ALPHA-ACTININ LOCALIZATION IN THE CLEAVAGE FURROW DURING CYTOKINESIS

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ABSTRACT

We used antibodies against alpha-actinin and myosin labeled directly with contrasting fluorochromes to localize these contractile proteins simultaneously in dividing chick embryo cells. During mitosis anti-alpha-actinin stains diffusely the entire cytoplasm including the mitotic spindle, while in the same cells intense antimyosin staining delineates the spindle. During cytokinesis both antibodies stain the cleavage furrow intensely, and until the midbody forms the two staining patterns in the same cell are identical at the resolution of the light microscope. Thereafter the anti-alpha-actinin staining of the furrow remains strong, but the antimyosin staining diminishes. These observations suggest that alpha-actinin participates along with actin and myosin in the membrane movements associated with cytokinesis.

KEY WORDS alpha-actinin · myosin · cytokinesis · cleavage furrow · fluorescent antibody

The identification of actin (12, 16) and myosin (3) in the cleavage furrow of dividing cells and the ability of microinjected antimyosin to inhibit cytokinesis (10) strongly suggest that the cytoplasmic contractile proteins generate the force for the membrane movements which partition the two daughter cells. Because the membrane is likely to be a passive participant in this process, it is essential to learn how it is connected to the contractile machinery. The molecular basis of such connections is not understood in any cell, although in the case of smooth muscle cells (14) and cultured fibroblasts (19) antibody staining has indicated that the muscle Z-line protein alpha-actinin is concentrated at or near sites where bundles of actin filaments attach to membranes. These observations prompted us to investigate alpha-actinin localization during the dynamic actin-membrane association which must occur during cytokinesis.

In this report we demonstrate that purified fluorescent antibodies against chicken gizzard alpha-actinin selectively stain the cytokinetic cleavage furrow of dividing tissue culture cells. Double staining with anti-alpha-actinin and anti-myosin reveals that alpha-actinin and myosin have nearly identical distributions in the cleavage furrow during cytokinesis; however, after the midbody forms, the intensity of the antimyosin staining in the furrow diminishes greatly while the intensity of the anti-alpha-actinin in the furrow remains strong. This departure of the myosin from the furrow probably indicates the end of tension development by the contractile ring, whereas the actin filaments (15) and alpha-actinin may remain because they are directly attached to the membrane. A preliminary account of this work was presented at the 17th Annual Meeting of the American Society for Cell Biology (5).

MATERIALS AND METHODS

Preparation of Alpha-Actinin

We extracted alpha-actinin from fresh chicken gizzards by a modification of methods previously used for cardiac muscle (18) suggested to us by Dr. R. M.

J. CELL BIOLOGY © The Rockefeller University Press · 0021-9525/78/1001-0268$1.00
Volume 79 October 1978 268–275

Robson of Iowa State University. The crude alpha-actinin was precipitated with 30% ammonium sulfate and then purified by column chromatography. In addition to ion exchange chromatography on DEAE-cellulose and hydroxyapatite (18), we employed gel permeation chromatography on 4% agarose (Bio-Rad A15m, 200–400 mesh, Bio-Rad Laboratories, Richmond, Calif.) in 0.6 M KI, 0.1 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, and 10 mM imidazole (pH 7.0). The columns were run in the following order: DEAE, agarose, hydroxyapatite, hydroxyapatite, and agarose. The purified alpha-actin was stored frozen in 10% sucrose or as a pellet in 35% ammonium sulfate at 4°C.

Preparation of Fluorescein Labeled Antibodies

Three white New Zealand rabbits were immunized with the purified alpha-actinin by methods described previously (3). The specificity of immune serum was determined by double diffusion and immunoelectrophoresis (3) using phosphate-buffered saline as the buffer (0.85% NaCl, 10 mM sodium phosphate, pH 7.4; phosphate-buffered saline [PBS]). Immune IgG was coupled with fluorescein isothiocyanate, and conjugates with fluorescein: IgG molar ratios of 1.3–4.0:1 were separated from other reactants as before (3).

Purification of Alpha-Actinin Antibodies

We purified anti-actinin from other antibodies in the immune serum by affinity chromatography on alpha-actinin-Sepharose 4B. We coupled 14.5 mg of purified alpha-actinin in 2 ml of 0.1 M KCl, 0.1 M borate, 1 mM $MgCl_2$, adjusted to pH 9 with KOH to 5 ml of CNBr-activated Sepharose 4B (11) by reaction for 20 h at 4°C. Pelleted beads were then reacted with 1 M glycine in the KCl-borate buffer, pH 8.5, for 18 h at 4°C, and washed successively with 50 ml of 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 10, 50 ml of 0.5 M NaCl, 0.1 M sodium acetate, pH 4, and several changes of PBS. Washed alpha-actinin-Sepharose 4B was stored at 4°C in PBS with 0.02% azide.

~15 mg of either immune IgG or fluorescein-immune IgG were passed twice through a 1 ml column of alpha-actinin-Sepharose 4B. The nonadherent IgG was used as absorbed immune IgG for a staining control. The column was washed with PBS until the absorbance of the effluent at 280 nm was zero. The anti-alpha-actinin was eluted with 0.2 M glycine HCl, pH 2.75, and neutralized (3). We recovered ~0.2 mg IgG as purified anti-alpha-actinin.

Antimyosin

The rhodamine-antimyosin (rabbit no. 8) and absorbed IgG used in this study have been described in detail (3).

Cells and Staining

Cells from the body wall of 11-day chick embryos were dissociated in 0.2% trypsin and grown on cover slips for 3–5 days in Eagle's Basal Medium with 10% fetal calf serum. We fixed, stained, and examined these cells as described previously (4). We also stained cells indirectly using unlabeled anti-alpha-actinin (purified antibody or immune IgG) and rhodamine-labeled goat anti-rabbit IgG (lot no. 10340, Cappel Laboratories, Inc., Cochranville, Pa.) diluted 250 times. The controls for staining specificity included: staining with absorbed immune IgG, blocking specific staining with unlabeled immune IgG or antisera, staining with specific antibodies in the presence of unlabeled preimmune IgG or serum, and indirect staining with preimmune serum using rhodamine labeled goat anti-rabbit IgG diluted 250 times.

Biochemical Methods

Alpha-actinin concentration was measured by the Hartree method (7) or by absorbance at 280 nm using an extinction coefficient of 1.1 $ml \cdot mg^{-1} \cdot cm^{-1}$. Gel electrophoresis in polyacrylamide was carried out in Tris-glycine buffer, pH 8.6, with or without 0.1% sodium dodecyl sulfate (19).

RESULTS

Characterization of Antigen and Antibodies

By two methods of gel electophoresis the purified gizzard alpha-actinin consists of a single component (Fig. 1) and by both double immunodiffusion and immunoelectrophoresis all of our antisera react with the same single component in both purified and crude alpha-actinin preparations (Figs. 2 and 3).[1]

Fluorescein-anti-alpha-actinin stains the Z-lines of cultured chick myotubes and the stress fibers of primary chick embryo cells, PtK cells, and HeLa cells (5). These staining patterns are specific for alpha-actinin judging by the criteria we have used previously (3) and will be described in detail elsewhere. The chick cells stained much more intensely than the other cells, so they were used to study the distribution of alpha-actinin in dividing cells.

[1] There is also evidence that these sera react with both native and denatured alpha-actinin from chicken skeletal, cardiac, and smooth muscles, though we have been unable to detect reaction with a 95,000 mol wt polypeptide from chicken brush border (Mooseker et al. Manuscript in preparation).

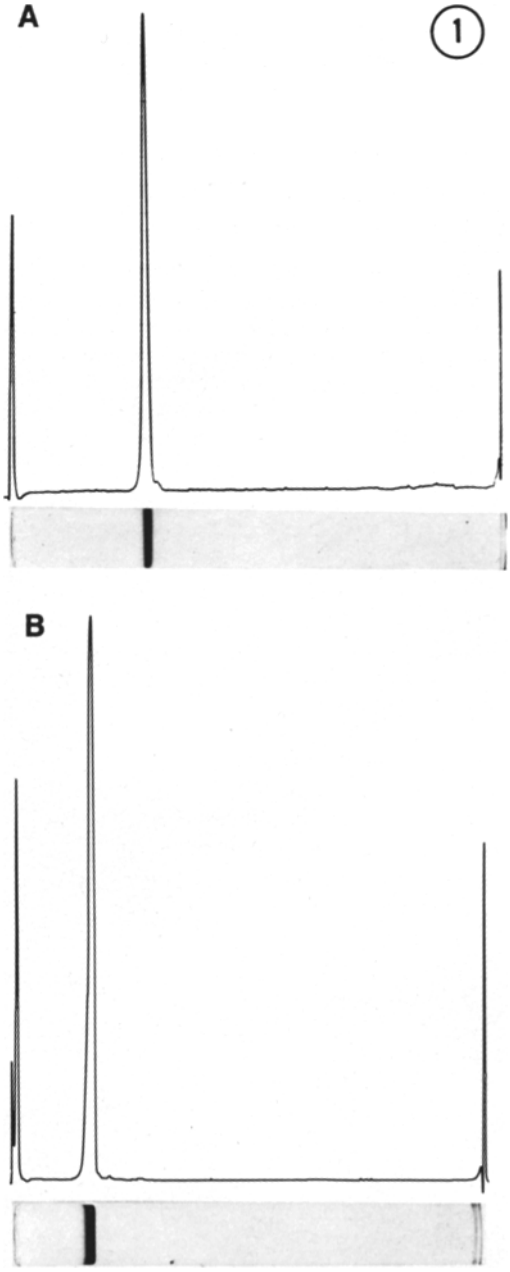
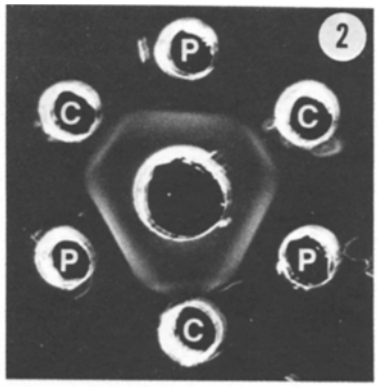


FIGURE 1 Polyacrylamide gel electrophoresis of purified alpha-actinin. Gels are stained and scanned at 550 nm. (*A*) Native alpha-actinin on a 5% gel. (*B*) Sodium dodecyl sulfate denatured alpha-actinin on a 7.5% gel.

Anti-Alpha-Actinin Staining of Dividing Cells

Fluorescein-anti-alpha-actinin IgG, purified fluorescein-anti-alpha-actinin and unlabeled anti-alpha-actinin (purified antibody or immune IgG) used with rhodamine-labeled goat anti-rabbit IgG stain the cytoplasm of mitotic cells uniformly and are concentrated in the concave portion of the cleavage furrow during cytokinesis (Fig. 4). From prophase through anaphase there is no contrast between the spindle and the surrounding cytoplasm. The chromosomes are unstained, whereas in the cortex the staining is often concentrated in intense spots of <1 μm. During cell cleavage the furrow was always stained much more intensely than any other region of the cell. By focusing through the cell, it is clear that this furrow staining is a cortical ring extending around the entire circumference of the furrow.

This staining of the cleavage furrow is specific for alpha-actinin by the following criteria. No cells stain with fluorescein labeled anti-alpha-actinin IgG absorbed with purified alpha-actinin (Fig. 4*h*) or fluorescein-labeled anti-alpha-actinin IgG in the presence of unlabeled immune IgG or antise-



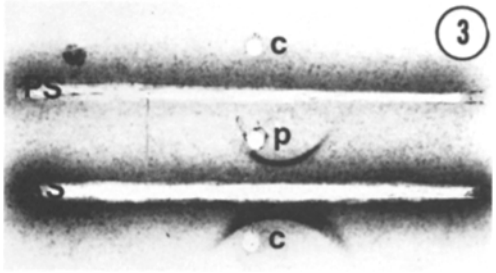


FIGURE 2 Double immunodiffusion in 1% agarose. A single precipitin band is detected between the antiserum (*center*) and purified alpha-actinin (*P*) or crude alpha-actinin consisting of the 30% ammonium sulfate precipitate of the original smooth muscle extract (*C*). Unstained.

FIGURE 3 Immunoelectrophoresis of purified alpha-actinin (*p*) and crude alpha actinin (*c*) against preimmune serum (*PS*) and immune serum (*AS*). Stained.

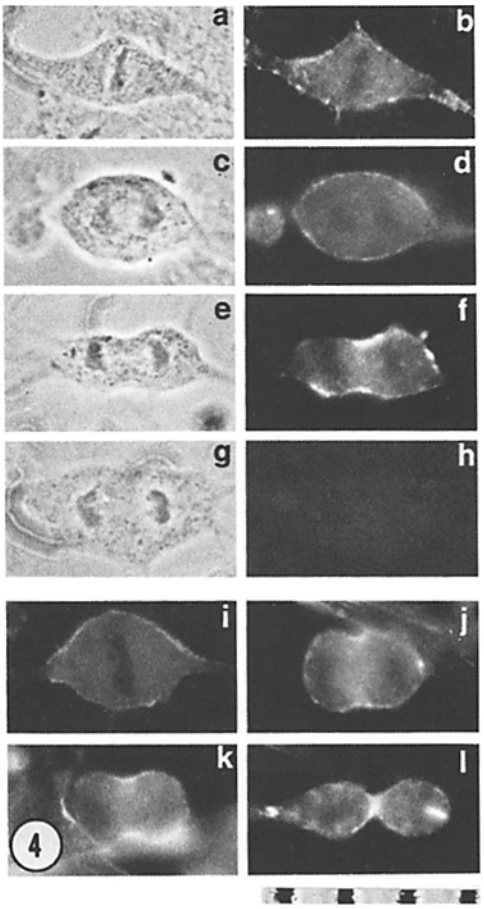


FIGURE 4 Anti-alpha-actinin staining of dividing chick embryo primary culture cells. The phase-contrast micrographs indicated *a*, *c*, *e*, and *g* correspond to the fluorescence micrographs indicated *b*, *d*, *f*, and *h*, respectively. During metaphase (*b* and *i*) and anaphase (*d*) the cytoplasm stains diffusely except for beaded staining in the cortex and no staining of chromosomes. During cytokinesis, the cleavage furrow stains intensely (*f*, *j*, *k*, and *l*). The beaded staining in the cortex is also present during cleavage. (*b* and *f*) stained indirectly using anti-alpha-actinin IgG (50 μg/ml) and rhodamine-labeled goat anti-rabbit IgG. (*d* and *i*) stained with fluorescein labeled pure antibody against alpha-actinin (20 μg/ml). (*h*) stained with fluorescein labeled anti-alpha-actinin IgG (150 μg/ml) absorbed with purified alpha-actinin. (*j* and *k*) stained indirectly using purified antibody against alpha-actinin (30 μg/ml) and rhodamine-labeled goat anti-rabbit IgG. (*l*) stained with fluorescein labeled anti-alpha-actinin IgG (60 μg/ml). Scale, 1 division = 10 μm.

rum. Using the indirect method, there is no staining with unlabeled preimmune IgG or serum. Specific staining is observed in cells stained with purified fluorescein labeled anti-alpha-actinin in the presence of unlabeled preimmune serum (Fig. 4*d*).

Double Staining of Dividing Cells with Fluorescein-Anti-Alpha-Actinin and Rhodamine-Antimyosin

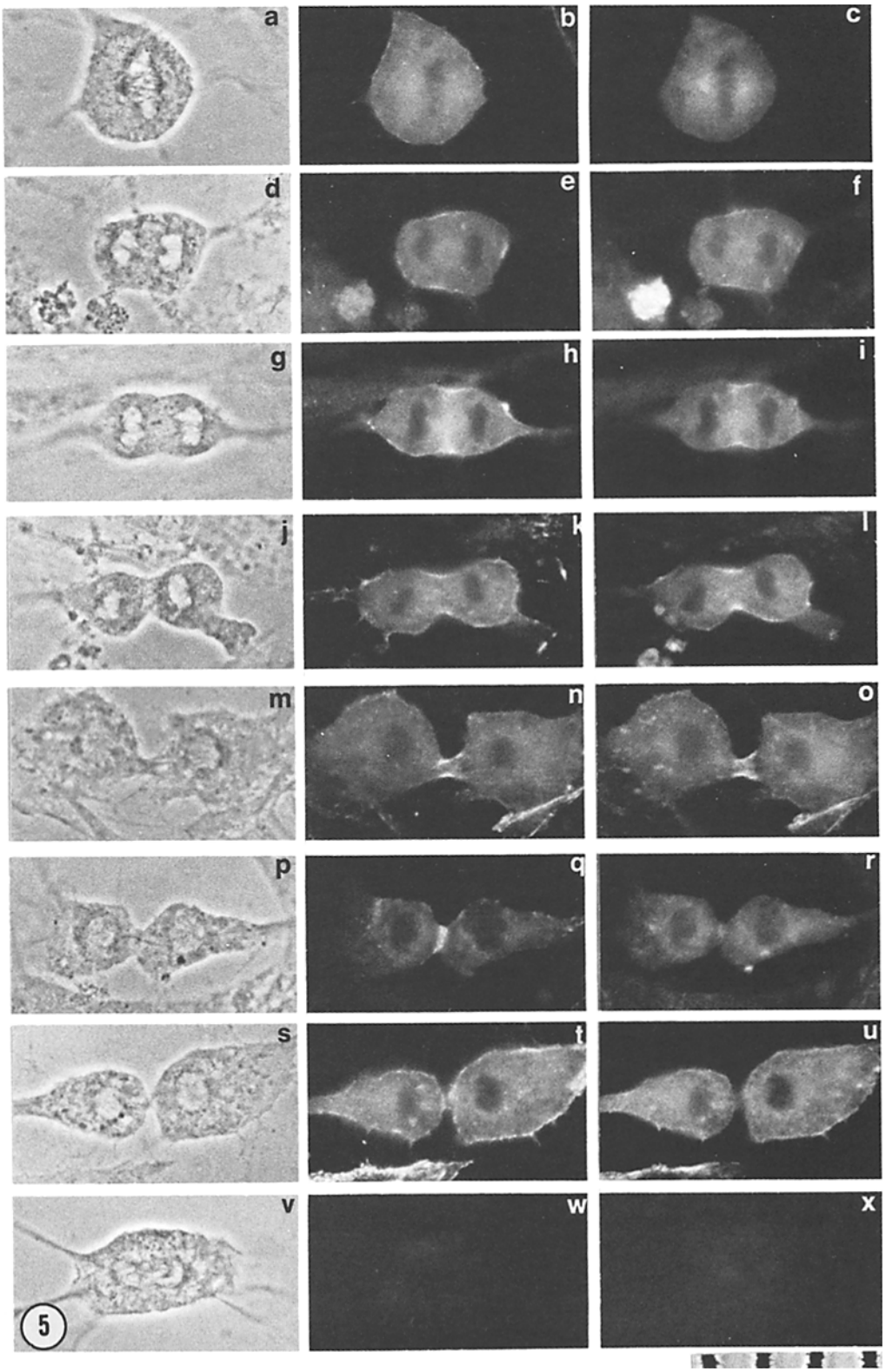
Double staining of dividing cells shows that alpha-actinin and myosin accumulate simultaneously in the cleavage furrow, but that at the completion of constriction myosin leaves the furrow before the alpha-actinin (Fig. 5). In metaphase, the location of the spindle is revealed by antimyosin staining (Fig. 5*c*), but not by anti-alpha-actinin staining (Figure 5*b*). In late anaphase, just before the establishment of the cleavage furrow, a cortical ring stained with both anti-alpha-actinin and anti-myosin appears around the equator of the cell (Fig. 5, *e* and *f*). From this early stage and throughout the process of cleavage furrow constriction, the two antibodies stain the concave portion of the cleavage furrow identically within the limits of resolution of the light microscope (Fig. 5, *d–o*). This coincident localization of anti-alpha-actin and antimyosin staining in the cleavage furrow ends when the midbody forms. Then the antimyosin staining decreases (Fig. 5*r*) while the anti-alpha-actinin staining remains strong (Fig. 5*q*). Later in telophase, the anti-myosin staining disappears altogether from the region of the midbody (Fig. 5*u*), while some anti-alpha-actinin staining persists (Fig. 5*t*).

We observed similar staining patterns in PtK and HeLa cells, but the intensity of the anti-alpha-actinin staining was weak, possibly reflecting a poor cross-reactivity of the antibody.

When cells are stained with labeled immune IgG absorbed with purified antigens, no staining is detectable (Fig. 5, *w* and *x*). The specific staining patterns can be blocked by the addition of unlabeled immune IgG or antisera during double staining, but not by unlabeled preimmune IgG or antisera.

DISCUSSION

Although alpha-actinin has not been purified from the cells used in this study, our purified directly labeled antibody reacts specifically with some component(s) by immunological criteria. The purified antibody, but not the absorbed immune IgG, stains nonmuscle cells. Moreover, the staining is blocked by unlabeled immune serum but not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by unlabeled preimmune serum, and preimmune IgG does not stain the cells, even by indirect staining. Thus, in the following paragraphs we will interpret the fluorescent anti-alpha-actinin staining patterns in terms of the distribution of alpha-actinin.

Unlike actin (reference 2 and footnote 2) and myosin (3, 4), which have been localized in the mitotic spindle with fluorescent antibodies, alpha-actinin does not appear to be concentrated in the mitotic spindle by the same criterion. Nonetheless, alpha-actinin is present in the spindle region, where its concentration appears to be the same as the surrounding regions of the cytoplasm. It is not, for example, excluded from the spindle like a 55,000 mol wt antigen possibly related to intermediate filaments (6). A uniform staining pattern is difficult to interpret, but it does not argue strongly for a role for alpha-actinin in mitotic spindle function. On the other hand, it does not rule it out.

The concentration of alpha-actinin and myosin in the concave portion of the cleavage furrow along with the circumferential bundle of actin filaments called the contractile ring suggests that these proteins are responsible, at least in part, for the membrane movement. The following features of the molecular architecture of the cleavage furrow are important for inferring molecular events during cytokinesis: (*a*) In at least some cells, preexisting actin filaments are present in all parts of the cortex including the site of the future cleavage furrow (1); (*b*) although an obvious circumferential bundle of actin filaments appears in the concave portion of the cleavage furrow during cytokinesis (16), actin molecules do not appear to be concentrated in this or any other region of the cortex, judging from staining of fixed whole cells with either fluorescent-heavy meromyosin or purified actin antibodies (reference 8 and footnote 2); (*c*) the restriction of the contractile ring to the concave portion of the furrow suggests that these aligned actin filaments are under tension and attached to the plasma membrane (17); (*d*) since the width and thickness of the contractile ring remain more or less constant as its circumference decreases, there must be some filament depolymerization (17); (*e*) the contractile ring and the concentration of alpha-actinin in the furrow have approximately the same distribution and lifetime during cytokinesis, suggesting that the alpha-actinin is associated with a stable fraction of the contractile ring actin; (*f*) myosin is concentrated around the equator from just before the appearance of the cleavage furrow (4) until the midbody forms; and (*g*) myosin appears to be necessary for furrowing (10).

All of these observations are consistent with a mechanism for cytokinesis similar to models proposed previously by Rappaport (13) and by Schroeder (17), now updated to include new information (Fig. 6). We suggest that the key event stimulated by the mitotic apparatus is the accumulation of myosin around the equator and/or the activation of contraction in this equatorial zone. As pointed out previously (13), tension generated in a limited zone around the equator by the interaction of myosin with preexisting membrane-bound actin filaments will align the fila-

[2] HERMAN, I., and T. D. POLLARD. Comparison of purified anti-actin and fluorescent-heavy meromyosin staining patterns in dividing cells. *J. Cell Biol.* Manuscript submitted for publication.

FIGURE 5 Dividing chick embryo primary culture cells in phase contrast (*a, d, g, j, m, p,* and *s*) stained simultaneously with fluorescein-labeled anti-alpha-actinin (*b, e, h, k, n, q,* and *t*) and rhodamine-labeled antimyosin (*c, f, i, l, o, r,* and *u*). The cell in *v* is stained with fluorescein labeled anti-alpha-actinin IgG absorbed with purified alpha-actinin (150 μg/ml) and rhodamine-labeled antimyosin IgG absorbed with purified myosin (100 μg/ml). One cell (*a*) is in metaphase and the spindle is clearly detectable by the antimyosin (*c*) but not by anti-alpha-actinin (*b*). One cell (*d*) is at the onset of cleavage and both anti-alpha-actinin (*e*) and antimyosin (*f*) stain the area of the cortex where the cleavage furrow will develop. The cleavage furrows of the three cells (*g, j,* and *m*) stain intensely with both anti-alpha-actinin (*h, k,* and *n*) and antimyosin (*i, l,* and *o*). Note the similarity of the staining patterns by the two antibodies in the same cell. Two cells (*p* and *s*) have formed the midbody, which is in focus in *p* but not in *s*, and the antimyosin staining is greatly reduced (*r* and *u*). The anti-alpha-actinin staining is strong in *q* and disappearing in *t*. Many dividing cells have strong beaded or patched staining with both antibodies, but the patches of the two antibodies are not necessarily coincident.

Antimyosin reagent was rhodamine-labeled antimyosin IgG (50 μg/ml) in all cases while anti-alpha-actinin reagent was fluorescein labeled anti-alpha-actinin IgG (90 μg/ml) in all except for *q* we used fluorescein labeled pure anti-alpha-actinin (20 μg/ml). Scale, 1 division = 10 μm.

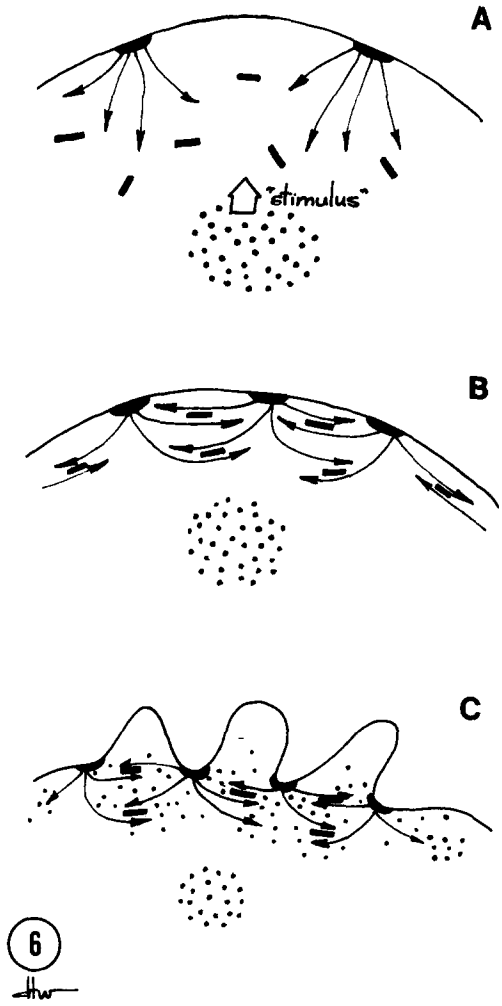

FIGURE 6 Idealized drawings depicting a possible mechanism of cytokinesis, which is described in detail in the text. An equatorial section of part of the cortex is shown at three successive times. Actin filaments attached to the membrane at their "barbed" ends are represented by lines with arrowheads. Alpha-actinin is associated with the base of these actin filaments in an unspecified manner. Small bipolar myosin filaments are represented by bars. The microtubules of the mitotic spindle are shown in cross section as a cluster of dots. (*A*) Early anaphase. The spindle sends a stimulus restricted to the equatorial cortex signaling activation of contraction. (*B*) Tension generated by interaction of myosin with membrane-bound actin filaments aligns actin filaments around the equator forming the contractile ring. (*C*) As the equatorial membrane is constricted by tension between actin-binding sites, the actin filaments disassemble from their free ends. Thus myosin, actin-binding sites, and alpha-actinin, but not actin, are concentrated in the furrow during cytokinesis.

ments to form the contractile ring. This tension could be generated by bipolar myosin filaments pulling pairs of oppositely polarized, plasma membrane-anchored actin filaments towards each other. Providing the tension on the actin and the membrane were restricted to the equatorial zone where the myosin appears to be concentrated, it would produce both a circumferential alignment of actin filaments and the concave furrow.

The model incorporates the suggestion that the mass of the contractile ring diminishes during cytokinesis by depolymerization of the actin filaments from their free "pointed" ends. This is in keeping with evidence that actin filament polymerization is favored at the "barbed" end (20) and by inference that depolymerization must be favored at the "pointed" end. The increase in concentration of alpha-actinin in the furrow during the course of cytokinesis is explained by its presence at or near actin-membrane attachment sites. This end of the actin filament may be the most stable. If the number of actin-membrane attachment sites associated with alpha-actinin remains constant as the circumference of the furrow decreases, their concentration would increase. Finally, although both a constricted bundle of actin filaments and their presumed membrane attachment sites seem to remain around the midbody, it seems unlikely that this contractile ring remnant continues to generate tension because little myosin is present. Although consistent with present information, this model remains to be tested by further experimentation.

The authors would like to thank Dr. Richard Robson of Iowa State University for allowing us to use his unpublished method for the isolation of alpha-actinin from chicken gizzard. We are also indebted to him for advice during our antigen preparation. We also thank Ms. Carol McKinley for preparing chick primary cultures and Ms. Diane Woodrum for making Fig. 6.

This work was supported by National Institutes of Health research grants GM 19654 and GM 23531, and by Research Career Development Award GM 70755.

Received for publication 19 June 1978, and in revised form 24 July 1978.

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