CELL STRUCTURE AND FUNCTION 15: 355–364 (1990)
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Fluorescence-Mediated Visualization of Actin and Myosin Filaments in the Contractile Membrane-Cytoskeleton Complex of *Dictyostelium discoideum*

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Key words: membrane-cytoskeleton complex/actin filament/myosin filament/fluorescence staining/*Dictyostelium discoideum*

ABSTRACT. **An intact complex that consisted of the cell membrane and cytoskeleton was prepared from *Dictyostelium* amoebae by an improved version of the method previously used by CLARKE *et al.* (1975). *Proc. Natl. Acad. Sci. USA.*, 72: 1758–1762. After cells had attached tightly to a polylysine-coated coverslip in the presence of a divalent cation, the upper portions of the cells were removed with a jet of microfilament-stabilizing solution squirted from a syringe. The cell membranes left on the coverslip were immediately stained with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments, and with antibody against myosin from *Dictyostelium* and a fluorescein-conjugated second antibody for staining of myosin. Networks of actin filaments and numerous rod-like structures of myosin (myosin filaments) aligned along them were observed on the exposed cytoplasmic surfaces of the cell membranes. These networks were similar to those observed in the cortex of fixed whole cells. Addition of ATP to these intact complexes of cell membrane and cytoskeleton caused the aggregation of both actin and myosin into several dot-like structures of actin on the cell membrane. Similar dot-like structures were also seen in the cortex of fixed whole cells, and their changes in distribution correlated with the motile activity of the cells. Transmission electron microscopy showed that these dot-like structures were composed of an electron-dense structure at the center, from which numerous actin filaments radiated outwards. These observations suggest that these novel dot-like structures are organizing centers for cortical actin filaments and may possibly be related to the adhesion of cells to the substratum.**

The interaction between actin filaments and the cell membrane is thought to be involved in the migration of cells, the maintenance of cellular shape, cytokinesis, the assembly of receptors, endocytosis, and adhesion (10, 11, 21, 22, 31). Moreover, the interaction between membrane-associated actin filaments and myosin must generate the forces required for such cellular activities via a sliding-filament mechanism analogous to that in muscle cells.

In *Dictyostelium* amoebae, a relatively large amount of information about the relationship between the cell membrane and the cytoskeleton has been published. An important initial study involved the copurification of actin and myosin with the membrane fraction (33). Then the interaction between purified membranes and purified actin was examined *in vitro* (19, 24). It was shown that actin could associate with purified membranes and that this association progressed in a saturable manner (16, 25). From biochemical and ultrastructural studies, two different types of association of actin filaments with the cell membrane were identified, that is, an end-on attachment of barbed ends of actin filaments and a lateral attachment mediated by rod-shaped bridges (3). The evidence that the ability of the cell membrane to be associated with actin filaments is sensitive to denaturation by heat and proteolysis suggested the participation of some integral membrane proteins in these associations (24).

Candidates for these membrane proteins were deduced from studies of some animal cells. The molecular organization of the membrane cytoskeleton of the red blood cell has been well characterized. Spectrin, one candidate molecule, was shown to bind actin filaments (23, 28, 36). Proteins similar to spectrin were identified in the cell membranes of other cells (8, 26, 32). Much information about the proteins that participate in the association between actin filaments and the cell membrane has also been obtained from studies of the microvilli of the intestinal brush border and of the adhesion plaques in which stress fibers terminate in cultured fibroblasts (5, 7, 12, 15, 18, 30).

Some membrane proteins have been also identified in *Dictyostelium* amoebae. Integral membrane proteins of 17 kDa (ponticulin) (38) and 24 kDa (34) were shown to have actin-binding activity. Antibodies against a protein of about 20 kDa (6) and against ponticulin inhibited actin-membrane binding.

Clarke *et al.* visualized actin filaments that lie under the cell membrane of *Dictyostelium* by removing the upper portion of cells attached to polylysine-coated electron-microscopic grids with a shearing stream of buffer (9). This method also allowed visualization of the membrane cytoskeletons of macrophages (17), leukocytes (4), erythrocytes (36), and animal eggs (27).

In the present study, we improved this visualization method and examined the networks of actin filaments under the cell membrane by fluorescence microscopy. Simultaneously, we also visualized individual myosin filaments (myosin rods) which were aligned with actin filaments in this unfixed preparation. Addition of ATP to this isolated membrane-cytoskeleton preparation resulted in aggregation of both actin and myosin into several dot-like structures of actin, which may correspond to the structures that organize the cortical actin filaments and are involved in the adhesion of cells to the substratum. This intact membrane-cytoskeleton preparation provides us with an excellent model for investigations of the molecular organization of the linkage between the cell membrane and the cytoskeleton. The data in this report have been presented elsewhere in preliminary form (42).

MATERIALS AND METHODS

Culture of cells. *Dictyostelium discoideum*, strain NC-4, was cultured in association with *Escherichia coli* (B/r) on nutrient agar that contained 10 g of peptone, 10 g of glucose and 20 g of agar in 1,000 ml of distilled water. Vegetative cells were harvested and freed from bacteria by washing three times with cold distilled water. The washed cells were spread on non-nutrient agar and incubated at 21°C until use.

Immunofluorescence microscopy. Indirect immunofluorescence staining of whole cells was performed by the "agar-overlay" method (39) with a little modification. In brief, cells were attached to a coverslip and overlaid with a thin sheet of agarose [0.15 mm thick, 8×8 mm^2, made of 2% agarose dissolved in 15 mM phosphate buffer (pH 6.4)]. The samples were fixed by rapid immersion in ethanol that contained 1% formalin at −15°C for 5 min. After washing with phosphate-buffered saline [PBS: 135 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 80 mM Na_2HPO_4 (pH 7.3)] for 20 min, the samples were incubated with monoclonal mouse antibody against myosin from *Dictyostelium* (DM-2) and 3 μg/ml tetramethylrhodamine-conjugated phalloidin (Sigma, St. Louis, MO, USA) for 60 min at room temperature. After washing with PBS for 30 min to remove the excess antibody and phalloidin, the samples were incubated with fluorescein-conjugated second antibody raised against mouse IgG (Sigma) for 60 min. After washing with PBS, the agarose sheet was removed from the sample. After a brief rinse with distilled water, the samples were mounted with mounting medium (10% polyvinyl alcohol, 20% glycerol, and 0.1% p-phenylene diamine in PBS).

For visualization of the membrane cytoskeleton of fixed cells, the upper portion of cells immunostained by the above procedure were sheared off with a jet of PBS from a 10-ml syringe with a 25-gauge needle.

Immunofluorescence-stained samples were observed under a Nikon epifluorescence microscope (XF-EFD2).

Preparation of the membrane cytoskeleton. Cells harvested from non-nutrient agar were suspended in a cold solution of 5 mM $MgCl_2$ in distilled water. An aliquot of the suspension was placed on a polylysine-coated coverslip. The coverslips had been prepared by treating carefully cleaned coverslips with polylysine (1 mg/ml in distilled water) for 5 min, rinsing them with distilled water, and drying them in air. After the cells had been allowed to spread for 5–8 min, the coverslips with cells were treated with a solution that contained 2 mg/ml polyacrylate (19) and 5 mM $MgCl_2$ for 30 sec to eliminate the non-specific binding of ruptured cell debris, and then a jet of chilled microfilament-stabilizing solution [10 mM PIPES, 5 mM EGTA, 15 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF (pH 7.5)] was squirted from a 50-ml syringe across the surface of the coverslips. Immediately after the rupture of the upper portion of cells by the jet of buffer, the samples were immersed in chilled microfilament-stabilizing solution for 5 min. They were then treated with tetramethylrhodamine-conjugated phalloidin and monoclonal myosin-specific antibody in a moist chamber at 25°C for 60 min, washed with the same buffer, and incubated for 60 min with fluorescein-conjugated second antibody raised in goat. After a rinse with microfilament-stabilizing solution, the samples were mounted in the same solution supplemented with 0.1% p-phenylene diamine and observed under the epifluorescence microscope.

For the ATP-contraction experiment with the membrane-cytoskeleton preparation, coverslips were inverted and fixed on slide glasses with double-sided tape, and microfilament-stabilizing solution that contained 1 mM ATP was perfused between the coverslips and the slide glasses.

The cell membrane of the membrane-cytoskeleton preparation, which could not be seen at all by phase-contrast microscopy, was observed by interference reflection microscopy.

Assessment of cell spreading. Coverslips were cleaned by wiping with a piece of gauze wetted with neutral detergent, rinsed in distilled water, and immersed in an aqueous solution of 1 N HCl. Before use, the coverslips were vigorously washed in distilled water and dried. Cells were washed by centrifugation with chilled distilled water, and then suspended in each test solution at a concentration of 2×10^6 cells/ml. An aliquot of 0.1 ml of each cell suspension was put on a cleaned coverslip, and the cells were allowed to fall down onto the coverslip for 10 sec. Then the time course of spreading of cells was observed under the phase-contrast microscope. The degree of cell spreading was assessed as described in the legend to Table I.

Transmission electron microscopy. Agar-overlaid cells were fixed and embedded in Spurr's resin for transmission

electron microscopy as described in a previous report (40). After the resin had polymerized, the coverslip was removed by rapidly cooling the resin on a block of dry ice. The sample was divided into pieces, and thin sections, parallel to the substratum, were cut on an ultratome. The sections were stained with 1% uranylacetate and Reynold's lead citrate, and then they were observed under a JEM 100-C electron microscope.

RESULTS

1. A network of microfilaments associated with the cell membrane of a fixed cell. Agar-overlaid interphase amoebae of *Dictyostelium* were fixed with ethanol that contained 1% formalin at −15°C, and treated with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments, and with monoclonal antibody against myosin from *Dictyostelium* followed by treatment with fluorescein-conjugated second antibody for staining of myosin (Fig. 1a, b). As previously reported, small and large pseudopods (arrows in Fig. 1a) were strongly stained with tetramethylrhodamine-conjugated phalloidin. Upon careful observation, the somewhat hazy but filamentous fluorescence of actin could be identified at the cortical region of the cells. Myosin was seen as discrete rod-like structures, which were shown in a previous study to be similar to synthetic filaments of myosin from *Dictyostelium* after polymerization *in vitro* (41).

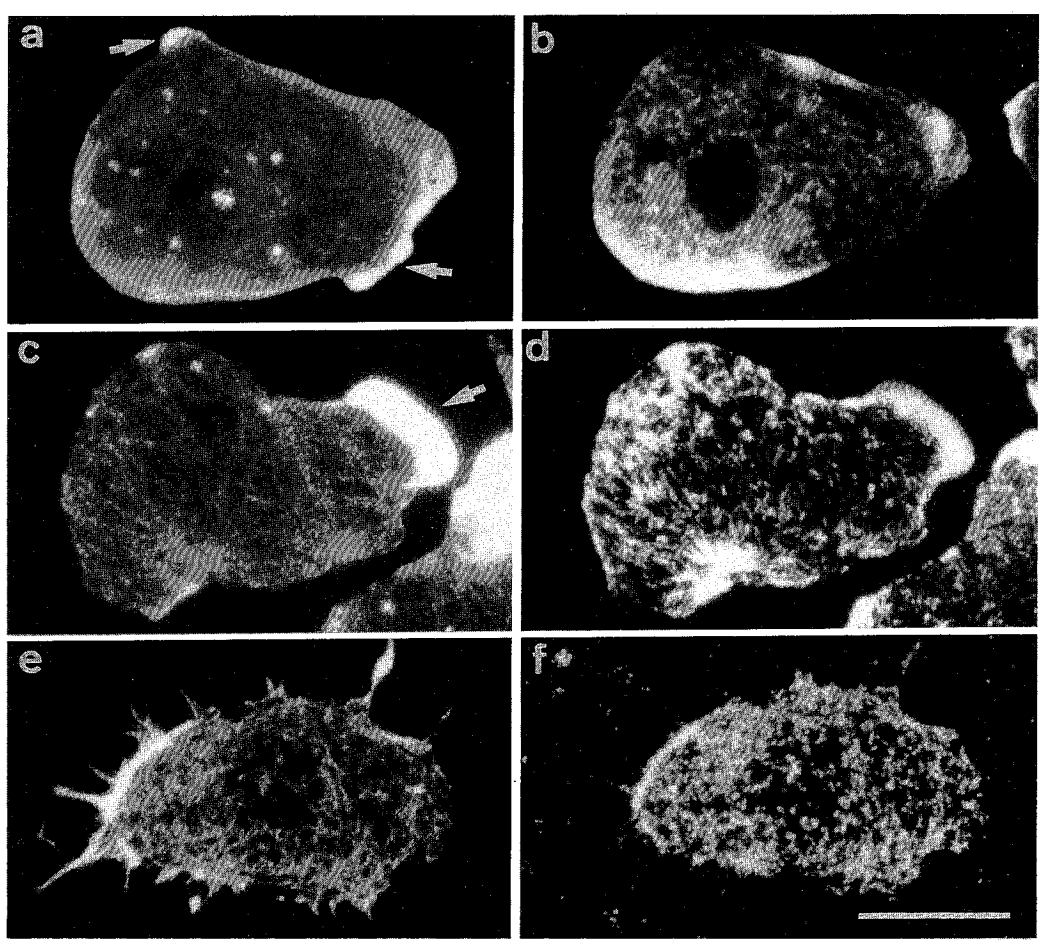


Fig. 1. Double immunofluorescence staining with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments (a, c, e) and with monoclonal antibody against myosin from *Dictyostelium* and fluorescein-conjugated second antibody for staining of myosin (b, d, f). (a, b) A fixed whole cell: (a) pseudopods (arrows) and several dot structures are strongly stained and, at the cortex, a network of actin filaments is seen as somewhat hazy, but filamentous staining. (b) Myosin is seen as discrete rod-like structures, or "myosin rods". (c, d) A membrane-cytoskeleton complex of a fixed cell; (c) a network of actin filaments is clearly seen, as compared with that in the whole cell shown in (a). Many long and continuous filaments run parallel to each other at the tail region of the cell. Note that the pseudopod remains (arrow). (d) "Myosin rods" are also clearly seen aligned along the actin filaments. (e, f) A membrane-cytoskeleton complex of an unfixed cell; (e) the network of actin filaments is evident and looks similar to that of the fixed cell shown in (c), except for the absence of pseudopods. (f) Myosin is seen as discrete "dots" along the actin filaments. Bar, 10 μm.

The upper regions of fixed and immunostained cells were removed by a jet of phosphate-buffered saline (PBS) squirted from a syringe. Networks of actin filaments were clearly observed on the exposed cytoplasmic surface of the cell membranes (Fig. 1c). It is plausible that individual filaments could be identified because of the removal of the overlapping of numerous filaments in the upper region of the cells. Many long and continuous actin filaments were seen running parallel to each other from one edge of the cell membrane to the other at the tail region of the cell. Each filament in the networks was shown to be composed of from one to several actin filaments by the electron microscopic observations of negatively stained samples (data not shown). In this paper, we hereafter refer to these fluorescent fibers as actin filaments, but they are not necessarily composed of only single filaments of actin. Many rod-like structures containing myosin (myosin rods) were observed along the actin filaments (Fig. 1d). The alignment of the myosin rods along the actin filaments was not necessarily continuous: there were many open spaces on the actin filaments. The density of the myosin rods associated with the actin filaments was lower in the anterior region than that in the tail region of a cell.

2. *Ionic conditions for and timing of cell spreading on a coverslip.* Living cells did not spread as widely and attach to the substratum as tightly as fixed "agar-overlaid" cells. To prepare the intact complex of cell membrane and actin filaments, we tried to identify conditions under which living cells would spread widely and attach tightly to coverslips (Table I). Cells suspended in distilled water or 15 mM phosphate buffer (pH 6.4) did not spread on the coverslips. It has been reported that cell-glass separations depend on salt concentrations and the valency of cations (14). Therefore, we examined the effects of several different cations on cell spreading. The results shown in Table I indicated that 5–8 mM concentrations of the divalent cations Ca^{2+}, Mg^{2+}, and Sr^{2+} were most effective for cell spreading. The same results were obtained in the case of polylysine-coated coverslips.

Cells were able to move even after spreading on polylysine-coated coverslips. About 10 min after contact with the surface of a polylysine-coated coverslip, the cells began to migrate, and further incubation of the coverslip in the moist chamber resulted in the aggregation of cells by chemotaxis and the formation of normal fruiting-bodies, as reported also by Clarke *et al.* (9). Cells were most widely spread about 5 to 8 min after contact with the substratum, and once they had begun to migrate, they never spread again.

3. *Microfilaments network associated with the intact cell membrane.* According to the results in the above section, cells suspended in 5 mM $MgCl_2$ were allowed to settle on polylysine-coated coverslips and then to spread for 5–8 min. Next, the upper portions of the widely spread cells were removed with a jet of microfilament-stabilizing solution squirted from a syringe. The cell membranes left on the coverslip were treated with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments, and with myosin-specific antibody and fluorescein-conjugated second antibody for staining of myosin. Networks of actin filaments were clearly visible (Fig. 1e), and these were identical to those observed on exposed cytoplasmic surfaces of the cell membranes of fixed cells (Fig. 1c, d), except for the absence of pseudopods in this unfixed preparation. Fluorescence due to myosin was seen as discrete structures aligned on the actin filaments (Fig. 1f). In this unfixed preparation of cell membrane, myosin appeared as "dots" rather than as the "rods" observed in the fixed cells. It is possible that myosin filaments in this unfixed sample might be somewhat compacted as a result of crosslinking by antibodies.

4. *Contractility of the intact membrane-cytoskeleton.* Detailed examination of the conditions for spreading and tight attachment of cells to the substratum, as described in section 2, enabled us consistently to obtain many cytoskeletons attached to the membranes (Fig. 2a, b). When 1 mM ATP was added to these fluorescence-stained membrane-cytoskeleton complexes (Fig. 2c, d), both actin and myosin aggregated within one second into several common foci to form large fluorescent dots (Fig. 2e, f). This process progressed as the network of actin filaments was broken up and gathered into each focus. Sometimes several smaller dots were formed, and then they were pulled together and fused to form larger fluorescent dots. Thus, these

Table I. EFFECTS OF IONIC CONDITIONS ON SPREADING OF CELLS.

Ionic condition	Degree of spreading[a]
Distilled water	0
Phosphate buffer	0
5 mM NaCl	0
10 mM NaCl	+
10 mM KCl	+
2 mM $MgCl_2$	++
5 mM $MgCl_2$	+++
8 mM $MgCl_2$	+++
10 mM $MgCl_2$	++
2 mM $CaCl_2$	++
5 mM $CaCl_2$	+++
5 mM $SrCl_2$	+++
5 mM $BaCl_2$	++
1 mM $LaCl_3$	+

[a] The degree of spreading is evaluated as 0, +, ++, +++, with (0) indicating the least spreading and (+++) indicating the most extensive spreading of the cells.

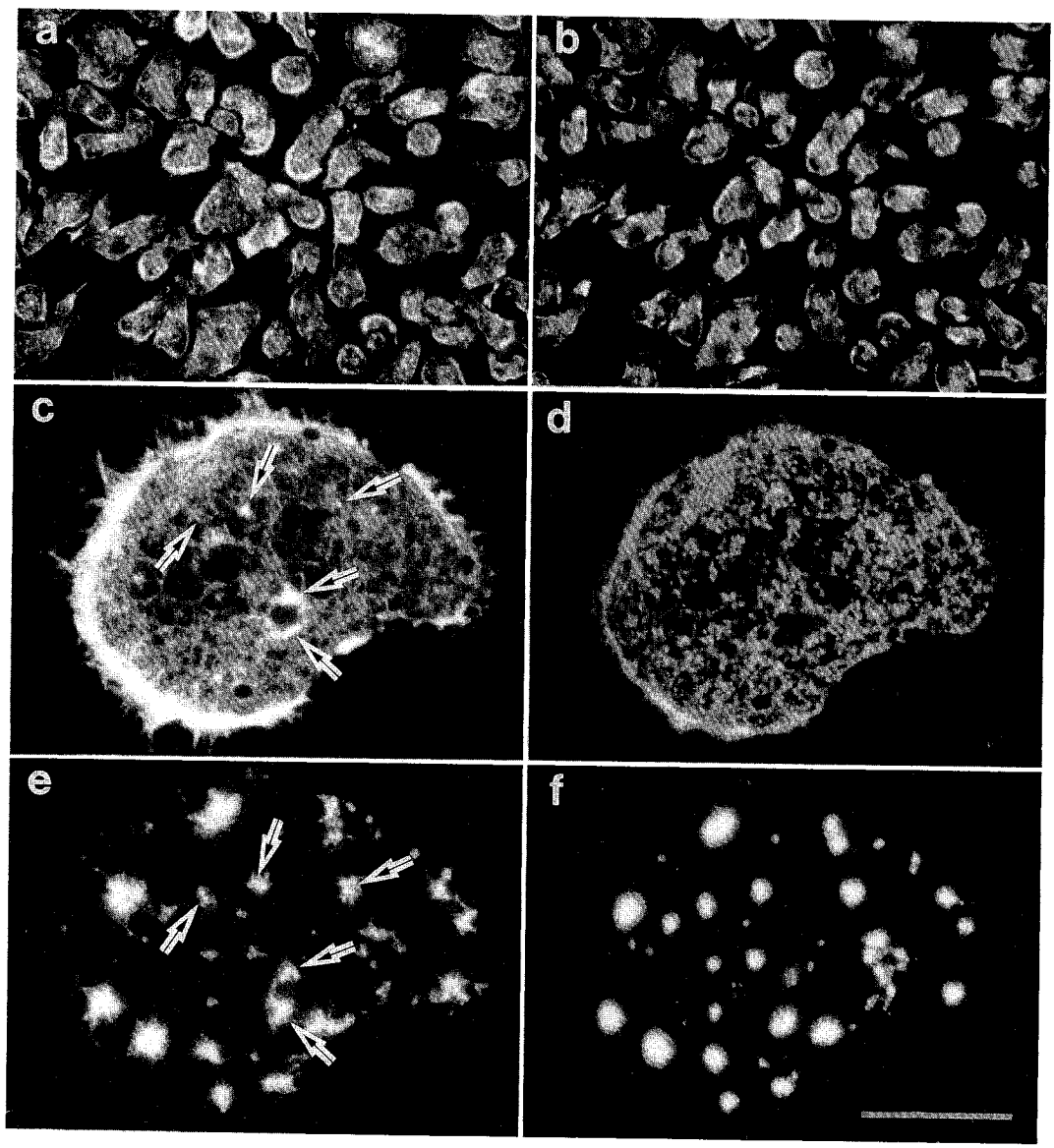


Fig. 2. Double immunofluorescence staining with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments (a, c, e) and with myosin-specific antidbody and fluorescein-conjugated second antibody for staining of myosin (b, d, f). (a, b) Low-magnification view of the networks of actin filaments (a) and myosin (b) of the intact membrane-cytoskeleton complexes prepared by our improved method. Using this method, we could easily obtain many intact complexes of the cell membrane and cytoskeleton with *in situ* organization. (c, d, e, f) Contraction of an intact membrane-cytoskeleton complex. Before (c, d), and after (e, f) the addition of 1 mM ATP. Both actin and myosin aggregated into several common foci that resulted in the formation of large fluorescent dots (arrows in e) within a second after the addition of ATP. Arrows in (c) show small dot-like structures of actin observed even before the contraction. Bar, 10 μm.

fluorescence dots were not constant in either size or number on each cell membrane.

Using an interference-reflection microscope, we examined whether the cell membrane itself was torn during the contraction of the networks of actin and myosin caused by the addition of ATP (Fig. 3). We were able to observe the image of the cell membrane itself, which was obscure under the phase-contrast microscope. We found that the contours of cell membrane itself did not change during the contraction (Fig. 3b, d). Therefore, the network of actin on the isolated cell membrane seems to be tightly associated with the limited areas of the cell membrane where the large fluorescent dots are formed by the additions of ATP.

5. Distribution of the dot-like structures in whole cells. The foci at which actin and myosin aggregated during the contraction of the network could be identified as small dot-like structures of actin on isolated cell

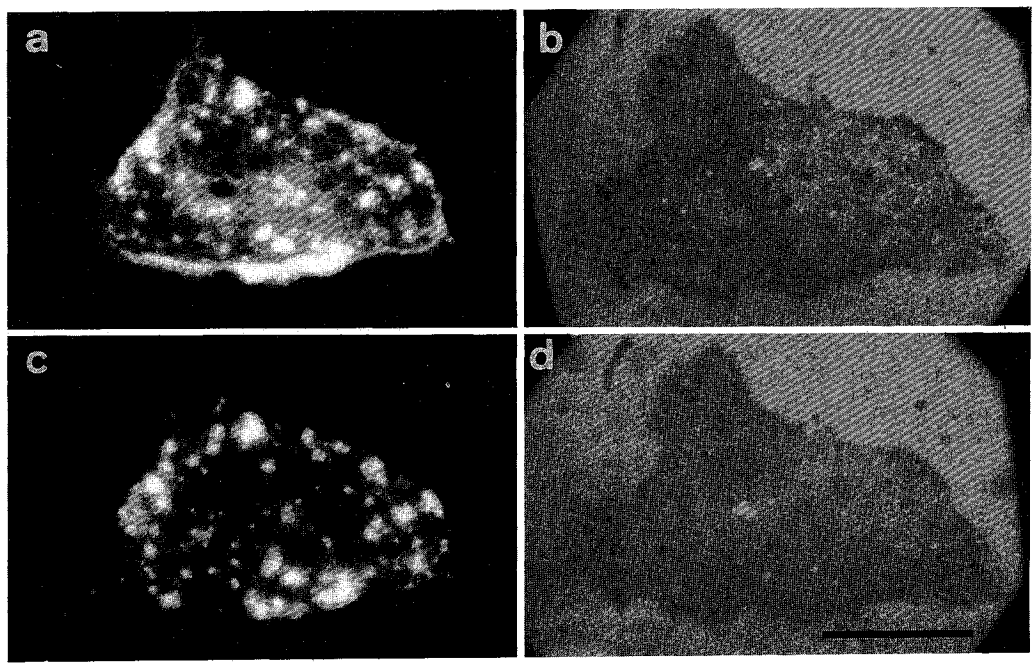


Fig. 3. Fluorescence (a, c) and interference reflection (b, d) micrographs of a membrane-cytoskeleton complex stained with tetramethylrhodamine-conjugated phallodin before (a, b) and after (c, d) the contraction caused by the additioin of ATP. The network of actin filaments on the exposed cytoplasmic surface of the cell membrane (a) has contracted to form several large fluorescent dots (c). Note that the cell membrane does not change its shape during the contraction (b, d). Bar, 10 μm.

membranes, even before the addition of ATP (arrows in Fig. 2c). Such actin structures were also observed in the cortical region of whole cell stained with tetramethylrhodamine-conjugated phalloidin (Fig. 4c, d, g, h). It was noted that myosin did not concentrate at these structures (Fig. 4b, f), unlike the large fluorescent dots formed after the contraction of the network on isolated cell membranes (Fig. 2f). In stationary cells, the small dot-like structures of actin were observed both in the ventral cortex attached to the substratum and in the dorsal cortex attached to the agar-sheet overlaid on the cell (Fig. 4g, h). In actively locomoting cells, the small dot-like structures were also observed and there were more of them near the tail region (Fig. 4c, d). When similar cells were compared, neither the size nor the number of the structures was constant; some cells had more than 50 dot-like structures of various sizes.

6. *Ultrastructures of the dot-like structures.* Cells were fixed by the agar-overlay method, embedded in Spurr's resin, and serial sections parallel to the substratum were prepared for electron microscopy. Figure 5 shows the cortical region of a cell near the substratum; in this region a novel structure corresponding to the small dot-like structure of actin was observed. This structure was composed of electron-dense material at the center with numerous microfilaments radiating from it, and no myosin filaments were seen at this region (Fig. 5). Similar structures have been seen on the cell membranes of spreading macrophages and polymorphonuclear leukocytes (4, 35), but they have never been reported in *Dictyostelium* amoebae. From all the above mentioned results, and by analogy to the macrophage and leukocyte, it is possible that the small dot-like structures of actin observed in *Dictyostelium* were actin-organizing centers on cell membrane which were involved in the adhesion of the cells to the substratum.

DISCUSSION

1. *Biological function of the dot-like structures.* We succeeded in the visualization, by epifluorescence microscopy, of the intact network of actin filaments which was associated with myosin rod-like structures, or myosin filaments, on exposed cytoplasmic surfaces of cell membranes of *Dictyostelium* amoebae that were attached to polylysine-coated coverslips. For the first time in *Dictyostelium* amoebae, we have observed novel dot-like structures, composed of a dense body at the center with radiating actin filaments (Fig. 5), and we suggest that they act as possible actin-organizing centers on the cell membrane. These dot-like structures must attach tightly to the cell membrane because they were not removed from it by the shearing force of the stream of buffer during the preparation process. The evidence

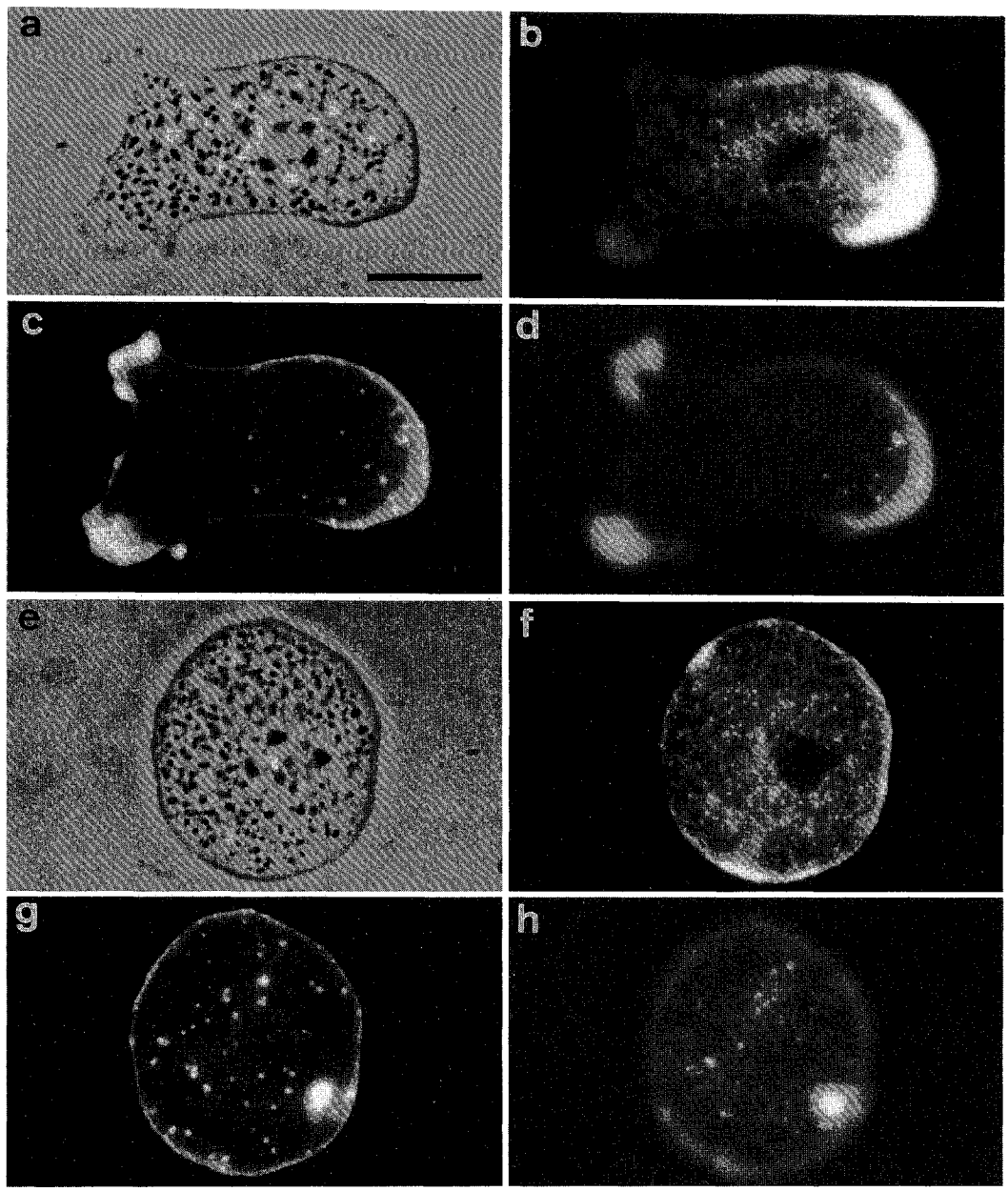


Fig. 4. Double immunofluorescence staining of whole cells with tetramethylrhodamine-conjugated phalloidin for staining of actin filaments (c, d, g, h), and with myosin-specific antibody and fluorescein-conjugated second antibody for staining of myosin (b, f). Panels (a) and (e) are phase-contrast micrographs of the same fields as those in (b–d) and (f–h), respectively. Panels (c, d) and (g, h) show the ventral (c, g) and dorsal (d, h) cortices of the same cells, respectively. Panels (b) and (f) show the fluorescence images on the same focal planes as those in panels (d) and (g), respectively. Small dot-like structures of actin can be seen in both the ventral and the dorsal cortical regions of whole cells (c, d, g, h). In the locomoting cell, the number of the dot-like structures is greater near the tail region (c, d); in the resting cell, more dot-like structures are visible in the ventral cortex (g) that in the dorsal cortex (h). Note that myosin did not concentrate the small dot-like structures of actin (b, f), in contrast with the large fluorescent dots that were formed by ATP-induced contraction (Fig. 2f). Bar, 10 μm.

that many actin filaments radiated from these dot-like structures, and that actin and myosin aggregated into them to form large fluorescent dots during the contraction caused by the addition of ATP, indicates that the dot-like structures were the organizing centers for actin filaments on the cell membrane. However, a different type of binding site for actin filaments must also exist on the cell membrane because some actin filaments remained on regions of the cell membrane other than the large fluorescent dots. However, such residual actin fila-

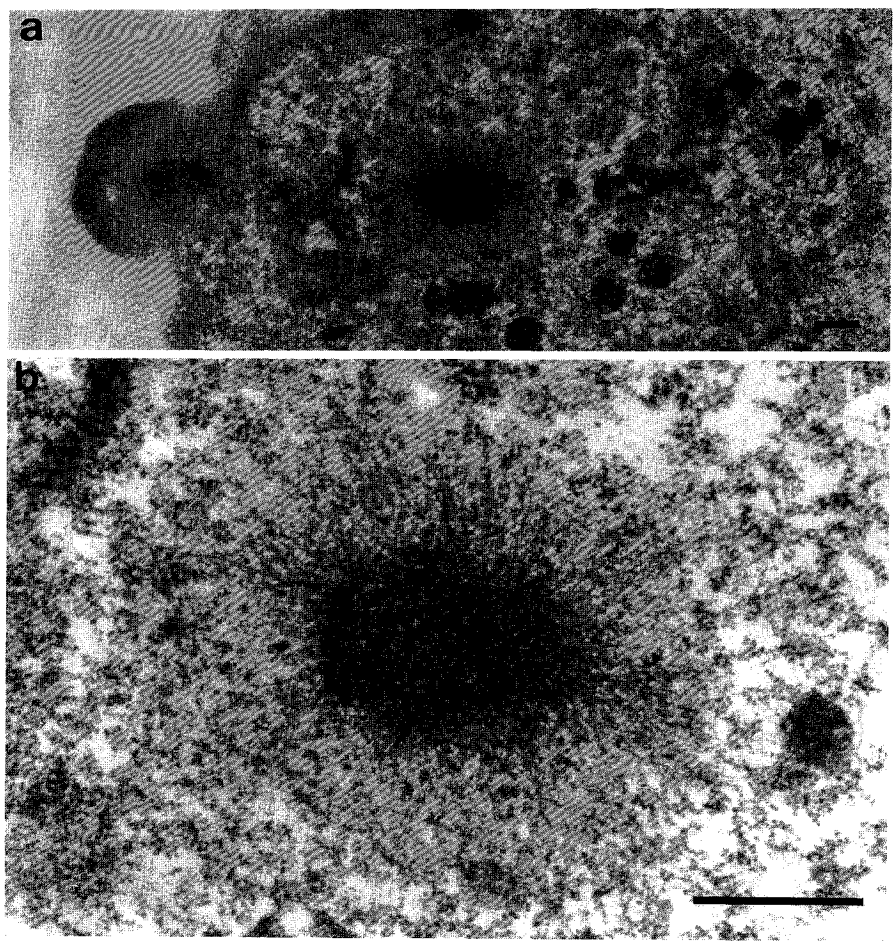


Fig. 5. Transmission electron micrographs of a section cut parallel to the subtratum showing a small dot-like structure of actin in the ventral cortex of a cell. Panel (b) is a high-magnification view of the small dot-like structure in (a), showing that many actin filaments radiate from the electron-dense structure at the center. Bar, 500 nm.

ments are not evident in Figure 2e because of the high contrast of the large fluorescent dots. The dot-like structures found in *Dictyostelium* look similar to the dense centers and the punctuate structures of macrophages and polymorphonuclear leukocytes (1, 4, 35). These structures are also the centers from which actin filaments radiate, and they are observed on the sub-plasma membrane when macrophages and polymorphonuclear leukocytes spread on the substratum. Focal contacts in cultured fibroblasts have also been described as sites at which bundles of actin filaments (stress fibers) terminate (2, 37). These specialized structures at the region of contact between the cell membrane and the substratum must play a role as sites that transduce or support the force produced by the interaction between actin filaments and myosin.

The dot-like structures observed in *Dictyostelium* amoebae could not be seen by interference reflection microscopy (Fig. 3), unlike the focal contacts in fibroblasts. The reason for this discrepancy may be that in the case of *Dictyostelium* there was a constant space between the cell membrane and the sustratum, which resulted in the loss of contrast in the interference. Gingell and Vince observed the process of spreading of *Dictyostelium* amoebae on several substrata by interference reflection microscopy, but they failed to find any evidence of focal points (13). It is plausible that the "foot" of the *Dictyostelium* amoebae is different from that of a fibroblast, because the former cell moved actively, in contrast with the latter cell, and the "foot" of a *Dictyostelium* amoebae must be generated and destroyed repeatedly and very rapidly. In this regard, it is important to compare the "foot" of a *Dictyostelium* amoebae with the "foot" of other amoeboid cells such as macrophages or leukocytes. Observations by interference reflection microscopy revealed that macrophages and leukocytes also do not make focal contacts and that they attach to the substratum over a relatively wide area (20). How-

ever, focal contacts were observed by interference reflection microscopy in the case of the protozoan amoebae *Naegleria gruberi*, from soil (29).

2. Does ATP-instigated contraction occur at the dot-like structures in living cells? Addition of ATP to the network of actin filaments on the exposed cell membrane that was attached to the polylysine-coated coverslip resulted in the aggregation of both actin and myosin into common foci to form the large fluorescent dots. In the process of this contraction, the network seemed to be torn between each member of a pair of small dot-like structures, because the actin filaments of the network bound strongly to the sites of the dot-like structures and the dot-like structures themselves were also strongly bound to the substratum through transmembrane linkages, probably as a result of the effect of polylysine. Does such contraction occur in living cells? It is, at the very least, unlikely that such "catastrophic" contraction occurs in a living cell because myosin did not concentrate at the dot-like structures observed in whole cells. If the cell membrane is not fixed by polylysine, the force generated from the contraction will cause a morphological change in the cell or in the cell membrae, but it will never result in the large-scale destruction of the network itself. The network of actin filaments is thought to be woven and organized by crosslinkages formed by several actin-binding proteins and, during the contraction in living cells, smaller-scale reactions must occur under the careful control of actin-binding proteins, in coordination with the morphological changes in the cells. In contrast, the contraction of the network on the isolated cell membrane, which resulted in the aggregation of a large amount of actin and myosin, seems to be out of control, probably because of the depletion of some actin-binding proteins which were present in the cells in a soluble form.

In *Dictyostelium* amoebae, regions from which myosin filaments are absent are sites of cell-cell adhesion, pseudopods, and mouths for phagocytosis; each of these structures is composed of a dense meshwork of actin filaments (39). In this study, we found that myosin was also absent from the dot-like structures of actin. It is noteworthy that the dot-like structures were different from the above mentioned structures in the sense that they were not composed of a meshwork but rather of radiating actin filaments (Fig. 5). Thus, the dot-like structure is a new type of structure that organizes actin filaments in *Dictyostelium*, and it is now necessary to ascertain what kinds of actin-binding protein are localized and function at this site.

The membrane-cytoskeleton complex prepared in this study should be almost intact and contain several actin-binding proteins. Thus, it should provide us with much information about the molecular organization of the interactions between the cell membrane and the cytoskeleton.

Acknowledgements. We thank Prof. Hiroh Shibaoka of the Department of Biology, Faculty of Science, Osaka University, for generously allowing us to use the ultratome. We thank Dr. Yoshio Fukui of the Department of Cell Biology and Anatomy, Northwestern University Medical School, for his encouragement. This work was supported by Grants 62780280 and 62790167 from the Japanese Ministry of Education.

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(Received for publication, July 12, 1990
and in revised form, October 2, 1990)