COMPARISON OF PURIFIED ANTI-ACTIN AND FLUORESCENT-HEAVY MEROMYOSIN STAINING PATTERNS IN DIVIDING CELLS

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ABSTRACT

We purified actin antibodies by affinity chromatography from the serum of rabbits immunized with glutaraldehyde-fixed chicken gizzard actin filaments and used this anti-actin to localize actin in myofibrils and fixed cultured cells at each stage of the cell cycle. By double immunodiffusion the anti-actin reacted with both smooth and skeletal muscle actin. Several blocking and absorption experiments demonstrated that the antibodies also bound specifically to actin in nonmuscle cells. The same structures stained using either the direct or the indirect fluorescent antibody technique; and, while the indirect method was more sensitive, the direct method was superior because there was no detectable nonspecific staining. As expected, anti-actin stained the I-band of myofibrils. It also stained stress fibers and membrane ruffles in HeLa cells. Some PtK-2 cells have straight stress fibers which stained with anti-actin, but in confluent cultures all PtK-2 cells have, instead, sinuous phase-dense fibers which stained with antibody. At prophase the whole cytoplasm stained uniformly with anti-actin. During metaphase and anaphase, anti-actin staining was concentrated diffusely in the mitotic spindle. In contrast, fluorescent heavy meromyosin stained discrete fine spindle fibers in these fixed cells. During cytokinesis, anti-actin stained the whole cytoplasm uniformly and was not concentrated in the cleavage furrow.

KEY WORDS anti-actin · purified · fluorescent · heavy meromyosin · dividing · cells

Staining cells with antisera from animals immunized with actin (14, 12, 8, 1, 15) can be a powerful way to study its intracellular distribution, providing the staining specificity can be demonstrated. Using an indirect fluorescent antibody staining technique, workers have found that immunoglobulins from these immune sera bind to a number of cellular structures including stress fibers (14, 12, 13, 1, 15), ruffled membranes (13, 18), and the mitotic spindle (1). In the case of stress fibers there is little doubt that actin is present, because these fibers consist of bundles of 6-nm filaments that bind heavy meromyosin (HMM) (19, 20, 9) and subfragment-1 (S-1) (7, 21). The evidence for actin in the mitotic spindle is more controversial. Although fluorescent HMM (19, 20, 9) and anti-actin sera (1) stain the spindle, relatively few 6-nm filaments are found in spindles prepared for electron microscope observation (4, 22, 10, 23, 21).

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The original studies with actin antisera employed unfractionated immune IgG and an indirect staining method (14, 12, 13, 1). The fluorescent staining was attributed to a reaction between immunoglobulins specific for actin and cellular actin molecules, because neither preimmune sera nor immune sera absorbed with actin stained strongly. However, these observations are also consistent with some or all of the staining's being a result of nonprecipitating antibodies to minor contaminants in the actin used for immunization and absorption, and even the actin antigen purified by gel electrophoresis was stated to be only 85% pure (14).

A second criticism which applies to the anti-actin work on the mitotic spindle is that detergent extraction of part of the cell's actin was necessary to detect fluorescence intensity differences between the spindle and its surrounding cytoplasm (1). This extraction could alter actin's distribution in the spindle just as it alters the cytoplasmic actin concentration.

In light of these problems with previous work and because it is important to determine with confidence which cellular structures contain actin and which do not, we have re-examined the patterns obtained from actin antibody staining of myofibrils and two different cultured cell lines, using several improvements in technique. First, we stained cells fixed by a method which retains 90% of the actin. Second, we used directly-labeled purified antibodies shown by precipitation to react only with actin in crude cellular extracts. We also compared this direct staining method with two indirect staining methods and found the direct method to be less sensitive, but noticeably "cleaner." In addition, we compared the anti-actin staining with fluorescent-heavy meromyosin spindle staining and found a subtle difference which may be attributable to the ability of these probes to discriminate between polymerized and unpolymerized actin.

Other workers are also aware that improved anti-actin reagents are necessary to increase the reliability of fluorescent antibody localization of actin. For example, Osborn et al. (15) have used purified anti-actin to stain stress fibers in recent experiments.

MATERIALS AND METHODS

Preparation of Chicken Gizzard Myofibrils for Actin Extraction

Myofibrils were obtained from fresh chicken gizzards by a slight modification from the method of Sobieszek and Bremel (24). Fresh gizzards were chilled on ice and cleaned. 300 g of ground muscle were suspended in 3.3 vol of a wash solution (24) containing 60 mM KCl, 20 mM imidazole-Cl, 1 mM diithiothreitol (DTT), 1 mM MgCl₂, 0.02% sodium azide, pH 6.9, at 0°C and homogenized in a Sorvall Omnimixer (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.) for 20 s at setting no. 9. The suspension was centrifuged for 30 min at 16,000 rpm in a Beckman J-21 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) equipped with a JA-20 rotor. This procedure was repeated five times, the first three washes containing 0.5, 0.5, and 0.3% of Triton X-100 (Sigma Chemical Co., St. Louis, Mo.). The last two washes had no detergent. The final buff-white pellet of myofibrils was washed four times with one liter of cold (4°C), deionized water at pH 8.4 (adjusted with 1 M sodium carbonate), dehydrated by washing twice with 400 ml of cold acetone and then dried overnight.

Extraction of Actin from Acetone Powder

Gizzard actin was purified by minor modifications of the method of Spudich and Watt (25). 10 g of acetone powder were dissolved and stirred into 200 ml of an extraction buffer (Buffer A) containing 2 mM Tris-Cl, 0.2 mM ATP, 0.5 mM DTT, 0.2 mM CaCl₂, pH 8.0, at 0°C for 15 min. Longer extraction yielded more contaminating proteins. The suspension was centrifuged in a JA-20 rotor for 30 min at 16,000 rpm. The supernate (110 ml) was filtered through glass wool and polymerized by adding KCl to 50 mM and MgCl₂ to 2 mM while stirring at room temperature for 45 min. After cooling to 4°C for 90 min, the solution was made 0.8 M in KCl by adding 4 M KCl dropwise while stirring. After 60 min, the viscous, opalescent solution was centrifuged for 2.5 h at 35,000 rpm in a 42.1 rotor cooled to 4°C. Pellet surfaces were washed, and the pellets homogenized in 10 ml of Buffer A. After dialysis for 3 d against several liters of Buffer A, the depolymerized G-actin was clarified by centrifugation for 2 h at 35,000 rpm in a 42.1 rotor at 4°C and repolymerized as described above; then it was depolymerized a second time. Twice-depolymerized actin was purified further by gel permeation chromatography on a 1.5 × 30 cm column of Sephadex-G150 equilibrated with Buffer A. The purified G-actin was lyophilized in Buffer A and stored dessicated at -20°C.

Preparation of Immunizing Agents and Immunization Schedule

1.0 mg of purified actin was dissolved in 2.0 ml of Buffer A at 0°C and polymerized by the addition of KCl to 100 mM and MgCl₂ to 2 mM. The polymerized actin was treated with 0.1% glutaraldehyde at 0°C for 1 h and then dialyzed against several changes of phosphate buffered saline (PBS) (15 mM phosphate, 0.85% NaCl, pH 7) over 3 d. The antigen was then emulsified with an...
equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Each white New Zealand rabbit (average weight 5 kg) received a total of 330 μg of actin in 20-30 subcutaneous injections distributed along the back and into each foot pad. After 4 wk, each rabbit was boosted with multiple subcutaneous injections totaling 300 μg of actin emulsified in Freund's incomplete adjuvant. Immune serum was obtained from bleedings 7-15 d after the boost.

**Double Immunodiffusion Analysis of Antisera**

Antisera were screened by double immunodiffusion (17) at room temperature for 1-3 d in 1.0% agarose (wt/vol) containing 0.02% sodium azide and 15 mM phosphate, 0.85% NaCl, pH 7.8. The plates were then washed, pressed, and dried before staining with Coomassie brilliant blue R.

**Preparation of Rhodamine Antiactin**

Rhodamine labeling was carried out by Fujiwara and Pollard's (5) modification of the method of Cebra and Goldstein (2) with 20 μg of rhodamine isothiocyanate per mg of IgG. The immunoglobulin-G (IgG) was precipitated from immune serum with 40% saturated ammonium sulfate, pH 7.0-7.5.

**Purification of Anti-actin from Immune IgG by Affinity Chromatography**

Actin antibodies were purified (either before or after conjugation with tetramethylrhodamine) by affinity chromatography on Sepharose-4B actin. Twice-depolymerized actin in 0.1 M KCl, 15 mM phosphate, pH 9.5, was coupled to CNBr-activated Sepharose-4B (3). 10 ml of immune IgG in 0.01 M phosphate buffer, pH 7.5, was then mixed with 2 ml of Sepharose-4B containing 1.5 mg of actin for 20 h at 4°C. The mixture was then poured into a 0.5 × 2.0 cm column, and 0.01 M phosphate buffer, pH 7.5, was passed through the column until A280 = 0. The fraction that did not bind to the column was used as absorbed IgG in control experiments. Anti-actin was eluted with 0.1 M glycine-HCl, pH 2.75, and 20-drop fractions were collected into ice cold tubes that contained two drops of 1 M phosphate buffer, pH 7.5, which immediately neutralized the pH. It was then dialyzed against PBS.

**Preparation of Myofibrils**

Small strips of chicken breast muscle myofibrils tied to wooden sticks were extracted for more than 1 mo at -20°C in 50% glycerol: 50% rigor buffer (9). 1/2-cm long strips were sheared for 5 s at 0°C in a Sorvall Omnimixer on setting no. 6 in rigor buffer. After filtering through cheesecloth, the myofibrils were pelleted and resuspended three times in rigor buffer.

**Tissue Culture Cells**

Rat kangaroo (PtK-2) and human HeLa cell lines were grown on 18 × 18 mm glass microscope cover slips as described previously (9).

**Staining Myofibrils with Fluorescent Actin Ligands**

A 0.1-ml pellet of myofibrils was resuspended in an equal volume of (a) 60 μg/ml purified rhodamine anti-actin, (b) 30 μg/ml purified anti-actin, or (c) 1:5, 1:15, or 1:45 dilution of immune serum for 60 min at 0°C. To remove unbound protein, the myofibrils were washed by pelleting three times in 30 ml of rigor buffer. In the case of b and c, the myofibrils were then mixed with an equal volume of fluorescein-goat anti-rabbit IgG (50 μg/ml, 1: 25 dilution Miles Lot no. $403) for an additional 60 min at 0°C. After washing with 30 ml of rigor buffer, the stained myofibrils were mounted under a cover slip and sealed with nail polish.

**Fixation of Cultured Cells for Fluorescence Microscopy**

Tissue culture cells were prepared for staining by fixation with formalin and extraction with acetone (9).

**Staining Fixed Tissue Culture Cells with Fluorescent Actin Ligands**

For indirect immune IgG and anti-actin staining, fixed HeLa and PtK-2 cells attached to glass cover slips were inverted onto a 100-μl drop of immune serum or 100 μg/ml of purified anti-actin IgG for 60 min at 37°C in a moist chamber. The cover slips were then washed by submersion in three changes of 40 ml of PBS. Cover slips with cells attached were then inverted onto a 100-μl drop of a 1:25 dilution (50 μg/ml) of fluorescein-goat anti-rabbit gamma globulin with a F/P = 4.01 (Miles Lab no. $403). The washing procedure was repeated and the cover slips were mounted onto a microscope slide in a 9:1 dilution of glycerol:PBS and sealed with nail polish.

For direct rhodamine-anti-actin staining, fixed cultures were treated with 60 μg/ml (F/P = 1.9) or 75 μg/ml (F/P = 2.0) of rhodamine-labeled anti-actin as described for the first step of the indirect technique.

**Fluorescent Heavy Meromyosin Staining of Cultured Cells**

Fixed HeLa and PtK cells were stained with either rhodamine-labeled or fluorescein-labeled HMM as described previously (9).

**Controls for Fluorescent Staining**

As controls for indirect staining with immune IgG, cells were reacted with either (a) immune serum that...
had been absorbed with 3 mg/ml purified actin for 24 h, or (b) preimmune serum and then stained with 50 μg/ml fluorescein-goat anti-rabbit IgG. Controls for indirect staining with purified anti-actin included staining with (a) adsorbed IgG (the fraction which passed directly through the anti-actin affinity column) or (b) preimmune serum, followed by fluorescein-labeled goat anti-rabbit IgG. Specificity of direct staining with rhodamine-anti-actin was demonstrated by incubating cells with (a) 100 μg/ml purified anti-actin followed by 60 μg/ml rhodamine-anti-actin, (b) 60 μg/ml rhodamine-labeled anti-actin that was adsorbed with purified actin for 24 h, (c) 100 μg/ml rhodamine-immune IgG that passed through the affinity column or by (d) incubation with preimmune serum followed by 60 μg/ml rhodamine-anti-actin.

For demonstrating specificity of myofibrillar staining, chicken breast muscle myofibrils were (a) stained with rhodamine-immune IgG adsorbed by affinity chromatography, or (b) incubated with 100 μg/ml purified anti-actin followed by reaction with 60 μg/ml rhodamine-anti-actin, or (c) incubated with 15 mg/ml preimmune serum followed by staining with 50 μg/ml fluorescein-goat anti-rabbit IgG.

Microscopy

Myofibrils and fixed tissue culture cells were observed with a Leitz Orthoplan microscope equipped with a Ploem vertical illuminator as described in detail elsewhere (5). Photographs were taken on Kodak Tri-X film developed in Microdol-X.

RESULTS

Antigen

The chicken gizzard actin used for immunization was >99% pure as assessed by gel electrophoresis in sodium dodecyl sulfate (SDS) (Fig. 1). Only a single band of stained protein could be detected when samples as small as 5 μg or as large as 100 μg were electrophoresed on 5, 7.5, or 10% polyacrylamide gels (data not shown).

Antibodies

All five rabbits injected with glutaraldehyde-fixed filaments of purified chicken gizzard actin produced antibodies reacting with actin, as shown by their ability to bind actin in the I-band of striated muscle myofibrils (Fig. 2). While this I-band staining could be demonstrated to be specific for actin by suitable controls (Fig. 2), it was apparent that the concentration of anti-actin in these immune sera was quite low since fluorescence intensity was low at dilutions >1:15 (Fig. 2). Moreover, these immune sera formed no stainable precipitin band after reaction with gizzard actin in agar. However, ~1% of the immune IgG could be recovered as purified anti-actin by affinity chromatography on Sepharose-4B-actin. The purified actin antibodies bound to the I-band of striated muscle (Fig. 2) and formed a single precipitated zone of equivalence after double immunodiffusion in agar with purified actin and crude extracts from skeletal or smooth muscle (Fig. 3). The precipitin lines all fused, indicating that the antigenic determinants from skeletal and smooth muscle actin are immunologically identical.

Actin Localization: Interphase Cells

We used both direct and indirect fluorescent-
FIGURE 2 Fluorescent antibody staining of chicken breast muscle myofibrils. Phase contrast (a) and fluorescence (b) micrographs of a myofibril stained with 60 μg/ml rhodamine-labeled purified anti-actin with a F/P = 1.9. Phase contrast (c) and fluorescence (d) micrographs of a myofibril reacted with 30 μg/ml purified anti-actin followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG with a F/P = 4.01. Phase contrast (e) and fluorescence (f) micrographs of a myofibril reacted with 1:5 immune serum followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG as in (c) and (d). Phase contrast (g) and fluorescence (h) micrographs of a myofibril reacted with 1:45 immune serum followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG as in (c) and (d). Phase contrast (i) and fluorescence (j) micrographs of a myofibril reacted with 1:15 immune serum followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG as in (c) and (d). Phase contrast (k) and fluorescence (l) micrographs of a myofibril stained with rhodamine-labeled immune IgG adsorbed by affinity chromatography. Phase contrast (m) and fluorescence (n) micrographs of a myofibril reacted with 100 μg/ml purified anti-actin before reaction with 60 μg/ml rhodamine-labeled anti-actin with F/P = 1.9. Phase contrast (o) and fluorescence (p) micrographs of a myofibril reacted with preimmune serum followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG as in (c) and (d). Bar, 1.0 μm.

antibody techniques with actin antibodies to study the distribution of actin in HeLa and PtK-2 cells fixed during interphase. All conclusions are based on the patterns observed after staining with purified actin antibodies directly conjugated with tetramethylrhodamine. The same structures could be stained indirectly with either unlabeled purified actin antibodies or immune serum; but there was also some nonspecific staining that was revealed by control experiments (see below).

The distribution of fluorescent anti-actin staining within HeLa cells depended to some extent on the cell density and culture conditions; but, there were always several different interphase staining patterns within a single culture. Rhodamine-labeled anti-actin stained cytoplasmic structures exclusively. Contrary to some previous reports (13, 14), no nuclear fluorescence was observed (Fig. 4d). Some cells, especially those that were well spread in areas of low cell density, had phase-dense stress fibers that stained with anti-actin as shown previously (13, 14). Some of these cells possessed fibers that varied from <1 μm in diameter near their tapered ends to 5 μm in regions where several fibers appeared to fuse (Fig. 4b). Smaller cells tended to have very fine fibers (0.5 μm) and some diffuse material that also stained with anti-actin (Fig. 4b). Virtually all PtK-2 cells that were observed after staining with fluorescent actin ligands had actin-containing cytoplasmic fibers, but their form and distribution varied considerably depending on culture conditions. In sparse cultures, many cells had prominent, straight phase-dense fibers that stained with anti-actin (Fig. 5a and b).
cell stained with anti-actin was shown by Cande et al. (1). In contrast, cells in confluent sheets have phase-dense, branched fibers that also stained with purified anti-actin (Fig. 5c and d). These sinuous, nemaline structures could also be stained indirectly with immune anti-actin serum (data not shown). Other workers have stained fibers similar to these in PtK cells with gamma globulin from nonimmunized animals (16) and with antibodies raised in rabbits against prekeratin (15).

**Actin Localization: Dividing Cells**

We confirmed that anti-actin stains the mitotic spindle of cultured cells (Figs. 6 and 8) using two technical improvements which insured that the spindle staining was solely a result of the presence of actin. First, we fixed the cells to reduce the likelihood that actin was redistributed within cells before staining, as might have been the case with cells prepared for staining by glycerination (19) or extraction with detergent (1). In fact, at least 90% of the actin is retained by cells that are fixed before staining, while 40% is lost during detergent extraction (9). Second, we used purified, directly labeled anti-actin which eliminates all detectable nonspecific staining (Fig. 10).

The images of metaphase spindles stained with either fluorescent anti-actin or fluorescent heavy meromyosin are generally similar, but there is one subtle, interesting difference shown in Figs. 6 and
FIGURE 5 PtK-2 cells fixed and stained during interphase with 60 μg/ml purified rhodamine-anti-actin. Phase contrast (a and c) and fluorescence (b and d) micrographs reveal straight (b) and sinuous (d) fibers stained with the antibody. Bars, 10 μm.

8 which is more fully appreciated by focusing through the spindle with the microscope. In both cases, the fluorescence is concentrated in the spindle compared with the diffusely stained, surrounding cytoplasm. In both cases, the region of bright fluorescence extends from the chromosomes to the poles, but there is a difference in the texture of staining. Fluorescent HMM is localized along the fine, discrete phase-dense spindle fibers. Anti-actin is more generally distributed within the spindle, making it difficult or impossible to detect individual spindle fibers.

During anaphase, actin-containing structures continue to be concentrated within the spindle both between the chromosomes and poles and in the interzone. Few anaphase cells were observed, making a detailed description of the staining patterns impossible at the present time.

In cleaving cells, both fluorescent anti-actin staining and fluorescent-HMM staining are spread relatively uniformly throughout the cytoplasm (Figs. 7, 8, and 9). Most importantly, neither of these actin-specific stains is concentrated in the cleavage furrow as are fluorescent anti-myosin (5) and fluorescent anti-alpha-actinin (6). Figs. 7h and 9a illustrate the extremes of cleavage furrow staining observed. In Fig. 7h the furrow region is stained less intensely than other parts of the cell, most likely because the cell is thinnest there; whereas, in Fig. 9a the cleavage furrow is stained more brightly than other cytoplasmic regions. In Fig. 9h the furrow region stains as brightly as any other region of the cell, but neither in this case nor in any other have we observed actin-specific staining concentrated in an equatorial cortical ring comparable to that observed with probes for
myosin (5) or alpha-actinin (6).

As daughter cells begin to spread out and move away from each other at the end of cell division, membrane ruffles stain intensely with these fluorescent actin ligands (Fig. 8f). This figure also illustrates a property of fluorescence micrographs which occasionally causes some confusion. The observed fluorescence intensity represents the two-dimensional projection of fluorescence from the three-dimensional cell. Hence the thickness of the cytoplasm at any point is a major determinant of the fluorescence intensity. In this case, the lower daughter cell is spread more than the upper daughter cell. The perinuclear fluorescence is more intense in the upper daughter, at least in part because of the greater thickness of the cytoplasm. In both cells, the extreme thinness of the cytoplasm between the perinuclear area and the peripheral ruffles must contribute to the weakness of the fluorescent signal in that area. A similar effect is seen in Fig. 4b. The same effect that cell thickness has upon fluorescence intensity accounts for the brightness of all rounded dividing cells compared with their flat neighbors (e.g., Fig. 7d).

Controls

A variety of control experiments were employed to insure that the fluorescent staining patterns observed were solely a result of the presence of cellular actin. Preimmune serum did not stain the cells (Fig. 10a and b). Direct staining of the HeLa cells with purified anti-actin could be blocked by preincubation with unlabeled purified antibodies (Fig. 10g and h) and prevented by absorption of the antibodies with purified actin (Fig. 10c-f). Identical results were obtained with PtK cells. The complete absence of staining in these control experiments demonstrated that all of the fluorescence in cells stained directly with rhodamine-labeled anti-actin was attributable to actin. In the case of indirect antibody staining, there was always some nonspecific staining of nuclear

![Image](https://example.com/image.png)
FIGURE 7 Cytokinesis series of fixed PtK-2 cells stained with five different fluorescent actin ligands. Phase contrast (a) and fluorescence (b) micrographs of a PtK-2 cell fixed during anaphase and reacted with immune serum followed by staining with 50 μg/ml fluorescein goat anti-rabbit IgG with a F/P = 4.01. Phase contrast (c) and fluorescence (d) micrographs of a PtK-2 cell fixed during telophase and stained with 0.67 mg/ml rhodamine heavy meromyosin with a F/P = 2.6. Phase contrast (e) and fluorescence (f) micrographs of a PtK-2 cell fixed and stained during telophase with 60 μg/ml purified rhodamine anti-actin with a F/P = 1.9. Phase contrast (g) and fluorescence (h) micrographs of a PtK-2 cell fixed during cytokinesis and stained with fluorescein-heavy meromyosin with a F/P = 4.3. Bars, 10 μm.

FIGURE 8 HeLa cells fixed during metaphase and cytokinesis and stained with four different fluorescent actin ligands. Phase contrast (a) and fluorescence (b) micrographs of a HeLa cell fixed during metaphase and stained with 0.52 mg/ml fluorescein-heavy meromyosin with a F/P = 4.3. Phase contrast (c) and fluorescence (d) micrographs of a HeLa cell fixed during metaphase and reacted with 100 μg/ml purified anti-actin followed by staining with 50 μg/ml fluorescein goat anti-rabbit IgG with a F/P = 4.01. Phase contrast (e) and fluorescence (f) micrographs of a HeLa cell fixed after cytokinesis and stained with 0.67 mg/ml rhodamine-heavy meromyosin with a F/P = 2.6. Bars, 10 μm.

structures, even after absorption with pure actin. Blocking controls are impossible with the indirect method. The specificity of fluorescent-HMM staining was demonstrated in cells stained in the
presence of 10 mM Mg-pyrophosphate (9) or by blocking staining by preincubating cells with unlabelled HMM (Fig. 10i and j).

**FIGURE 9** HeLa and PtK cells fixed and stained during cytokinesis. Fluorescence images of HeLa (a) and PtK-2 (b) cells stained with 60 μg/ml purified rhodamine-anti-actin. Bar, 10 μm.

**Fluorescent Reagents**

Three direct and two indirect fluorescent actin ligands were compared for their abilities to stain actin, i.e., fluorescein- and rhodamine-labeled heavy meromyosin and purified rhodamine-labeled anti-actin vs. indirect staining with immune serum or purified anti-actin. As in other cases (9), rhodamine was the superior fluorochrome because it did not bleach; and, while our indirect antibody methods were more sensitive, direct staining was "cleaner" and results were more convincing because there was no nonspecific staining and because blocking control experiments could be performed in addition to absorption.

**DISCUSSION**

The objective of staining cells with fluorescent actin ligands is to determine the natural distribution of actin within cells. As in other experimental work, the interpretation of fluorescent staining patterns depends heavily upon adequate controls.

**FIGURE 10** Control experiments on HeLa cells fixed during metaphase and cytokinesis. Phase contrast (a) and fluorescence (b) micrographs of a HeLa cell fixed during metaphase and reacted with preimmune serum followed by staining with 50 μg/ml fluorescein-labeled goat anti-rabbit IgG with a F/P = 4.01. Phase contrast (c) and fluorescence (d) micrographs of a HeLa cell fixed during metaphase and reacted with absorbed immune serum. Phase contrast (e) and fluorescence (f) micrographs of a HeLa cell fixed during metaphase reacted with immune IgG absorbed by affinity chromatography and then stained indirectly with 50 μg/ml fluorescein-goat anti-rabbit IgG as in a and b. Phase contrast (g) and fluorescence (h) micrographs of HeLa cells fixed and reacted with 100 μg/ml purified anti-actin followed by incubation with 60 μg/ml purified rhodamine-anti-actin with a F/P = 1.9. Phase contrast (i) and fluorescence (j) micrographs of a HeLa cell fixed during metaphase and reacted with 4.0 mg/ml unlabeled HMM before incubation with 0.67 mg/ml rhodamine-labeled heavy meromyosin with a F/P = 2.6. Bars, 10 μm.
and the validity of any assumptions. We have interpreted our micrographs by assuming that: (a) all fluorescence is a result of fluorescent actin ligand bound to actin, (b) fluorescence intensity is proportional to the local concentration of actin, and (c) all cellular actin remains in its natural position and is both accessible to and reactive with the fluorescent ligand.

We believe that these three assumptions are correct for the following reasons. The first premise is very likely to be true because control experiments (Fig. 10) rule out most causes of nonspecific staining. Although these controls cannot formally eliminate the possibility that some of the antibody staining is a result of low concentration of antibody against a minor contaminant, it is unlikely that a low titre of such an antibody would contribute much to the fluorescence intensity, because the intensity is proportional to the concentration of antibody applied to the specimen. This observation also indicates that the second premise is true. The third premise is true to the extent that our fixation-dehydration protocol extracts <10% of the actin from the cell before staining (9).

Although it has not been demonstrated experimentally that the cellular actin retains its antigenicity and its natural position, we assume that it has in the following discussion.

Our work confirms previous reports that actin antisera stain stress fibers (14, 12, 18, 15), surface membrane ruffles (14, 13), and the mitotic apparatus (1); and, in addition, it includes sufficient controls to establish that cellular staining is exclusively a result of anti-actin. Rhodamine-labeled anti-actin did not stain nuclei, suggesting that previously reported nuclear staining (1, 13) was an artifact. Two new observations are that actin is present, but not concentrated in the cleavage furrow and that actin is present in the sinuous, dense fibers of confluent PtK-2 cells.

Our observations on stained metaphase cells require special comment. Bright fluorescence contrast was observed between the mitotic spindle and its surrounding cytoplasm in formalin-fixed cells rendered permeable to fluorescent actin ligands by acetone extraction. Previously, investigators found it necessary to extract part of the cytoplasmic actin to reveal such fluorescence intensity differences (1). In our work, extraction

1 T. D. Pollard, unpublished observation on platelets stained with rhodamine anti-myosin measured with a fluorescence microscope photometer.

may have been unnecessary because of the complete absence of nonspecific staining; whereas, in the previous study some nonspecific cytoplasmic staining may have reduced the fluorescence contrast of the spindle in unextracted cells. An additional remarkable difference is that in the previous report (1) anti-actin containing serum stained fibers in the spindle; whereas, our purified rhodamine-labeled anti-actin stained the spindle more diffusely. A possible explanation for this difference is that a diffuse actin component was selectively extracted from the spindles by detergent lysis, leaving behind a less soluble fibrous fraction of spindle actin. In fixed and acetone-treated cells which retain >90% of their actin, such a diffuse component lying between actin-containing fibers could obscure the fibers. The fact that spindle fibers were readily visible in cells stained with fluorescent-HMM, but not in those stained with fluorescent anti-actin, suggests that the fibrous component is polymerized actin and that the diffuse component is unpolymerized actin. This interpretation assumes that heavy meromyosin binds preferentially to actin filaments and that anti-actin binds to polymers as well as monomers of actin. If these speculations are true, then spindle actin filaments are aligned predominantly parallel to spindle microtubules. Many actin filaments observed within the mitotic spindle by electron microscopy (11) actually do have this orientation (4, 21). Actin filaments with this orientation together with myosin (5) could generate force along the axis of the spindle fibers which is, of course, parallel to the path of chromosome movement.

Judging from fluorescent-HMM and anti-actin staining patterns of cultured cells fixed during cytokinesis, actin is not concentrated in the cleavage furrow. Previous reports of bright fluorescent-HMM staining of the cleavage furrow of similar cultured cells (20) may be the result of selective actin extraction from the adjacent cytoplasm during glycerination. There is no question that a circumferential band of actin filaments, the contractile ring, is concentrated in the cleavage furrow (22, 20). It seems possible that cortical actin, adjacent to the contractile ring, has gone unnoticed by electron microscopy because it is poorly preserved or less well organized than the contractile ring actin filaments. Perhaps the contractile ring filaments are stabilized during fixation by alpha-actinin, which has been shown to be concentrated in the cleavage furrow (6).
These observations suggest that formation of the contractile ring may not require recruitment of actin filaments to the cleavage furrow, but rather more simply, may require circumferential alignment of preexisting actin filaments. Since myosin is concentrated in the cleavage furrow (5), localized equatorial tension generated by its interaction with membrane-bound actin filaments may be responsible for orientation of these contractile ring filaments. Judging from the example of cytokinesis where myosin, but not actin, is concentrated at the site of tension development, it may be more informative to use myosin antibodies rather than actin antibodies to identify other regions of motile force production in nonmuscle cells.

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