Relation between Cell Activity and the Distribution of Cytoplasmic Actin and Myosin

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ABSTRACT We documented the activity of cultured cells on time-lapse videotapes and then stained these identified cells with antibodies to actin and myosin. This experimental approach enabled us to directly correlate cellular activity with the distribution of cytoplasmic actin and myosin. When trypsinized HeLa cells spread onto a glass surface, the cortical cytoplasm was the most actively motile and random, bleb-like extensions (0.5-4.0 μm wide, 2-5 μm long) occurred over the entire surface until the cells started to spread. During spreading, ruffling membranes were found at the cell perimeter. The actin staining was found alone in the surface blebs and ruffles and together with myosin staining in the cortical cytoplasm at the bases of the blebs and ruffles. In well-spread, stationary HeLa cells most of the actin and myosin was found in stress fibers but there was also diffuse actin staining in areas of motile cytoplasm such as leading lamellae and ruffling membranes. Similarly, all 22 of the rapidly translocating embryonic chick cells had only diffuse actin staining. Between these extremes were slow-moving HeLa cells, which had combinations of diffuse and fibrous actin and antmyosin staining. These results suggest that large actomyosin filament bundles are associated with nonmotile cytoplasm and that actively motile cytoplasm has a more diffuse distribution of these proteins.

In this study we documented the motility of several different types of living cells on time-lapse videotapes before fixation and staining of the same cells for actin and myosin with fluorescent antibodies. With this unique approach we were able to correlate directly the activities of living cells with the distribution of cytoplasmic actin and myosin.

MATERIALS AND METHODS

Antibody Preparation

ACTIN: Rabbit antibodies were prepared against chicken gizzard (smooth muscle) actin, labeled with fluorescein and purified by affinity chromatography (22).

MYOSIN: Dr. Keigi Fujiwara immunized a goat with the purified platelet myosin rod exactly as described for immunizing rabbits (15). Immune Ig was labeled with rhodamine, and antmyosin IgG was purified by affinity chromatography (15).

Cell Preparation

LIVING CELLS

HELa CELL CULTURES: HeLa cells were grown on glass microscope cover slips exactly as described previously (21).
EMBRYONIC CULTURES: Cells were obtained from the skin and wing buds of 11-d-old chick embryos. Embryonic tissue was minced and digested for 10 min at 37°C in 2.5 mg/ml trypsin in phosphate-buffered saline (PBS; 0.015 M sodium phosphate, 0.15 M NaCl, pH 7.4). Dissociated cells were grown for 2-3 d on 18 × 18 mm glass cover slips in Dulbecco's minimum essential medium (DMEM) supplemented with 1% chick serum and 1% fetal calf serum.

TIME-LAPSE VIDEOTAPE RECORDS: Living cells were observed while attached to cover slips submerged in growth medium in a 35-mm petridish sealed with Parafilm to maintain a 5% CO₂ atmosphere. The petridish, containing cells growing on glass coverslips, was placed in a 37°C air curtain on a stage of an Nikon inverted light microscope equipped with phase-contrast optics. Time-lapse videotapes were recorded with a Panasonic NV-8030 VTR. Although photographs of the TV screen do not reproduce well, these videotapes allowed us to evaluate the activity of the cells in some detail. Locomotion, pseudopod extension and retraction, mitosis, cytokinesis, and membrane ruffling were all easily observed. Intracellular movements of small organelles could not be evaluated.

CELL SPREADING: Confluent cultures of HeLa cells were briefly washed in a Ca²⁺/Mg²⁺-free PBS and trypsinized for 1.5 min at 37°C with 1 mg/ml trypsin in PBS. Trypsinized cells were shaken from the growth substrate, suspended in fresh DMEM and 10% fetal calf serum, incubated at 37°C for 5 min. Samples were then pelleted by centrifugation at 10,000g for 2 min at 25°C and 30 μl was applied in each lane. Tissue or slab SDS gelelectrophoresis was carried out in a 14% polyacrylamide resolving gel (29) in a slab gel apparatus. Gel electrophoresis was carried out for 2 min at 37°C, and gels were stained with 0.5% Coomassie Blue R-250 and scanned at 600-250 nm. Areas under peaks were extrapolated to the baseline and measured by cutting and weighing. The mean weight of the duplicate or triplicate samples was calculated with the standard deviation from the mean values. To detect myosin in these gels we used a modification of the antibody overlay technique of Adair et al. (3). Gels stained with actin antibody were not fixed. Gels were washed and equilibrated with 0.15 M sodium chloride, 0.05 M Tris, pH 7.8, 1 mg/ml Triton X-100, and 1 mg/ml BSA (TTX-BSA) overnight. They were then incubated with 100 μg/ml rabbit antimyosin Ig fraction (12) for 4 h. Gels were washed with several changes of 1 liter of TTX-BSA and then incubated with 20 ml of 111-labeled protein A in TTX-BSA (0.6 × 10⁶ cpm/ml) for 3 h (sp act, 1 × 10⁶ cpm/μg). Gels were washed with TTX-BSA and dried on filter paper. Radioautograms were exposed on x-ray film (Cronex 2DC) at −70°C with an intensifying screen for 4 d.

RESULTS

Preparation of Cells for Antibody Staining

Our objective was to correlate the activity of living cells with the distribution of actin and myosin in the cytoplasm, so it was necessary to establish that neither cellular morphology nor protein composition was altered by fixation, dehydration, or incubation with fluorescent antibody. We show that the gross morphology of the living cultured cells was preserved during antibody staining. Furthermore, the same fixation-dehydration procedure preserved the ultrastructure of these cells as well (23). To establish that the actin and myosin were retained in the cells carried through the fluorescent antibody procedure, we examined by gel electrophoresis the polypeptide composition of the fixed cells and the various solutions used to prepare these cells (Fig. 1). Remarkably, the polypeptide composition of the cells was altered very little by formaldehyde-acetone, formaldehyde-acetone, or methanol fixation/permeabilization. With methanol, there was some aggregated material at the top of the gel. None of the fixation or wash solutions contained more than traces of polypeptides. In the case of PtK₂ cells, 92% of the stained actin band was retained in the cell pellet by formaldehyde-acetone, 88% by formaldehyde-acetone, and 82% by methanol. Because myosin accounts for <1% of HeLa protein (41), the myosin heavy chain was difficult to identify with certainty on the gels. A faint 200,000 mol wt band was found in the fixed cell pellets, but, to be certain that it was myosin and that no myosin was extracted, we stained the gels with antimyosin. In the fixed cell pellet, both the 200,000 mol wt band and some higher molecular weight material, presumably aggregated myosin, bound the antimyosin, but no myosin was detected in this way in the buffer wash of these cells (Fig. 1).

Antibody Staining

CELL SPREADING: We investigated HeLa cell spreading on a glass surface by correlating motile activity and fluorescent staining for actin and myosin in the same cells. In the first few minutes during which spherical HeLa cells began to settle on the glass surface, many transient and rounded blebs 0.5-4.0 μm wide and 2-5 μm long formed and retracted at the cell surface. These surface blebs were first described by Holtfreter (24), and later Taylor (38) described this behavior as stage I in the spreading of conjunctiva cells onto a glass surface. When speeded up by time-lapse these hyaline blebs of cytoplasm appeared to protrude and retract while moving around the cell perimeter, in an almost circular, wave-like motion. This surface blebbing continued until the cells began to attach more firmly onto the glass. This coincides with stage II of Taylor's scheme and occurs ~20-40 min after contact with the glass substrate. Later, as these cells continued to flatten and spread out, the nuclei of the cells also flattened and became visible in phase contrast. This corresponded to stage III in Taylor's scheme.
FIGURE 1. Proteins present in fixed HeLa cells. Samples were boiled in 2% SDS and 10% β-mercaptoethanol before electrophoresis in a 7.5–15% polyacrylamide gradient slab gel. Lanes A–H, Coomassie Blue staining; lanes I and J, radioautography. (A) Molecular weight standards. (B) Unfixed HeLa cells. (C) Polypeptides lost from formaldehyde-fixed and acetone-treated HeLa cells during a 1-h incubation with PBS to simulate antibody staining. (D) Final cell pellet of formaldehyde-fixed and acetone-treated HeLa cells. (E) Polypeptides lost from formaldehyde-fixed and ethanol-treated HeLa cells during a 1-h incubation with PBS to simulate antibody staining. (F) Final cell pellet of formaldehyde-fixed and ethanol-treated HeLa cells. (G) Polypeptides lost from methanol-fixed HeLa cells during a 1-h incubation with PBS to simulate antibody staining. (H) Final cell pellet of methanol-fixed HeLa cells. (I and J) Identical to C and D but treated with antimyosin and 125I-labeled protein A rather than being stained. (I) No radioactivity is seen, indicating that myosin is not lost during simulated antibody staining of cells. (J) Radioactivity is present in the fixed HeLa cells at a mobility corresponding to the heavy chain of myosin. High molecular weight material at the top of the gel may be aggregated myosin.

In 24 HeLa cells with documented surface blebbing, the most intense fluorescent staining with both antiactin and antimyosin was in the cell periphery, but the distribution and texture of staining for actin and myosin were distinctive (Figs. 2 and 3). The blebs themselves were outlined with an intense ring of antiactin fluorescence juxtaposed to the plasma membrane (Fig. 2A). The antimyosin fluorescence was weak in the surface blebs but was intensely localized in a subcortical belt that also stained intensely with antiactin (Figs. 2 and 3).

Later, as spreading onto the glass surface continued, membrane ruffling was always associated with discrete regions of the cell perimeter, especially in the direction of movement (Fig. 3, cell 3; Fig. 3, cells 4 and 5, arrows). As in other well-documented cases (1–3), the ruffling membrane of spreading HeLa cells appears to build up at the margins and then retreat toward the cell center. In all cells with documented activity, ruffles were stained intensely with antiactin (Figs. 3–5). Others have also shown antiactin staining of membrane ruffles in spreading cells fixed during stage III of the Taylor scheme (25, 31, 38). On the other hand, antimyosin either did not stain or only weakly stained these regions of membrane ruffling (Fig. 3). Punctate antimyosin staining was concentrated in a circular subcortical zone together with antiactin staining (Fig. 3, cell 4). The antimyosin fluorescence remained radially arranged until very fine punctate fibers could be seen traversing the cell.

FIGURE 2. Double antibody staining of spreading HeLa cells. Fluorescent antibody staining of six spreading HeLa cells stained simultaneously with 50 μg/ml fluorescein-antiactin (A, C, E, G) and 20 μg/ml rhodamine-antimyosin (B, D, F, H). The cells appearing in A–F were fixed <20 min after plating onto glass cover slips. Notice the prominent surface blebbing outlined by actin fluorescence in A and E. Antimyosin weakly stains the surface blebs but is concentrated in a zone juxtaposed to the blebbing. The cell in G and H was fixed nearly 2 h after trypsinization, and it is considerably flatter than cells in A, C, and E. Bar, 4 μm.
interior (Fig. 3, cell 4). This occurred 1–2 h after the cells settled onto the glass during stage III of Taylor’s scheme. This bright, peripheral staining in the spreading cells was particularly impressive because this region of the cytoplasm was thinnest, making it unlikely that this intense staining of the cell periphery was the result of a superimposed signal through a long path length.

INTERPHASE CELLS: That interphase HeLa cells were quite heterogeneous in size, shape, motility, and contractile protein distribution gave us the opportunity to analyze a variety of cells. Because of this diversity, cell morphology alone was an inadequate indicator of motile activity, and the time-lapse records of the living cells were necessary for the interpretation of the fluorescence micrographs.

At one extreme, ~10% of interphase HeLa cells were highly flattened and contained prominent stress fibers. All nine of the flat cells that we videotaped failed to move in 0.9–1.2 h (Figs. 4 and 5). Virtually all of the antiactin fluorescence in these flat nonmotile cells was confined to stress fibers, although there was also some intense diffuse staining of membrane ruffles at the cell periphery (Fig. 4 and cell J in Fig. 5) as in the spreading cells. Myosin was also localized in the same stress fibers of these cells (not shown).

At the other extreme, there were a number of small, wedge-shaped HeLa cells that moved rapidly (~0.5–3 μm/min). These cells were usually lost during fixation and staining, which accounts for their absence in previous studies. In general they were similar to the actively motile chick embryo cells described below, because the antiactin and antimyosin gave diffuse staining.

Between these extremes there were many medium-sized cells that were not highly motile but slowly changed their shapes such as cells 2, 4, and 5 in Fig. 5. These cells usually had a combination of fibrous and diffuse staining with antiactin and antimyosin. The diffuse component was usually spread throughout the cytoplasm (Fig. 5 E) but was especially intense in leading lamellae (Fig. 5 F, arrow).

There were three major types of cells in cultures of embryonic chicken, skin and wing buds. Most of the cells were spindle-shaped, often 50–100 μm long, and probably were myoblasts. Other cells, presumably fibroblasts, were A- or wedge-shaped. The third major cell type was small (10–20 μm
FIGURE 5  Relationship between HeLa cell activity and cytoplasmic actin. (A–C) Phase-contrast images of the living HeLa cells. The time is recorded in hours:minutes:seconds in the lower left-hand corner of the TV monitor. (D–F) Fluorescence micrographs of the same cells after fixation and staining with 75 μg/ml fluorescein-antiactin. As labeled in C, 1 was a well-spread cell with a circular profile. Before fixation, the left-hand border actively ruffled. After staining, this region had bright, diffuse fluorescence. 2 retracted a microspike on its right side (B) during taping and had extended another toward 6:00 (C, *) 8 min later. The antiactin fluorescence was both diffuse and fibrous (E). 3 remained essentially immobilized and the majority of its actin fluorescence was fibrous (F). 4 was spindle-shaped and during the taping became stretched out. It elongated and had a diffuse actin fluorescence (F, top left). Some cells, such as 5, possessed a leading lamella that advanced as a sheetlike, mobile projection that varied in size as it was elaborated from the cell perimeter. When this cell was fixed and stained for actin, this mobile lamellipodium was intensely fluorescent (F). Bars (A–C), 10 μm; (D and E), 3.0 μm and 10 μm, respectively.

The velocities of movements varied with time from 0 to 3.5 μm/min at the maximum. The three cells in Fig. 6 illustrate the variety of movements and the variability of the movement velocities (rates). Cell • (Fig. 6) had five major lamellipodia each of which possessed regions of ruffling at the beginning of the taping. After the cell had stretched itself to 1.5 times its original length, all its lamellipodia joined to form one leading lamella and, when this moving cell was fixed and stained with fluorescein-antiactin, only diffuse fluorescence was observed (Fig. 6). Cell ■ (Fig. 6) changed its direction of movement four times during videotaping. Each time it contacted a neighboring cell with its wavelike lamella, its movement forward was inhibited. Between the end of videotaping and the time the cells were fixed (<60 s), this cell ■ changed its shape slightly by releasing its attachment to a neighbor (cf. lower left cell ■ in E and F with G and H in Fig. 6) and the direction of lamellar movement (cf. cell ■ lamellae in E and F with G and H in Fig. 6). This moving cell ■ had diffuse antiactin fluorescence. Cell ▲ (Fig. 6) moved randomly during taping, showing few directed movements. As with cell ■, it is apparent that cell ▲ was moving at the time of fixation because there are subtle differences in morphology between the living cell and the fixed cell. In particular, the top, left-hand ruffling membrane spread out slightly (cf. E and F with H and K in Fig. 6), the lower left-hand ruffling membrane is redistributed, and the tail portion (indicated by ▲ in Fig. 6 F) has pulled in slightly. Again, this moving cell had diffuse antiactin fluorescence after fixation.

The movements observed with HeLa and embryonic chicken cells were representative of the movements and fluorescent antibody staining patterns seen in other cell types including migrating neural crest cells (10) and human peripheral blood cells (see Table I). The large, stationary, and spread-out cells that we studied in HeLa cultures were absent from the blood cell and neural crest cell preparations; and, in general, these moving cells had diffuse fluorescence.

DISCUSSION

Using time-lapse videotapes, we documented the activity of living cells (see Table I) and correlated this information with fluorescent antibody staining for actin and myosin. The movements we observed during cell spreading and locomotion on glass surfaces were similar to those reported earlier (1, 4, 7, 25, 31, 38). After the cells with documented activity were fixed and stained with fluorescent antibodies, their morphology seemed normal, based on light and electron microscopy (23). Moreover, most, if not all, of the actin and myosin was retained in these cells prepared for antibody staining; and actin and myosin remained reactive with their antibodies after fixation. Although we cannot prove that all of the antigens remained in their natural cellular locations during the fixation/dehydration pro-
FIGURE 6  Relationship between the activity of chick embryo cells and cytoplasmic actin. Phase-contrast images (A–F) of living chick embryo cells taken from a continuous videotape as described in Figs. 3–5. Fluorescence (G–H) and phase-contrast (J–L) micrographs of three of these cells (▲, ■, *) after fixation and staining with 75 μg/ml fluorescein-antiactin. All of these motile cells (▲, ■, *) possess only diffuse actin fluorescence (G–I). Bars, (A–F), 20 μm; (G–L), 10 μm.

TABLE I
Summary of Observations

| Cell type          | n   | Morphology                              | TV-documented movements                  | Actin                        | Myosin                        |
|--------------------|-----|-----------------------------------------|------------------------------------------|------------------------------|-------------------------------|
| HeLa               | 9   | Fully spread                            | − (General cytoplasm)                    | Fibrous                      | Fibrous                       |
|                    |     |                                        | + (Membrane ruffling)                    | Diffuse                      | Absent from ruffle perimeter  |
|                    | 21  | Spread with lamella, ruffles, and pseudopodia | + (Movement at cell perimeters)         | Fibrous, diffuse in ruffles  | Fibrous, absent from ruffle perimeter |
|                    | 4   | Spindle-shaped                          | +++                                      | Diffuse                      | Diffuse                       |
|                    | 7   | Dividing                                | ++ (Chromosomal)                        | Diffuse, spindle             | Diffuse, cortical             |
|                    | 7   | Cytokinetic                             | ++ (Peripheral membrane; ruffling; daughters move) | Diffuse                      |                               |
|                    | 24  | Spreading with surface blebbing         | + (Surface blebbing, pseudopodial)      | Cortical: diffuse, fibrous   | Cortical: diffuse belt        |
| Chicken embryo cultures | 4   | Wedge-shaped with lamella and pseudopodia | +                                        | Fibrous, Diffuse             | No cross-reaction with myosin antibodies |
|                    | 17  | Spindle-shaped                          | +++                                      | Diffuse only                 |                               |
|                    | 5   | Amoeboid-like                           | +++                                      | Diffuse only                 |                               |
| Neural crest       | 23  | Spindle-shaped                          | +++                                      | Diffuse only                 | No cross-reaction             |
| Human blood        |     |                                        |                                          |                              |                               |
| Granulocytes       | 6   | Amoeboid                                | +++ (Pseudopods move)                    | Diffuse                      | Diffuse                       |
| Monocytes          | 1   |                                        | ++                                       | Diffuse                      | Diffuse                       |

Fibrous, stress fiber fluorescence; diffuse, uniform fluorescence without stress fiber staining; −, not moving; +, membrane ruffling + other subcellular movements with no translocation; ++, translocating (0–0.5 μm/min); ++++, translocating (0.5–3.5 μm/min).
cEDURE, this seems likely from the lack of antigen extraction and the good morphological preservation.

The results obtained by correlating the cellular activity with fluorescent antibody staining were:

(a) Motile cells or motile parts of cells stain diffusely with antibodies to actin and myosin. This includes spreading HeLa cells, moving chick embryo cells and blood cells, ruffling membranes of both chick embryo and HeLa cells, and dividing HeLa cells.

(b) When most of the fluorescent-antiactin staining is confined to prominent stress fibers, cells grown on glass move about very slowly, if at all. However, there are active movements at ruffled membranes in the periphery of these cells. Such active regions stain diffusely with fluorescein-antiactin.

(c) Combinations of diffuse and fibrous antiactin (and antimyosin) staining can be found in cells that are stationary or change shape slowly. These slow shape changes can lead to slow locomotion.

(d) Nonlocomoting cells can have any antiactin and antimyosin staining pattern.

These observations have led us to conclude that (a) only in the cases of ruffled membranes, mitosis, and cytokinesis is it possible to predict cellular activity from morphology and antibody staining; (b) stress fibers are not essential for motility; and (c) cellular motility is usually associated with a diffuse distribution of actin and myosin.

On the basis of morphology alone, we (15, 16, 21, 22) and others (8, 19, 25, 30–32) have argued that actin (and/or myosin) was present in parts of cells that were moving. We believe that this is true for ruffling membranes, the cleavage furrow, and the mitotic apparatus. Only these events have cellular morphology or antibody staining patterns distinctive enough to conclude that motion was occurring at the time of fixation. For example, both stationary and slowly locomoting HeLa cells (Fig. 4) and chick embryo cells (Fig. 6) can have diffuse, or a mixture of diffuse and fibrous antiactin staining.

Although there is good evidence that isolated stress fibers (26) and the stress fibers of permeable cell models (28) can contract in Mgubre-Ar-ATP, our observations on living HeLa cells show that stress fibers are not essential for motility. In cells or parts of cells with prominent stress fibers, little or no motion occurs. We never observe locomotion of well-spread HeLa cells when most of the antiactin and antimyosin fluorescence is confined to stress fibers. Although motion was inferred, not documented, Badley et al. (8) made similar observations on cultured chick fibroblasts. Given these observations and the evidence that many stress fibers terminate on substrate attachment plaques (17, 27), it seems likely that most stress fibers have a structural role in anchoring the cytoplasmic matrix to the substrate, rather than being contractile. Their contractile potential is expressed only when one or both of their terminal attachment plaques is released from the substrate, such as in experimental manipulation (26, 28). Actually, neither the function of these photogenic actomyosin bundles in vitro nor their very existence in vivo is established.

Motile regions of cells always had diffuse antiactin fluorescence, suggesting that their motility was probably brought about by a diffuse actin network in the cytoplasm. Taylor and his co-workers (40) injected fluorescent-actin into rapidly moving amoebas and found that it also distributed diffusely in the streaming cytoplasm and the stationary cortex. These light microscope observations do not mean, however, that actin filaments are not involved, because electron micrographs reveal actin filaments in the cortex of amoeba (13), as well as the cytoplasm of ruffling membranes (4, 11, 12, 37, 42) and the cleavage furrow (36).

In general, myosin is also dispersed throughout the cytoplasm of motile cells (Figs. 2 and 3) as well as the mitotic apparatus (15, 16) and is concentrated in the cleavage furrow (15, 16, 20). One exception is the apparent absence of myosin from the margins of ruffling membranes (Fig. 2 and 3) (15, 19) and from the interior cytoplasm of spreading HeLa cells (Figs. 2 and 3). If myosin does participate in moving ruffles, it must do so in the cytoplasm at the base of the ruffles where it is found with actin (Figs. 2 and 3). This seems similar to the situation in the brush border, where myosin is confined in the terminal web (9, 14, 23, 34). The existence of a morphologically dispersed form of the cell’s contractile apparatus explains why all regions of the cytoplasm are potentially contractile (39).

The combination of time-lapse recording and fluorescent antibody staining has provided some new information about cytoplasmic actin and myosin and cell activity; however, the approach has limitations. First, the fluorescence micrographs provide no information about the physical state of actin or myosin except when actin is assembled into stress fibers. In diffusely stained cells, actin could be either monomeric or polymerized and the filaments could be arranged in either a random network or in small bundles like the contractile ring (36). It is entirely possible that in diffusely stained cells (Fig. 6) some of the actin filaments are also aligned in bundles by tension, like those of the contractile ring. These small bundles are not detected by fluorescent antibody staining, because the actin concentration in the bundles and the surrounding cytoplasm is the same. A second limitation is that the apparent absence of fluorescence from part of a cell does not mean that the antigen is missing there. For example, by electron microscopy we found low concentrations of ferritin-labeled antimyosin in regions of stress fibers that appear nonfluorescent when stained with rhodamine-antimyosin (23). Consequently, one can conclude from fluorescent antibody staining that the ratio of actin to myosin is much higher in the margin than at the base of the ruffles, not that myosin is absent from membrane ruffles.

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