Filamin Concentration in Cleavage Furrow and Midbody Region: Frequency of Occurrence Compared with That of Alpha-Actinin and Myosin

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ABSTRACT Affinity-purified rabbit antibody to purified chicken gizzard filamin was used in indirect immunofluorescence to localize filamin in dividing chick embryo cells. The antibody was shown to bind only chick embryo cell filamin when whole cell extracts were analyzed by the sensitive sodium dodecyl sulfate-polyacrylamide gel electrophoresis overlay technique described by Adair et al. (1978, *J. Cell Biol.* 79:281–285). The results show that filamin is located in stress fibers and membrane ruffles during interphase. As cells enter prophase, the condensing chromosomes are surrounded by diffuse antifilamin staining. No stress fibers are apparent. During metaphase and anaphase, the staining is bright but diffuse. There is often peripheral membrane staining. Filamin is not concentrated in the spindle region but neither is it excluded from the spindle. During cytokinesis, filamin is found highly concentrated in the cleavage furrow in 16 out of 100 cells examined. This frequency of concentration in the furrow is comparable to that observed for alpha-actinin (14%). Myosin concentration in the furrow is more frequent; it is observed in 37% of the cells examined. Neither myosin, alpha-actinin, nor filamin is observed concentrated in the furrow 100% of the time. We conclude that the results are consistent with, but not sufficient to prove, the hypothesis that alpha-actinin and filamin are essential components of the mechanism of cytokinesis.

Filamin is a high molecular weight (250,000 mol wt) actin-binding protein that was first isolated from chicken smooth muscle (37). Filamin may be functionally homologous to the high-molecular-weight actin-binding protein isolated from rabbit alveolar macrophages as described by Hartwig and Stossel (18). A molecule that is antigenically as well as functionally homologous to filamin has since been found in mammalian smooth muscle, platelets, fibroblasts, macrophages, kidney, and liver and in avian skeletal muscle (3, 19, 35). The name filamin derives from the filamentous staining pattern given by indirect immunofluorescent staining of cultured cells with antifilamin antibodies (37). Because purified filamin does not form filaments in vitro, its filamentous intracellular distribution is thought to be attributable to its association with actin-containing stress fibers (19).

The in vitro actin-binding properties of filamin have been partially characterized. Filamin cross-links F-actin filaments, causing the formation of a gel. Gelation requires F-actin (G-actin will not substitute) (39). Filamin has also been shown to inhibit the actin-activation of myosin ATPase (9). Davies and co-workers (10, 36) have demonstrated that filamin is a phosphoprotein whose phosphorylation in cell extracts is stimulated by cyclic AMP. However, the significance of the phosphorylation of filamin with respect to its in vitro properties has not been demonstrated.

Although some details are known about the in vitro interaction of actin and filamin, there is far less information concerning the possible interaction of these proteins in vivo. By immunofluorescent staining, filamin has been found in several cellular structures known to contain actin, i.e., stress fibers, microspikes, and membrane ruffles (19). Filamin has also been found in the terminal web of intestinal epithelial cells (4), a region shown to be enriched in contractile proteins such as actin (34), myosin (27), and alpha-actinin (4, 8, 16). The

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localization of filamin in cellular structures known to contain actin suggests that filamin interacts with and possibly regulates the state of actin in vivo.

We decided to take these immunolocalization studies a step further by determining whether filamin redistributes during a contractile process thought to involve actin. Mitosis and cytokinesis are dynamic cellular processes in which actin has been postulated to play an important mechanistic role (12, 15, 32). In this paper we report observations on the distribution of filamin during the cell cycle.

MATERIALS AND METHODS

Purification of Filamin

Filamin was purified from frozen (−20°C), glycerinated chicken gizzards (Pel-Freeze Farms, Inc., Rogers, Ark.) according to a modification of the method of Shizuta et al. (33). Modifications of the published procedure included dialysis of the high-salt extract, before $(NH_4)_2SO_4$ fractionation, against a no-salt buffer to precipitate myosin. Sepharose 4B chromatography was done in the presence of 0.6 M KCl to decrease protein aggregation. Purified filamin was stored at −20°C in 50% glycerol.

Antibodies

ANTIFILAMIN: Antifilamin antibody was raised in rabbits and affinity purified on cyanogen bromide–activated Sepharose 2B (29) that was coupled to highly purified chicken gizzard filamin. The antibody was eluted at 4°C with 0.05 M acetic acid, pH 4.0, neutralized with 2 M Tris-HCl, pH 8.0, and then dialyzed against 0.01 M PO_4 buffer containing 0.15 M NaCl, pH 7.0. The antibody was stored at 4°C in 0.02% NaN_3.

RHODAMINE-LABELED GOAT ANTI-RABBIT IgG (Fc FRAGMENT): Goat anti-rabbit IgG (Fc fragment) whole serum was a gift of Dr. John Cebra, The Johns Hopkins University, Baltimore, Md. Purified immune IgG was labeled with tetramethylrhodamine isothiocyanate (N. L. Cappel Labs, Cochranville, Pa.) and subsequently fractionated as described by Cebra and Goldstein (7).

ANTI-MYOSIN: Whole goat serum directed against mouse L cell myosin (generous gift of Dr. Ira Pastan, National Institutes of Health, Bethesda, Md.) was tested for its ability to recognize myosin in chick embryo cell lysates by indirect immunoprecipitation with heat- and formalin-fixed *Staphylococcus aureus*. The goat serum specifically precipitated a protein the size of the myosin heavy chain from the chick cell lysates (data not shown).

ANTI-ALPHA-ACTININ: Two anti-alpha-actinin antibodies were used in these studies; both antibodies were directed against chicken gizzard alpha-actinin. Whole rabbit serum directed against alpha-actinin was the generous gift of Dr. Keigi Fujiwara, Harvard Medical School, Boston, Mass., and was previously characterized as to its reactivity against chick embryo cells (14). The other anti-alpha-actinin antibody used was affinity purified on Sepharose-alpha-actinin and has been previously characterized by Craig and Pardo (8).

Indirect Immunoprecipitation of Filamin from Gizzard Extracts

Low- and high-salt extracts of chicken gizzards were prepared (33) and used in indirect immunoprecipitation studies with the affinity-purified antifilamin antibody. Indirect precipitation of the antigen-antibody complexes with heat- and formalin-fixed *Staphylococcus aureus* was done, following Kessler's procedure (22), except that immune complexes were eluted from the *S. aureus* by incubation at 37°C for 15 min in Fairbanks' sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (11).

Chick Embryo Cells

Chick embryo cell cultures were prepared from 11-d-old chick embryos (23). Cells were grown in medium 199 (Grand Island Biological Co., Grand Island, N. Y. [GIBCO]) supplemented with 10% fetal calf serum (GIBCO) and 2% penicillin-streptomycin mixture (5,000 U potassium penicillin G/ml, 5,000 μg streptomycin sulfate/ml; GIBCO) in plastic tissue culture dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) containing 22- × 22-mm glass microscope coverslips.

Preparation of Chick Embryo Cell Lysates and Antibody Gel Overlay

Chick embryo cells (3×10^7 cell/ml) were lysed by homogenization in an equal volume of lysis buffer; 4 mM EDTA, 1 mM EGTA, 50 mM KPO_4, pH 6.9, 0.5% Nonidet P-40 (NP-40; Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.), 10 IU/ml Trasylol (Mobay Chem Corp., New York), .0157 mg/ml Benzamidine (Sigma Chemical Co., St. Louis, Mo.), 0.001 mg/ml pepstatin A (Sigma Chemical Co.), 0.032 mg/ml leupeptin and .001 mg/ml antipain (gift of Dr. Walter Troll, New York University). The cells were disrupted in lysis buffer at 4°C by 50 strokes in a Dounce homogenizer (Kontes Co., Vineland, N. J.). The lysates were spun at 90,000 *g* for 45 min at 4°C. The supernates were electrophoresed on mini (0.5 mm × 89 mm × 102 mm) SDS-polyacrylamide slab gels and analyzed by the sensitive antibody overlay technique described by Adair et al. (1). In brief, after electrophoresis the gels were fixed in 25% isopropanol, 10% acetic acid overnight, washed, equilibrated in NP-40 buffer (0.05 M Tris, 0.5% NP-40, 0.15 M NaCl, 0.02% NaN_3, 0.1% bovine serum albumin, pH 8.0), and then incubated overnight with the appropriate affinity-purified antibody solution (110 μg/ml in NP-40 buffer). After repeated washings in the above buffer, gels were incubated overnight with ^{125}I-protein A (sp act 5.5×10^5 cpm/μg), then extensively washed, stained with Coomassie Blue, dried, and exposed to Kodak x-ray film for 2–48 h. Protein A was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J., and iodinated by the chloramine T method (17).

PAGE

Cylindrical gels containing 4% acrylamide monomer and 0.15% *bis*-acrylamide were run according to the Fairbanks' protocol (11). Slab gels were run with a Tris-glycine buffer system according to Laemmli (24), with a 7.5% acrylamide running gel and a 4.5% stacking gel.

Indirect Immunofluorescence

Cells grown on coverslips were prepared for indirect immunofluorescence by fixation for 10 min in 3.7% formaldehyde in phosphate-buffered saline (PBS) at room temperature. After washing in PBS, the fixed cells were made permeable by incubating in 0.1% Triton X-100 in PBS for 2 min, or 0.05% digitonin in PBS for 2 min, or −20°C acetone for 5 min, and then air-dried. Then, 10–20 μl of affinity-purified antifilamin (110 μg/ml) was applied directly to the coverslip. A clean coverslip was placed on top. Coverslips were incubated for 30–40 min in a moist chamber and then washed for 15 min in several changes of PBS. The cells were then stained with 20 μl of rhodamine-conjugated goat anti-rabbit IgG (R-GARIgG) (1 mg/ml) in a manner identical to that in the first staining. After two 8-min washes in PBS, coverslips were mounted onto a microscope slide with a drop of phosphate-buffered glycerol (9% glycerol). Alternatively, cells were fixed with absolute methanol (−20°C) for 5 min, rinsed with PBS, and stained as described above.

Stained cells were examined with a Leitz Ortholux II (E. Leitz, Inc., Rockleigh, N. J.) fluorescent microscope equipped with a Ploem vertical illuminator and × 40 (numerical aperture [NA] = 1.0) and × 63 (NA = 1.4) oil immersion lenses. Photographs were taken on Kodak Ektachrome 400 film or Kodak Tri X black and white film, ASA 400.

RESULTS

Purity of Filamin Antigen

The chicken gizzard filamin used for immunization was >90% pure as determined by SDS-PAGE (Fig. 1 *a*). This highly purified protein was determined to be filamin on the basis of its size (250,000 monomer mol wt), its ability to gel F-actin, and its inhibition of the actin-activated heavy meromyosin (HMM) ATPase (data not shown). The filamin covalently coupled to Sepharose 2B used to affinity purify antifilamin antibodies was >95% pure by SDS-PAGE (Fig. 1 *b*).

Specificity of Purified Antifilamin Antibody

INDIRECT IMMUNOPRECIPITATION: The specificity of the affinity-purified antifilamin antibody was tested by incubating low- and high-salt extracts of chicken gizzard with antibody and precipitating the immune complexes with heat- and formalin-fixed *S. aureus*. These gizzard extracts are identical to those used in the preparation of the filamin antigen and therefore contain proteins that may have contaminated the original antigen. In Fig. 2, it can be seen that in both the low- (lane *C*) and high- (lane *G*) salt extracts, filamin is the only

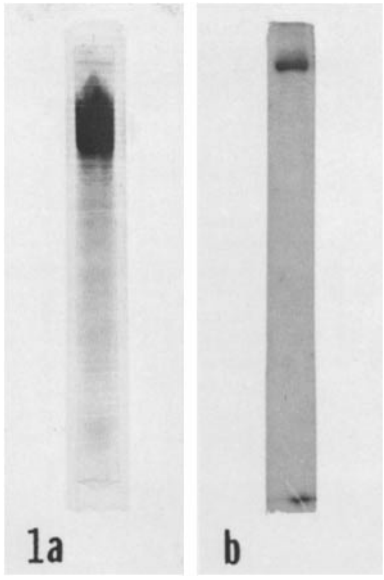

FIGURE 1 Coomassie Blue-stained SDS-polyacrylamide gels of purified chicken gizzard filamin. (*a*) Electrophoresis of filamin antigen (70 μg) on 4% acrylamide cylindrical gel. (*b*) Electrophoresis on 7.5% acrylamide slab gel of filamin (3 μg) that was coupled to Sepharose 2B and used to affinity-purify antifilamin. As little as 0.1 μg of protein can be detected by Coomassie Blue on the 7.5% acrylamide slab gel, indicating that the filamin in *b* is at least 95% pure. India ink marks dye front in *b*.

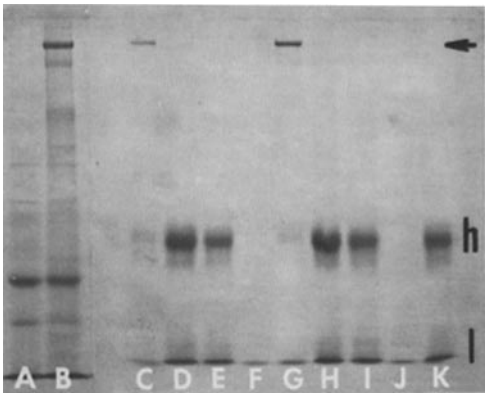

FIGURE 2 Immunoprecipitation. Polyacrylamide gel analysis of immune complexes formed between purified antifilamin antibody and gizzard extracts. Lane *A*, low-salt extract. Lane *B*, high-salt extract. Lane *C*, low-salt extract + antifilamin + *S. aureus*. Lane *D*, low-salt extract + nonadherent IgG + *S. aureus*. Lane *E*, low-salt extract + NRS + *S. aureus*. Lane *F*, low-salt extract + *S. aureus*. Lane *G*, high-salt extract + anti-filamin + *S. aureus*. Lane *H*, high-salt extract + nonadherent IgG + *S. aureus*. Lane *I*, high-salt extract + NRS + *S. aureus*. Lane *J*, high-salt extract + *S. aureus*. Lane *K*, NRS + *S. aureus*. The positions of the heavy and light chains of IgG are marked with the black *h* and *l*, respectively. The filamin position is marked with a small black arrow.

protein precipitated by the antifilamin *S. aureus* complex. Controls for this indirect immunoprecipitation included incubation of the low- and high-salt extract with *S. aureus* extract in the absence of antibody (Fig. 2, lanes *F* and *J*) and substitution of antifilamin with either normal rabbit serum (NRS) (Fig. 2, lanes *E* and *I*) or nonadherent IgG (the fraction of immune serum that passed directly through the filamin-Sepharose column; Fig. 2, lanes *D* and *H*). All controls were negative; that is, nonspecific precipitation of filamin did not occur. The indirect immunoprecipitation technique detects both precipitating and nonprecipitating antibodies.

ANTIBODY OVERLAY: When tested against total proteins of chick embryo cells, the purified antifilamin antibody recognized only a filamin-sized molecule (Fig. 3). The antibody overlay technique is so sensitive that protein bands not detected by Coomassie Blue (<1 ng of protein/band) can be detected by autoradiography. Fig. 3*a* is a Coomassie Blue–stained gel of purified filamin and varying concentrations of cell lysate subjected to the antibody overlay technique. Fig. 3*b* is the corresponding autoradiograph of that gel. The only protein in the chick embryo cell lysates that bound the purified antifilamin antibody comigrates with chicken gizzard filamin. If this technique is applied to chick embryo cells that are solubilized directly into SDS-electrophoresis sample buffer, identical results are obtained (data not shown).

Cell Fixation and Staining

Many different fixatives were tested in these immunofluorescence studies. The fixation procedures that gave the best preservation of cell morphology and staining intensity are: (*a*) 3.0–3.7% formaldehyde (10 min) followed by either −20°C acetone (5 min), 0.05% digitonin (2 min), or 0.1% Triton X-100 (2 min); (*b*) absolute methanol (−20°C) for 5 min.

Examples of the type of staining patterns observed with these two basic fixation methods are seen in Figs. 4–6. In Fig. 5, all of the cells were fixed with the cold methanol procedure. Fig. 6 shows cells fixed by formaldehyde followed by acetone (Fig. 6*c*, *d*, and *k*), Triton (Fig. 6*e*, *f*, and *l*), or digitonin (Fig. 6*g*, *h*, *i*, and *j*) and fixed by methanol (Fig. 6*a* and *b*). In general, the various fixation procedures gave comparable staining patterns. A qualitative difference with the methanol fixation was noticed, however. Cells fixed with methanol gave greater contrast between stained and unstained regions (note fine stress fiber detail in Fig. 4*b*). This difference in contrast may be attributable to the fact that methanol may not "fix" proteins that are

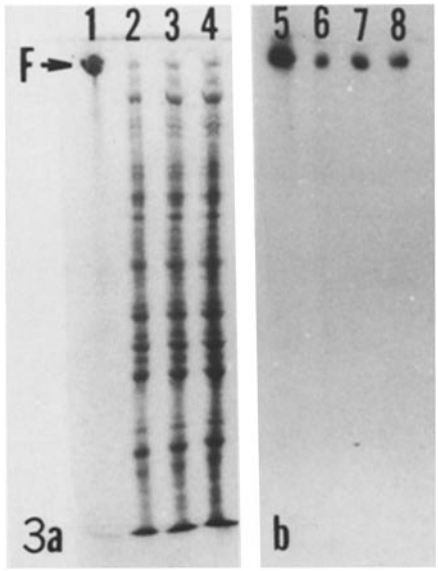

FIGURE 3 Antibody gel overlay. SDS-polyacrylamide minislab gel of filamin standard (lanes *1* and *5*) and varying concentrations of NP-40 chick embryo cell extract (lanes *2–4* and *6–8*) subjected to antifilamin and ^{125}I-protein A overlay. (*a*) Coomassie Blue-stained gel; (*b*) corresponding autoradiogram. Filamin position is marked by *F*.

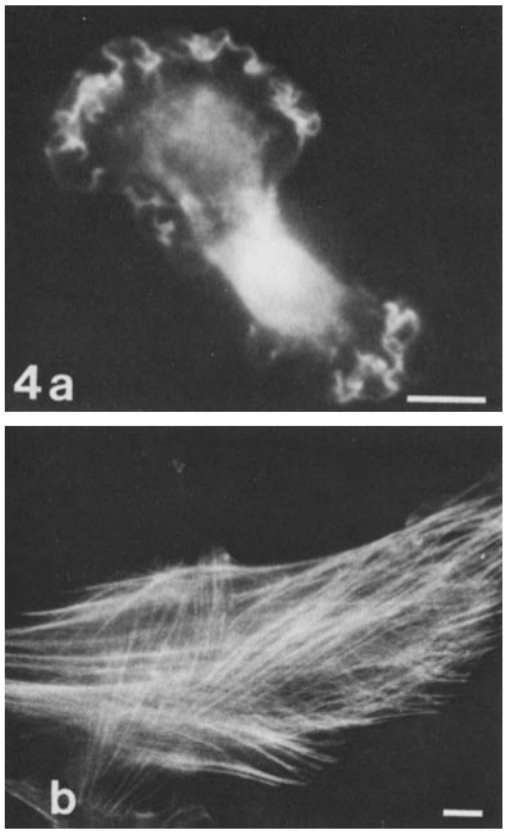

FIGURE 4 Interphase. Primary chick embryo cells stained with antifilamin and R-GARIgG showing two distinctive staining patterns. In motile cells (*a*), filamin is localized in membrane ruffles (cell fixed with formaldehyde/Triton). In *b*, cytoplasmic stress fibers stain brightly with antifilamin (cell fixed with methanol). Bars, 10 μm.

part of the soluble cell matrix and they may therefore be washed out during the staining procedure, resulting in a darker background.

Localization of Filamin by Indirect Immunofluorescence

INTERPHASE: There are two distinctive filamin staining patterns in interphase cells. Filamin is found in membrane ruffles (Fig. 4*a*) and stress fibers (Fig. 4*b*). Our data on filamin localization in interphase cells confirm the findings of Heggeness et al. (19).

MITOSIS: In prophase the cell rounds up and stress fibers are no longer present (data not shown). Filamin distribution during metaphase is shown in Fig. 5*a*. As in prophase, the filamin staining is bright, but diffuse. There is often a slight concentration of filamin around the periphery of the cell. Filamin is not concentrated in the spindle but neither is it excluded from the spindle region. In contrast to actin (6, 21), there is no indication that filamin is organized into spindle fibers. In anaphase (Fig. 5*c*), the filamin distribution is similar to that during metaphase. The staining is bright, but is not organized into any distinctive structure.

CYTOKINESIS: The cells shown in Fig. 5*e–n* depict filamin distribution from early cleavage through the completion of cell division. The antifilamin staining shows that filamin is associated with the membrane in the region of the furrow (Fig. 5*e* and *g*), but also that filamin appears to be concentrated in the cytoplasm near the furrow. This staining pattern differs somewhat from that seen for alpha-actinin and myosin (14), in that

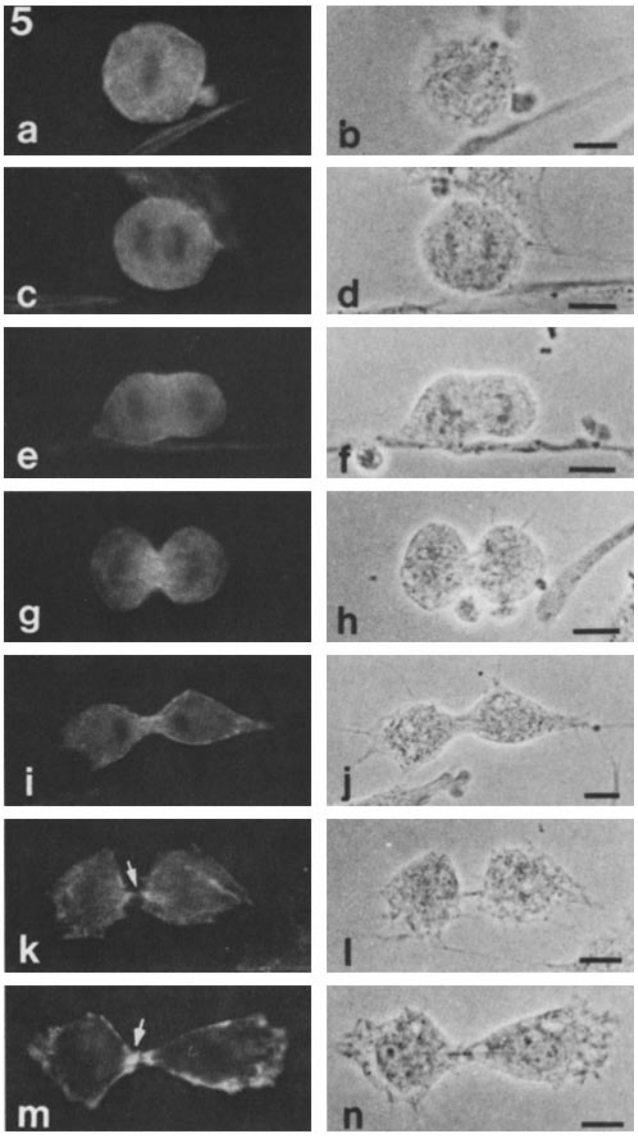

FIGURE 5 Mitosis and cytokinesis. Chick embryo cells fixed with absolute methanol (−20°C) seen by phase contrast (*b, d, f, h, j, l,* and *n*) and stained with antifilamin and R-GARIgG (*a, c, e, g, i, k,* and *m*). In metaphase (*a*), antifilamin staining is bright but diffuse. Spindle fibers are not specifically stained (spindle is visible in corresponding phase photo *b*). During anaphase (*c* and *d*), antifilamin staining is bright throughout the cytoplasm and is not localized specifically to the spindle region. In early telophase (*f*), filamin appears to be concentrated along the membrane and in the developing cleavage furrow (*e*). As furrowing continues (*h*), antifilamin staining is bright in the furrow region (*g*). Antifilamin staining (*i*) of cell in early midbody stage (*j*) shows concentration in midbody, but also increased punctate staining of the daughter cells. In late midbody stage (*l*), antifilamin staining is very bright in the midbody region, although the intercellular bridge is not completely stained (*k*, arrow). In a very late stage, the daughter nuclei have reformed and the cells are held together by a narrow intercellular bridge (*n*). Antifilamin staining (*m*) shows very bright staining of the cytoplasm adjacent to the intercellular bridge (arrow); the bridge itself is unstained. Note also the brightly fluorescent ruffles. Bars, 10 μm.

membrane staining is not so intense. As furrowing continues (Fig. 5*i*), filamin remains associated with the cleavage furrow region. At the completion of cell division, filamin remains associated with the midbody region (Fig. 5*k* and *m*); however,

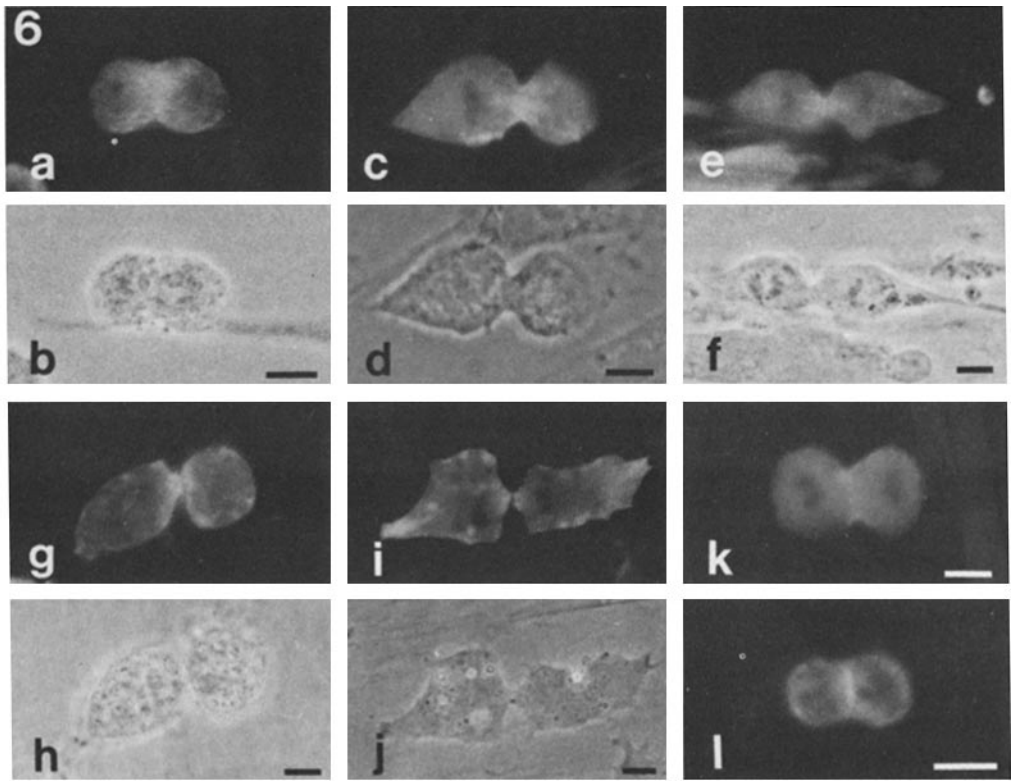

FIGURE 6 Cytokinesis. Chick embryo cells during cytokinesis as seen by phase contrast (*b*, *d*, *f*, *h*, and *j*) and by fluorescence, stained with antifilamin antibody and R-GARIgG (*a*, *c*, *e*, *g*, *i*, *k*, and *l*). Cells were fixed with methanol (*a* and *b*), formaldehyde/acetone (*c*, *d*, and *k*), formaldehyde/Triton (*e*, *f*, and *l*) or formaldehyde/digitonin (*g*, *h*, *i*, and *j*). Cells in mid-cleavage furrow stage (*a*, *k*, and *l*) show a high concentration of filamin in furrow region. Antifilamin staining of the furrow region is intense in later stages of cytokinesis (*c* and *e*). At the midbody stage (*g* and *i*), the antifilamin intensely stains the cytoplasm adjacent to the intercellular bridge, but not the bridge itself. Plane of focus in (*h*) is at the level of the chromosomes, the intercellular bridge is not visible. Bars, 10 μm.

in the majority of cases the filamin is not concentrated in the midbody but rather in the adjacent cytoplasm (arrows in Fig. 5 *k* and *m*). This is particularly apparent in Fig. 5 *m*, where the cells are held together by a narrow intercellular bridge, which is devoid of detectable filamin.

Fig. 6 shows other examples of filamin concentration in the cleavage furrow and in the midbody region. These cells have been fixed in a variety of ways as indicated in the legend. These photographs show clearly that in these cells the cleavage furrow is the most intensely stained region of the cell. The poles of the cells before midbody formation are less intensely stained (Fig. 6 *a*, *c*, *e*, *k*, and *l*). The increased staining of the furrow is often diffuse (Fig. 6 *a*, *c*, and *e*). However, it has also been observed that the antifilamin appears to stain the membrane in the furrow region (Fig. 6 *k* and *l*).

CONTROLS FOR INDIRECT IMMUNOFLUORESCENT STAINING: Controls for the indirect immunofluorescent staining included substituting normal rabbit IgG for the purified antifilamin antibody (data not shown) and staining the cells with only rhodamine-labeled goat anti-rabbit IgG (Fig. 7). All controls were negative, i.e., there is no nonspecific staining of the cells. Therefore, antifilamin staining results from the specific interaction of the purified antifilamin antibody with filamin *in situ*.

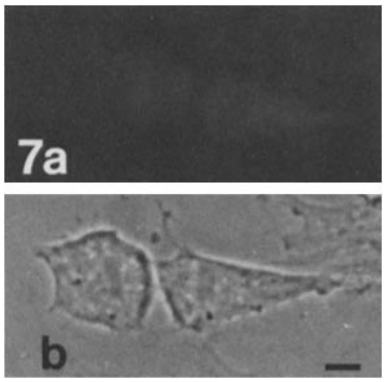

FIGURE 7 Control. Dividing chick embryo cell in midbody stage, fixed by formaldehyde/acetone, shown in phase contrast (*b*) and stained with rhodamine-labeled GARIgG (*a*). There is little nonspecific staining of the cell. Bar, 10 μm.

Frequency of Filamin, Alpha-Actinin, and Myosin Association with the Cleavage Furrow and Midbody Regions

During the course of these studies we noticed that the intensity of filamin staining of the cleavage furrow varied from cell to cell. The range of staining intensity was from no apparent concentration of filamin in the furrow region to high concentration as depicted in Fig. 5 *g* and 6 *a*, *c*, *k*, and *l*. To further analyze the frequency of association of filamin with the cleavage furrow, we decided to compare it with the frequency of association of alpha-actinin and myosin with the cleavage furrow. We chose to look at alpha-actinin and myosin because Fujiwara and co-workers (13, 14) have previously reported that these two proteins are associated with the cleavage furrow.

Tables I and II list data obtained with chick embryo cells

TABLE I

Frequency of Cells Showing Concentration of Filamin, Alpha-Actinin, or Myosin in the Cleavage Furrow *

	Filamin		Alpha-actinin‡		Myosin	
Degree of localization in cleavage furrow§	Number of cells	% Total	Number of cells	% Total	Number of cells	% Total
High contrast	16	16	21	13.5	37	37
No contrast	45	45	86	55	34	34
Marginal contrast	39	39	49	31	29	29
Total cells	100		156		100	

* Cells were fixed with 3.7% formaldehyde followed by acetone treatment as described in Materials and Methods. To simplify the analysis all stages of cleavage furrow were combined. Data were obtained from eight independent staining experiments.

‡ The alpha-actinin data were obtained with two antibodies as described in Materials and Methods. The data obtained with our anti-alpha-actinin gave 12% high contrast (74 cells); the data obtained with anti-alpha-actinin supplied by Dr. K. Fujiwara gave 16% high contrast (82 cells).

§ A cell was scored as *high contrast* if the cleavage furrow was more intensely stained than the surrounding regions of the cell. Cells classified as *no contrast* showed uniform staining of the cytoplasm; that is, no preferential staining of the furrow was apparent. The category *marginal contrast* was used for cells that showed only a marginal increase in staining intensity of the furrow.

TABLE II

Frequency of Cells Showing Concentration of Filamin, Alpha-Actinin, or Myosin in the Midbody Region *

	Filamin		Alpha-actinin‡		Myosin	
Degree of localization in midbody region§	Number of Cells	% Total	Number of Cells	% Total	Number of Cells	% Total
High contrast	38	35	74	50	20	19
No contrast	12	11	38	26	40	38
Marginal contrast	60	54	36	24	45	43
Total cells	110		148		105	

* Cells were fixed with 3.7% formaldehyde followed by acetone treatment as described in Materials and Methods. Data were obtained from eight independent staining experiments.

‡ The alpha-actinin data were obtained with two antibodies as described in Materials and Methods. The data obtained with our anti-alpha-actinin gave 43% high contrast (90 cells); the data obtained with anti-alpha-actinin supplied by Dr. K. Fujiwara gave 60% high contrast (58 cells).

§ A cell was scored as *high contrast* if the cytoplasm adjacent to the intercellular bridge was more intensely stained than the surrounding region of the cell. Examples are seen in Fig. 6 *g* and *i*. The term *no contrast* was used for cells that had uniform cytoplasmic staining; that is, no preferential staining of the midbody region was apparent. The category *marginal contrast* was used for cells that showed only a slight increase in staining intensity of the midbody region compared with the rest of the cell. It is important to note that, in the majority of cases, the intercellular bridge was unstained.

stained with either affinity-purified antifilamin (110 μg/ml), affinity-purified anti-alpha-actinin (110 μg/ml), whole rabbit serum (used at 1:100) directed against chick gizzard alpha-actinin, or whole goat serum (used at 1:25) directed against mouse L cell myosin. These cells were scored for the concentration of the particular protein in the cleavage furrow or midbody region. Assignment to the specific categories of "high contrast," "no contrast," or "marginal contrast" depended upon the intensity of staining of the furrow or midbody. Cells were classified as high contrast only if the cleavage furrow or midbody region was more intensely stained than surrounding regions of the cell. (Examples are shown in Figs. 5 and 6.) In cases where the furrow or midbody was stained to the same degree as the rest of the cell, the cell was classified as having no contrast in the furrow or midbody region. The category marginal contrast was used for those cells that showed only a slight increase in staining intensity of the cleavage furrow or midbody region over the rest of the cell. To simplify the analysis, all stages of cleavage furrow (Table I) or midbody region (Table II) were combined.

It was found (Table I) that filamin was concentrated in the cleavage furrow in 16% of the cells examined. There was no apparent concentration of filamin in 45% of the cells. These numbers are very similar to those observed for alpha-actinin. Alpha-actinin appeared to be concentrated in the cleavage furrow ~14% of the time; there was no apparent concentration in 55% of the cells. The alpha-actinin data include cells stained with both our anti-alpha-actinin antibody and an anti-alpha-actinin previously used by Fujiwara et al. (14). Comparable results were seen with both antibodies. The myosin data differ from both the filamin and alpha-actinin data. Myosin was found concentrated in the cleavage furrow 37% of the time; this frequency is 2–2.5 times that observed for filamin or alpha-actinin. Myosin did not appear to be specifically concentrated in the cleavage furrow in 34% of the cells we examined. We found some instances where myosin, alpha-actinin, or filamin appeared to be excluded from the furrow, but these instances occurred <5% of the time in all three cases.

The results presented in Table II show that alpha-actinin was found highly concentrated in the midbody region in half of the cells observed. There was no apparent concentration of alpha-actinin in the midbody region in 26% of the cells. Filamin was highly concentrated in the midbody region less frequently than alpha-actinin, at only 35% of the time. However, only 11% of the cells showed no apparent concentration of filamin. Myosin was found highly concentrated in the midbody region in only 19% of the cells; there was no apparent concentration in nearly 40% of the cells. Again, it is important to note that it is not the intercellular bridge that is stained in most cases, but rather the adjacent cytoplasm. Because the midbody stage is quite long, these results suggest that filamin and alpha-actinin may remain associated with the midbody region longer than myosin, an observation previously made for myosin and alpha-actinin by Fujiwara et al. (14).

DISCUSSION

Filamin Distribution during the Cell Cycle

The significance of the distribution of filamin during mitosis and cytokinesis can be better appreciated if compared to the distribution of other microfilament-associated proteins during these processes. Unlike myosin, which is concentrated in the mitotic spindle (13), and actin, which appears organized into distinct spindle fibers (6, 20, 21), filamin is not concentrated in the mitotic spindle. During mitosis the distribution more closely resembles that of alpha-actinin, although membrane staining is not so intense (14). Neither anti-alpha-actinin nor antifilamin staining of the spindle region indicates increased contrast of the spindle or distinct spindle fibers; however, the staining is not excluded from the spindle and, therefore, filamin and alpha-actinin may be associated with it.

In contrast to the diffuse antifilamin staining during mitosis, filamin is often seen to be nonuniformly distributed during cytokinesis. As seen in Figs. 5 and 6, filamin is concentrated in the cleavage furrow of dividing cells. This localization is similar to that of alpha-actinin (14) and myosin (13, 14). Toward the end of cytokinesis, filamin is very often concentrated in the cytoplasm adjacent to the intercellular bridge (Figs. 5 *k* and *m* and 6 *g* and *i*). This localization is again similar to that for alpha-actinin (14) and myosin (13, 14), although myosin seems to leave this area sooner than alpha-actinin or filamin.

Although the results are straightforward, the appropriate interpretation is much less evident. Authors of analogous studies with other microfilament-associated proteins have interpreted preferential concentration of a protein in a particular structure of the cell as evidence for participation of the protein in the architecture or function of the cell structure. For example, it has been proposed that the concentration of myosin in the cleavage furrow indicates that myosin interacts with the microfilaments of the contractile ring to provide the force required for cleavage (14). In fact, subsequent support for this proposal has come from studies in which microinjection of antimyosin into cells of starfish blastulae was found to prevent furrowing and subsequent cell cleavage (25). By analogy, we might suggest, on the basis of studies that show a concentration of filamin (this paper) or alpha-actinin (14) in the cleavage furrow, that these proteins are also involved in the cleavage process. However, the results of a frequency analysis of myosin, alpha-actinin, and filamin concentration in the cleavage furrow make it risky to draw such a conclusion from immunofluorescence data alone.

Frequency Analysis of the Association of Filamin, Alpha-Actinin, and Myosin with the Cleavage Furrow and Midbody Regions

Previous reports of immunofluorescence localization of microfilament-associated proteins in dividing cells did not include quantitation of the frequency of association between the various proteins and the cleavage furrow (2, 13, 14, 20, 21, 30). In the absence of such data, we assumed, as perhaps others did, that microfilament-associated proteins, such as myosin, alpha-actinin, and tropomyosin, are always concentrated in the cleavage furrow. We were concerned, therefore, when we observed that the degree of filamin concentration in the cleavage furrow was quite variable. At first, we thought that the increased association between filamin and the cleavage furrow that was observed in many cases might be an artifact. To explore this problem, we compared the frequency of filamin concentration in the cleavage furrow and midbody regions with that of alpha-actinin and myosin. Because independent evidence exists for the involvement of myosin in furrowing (25), we were particularly interested in determining the number of times that myosin was found to be concentrated in the cleavage furrow. The results demonstrated that filamin is concentrated in the cleavage furrow as often as alpha-actinin (~15% of the time), but only half as frequently as myosin. However, the most important point is that none of these proteins are found preferentially concentrated in the cleavage furrow 100% of the time.

It is important to distinguish between preferential concentration of a protein and presence of the protein. Preferential concentration means that the staining is brighter in a particular region, compared with surrounding regions. If the particular region is stained but is not brighter than the surrounding areas, it cannot be determined whether the protein is specifically associated with the structure in question. At the light microscope level, mere presence of a protein in a general region is not very informative. In almost all of the dividing cells we observed, the various proteins analyzed were detectable in the cleavage furrow, but they were preferentially concentrated there at a frequency of only 15–40%.

The implications of the frequency analysis are varied. First, it questions the validity of presenting immunofluorescence localization data as evidence for the participation of a protein in a specific cellular activity, if there is no analysis of either the time-course of association or the frequency of association to enable informed evaluation of the findings. Second, it shows that the interpretation of immunofluorescence localization is not trivial. For example, how cogent would the suggestion that myosin is required for cleavage have been, if it had been known that only 37% of cells in cleavage showed concentration of myosin in the furrow and if the subsequent corroborative evidence from microinjection of antimyosin (25) were not available? Third, quantitative analysis might help to explain some of the current controversies in the literature. For example, there is some disagreement at this time as to whether the concentration of actin in the cleavage furrow is significantly higher than in other parts of the cell cortex. An early report by Sanger (30), using fluorescent HMM labeling, indicated that actin was concentrated in the cleavage furrow, presumably in the contractile ring. These findings have been corroborated by Aubin et al. (2). However, reports by Herman and Pollard (20, 21), using both fluorescent antiactin and HMM, have stressed that actin is not significantly concentrated in the cleavage furrow. Nevertheless, one of these papers (21) does present a figure indicating the range of actin concentration in the cleavage furrow. Herman and Pollard suggested that the cortical actin concentration is constant throughout the cell and that the contractile ring represents only a specialized realignment or repacking of preexisting filaments. Wang and Taylor (40) have reported on the distribution of fluorochrome-labeled skeletal muscle actin that was injected into sea urchin eggs. Their results show that, immediately after fertilization, the fluorochrome-labeled actin is concentrated in the membrane-cortical regions. However, during cell cleavage there is no distinctly fluorescent cleavage furrow. It is possible, in light of our findings with filamin, alpha-actinin, and myosin, that the different results obtained for actin localization may in part be attributable to a not fully recognized variability of actin concentration in the furrow from cell to cell and to differences in interpretation of its importance by the researchers.

Why Is There Variation in the Apparent Concentration of Actin-binding Proteins in the Cleavage Furrow Region?

There are at least two explanations for the variability in the concentration of filamin, alpha-actinin, and myosin in the cleavage furrow. There may be differential extraction of these proteins during the fixation and staining procedures. However, if there is differential extraction, it occurs under many fixation conditions and suggests that these proteins are often in a less extractable form when associated with the cleavage furrow, as they seem to be during interphase when they are associated with stress fibers. Unfortunately, our attempts to analyze the extraction of filamin during fixation and staining were not

successful, probably because filamin makes up a very small percentage of the total cellular protein and it is highly sensitive to proteolysis. Another possible explanation arises from the transient nature of the cleavage furrow. Actin-binding proteins may be needed and thus concentrated in the furrow for a very brief time; therefore, only a small percentage of dividing cells would be seen with good preferential localization. A detailed time-course analysis of localization in a highly synchronized population of cells would permit an investigation of this second possibility.

If Filamin Is Required for Cleavage, What Functions Might It Perform?

The in vitro properties of filamin, i.e., that it is a phosphoprotein that induces the gelation of F-actin and inhibits the actin-activation of myosin ATPase, are the only facts that we have with which to speculate on the role of filamin in cell cleavage. These properties indicate that filamin may be involved in the increase in cortical gel strength that has been reported to occur before cleavage (26). Alternatively, filamin may act to regulate the interaction of actin with myosin.

Another possible role for filamin is suggested by a recent report by Schollmeyer et al. (31), which indicates that actin-binding protein (ABP) and alpha-actinin together may be capable of orienting actin filaments into parallel arrays. These workers used purified human-platelet actin filaments, stabilized by porcine skeletal muscle troponin (TN) and tropomyosin (TM), to analyze the types of macromolecular structures that are formed when these filaments are allowed to react with porcine muscle alpha-actinin or platelet ABP (a protein that appears to be homologous to filamin in several respects) (5, 35, 38). When added individually, alpha-actinin and ABP were both found to gel actin and, by electron microscope examination, the actin-TM/TN filaments were seen to be randomly cross-linked. However, when alpha-actinin and ABP were added sequentially to the actin-TM/TN filaments, gelation occurred and the actin filaments were organized into bundles of parallel filaments. The similarities in microfilament organization between the contractile ring (32) and the in vitro complex formed by actin, alpha-actinin, and ABP suggest that filamin (possibly analogous to ABP) and alpha-actinin may be concentrated in the cleavage furrow because they interact with actin to form the highly organized, parallel microfilaments of the contractile ring.

Although it is easy to imagine possible roles for alpha-actinin and filamin in cytokinesis, it is clear from this immunofluorescence study that the next objective must be to obtain more direct evidence that these proteins are actually required for cytokinesis.

We thank Dr. Tom Pollard for critically reading the manuscript and for use of his Leitz Ortholux microscope. We also thank Dr. Keigi Fujiwara for his willingness to exchange anti-alpha-actinin antibodies and for allowing M. H. Nunnally to confirm our alpha-actinin localization results in his laboratory.

Preliminary phases of this work were supported by the Jane Coffin Childs Fund project 324 and the American Cancer Society, Maryland Division, grant 76-27. The research is currently funded by U. S. Public Health Service grant A1-A13700. Mary H. Nunnally and Janet D'Angelo are doctoral candidates supported by 5T-32GM07445.

A preliminary account of some of the work presented in this paper has appeared previously (28).

Received for publication 17 April 1980, and in revised form 11 June 1980.

REFERENCES

1. Adair, W. S., D. Jurivich, and U. W. Goodenough. 1978. Localization of cellular antigens in sodium dodecyl sulfate-polyacrylamide gels. *J. Cell Biol.* 79:281–285.
2. Aubin, J. E., K. Weber, and M. Osborn. 1979. Analysis of actin and microfilament-associated proteins in the mitotic spindle and cleavage furrow of PtK2 cells by immunofluorescence microscopy. *Exp. Cell Res.* 124:93–109.
3. Bechtel, P. J. 1979. Identification of a high molecular weight actin-binding protein in skeletal muscle. *J. Biol. Chem.* 254:1755–1758.
4. Bretscher, A., and K. Weber. 1978. Localization of actin and microfilament-associated proteins in the microvilli and terminal web of the intestinal brush border by immunofluorescence microscopy. *J. Cell Biol.* 79:838–845.
5. Brotschi, E. A., J. H. Hartwig, and T. P. Stossel. 1978. The gelation of actin by actin-binding protein. *J. Biol. Chem.* 253:8988–8993.
6. Cande, W. Z., E. Lazarides, and J. R. McIntosh. 1977. A comparison of the distribution of actin and tubulin in the mammalian mitotic spindle as seen by indirect immunofluorescence. *J. Cell Biol.* 72:552–567.
7. Cebra, J. J., and G. Goldstein. 1965. Chromatographic purification of tetramethylrhodamine immune globulin conjugates and their use in the cellular localization of rabbit gamma globulin polypeptide chain. *J. Immunol.* 95:230–245.
8. Craig, S. W., and J. V. Pardo. 1979. Alpha-actinin localization in the junctional complex of intestinal epithelial cells. *J. Cell Biol.* 80:203–210.
9. Davies, P., P. Bechtel, and I. Pastan. 1977. Filamin inhibits actin activation of heavy meromyosin ATPase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 77:228–232.
10. Davies, P., Y. Shizuta, K. Olden, M. Gallo, and I. Pastan. 1977. Phosphorylation of filamin and other proteins in cultured fibroblasts. *Biochem. Biophys. Res. Commun.* 74: 300–307.
11. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606–2617.
12. Forer, A. 1974. Possible roles of microtubules and actin-like filaments during cell-division. *In* Cell Cycle Controls. G. M. Padilla, I. L. Cameron, and A. Zimmerman, editors. Academic Press, Inc., New York. 319–336.
13. Fujiwara, K., and T. D. Pollard. 1976. Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow, and mitotic spindle of human cells. *J. Cell Biol.* 71:848–875.
14. Fujiwara, K., M. E. Porter, and T. D. Pollard. 1978. Alpha-actinin localization in the cleavage furrow during cytokinesis. *J. Cell Biol.* 79:268–275.
15. Gawadi, N. 1974. Characterization and distribution of microfilaments in dividing locust testis cells. *Cytobios.* 10:17–25.
16. Geiger, B., K. T. Tokuyasu, and S. J. Singer. 1979. Immunocytochemical localization of alpha-actinin in intestinal epithelial cells. *Proc. Natl. Acad. Sci. U. S. A.* 76:2833–2837.
17. Greenwood, F. C., W. M. Hunter, and J. S. Glover. 1963. The preparation of ^{125}I-labelled human growth hormone of high specific radioactivity. *Biochem. J.* 89:114–123.
18. Hartwig, J. H., and T. P. Stossel. 1975. Isolation and properties of actin, myosin, and a new actin-binding protein in rabbit alveolar macrophages. *J. Biol. Chem.* 250:5696–5705.
19. Heggeness, M. H., K. Wang, and S. J. Singer. 1977. Intracellular distributions of mechanochemical proteins in cultured fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* 74:3883–3887.
20. Herman, I. M., and T. D. Pollard. 1978. Actin localization in fixed dividing cells stained with fluorescent heavy meromyosin. *Exp. Cell Res.* 114:15–25.
21. Herman, I. M., and T. D. Pollard. 1979. Comparison of purified anti-actin and fluorescent-heavy meromyosin staining patterns in dividing cells. *J. Cell Biol.* 80:509–520.
22. Kessler, S. W. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antigen-antibody complexes with protein A. *J. Immunol.* 115:1617–1624.
23. Kruse, P. F., and M. K. Patterson, editors. 1973. Tissue Culture Methods and Applications. Academic Press, Inc., New York. 119–122.
24. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
25. Mabuchi, I., and M. Ikuno. 1977. The effect of myosin antibody on the division of starfish blastomeres. *J. Cell Biol.* 74:251–263.
26. Marsland, D., and J. V. Landau. 1954. The mechanism of cytokinesis: temperature-pressure studies on the cortical gel system in various marine eggs. *J. Exp. Zool.* 125:507–539.
27. Mooseker, M. S., T. D. Pollard, and K. Fujiwara. 1978. Characterization and localization of myosin in the brush border of intestinal epithelial cells. *J. Cell Biol.* 79:444–453.
28. Nunnally, M. H., J. M. D'Angelo, and S. W. Craig. 1979. Localization of filamin in the cleavage furrow of dividing chick embryo fibroblasts. *J. Cell Biol.* 83(2, Pt. 2): 371*a* (Abstr.).
29. Parikh, I., S. March, and P. Cuatrecasas. 1974. Topics in the methodology of substitution reactions with agarose. *Methods Enzymol.* 34:77–102.
30. Sanger, J. W. 1975. Changing patterns of actin localization during cell division. *Proc. Natl. Acad. Sci. U. S. A.* 72:1913–1916.
31. Schollmeyer, J. V., G. H. R. Rao, and J. G. White. 1978. An actin-binding protein in human platelets. *Am. J. Pathology.* 93:433–446.
32. Schroeder, T. E. 1975. Dynamics of the contractile ring. *In* Molecules and Cell Movements. S. Inoue and R. E. Stephens, editors, Raven Press, New York. 305–332.
33. Shizuta, Y., H. Shizuta, M. Gallo, P. Davies, I. Pastan, and M. S. Lewis. 1976. Purification and properties of filamin, an actin-binding protein from chicken gizzard. *J. Biol. Chem.* 251:6562–6567.
34. Tilney, L. G., and M. Mooseker. 1971. Actin in the brush borders of epithelial cells of the chicken intestine. *Proc. Natl. Acad. Sci. U. S. A.* 68:2611–2615.
35. Wallach, D., P. J. A. Davies, and I. Pastan. 1978. Purification of mammalian filamin. *J. Biol. Chem.* 253:3328–3335.
36. Wallach, D., P. J. A. Davies, and I. Pastan. 1978. Cyclic AMP-dependent phosphorylation of filamin in mammalian smooth muscle. *J. Biol. Chem.* 253:4739–4745.
37. Wang, K., J. F. Ash, and S. J. Singer. 1975. Filamin, a new high-molecular weight protein found in smooth muscle and non-muscle cells. *Proc. Natl. Acad. Sci. U. S. A.* 72:4483–4486.
38. Wang, K. 1977. Filamin, a new high-molecular-weight protein found in smooth and non-muscle cells. Purification and properties of chicken gizzard filamin. *Biochemistry* 16:1857–1865.
39. Wang, K., and S. J. Singer. 1977. Interaction of filamin with F-actin in solution. *Proc. Natl. Acad. Sci. U. S. A.* 74:2021–2025.
40. Wang, Y., and D. L. Taylor. 1979. Distribution of fluorescently labelled actin in living sea urchin eggs during early development. *J. Cell Biol.* 82:672–679.