

Actin-binding Proteins from *Drosophila* Embryos: A Complex Network of Interacting Proteins Detected by F-actin Affinity Chromatography

Kathryn G. Miller, Christine M. Field, and Bruce M. Alberts

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143

Abstract. By using F-actin affinity chromatography columns to select proteins solely by their ability to bind to actin filaments, we have identified and partially purified >40 proteins from early *Drosophila* embryos. These proteins represent ~0.5% of the total protein present in soluble cell extracts, and 2 mg are obtained by chromatography of an extract from 10 g of embryos. As judged by immunofluorescence of fixed embryos, 90% of the proteins that we have detected in F-actin column eluates are actin-associated in vivo (12

of 13 proteins tested). The distributions of antigens observed suggest that groups of these proteins cooperate in generating unique actin structures at different places in the cell. These structures change as cells progress through the cell cycle and as they undergo the specializations that accompany development. The variety of different spatial localizations that we have observed in a small subset of the total actin-binding proteins suggests that the actin cytoskeleton is a very complex network of interacting proteins.

THE *Drosophila* embryo develops as a large highly organized syncytial cell during the first 14 nuclear division cycles. Within this large cell, regions of the cytoplasm differ from each other in organization and structure, and cytoplasmic and organellar movements occur in a precisely timed and coordinated fashion as the embryo develops. This high degree of organization is mediated by the cytoskeleton, with actin filaments and microtubules playing many important roles (Foe and Alberts, 1983; Edgar et al., 1988).

The actin cytoskeleton in early *Drosophila* embryos has been studied by immunofluorescence techniques (Karr and Alberts, 1986; Warn et al., 1984). Actin is highly concentrated in the cortex of the embryo, where large organelles are excluded from a special region of the cytoplasm. During the syncytial blastoderm stage (nuclear cycles 10–14), nuclei are present in an evenly spaced monolayer in the cortex of the cell, and actin forms distinct structures that change during the cell cycle and with developmental time. During interphase, actin filaments appear to help hold nuclei in their proper cortical location and are concentrated just below the plasma membrane in a patch above each nucleus. During mitosis, transient membrane furrows form between closely spaced nuclei in the syncytium, which presumably helps to keep the neighboring mitotic spindles separate from each other (Stafstrom and Staehelin, 1984). At this stage of the nuclear cycle, actin filaments form a continuous layer beneath the entire plasma membrane, including the membrane fur-

rows. However, the actin underlying the furrows plays an important role in their formation, and it differs in function, organization and regulation from the actin filaments underlying the rest of the membrane.

Early *Drosophila* embryos are large syncytial cells that are available in sufficient quantities for biochemical analyses and can be readily manipulated by both genetic and microinjection techniques. They therefore provide an especially accessible system for analyzing the structure and function of the many proteins associated with actin filaments (called actin-binding proteins or ABPs).¹ To begin a characterization of proteins that are important for actin filament organization and function in these embryos where relatively few ABPs have been previously characterized, we have developed an F-actin affinity chromatography method (Miller and Alberts, 1989). Beginning with the ABPs identified in this way, we hope to gain insight into the many actin-mediated processes involved in the structure, growth, and development of these fascinating cells. Selected aspects of this work have been reported in a preliminary form (Miller et al., 1985).

Materials and Methods

Materials

All chemicals used were reagent grade. Phalloidin, leupeptin, pepstatin, aprotinin, suberimidate, and NP-40 were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies to *Drosophila* cytoplasmic myosin and spectrin were provided by Dan Kiehart. Mono Q column was from Pharmacia Fine Chemicals (Piscataway, NJ).

1. *Abbreviations used in this paper:* ABP, actin-binding proteins; DAPI, 4,6-diamidino-2-phenylindole.

K. G. Miller's present address is the Department of Biology, Washington University, St. Louis, MO 63130.

Buffers

E buffer: 5 mM K-Hepes, pH 7.5, 0.05% NP-40, 0.5 mM Na₃EDTA, 0.5 mM Na₃ EGTA, 10 µg/ml each leupeptin, pepstatin, and aprotinin. A buffer: 50 mM K-Hepes, pH 7.5, 2 mM DTT, 0.5 mM Na₃ EDTA, 0.5 mM Na₃ EGTA, 0.05% NP-40, 10 µg/ml each of leupeptin, pepstatin, and aprotinin. SDS polyacrylamide gel sample buffer: 62 mM Tris-Cl, pH 6.8, 3% SDS, 5% β-mercaptoethanol, 10% glycerol. TBS: 0.02 M Tris-Cl, pH 7.5, 0.5 M NaCl. PBS: 20 mM potassium phosphate, pH 7.3, 0.15 M NaCl.

Preparation of a *Drosophila* Embryo Extract

Embryos were collected from Oregon R flies in population cages by letting the flies lay eggs on fresh corn meal agar plates for a period of up to 4 h. Embryos were washed off the plates with 0.15 M NaCl, 0.05% Triton X-100 (Triton-NaCl wash solution) and briefly dechorionated with 50% Clorox (bleach). After extensive rinsing with Triton-NaCl, the embryos were washed with E buffer and blotted dry. The embryos were weighed and suspended in 10 vol (wt/vol) of E buffer. After PMSF was added to 1 mM, the embryos were homogenized at 4°C with 5 strokes of a motor-driven, loose fitting teflon-glass homogenizer (Wheaton Instruments, Millville, NJ). The homogenate was centrifuged at 10,000 g for 20 min and the supernatant brought to 2 mM DTT and 50 mM K-Hepes (pH 7.5). After centrifugation at 100,000 g for 1 h the supernatant was loaded onto the appropriate columns at 4°C.

Affinity Chromatography of Extracts

Affinity chromatography columns were prepared as described (Miller and Alberts, 1989), using actin filaments stabilized with phalloidin. F-actin, G-actin, and control (albumin) columns of equal bed size and protein content were equilibrated with A buffer containing 10% glycerol (or another indicated loading buffer) at 4°C. Equal volumes of the same extract were applied to all of the columns in each experiment (unless otherwise noted), using a flow rate of 1 column vol/h or less. After loading, all of the columns were rinsed at 1–2 column vol/h with A buffer containing 10% glycerol until protein in the eluate had reached background levels for the F-actin columns (<10 µg/ml protein), which generally required more rinsing than the G-actin or control columns. The elution was then carried out step-wise with A buffer containing 10% glycerol plus added KCl and/or 1 mM ATP plus 3 mM MgCl₂, as indicated in each experiment. Protein was determined by the method of Bradford (1976). The fractions containing protein in each elution step from F-actin columns were pooled, as were equivalent fractions from control or G-actin columns (which often had no detectable protein peak). The protein present in an aliquot of each pool was then precipitated with 10% TCA. After the precipitate was resuspended in SDS polyacrylamide gel sample buffer and neutralized with 2 M Tris base, volumes representing the same proportion of the eluates from each column were loaded onto the successive lanes of a 5–15% polyacrylamide gradient gel (or a 8.5% polyacrylamide gel) and electrophoresed in SDS by standard techniques (Laemmli, 1970). Proteins were visualized by Coomassie blue staining of the gels.

Protein Immunoblotting

To detect specific proteins with antibodies, samples were electrophoresed, transferred to a nitrocellulose sheet by blotting (Towbin et al., 1979), and detected by standard techniques (except that proteins were transferred in 25% methanol, 0.15 M glycine, 0.02% SDS, pH 7.5). Peroxidase-conjugated, goat anti-mouse antibody (Cappel Laboratories, Malvern, PA) or goat anti-rabbit antibody (Bio-Rad Laboratories, Cambridge, MA) were used to visualize proteins.

Sucrose Gradient Sedimentation

The *Drosophila* actin-binding proteins eluted from a preparative size F-actin column in a single elution step (1 M KCl, 2 mM ATP, 3 mM MgCl₂ in A-buffer containing 10% glycerol) were concentrated to ~1 mg/ml and dialyzed against 0.1 M KCl, 50 mM Tris-Cl, pH 7.7, 0.5 mM Na₃ EDTA, 0.5 mM Na₃ EGTA, 2 mM DTT. After centrifugation at 15,000 g for 15 min to remove aggregates, a volume of 200 µl was loaded onto a 5–20% sucrose gradient prepared in the above dialysis buffer. Centrifugation was at 50,000 rpm in a rotor (SW60; Beckman Instruments, Inc., Palo Alto, CA) for 15 h. An identical gradient containing protein markers of known size was run in parallel. After the gradients were collected drop-wise from the bottom, the

proteins in each fraction were analyzed by electrophoresis through an 8.5% SDS polyacrylamide gel.

Generation of Mouse Polyclonal Antibodies to Gel-Purified Proteins

We have produced mouse polyclonal antibodies using procedures modified from those of Amero et al. (1987). A total of about 1.6 mg of affinity-purified ABPs were separated on two 14.5 cm × 20 cm preparative 5–15% polyacrylamide gradient gels containing SDS (0.82-mm thick). The gels were stained with Coomassie blue, destained in methanol/acetic acid/H₂O (5:1:5) for 0.5 h, and treated with a solution of 2% glutaraldehyde in water for 1 h. The gels were then transferred to 7% acetic acid to complete the destaining procedure. After soaking in water to remove the acetic acid, protein bands of interest were excised with a razor blade and homogenized with a motor-driven homogenizer in the presence of a small amount of water. The homogenized gel bands were then lyophilized and resuspended in 1.5 ml of sterile PBS.

Each gel slice was used for a total of four immunizations spaced at 2 wk intervals. The first immunization was with 0.45 ml of homogenized gel slice slurry and 0.1 ml of complete Freund's adjuvant, while the remaining three immunizations used 0.34 ml of the slurry and 0.1 ml of incomplete Freund's adjuvant. The adjuvant mixtures were warmed to 37°C and vortexed vigorously before loading into syringes. All immunizations were given intraperitoneally with a 22-gauge needle. Beginning 1 wk after the final immunization, the mice were bled intraorbitally every 7–10 d, and the sera were tested for the presence of specific antibodies. Mice were anesthetized with ether before all immunizations and bleeds.

Localization of ABPs Using Indirect Immunofluorescence

Drosophila embryos that were collected from population cages for up to 3.5 h were harvested and dechorionated as described above for extract preparation. The dechorionated embryos were fixed by two different methods. As our standard procedure, embryos were fixed with formaldehyde by the method of Karr and Alberts (1986). However, some antigenic determinants were not well preserved by this method and were better visualized after heat treatment followed by devitelinization in methanol. For the latter purpose, embryos were added to 10 ml Triton-NaCl wash solution at 90°C in a boiling water bath (total time at 90°C is <5 s). The solution was immediately vortexed, mixed with 40 ml of cold Triton-NaCl, and plunged into an ice-water bath. After removal of the Triton-NaCl solution, embryos were shaken with heptane: 95% methanol, 25 mM Na₃ EGTA (1:1) for 30 s. Devitelinized embryos sink in the methanol solution and were collected with a Pasteur pipette, rinsed several times with 95% methanol: 25 mM Na₃ EGTA and incubated for at least 1 h in this solution. The embryos were then rehydrated by successive rinses with 40 ml each of: 95% methanol/5% PBS, 60% methanol/40% PBS, 40% methanol/60% PBS, 20% methanol/80% PBS, and PBS. Both formaldehyde-fixed and heat-fixed embryos were incubated in PBS containing 5% BSA and 0.05% Tween detergent before staining with antibodies.

Whole fixed embryos were stained with polyclonal antisera or monoclonal antibodies at dilutions determined empirically to give the clearest images. The bound antibodies were then visualized by treatment with a rhodamine-conjugated goat anti-mouse antiserum (Cappel Laboratories) that had been previously absorbed on whole fixed *Drosophila* embryos to reduce the nonspecific background. All embryos were also labeled with the DNA specific dye DAPI (4, 6-diamidino-2-phenylindole) so that developmental age and cell cycle stage could be assessed precisely (Foe and Alberts, 1983). The stained embryos were observed and photographed using both rhodamine and DAPI filter sets on a standard microscope (Carl Zeiss, Inc., Thornwood, NY) or a scanning confocal microscope (Bio-Rad Laboratories, Cambridge, MA).

Results

To understand better the role of the actin cytoskeleton in the spatial organization of the cytoplasm during embryonic development, we need to characterize the proteins associated with actin filaments in embryos. To facilitate such a study, we have developed a simple method of F-actin affinity chro-

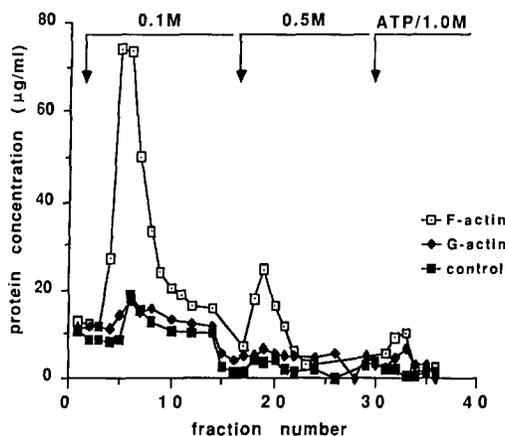


Figure 1. Elution profiles observed when embryo extracts are chromatographed on F-actin, G-actin, and control (albumin) columns. The protein concentration in each fraction of 0.5 ml is plotted against the fraction number in each case. In this experiment, 10 ml of clarified extract containing 3.3 mg/ml of protein was loaded onto each 3-ml column; elutions were with A buffer containing the indicated concentration of KCl, plus 1 mM ATP and 3 mM MgCl₂ where noted.

matography (Miller and Alberts, 1989). When this technique is used to analyze extracts of early *Drosophila* embryos, ~15 major proteins and at least 30 more minor ones are observed to bind and elute specifically from F-actin columns (Figs. 1 and 2). These proteins represent ~0.5% of the total soluble protein, whereas G-actin and control columns bind <0.05%. We have identified one of the proteins as *Drosophila* cytoplasmic myosin (205,000-D protein in an ATP elution) (Kiehart and Feghali, 1986) and another as *Drosophila* spectrin (~230,000-D protein present in the salt elutions) (Dubreuil et al., 1987) by immunoblotting proteins separated by SDS polyacrylamide gel electrophoresis with antibodies provided by Dan Kiehart (data not shown). Numerous other major proteins elute from the F-actin columns and not from G-actin or control columns. These include a 150,000-D protein in an ATP elution and a 130,000-D protein present in a 0.5 M salt elution. In addition, salt elutes two proteins of ~220–240,000 D, two or three proteins >250,000 D, several 100,000-D proteins, and a 60,000-D protein. A prominent group of proteins in the 35–45,000-D size range is seen as well (Fig. 2; see also Figs. 3 and 4). All of these major protein species are always present in the F-actin column eluates, but they often vary in relative abundance from preparation to preparation. In part, this may be because of variable levels of proteolysis that affect some proteins more than others (Fig. 5).

A few minor species bind to both G-actin and F-actin columns. Some known ABPs also have some affinity for G-actin (Bretscher and Weber, 1980). However, most of the *Drosophila* proteins we detect bind only to F-actin (Fig. 2 A).

The proteins that elute from F-actin columns can be rebound to a second F-actin column specifically, revealing that at least some features of their native structure are maintained under our elution conditions (Fig. 2 B). The protein in the unbound fraction ("flow-through") in such a rebinding experiment is dominated by the abundant embryo yolk proteins (43–45,000 D), a small fraction of which often contaminates the initially selected ABPs. In addition, the ATP-eluting pro-

teins rebound to the F-actin column only partially, probably because of some residual ATP in the dialyzed ABP eluate that was reloaded onto F-actin (Fig. 2 B).

Preparative Quantities of ABPs Can Be Obtained by F-Actin Affinity Chromatography

A *Drosophila* ABP that is quantitatively bound and eluted from an F-actin column will be purified more than 200-fold from the extract in this single chromatography step. We have routinely used columns as large as 25-ml packed volume (containing ~25 mg of phalloidin-stabilized F-actin) to fractionate the extract from 10 g of embryos; this yields approximately 2 mg of total *Drosophila* ABPs. The proteins obtained can be stored at -70°C in A buffer containing 30% glycerol and accumulated for the further fractionations to be described next.

The Behavior of the *Drosophila* ABPs during Subsequent Fractionation Steps

Eventually, each of the *Drosophila* ABPs must be purified to homogeneity in active form to permit in vitro characterizations. We have begun such purification using, as a first step, ion-exchange chromatography on an HPLC column. As judged by the SDS polyacrylamide gel electrophoresis analysis in Fig. 3, two major proteins are ~90% homogeneous after this ion-exchange step (the 130,000-D protein eluting in fractions 10–12, and the 230,000-D protein in fractions 23–26). Other proteins appear to coelute from the ion-exchange column, such as the group of proteins between 35,000 and 45,000 D in fractions 4–7. Some proteins seem to appear in two different places in the salt gradient, which may indicate that they can exist both free and in association with other proteins; however, additional studies are needed to test whether ABPs of the same molecular weight are actually the same protein.

To test further the possibility that some of the *Drosophila* ABPs are present in larger complexes in our eluates, we have analyzed the ABP mixture by sucrose gradient centrifugation (Fig. 4). This technique fractionates each species according to its sedimentation rate and provides information on the size and/or shape of the molecules in their native state. Sedimentation of the mixture of ABPs through a 5–20% sucrose gradient reveals that the group of 35,000–45,000-D proteins that coelute after HPLC ion-exchange chromatography exist as a complex. This group of proteins cosediments at ~11S, a rate that is much faster than expected for the size of the individual components (fractions 17–20; an apparent molecular weight of ~200,000 when compared to globular proteins run as standards on a parallel gradient). These actin-binding proteins sediment similarly even in 1 M KCl (data not shown). Several other actin-binding proteins sediment at rates slower than that expected on the basis of their molecular weight, indicating that the proteins are asymmetrically shaped. Thus, one of the 100,000-D proteins (peak at fraction 7) sediments significantly slower than the 43,000-D ovalbumin (3.5S) standard. The 130,000-D protein sediments only slightly ahead of albumin (4.3S, 68,000 D), and several ~230,000-D proteins also sediment considerably slower than expected (peak at fraction 14 for the largest; peak at fraction 16 for several others). Asymmetric shape is a common characteristic of

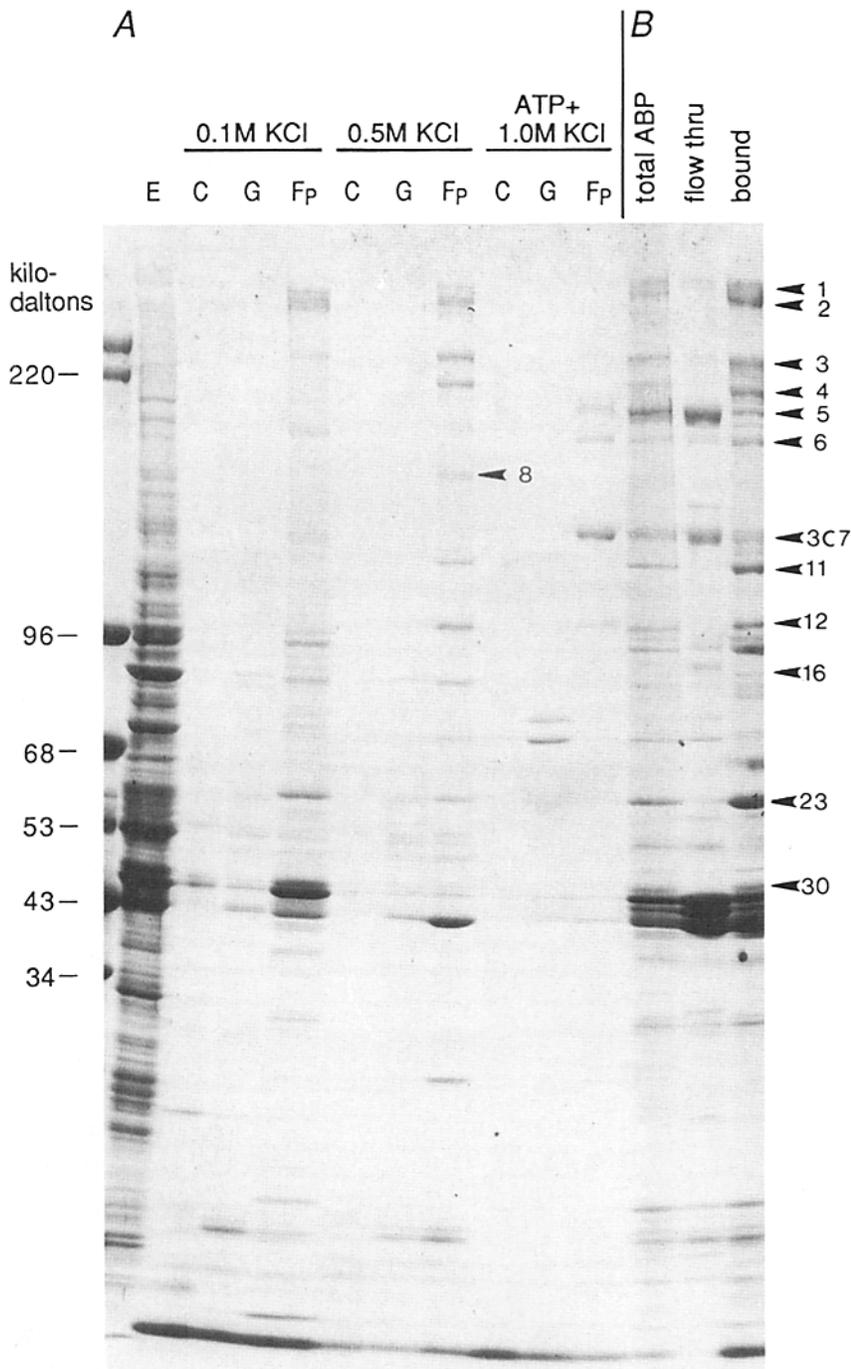


Figure 2. Analysis by SDS-PAGE of the proteins eluting from columns loaded with a *Drosophila* embryo extract. (A) The proteins eluted from F-actin, G-actin, and control columns loaded and run in parallel. Lanes are marked as follows: E, extract; C, control (albumin column); G, monomeric actin column (G-actin); F_p, phalloidin-stabilized filamentous actin (F-actin) column. The same proportion of the total sample was loaded onto the gel for each eluate. (B) Re-binding to a second F-actin column of pooled *Drosophila* ABPs isolated by elution with 1.0 M KCl, 1 mM ATP, 3 mM MgCl₂ in A buffer containing 10% glycerol. This eluate was dialyzed for 4 h against A buffer and then centrifuged to remove any precipitate before reloading onto the F-actin column. The lane marked *flow-through* is the fraction that did not rebind; the lane marked *bound* contains the protein that rebound and eluted from the second column. The *total ABP* lane contains one-third as much of the total sample than does the other two lanes. When uncontaminated by the yolk proteins, generally 70% of the total protein isolated from an initial F-actin column will rebind to a second one. The numbers to the right of the figure correspond to the bands used for immunization of mice to generate the antisera shown in Table I. Band 5 is cytoplasmic myosin.

known ABPs (Stossel et al., 1985); for example, spectrin, α -actinin, and filamin.

Distribution of ABPs in *Drosophila* Embryos

To determine if the large number of proteins in *Drosophila* embryos that bind to F-actin affinity chromatography columns are associated with actin filaments in fixed embryos, we have raised antibodies against some of the proteins and used these antibodies to locate them in early embryos. After immunizing mice with more than 30 protein bands from preparative SDS polyacrylamide gels (numbered in order

of decreasing molecular mass), we obtained mono-specific polyclonal antisera against 13 different protein species. When used to probe protein (Western) blots of salt eluates, these antibodies react with the protein species used for the immunization, as illustrated for some of these antibodies in Fig. 5. The proteins recognized by antisera 2, 3, 4, 11, 12, 16, 23, and 30 (Fig. 5) and by antisera 1, 5, 6, and 8 (data not shown) are present in F-actin column eluates and absent from the eluates of control columns. Some antibodies also react with lower molecular weight species (e.g., antiserum 4, see Fig. 5 legend); these are variably observed in ABP preparations and presumably are proteolysis products of the larger species.

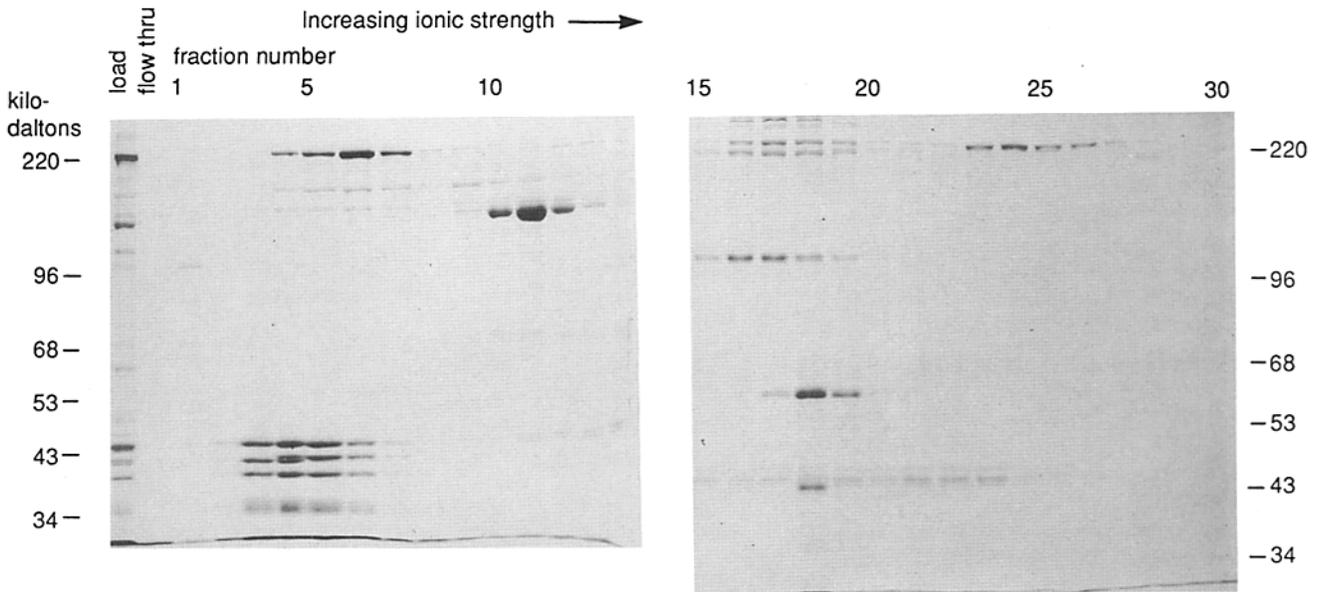


Figure 3. An analysis by SDS-PAGE of *Drosophila* ABPs fractionated by ion exchange chromatography on a positively charged HPLC resin. The total ABPs eluted from an F-actin column (0.6 mg) were dialyzed into 50 mM Tris-Cl, pH 7.7, 50 mM KCl, 0.5 mM DTT, 5% glycerol, 1 μ g/ml each of aprotinin, leupeptin, and pepstatin, and loaded onto a 1-ml Mono Q column (*load*). After washing at 4°C, the column was eluted with a 40-ml gradient of NaCl (50 mM to 1 M) at 1 ml/min. Proteins eluting in each step were precipitated with TCA and prepared for SDS-PAGE as described in Materials and Methods. The flow-through is the fraction that did not bind to the column. The column fractions are indicated by fraction numbers in order of increasing ionic strength; each fraction contained 0.5 ml. Note that these fractions have been analyzed on two separate gels in which molecular mass calibrations differ. This experiment was performed by Enrique Amaya.

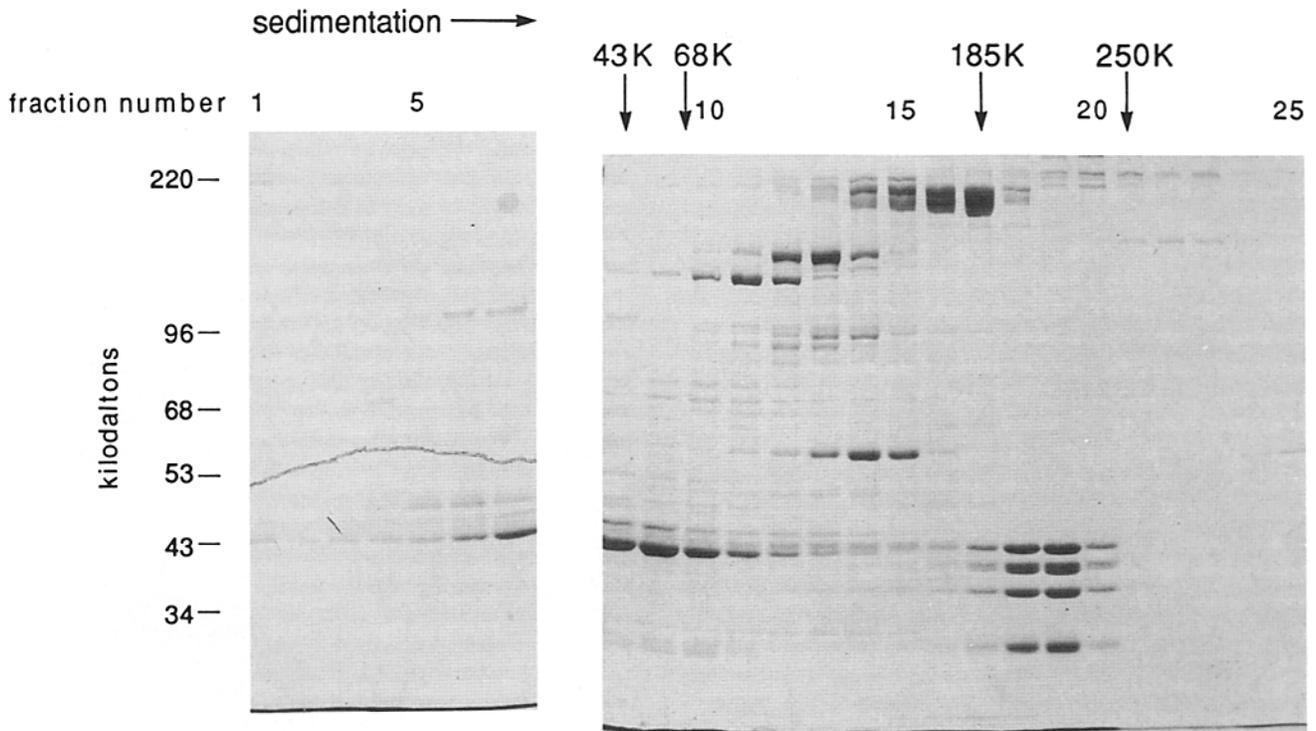


Figure 4. Analysis by SDS-PAGE of total *Drosophila* ABPs fractionated by sedimentation through a 5–20% sucrose gradient. Gradients were run as described in Materials and Methods, and fractions are numbered from the top of the gradient. The positions of the roughly spherical marker proteins sedimented on an identical gradient run in parallel are marked and denoted by their native molecular masses; these markers are catalase (250,000 D; 11.3S), phosphorylase b (185,000 D; 8.4S), BSA (68,000 D; 4.3S), and ovalbumin (43,000 D; 3.5S).

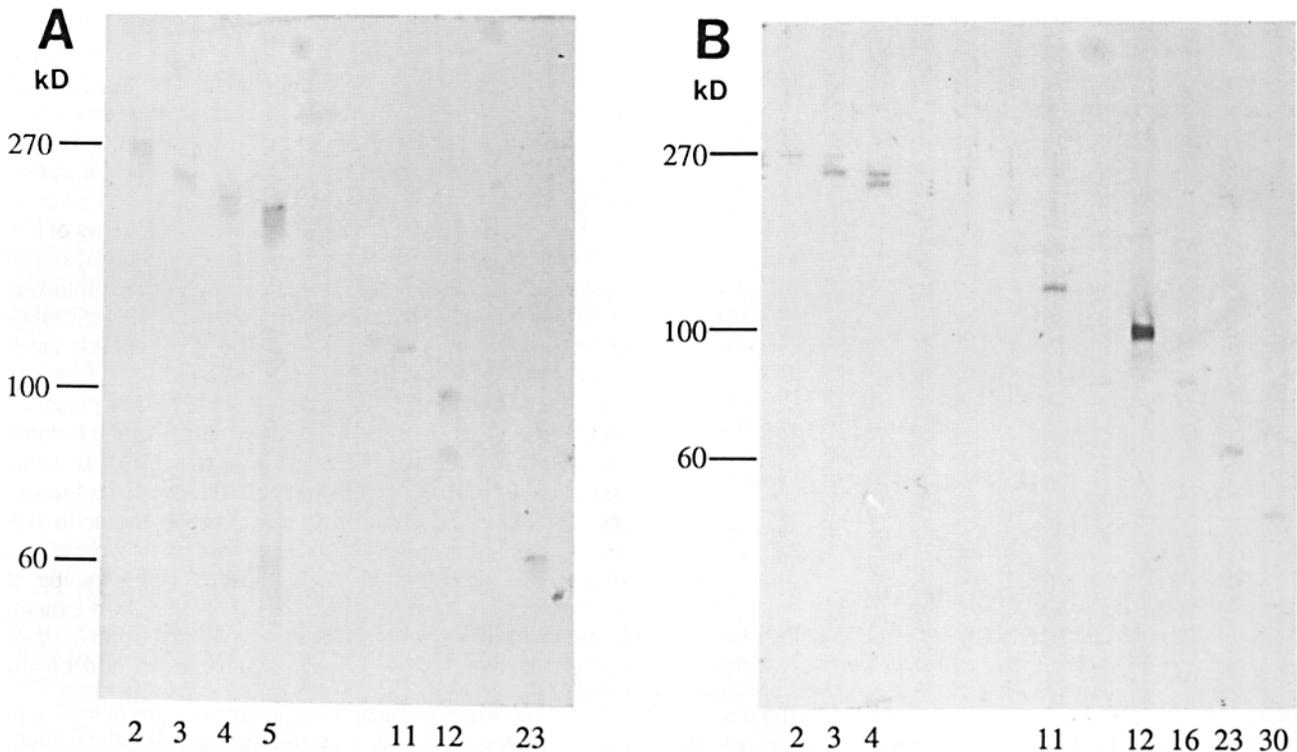


Figure 5. Immunoblot analysis of polyclonal mouse antibodies to *Drosophila* ABPs. *Drosophila* whole embryo homogenate (*A*, 30 µg protein/lane) or proteins that elute from F-actin affinity columns with 0.5 M KCl (*B*, 1 µg protein/lane) were heated in SDS, electrophoresed on 7.5% polyacrylamide gels, blotted onto nitrocellulose, and probed with the antisera listed below each lane (see Table I). Antisera 8, 16, and 30 do not identify any protein on blots of whole embryo extract (*A*). Antiserum 1 is not shown on the blot in *A*. Antiserum 4 labels two species in the actin-binding fraction (*B*). This antiserum was raised by immunization with the lower molecular mass species, an apparent proteolysis product of the higher molecular mass species, since the relative amounts of these two species varies in different preparations. The higher molecular mass species, although similar in size to the protein identified by antiserum 3, can be resolved from it. Therefore, antisera 3 and 4 identify different proteins. The antigens recognized by antiserum 5 and mA 3C7 are absent in 0.5 M KCl eluates; these two proteins elute from F-actin in the presence of ATP (not shown). Antisera 1, 6, and 8 are not shown on the blot in *B*.

When used to probe blots of extracts of whole *Drosophila* embryos, 9 of the 12 antibodies tested react with a single band of the identical molecular weight as the species detected in ABP preparations (for antisera 2, 3, 4, 5, 11, 12, 23, see Fig. 5 *A*; data for antiserum 1 and mAb 3C7 not shown). The remaining three antisera (8, 16, 30) fail to detect any protein in whole embryo extracts, even on heavily loaded blots, suggesting that they recognize minor protein species. Antibodies obtained against one protein do not react with other bands used for immunization, indicating that each protein band is unique. Since 30 times more protein was loaded in each lane for the extract (Fig. 5 *A*) compared to the ABP eluate (Fig. 5 *B*), these experiments also show that each of the antigens recognized by our antibodies is greatly enriched in the F-actin column eluates relative to the extract.

We have previously produced monoclonal antibodies against several of the *Drosophila* ABPs and used them to determine the distribution of the corresponding ABPs (Miller et al., 1985) relative to that of actin during the syncytial blastoderm stages of *Drosophila* embryonic development (Karr and Alberts, 1986; Warn et al., 1984). The polyclonal antibodies have enabled us to extend this analysis, as summarized in Table I, where the distribution of each antigen during nuclear cycles 11 through 13 is presented. To obtain this data, embryos were observed both by conventional fluorescence microscopy (which clearly reveals surface structures) and by

scanning confocal microscopy (which produces optical sections showing the interior of these thick specimens).

To prepare Table I, we examined both formaldehyde-fixed and heat-fixed embryos at all stages during nuclear cycles 11–14. Several antisera were used to stain each batch of fixed embryos (using ~150 embryos/antiserum) and anti-actin staining was also performed as a control for proper fixation. Many embryos at all stages of the cell cycle during this time period were observed, with a large number of fixations performed for each antiserum or monoclonal antibody. These embryos can be staged to within ± 1 min by observation of the nuclear density and chromosome morphology (Foe and Alberts, 1983), which are readily visualized by the DAPI staining pattern of each fixed embryo. Thus, we can be sure that embryos of exactly the same age were observed with each antiserum. For four different antisera or monoclonal antibodies, double label immunofluorescence with anti-actin antibody or fluorescent phalloidin was examined to confirm our determination of location of the antigen relative to actin (data not shown).

During interphase of nuclear cycles 11 to 13, actin filaments are present in the cortical cytoplasm in patches or caps (cortical caps) over the syncytial nuclei. These caps are thought to help hold the nuclei in an even cortical layer, since the nuclei fall away from the surface into an irregular array when the actin is disrupted (Zalokar and Erk, 1976; Foe and

Table I. The Spatial Distribution of *Drosophila* ABPs, as Determined by Immunofluorescence Staining during the Syncytial Blastoderm Stages of Embryo Development*

Antibody number	Mass in kilodaltons	Distribution in interphase	Distribution in metaphase
1	296	Cap-borders	Furrows
2	270	Cap-borders with diffuse cytoplasmic staining	Furrows‡
§13G5	230	Cortical caps	Caps
3	230	Cap-borders	Furrows‡
4	220	Cap-borders	Furrows‡
5	205	Cytoplasmic	Furrows
6	200	Nuclear envelope	
8	185	Concentrated in cortex, excluded from cortical cap	Furrows‡ (only at tips)
§3C7	150	Cortical cap with punctate cytoplasmic staining	Furrows
11	130	Cortical cap with punctate cytoplasmic staining	Furrows (plus a cytoplasmic component)
12	100	Cortical caps	Furrows
16	87	Not in caps, concentrated in cortex, punctate	Furrows‡
23	60	Cortical caps	Furrows
30	49	Not in caps, cytoplasmic, punctate, somewhat concentrated in cortex	Furrows
Actin	43	Cortical caps	Furrows

Furrows, staining underlies transient membrane furrows that divide neighboring cytoplasmic domains in metaphase of cycles 11–13. *Cortical caps*, staining is concentrated in a patch just below the plasma membrane above each syncytial nucleus in cycles 10–14. *Cytoplasmic*, staining is detected throughout the cytoplasmic domain that surrounds each syncytial nucleus. *Cap-borders*, staining appears to be concentrated at the edges of the cortical caps when an embryo is viewed from the top surface in interphase.

* Also see Fig. 10.

‡ Staining at cellularization front during cycle 14.

§ These are monoclonal antibodies: 3C7 recognizes the same antigen as antibody 10.

Alberts, 1983). During metaphase, transient membrane furrows form that divide neighboring mitotic spindles from each other; at this time the actin is present in a continuous layer in the cortex, and it lies beneath the membrane in each of these furrows. Surface views of these distributions have been previously described in detail (Karr and Alberts, 1986); Fig. 6 (*top*) presents the cross-sectional view of these actin structures that is obtained by scanning confocal microscopy (White et al., 1987).

Most of the *Drosophila* ABPs that we have characterized by immunocytochemical methods produce staining patterns that can be distinguished from each other, either at different developmental stages or during part of the highly dynamic nuclear cycle. However, most of them can be roughly classified into one of three groups: (a) like actin filaments at all times; (b) like actin filaments only during metaphase; (c) or like actin filaments only during interphase. Surface views of antibody-stained embryos showing one example in each group are presented in Fig. 7. Note that, in these surface views, those proteins that colocalize with actin during metaphase appear to be concentrated in a series of interconnected hexagonal rings, because of the deep furrows lined with actin that surround each spindle (see Fig. 6, *top*).

The most striking feature of the protein localizations ob-

served, and the one that most makes us believe that all of the proteins described in Table I (except number 6) are actin-associated in the cell, is their predominant localization just beneath the plasma membrane, a property that they share with actin filaments. This aspect of their localization is most clearly seen with a scanning confocal fluorescence microscope, which readily provides optical sections perpendicular to the plasma membrane. Immunofluorescence views of five ABPs taken during interphase and mitosis in the syncytial blastoderm (cycles 10 to 13) are shown in Fig. 8, while Fig. 9 presents similar views taken during the process of cellularization (mid-cycle 14). The noncortical (i.e., nuclear envelope) location of the antigen recognized by serum 6 (see Fig. 6, *bottom*) demonstrates that antibodies can readily penetrate deeply into the embryo and provides a control for the staining methods used (see also Karr and Alberts, 1986). In some cases (see antiserum 11 in Fig. 8; also antisera 5, 16, and 30, not shown), staining intensity is greatest in the actin-rich cortex, but general cytoplasmic staining is also observed. Thus, some of the proteins that colocalize with actin appear to extend further into the cytoplasm from the plasma membrane than do most actin filaments. ABPs identified in other systems, such as *acanthamoeba* α -actinin (Pollard et al., 1986), show similar distributions.

*Distinct Features in the Localizations of *Drosophila* ABPs*

Most of the antigens we detect are present in the cortex in an actinlike distribution during mitosis, displaying the characteristic hexagonal surface staining pattern that results when a protein underlies the plasma membrane in the furrows. During interphase, many of these antigens differ in their arrangement both from actin and from each other. Several antigens (1, 2, 3, and 4) stain the regions between nuclear domains where the furrows have retracted (see Figs. 7 and 8), giving them the "cap border" pattern when viewed from the surface. In contrast, actin and antigens that mimic actin, such as the protein recognized by antiserum 23 (Fig. 7), antiserum 12 (Fig. 8), and mAb 3C7 (not shown) have reformed into caps and disappeared from the cap borders. Even if we confine our attention to the antigens that are present in cap borders at interphase, there are clear differences in spatial distributions. For example, at interphase, antigen 2 only weakly labels the cap region itself in contrast to antigen 4 that stains both the cap and cap borders with equal intensity (see Fig. 8, *left*). Our preliminary staining of whole egg chambers (containing nurse cells, follicle cells, and developing oocytes) reveal distinct spatial distributions for many of these antigens (data not shown), further distinguishing them from each other and from actin. Other antigens (*Antiserum II* in Fig. 8; 5, 16, and 30, not shown) that colocalize with actin during metaphase have a cytoplasmic distribution during interphase. These antigens are often more concentrated in the cortex but also are present in the cytoplasmic domain that surrounds each nucleus.

The distribution of antigen 8 is especially interesting, because it suggests that this protein might be important for generating the actin structures that underlie the furrow at metaphase. This antigen, which during interphase of the syncytial blastoderm stages is only present in the region where the furrow will subsequently form, is concentrated at the inward growing tips of the furrows during metaphase (Fig. 8).

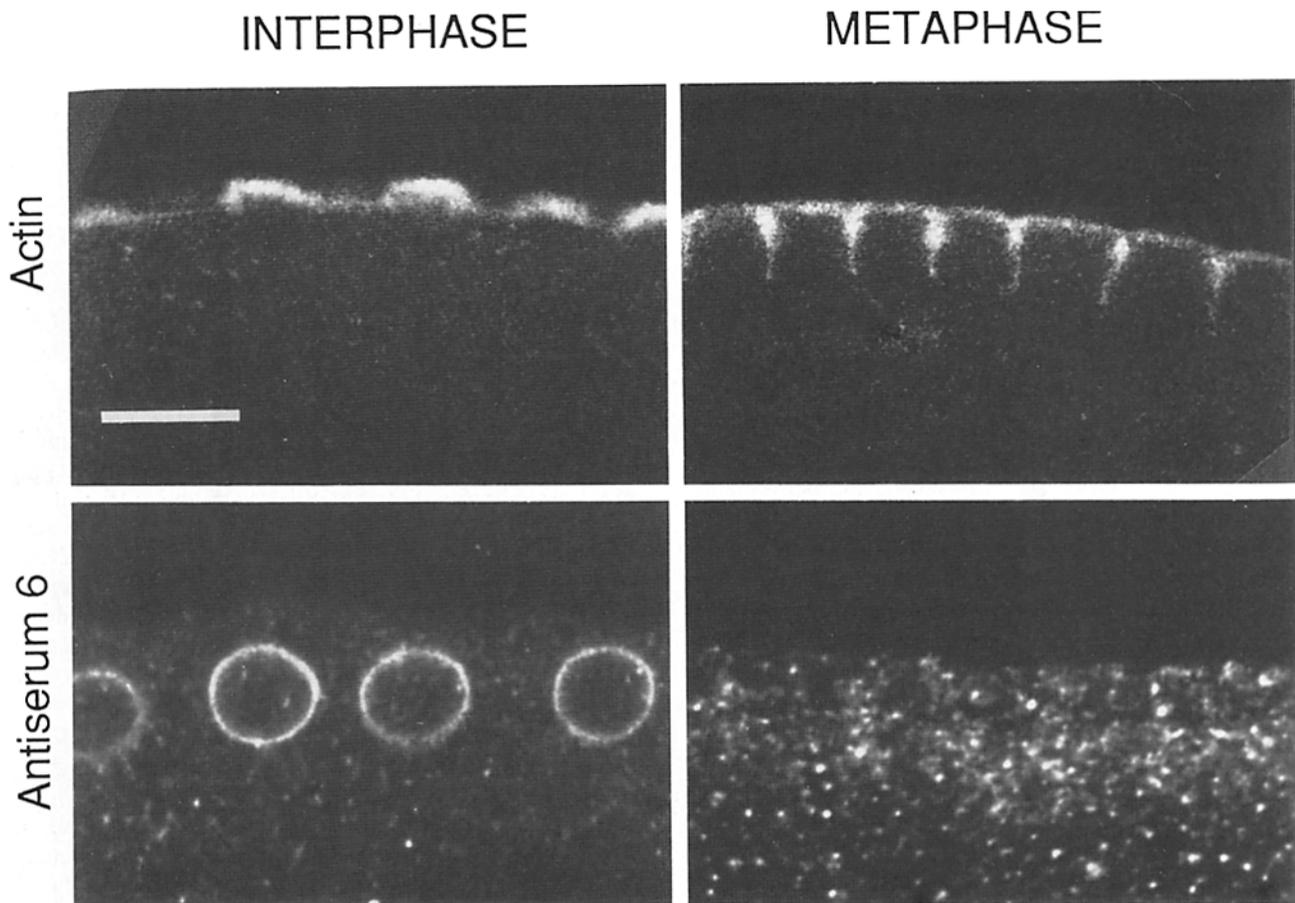


Figure 6. Optical sections of whole mount *Drosophila* embryos stained with the indicated antibodies as visualized using the scanning confocal microscope. Embryos are shown in interphase (left) or metaphase (right) with anti-actin antibody or polyclonal antiserum 6. Antiserum 6 stains the interphase nuclear envelope, and dissociates from this envelope during mitosis (see Table I). Bar, 10 μm .

A similar distinction between different actin binding proteins can be detected by staining the synchronously invaginating plasma membrane furrows that divide nuclei into cells at the cellular blastoderm stage (cycle 14). As expected by their behavior at earlier stages, antigen 8 is present only at the cellularization front, while antigen 4 and 11 are present both in the cortex and at the cellularization front (Fig. 9).

In contrast to the polyclonal antibodies that stain membrane furrows preferentially, several monoclonal antibodies that we have raised against the F-actin column eluates detect proteins that remain in cortical caps during both interphase and metaphase. An example of such a staining pattern for a 230,000 mol wt antigen is shown Fig. 7 (e and f). We attempted to generate polyclonal antisera to all of the Coomassie-stained protein species in this molecular weight range; since none of these antibodies stained cortical caps during

metaphase, we believe that the protein recognized by the monoclonal antibody is an actin-binding protein of relatively low abundance.

A schematic diagram that illustrates all of the distributions we have observed for ABPs in the syncytial blastoderm embryos is presented in Fig. 10.

Discussion

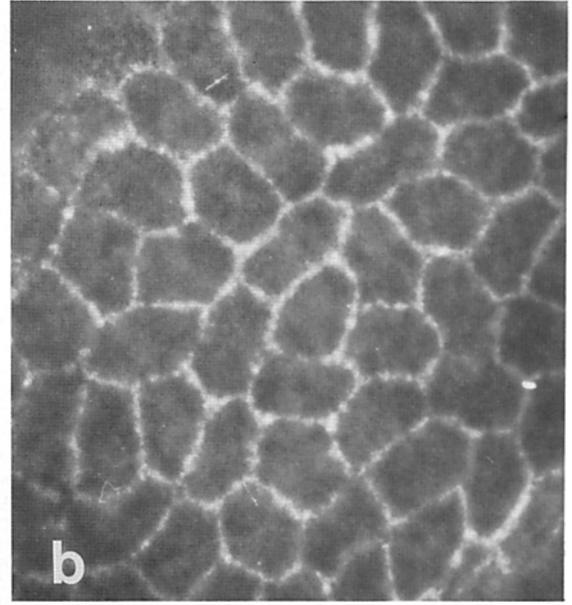
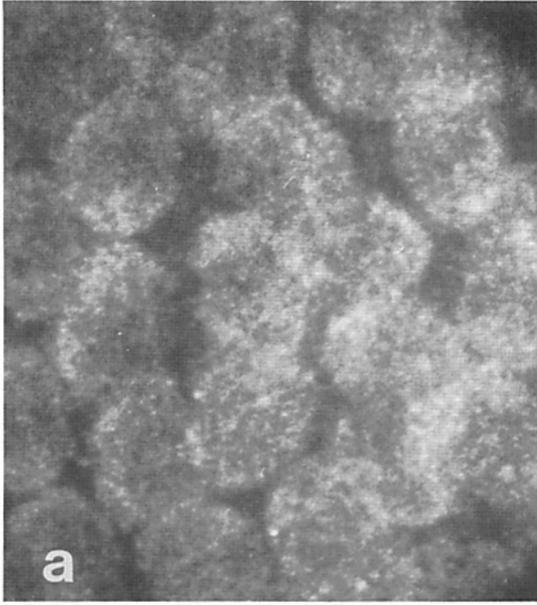
Our long range goal is to understand how the actin cytoskeleton functions in the early *Drosophila* embryo, a large, highly organized syncytium that contains up to 6,000 nuclei in a single cell. Many of its highly organized processes (such as precisely timed nuclear migrations, periodic yolk contractions, the even spacing of nuclei in a monolayer in the syncytial blastoderm, and local cell movements and shape changes

Figure 7. Immunofluorescence staining of *Drosophila* embryos with antibodies to actin-binding proteins, viewed looking down on the embryo surface by conventional fluorescence microscopy. The surface of syncytial blastoderm embryos at interphase (left, a, c, and e) and metaphase (right, b, d, and f) stained with antibodies to three different classes of actin-binding proteins is shown. a and b show an actin-binding protein that co-distributes with actin filaments throughout the cell cycle (polyclonal antiserum 23; see Table I). c and d show an actin-binding protein that is concentrated in the membrane invaginations (furrows) between nuclear domains during metaphase (polyclonal antiserum 2; see Table I). Finally, e and f show an actin-binding protein that remains confined to cortical caps throughout the nuclear cycle (monoclonal antibody 13G5). Bar, 10 μm .

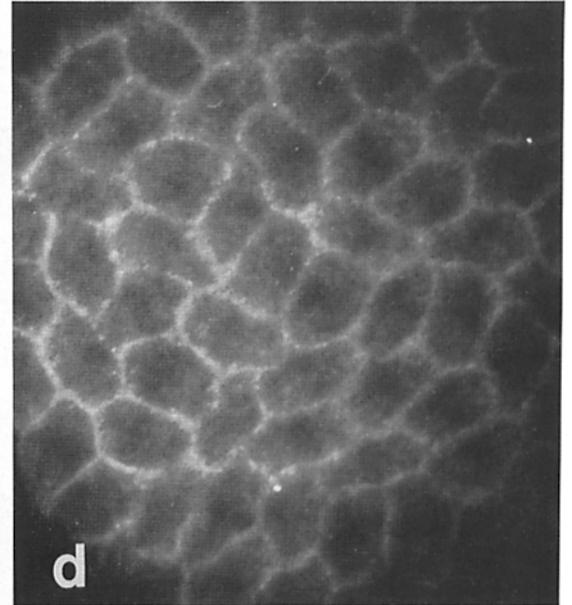
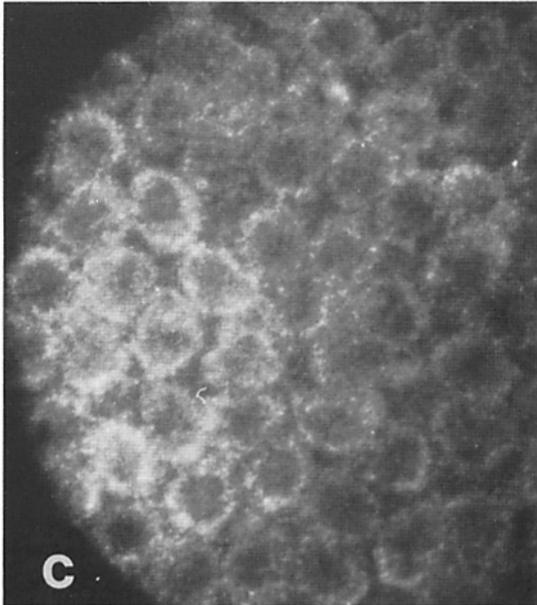
INTERPHASE

METAPHASE

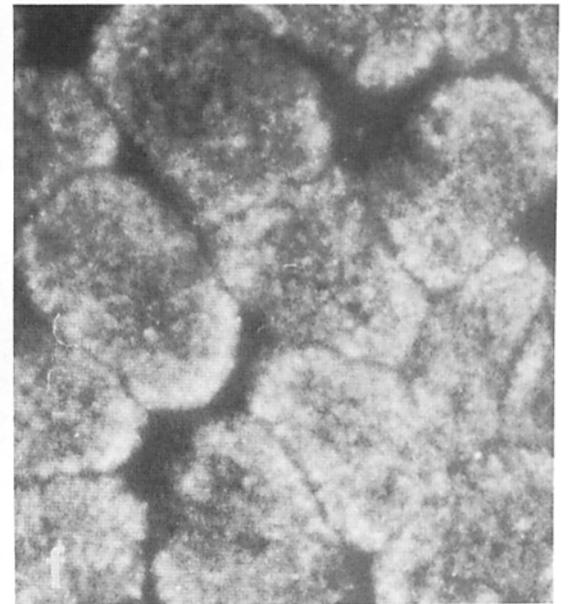
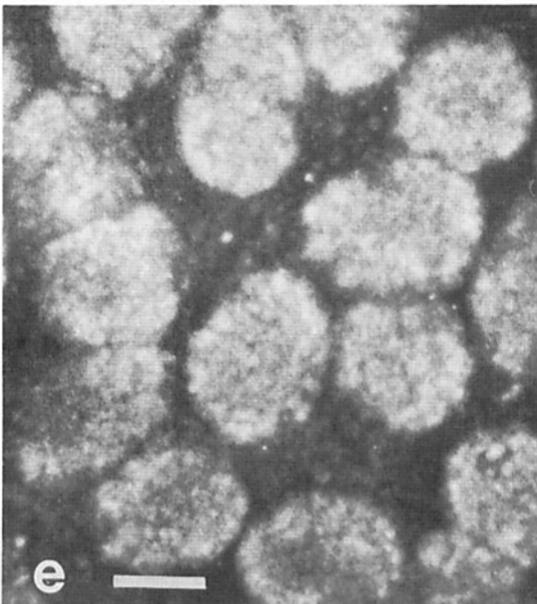
Antiserum 23



Antiserum 2



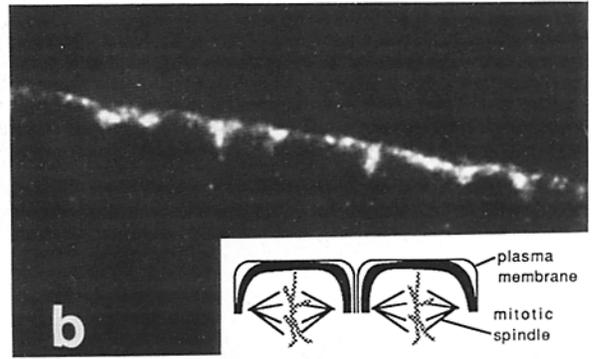
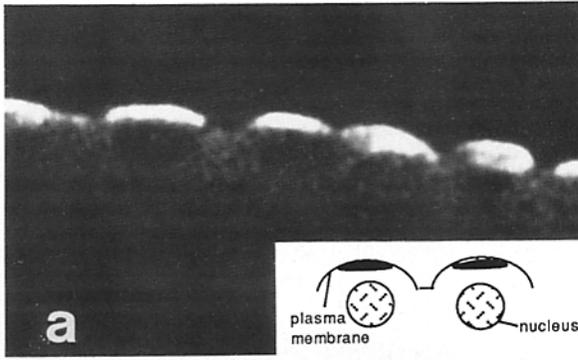
Monoclonal 13G5



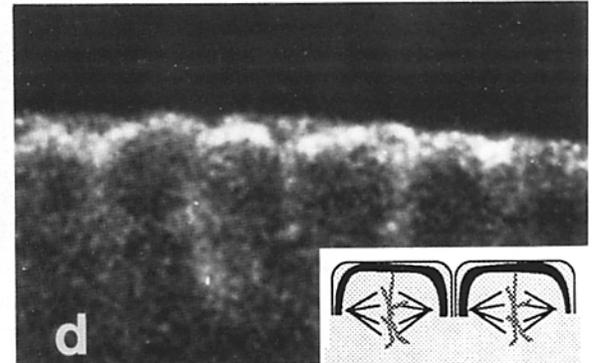
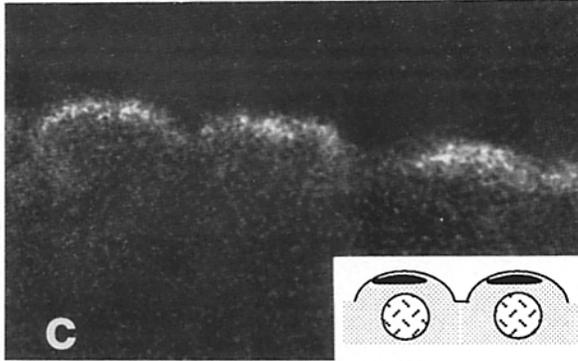
INTERPHASE

METAPHASE

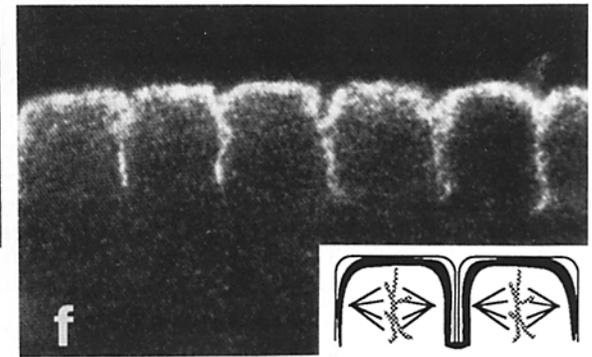
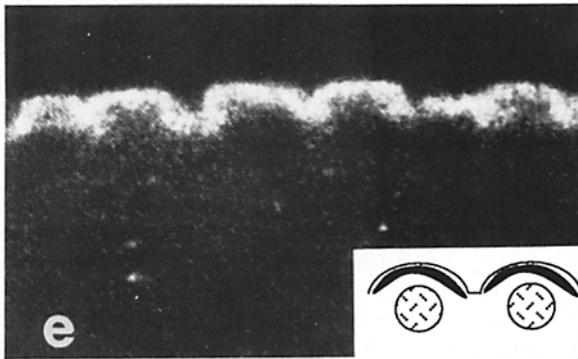
Antiserum 12



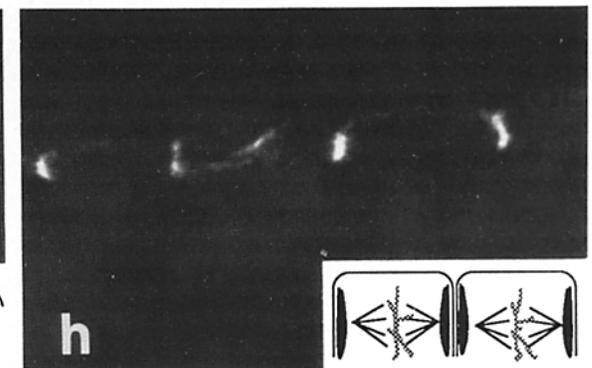
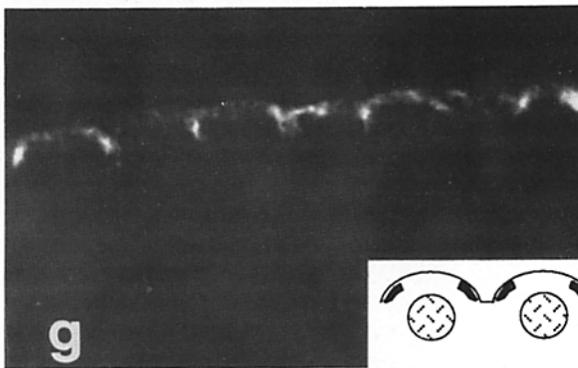
Antiserum 11



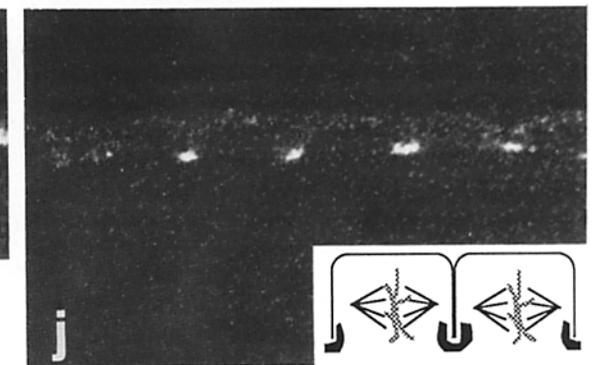
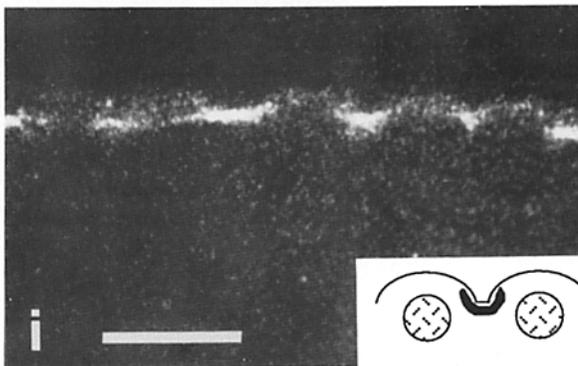
Antiserum 4



Antiserum 2



Antiserum 8



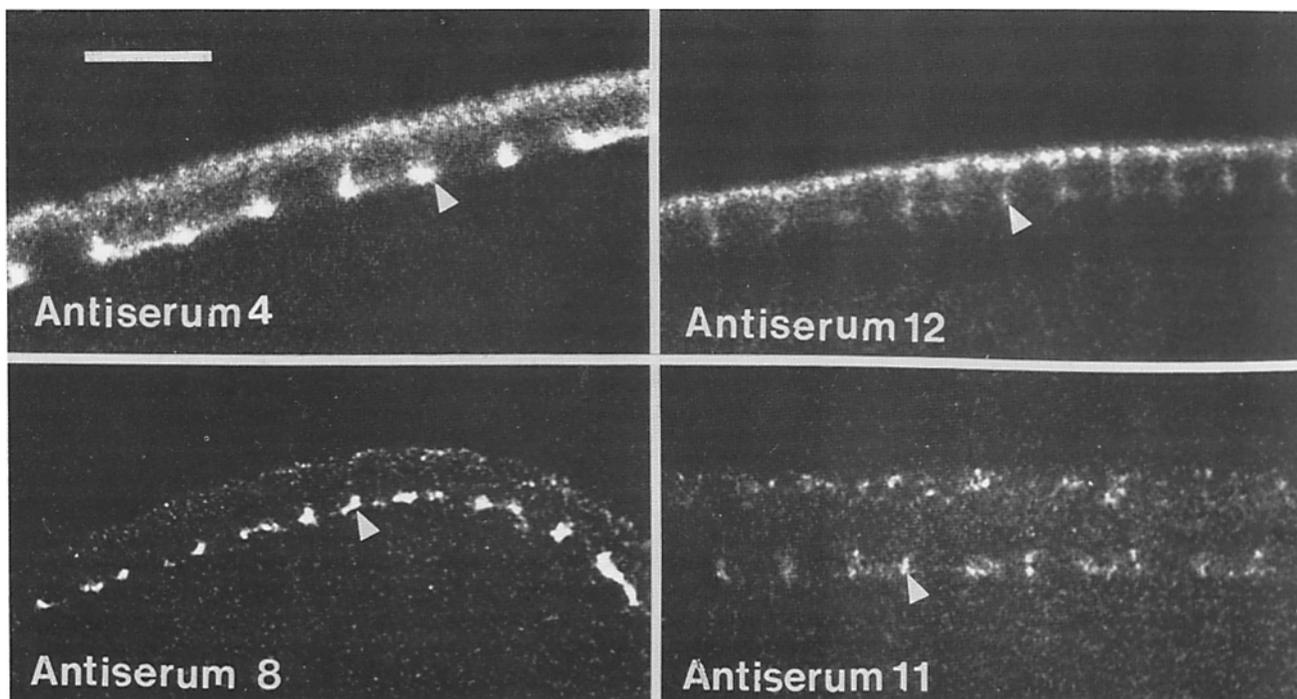


Figure 9. Distribution of some actin-binding proteins during formation of the cellular blastoderm. *Drosophila* embryos in nuclear cycle 14 stained with each of the indicated polyclonal antisera are shown, with the tip of the white arrow marking the front of the membrane furrows (these have progressed inward to a different extent in each embryo). The proteins recognized by antisera 4 and 8 are concentrated at the tip of the inward-growing furrow front (see Table I), whereas the staining with antiserum 12 most resembles actin-filament staining. Bar, 10 μ m.

during gastrulation) depend on the cytoskeleton (Foe and Alberts, 1983; Karr and Alberts, 1986; Warn et al., 1984; Warn and Warn, 1986). It is perhaps not surprising, therefore, that the actin cytoskeleton is a very complex network of interacting proteins, as judged by the large number of ABPs detected by F-actin affinity chromatography and the complexity of their distributions in fixed embryos.

In total, 12 of 13 antigens to which polyclonal antisera have been obtained colocalize with F-actin structures at sometime during the syncytial blastoderm divisions (see Fig. 10). The majority have the characteristic furrow arrangement at metaphase (the exception is a 200,000-D protein that is present in nuclear membranes [Fig. 6], which we have considered to be a nonactin-binding contaminant, but which could also be the myosinlike molecule reported in the nuclear envelope [Berrios and Fischer, 1986]). Our immunofluorescence survey of the major proteins that bind to F-actin affinity columns thus indicates that >90% of the proteins selected by this method colocalize with actin structures at some point during early development. Since the 13 antigens can be viewed as a random sample (all that was required in their selection was that their antisera produced a positive Western blot to the F-actin eluate), this suggests to us that

the bulk of the proteins isolated with F-actin columns from *Drosophila* embryos are actin-associated within the cell.

In the process of generating these 13 polyclonal antisera, we immunized mice with 42 different actin-binding proteins. Only in the 13 cases shown did the antisera react with *Drosophila* proteins by immunofluorescence or protein blotting. This reassures us that these are not preimmune responses and represent reaction to the immunogen. In similar experiments using 50 microtubule-binding proteins isolated on microtubule affinity columns, no actinlike staining was obtained (Kellogg et al., 1989).

The extraction conditions that we have used should solubilize most cytoplasmic ABPs, but ABPs from unusually stable structures (such as brush border microvilli or stress fibers) may be removed in the pellet when our extracts are clarified by high speed centrifugations. Not surprisingly, different extraction conditions allow additional ABPs to be isolated from these embryos (Miller et al., 1985; K. G. Miller and C. M. Field, unpublished results). Thus, 40 proteins represents our minimal estimate for the number of ABPs present in the *Drosophila* embryo.

The antibodies to individual ABPs have provided some insight into the complex regulation of the actin cytoskeleton in

Figure 8. Optical sections of whole-mount *Drosophila* embryos stained with antibodies to actin-binding proteins, as visualized in the scanning confocal microscope. Embryos are shown in interphase (left) or metaphase (right) after staining with five different polyclonal antibodies, as indicated to the left of each pair of micrographs (see Table I). Embryos shown are in nuclear cycles 11–13. The cell cycle and developmental stage of the embryos pictured vary from micrograph to micrograph. A schematic diagram of each staining pattern relative to the membrane is presented in the bottom right corner of each photograph. Bar, 10 μ m.

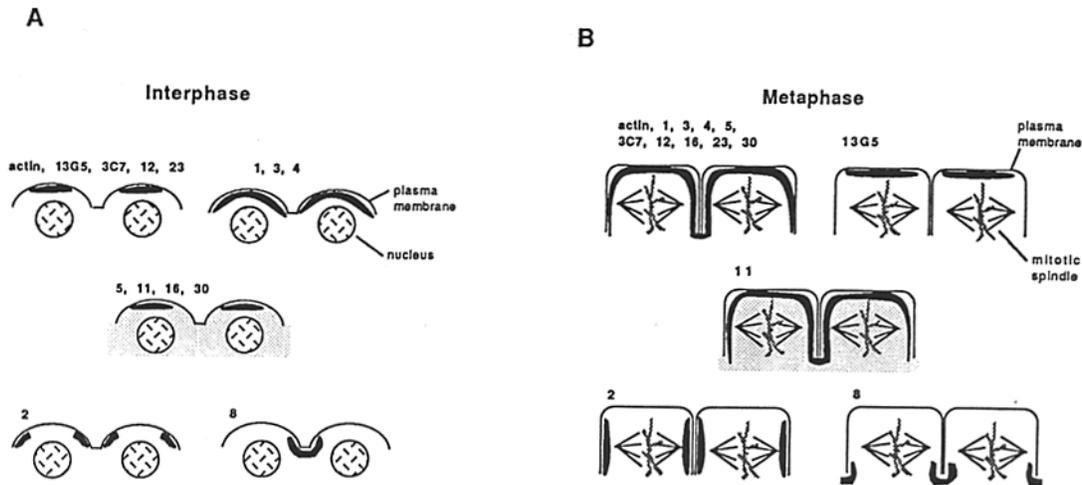


Figure 10. Schematic diagram of ABP distributions (black shading) observed during the syncytial blastoderm stages of *Drosophila* development. Antisera or monoclonal antibodies that display the diagrammed distributions are indicated above each illustration for embryos in interphase (A) and metaphase (B). The stippling in the interphase cytoplasm for antisera 5, 11, 16, and 30 and the metaphase cytoplasm for antiserum 11 denotes a partial cytoplasmic location.

the early embryo. Most of these ABPs seem to discriminate between different actin structures in the cell. Their proclivity for metaphase membrane “furrows” could in part reflect the procedures we have used to chromatograph extracts, since our solubilization conditions might preferentially extract proteins from these transient structures relative to the cortical caps. Nevertheless, some of our monoclonal antibodies stain cortical cap structures and not furrows (see Fig. 7 and Miller et al., 1985), suggesting that their antigens are minor, unusually immunogenic protein species in the eluates from our F-actin columns.

The antigens that recognize furrows appear to fall into groups. One group of antigens colocalizes with actin at all times (3C7, 12, 23; see Fig. 10). These antigens might be general stabilizers of actin and bind all actin filaments in the embryo. Other furrow antigens (1, 3, 4) remain in the furrow region during interphase after actin has disappeared, forming an apparently continuous layer underlying the plasma membrane, both at interphase and metaphase. These proteins could be important regulators of actin’s association with the membrane. In contrast to these antigens, antigen 2 is not present in the cap structures (see Figs. 8 and 10) and stains only the cap border region. Antigen 8 is primarily present at the leading edge of membrane furrows, suggesting a role in the initiation of furrowing. These different proteins could cooperate to form macromolecular assemblies that regulate the position and function of actin structures. For example, interactions between antigen 2, antigen 8, and the group of antigens (1, 3, 4) that are present in a continuous layer underlying the plasma membrane, could be responsible for forming the actin structures required in the furrows, with the activities of some of these proteins (like 8 and/or 2) regulated by cell cycle-related cues.

Our initial attempts at further purification of the ABPs from *Drosophila* embryos supports the idea that some of them are members of larger complexes (see Fig. 3). Such complexes can be purified and examined *in vitro* for novel activities. Once purified, both complexes and individual

ABPs can also be used to make an affinity matrix to probe for higher levels of cytoskeletal organization. This should be especially informative in the case of proteins whose *in vivo* localization suggests that they may be involved in linking actin filaments to other important structures, such as membranes.

Our antibodies to ABPs have been used to probe the function of individual ABPs during embryonic development by injecting the antibodies into live embryos and analyzing the disruption of cell function that results (K. G. Miller, unpublished results). Thus, these antibodies promise to provide important information on ABP functions in the embryo.

Conclusion

Using F-actin affinity chromatography, a large number of potentially interesting ABPs have been identified from early *Drosophila* embryos, with no bias with regard to their ultimate function. By combining this method of identification with immunocytological techniques employing monoclonal and polyclonal antibodies, we have detected proteins that may regulate the formation of actin structures at appropriate times and positions in the embryo. The behavior of these antigens as judged by immunofluorescence during early development, and the formation of multiprotein complexes by some of them, suggest that regulation of actin structures in these cells involves the activities and interactions of a complicated network of different proteins. Some of the proteins we have identified could represent new types of proteins involved in the regulation of actin structure and function in cells.

We thank Enrique Amaya for performing the HPLC fractionation experiment. We are indebted to Tom Pollard for useful discussions. We also thank Dan Kiehart for providing antibodies to *Drosophila* proteins, Doug Kellogg for many stimulating discussions, and Doug Kellogg, Jon Minden, William Sullivan, Scott Smiley, and Elaine Bearer for critical reading of the manuscript. We are also grateful to David States for skillful manuscript preparation.

This work was supported by National Institutes of Health (NIH) grant GM23928 and successive postdoctoral fellowships to K. G. Miller from NIH (GM08740) and California American Cancer Society (5-40-85).

Received for publication 11 April 1989 and in revised form 28 August 1989.

References

- Amero, S. A., T. C. James, and S. C. R. Elgin. 1987. Raising antibodies to protein bands in gels. In *Methods in Molecular Biology*, Volume 3. J. M. Walker, editor. Humana Press Inc., Clifton, NJ. 355-362.
- Berrios, M., and P. A. Fisher. 1986. A myosin heavy chain-like polypeptide is associated with the nuclear envelope in higher eucaryotic cells. *J. Cell Biol.* 103:711-724.
- 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.
- Bretscher, A., and K. Weber. 1980. Villin is a major protein of the microvillus cytoskeleton which binds both G and F actin in calcium-dependent manner. *Cell.* 20:839-847.
- Dubreil, R., T. J. Byers, D. Branton, L. S. B. Goldstein, and D. P. Kiehart. 1987. *Drosophila* spectrin I. Characterization of the purified protein. *J. Cell Biol.* 105:2095-2102.
- Edgar, B. A., G. M. Odell, and G. Schubiger. 1987. Cytoarchitecture and the patterning of fushi tarazu expression in the *Drosophila* blastoderm. *Genes & Dev.* 1:1226-1237.
- Foe, V. E., and B. M. Alberts. 1983. Studies of nuclear and cytoplasmic behavior during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* 61:31-70.
- Karr, T. L., and B. M. Alberts. 1986. Organization of the cytoskeleton in early *Drosophila* embryos. *J. Cell Biol.* 102:1494-1509.
- Kellogg, D. R., C. M. Field, and B. M. Alberts. 1989. Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. *J. Cell Biol.* 109:2977-2991.
- Kiehart, D. P., and R. Feghali. 1986. Cytoplasmic myosin from *Drosophila melanogaster*. *J. Cell Biol.* 103:1517-1525.
- Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Miller, K. G., and B. M. Alberts. 1989. F-actin affinity chromatography: technique for isolating previously unidentified actin-binding proteins. *Proc. Natl. Acad. Sci. USA.* 13:4808-4812.
- Miller, K. G., T. L. Karr, D. R. Kellogg, I. J. Mohr, M. Walter, and B. M. Alberts. 1985. Studies on the cytoplasmic organization of early *Drosophila* embryos. *Cold Spring Harbor Symp. Quant. Biol.* 50:79-90.
- Pollard, T. D., P. C-H. Tseng, D. L. Rimm, D. P. Bichell, R. C. Williams, Jr., J. Sinard, and M. Sato. 1986. Characterization of α -actinin from *Acanthamoeba*. *Cell Motil. Cytoskeleton.* 6:649-661.
- Stafstrom, J. P., and L. A. Staehelin. 1984. Dynamics of the nuclear envelope and of nuclear pore complexes during mitosis in the *Drosophila* embryo. *Eur. J. Cell Biol.* 34:179-189.
- Stossel, T. P., C. Chaponnier, R. M. Ezzel, J. H. Hartwig, P. A. Janmey, D. J. Kwiatkowski, S. F. Lind, D. B. Smith, F. S. Southwick, H. L. Yin, and K. S. Zaner. 1985. Nonmuscle actin-binding proteins. *Annu. Rev. Cell Biol.* 1:353-402.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
- Warn, R. M., and A. Warn. 1986. Microtubule arrays present during the syncytial and cellular blastoderm stages of the early *Drosophila* embryo. *Exp. Cell Res.* 163:201-210.
- Warn, R. M., R. Magrath, and S. Webb. 1984. Distribution of F-actin during cleavage of the *Drosophila* syncytial blastoderm. *J. Cell Biol.* 98:156-162.
- White, J. G., W. B. Amos, and M. Fordham. 1987. An evaluation of confocal versus conventional imaging of biological structures by fluorescence light microscopy. *J. Cell Biol.* 105:41-48.
- Zalokar, M., and I. Erk. 1976. Division and migration of nuclei during early embryogenesis of *Drosophila melanogaster*. *J. Microbiol. Cell.* 25:97-106.