (831 bp) was subcloned into the vector pGEX.1, which directs expression of proteins fused to the enzyme GST (Smith and Johnson, 1988). We have called the 73-kDa fusion protein expressed from this construct GST/190-c. The GST portion of this protein (26 kDa) allows its rapid purification on a glutathione-agarose column (Smith and Johnson, 1988). Affinity-purified rabbit polyclonal antibodies raised against the purified GST/190-c fusion protein recognize a 190-kDa *Drosophila* embryo protein that is quantitatively retained on microtubule-affinity columns (Figure 1), and the antibodies give cell cycle-specific staining of the centrosome in a manner identical to the original mouse polyclonal serum (Figure 2). The staining of the centrosome is weakest during interphase and increases dramatically during mitosis, reaching maximal levels during anaphase and telophase.

The affinity-purified rabbit polyclonal antibody also gives a very weak staining of the mitotic spindle and a nuclear staining during interphase in embryos older than nuclear cycle 12. For unknown reasons, the original mouse antibody does not reveal these spindle and nuclear localizations of DMAP 190. We have recently found that the gene that encodes DMAP 190 is identical to the gene that encodes the protein recognized by the Bx63 monoclonal antibody (Whitfield *et al.*, 1988). This conclusion is based on a comparison of our partial sequence for the DMAP 190 cDNA clone with sequences from the Bx63 clone (Whitfield and Glover, personal communication). The Bx63 monoclonal antibody was originally isolated in a screen for monoclonal antibodies that recognize nuclear antigens in the early *Drosophila* embryo (Frasch *et al.*, 1986).

The Use of Low-Affinity Antibodies to Purify DMAP 190 and Its Associated Proteins

The further characterization of DMAP 190 must address two fundamental questions: what is the function of this protein and how is it localized to the centrosome in a cell cycle-specific manner? As a first step toward addressing these questions, we wanted to purify DMAP 190 and identify proteins that interact with it. The purified DMAP 190 could be studied in vitro to determine whether it has any activities that affect microtubule dynamics, and it could be fluorescently labeled and injected back into embryos to study its behavior in vivo. Proteins that interact with DMAP 190 could be studied in a similar manner. The identification of proteins that interact with DMAP 190 is of particular importance, because most proteins function within the cell as components of multiprotein complexes, rather than as isolated proteins.

We have used an immunoaffinity chromatography approach to purify DMAP 190 and its associated proteins. For this procedure, polyclonal antibodies that recognize DMAP 190 with low affinity are isolated and used to construct an immunoaffinity column. This is done by immunizing rabbits with the GST/190-c fusion protein. The serum from these rabbits contains antibodies that recognize the GST portion of the fusion protein, as well as antibodies that recognize the DMAP 190 portion. The antibodies in the serum that recognize GST are removed by passing the serum repeatedly over a column matrix that has purified GST covalently bound to it. The antibodies that are specific for the DMAP 190 portion of the fusion protein are then isolated by passing the serum over a column containing covalently bound GST/190-c fusion protein. After washing this column extensively, the antibodies that recognize DMAP 190 with low affinity are eluted with a buffer containing 50 mm HEPES, pH 7.6, 1.4 M $MgCl_2$, and 10% glycerol. The high-affinity antibodies remain on the column and are eluted with 0.5% acetic acid, 0.15 M NaCl. The yield of low- and high-affinity antibodies from 300 ml of serum was 16 and 42 mg, respectively.

We construct immunoaffinity columns for the purification of DMAP 190 by covalently linking 3 mg of the low-affinity antibodies to 1.0 ml of protein A Sepharose. A control IgG column is constructed by linking 3 mg of preimmune IgG antibodies to the same column matrix (see MATERIALS AND METHODS).

Immunoaffinity Purification of a Multiprotein Complex Containing the DMAP 190 Protein

A cytoplasmic extract made from 18 g of early *Drosophila* embryos is loaded onto both an anti-DMAP 190 im-

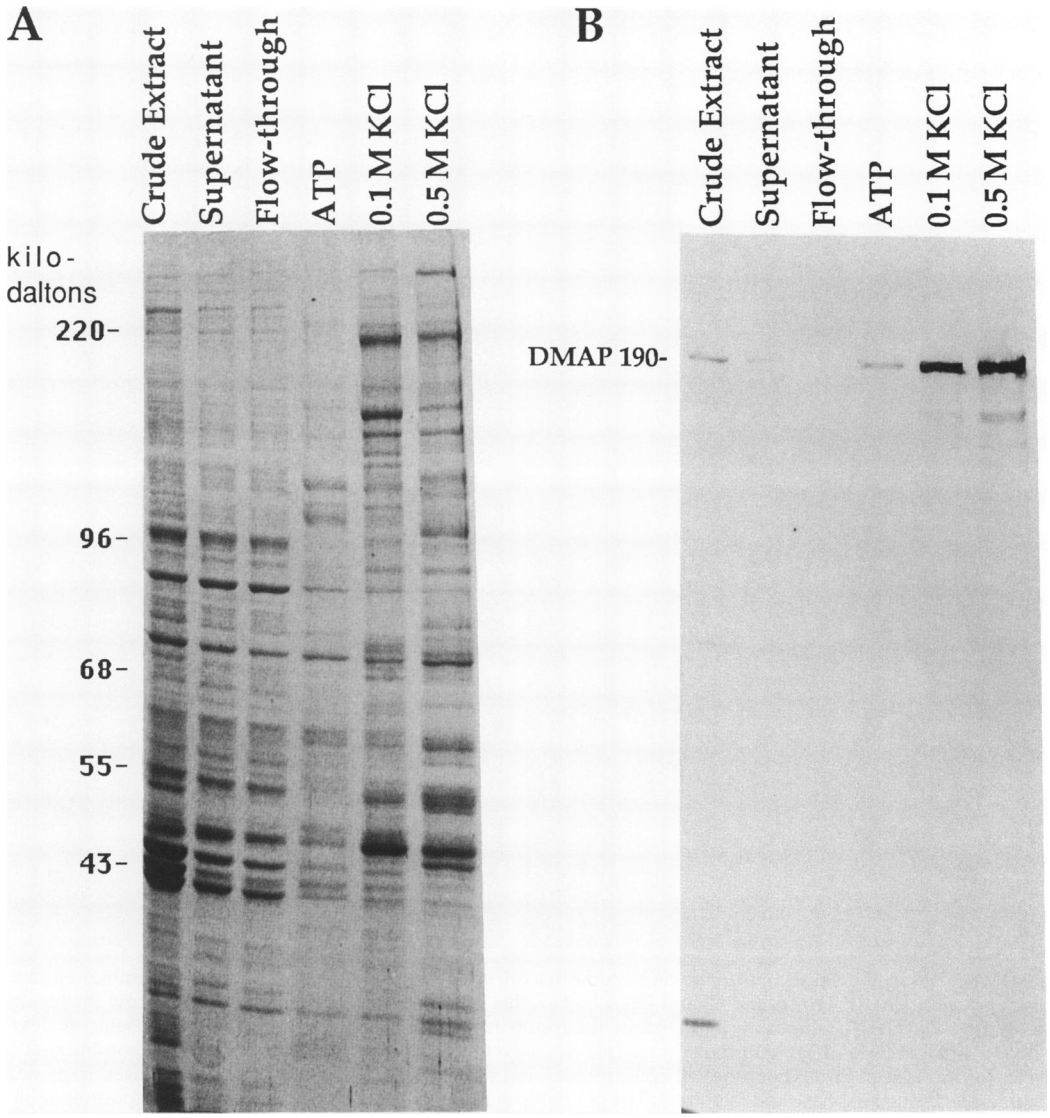

Figure 1. Antibodies raised against the DMAP 190 fusion protein recognize a 190-kDa protein that is retained on a microtubule-affinity column. (A) An 8.5% polyacrylamide gel showing the *Drosophila* embryo proteins that bind to a microtubule-affinity column. Microtubule-affinity chromatography was carried out as described previously (Kellogg *et al.*, 1989). Lanes: 1, Crude extract, 20 μg; 2, high-speed supernatant (column load), 15 μg; 3, column flow-through (unbound proteins), 15 μg; 4, ATP elution, 10 μg; 5, 0.1 M KCl elution, 28 μg; and 6, 0.5 M KCl elution, 30 μg. The gel is stained with Coomassie blue. (B) A western blot showing the behavior of DMAP 190 in a microtubule-affinity chromatography experiment. A polyacrylamide gel identical to the one shown in A was transferred to nitrocellulose and probed with the affinity-purified rabbit polyclonal antibody that recognizes DMAP 190. Note that DMAP 190 is quantitatively depleted from the extract by the microtubule-affinity column (compare the supernatant with the column flow-through). In the crude extract, the anti-DMAP 190 antibody reproducibly stains a protein band at 25 kDa, as well as DMAP 190 at 190 kDa. Because DMAP 190 is enriched by microtubule-affinity chromatography and we have been able to isolate an additional centrosomal protein that interacts with it (see below), the 25 kDa protein is unlikely to be the protein that is recognized by the anti-DMAP 190 antibody in the centrosome. Because the 25 kDa protein is insoluble (compare crude extract with supernatant gel lanes), we could not follow its behavior on affinity columns.

munoaffinity column and a control IgG column. After washing extensively with column buffer, the columns are eluted with the same buffer containing 1.0 M KCl, followed by buffer containing 1.5 M $MgCl_2$. The 1.0 M KCl disrupts most interactions between proteins but it does not disrupt the antibody-antigen interaction. The 1.5 M $MgCl_2$ buffer disrupts the antibody-antigen interaction and releases DMAP 190 from the immunoaffinity column. Figure 3 shows an elution profile that compares the amount of protein eluted from the anti-DMAP 190 column with that eluted from the control column. The 1.0 M KCl eluate from the anti-DMAP 190 immunoaffinity column contains 1.6 mg of protein, whereas the 1.5 M $MgCl_2$ eluate contains 0.25 mg of

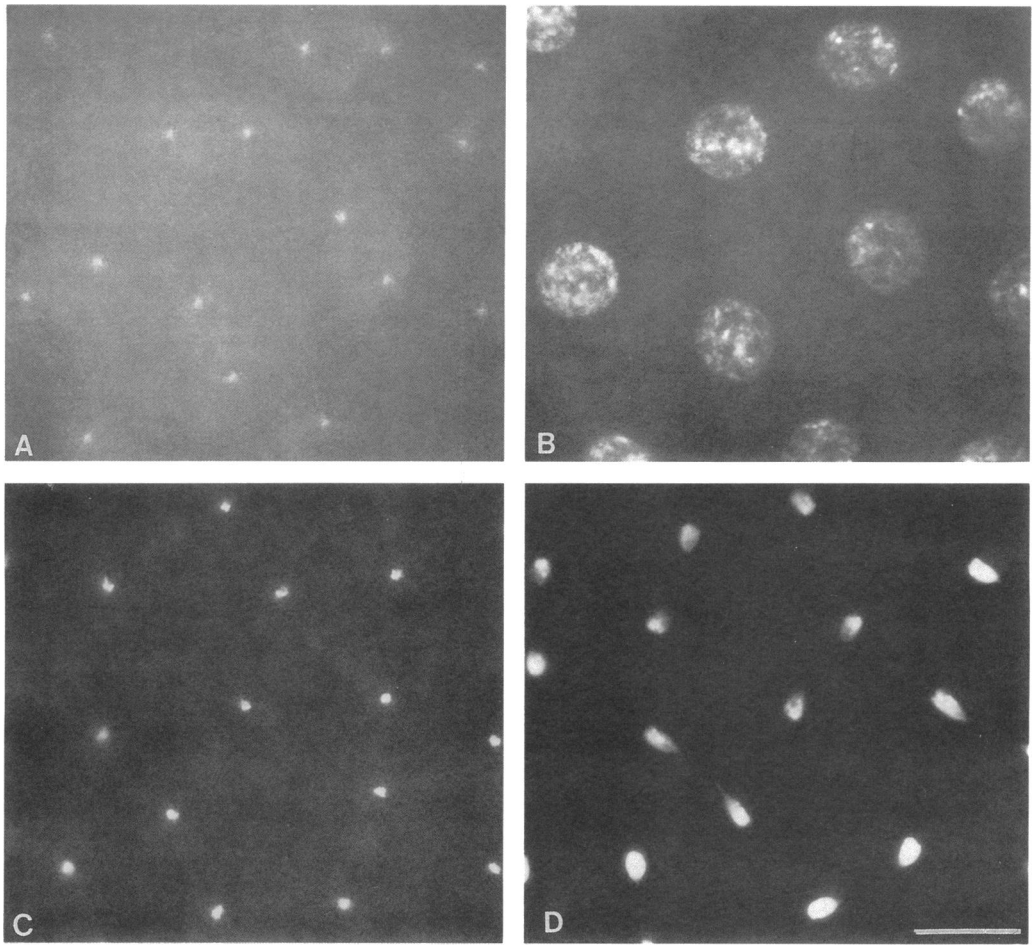

Figure 2. Anti-DMAP 190 antibodies recognize the centrosome in a cell cycle-specific manner. A double-label immunofluorescence experiment showing the distribution of DMAP 190 (A and C) and DNA by Hoechst 33258 staining (B and D). A and B show the results for an embryo in early prophase of nuclear cycle 10, whereas C and D show the results for an embryo in late anaphase of the same nuclear cycle. The photomicrographs show a small section of the embryo surface (see Kellogg *et al.*, 1988, for the distribution of microtubules at this stage). Similar staining of the centrosome is seen throughout the cell cycle; the staining is weakest during interphase and increases dramatically during mitosis, reaching maximal levels during anaphase and telophase. The photomicrographs were produced using identical exposures and printing conditions. The relatively high level of cytoplasmic staining seen during early prophase may represent DMAP 190 that has not been incorporated into the centrosome. Bar is 10 μm.

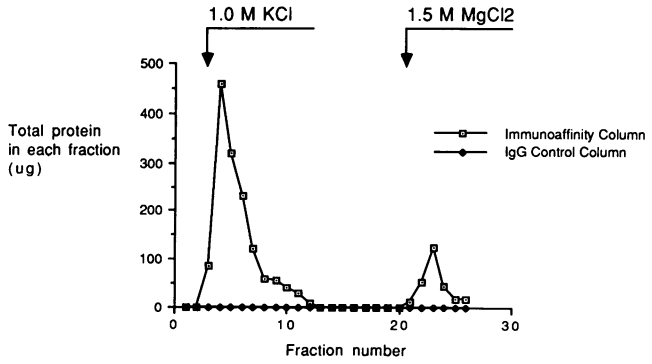

Figure 3. An elution profile comparing the amount of *Drosophila* embryo protein that elutes from the anti-DMAP 190 immunoaffinity column and a control IgG column. Chromatography was carried out as described in the text. Each column fraction has a volume of 0.3 ml.

protein. Very little protein is retained on the control IgG column.

An SDS polyacrylamide gel analysis of the proteins contained in these fractions is shown in Figure 4A. The 1.5 M $MgCl_2$ elution contains the DMAP 190 centrosomal protein in highly purified form, plus small amounts of a 70-kDa protein that can be ignored, because it is also retained on the control column. The 1.0 M KCl elution contains ~10 major protein bands, as well as a number of minor ones. We present evidence below that these proteins are retained on the immunoaffinity column by virtue of their interactions with DMAP 190. Because the immunoaffinity column is washed with 75 column volumes of buffer before elution, these proteins must interact with DMAP 190 with high affinity to be retained. The same group of proteins is seen in the 1.0 M KCl eluate when the immunoaffinity

column is constructed with the high-affinity antibodies that recognize DMAP 190; however, in this case DMAP 190 cannot be eluted with 1.5 M $MgCl_2$.

Additional proteins can be detected in the 1.0 M KCl eluate when larger amounts of total protein are loaded on the gel. These may represent proteins that interact with DMAP 190 with lower affinity or proteins that are retained on the column by virtue of secondary interactions with the proteins that interact directly with DMAP 190. It is also possible that some of these proteins bind to DMAP 190 in vitro, but not in vivo, as we have not tested most of them for their in vivo localization. Finally, some proteins could be retained on the immunoaffinity column because they cross-react weakly with the anti-DMAP 190 antibody. However, this seems unlikely because no cross-reacting proteins are detected in the 1.0 M KCl eluate by western blotting (Figure 4B, see below).

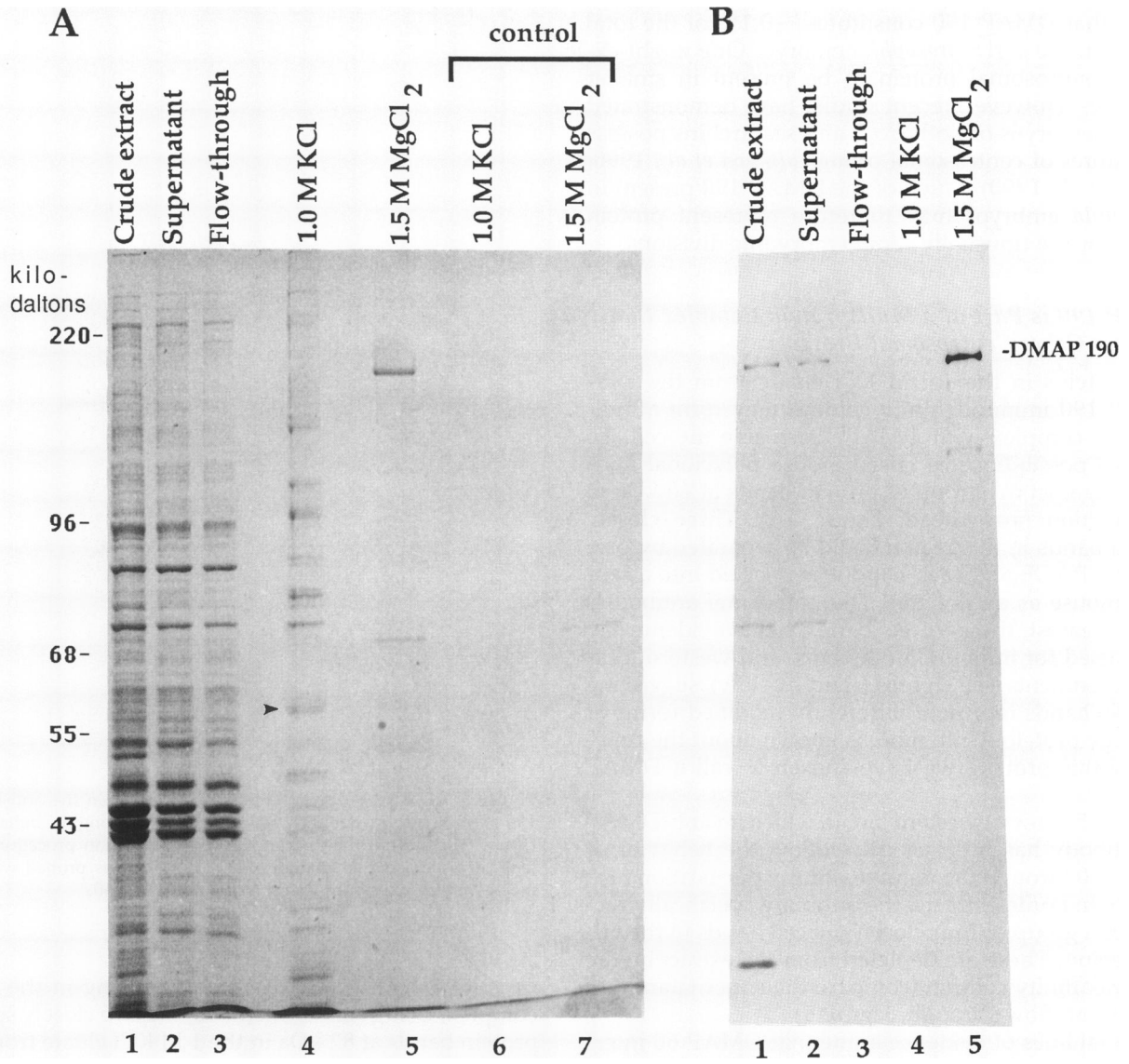

Figure 4. Chromatography of a *Drosophila* embryo extract on anti-DMAP 190 and control IgG immunoaffinity columns. (A) Analysis of the proteins that bind to the immunoaffinity columns by electrophoresis through an SDS-containing 8.5% polyacrylamide gel. Lanes 1–5 show results for the anti-DMAP 190 column: 1, Crude extract, 20 μg; 2, high-speed supernatant (column load), 15 μg; 3, column flow-through (unbound proteins), 15 μg; 4, 1.0 M KCl elution, 7 μg; and 5, 1.5 M $MgCl_2$ elution, 2 μg. Lanes 6 and 7 show the 1.0 M KCl and 1.5 M $MgCl_2$ elutions, respectively, from the control IgG column. Lanes carrying the salt elution fractions are separated by blank lanes to avoid distortion of adjacent lanes by the high amount of salt in these fractions. The gel is stained with Coomassie blue. (B) A western blot showing the behavior of DMAP 190 through the immunoaffinity purification procedure. Proteins were resolved on an SDS-containing 8.5% polyacrylamide gel and then transferred to nitrocellulose. The protein samples used for the western blot were identical to the ones used in Lanes 1–5 of A, except that the KCl elution lane is loaded with 0.7 μg protein and the $MgCl_2$ elution lane is loaded with 0.2 μg protein. The blot was probed with the high-affinity rabbit polyclonal antibodies that recognized DMAP 190.

Figure 4B is a western blot that shows the behavior of DMAP 190 through the immunoaffinity purification procedure. DMAP 190 is a soluble protein (compare the crude extract with the clarified supernatant), and the immunoaffinity column depletes the extract of DMAP 190 (compare the supernatant with the flow-through fraction). We have estimated the amount of DMAP 190 present in embryo extracts by comparing the intensity of the signal for DMAP 190 in the crude extract with standards prepared by testing varying amounts of the purified protein on western blots. We can thereby estimate that DMAP 190 constitutes ~0.1% of the total protein in the early *Drosophila* embryo. One might expect a centrosomal protein to be present in smaller quantities. However, recent studies have demonstrated that the embryos of both frogs and sea urchins possess large stores of centrosomal proteins (Gard *et al.*, 1990; Sluder *et al.*, 1990). Much of the DMAP 190 present in *Drosophila* embryos may therefore represent protein stored for the unusually rapid embryonic divisions.

DMAP 190 is Part of a Multiprotein Complex that Includes Other Centrosomal Proteins

The proteins in the 1.0 M KCl eluate from the anti-DMAP 190 immunoaffinity column may form a multiprotein complex with DMAP 190 inside the cell. To test this possibility, we raised mouse polyclonal antibodies against the group of protein bands at 60 kDa in this fraction (arrowhead, Figure 4A). Three closely spaced bands in this region could be separated by preparative PAGE and each band was injected into a separate mouse as an antigen. The polyclonal antibodies raised against these three bands behave identically when used for immunofluorescence and western blotting experiments (see Figures 6 and 7 below), suggesting that the bands represent differently modified forms of the same protein. Until more is known about the function of this protein, we have chosen to call it DMAP 60.

Figure 5 shows a western blot in which an anti-DMAP 60 antibody has been used to follow the behavior of DMAP 60 through the immunoaffinity purification procedure. In crude extracts, the antibody specifically recognizes a group of four closely spaced bands in the 60-kDa region. These are depleted from the extract by the immunoaffinity column (compare the supernatant and the column flow-through, Figure 5).

Several lines of evidence argue that DMAP 60 interacts with DMAP 190 inside the cell. Antibody staining of early *Drosophila* embryos shows that DMAP 60 localizes to the centrosome during anaphase in a manner identical to DMAP 190 (Figure 6). In addition, western blotting reveals that DMAP 60 is retained on microtubule-affinity columns, thus indicating that it is a MAP (Figure 7). Note that DMAP 60 behaves in an identical manner to DMAP 190 on microtubule-affinity columns,

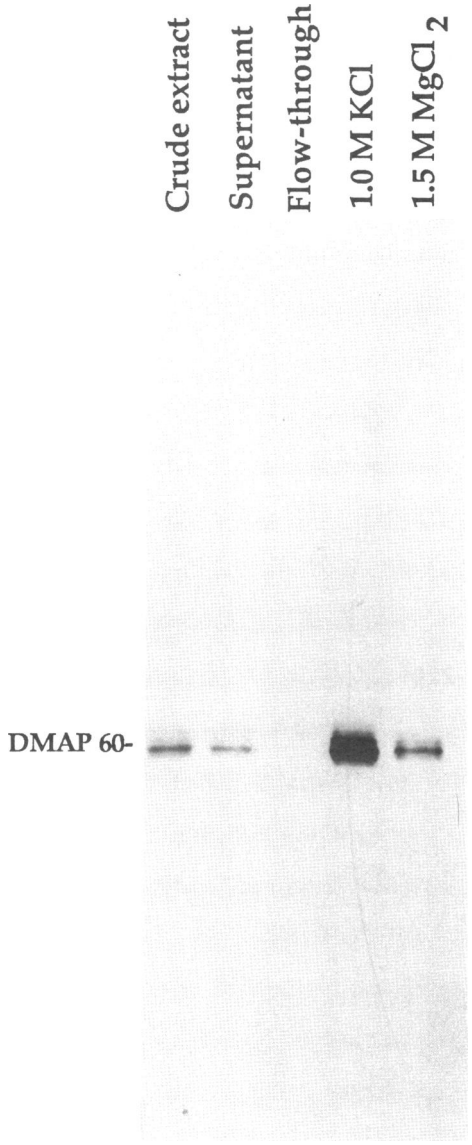

Figure 5. DMAP 60 is quantitatively retained on the anti-DMAP 190 immunoaffinity column. A western blot showing the behavior of DMAP 60 through the immunoaffinity purification procedure. The lanes are the same as in Figure 4B. The blot was probed with the mouse polyclonal serum that recognizes DMAP 60.

consistent with the two proteins existing in the same complex. Using similar methods, the two closely spaced protein bands at 82 kDa in the 1 M KCl eluate from the anti-DMAP 190 immunoaffinity column have also been shown to bind to microtubule-affinity columns and to be associated with centrosomes and mitotic spindles in fixed embryos (J. Raff and B. A. Alberts, unpublished observations).

We have cloned a cDNA for DMAP 60 and have used it to produce a GST fusion protein. Using the approaches described above, we purified low-affinity an-

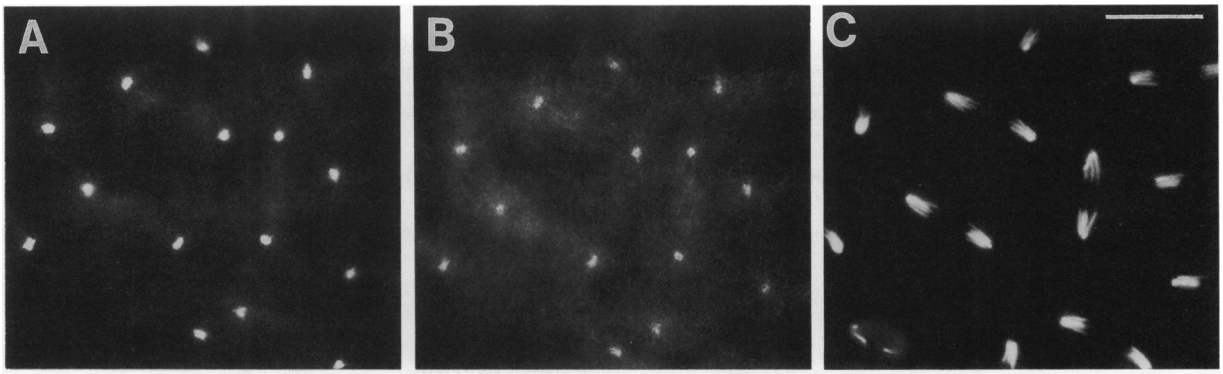

Figure 6. The DMAP 190 and DMAP 60 proteins colocalize in embryos. A triple-label immunofluorescence experiment shows the distribution of DMAP 190 (A), DMAP 60 (B), and DNA by Hoechst 33258 staining (C) in the same embryo. The photomicrographs show a small section of the surface of an embryo in anaphase of nuclear cycle 10 (see Kellogg *et al.*, 1988, for the distribution of microtubules at this stage). Bar is 10 μm.

tibodies that recognize DMAP 60. When a *Drosophila* embryo extract is passed over a column constructed with these antibodies, we find that DMAP 190, as well as all the major proteins in the DMAP 190 complex in Figure 4, are retained on the column and eluted with 1.0 M KCl. These results show that the DMAP 190 complex described in this report can also be isolated with an antibody that recognizes a different protein in the complex. This data, along with a more detailed characterization of DMAP 60, will be presented elsewhere (Kellogg and Alberts, unpublished observations).

DISCUSSION

We have cloned the gene for DMAP 190, a MAP from the *Drosophila* embryo that is localized to the centrosome. In addition, we have used low-affinity antibody chromatography to purify both DMAP 190 and its associated proteins.

Immunoaffinity chromatography with low-affinity polyclonal antibodies should be generally useful for purifying proteins and protein complexes. This approach has a number of advantages over the conventional methods used for protein purification. Proteins that are present in very minor quantities can be purified in a single step and interacting proteins can be easily identified and purified. An advantage of this approach to immunoaffinity chromatography is that the conditions used to elute an antigen from an immunoaffinity column can be chosen in advance when purifying the low-affinity antibodies. In our experiments, we have selected 1.5 M $MgCl_2$, pH 7.6, as an elution condition for purification of low-affinity antibodies. However, it should also be possible to purify low-affinity polyclonal antibodies using a variety of other conditions that have proven to be useful for immunoaffinity purification of native proteins using weak-binding monoclonal antibodies (e.g., with 1 M $MgCl_2$, pH 8.0 [Chang and Bollum, 1986]; 3.2 M $MgCl_2$, pH 8.0 [Chang *et al.*, 1984]; 5 M NaCl, pH 7.5 [Reyland and Loeb, 1987]; 20 mM triethylamine, pH 10.8 [Simanis and Lane, 1985]; 50% ethylene glycol, 0.5 M NaCl, pH 8.0 [Ishimi *et al.*, 1988]). This would allow one to alter the elution conditions, if necessary, to preserve the activity of a particular protein or to enhance its solubility. (For more general considerations regarding immunoaffinity chromatography, see Wilchek *et al.*, 1984; Harlow and Lane, 1988).

Our approach to low-affinity antibody chromatography requires only that the gene for the protein of interest be cloned so that coding sequences from the gene can be used to express a stable soluble fusion protein. The pGEX expression vectors developed by Smith and Johnson (1988) are particularly well suited for the expression of the fusion proteins used in such experiments. These vectors direct expression of proteins joined to the enzyme GST, which tend to be soluble and stable, perhaps because GST itself is small (26 kDa) and highly soluble. Furthermore, large amounts of these fusion proteins can be conveniently isolated in a single step by exploiting the affinity of GST for glutathione-agarose.

The finding that DMAP 190 interacts with a number of other MAPs to form a multiprotein complex is an important step toward understanding the cellular functions carried out by this protein. Many, if not most, cellular functions are carried out by multiprotein complexes, including DNA replication, transcription, protein synthesis, and protein translocation across the membrane of the endoplasmic reticulum. The study of such complexes can be considerably more informative than the study of their isolated components. The DMAP 190 complex and its individual components can now be tested in a variety of in vitro assays to identify activities that affect centrosome or microtubule behavior. In addition, antibodies that recognize these centrosomal proteins can be injected into embryos in attempts to disrupt protein function. If the antibodies are coinjected with

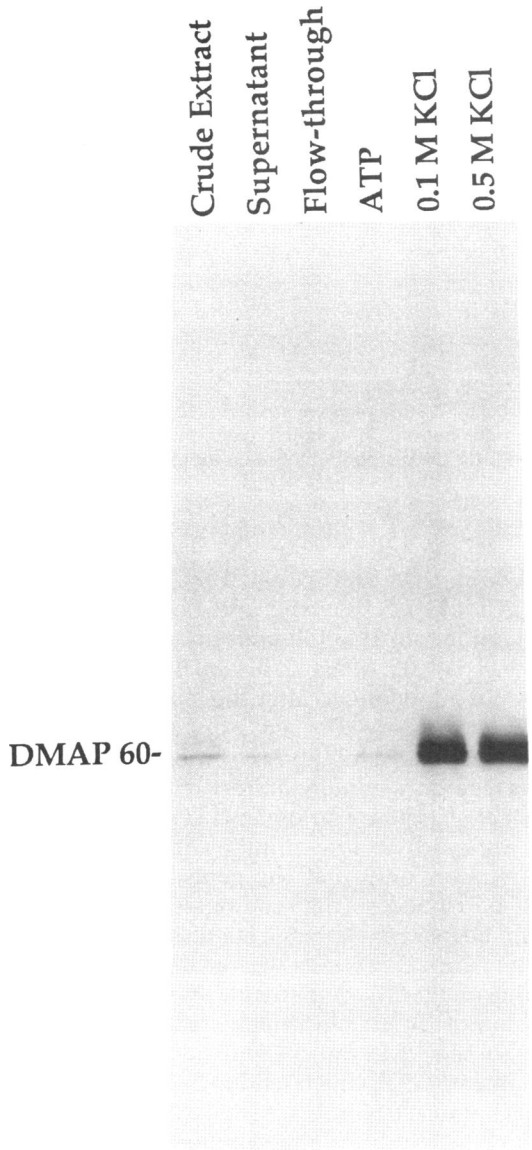

Figure 7. DMAP 60 is retained on a microtubule-affinity column. A western blot showing the behavior of DMAP 60 through a microtubule-affinity chromatography experiment. A polyacrylamide gel identical to the one shown in Figure 1A was transferred to nitrocellulose and probed with the mouse polyclonal antibody that recognizes DMAP 60.

fluorescently labeled tubulin subunits, the behavior of microtubules can be followed in real time (Kellogg *et al.*, 1988). This should allow the identification of specific defects in microtubule behavior that result from the antibody-induced disruption of a centrosomal protein function.

Once the cDNA for a new centrosomal protein has been cloned, low-affinity antibody chromatography can be used to isolate the protein individually and to identify and purify additional proteins that interact with it. By continuing these kinds of approaches, we hope to accomplish a detailed dissection of centrosome structure and function.

ACKNOWLEDGMENTS

Karen Oegema provided assistance and helpful discussions during the course of this work. Karen Butner, Steve Doxsey, Len Freedman, and Christian Lehner also provided helpful advice. This work was supported by NIH grant GM-23928.

REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.

Brinkley, B.R. (1985). Microtubule organizing centers. Annu. Rev. Cell Biol. *1*, 145–172.

Chang, L.M.S., and Bollum, F.J. (1986). Molecular biology of terminal transferase. CRC Crit. Rev. Biochem. *21*, 27–52.

Chang, L.M.S., Rafter, E., Augl, C., and Bollum, F.J. (1984). Purification of a DNA polymerase-DNA primase complex from calf thymus glands. J. Biol. Chem. *259*, 14679–14687.

Frasch, M., Glover, D., and Saumweber, H. (1986). Nuclear antigens follow different pathways into daughter nuclei during mitosis in early *Drosophila* embryos. J. Cell Sci. *82*, 155–172.

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

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Huynh, T.V., Young, R.A., and Davis, R.W. (1985). Constructing and screening cDNA libraries in lambda gt10 and lambda gt11. In: DNA Cloning: A Practical Approach, vol. 1, ed. D. Glover. IRL Press: Oxford. Washington. 49–78.

Ishimi, Y., Claude, A., Bullock, P., and Hurwitz, J. (1988). Complete enzymatic synthesis of DNA containing the SV40 origin of replication. J. Biol. Chem. *263*, 19723–19733.

Karsenti, E., and Maro, B. (1986). Centrosomes and the spatial organization of microtubules in animal cells. Trends Biochem. Sci. *11*, 460–463.

Kellogg, D.R., Field, C.M., and Alberts, B.M. (1989). Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. J. Cell Biol. *109*, 2977–2991.

Kellogg, D.R., Mitchison, T.J., and Alberts, B.M. (1988). Behaviour of microtubules and actin filaments in living *Drosophila* embryos. Development *103*, 675–686.

Laemmli, U.K. (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Miller, K.G., and Alberts, B.M. (1989). F-actin affinity chromatography: technique for isolating previously unidentified actin-binding proteins. Proc. Natl. Acad. Sci. USA *86*, 4808–4812.

Reyland, M.E., and Loeb, L.A. (1987). On the fidelity of DNA replication. Isolation of high fidelity DNA polymerase-primase complexes by immunoaffinity chromatography. J. Biol. Chem. *262*, 10824–10830.

Simanis, V., and Lane, D.P. (1985). An immunoaffinity purification procedure for SV40 large T antigen. Virology *144*, 88–100.

Sluder, G., Miller, F.J., Cole, R., and Rieder, C.L. (1990). Protein synthesis and the cell cycle: centrosome reproduction in sea urchin eggs is not under translational control. J. Cell Biol. *110*, 2025–2032.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene *67*, 31–40.

Steinhauer, W.R., Walsh, R.C., and Kalfayan, L.J. (1989). Sequence and structure of the *Drosophila melanogaster* ovarian tumor gene and generation of an antibody specific for the ovarian tumor protein. Mol. Cell. Biol. *9*, 5726–5732.

Towbin, H., Staehlin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA *80*, 4350–4354.

Vorobjev, I.A., and Nadezhdina, E.S. (1987). The centrosome and its role in the organization of microtubules. Int. Rev. Cytol. *106*, 227–293.

Whitfield, W.G., Millar, S.E., Saumweber, H., Frasch, M., and Glover, D.M. (1988). Cloning of a gene encoding an antigen associated with the centrosome in *Drosophila*. J. Cell Sci. 467–480.

Wilchek, M., Miron, T., and Kohn, J. (1984). Affinity chromatography. Methods Enzymol. *104*, 3–69.