Studies on the Centrosome and Cytoplasmic Organization in the Early *Drosophila* Embryo

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The cytoskeleton plays a central role in such diverse cellular processes as cell division, chromosome segregation, neurite outgrowth, cell motility, and the generation of cytoplasmic order and cell shape. The study of microtubule and actin filament networks is still in its infancy. The molecular mechanisms that lead to the assembly of complex cellular structures like a cleavage furrow or a mitotic spindle remain largely unknown, and the elucidation of such mechanisms represents a major challenge in understanding cellular form and function.

The early *Drosophila* embryo is a highly organized and dynamic single cell that provides an especially good model system for an analysis of the cytoskeleton. The embryo begins development as a giant syncytial cell (Zalokar and Erk 1976; Foe and Alberts 1983). The earliest nuclear divisions occur in the interior of the embryo and are extremely rapid, taking place at intervals of 8–10 minutes. After seven nuclear divisions, most of the nuclei begin to migrate to the embryo cortex. These nuclei arrive at the cortex at the beginning of nuclear cycle 10 and form an evenly spaced monolayer. The nuclei in this monolayer undergo four more rapid synchronous divisions and then become synchronously cellularized by invaginations of the plasma membrane to form the cellular blastoderm.

All of these events occur in a highly reproducible manner, requiring many cytoplasmic changes that are both spatially and temporally ordered within a single cell. The early embryo is readily amenable to immunofluorescence, microinjection, and genetic studies. In addition, large quantities of these embryos are available for biochemical analyses. A wide range of technical approaches is thus available for studying the cytoskeleton in these giant cells.

Our recent work has focused on the identification and characterization of the *Drosophila* embryo proteins that mediate the many functions of microtubules. We are employing both biochemical and genetic approaches to this problem. We began the biochemical studies by using microtubule affinity chromatography to isolate proteins that bind to microtubules (Kellogg et al. 1989). This approach has allowed us to identify a large number of novel microtubule-associated proteins (MAPs), some of which are localized to the centrosome. The identification of centrosomal MAPs has enabled us to begin a biochemical analysis of this fascinating and important organelle.

To initiate a genetic characterization of microtubule behavior and function, we have identified maternal effect lethal mutations that specifically disrupt cytoskeletal organization in the early *Drosophila* embryo. The first of these mutants has been characterized in detail, and its primary defect appears to be in centrosome separation during nuclear division (Sullivan et al. 1990). A screen through 73 female sterile lines has identified five additional mutations with related phenotypes. Characterization of the proteins encoded by these genes is likely to provide new insights into cytoskeletal function and behavior.

The Centrosome

Much of our current work is focused, directly or indirectly, on the centrosome. We have chosen this focus because the centrosome plays a central role in a variety of cellular events, including cell division, chromosome segregation, directed cell movement, and the overall organization of the cytoplasm (for reviews, see Karsenti and Maro 1986; Vorobjev and Nadezhdina 1987). The centrosome was first described more than 100 years ago, when cytologists using the light microscope were able to observe a densely staining dot (or a pair of dots) associated with the interphase nucleus in animal cells (Wilson 1925). The dots were usually surrounded by an amorphously stained cloud of material that appeared to be the source of a system of fibers reaching throughout the cytoplasm. The densely staining dots were eventually given the name of centrioles, whereas the centrioles together with their surrounding cloud were called the centrosome. The early cytologists viewed the ordered duplication of centrosomes during each cell cycle as a central mechanism for cell reproduction and inheritance.

More recent studies have shown that the centrosome nucleates the growth of microtubules and is the primary microtubule-organizing center within cells. Studies with the electron microscope have revealed that the centrosome is structurally complex (Rieder and Borisy 1982; Vorobjev and Chentsov 1982; Vorobjev and Nadezhdina 1987). During interphase, the centrosome contains a pair of centrioles, each composed of nine triplet microtubules arranged in a characteristic barrel configuration. The centrioles are surrounded by an amor-

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phous electron-dense halo called the pericentriolar material (PCM), which appears to be the source of the hundreds of microtubules that are nucleated from the centrosome in animal cells. A variety of studies have shown that the PCM alone, without centrioles, can function as the major microtubule-organizing center in cells; the function of the centrioles is therefore unclear (see, e.g., Keryer et al. 1984).

By means of the microtubule network that it organizes, the centrosome plays a central role in the overall organization of the cytoplasm. For example, by acting on the actin filament network at the cell cortex, the centrosome is able to cause spatially localized changes in the plasma membrane. Perhaps the most general of these events occurs just before cytokinesis, when signals that emanate from the two spindle poles determine the plane of the subsequent cleavage furrow, a structure that is based on actin filaments (for review, see Rappaport 1986). As illustrated in Figure 1A, cleavage furrows are always initiated precisely halfway between two centrosomes, even when there is no chromatin or mitotic spindle between them. The analogous processes in the early *Drosophila* embryo are the actin-dependent pulling down of plasma membrane that causes cellularization at cycle 14 and the transient furrowing of membrane around the syncytial nuclei during mitosis of cycles 11 through 13 (Stafstrom and Staehelin 1984; Karr and Alberts 1986).

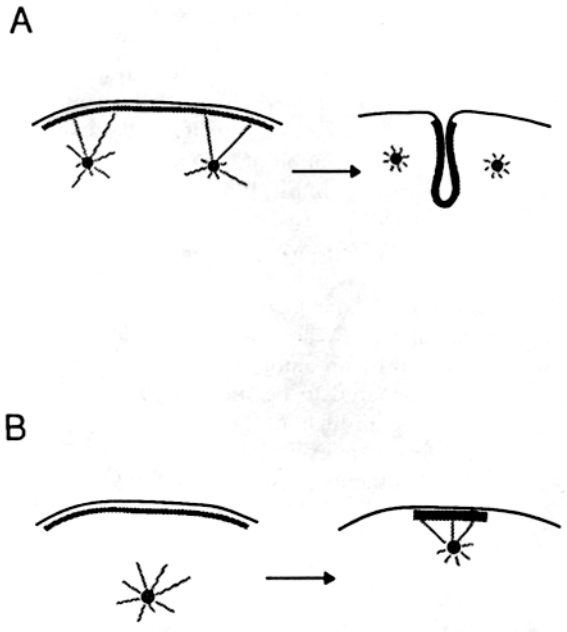

Figure 1. Centrosomes can reorganize cortical actin and the plasma membrane. (*A*) The cortical actin and plasma membrane are induced to form a cleavage furrow by the centrosomes at the spindle pole (for review, see Rappaport 1986). This induction occurs at metaphase in many different eukaryotic cells and leads to the formation of a cleavage furrow in anaphase. (*B*) In the early *Drosophila* embryo, an initially uniform layer of cortical actin is induced to form actin "caps" when centrosomes migrate to the embryo cortex (Karr and Alberts 1986; Raff and Glover 1989). Stippled area represents the cortical actin.

The ability of the centrosome to affect the cortical actin and the plasma membrane is especially clear in the *Drosophila* embryo, where centrosomes can be dissociated from nuclei during the early nuclear divisions by injecting embryos with the DNA synthesis inhibitor aphidicolin (Raff and Glover 1988). Remarkably, the centrosomes in these embryos continue to divide and migrate to the embryo cortex, where they organize actin "caps" and create plasma membrane domains that are similar to those in normal embryos (Fig. 1B). In addition, those centrosomes that migrate to the posterior end of the embryo are able to organize the formation of "pole cells" in the absence of any nuclei, meaning that the centrosomes induce the cortical actin with its overlying plasma membrane to wrap around them and pinch off to form separate cell-like units (Raff and Glover 1989). These and other observations demonstrate that centrosomes are able to determine the organization of actin filaments and that these actin filament networks in turn shape the plasma membrane.

The processes just discussed suggest a pathway: centrosomes → microtubules → actin filaments → plasma membrane. There is also substantial evidence for a

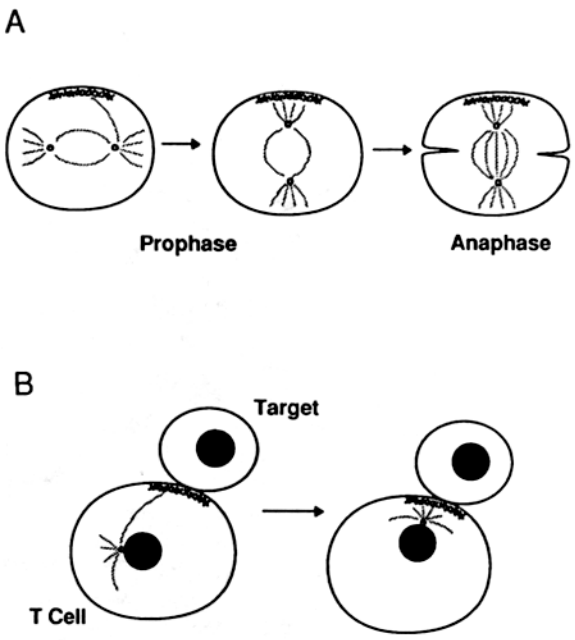

Figure 2. The cell cortex can determine the position of centrosomes. (*A*) In the early *Caenorhabditis elegans* embryo, centrosomal microtubules that interact with the cell cortex induce a rotation of the spindle axis that determines the subsequent plane of cleavage (Hyman and White 1987; Hyman 1989). A similar spindle rotation occurs in selected groups of cells during mitosis of nuclear cycle 14 in the *Drosophila* embryo (Foe 1989). The nature of the signal at the cortex (represented by crosshatches) is unknown. (*B*) A reorientation of the internal contents of killer T cells appears to be initiated by centrosomal microtubules that contact the point of recognition between the killer cell and its target cell (Kupfer and Singer 1989a,b). The crosshatched area marks a change in the actin cortex that precedes the reorientation. As a result, the T cell becomes polarized to secrete specifically at the target cell.

reverse pathway, in which signals emanating from a local region of the plasma membrane position the centrosome by interacting with its microtubules, thereby polarizing the cell. As illustrated in Figure 2, two well-studied examples of this phenomenon are the repositioning of the mitotic spindle in *Caenorhabditis elegans* blastomeres (Hyman and White 1987; Hyman 1989) and the polarization of a killer T cell toward its target cell (Kupfer and Singer 1989a,b).

In the most general sense, the centrosome and its associated microtubules may be viewed as an apparatus for appropriately positioning each cell's internal organelles (chromosomes, Golgi apparatus, endoplasmic reticulum, cleavage furrow, mitotic spindle, and so on). Although the centrosome is sometimes considered to be involved merely in the nucleation of microtubules and the stabilization of their minus ends, the recent finding that the kinetochore-to-centrosome microtubules in mammalian tissue culture cells depolymerize continuously at the centrosome during mitosis demonstrates that the centrosome is more complicated (Mitchison 1989). The centrosome might even carry out many additional functions that have not yet been characterized. For example, the centrosome might be intimately involved in responding to cell cycle or positional clues—perhaps by generating signals that travel along microtubules to the plasma membrane or by guiding the assembly of specific types of MAPs onto selected microtubules. Clearly, there is still a great deal to be learned about this fascinating organelle.

Centrosomal Changes during the Cell Cycle

Two aspects of centrosomal behavior are of special relevance to a symposium focused on the cell cycle. First, there is evidence that there is a mechanism for the ordered series of centrosomal duplication events that can be uncoupled from the cyclin/$p34^{cdc2}$ clock mechanism. Thus, in both early *Xenopus* and sea urchin embryos, a cyclohexamide or emetine treatment that stops the cyclin/$p34^{cdc2}$ cycle allows centrosome duplication to continue, albeit with a slower cycle time and in an asynchronous fashion (Gard et al. 1990; Sluder et al. 1990). (In contrast, the centrosomes in *Drosophila* embryos stop dividing in the presence of protein synthesis inhibitors [J. Raff and W. Sullivan, unpubl.].) How the two mechanisms are coordinated in normal cells is not known.

A second aspect of centrosome function related to the cell cycle starts with the observation that the number of microtubules nucleated by the centrosome is several times greater at metaphase than in interphase. This appears to be a property of the centrosome itself, rather than the cytoplasmic environment, since the ability of centrosomes to nucleate more microtubules in metaphase can also be observed in lysed cytoplasts (Kuriyama and Borisy 1981). The increase in the number of microtubules nucleated by the centrosome is correlated with a large increase in the amount of pericentriolar material (Rieder and Borisy 1982). In addition to a change in quantity, there may also be a qualitative change in the pericentriolar material as the cell cycle proceeds.

Protein Components of the Centrosome

A biochemical approach to the characterization of the centrosome has been difficult due to the low abundance of centrosomes within cells. Genetic approaches to identifying centrosomal proteins have also gone slowly, perhaps because it is difficult to predict the phenotypes of mutations in centrosomal proteins. Recently, however, a number of experiments have opened the way to a biochemical and genetic characterization of centrosomal proteins in a variety of different organisms.

The yeast *Saccharomyces cerevisiae* and other fungi possess a spindle pole body (SPB) rather than a centrosome. The SPB is functionally similar to the centrosome in that it is the primary microtubule-organizing center within the cell, but it is structurally distinct from the centrosome, being composed of several electron-dense layers that are embedded in the nuclear envelope. A protocol has recently been developed that allows a 600-fold enrichment of SPBs from the yeast *S. cerevisiae* (Rout and Kilmartin 1990 and this volume). Monoclonal antibodies that recognize two of the proteins in this enriched fraction specifically stain the SPB when used for immunofluorescence and immunogold labeling experiments. Other yeast proteins that function as components of the SPB may have been identified in screens for *cdc* mutants that affect duplication of the spindle poles (Baum et al. 1988; Uzawa et al. 1990). Proteins involved in centriolar function may also have been identified in genetic screens for defects in basal body function in *Chlamydomonas reinhardtii* (Dutcher and Lux 1989).

A recently identified tubulin variant called γ-tubulin has been identified as a component of the SPB in the fungus *Aspergillus nidulans*. γ-tubulin, which is 35% homologous to α and β tubulins, was originally identified in *A. nidulans* as the product of an extragenic suppressor of a mutation in β-tubulin (Oakley and Oakley 1989; Oakley et al. 1990). A null mutation in the γ-tubulin gene is a recessive lethal that strongly inhibits nuclear division and weakly inhibits nuclear migration. The null mutation also causes a reduction in the number and length of cytoplasmic microtubules and a complete loss of the mitotic spindle. Antibodies raised against γ-tubulin specifically recognize the SPB in *A. nidulans*. The antibodies also recognize the SPB in the yeast *Schizosaccharomyces pombe* and the centrosome in a wide variety of higher animal cells. γ-tubulin genes have recently been cloned from *S. pombe*, *Drosophila*, and humans (Stearns et al. 1991; Zheng et al. 1991). The function of this minor tubulin remains unknown, although its localization to centrosomes and SPBs has raised speculation that it may function in the nucleation of microtubule growth.

A variety of antibodies that stain the centrosome of

higher eukaryotes have been obtained (Calarco-Gillam et al. 1983; Bornens et al. 1987; Whitfield et al. 1988; Buendia et al. 1990; Baron et al. 1991; Tousson et al. 1991). In addition, procedures have been developed that allow centrosomes to be substantially purified as very large complexes of proteins (Mitchison and Kirschner 1984; Bornens et al. 1987).

The biochemical approach that our laboratory has taken to the identification and characterization of centrosomal proteins is suggested by the observations of McNiven and Porter (1988). In these studies, the edge of a very large flattened cell was cut away from the cell body, leaving the centrosome behind with the nucleus. After a short incubation, the microtubules in these cytoplasmic fragments were found to reorganize around a new cell center, in which their minus ends were embedded. These results suggest that the subunits of the pericentriolar material are present in the cytoplasm and can rapidly assemble to form a new organizing center for microtubules, without requiring a preexisting centrosome to act as a template. This provides a rationale for attempts to identify and isolate such subunits from the cytoplasm of developing embryos (where preexisting subunits would be expected to be stored in large quantities), as well as for the hope that they will provide a pathway for reassembling the essential components of a functioning centrosome.

METHODS

Buffers. *Extract Buffer:* 50 mM HEPES (pH 7.6), 50 mM KCl, 1.0 mM Na_3EGTA, 1.0 mM Na_3EDTA, and 0.05% Nonidet P-40. *Protease Inhibitor Stock:* 1 mM benzamidine-HCl, 0.1 mg/ml phenanthroline, and 1 mg/ml each of aprotinin, leupeptin, and pepstatin A (this stock is used at dilutions of 1/100 to 1/1000, as noted). *Column Buffer:* 50 mM HEPES (pH 7.6), 50 mM KCl, 1.0 mM Na_3EGTA, 1.0 mM $MgCl_2$, and 10% glycerol. *Tris-buffered Saline (TBS):* 20 mM Tris-HCl (pH 7.5) and 0.5 M NaCl. *Phosphate-buffered saline (PBS):* 10 mM sodium phosphate (pH 7.3) and 0.15 M NaCl. *Polyacrylamide Gel Sample Buffer*: 63 mM Tris-HCl (pH 6.8), 3% SDS, 5% β-mercaptoethanol, and 10% glycerol.

DNA cloning. To clone D-MAP 190, we screened a λgt11 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 essentially as described by Huynh et al. (1985). Approximately 600,000 phage plaques were screened, and we obtained five potential positive clones. One of these phage clones carried an insert that yielded three fragments when cut with *Eco*RI. We subcloned the smallest of these fragments (831 bp) into the *Eco*RI 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). We have called the 73-kD fusion protein synthesized by this construct GST/190-c. Rabbit polyclonal antibodies raised against this fusion protein (see below) recognize a *Drosophila* 190-kD 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.

Construction of GST and GST/190-c protein affinity columns. The purification of polyclonal antibodies specific for D-MAP 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 were purified as described by Smith and Johnson (1988), except that the proteins were eluted from the glutathione agarose resin by flow through a column, rather than batchwise, in order to obtain a more concentrated protein solution. From 5 g of packed *Escherichia coli* cells, we obtained 125 mg of GST and 45 mg of GST/190-c. The purified proteins were dialyzed into 50 mM HEPES (pH 7.6) and 25 mM KCl and coupled to Affigel-10 agarose (Bio-Rad) at 5–10 mg protein per milliliter of agarose, according to the manufacturers instructions.

Isolation of low-affinity antibodies that recognize D-MAP 190. To generate antibodies against D-MAP 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. Before antibodies that are specific for the D-MAP 190 portion of the fusion protein could be isolated, it was necessary to remove the antibodies that recognize the GST portion of the protein. This was accomplished by passing the serum over a column containing covalently bound purified GST. The serum was passed over the column several times, until no more antibody was bound. To regenerate the column between passes, the anti-GST antibodies were 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 antibodies in the serum that are specific for the D-MAP 190 portion of the protein were then isolated by passing the depleted serum over a column containing 25 mg of covalently bound GST/190-c fusion protein. The column was washed with TBS until no protein could be detected in the flowthrough (~25 column volumes), the low-affinity anti-D-MAP 190 antibodies were eluted with 1.4 M $MgCl_2$, 10% (v/v) glycerol, and 50 mM HEPES (pH 7.6). Bovine serum albumin was added to the purified antibodies as a carrier protein (5 mg albumin for each milligram of antibody), and the antibodies were then dialyzed into PBS containing 0.02% sodium azide and 50% (v/v) glycerol. The low-affinity antibodies were stored at −20°C until use.

After elution of the low-affinity antibodies, the high-affinity anti-D-MAP 190 antibodies were eluted from the column with 0.5% acetic acid and 0.15 M NaCl. 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 (data not shown). Additional high-affinity antibodies can be obtained by eluting the column with 100 mM triethylamine (pH 11.5). 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 D-MAP 190 were coupled to protein A–Sepharose as described by 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).

A clarified *Drosophila* embryo extract was prepared from 15–20 g of *Drosophila* embryos (age 0–4 hr) as described previously (Miller and Alberts 1989), except that the extract buffer described above was used. The extract was then passed through three different columns connected in series. The first column is a 15-ml column that was constructed with Sepharose CL-4B (Pharmacia). This column serves 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-D-MAP 190 immunoaffinity column. After loading at a flow rate of 20 ml/hr, the three columns were disconnected from each other and the control column and the immunoaffinity column were washed with 75 column volumes of column buffer containing protease inhibitors (1/200) and 0.05% Nonidet P-40. The wash step was carried out overnight using gravity to produce a flow rate of 10–30 ml/hr. All columns were run at 4°C.

Before elution, the columns were washed with several column volumes of column buffer in order to remove the Nonidet P-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 column fractions were assayed for protein according to the method of Bradford (1976), and small samples were removed from the peak fractions and mixed with 4 × gel sample buffer for analysis by SDS-PAGE (Laemmli 1970).

The maximal yield of D-MAP 190 from our column was 0.25 mg, which represented the capacity of the column. This was only about 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.

Generation of mouse polyclonal antibodies against D-MAP 60. Mouse polyclonal antibodies that specifically recognize D-MAP 60 were generated by immunizing mice with polyacrylamide gel-purified protein as described previously (Kellogg et al. 1989). Approximately 100 μg of D-MAP 60 was resolved from the other proteins in the 1.0 M KCl fraction by preparative polyacrylamide gel electrophoresis. Alter staining the gel with Coomassie blue, three separate protein bands could be resolved in the 60-kD region. Each of these three bands was excised and used to immunize a separate mouse.

Western blotting and immunofluorescence. Western blotting and immunofluorescence were carried out as described previously (Towbin et al. 1979; Kellogg et al. 1988).

Screening for maternal effect lethal mutations that disrupt cytoskeletal organization in the early embryo. Collections of maternal effect lethal mutants were generously provided by the laboratories of Y.-N. Jan and A. Spradling. The mutants were generated by the mobilization of single P transposable elements (Cooley et al. 1989). To screen for defects in cytoskeletal organization, 0–3-hr embryos from homozygous mutant females were collected, fixed, and fluorescently labeled for actin, tubulin, and nuclei. Actin and tubulin were labeled using monoclonal antibodies (Amersham), and nuclei were labeled with Hoechst dye. Confocal microscopy was performed using a Bio-Rad MRC 600 confocal head mounted on a Nikon photoscope with a 60 × planapo lens (na 1.4). Embryos were screened for uneven nuclear spacing, spindle abnormalities, and abnormally high numbers of yolk nuclei during the cortical divisions.

RESULTS

Use of Microtubule Affinity Chromatography to Identify Novel MAPs

An important step toward understanding the behavior and function of complex microtubule arrays is the identification and characterization of their individual components. Toward this end, we have developed affinity chromatography procedures that allow purification of proteins that interact with microtubules from *Drosophila* embryo extracts (Kellogg et al. 1989; Miller et al. 1991). In this kind of experiment, an extract is made from early *Drosophila* embryos and is loaded onto a microtubule affinity column. The affinity column is constructed by covalently linking taxol-stabilized microtubules to an inert agarose matrix. After loading the extract, the column is washed extensively with buffer, and the MAPs that remain bound to microtubules on the column are eluted by sequential washes with buffer containing 2.0 mM ATP, 0.1 M KCl, or 0.5 M KCl. The results of an experiment of this kind are shown in Figure 3. We find that a large number of different proteins bind to a microtubule affinity col-

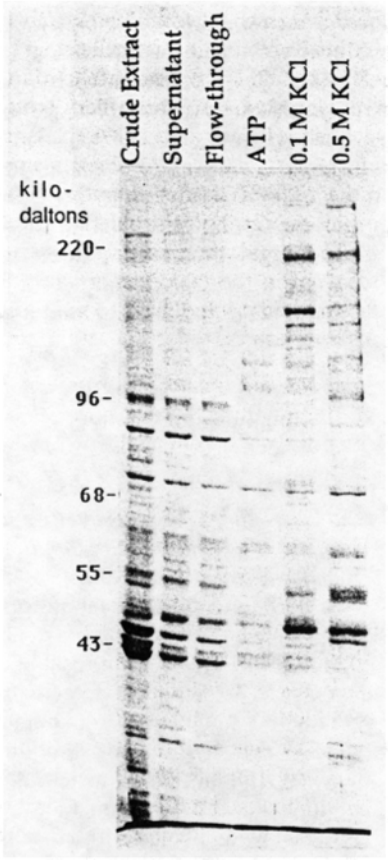

Figure 3. The *Drosophila* embryo proteins that bind to a microtubule affinity column. An 8.5% polyacrylamide gel showing the *Drosophila* embryo proteins that bind to a microtubule affinity column. Microtubule affinity chromatography was carried out as described previously (Kellogg et al. 1989; Miller et al. 1991). Lanes: Crude extract, 20 μg; high-speed supernatant (column load), 15 μg; column flowthrough (unbound proteins), 15 μg; ATP elution, 10 μg; 0.1 M KCl elution, 28 μg; 0.5 M KCl elution, 30 μg. The gel is stained with Coomassie blue. None of these proteins bind to a control column containing covalently bound bovine serum albumin instead of microtubules.

umn, none of which bind to a control column constructed with bovine serum albumin. To begin to characterize these proteins, we generated individual mouse polyclonal antibodies that specifically recognize 24 of them (Kellogg et al. 1989). As judged by immunofluorescence, some of the antigens recognized by these antibodies localize to the mitotic spindle in the early *Drosophila* embryo, whereas 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 many proteins that bind to microtubule affinity 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.

The MAPs that localize to the centrosome are of particular interest to us, since these represent the first biochemically defined MAPs that localize to this important microtubule-organizing center. The identification of these MAPs has provided us with a good starting point for a detailed characterization of the centrosome, which is the focus of the work described below.

Isolation of a Partial cDNA Clone Encoding D-MAP 190, a 190-kD MAP Localized to the Centrosome

In the study described above, we obtained a mouse polyclonal antibody that recognizes a 190-kD protein that binds to microtubules and is localized to the centrosome in embryos (initially designated as S1-4 in Kellogg et al. [1989]). Until more is known about the function of this protein, we have chosen to call it D-MAP 190, indicating that it is a 190-kD MAP from *Drosophila melanogaster*.

A partial cDNA clone encoding D-MAP 190 was isolated by screening an ovarian λgt11 cDNA expression library with the mouse polyclonal antibodies. An 831-bp DNA fragment from one of the phage clones identified in the screen was fused to the coding se-

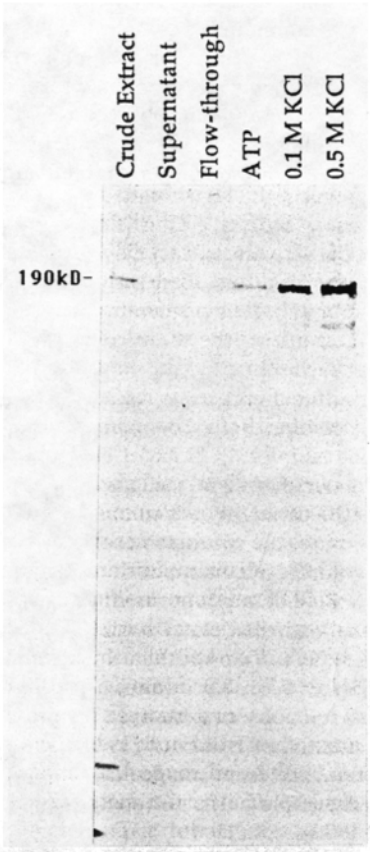

Figure 4. D-MAP 190 is a MAP. Western blot showing the behavior of D-MAP 190 through a microtubule affinity chromatography experiment. A polyacrylamide gel identical to the one shown in Fig. 3 was transferred to nitrocellulose and probed with affinity-purified rabbit polyclonal antibody that recognizes D-MAP 190.

quences for the enzyme GST, allowing large amounts of the protein sequence encoded by the cDNA to be isolated in pure form (Smith and Johnson 1988). Rabbit polyclonal antibodies raised against this fusion protein recognize a 190-kD protein that is enriched by microtubule affinity chromatography, as illustrated by the data in Figure 4. In addition, the antibodies stain the centrosome in a manner identical to the original mouse polyclonal serum at metaphase. These results confirm the correct identity of the cDNA clone. The rabbit polyclonal antibody also gives a very weak staining of the spindle (not shown) and a nuclear staining during interphase in embryos older than nuclear cycle 12 (not shown). For unknown reasons, the original mouse antibody does not reveal these spindle and nuclear localizations of D-MAP 190.

Use of Low-affinity Antibodies to Purify D-MAP 190 Along with Its Associated Proteins

The further characterization of D-MAP 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 D-MAP 190 and to identify any proteins that interact with it to form a multiprotein complex. The purified D-MAP 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 D-MAP 190 could be studied in a similar manner. The identification of proteins that interact with D-MAP 190 is of particular importance, since many proteins are known to function within the cell as components of multiprotein complexes, rather than as isolated proteins. The study of such complexes can be considerably more informative than the study of their isolated components and might provide access to a subunit of the centrosome.

We have used an immunoaffinity chromatography approach to purify D-MAP 190 and its associated proteins. Immunoaffinity chromatography is often not useful for the purification of proteins in their native state, since 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 relatively low affinity (Chang et al. 1984; Chang and Bollum 1986). Because antigens can be dissociated from these antibodies under relatively mild conditions (e.g., with 1.0–3.2 M $MgCl_2$, pH 8.0), immunoaffinity columns constructed with them can be very effective in the purification of proteins (Chang et al. 1984; Chang and Bollum 1986).

We reasoned that similar low-affinity antibodies should exist in a typical polyclonal serum, and thus we designed a procedure to isolate such antibodies. For this procedure, polyclonal antibodies that recognize D-MAP 190 were generated by immunizing rabbits with the D-MAP 190–GST fusion protein described above. The serum from these rabbits contained antibodies that recognize the GST portion of the fusion protein, as well as antibodies that recognize the D-MAP 190 portion. The antibodies that recognize GST were removed by passing the serum over a column matrix that has purified GST bound to it. The antibodies that are specific for the D-MAP 190 portion of the D-MAP 190–GST fusion protein were then isolated by passing the serum over a column containing bound fusion protein. After washing this column extensively, the low-affinity antibodies were eluted with a buffer containing 50 mm HEPES (pH 7.6), 1.4 M $MgCl_2$, and 10% glycerol. The high-affinity antibodies were then eluted from the column with 0.5% acetic acid and 0.15 M NaCl. The yield of low- and high-affinity antibodies from 300 ml of rabbit serum was 16 and 42 mg, respectively.

We constructed immunoaffinity columns for the purification of D-MAP 190 by covalently linking 3 mg of the low-affinity antibodies to 1.0 ml of protein A–agarose. A control IgG column was constructed by linking 3 mg of preimmune IgG antibodies to the same column matrix.

To purify D-MAP 190, a clarified cytoplasmic extract was made from 15–20 g of early *Drosophila* embryos (0–2.5 hr postfertilization) and was loaded onto an anti-D-MAP 190 immunoaffinity column and the control IgG column. After washing extensively with buffer, the columns were eluted with 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 D-MAP 190 from the immunoaffinity column. Figure 5 shows an elution profile that compares the amount of protein eluted from the anti-D-MAP 190 column and from the control column. The 1.0 M KCl elution of the anti-D-MAP 190 immunoaffinity column contains 1.6 mg of protein, and the 1.5 M $MgCl_2$ elution contains 0.25 mg of protein. Little or no protein is retained on the control IgG column.

An SDS-polyacrylamide gel analysis of the proteins contained in these fractions is shown in Figure 6A. The 1.5 M $MgCl_2$ elution contains the D-MAP 190 centrosomal protein in highly purified form, plus small amounts of a 70-kD protein that is also retained on the control column. The 1.0 M KCl elution contains approximately ten 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 D-MAP 190. Since the immunoaffinity column is washed with 75 column volumes of buffer before elution, these proteins must interact with D-MAP 190 with high affinity to be retained.

Figure 6B is a Western blot that shows the behavior of D-MAP 190 through the immunoaffinity purification procedure. D-MAP 190 is a soluble protein (compare

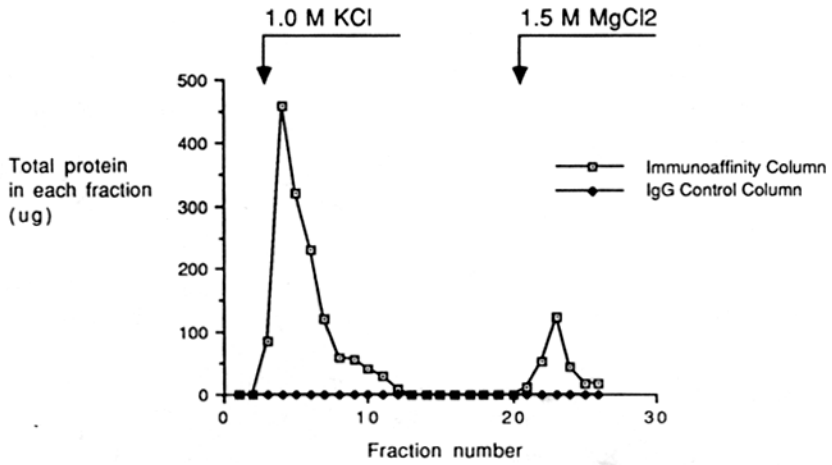

Figure 5. An elution profile comparing the amount of *Drosophila* embryo protein that elutes from the anti-D-MAP 190 immunoaffinity column and a control IgG column. Chromatography was carried out as described in the text. Each column fraction has a volume of 0.3 ml.

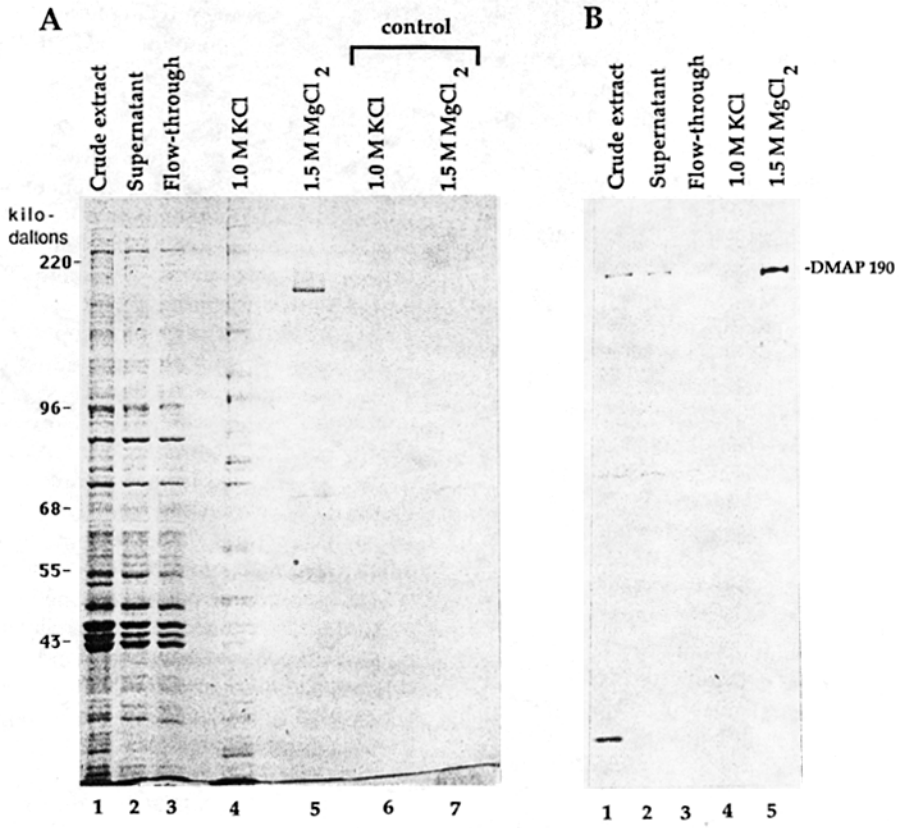

Figure 6. Proteins in a *Drosophila* embryo extract that bind to anti-D-MAP 190 and control IgG immunoaffinity columns. (*A*) Analysis of the proteins that bind to the immunoaffinity columns by electrophoresis on an 8.5% polyacrylamide gel. Lanes *1–5* show the results for the anti-D-MAP 190 column. (*1*) Crude extract, 20 μg; (*2*) high-speed supernatant (column load), 15 μg; (*3*) column flowthrough (unbound proteins), 15 μg; (*4*) 1.0 M KCl elution, 7 μg; (*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 D-MAP 190 through the immunoaffinity purification procedure. Proteins were resolved on an 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 Fig. 2A, except that the KCl elution lane was loaded with 0.7 μg protein and the $MgCl_2$ elution lane was loaded with 0.2 μg protein. The blot was probed with the high-affinity rabbit polyclonal antibodies that recognize D-MAP 190.

lanes 1 and 2), and the immunoaffinity column largely depletes the extract of D-MAP 190. We have estimated the amount of D-MAP 190 that is present in embryo extracts by comparing the intensity of the signal for D-MAP 190 in the crude extract with a standard curve prepared by loading varying amounts of the purified protein onto Western blots. We can thereby estimate that D-MAP 190 constitutes approximately 0.1% of the total protein in the early *Drosophila* embryo.

D-MAP 190 Is Part of a Multiprotein Complex That Includes Other Centrosomal Proteins

The proteins in the 1.0 M KCl elution of the anti-D-MAP 190 immunoaffinity column represent candidates for proteins that form a multiprotein complex with D-MAP 190. As a first step in their characterization, we have raised mouse polyclonal antibodies against the group of protein bands at 60 kD in this elution. The three closely spaced bands that can be discerned in this region were separated by preparative polyacrylamide gel electrophoresis, and each band was used to raise mouse polyclonal antibodies. The antibodies raised against these three bands behave identically when used for immunofluorescence and Western blotting experiments (see below), suggesting that they represent differently modified forms of the same protein. Until more is known about the function of this protein, we have chosen to call it D-MAP 60.

Several lines of evidence argue that D-MAP 60 interacts with D-MAP 190 inside the cell. Immunolocalization data with the anti-D-MAP 60 antibody show that D-MAP 60 localizes to the centrosome during anaphase in a manner similar to that of D-MAP 190 (Fig. 7). In addition, Western blotting reveals that D-MAP 60 is retained on microtubule affinity columns, thus indicating that it is a MAP (Fig. 8). By similar means, the two closely spaced protein bands at 82 kD in the 1.0 M KCl

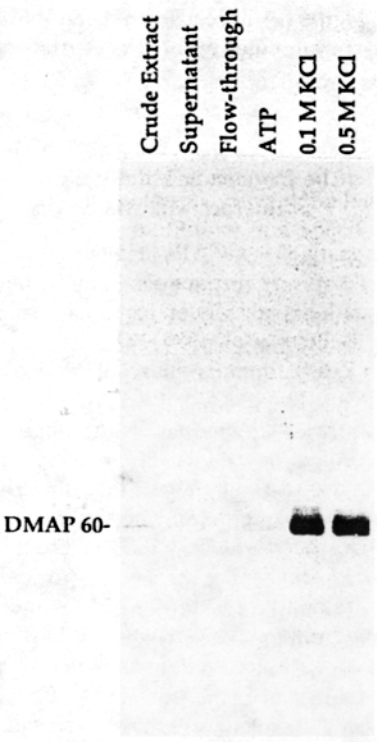

Figure 8. D-MAP 60 is a MAP. Western blot showing the behavior of D-MAP 60 through a microtubule affinity chromatography experiment. A polyacrylamide gel identical to the one shown in Fig. 3 was transferred to nitrocellulose and probed with the mouse polyclonal antibody that recognizes D-MAP 60.

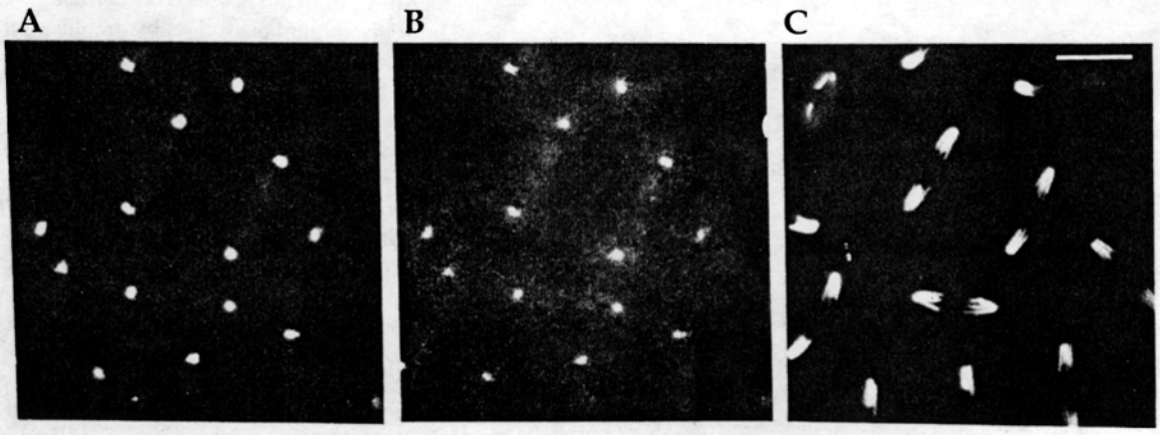

Figure 7. The D-MAP 190 and D-MAP 60 proteins colocalize in embryos. A triple-label immunofluorescence experiment showing the distribution of D-MAP 190 (*A*), D-MAP 60 (*B*), and DNA (*C*; Hoechst 33258 staining) in the same embryo. The microphotographs show a small section of the surface of an embryo in anaphase of nuclear cycle 10 (for the distribution of microtubules at this stage, see Kellogg et al. 1988).

elution of Figure 6A have also been shown to be associated with microtubules and centrosomes (not shown).

The *Drosophila* Mitotic Cyclins Interact with MAPs

The majority of the MAPs that we have identified by microtubule affinity chromatography undergo dramatic changes in their subcellular locations during the cell cycle (see Kellogg et al. 1989). This is particularly true for D-MAP 190. Immunofluorescence data show that during mitosis, D-MAP 190 is very strongly localized to the centrosome, whereas at interphase, its presence at the centrosome is weakly detectable (not shown). These observations suggest that the behavior of MAPs must somehow be controlled by the fundamental cell cycle control network. To address this question, we have carried out experiments to examine whether either the mitotic cyclins or $p34^{cdc2}$ interact with microtubules. When we used Western blotting to probe the MAP-containing fractions obtained by microtubule affinity chromatography for the presence of these proteins, the results were most striking for *Drosophila* cyclin B. Figure 9 shows that the majority of cyclin B in the crude extract is pelleted in the centrifugation steps used to prepare the extract for affinity chromatography (compare the crude extract with the supernatant). But the cyclin B that remains in the supernatant is depleted from the extract as it passes over the microtubule affinity column (compare the supernatant with the flow-through). Approximately one half of the cyclin B that binds to the column is eluted with 0.1 M KCl, whereas the remainder is eluted with 0.5 M KCl. None of the cyclin B is eluted with ATP.

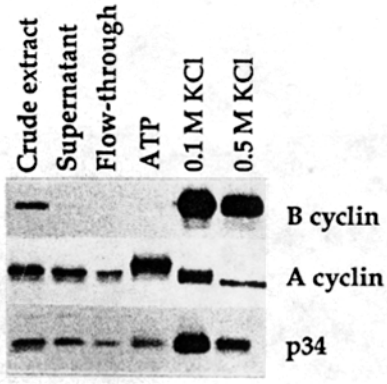

Figure 9. Mitotic cyclins and $p34^{cdc2}$ interact with microtubules. Polyacrylamide gels identical to the one shown in Fig. 3 were transferred to nitrocellulose and probed with antibodies specific for the *Drosophila* A and B cyclins and $p34^{cdc2}$. The antibodies that recognize the A and B cyclins were obtained from C. Lehner and P. O'Farrell and have been characterized previously (Lehner and O'Farrell 1990). The antibody that recognizes $p34^{cdc2}$ was obtained from M. Kirschner and M. Solomon and was generated by immunizing rabbits with the PSTAIR peptide.

The results obtained for cyclin A are more complex. The form of cyclin A that migrates slowly on SDS-polyacrylamide gels is quantitatively retained on the column, whereas the faster-migrating forms are only partially retained. Moreover, the slow-migrating form of cyclin A is specifically eluted with ATP, whereas the other forms are eluted with increasing concentrations of salt. Only a small fraction of the total $p34^{cdc2}$ in *Drosophila* embryo extracts is retained on microtubule affinity columns. Controls show that neither the cyclins nor $p34^{cdc2}$ binds to an identical column containing covalently bound bovine serum albumin instead of microtubules (not shown).

These results do not allow one to distinguish whether the cyclins bind directly to microtubules or whether they bind indirectly via interactions with other MAPs. To address this question, we made a fusion protein that includes the full-length *Drosophila* cyclin B fused to the enzyme GST (Smith and Johnson 1988). The fusion protein was expressed in bacteria, purified, and tested for its ability to interact with microtubules in the presence and absence of MAPs. The results of this experiment showed that the cyclin B fusion protein binds to a microtubule affinity column only in the presence of MAPs (data not shown). The most straightforward interpretation of this result is that cyclin B interacts directly with MAPs, rather than with microtubules.

An interaction between cyclins and microtubules was previously implied by the finding of an allele of the *S. pombe* cyclin B gene that causes the cell to become hypersensitive to the microtubule inhibitor thiabendazole (Booher and Beach 1988). Similarly, immunofluorescence studies in *S. pombe* have shown that cyclin B is localized to the spindle poles during mitosis (Alfa et al. 1990), and the work presented by Buendia et al. (this volume) demonstrates that the A and B cyclins differ in their ability to cause cell-cycle-specific changes in microtubule dynamics in frog extracts.

There are a number of possible implications of these results. The cyclins may be cell-cycle-specific adapter proteins that direct the activity of the $p34^{cdc2}$ protein kinase to specific substrates or subcellular locations. In this view, the presence of numerous different cyclin proteins in the cell adds a great potential for regulating the activity of the $p34^{cdc2}$ kinase. Alternatively, the cyclins and $p34^{cdc2}$ might interact with microtubules simply to localize these two important proteins to specific sites within the cell. However, it seems more likely that the strong interaction of the cyclins with microtubules and MAPs reflects a direct link to cytoskeletal function. The functional studies presented by Buendia et al. (this volume) strongly support this possibility. We are currently carrying out experiments to determine whether the cyclins interact directly with any of the proteins present in the centrosomal complex described above.

Identification of Maternal Effect Lethal Mutations That Disrupt Cytoskeletal Organization in the Early *Drosophila* Embryo

Genetic analyses have provided numerous important insights into complex biological processes. To initiate a genetic approach to cytoskeletal organization in the early *Drosophila* embryo, we are identifying and characterizing maternal effect lethal mutations that specifically disrupt the organization of the syncytial blastoderm. Since the syncytial blastoderm represents a unique stage in the *Drosophila* life cycle, it seems likely that there are special requirements for gene products during this stage and that mutations should be obtainable that disrupt these gene products. We initially described an ethylmethanesulfonate (EMS)-induced maternal effect mutation, *daughterless-abo-like (dal)*, that disrupts centrosomal separation during the late blastoderm divisions (Sullivan et al. 1990). Observations of living embryos that have been injected with both fluorescently labeled histone and tubulin show that the embryos derived from homozygous *dal* mothers develop normally until the nuclei reach the surface at nuclear cycle 10. However, during the surface divisions (cycles 10, 11, 12, and 13), the centrosomes associated with approximately half of the nuclei fail to complete their migration to opposite poles prior to assembly of the mitotic spindle. This causes the assembly of aberrant spindles that are incapable of normal chromosome segregation. The resulting abnormal nuclei dissociate from their centrosomes and sink into the center of the embryo prior to the formation of the cellular blastoderm.

The easily detectable phenotype of *dal* embryos has encouraged us to screen existing collections of P-element-induced maternal effect lethal mutations to identify additional mutations that specifically disrupt the cytoskeleton during the syncytial blastoderm divisions. This analysis has led to the identification of a class of mutations that do not affect early nuclear divisions or the migration of nuclei to the surface, yet severely disrupt the organization of the embryo after the nuclei reach the surface at nuclear cycle 10 and interact with the cortical cytoskeleton (Fig. 10).

One of these mutations, currently designated as *female sterile 37 (fs37)*, is of particular interest. Embryos from homozygous *fs37* mothers develop normally until completion of nuclear migration. During the surface divisions (cycles 10–13), however, the cortical region of these embryos becomes increasingly disorganized. In wild-type embryos, mitotic spindles are regularly spaced (Fig. 11a), and the cortical actin is organized in domains around each spindle (Fig. 11b). In mutant embryos, the spindles are unevenly distributed and are often fused to each other (Fig. 11c), and the actin filaments are no longer organized in distinct domains over each spindle (Fig. 11d). Real-time analysis of the cytoskeleton in *fs37* embryos demonstrates that

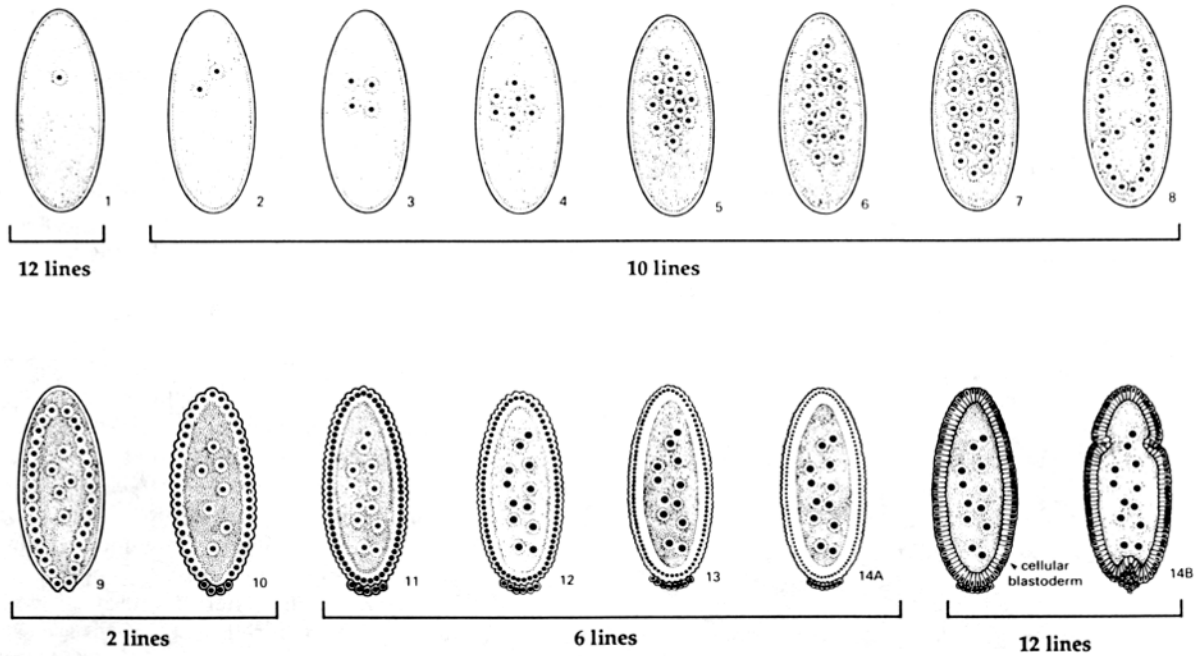

Figure 10. Results of a screen for maternal effect lethal mutations that disrupt cytoplasmic organization. A total of 73 lines carrying maternal effect lethal mutations induced by single P elements were screened for defects in nuclear and cytoskeletal organization. Mutant embryos were classified by the nuclear cycle at which they arrested or first showed abnormal nuclear behavior. These classes are indicated by the brackets, along with the number of lines recovered. Note that six of these mutations were normal through cycle 10, and only then began to show abnormal nuclear spacing and defects in actin filament and spindle organization. Only 44 of the lines laid normal or near-normal numbers of eggs. The remaining lines produced reduced numbers of eggs that were often morphologically abnormal. These were not screened cytologically.

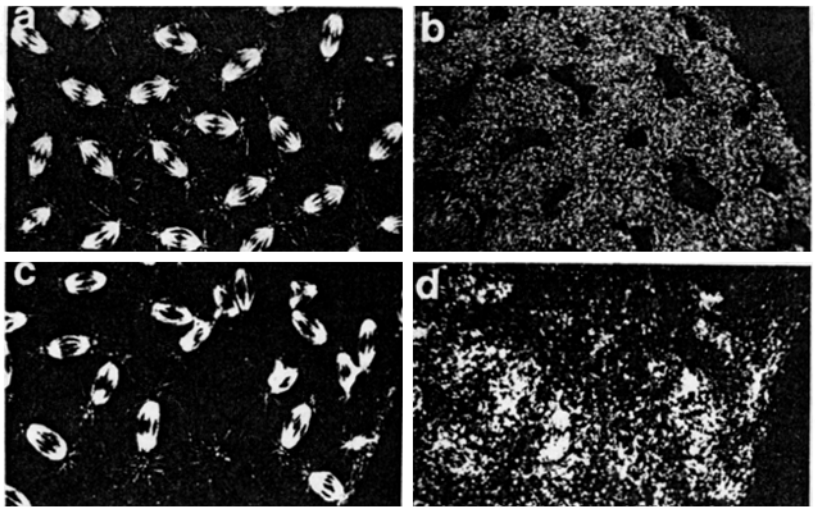

Figure 11. *fs37* maternal effect mutation causes a defect in cortical actin organization. Embryos derived from wild-type and homozygous *fs37*$^-$ females were fixed and stained to reveal the distribution of both microtubules and actin filaments. (*a*) Distribution of microtubules in a wild-type embryo at metaphase of nuclear cycle 12; (*b*) distribution of actin just beneath the surface of the plasma membrane in the same embryo. (The actin that surrounds each spindle at metaphase [Karr and Alberts 1986] cannot be seen in this focal plane.) In this wild-type embryo, the spindles are evenly spaced, and the cortical actin is organized in distinct domains over each spindle. (*c,d*) Corresponding stage in an embryo from an *fs37*$^-$ homozygous mother. The spindles are no longer evenly spaced, and the cortical actin is no longer organized in distinct domains over each spindle.

the defect in actin organization occurs specifically during mitosis and is likely to be the primary cause of the defects. The normal actin cap structures form at interphase in these embryos, but the furrows that normally separate adjacent spindles at metaphase are absent (data not shown).

In normal embryos, the mitotic actin furrows appear to prevent spindle fusions by providing a physical barrier between adjacent spindles (Karr and Alberts 1986; Sullivan et al. 1990). The molecular defect in *fs37* could be either in a regulatory function required to induce metaphase changes in actin, in a signaling mechanism associated with the centrosome that causes furrowing (see Fig. 1A), or in a structural component of the furrow itself. Cellular and molecular analyses of this and other mutations in the same class can readily be carried out since the mutations are induced by single P-element insertions (Cooley et al. 1989). We should therefore soon know the nature and cellular location of the *fs37* gene product. This type of genetic analysis should provide valuable insights concerning the structure, function, and regulation of the cytoskeleton in early embryos.

CONCLUSIONS

The study of the cytoskeleton presents a number of unique experimental challenges. In other systems, major progress toward understanding cellular function has generally been made by reconstituting functions in vitro and by the use of genetic screens to help identify the proteins involved. The study of the cytoskeleton has only a limited access to these approaches. The cytoskeleton is highly complex, and it is difficult to devise in vitro assays and genetic screens. For instance, we are largely ignorant of the many molecular steps that must be involved in the assembly of a mitotic spindle, but we cannot assemble spindles with partially purified components to begin to identify these steps, nor are there known genetic screens that will specifically identify genes involved in this pathway.

Nevertheless, there has been encouraging progress recently toward understanding the cytoskeleton. In our laboratory, we have attempted to develop new approaches for the identification and characterization of cytoskeletal proteins and protein complexes. We began our studies by developing affinity chromatography methods for the identification of proteins that interact with microtubules (Kellogg et al. 1989). This resulted in the identification and partial characterization of a large number of novel MAPs. We have focused our recent work on a 190-kD MAP that is localized to the centrosome, and we are using this protein as a starting point for a detailed molecular dissection of this important and yet poorly understood organelle. We first obtained a cDNA clone for D-MAP 190 and then used materials produced from this clone to develop a low-affinity antibody chromatography method that has allowed us to purify both D-MAP 190 and a number of additional MAPs that interact with it.

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 finding that D-MAP 190 interacts with a number of other MAPs to form a multiprotein complex suggests a simplification of the problem of studying the large number of different MAPs that bind to micro-

tubules. Rather than studying the functions of the many individual proteins, it may be possible to study the functions of a smaller number of multiprotein complexes.

What is the function of the complex of centrosomal proteins that we have isolated? The centrosomal location of these proteins suggests that they might be involved in a known centrosome function, such as the nucleation of microtubule growth, centrosomal duplication, and the stabilization of the minus ends of microtubules. Alternatively, these proteins might perform an uncharacterized centrosomal function or they might bind to the centrosome only to ensure their equal segregation at mitosis. In any case, the purified proteins can now be tested in a variety of in vitro assays to determine whether they possess activities that affect microtubules or centrosomes, and antibodies that recognize these centrosomal proteins can be injected into embryos in attempts to disrupt protein function. If the antibodies are coinjected with 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 dynamics that result from the injection of antibodies that disrupt the function of a centrosomal protein.

We have also screened through collections of maternal effect lethal mutations and discovered a number of novel mutations that specifically disrupt cytoskeletal organization in the syncytial blastoderm embryo. These kinds of mutations are likely to make new contributions to our understanding of both the functions of cytoskeletal proteins and the organization and function of cytoskeletal networks. The fact that these mutations have been induced by single P-element insertions will greatly facilitate their characterization (Cooley et al. 1989). Thus, the genes affected by the mutations can be conveniently cloned, and antibodies raised against the gene products can be used to determine their subcellular localizations.

Once the cDNA for a new cytoskeletal protein has been cloned, low-affinity antibody chromatography can be used both to isolate the protein and to identify and purify additional proteins that interact with it. We expect that the biochemical and genetic approaches that we have described will eventually be complementary, resulting in the identification of overlapping sets of proteins. By continuing these kinds of approaches, we hope to accomplish a detailed dissection of cytoskeletal organization and function in the early *Drosophila* embryo.

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