Release of Myosin II from the Membrane–Cytoskeleton of *Dictyostelium discoideum* Mediated by Heavy-Chain Phosphorylation at the Foci within the Cortical Actin Network

Shigehiko Yumura and Toshiko Kitanishi-Yumura

Biological Institute, Faculty of Science, Yamaguchi University, Yamaguchi 753, Japan

Abstract. Membrane–cytoskeletons were prepared from *Dictyostelium* amebas, and networks of actin and myosin II filaments were visualized on the exposed cytoplasmic surfaces of the cell membranes by fluorescence staining (Yumura, S., and T. Kitanishi-Yumura. 1990. *Cell Struct. Funct.* 15:355–364). Addition of ATP caused contraction of the cytoskeleton with aggregation of part of actin into several foci within the network, but most of myosin II was released via the foci. However, in the presence of 10 mM $MgCl_2$, which stabilized myosin II filaments, myosin II remained at the foci. Ultrastructural examination revealed that, after contraction, only traces of monomeric myosin II remained at the foci. By contrast, myosin II filaments remained in the foci in the presence of 10 mM $MgCl_2$. These observations suggest that myosin II was released not in a filamentous form but in a monomeric form. Using [γ^{32}P]ATP, we found that the heavy chains of myosin II released from membrane–cytoskeletons were phosphorylated, and this phosphorylation resulted in disassembly of myosin filaments. Using ITP (a substrate for myosin II ATPase) and/or ATPγS (a substrate for myosin II heavy-chain kinase [MHCK]), we demonstrated that phosphorylation of myosin heavy chains occurred at the foci within the actin network, a result that suggests that MHCK was localized at the foci. These results together indicate that, during contraction, the heavy chains of myosin II that have moved toward the foci within the actin network are phosphorylated by a specific MHCK, with the resultant disassembly of filaments which are finally released from membrane–cytoskeletons. This series of reactions could represent the mechanism for the relocation of myosin II from the cortical region to the endoplasm.

MYOSIN II, which is one of the major components of the cytoskeleton in nonmuscle cells, produces the motive force necessary for cell movements and cytokinesis via interactions with actin filaments. Myosin II isolated from *Dictyostelium* amebas can assemble into bipolar thick filaments in vitro (28). Immunofluorescence studies and immunoelectron microscopy have shown that myosin II in *Dictyostelium* amebas, similar to myosin in muscle cells, forms filaments in vivo (30, 31). In addition, most of the myosin II in Triton-X100–insoluble cytoskeletons of *Dictyostelium* amebas is in the filamentous form (4, 27). All these observations suggest that, in *Dictyostelium* amebas, actin and myosin II generate the motive force by a mechanism analogous to the sliding-filament model of actomyosin in muscle cells. However, actin and myosin filaments in *Dictyostelium* amebas, unlike those in muscle cells, show no evidence of any regular arrangement such as that observed in the sarcomere in muscle cells and, in addition, they do not stay at a single site but can relocate within a cell. For example, myosin II filaments are concentrated at the tail region during locomotion, while they are concentrated in the furrow region to form the contractile ring during cell division (12, 30, 33). Upon chemotactic stimulation of cells at the aggregation stage with the chemoattractant cAMP, myosin filaments in the endoplasm are translocated to the ectoplasm (cortical region) and then return to the endoplasm (30). The velocity of the relocation of myosin filaments within the cell seems to be very high. In the case of chemotactic stimulation, it takes only 2 min at 4°C and only 30 s at room temperature for myosin filaments to relocate from the endoplasm to the ectoplasm (cortical region) (23).

Myosin II filaments in the cortical region are considered to be essential to the generation of motive force, in view of their accumulation in the cortical region in actively locomoting cells and during cytokinesis. About 30 s after chemotactic stimulation with cAMP, when myosin filaments have moved from the endoplasm to the cortical region, cells contract and become spherical in shape, and they are referred to as being in the "cringing phase" (9, 24). In parallel with this relocation of myosin filaments, phosphorylation of the heavy chains of myosin, in addition to phosphorylation of the light chains, occurs transiently (1, 2, 25). With regard to heavy-chain phosphorylation, it has been shown in studies with the isolated kinase and the phosphatase that phosphorylated myosin does not assemble and remains in a monomeric form under physiological conditions that allow dephosphor-

ylated myosin to assemble into filaments (15, 16). Therefore, we can ask two questions. Does the phosphorylation of heavy chains regulate the assembly and disassembly of myosin II in vivo and, furthermore, is such regulation via heavy-chain phosphorylation related to the relocation of myosin filaments in the cell?

In the present study, we prepared membrane–cytoskeletons, which consisted of cell membranes and networks of actin filaments decorated with myosin II filaments, from *Dictyostelium* amebas by an improved version (32) of the method originally reported by Clarke et al. (5). Addition of ATP caused contraction with resultant aggregation of part of the actin into several foci within the actin networks on the membrane–cytoskeletons. In contrast to the actin, the myosin II was released from the membrane–cytoskeletons during contraction and, at the same time, phosphorylation of myosin heavy chains occurred, which resulted in the disassembly of myosin filaments. From results of experiments with ITP as a substrate for myosin ATPase and/or ATPγS as a substrate for myosin heavy-chain kinase (MHCK)[1], we determined that phosphorylation of myosin II heavy chains occurred at the foci within the actin network, which suggests that the MHCK was localized at the foci. All these results together indicated that, in living cells, myosin filaments in the cortical region return to the endoplasm not in a filamentous form but in a monomeric form after contraction, and that phosphorylation of myosin heavy chains participates in this process. This series of reactions could explain the dynamic relocation of myosin II from the ectoplasm (cortical region) to the endoplasm during chemotaxis.

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 (3). Vegetative cells were harvested and freed from bacteria by washing three times with cold distilled water. Transformed myosin null mutants, HS2206, with the plasmid pSB3 were cultured according to Egelhoff et al. (7). The washed cells were spread on nonnutrient agar and incubated at 21°C until use.

Preparation of Membrane–Cytoskeletons

Cells harvested from nonnutrient agar were suspended in a cold solution of 5 mM $MgCl_2$ in distilled water. As described previously, 5 mM $MgCl_2$ was most effective for cell spreading (32). An aliquot of the suspension was placed on a polylysine-coated coverslip. The coverslips had been prepared by treating well-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 (11) and 5 mM $MgCl_2$ for 30 s to eliminate the nonspecific binding of ruptured cell debris, and then a jet of chilled microfilament-stabilizing solution (MFSS) (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 with a 25-gauge needle across the surface of the coverslips. Immediately after the rupture of the upper portion of cells by the jet of MFSS, the samples were immersed in chilled MFSS for 5 min. For visualization of actin filaments and myosin II, membrane–cytoskeletons on the coverslips were treated with tetramethylrhodamine-conjugated phalloidin and monoclonal myosin II–specific antibody (DM-2) in MFSS at 25°C for 30 min, washed with MFSS, and incubated at 25°C for 30 min with fluorescein-conjugated second antibody raised in goat. For the ATP-contraction experiments, with the exception of those for which results are shown in Fig. 1 (*a–d*), membrane–cytoskeletons on the coverslips were first stained only with tetramethylrhodamine-conjugated phalloidin for 10 min. Next, they were rinsed with MFSS or with a test solution, and then they were treated with the same solution supplemented with 0.1 mM ATP for 5 min. In the analysis of the possible MHCK activity in membrane–cytoskeletons, 1 mM ITP and/or 4 mM ATPγS were used in place of ATP. Then the samples were immunostained with myosin II–specific antibody and fluorescein-conjugated second antibody for visualization of myosin II. After a rinse with MFSS, the samples were mounted in the same solution supplemented with 10% polyvinyl alcohol and 0.1% *p*-phenylene diamine and observed under an epifluorescence microscope (Nikon XF-EFD2).

1. *Abbreviations used in this paper*: MFSS, microfilament stabilizing solution; MHCK, myosin II heavy chain kinase.

Transmission Electron Microscopy

Membrane–cytoskeletons on plastic coverslips (Lux Scientific Corporation, Newbury Park, CA) were prefixed in methanol that contained 1% formalin at −15°C for 5 min, and then they were fixed with 0.05% glutaraldehyde and 1% formaldehyde plus 0.01% tannic acid in MFSS on ice for 30 min. After washing with MFSS for 20 min on ice, the samples were postfixed with 1% osmium tetroxide on ice for 30 min, and then washed with distilled water. Next, the samples were dehydrated in a graded ethanol series, substituted with propylene oxide, and embedded in Spurr's resin. In the preparations for the immunoelectron microscopy, membrane–cytoskeletons were sequentially treated with monoclonal myosin II–specific antibody (DM-2) in MFSS for 1 h at 25°C and second antibody conjugated with 5-nm colloidal gold particles (Janssen Ltd., Beerse, Belgium) in MFSS for 1 h at 25°C before fixation. After the resin had polymerized, the coverslip was removed by rapidly cooling the resin on a block of dry ice. The resin block was divided into pieces, mounted, and thin-sectioned parallel to the substratum on an ultratome. The sections were stained with 1% uranyl acetate and Reynold's lead citrate, and then they were observed under a JEM 100-C electron microscope.

SDS-PAGE and Autoradiography

To prepare larger amounts of membrane–cytoskeletons, polylysine-coated glass slides (26 × 25 mm) were used in place of the polylysine-coated coverslips. Membrane–cytoskeletons were treated with 3.3 ng/ml phalloidin in MFSS for 20 min at 25°C and then 0.1 mM [γ^{32}P]ATP (1 μCi, 100 mCi/mmol) in MFSS was applied to membrane–cytoskeletons on the glass slides to cause contraction. After a 5-min incubation at 25°C, the solution on the slides, which contained proteins released from membrane–cytoskeletons during contraction, was carefully collected into a microtube and 5 μg of BSA and 1 μg of myosin II purified from *Dictyostelium* were added to it. Proteins in the solution collected in the microtube were precipitated by addition of TCA and separated by SDS-PAGE as described by Laemmli (18). Finally, the proteins were stained with Coomassie brilliant blue G and dried gels were subjected to autoradiography with Kodak X-Omat AR film.

For the quantitative assay, much larger amounts of membrane–cytoskeletons were prepared, and carriers were not added. Bands of myosin II heavy chain on Coomassie brilliant blue–stained gels were cut out and their radioactivities were measured. The protein amount of myosin heavy chain was calculated by densitometry of Coomassie brilliant blue–stained gel.

Assay for Myosin II Heavy Chain Kinase

The membrane–cytoskeletons (equivalent to two slide glasses) were scraped by a silicon scraper and collected in a microtube. The sample was homogenized by a sonicator. 1 mg of *Dictyostelium* myosin II was added and ATP, ITP, or ATPγS added to a final concentration of 1 mM. After the incubation for 30 min, the protein was precipitated by the addition of 6% TCA and washed three times with 6% TCA. The amount of phosphorus that the precipitated proteins contained was estimated by the method of Lowry et al. (18) after the ashing in H_2SO_4 and $HClO_4$. KH_2PO_4 was used as standard. The amount of phosphorous incorporated in myosin II was calculated by the subtraction of the value of the reaction mixture which did not contain myosin II from the value of the reaction mixture.

Results

Myosin II Is Released from Membrane–Cytoskeletons via Foci within the Actin Network

We prepared complexes that consisted of the cell membrane

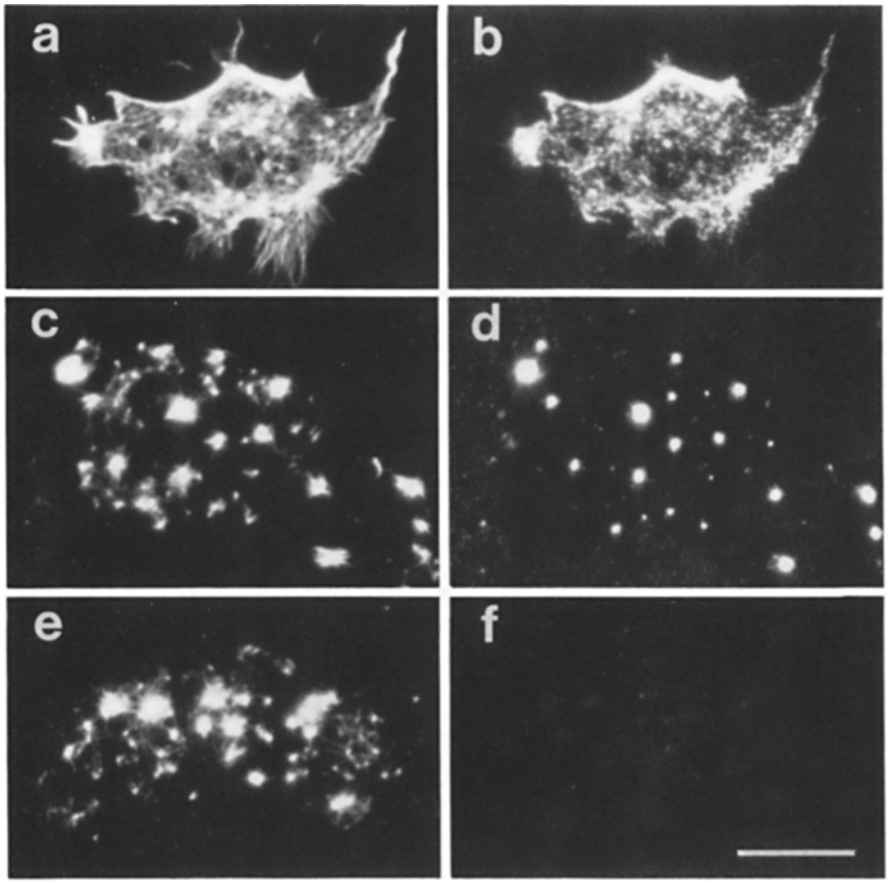

Figure 1. Double-immunofluorescence staining of unfixed membrane–cytoskeletons with tetramethylrhodamine-conjugated phalloidin, for staining of actin filaments (*a*, *c*, and *e*), and with antibody against myosin II from *Dictyostelium* and fluorescein-conjugated second antibody, for staining of myosin II (*b*, *d*, and *f*), before (*a* and *b*) and after (*c–f*) contraction caused by the addition of 0.1 mM ATP. (*a* and *b*) A membrane–cytoskeleton before contraction. A network of actin filaments (*a*) and numerous myosin filaments (*b*) are seen on the exposed cytoplasmic surface of the membrane–cytoskeleton. (*c–f*) Membrane–cytoskeletons after contraction. (*c* and *d*) ATP was added after the immunostaining with myosin II–specific antibody and fluorescein-conjugated second antibody. Part of actin (*c*) and almost all of myosin II (*d*) have aggregated into several foci within the actin network to form large fluorescent dots. (*e* and *f*) ATP was added before the immunostaining with antibodies. Part of actin has aggregated at several foci within the actin network (*e*), but almost all of myosin II has disappeared (*f*). Bar, 10 μm.

and cytoskeleton from *Dictyostelium* amebas by the method described previously (32). In brief, the upper portions of cells that had become tightly attached to a polylysine-coated coverslip were removed with a jet of MFSS squirted from a syringe, and then the cell membranes left on the coverslip were immediately stained with tetramethylrhodamine–conjugated phalloidin, for staining of actin filaments, and with antibody against myosin II from *Dictyostelium* and fluorescein-conjugated second antibody, for staining of myosin II. On the exposed cytoplasmic surface of the cell membranes, networks of actin filaments (Fig. 1 *a*) and numerous rod-like structures of myosin II, or myosin II filaments in situ (31), aligned along the actin filaments (Fig. 1 *b*) were observed. We call these intact complexes of the cell membrane and cytoskeleton "membrane–cytoskeletons."

As described previously (32), addition of 0.1 mM ATP to

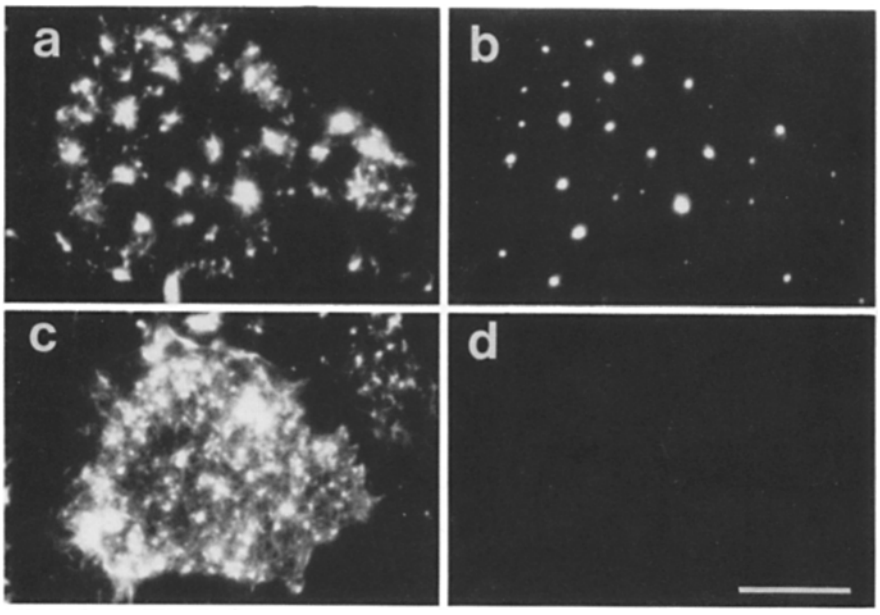

Figure 2. Double-immunofluorescence staining of unfixed membrane–cytoskeletons with tetramethylrhodamine-conjugated phalloidin, for staining of actin filaments (*a* and *c*), and with antibody against myosin II from *Dictyostelium* and fluorescein-conjugated second antibody, for staining of myosin II (*b* and *d*), after contraction in the presence of 10 mM $MgCl_2$ (*a* and *b*), or in the presence of 200 mM KCl (*c* and *d*). (*a* and *b*) Myosin II remains at the contracted actin dots after contraction in the presence of 10 mM $MgCl_2$ (*b*). (*c* and *d*) Myosin II has been released and can not be seen at the contracted actin dots after contraction in the presence of 200 mM KCl (*d*). Bar, 10 μm.

the membrane-cytoskeletons after the treatment with tetramethylrhodamine-conjugated phalloidin and with myosin II-specific antibody and fluorescein-conjugated second antibody caused the aggregation of part of actin and almost all of myosin II into several foci within the actin network, with the formation of large fluorescent dots, or "contracted actin dots", on the cell membrane within a second (Fig. 1, *c* and *d*), though the contour of the cell membrane was not altered. Contraction of the membrane-cytoskeletons occurred independently of the presence of absence of Ca^{2+} ions (data not shown). When 0.1 mM ATP in MFSS was added to the membrane-cytoskeletons before the treatment with myosin II-specific antibody and fluorescein-conjugated second antibody, part of actin aggregated into several foci within the actin network (Fig. 1 *e*) but, unexpectedly, almost all of myosin II disappeared (Fig. 1 *f*). When 0.1 mM ATP was added to the membrane-cytoskeletons after the treatment with fluorescein-conjugated Fab fragment of myosin II-specific antibody, we were able to observe the myosin filaments moving toward the actin foci and releasing at the foci. These observations suggest that, during contraction caused by the addition of ATP, myosin filaments in the membrane-cytoskeletons first move toward the foci and then, via these foci, they are released from the membrane-cytoskeletons. However, in the case of contraction after the decoration with the myosin II-specific antibody and fluorescein-conjugated second antibody, it is conceivable that complexes which consisted of myosin II molecules and antibodies might have hindered the release of myosin II from the foci and, as a result, myosin II remained at the foci.

Myosin II Filaments Are Disassembled and Released from Membrane-Cytoskeletons

When 0.1 mM ATP was added to the membrane-cytoskeletons in the presence of 10 mM $MgCl_2$, in place of 2 mM $MgCl_2$, in MFSS and then the sample was immunostained, myosin II remained at the contracted actin dots formed during contraction (Fig. 2, *a* and *b*). By contrast, when 0.1 mM ATP was added to the membrane-cytoskeletons in the presence of 200 mM KCl, in place of 15 mM KCl, in MFSS and then the sample was immunostained, myosin II could not be seen at the actin foci (Fig. 2, *c* and *d*), though there was only a small scale rearrangement of the actin filaments. Neither rearrangements of the actin filaments nor decrease of myosin II were observed by the treatment with only 10 mM $MgCl_2$ or 200 mM KCl in MFSS which did not contain ATP. These results suggest that the filamentous form of myosin II, which was stabilized in the presence of 10 mM $MgCl_2$, could not be released from the membrane-cytoskeletons, while monomeric myosin II in the presence of 200 mM KCl was released from the membrane-cytoskeletons during contraction. Thus, it appears that during contraction caused by the addition of ATP, myosin filaments in the membrane-cytoskeletons disassemble and then are released via the contracted actin dots in monomeric form.

Ultrastructure of Contracted Actin Dots in Membrane-Cytoskeletons

The membrane-cytoskeletons before and after contraction were fixed, embedded, thin-sectioned parallel to the substratum, and examined under the electron microscope. The samples were fixed by the method described in our previous report to preserve the ultrastructure of myosin II filaments. Myosin II filaments are susceptible to chemical fixatives that are conventionally used during preparation for EM (31). When the membrane-cytoskeletons were fixed before contraction, filaments of ~12 nm in thickness and <0.5 μm in length were observed in the sections near the cell membrane (Fig. 3 *a*). We showed previously by immunoelectron microscopy that these 12-nm-thick filaments are myosin II filaments in situ (31). However, also as described previously, we were unable to preserve the ultrastructure of actin filaments effectively under these conditions and, therefore, the actin filaments seemed to be somewhat tattered. When the membrane-cytoskeletons were fixed after contraction in MFSS, characteristic dense dot-like structures were observed in the sections near the cell membrane, but no myosin filaments were seen at all. By contrast, in the membrane-cytoskeletons fixed after contraction in the presence of 10 mM $MgCl_2$, 12 nm-thick myosin filaments were observed in similar dense dot-like structures (Fig. 3, *b* and *c*). Immunogold labeling of the membrane-cytoskeletons was then carried out using myosin II-specific antibody and colloidal gold-conjugated second antibody after contraction in MFSS. In immunogold-labeled preparations, some deposits of colloidal gold were observed in the dense dot-like structures (Fig. 3 *d*), which may represent the remnants of myosin II. However, myosin II remaining in the dense dot-like structures seemed not to be filamentous but to be in the monomeric form, unlike myosin II observed in the membrane-cytoskeletons that were fixed after contraction in the presence of 10 mM $MgCl_2$ in which myosin filaments were preserved (Fig. 3, *b* and *c*). These results indicate that the characteristic dense dot-like structures correspond to the contracted actin dots visualized by the fluorescence staining (Fig. 1, *c-f*), and that disassembly of myosin filaments occurs during contraction in MFSS (Fig. 3 *d*). By contrast, in the case of contraction in the presence of 10 mM $MgCl_2$, disassembly of myosin filaments is suppressed and a substantial number of myosin filaments remain in the contracted actin dots.

Heavy Chains of Myosin II Released from Membrane-Cytoskeletons Are Phosphorylated

Assembly of purified myosin II molecules from *Dictyostelium* is regulated by the phosphorylation of their heavy chains in vitro, and the phosphorylation is catalyzed by a specific kinase, the MHCK. The equilibrium between monomers and filaments has been examined under various ionic conditions with both phosphorylated and dephosphorylated myosin II (16), and phosphorylated myosin II is known to be in the monomeric form in MFSS, while dephosphorylated myosin II forms filaments in MFSS. It is also shown by in vitro studies that high concentrations of ATP promote partial disassembly of myosin II filaments (16). However, 0.1 mM ATP in the presence of 2 mM $MgCl_2$ in MFSS did not promote disassembly of myosin II filaments (data not shown).

Proteins which were released from the membrane-cytoskeletons during contraction were analyzed by SDS-PAGE, and actin and myosin II were found to be major components of the protein released from the membrane-cytoskeletons (data not shown). 90% of the total myosin II and 15% of the total actin were released. But, we find it difficult to estimate accurately the amount of actin and myosin II remaining in

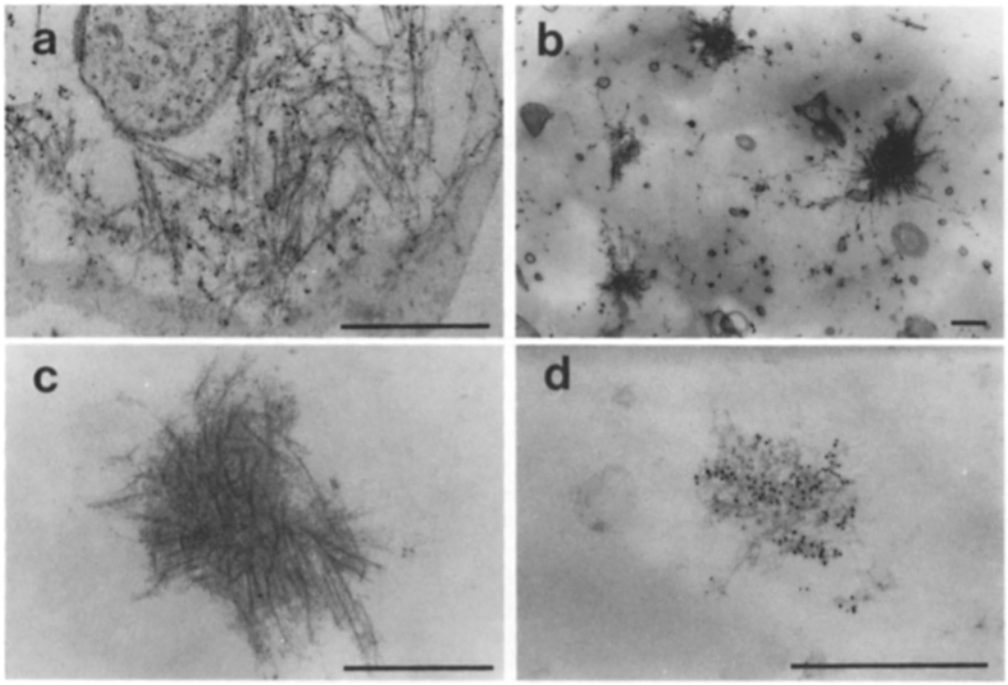

Figure 3. Transmission electron micrographs of membrane-cytoskeletons before (*a*) and after (*b–d*) contraction. (*a*) A membrane-cytoskeleton fixed before contraction. Filaments of ~12 nm in thickness and <0.5 μm in length are observed in the sections near the cell membrane, and these are myosin II filaments in situ. (*b* and *c*) Membrane-cytoskeletons fixed after contraction in the presence of 10 mM $MgCl_2$. Characteristic dense dot-like structures (*b*) are observed in the sections near the cell membrane, and myosin II filaments (*c*) are observed in the dense dot-like structures. (*d*) Immuno-gold labeling of a membrane-cytoskeleton with myosin II-specific antibody and colloidal gold-conjugated second antibody after contraction in MFSS. Myosin II filaments are not seen at all and only some deposits of colloidal gold can be seen in the dense dot-like structures. Bar, 500 nm.

the membrane-cytoskeletons because we observed by a fluorescence microscopy that some fraction of released actin and myosin II tends to re-adhere to a polylysine-coated glass. Next, contraction was performed in the presence of [γ^{32}P]-ATP, and the phosphorylated proteins released from the membrane-cytoskeletons were analyzed by autoradiography of a gel after SDS-PAGE. In this experiment, BSA and purified myosin II from *Dictyostelium* were used as carriers because the amount of protein released from the membrane-cytoskeletons was very small (this modification was suggested by Dr. S. Takahashi). In addition, the use of purified myosin II from *Dictyostelium* provided a marker for the myosin II heavy chains. The major phosphorylated protein was the myosin II heavy chain. No phosphorylation of light chains of myosin II was detected (Fig. 4, lane *5*) even though the sample was run on a higher percentage SDS polyacrylamide gel. Quantitative assay showed that 0.05 mol of phosphate was incorporated per mole of myosin heavy chain. In the case of contraction in the presence of 10 mM $MgCl_2$, no phosphorylated heavy chains of myosin II were detected in the protein released from the membrane-cytoskeletons (Fig. 4, lane *6*). Less than 5% of the total myosin II was released in this case. It was estimated that 0.05 mol of phosphate was incorporated per mole of myosin heavy chain left behind unless any fraction of released myosin II from the membrane-cytoskeletons could re-adhere to a polylysine-coated glass. These results show that the heavy chains of myosin II released from the membrane-cytoskeletons are phosphorylated during contraction.

Phosphorylation of Myosin II Heavy Chains Is Prerequisite to the Release of Myosin II from Membrane-Cytoskeletons

Recently, Egelhoff et al. (7) have transformed myosin null *Dictyostelium* cells with a vector expressing an altered myosin II gene that eliminates the terminal 34-kD of the tail of myosin II heavy chain (the site of myosin II heavy chain phosphorylation) (29). These transformants display excessive localization of truncated myosin II in the cortical cytoskeleton. We examined whether or not truncated myosin II was released from the membrane-cytoskeletons prepared from the transformants. Rod-like structures of truncated my-

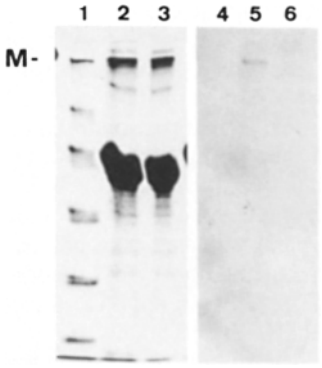

Figure 4. Analysis of the phosphorylation of proteins released from membrane-cytoskeletons during contraction by autoradiography (lanes *4–6*) of a gel stained after SDS-PAGE (lanes *1–3*). BSA and purified myosin II from *Dictyostelium* were added to the samples in lanes 2 and 3 as carriers. (Lane *1*) Molecular mass markers, *Dictyostelium* myosin II heavy chain (215 kD), phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), from the top; (lane *2*) proteins released from membrane-cytoskeletons during contraction; (lane *3*) proteins released from membrane-cytoskeletons during contraction in the presence of 10 mM $MgCl_2$; (lane *4*) autoradiography of lane 1; (lane *5*) autoradiography of lane 2 (the major phosphorylated proteins were myosin II heavy chains); (lane *6*) autoradiography of lane 3 (no phosphorylated myosin II heavy chains were detected); M, heavy chains of *Dictyostelium* myosin II. BSA and *Dictyostelium* myosin II that have been added as carriers were seen in Coomassie blue-stained gel (lanes *2* and *3*).

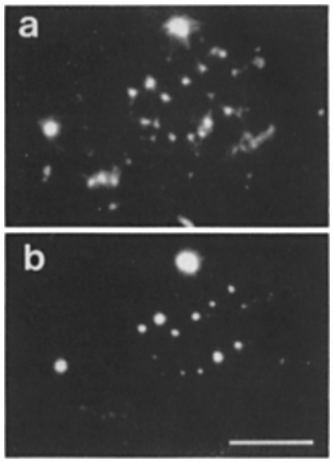

Figure 5. Double-immunofluorescence staining of membrane-cytoskeletons prepared from pBS3 transformants with tetramethylrhodamine-conjugated phalloidin (*a*), and with antibody against myosin II from *Dictyostelium* and fluorescein-conjugated second antibody (*b*), after contraction caused by the addition of 0.1 mM ATP. Note that myosin II remains at the contracted actin dots after contraction. Bar, 10 μm.

osin filaments were observed when the membrane-cytoskeletons prepared from the transformants were stained with antibody against myosin II (data not shown). When 0.1 mM ATP in MFSS was added to the membrane-cytoskeletons before the treatment with antibody against myosin II, myosin II was not released from the membrane-cytoskeletons and remained at the contracted actin dots (Fig. 5, *a* and *b*). This observation indicates that the phosphorylation of myosin II heavy chains is prerequisite to the release of myosin II from the membrane-cytoskeletons.

Myosin II Is Phosphorylated at the Foci within the Actin Network

As indicated above, phosphorylation of the myosin II heavy chains occurred during contraction of the membrane-cytoskeletons, with the resultant disassembly of myosin filaments into monomers that could be released from the membrane-cytoskeletons via the foci within the actin network. Where does this phosphorylation of myosin heavy chains occur? To date, the location of MHCK in cells has not been determined. It is possible that MHCK is associated with myosin filaments or myosin molecules and that phosphorylation of myosin heavy chains occurs in the course of the movement of myosin filaments toward the foci within the actin network during contraction. Alternatively, MHCK may be localized at the foci within the actin network, so that phosphorylation of myosin heavy chains would then occur at the foci after contraction. To ascertain which possibility is more likely, we used ITP and/or ATPγS in place of ATP for contraction of the membrane-cytoskeletons and examined whether or not myosin II was released from the membrane-cytoskeletons. Kuczmarski et al. (17) reported that ITP but not ATPγS can act as the substrate for the myosin II. ATPγS but not ITP can act as the substrate for MHCK. The amount of incorporated phosphorus in myosin II increased at 4 ng/30 min/mg when the homogenized membrane-cytoskeletons and isolated myosin II were incubated with ATPγS. The value was 6 ng/30 min/mg with ATP. However, there was no increase of phosphorus in myosin II in the case of ITP. When 1 mM ITP was added to the membrane-cytoskeletons, contraction of the membrane-cytoskeletons occurred. Recently, Kuczmarski et al. (17) reported that 0.5 mM ITP induced little contraction of the Triton-insoluble cytoskeletons of *Dictyostelium* although ITP could be hydrolyzed by myosin II. In our studies, <1 mM ITP did not induce contraction of the membrane-cytoskeletons. It is plausible that more ITP might be necessary for the contraction because ITP is a poor substrate for myosin II. In the case of ITP, myosin II was not released from the membrane-cytoskeletons and remained at the contracted actin dots (Fig. 6, *a* and *b*). By contrast, when 4 mM ATPγS and 1 mM ITP were added to the membrane-cytoskeletons, myosin II seemed to be released from the membrane-cytoskeletons and was no longer found at the contracted actin dots (Fig 6, *c* and *d*). Next, after myosin II had aggregated at the contracted actin dots as a result of the addition of 1 mM ITP, 0.1 mM ATP was added to the contracted membrane-cytoskeletons. Myosin II was released from the membrane-cytoskeletons and was no longer found at the contracted actin dots (Fig. 6, *e* and *f*). When the membrane-cytoskeletons were treated with 4 mM ATPγS only, contraction did not occur (data not shown). Finally, the membrane-cytoskeletons were treated first with 4 mM ATPγS and then, after a rinse with MFSS, with 1 mM ITP. In this case, contraction occurred, but myosin II was not released and remained at the contracted actin dots (Fig. 6, *g* and *h*).

These results indicate that phosphorylation of myosin heavy chains occurs at the foci within the actin network, and that phosphorylation does not occur before the arrival of myosin II filaments at these foci. This conclusion suggested that MHCK is localized at the foci within the actin network and that phosphorylation of myosin heavy chains occurs at the foci after contraction. Thus, the second possibility mentioned above seems most plausible.

To summarize all the results obtained here, a model of the way in which myosin II filaments in the membrane-cytoskeletons (cortical region) return to the endoplasm is presented in Fig. 7. During the ATP-dependent contraction, myosin II filaments in the membrane-cytoskeletons slide on actin filaments toward the foci within the actin network. At the foci, where specific MHCK is located, phosphorylation of myosin II heavy chains occurs and, as a result, the myosin II filaments disassemble into monomers. Then myosin II in its monomeric form is released from the foci and returns to the endoplasm. Actin filaments were recruited into the actin foci to form large "contracted actin dots" upon addition of ATP though this is not drawn in Fig. 7. Since the actin filaments are thought to be crosslinked and woven by several species of actin-binding proteins, the crosslinked actin filaments might be pulled into the actin foci during the contraction. It is plausible that smaller-scale reactions of actin filaments must occur under the careful control of actin-binding proteins in vivo (32).

Discussion

Cyclical Assembly and Disassembly of Myosin II Regulated by Heavy-chain Phosphorylation

In this study we found that, during contraction caused by the addition of ATP to the membrane-cytoskeletons of *Dictyostelium* amebas, myosin II filaments in the membrane-cytoskeletons first moved toward a number of foci within the actin network, and then they were released from the membrane-cytoskeletons as myosin II monomers. We also found that, in parallel with this process, phosphorylation of myosin heavy chains occurred, with the resultant disassembly of

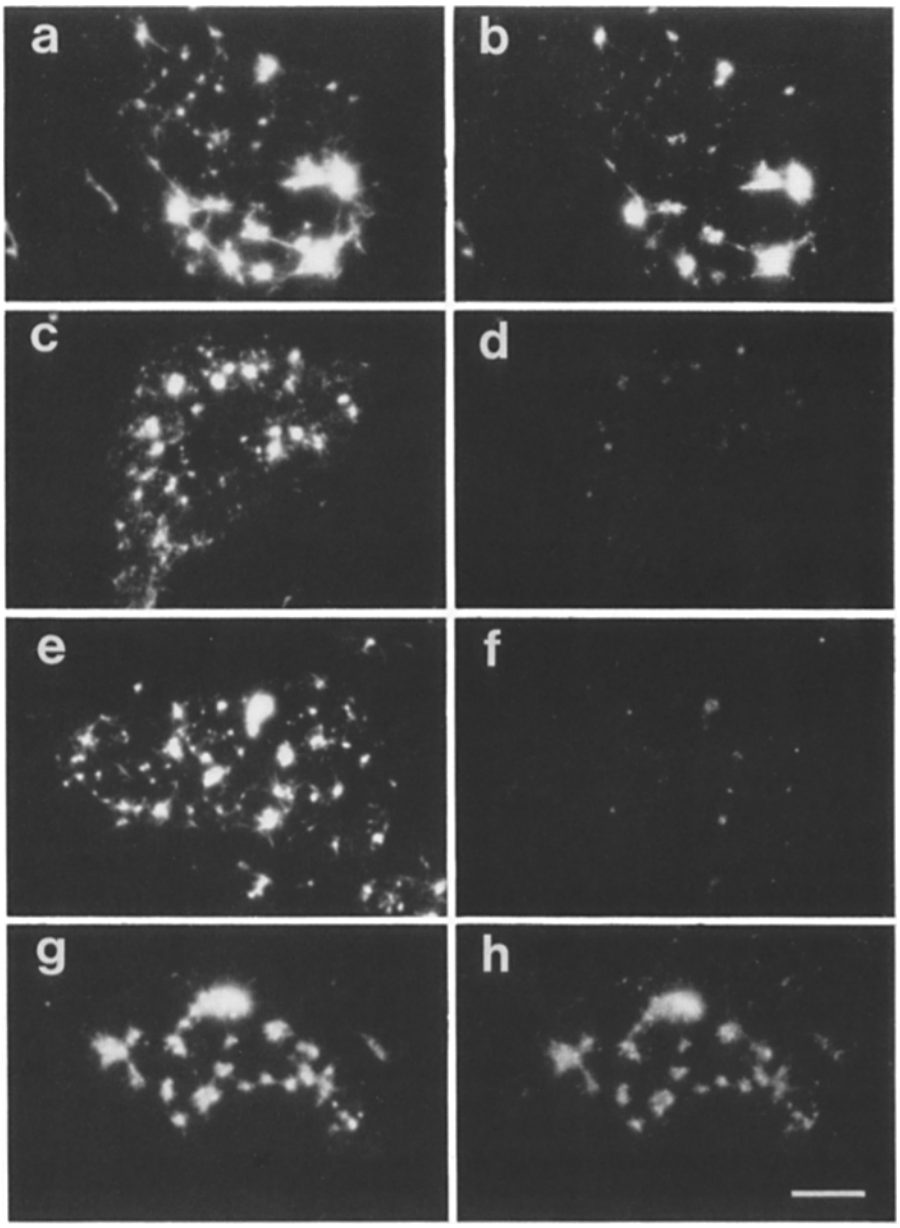

Figure 6. Double-immunofluorescence staining of unfixed membrane–cytoskeletons with tetramethylrhodamine-conjugated phalloidin, for staining of actin filaments (*a*, *c*, *e*, and *g*), and with antibody against myosin II from *Dictyostelium* and fluorescein-conjugated second antibody, for staining of myosin II (*b*, *d*, *f*, and *h*), after the treatments with ITP (substrate for myosin II ATPase) and/or ATPγS (substrate for myosin II heavy-chain kinase) in place of ATP. (*a*, *b*) A membrane–cytoskeleton treated with 1 mM ITP. Contraction occurred, but myosin II was not released from the membrane–cytoskeleton and remained at the contracted actin dots. (*c* and *d*) A membrane–cytoskeleton treated with a mixture of 4 mM ATPγS and 1 mM ITP. Myosin II was released from the membrane–cytoskeleton and did not remain at the contracted actin dots. (*e* and *f*) A membrane–cytoskeleton treated first with 1 mM ITP and then with 0.1 mM ATP. Myosin II was released from the membrane–cytoskeleton and did not remain at the contracted actin dots. (*g* and *h*) A membrane–cytoskeleton treated first with 4 mM ATPγS and then with 1 mM ITP. Contraction occurred but myosin II was not released and remained at the contracted actin dots. Bar, 10 μm.

myosin filaments. The possible mechanism of the release of cortical myosin II mediated by the heavy chain phosphorylation was firmly supported by the experiments using the membrane–cytoskeletons of truncated myosin II transformants whose myosin II is devoid of the site of heavy chain phosphorylation (Fig. 5).

The amount of phosphate incorporated into the released myosin II was unexpectedly small (0.05 mol phosphate per mole of myosin heavy chain). Based on in vitro studies, complete disassembly of *Dictyostelium* myosin filaments requires at least 1 mol of phosphate incorporated per mole of myosin heavy chain, though it might vary depending on the buffer conditions. One possible explanation is that myosin phosphatase activity associated with the membrane–cytoskeleton might rapidly remove phosphate from the phosphorylated myosin heavy chains. However, the ratio of incorporated phosphate and myosin heavy chain was constant in our experiments. In addition, the use of phosphatase inhibitors did not increase the amount of phosphate incorporated into the myosin heavy chain (data not shown). Another explanation is given below. The ratio (0.05 mol phosphate per mole of myosin heavy chain) indicates that one phosphate is incorporated into each myosin filament which consists of ~10 molecules of myosin (i.e., 20 molecules of myosin heavy chain), as revealed by our immunoelectron microscopic study (31). So, it is plausible that a myosin filament might disassemble transiently at the time of its passage through the actin foci by the incorporation of only one phosphate per myosin filament.

Berlot et al. (1) described that myosin II incorporates only 0.05 mol of phosphate per mole of heavy chain when developed *Dictyostelium* cells are labeled with [^{32}P] orthophosphate. Furthermore, the value increases by a factor of 1.8 when cells are stimulated with a chemoattractant. These results indicate that only a small fraction of myosin II molecules can be phosphorylated during the translocation of myosin II in a cell.

Myosin II filaments in *Dictyostelium* amebas can relocate

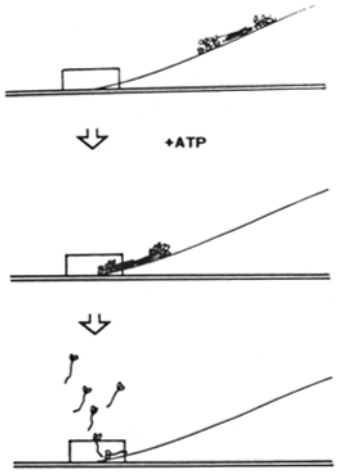

Figure 7. Schematic illustration of the proposed model of the way in which myosin II filaments in the cortical region return to the endoplasm as a result of heavy-chain phosphorylation during ATP-dependent contraction. Double lines and boxes on them in the illustration show the cell membrane and the foci within the actin network, respectively. Myosin II filaments in the cortical region (*top*) slide on actin filaments toward the foci within the actin network (*middle*). At the foci, where a specific MHCK is localized, phosphorylation of myosin heavy chains occurs, resulting in the disassembly of myosin filaments into myosin monomers, which can be released from the foci and return to the endoplasm (*bottom*).

within a cell to support the particular behavior of the cell. For example, in an actively locomoting cell, they are accumulated in the tail cortex, while during cytokinesis they are concentrated in the furrow region to form the contractile ring. We reported previously, as another example of such dynamic relocation of myosin filaments in the cell, that upon the chemotactic stimulation of *Dictyostelium* amebas at the aggregation stage with the chemoattractant cAMP, myosin filaments in the endoplasm move to the cortical region and then return again to the endoplasm (30). We recently observed that myosin filaments moved toward the actin foci of the cortical actin network and were released to the endoplasm during the chemotactic stimulation (our manuscript in preparation). The release of myosin filaments from the membrane–cytoskeletons, as observed in this study, appears to correspond to the relocation of myosin filaments from the cortical region to the endoplasm.

The heavy chains of myosin II that were released from the membrane–cytoskeletons were phosphorylated. Probably, as the next step in the cell, they are dephosphorylated again in the endoplasm by myosin heavy-chain phosphatase, with resultant reassembly into filaments. Usually, myosin filaments are present in the endoplasm as well as in the cortical region. In addition, as part of the chemotactic response, myosin filaments disappear from the endoplasm but soon reappear. These observations also suggest that the assembly of myosin occurs in the endoplasm. Kuczmarski and Pagone (14) have, in fact, isolated a myosin heavy-chain phosphatase from *Dictyostelium* amebas and they have also shown that a myosin heavy-chain phosphatase is present in the cell supernatant.

Immediately after their assembly, newly formed myosin filaments must become associated with actin filaments. However, it is also possible that myosin monomers are first associated with actin filaments and then the assembly of myosin filaments occurs on the actin filaments. In favor of this possibility, it was reported recently that actin filaments promote the assembly of myosin II (20). Myosin filaments associated with actin filaments move to the cortical region. Then, after contraction in the cortical region, they are disassembled via heavy-chain phosphorylation at the foci within the actin network, and they are again released from the cortical region as myosin monomers. Such cyclical assembly and disassembly of myosin molecules, mediated by heavy-chain phosphorylation, could explain the mechanism responsible for the relocation of myosin filaments between the cortical region and the endoplasm during the chemotactic response. In support of this possibility, the time course of the phosphorylation of myosin heavy chains corresponds closely to that of the relocation of myosin filaments during the chemotactic response (2).

Light Chains of Cortical Myosin II Are Phosphorylated

In *Dictyostelium*, unlike the case in other nonmuscle cells, phosphorylation of the light chains does not regulate the assembly of myosin II (10). However, the actin-activated ATPase activity of myosin II is regulated by the phosphorylation of light chains, that is, the actin-activated ATPase activity increases when the light chains of myosin II are phosphorylated, and dephosphorylation decreases the actin-activated ATPase activity. This phenomenon was also demonstrated in an experiment with opened *Nitella* cells in which beads coated with myosin II molecules that had been phosphorylated on their light chains could move along the bundles of actin filaments, but beads coated with dephosphorylated myosin II could not move. Phosphorylation of myosin light chains can be 50% inhibited by 1–2 mM Ca^{2+} ions (10). As shown in the present study, the membrane–cytoskeletons can contract independently of the presence of Ca^{2+} ions. In addition, when [γ^{32}P]ATP was used as substrate for contraction of the membrane–cytoskeletons, phosphorylation of myosin light chains was not detected (Fig. 4). These results suggest that the light chains of myosin in the membrane–cytoskeletons have already been phosphorylated or are in an "activated form". When does the phosphorylation of myosin light chains occur? Phosphorylation may occur before the association of myosin with the cortical actin filaments and, thus, light chains of myosin on the cortical actin filaments would always be phosphorylated.

Experiments using Triton-insoluble cytoskeletons of cAMP-stimulated *Dictyostelium* cells revealed that the time course of the phosphorylation of myosin light chains coincided with the time course of the relocation of myosin filaments during the chemotactic response (2), suggesting a correlation between the association of myosin filaments with the cortical actin filaments and the phosphorylation of their light chains. Therefore, the above possibility appears the more likely one at present. In addition, a correlation between the association of myosin with the cortical cytoskeleton and the phosphorylation of the myosin light chains has been found in platelets (8, 22).

Characterization of the Cortical Myosin II Heavy Chain-Kinase

MHCKs have been isolated from *Dictyostelium* by several workers. Maruta et al. (21) isolated a MHCK from *Dictyostelium* cells in the developmental phase, and it was a protein of 70 kD whose activity was inhibited by Ca^{2+} ions and calmodulin. MHCK has also been purified (6) or partially purified (13) from the soluble fraction of vegetative *Dictyostelium* cells. The enzyme purified from vegetative cells was shown to have a molecular mass of 130 kD. Starting with

the membrane fraction of *Dictyostelium* cells, Ravid and Spudich (26) isolated a MHCK with a molecular mass of 84 kD by SDS-PAGE and 240 kD by gel filtration. The presence of several species of MHCK in *Dictyostelium* cells may reflect their functional differences, and such differences may be related to their distribution in the cell. In the case of vegetative cells, in which most of myosin filaments are found in the cortical region and only a few are found in the endoplasm (30), the presence of an endoplasmic MHCK, in addition to a membrane-bound cortical MHCK, is quite plausible.

The activity of MHCK associated with the membrane–cytoskeletons prepared in this study may correspond to that of the MHCK isolated by Ravid and Spudich (26) since both were found in the membrane fraction. However, the other MHCKs cannot be excluded as possible candidates for the MHCK activity in the membrane–cytoskeletons. The present results suggest that MHCK activity is localized in a limited region on the cell membrane, or at the foci within the actin network. The molar ratio of MHCK to myosin molecules was 1:37 in the case of the MHCK isolated by Côté et al. (6) and 1:139 in the case of the MHCK isolated by Ravid and Spudich (26). These relatively low ratios of MHCK to myosin molecules also support their specific localization rather than their direct association with the myosin molecules. A model summarizing our results is presented in Fig. 7. Myosin II filaments are first accumulated at specific sites where MHCK is localized, namely, the foci within the actin network, and then they are phosphorylated. This process seems to be highly efficient and, since the number of foci within the actin network is countable, it seems likely that not one but several MHCK molecules are present at each actin focus.

We thank Dr. Thomas T. Egelhoff of the Department of Cell and Developmental Biology, Stanford University School of Medicine, for kindly providing us with truncated myosin mutants. 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. Susumu Takahashi of the Department of Biology, Faculty of General Education, Yamaguchi University, for useful suggestion for autoradiography. We thank Dr. Taro Q. P. Ueda of the Department of Cell and Developmental Biology, Stanford University School of Medicine, for valuable discussion. We thank Dr. Yoshio Fukui of the Department of Cell Biology and Anatomy, Northwestern University Medical School, for his encouragement.

Received for publication 12 August 1991 and in revised form 28 February 1992.

References

1. Berlot, C. H., J. A. Spudich, and P. N. Devreotes. 1985. Chemoattractant-elicited increases in myosin phosphorylation in *Dictyostelium*. *Cell.* 43:307–314.
2. Berlot, C. H., P. N. Devreotes, and J. A. Spudich. 1987. Chemoattractant-elicited increases in *Dictyostelium* myosin phosphorylation are due to changes in myosin localization and increase in kinase activity. *J. Biol. Chem.* 262:3918–3926.
3. Bonner, J. T. 1947. Evidence for formation of cell aggregation by chemotaxis in the development of *Dictyostelium discoideum*. *J. Exp. Zool.* 106:1–26.
4. Clarke, M., and A. Baron. 1987. Myosin filaments in cytoskeletons of *Dictyostelium* amoebae. *Cell Motil. Cytoskeleton.* 7:293–303.
5. Clarke, M., G. Schatten, D. Mazia, and J. A. Spudich. 1975. Visualization of actin fibers associated with the cell membrane in amoebae of *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. USA.* 72:1758–1762.
6. Côté, G. P., and U. Bukiejko. 1987. Purification and characterization of a myosin heavy chain kinase from *Dictyostelium discoideum*. *J. Biol. Chem.* 262:1065–1072.
7. Egelhoff, T. T., S. S. Brown, and J. A. Spudich. 1991. Spatial and temporal control of nonmuscle myosin localization: identification of a domain that is necessary for myosin filament disassembly *in vivo*. *J. Cell Biol.* 112:677–688.
8. Fox, J. E. B., and D. R. Phillips. 1982. Role of phosphorylation in mediating the association of myosin with the cytoskeletal structure of human platelets. *J. Biol. Chem.* 257:4120–4126.
9. Futrelle, R. P., J. Traut, and W. G. McKee. 1982. Cell behavior in *Dictyostelium discoideum*: preaggregation response to localized cyclic AMP pulses. *J. Cell Biol.* 92:807–821.
10. Griffith, L. M., S. M. Downs, and J. A. Spudich. 1987. Myosin light chain kinase and myosin light chain phosphatase from *Dictyostelium*: effects of reversible phosphorylation on myosin structure and function. *J. Cell Biol.* 104:1309–1323.
11. Jacobson, B. S. 1980. Actin binding to the cytoplasmic surface of the plasma membrane isolated from *Dictyostelium discoideum*. *Biochem. Biophys. Res. Commun.* 97:1493–1498.
12. Kitanishi-Yumura, T., and Y. Fukui. 1989. Actomyosin organization during cytokinesis: reversible translocation and differential redistribution in *Dictyostelium*. *Cell Motil. Cytoskeleton.* 12:78–89.
13. Kuczmarski, E. R. 1986. Partial purification of two myosin heavy chain kinases from *Dictyostelium discoideum*. *J. Muscle Res. Cell Motil.* 7:501–509.
14. Kuczmarski, E. R., and J. Pagone. 1986. Myosin specific phosphatases isolated from *Dictyostelium discoideum*. *J. Muscle Res. Cell Motil.* 7:510–516.
15. Kuczmarski, E. R., and J. A. Spudich. 1980. Regulation of myosin self-assembly: phosphorylation of *Dictyostelium* heavy chain inhibits formation of thick filaments. *Proc. Natl. Acad. Sci. USA.* 77:7292–7296.
16. Kuczmarski, E. R., S. R. Tafuri, and L. M. Parysek. 1987. Effect of heavy chain phosphorylation on the polymerization and structure of *Dictyostelium* myosin filaments. *J. Cell Biol.* 105:2989–2997.
17. Kuczmarski, E. R., L. Palivos, C. Aguado, and Z. Yao. 1991. Stopped-flow measurement of cytoskeletal contraction: *Dictyostelium* myosin II is specifically required for contraction of amoeba cytoskeletons. *J. Cell Biol.* 114:1191–1199.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 277:680–685.
19. Lowry, O. H., N. R. Roberts, K. Y. Leiner, M. Wu, and A. L. Farr. 1954. The quantitative histochemistry of brain. I. Chemical methods. *J. Biol. Chem.* 207:1–17.
20. Mahajan, R. K., K. T. Vaughan, J. A. Johns, and J. D. Pardee. 1989. Actin filaments mediate *Dictyostelium* myosin assembly *in vitro*. *Proc. Natl. Acad. Sci. USA.* 86:6161–6165.
21. Maruta, H., W. Baltes, P. Dieter, D. Marmé, and G. Gerisch. 1983. Myosin heavy chain kinase inactivated by Ca^{2+}/calmodulin from aggregating cells of *Dictyostelium discoideum*. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:535–542.
22. Nachmias, V. T., J. Kavaler, and S. Jacubowitz. 1985. Reversible association of myosin with the platelet cytoskeleton. *Nature (Lond.).* 313:70–72.
23. Nachmias, V. T., Y. Fukui, and J. A. Spudich. 1989. Chemoattractant-elicited translocation of myosin in motile *Dictyostelium*. *Cell Motil. Cytoskeleton.* 13:158–169.
24. Newell, P. C. 1986. The role of actin polymerization in amoebal chemotaxis. *Bioassays.* 5:208–211.
25. Rahmsdorf, H. J., D. Malchow, and G. Gerish. 1978. Cyclic AMP-induced phosphorylation in *Dictyostelium* of a polypeptide comigrating with myosin heavy chains. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:322–326.
26. Ravid, S., and J. A. Spudich. 1989. Myosin heavy chain kinase from developed *Dictyostelium* cells. *J. Biol. Chem.* 264:15144–15150.
27. Reines, D., and M. Clarke. 1985. Immunochemical analysis of the supramolecular structure of myosin in contractile cytoskeletons of *Dictyostelium* amoebae. *J. Biol. Chem.* 260:14248–14254.
28. Spudich, J. A. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells. *J. Biol. Chem.* 249:6013–6020.
29. Vaillancourt, J. P., C. Lyons, and C. P. Côté. 1988. Identification of two phosphorylated threonines in the tail region of *Dictyostelium* myosin II. *J. Biol. Chem.* 263:10082–10087.
30. Yumura, S., and Y. Fukui. 1985. Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in *Dictyostelium*. *Nature (Lond.).* 314:194–196.
31. Yumura, S., and T. Kitanishi-Yumura. 1990. Immunoelectron microscopic studies of the ultrastructure of myosin filaments in *Dictyostelium discoideum*. *Cell Struct. Funct.* 15:343–354.
32. Yumura, S., and T. Kitanishi-Yumura. 1990. Fluorescence-mediated visualization of actin and myosin filaments in contractile membrane-cytoskeleton complex of *Dictyostelium discoideum*. *Cell Struct. Funct.* 15:355–364.
33. Yumura, S., H. Mori, and Y. Fukui. 1984. Localization of actin and myosin for the study of amoeboid movement in *Dictyostelium* using improved immunofluorescence. *J. Cell Biol.* 99:894–899.