Localization of Actin and Myosin for the Study of Ameboid Movement in *Dictyostelium* Using Improved Immunofluorescence

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ABSTRACT The distribution of actin and myosin in *Dictyostelium* amebae at different developmental stages was studied by improved immunofluorescence ("agar-overlay" technique). Both were localized at the cortical region of amebae in all early developmental stages. In amebae with polarized morphology, bright fluorescence with antiactin was seen in the anterior pseudopode. The cortex in the posterior end was also stained with antiactin. On the other hand, very specific crescent-shaped staining with antimyosin was seen at the posterior cortex. In cells in contact with each other, actin was concentrated at the contact region, whereas myosin was localized specifically in the cortex on the other side of the contact region. At the aggregation stage, when monopodial amebae migrate forming streams, actin staining was seen all around the cell periphery, with intense fluorescence in the anterior pseudopode. On the other hand, specific staining of myosin was seen only at the posterior cortex. The cleavage furrow of cells performing cytokinesis displayed distinct myosin staining, and this staining represented the filamentous structure aligned in parallel to the axis of constriction. These findings indicate that myosin staining reflects the portion of the cell cortex where contraction occurs and the motive force of ameboid movement is generated at the posterior cortex of a migrating cell.

Actin and myosin are thought to play significant roles in the biological machinery in nonmuscle cell movement. However, the locomotory mechanism of a cell has not been totally elucidated. *Dictyostelium* is a good material for studying cell motility because it migrates by ameboid movement, one of the most conservative mechanisms of cell motility. Biochemical and electron microscopic studies have revealed the significant concomitance of actin, myosin, and their associated proteins in cellular motile events of *Dictyostelium* (6, 12). The solation-contraction coupling hypothesis of Hellewell and Taylor (13) suggests a structural requirement of local breakdown of the gel for contraction in the motile extract of *Dictyostelium*. However, the structural organization of the contractile components in intact cells has not been fully clarified.

Initial immunofluorescence using antiactin showed that vegetative amebae were stained uniformly whereas actively migrating cells are stained strongly at their leading edges (8). Recently, Bazari and Clarke (2) demonstrated that calmodulin and myosin are localized in the peripheral region. Condeelis et al. (5) and Brier et al. (3), using conventional immunofluorescence, found that 120- and 95-kdalton actin-binding proteins are also localized at the cell periphery.

Partially because of the round shape and small size of *Dictyostelium* amebae, no detailed information on the spatial organization of cytoskeletal components has been provided by conventional immunofluorescence. We thus improved the technique and identified the localization of microtubules (17, 30). In the present study, we document the localization of actin and myosin in various developmental stages of amebae revealed by the improved agar-overlay technique using rabbit antiactin and newly obtained monoclonal anti-*Dictyostelium* myosin antibodies. The agar-overlay technique can preserve cellular structures during the fixation and prevent their disruption during sample preparation.

Interestingly, very specific myosin staining was observed at the posterior cortex of the migrating amebae, whereas actin staining was localized in the anterior pseudopodes and the

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posterior cortex. This finding suggested that the motive force of ameboid movement is generated at the posterior cortex of migrating cells. The possible role of actin in the anterior pseudopode is discussed in relation to speculative coupling in the organization of cytoskeletal elements involved in the ameboid movement.

MATERIALS AND METHODS

Cells and Cultures: *Dictyostelium discoideum* (Ax-2) was cultured in HL-5 (29) medium for the purification of myosin. The wild type (NC-4) was grown with *Escherichia coli* (B/r) on nutrient agar and used for all the immunofluorescence. The cells were suspended and washed twice with 15 mM Na/K-phosphate buffer (pH 6.4), then inoculated on 2% agar. They were allowed to develop until the appropriate stages and then harvested prior to the preparation of immunofluorescent samples.

Derivation of Hybridomas: *Dictyostelium* myosin was purified according to the methods of Clarke and Spudich (4) and Mockrin and Spudich (22). 4–5-wk-old mice were intraperitoneally injected with 100 μg of myosin in Freund's complete adjuvant. 2 wk later, a booster of 100 μg of myosin mixed with incomplete adjuvant was given intraperitoneally. 1 wk later, the antibody activity was examined by enzyme-linked immunosorbent assay (9) and all the mice gave positive results. 4 wk after the primary immunization, 200 μg of myosin in PBS (138 mM NaCl, 2.7 mM KCl, 8 mM Na/K-phosphate buffer [pH 7.2]) was injected intraperitoneally, and 2 d later 100 μg of myosin in PBS was injected both intraperitoneally and intravenously. 2 d after the final immunization, the spleen cells were fused with Sp2/0-Ag 14 mouse myelomas (26). Hybridomas producing antibodies against myosin were screened by enzyme-linked immunosorbent assay, and the final screening was performed by indirect immunofluorescence.

After cloning by limiting dilution, two hybridomas were established and named DM-2 or DM-6 (DM: *Dictyostelium* myosin[1]). The monoclonal DM-2 antibody was mainly used in the present study. Whole sera of the positive mice were collected and used for the polyclonal control staining.

Indirect Immunofluorescence: Cells at each developmental stage were harvested and suspended in the phosphate buffer, and an aliquot of the suspension was placed on a coverslip. A thin agarose sheet (0.15 mm thick, 8 × 8 mm square wide, made of 2% agarose [immunological grade] dissolved in the phosphate buffer) was put on the cells. Excess buffer was removed using small pieces of filter paper, and the sample was observed under a phase-contrast microscope. When the cells were at the appropriate condition, the buffer was removed from the surface of the agarose until the cells became very flat due to the mechanical pressure. Details of this technique will be furnished on request. The samples were immersed in −10°C methanol and fixed for 5 min. After a brief rinse with PBS, the samples were incubated with the primary antibody (DM-2 culture medium or rabbit anti-chicken gizzard actin serum [1:20]) for 30 min at 37°C. The samples were washed for 30 min with PBS (this washing was indispensable to prevent background staining). They were then incubated with fluorescein isothiocyanate-labeled second antibody preadsorbed with *Dictyostelium* cell lysate.

The preadsorption was done as follows. The cell pellet (0.1 ml) was fixed for 5 min with cold methanol (−10°C), washed three times with PBS, and resuspended in 500 μl of the second antibody ([1:25] diluted with PBS containing 0.1% (wt/vol) NaN_3; Cappel or Miles-Yeda). The suspension was incubated for 30 min at 36°C, then centrifuged for 30 min at 13,000 rpm, and finally the adsorbed antibody was carefully collected using a Pasteur pipette. The adsorbed antibody could be kept for as long as one month in a refrigerator.

The samples were finally washed with PBS, briefly rinsed with distilled water, and mounted with Gelvator (24) containing 1 mg/ml *p*-phenylenediamine (15). The fluorescent micrographs were taken under an Olympus epifluorescence microscope (BH-RFL) equipped with × 100 lens (NA 1.25) using Kodak Tri-X film and developed with Acufine (Acufine, Inc., Chicago, IL).

Immunoblotting: SDS PAGE was performed according to Laemmli (20) on 10% slab gel, and the protein was electrophoretically transferred (28) to nitrocellulose paper (Bio-Rad Laboratories, Richmond, CA) for 22 h at 0.1 A (3 V/cm) in the buffer containing 25 mM Tris-base, 192 mM glycine, 0.1% SDS, and 20% methanol. The paper was blocked with 3% gelatin (Bio-Rad Laboratories), and sequentially incubated with DM-2 antibody (1:1 culture medium containing 3% BSA), TTBS (0.05% Tween-20 in Tris-buffered saline [pH 7.5]), peroxidase-labeled second antibody (HRPO-rabbit anti-mouse IgG [Litton]; 1:330 diluted with TBS containing 3% BSA and 1% gelatin), TTBS, and finally with 0.5 mg/ml 4-chloro-1-naphthol and 0.5 μl/ml H_2O_2 (30%) for color development.

[1] *Abbreviation used in this paper:* DM, *Dictyostelium* myosin.

RESULTS

Specificity of Antibodies

The antibody of DM-2 hybridoma was of the IgG class as shown by the Ouchterlony double diffusion test (data not shown), and the specificity was tested by immunoblotting. DM-2 antibody was reactive to the heavy chain of purified *Dictyostelium* myosin (Fig. 1, *c* and *g*) as well as the corresponding band in the whole cell lysate (Fig. 1, *b* and *f*). However, DM-2 crossreacted with neither rabbit skeletal muscle myosin (Fig. 1, *d* and *h*) nor chicken gizzard smooth muscle myosin (210-kdalton band in Fig. 1, *a* and *e*).

In the initial experiments, some minor bands ranging 170–180 kdaltons represented positive reaction with DM-2. We could not totally eliminate the contamination of these minor bands from the myosin preparation by adding protease inhibitors to the purification buffers (50 μg/ml leupeptin, 1 mM PMSF [phenylmethylsulfonyl fluoride], and 0.1 mM TLCK [*p*-tosyl-L-lysine chloromethyl ketone hydrochloride]). These bands were likely to be degradation products of the myosin heavy chain since the amount of these proteins included in the whole cell lysate decreased significantly by solubilizing the cells by adding prewarmed SDS sample buffer to the cell pellet (Fig. 1 *b*).

The specificity of the immunofluorescence was tested by several control experiments including (*a*) the staining without the primary antibody or (*b*) with preimmune mouse serum for the primary antibody. In the initial experiments, some background staining was occasionally observed even by the control staining. This nonspecific staining was apparently caused from the second antibody, and could be eliminated by the preadsorption of the fluorochrome-labeled antibody with *Dictyostelium* lysate as described in Materials and Methods.

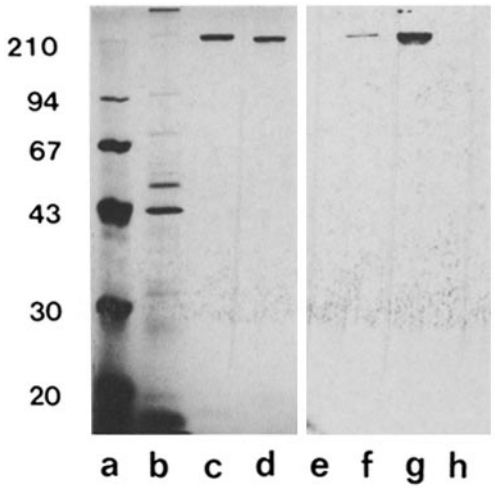

FIGURE 1 Western blot of the monoclonal anti-*Dictyostelium* myosin (DM-2) antibody. The samples were electrophoresed on 10% SDS PAGE and stained with Coomasie Blue (*a–d*) or after blotting to nitrocellulose paper, labeled with peroxidase-labeled anti-mouse IgG subsequent to the incubation with DM-2 (*e–h*). Lanes *a* and *e*, molecular weight markers; lanes *b* and *f*, *Dictyostelium* cell lysate (25 μg); lanes *c* and *g*, *Dictyostelium* myosin (5 μg); lanes *d* and *h*, rabbit skeletal muscle myosin (5 μg). DM-2 specifically bound to the heavy chain of *Dictyostelium* myosin (*f* and *g*) but neither to rabbit skeletal (*d* and *h*) nor chicken gizzard smooth muscle myosin (210-kdalton band in *a* and *e*). Values are molecular weight × 10^{-3}.

Preservation of Cell Structure by the Agar-overlay Technique

Migrating *Dictyostelium* amebae are not large enough (8-μm diam) or flat enough to allow light microscopic observation of their cellular structures, as shown by scanning electron microscopy (see Fig. 2 *A* of reference 30). Furthermore, their cytoskeletal machinery are very small and no stable large structures (such as stress fibers) are visible. In addition, their cytoskeletons are susceptible to the mechanical disturbances that are inevitable during preparation of samples for immunofluorescence. We tried to overcome these problems by laying a thin agarose sheet over the cells before fixation and performing the immunofluorescence procedure under this condition. The amebae could migrate normally and undergo aggregation under the agarose sheet. Microscopic images of the cells improved greatly with this technique, and cellular organelles such as pseudopodes, mitochondria, nuclei, nucleoli, and even nuclear-associated bodies (19, 25) could be identified under a phase-contrast microscope. In addition, the mechanical support by the agarose sheet resulted in good preservation of the micro- as well as macromorphology of the cells, as shown by comparative phase-contrast microscopy before and after fixation with cold methanol (Fig. 2).

Actin and Myosin in Vegetative Cells

In the vegetative stage, the cells feed on *Escherichia coli*, divide by binary fission every 3 h, and have a round shape with a few small pseudopodes. Actin staining caused a diffuse fluorescence all over the cytoplasm, whereas antimyosin specifically stained the periphery of the cells (Fig. 3, *a–d*). The different distributions of actin and myosin were not unexpected, since cortical motile activity is not prominent at this stage and the cortical machinery is likely to be organized at the same time as the activation of the motile event. This idea was supported by the observed changes in the pattern of the actin staining during the transformation from the vegetative to migratory amebae; both actin and myosin stainings were evident at the cell cortex at this stage (Fig. 3, *e–h*).

To assess the possible involvement of actin and myosin in specific motile activities, special attention was paid to the cells performing cytokinesis or phagocytosis. The polar pseudopodes in dividing cells were stained with antiactin (Fig. 3, *i* and *j*). However, very specific staining with antimyosin was observed at the constricted region of the dividing cells. Interestingly, this myosin staining showed a filamentous structure aligned parallel to the axis of constriction (Fig. 3, *k* and *l*), although no specific actin-containing structure was seen in

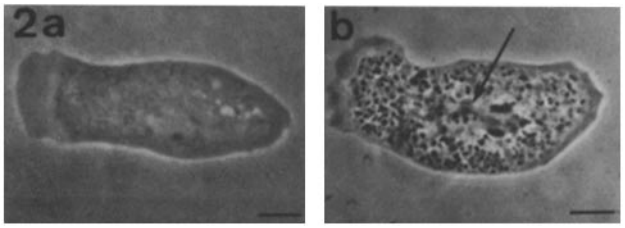

FIGURE 2 Comparative phase-contrast microscopy of *Dictyostelium* amebae before (*a*) and after (*b*) fixation by the agar-overlay technique. Cell structure was well preserved and the arrow indicates the site of the nuclear-associated body (microtubule-organizing center). Bars, 5 μm.

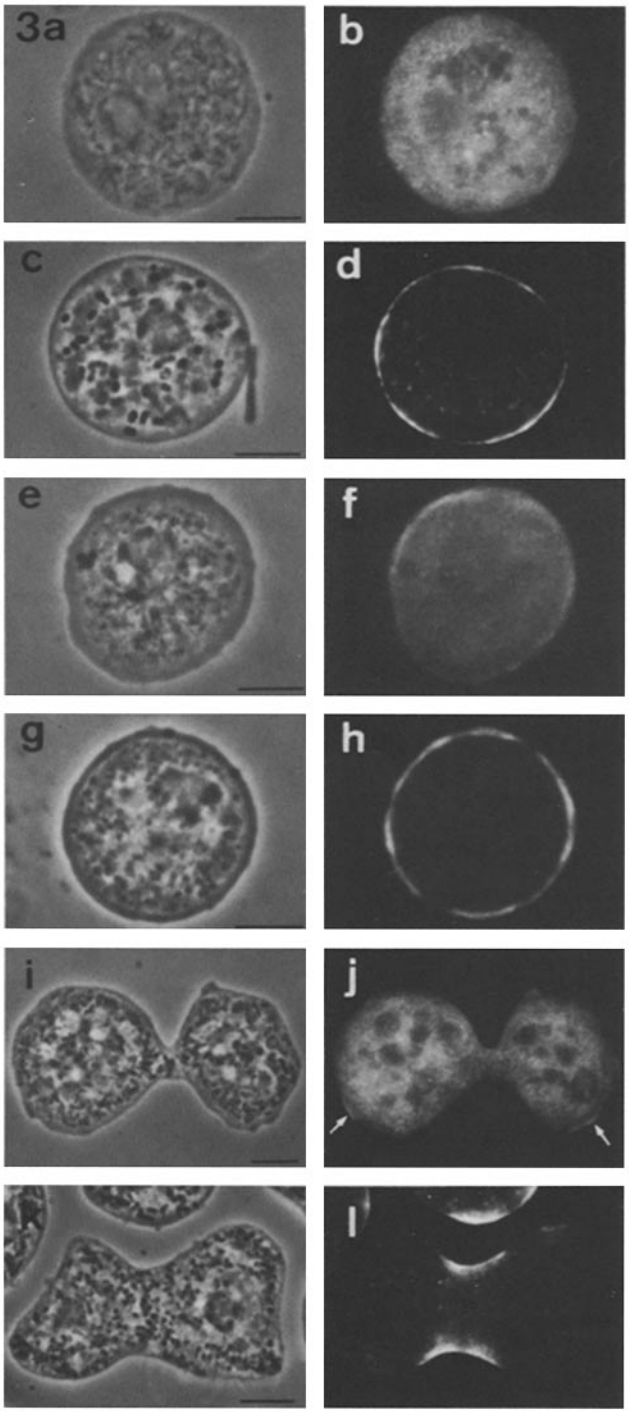

FIGURE 3 Indirect immunofluorescence of vegetative *Dictyostelium* amebae prepared by the agar-overlay technique showing the distribution of actin and myosin. (*a* and *b*) A vegetative amebae stained with antiactin showing diffuse fluorescence in the cytoplasm. (*c* and *d*) A vegetative ameba stained with monoclonal antimyosin (DM-2) showing peripheral staining. (*e* and *f*) A round cell that was allowed to develop for 2 h on agar plate was then fixed and stained with antiactin. Peripheral staining became evident by 2 h of incubation. (*g* and *h*) A round cell like that of *e*, stained with DM-2, and showing peripheral staining. (*i* and *j*) A dividing (telophase) cell stained with antiactin. Note the staining of pollar small pseudopodes (arrows in *j*). (*k* and *l*) A dividing cell stained with DM-2. Note the specific staining at the cleavage furrow. The staining pattern suggests that the filamentous structure is aligned parallel to the axis of constriction. Bar, 5 μm.

this region (Fig. 3*j*). Staining of the phagocytic cup was similar to that of the anterior pseudopode; i. e., brightly stained with antiactin, but not with antimyosin (data not shown).

Actin and Myosin in Locomotory Cells

When the amebae were harvested and inoculated onto the agar plate, they started active locomotion in 2–3 h. At this stage, both the anterior pseudopode and the posterior cortex were stained with antiactin whereas only the posterior cortex was stained with antimyosin (Fig. 4, *a–d*). Clearly, two classes of anterior pseudopodes were present, appearing as dark and light regions under a microscope equiped with dark-contrast phase optics. The dark region was stained strongly with antiactin but the light region was not. The light pseudopode looked like a hyaline region, and the boundary between this region and the ground cytoplasm showed actin staining that might be due to a cortical actin layer (arrowhead in Fig. 4 *f*). The differences in the staining of pseudopodes may indicate differences in the actin organization of these pseudopodes.

In cells in contact with each other, the contact sites were stained strongly with antiactin (Fig. 4, *g* and *h*), whereas no staining was observed with antimyosin. This may be due to the well-developed microfilament mesh found by transmission electron microscopy (see Fig. 3*c* of reference 11). The periphery on the other side of the contact region was specifically stained with antimyosin (Fig. 4, *i* and *j*).

Actin and Myosin in Aggregating Cells

In the aggregation stage, when the amebae aggregate in streams, elongated cells migrate forming head-to-tail as well as side-by-side associations. In this stage, not only the pointed pseudopode and posterior region but also the lateral cortical region were stained with antiactin (Fig. 5, *a* and *b*). Antimyosin only stained the lateral and the posterior cortex (Fig. 5, *f* and *g*). In the tip of the pseudopode, peculiar rod-like staining with antiactin, not seen in other stages, was prominent (Fig. 5, *c* and *d*).

We obtained good macroscopic images of the aggregate by the agar-overlay technique (Fig. 5, *a–e*). Very bright myosin staining was particularly observed at the posterior end next to the lateral cortex of the cells at the periphery of the aggregate (Fig. 5 *e*). This myosin staining suggested that a centripetal force was generated at the outermost cortex of the peripheral cells and this force might be required for the formation of the aggregate. This idea was supported by evidence for myosin localization in contacting cells that looked as though they were pushing each other (Fig. 4*j*).

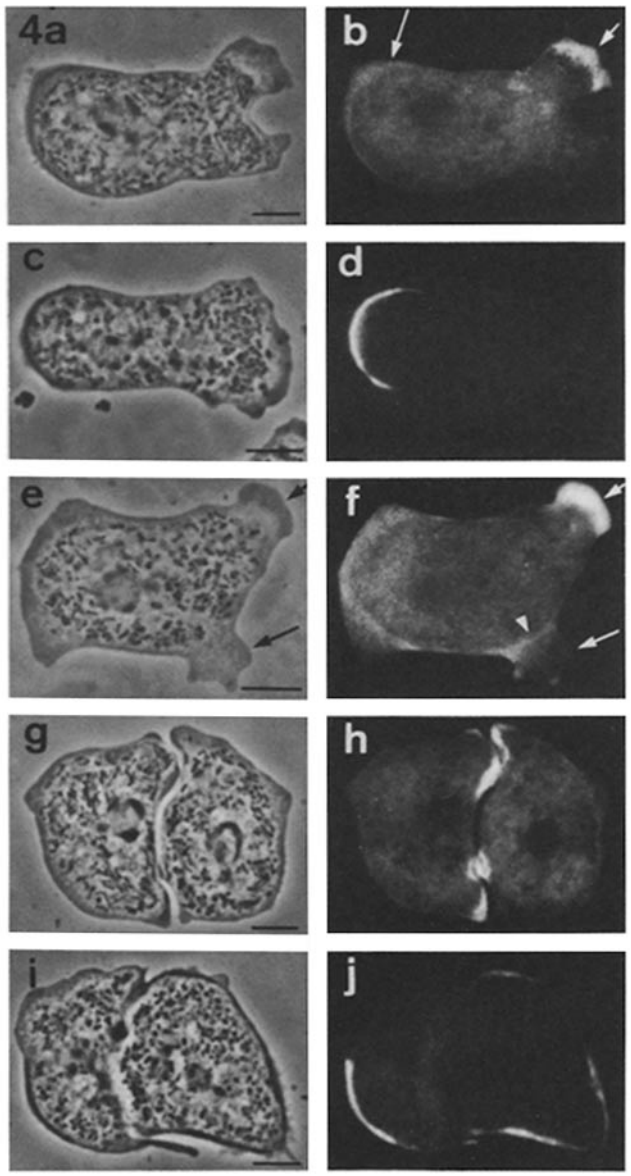

FIGURE 4 Distribution of actin and myosin in locomotory amebae prepared by the agar-overlay technique. (*a* and *b*) An actively locomoting cell stained with antiactin. Note the bright staining of the anterior pseudopode (short arrow in *b*). Staining of the posterior cortex (long arrow) was also prominent. (*c* and *d*) A monopodial cell stained with monoclonal antimyosin (DM-2). The cortex in the posterior end was specifically stained. (*e* and *f*) A cell with actively extending pseudopodes stained with antiactin. The dark pseudopode (short arrows) was stained brightly whereas the light pseudopode (long arrows) was not. Note the staining at the boundary of the light pseudopode and the ground cytoplasm (arrowhead in *f*). (*g* and *h*) Two cells in contact with each other stained with antiactin. Bright staining was prominent in the contact regions. (*i* and *j*) Two cells in contact with each other stained with DM-2. The cortices on the other side of the contact regions were stained. Bars, 5 μm.

DISCUSSION

Assessment of the Agar-overlay Technique

Extraction of plasmodium placed between two sheets of agar with glycerol was first reported with *Physarum polycephalum* (16). This method was recently applied for the immunofluorescence of *Physarum* plasmodium and was shown to be efficient for providing good immunofluorescent images (23). In the present study, we found that the agar-overlay technique is very useful for either phase-contrast or immunofluorescent microscopy of *Dictyostelium* amebae as small as 8-μm diam. The drawback of this technique is a possible artifact arising from mechanical stress from the agarose, which might cause peripheral staining of actin and myosin. To circumvent this problem, we compared the staining patterns of flat cells prepared by the agar-overlay technique with those of round cells prepared by conventional fixation procedure. We found that fixation with cold methanol totally disrupted the structure of suspended round cells. Thus, conventional fixation with 3.7% formaldehyde in PBS or 15 mM phosphate buffer is not suitable because it results in drastic shrinkage of the cells. Careful reconsideration of the fixation protocol for

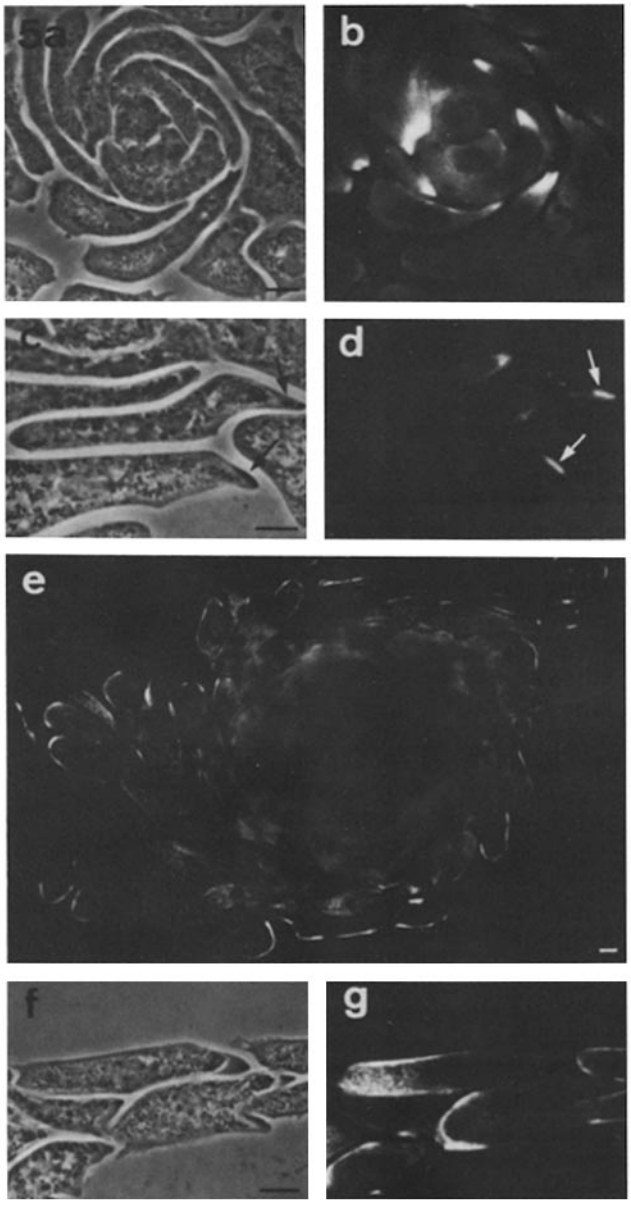

FIGURE 5 Distribution of actin and myosin in aggregating amebae prepared by the agar-overlay technique. (*a* and *b*) Staining with antiactin of cells undergoing spiral movement to the aggregation center. The anterior pseudopode and the cell cortex were stained. (*c* and *d*) High magnification micrographs of cells in the aggregation stream stained with antiactin. Note the rod-like staining in the anterior pseudopode and a comparative phase-contrast image (arrows). (e) Macroscopic immunofluorescence of the aggregation center stained with monoclonal antimyosin (DM-2). Staining of the lateral as well as the posterior cortex was prominent in the cells at the outermost periphery of the aggregate. (*f* and *g*) High magnification micrographs of cells in the aggregation stream stained with DM-2. Note the specific staining of the lateral as well as the posterior cortex. Bars, 5 μm.

suspended cells showed that fixation with 2.5% formaldehyde in the phosphate buffer for 10 min followed by extraction with −10°C methanol for 5 min resulted in good preservation of the cellular morphology. Actin and myosin were also localized mainly at the cortical region of the suspended cells (Fig. 6, *a* and *b*). This observation, together with the evidence that the amebae could undergo normal aggregation, encouraged us to apply the agar-overlay technique for the immunofluorescence. We were rewarded with well-preserved cellular structures and high-resolution fluorescent images.

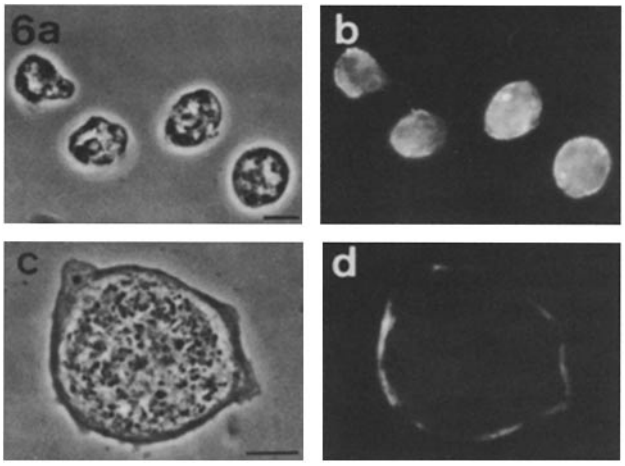

FIGURE 6 (a and *b*) Immunofluorescence of suspended cells prepared by a conventional method. The cells were fixed with formaldehyde, extracted with methanol, then stained with antiactin. The cell periphery was stained most brightly, showing the plausibility of cortical staining after the agar-overlay procedure. (c and *d*) Immunofluorescence using polyclonal antimyosin serum, showing the staining pattern was essentially identical to that with DM-2. Bars, 5 μm.

Evaluation of the Fluorescent Staining

Cellular structures were well preserved by the agar-overlay technique despite the fact that no strong cross-linking fixatives are allowed in immunofluorescence. The good preservation probably resulted from (*a*) rapid fixation by dipping the samples into cold methonal and (*b*) the prevention of disruption during the staining steps.

We next considered whether the crescent-shaped fluorescence by monoclonal DM-2 antibody reflects the specific supramolecular architecture of myosin, since monoclonal antibodies only recognizing specific epitopes are not unusual (7). We found that the staining patterns by polyclonal antimyosin sera were essentially identical to those by DM-2 (Fig. 6, *c* and *d*). This indicated that the fluorescence by DM-2 must correctly reflect the localization of myosin.

Several different patterns were observed in the staining with polyclonal antiactin. Very dense patch-like fluorescence (Fig. 4*h*) might reflect the well-developed microfilament meshworks revealed by electron microscopy (see Fig. 3*c* of reference 11). Peripheral staining might represent the loose microfilament layers localized beneath the plasma membrane (see Fig. 3*D* of reference 11). The diffuse fluorescence in the cytoplasm by antiactin (Fig. 3*b*) probably reflect the loose cytoplasmic mesh of microfilaments (represented by *Cf* in Fig. 2 of reference 10). The diffuse fluorescence probably does not reflect the monomeric form of actin, since soluble proteins are not likely to remain after the methanol fixation. Thus, the differential patterns of fluorescence seem to represent the different modes of organization of actin filaments.

We recognize that we must carefully evaluate the influence of the accessible volume on the amount of fluorescence (1). For example, we cannot exclude the possible influence of the difference in the pathlength caused by the included organelles (excluded volumes), although the thickness of the cells is mostly constant because of the overlaid agarose. However,

the totally different staining patterns of actin and myosin and the difference in the antiactin staining in some pseudopodes (Fig. 4, *e* and *f*) suggest that the amount of the fluorescence could not be totally, if any, interpreted by the accessible volume.

Significance of the Differential Organization of Actin and Myosin

An explanation was sought for the weak fluorescence of actin that was observed in regions where very strong and specific localization of myosin was found. The actin filaments may be organized to form only diffuse mesh works or bundles at the site where the force will be generated by a speculative sliding mechanism in analogy to the "sliding filament" theory (14) in skeletal muscle. This organization may be related to the contractile state of actin as proposed by Hellewell and Taylor (13) and might exhibit a very weak fluorescence. Conversely, the dense fluorescence by actin at the anterior pseudopode or at the contact region may reflect totally different forms of actin which might be involved in novel functions of *Dictyostelium* actin in terms of the determination of cellular polarity (30) as well as cell adhesion.

The differential organization of cytoskeletal elements in the cell cortex has been suggested by our previous study (30) and must be related to the multiforms of the regulatory compartments of *Dictyostelium*. Cortical distribution of some actin-binding proteins has been reported by Condeelis et al. (5) and Brier et al. (3), and Bazari and Clarke (2) have reported on a calmodulin localization in the cortical region. Our agar-overlay technique should be useful for studying the differential distribution of these regulatory components in the cell cortex. Specific fluorescence by antimyosin at the posterior cortex may represent the polymerized form of myosin regulated by dephosphorylation of its heavy chains, as revealed by Kuczmarski and Spudich (18).

Possible Coordination of the Differential Cortical Organization

The present study has suggested that there are different forms of actin in the supramolecular organization in the cell cortex. The posterior accumulation of myosin suggested that the motive force of the ameboid movement is generated at the posterior end, supporting the idea originally proposed by Mast (21) for *Ameba*. Also, this localization of myosin might be coupled with the formation of the anterior pseudopode which showed bright actin staining. This suggestion led us to speculate that the differential organization of cytoskeletal elements is coordinated with physiological activities, i.e., chemotactic movement. This coordination has been suggested by a sophisticated study by Swanson and Taylor (27) in chemotactically activated amebae. The high resolution immunofluorescence by the agar-overlay technique should provide significant information along this line of studies.

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Note Added in Proof: During the preparation of the manuscript, Rubino and co-workers (Rubino, S., M. Fighetti, E. Unger, and P. Cappuccinelli, 1984, *J. Cell Biol.*, 98:382–390.) published an article on the immunofluorescent localization of actin, myosin, and tubulin of *Dictyostelium*.

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