

# Stopped-Flow Measurement of Cytoskeletal Contraction: *Dictyostelium* Myosin II Is Specifically Required for Contraction of Amoeba Cytoskeletons

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**Abstract.** Cytoskeletons provide valuable information on the composition and organization of the cell's contractile machinery, and in many cases these cell models retain the ability to contract. To quantitate contraction rates, we developed a novel stopped-flow assay permitting simultaneous analysis of thousands of *Dictyostelium* cytoskeletons within milliseconds of mixing with Mg-ATP. Cytoskeletons were placed in one syringe of the stopped flow apparatus and the appropriate buffer was placed in the second syringe. Mixing with Mg-ATP caused an immediate increase in the absorbance at 310 nm. Rapid fixation of the cytoskeletons during the reaction confirmed that this change in absorbance was highly correlated with contraction of the cytoskeletons. This spectroscopic change was used to measure the effects of temperature, pH, ionic strength,

and nucleotides on contraction rate. Treatment with high salt and ATP removed most of the myosin, some actin, and small amounts of minor proteins. These extracted cytoskeletons lost the ability to contract, but after the addition of purified *Dictyostelium* myosin they regained full function. In contrast, rabbit skeletal muscle myosin was unable to restore contractility, even though it bound to the extracted cytoskeletons. Cytoskeletons prepared from a myosin-null mutant did not contract. Upon the addition of purified amoeba myosin, however, they became contractile. These results suggest that filamentous *Dictyostelium* myosin II is essential for contraction, and that the actin cytoskeleton and associated proteins retain their functional organization in the absence of myosin.

CELL "models" have provided valuable insight into the workings of the contractile apparatus. Early work by Hoffman-Berling demonstrated that glycerol-extracted fibroblasts could contract in the presence of Mg-ATP and calcium (17). Subsequent work with cytoskeletons prepared with nonionic detergents suggested that contraction was caused by "stress fibers" (22), structures known to contain a variety of contractile proteins including actin filaments, myosin, tropomyosin, and alpha-actinin. Double-label immunofluorescence revealed that the distribution of myosin and alpha-actinin alternated along the length of the stress fiber (28), and electron microscopic immunogold studies suggested that the myosin was organized into bipolar thick filaments (26). The calcium requirement appeared to be due to calcium-calmodulin-dependent light chain kinase (33, 18), and both calmodulin (9) and the kinase (8) are components of the stress fibers. Another cytoskeletal structure, the contractile ring, can also be reactivated in vitro. Cande observed ATP-dependent contraction in permeabilized mitotic cells (4), and Mabuchi et al. recently isolated functional contractile rings from oocytes (29). It has been suggested that less highly organized actin meshworks can also contribute to contraction of tissue culture (32), and intestinal brush border cells (19).

Typical stress fibers do not appear to be present in *Dictyostelium* amoebae growing in suspension; rather there is an actin meshwork (13) within which myosin thick filaments are dispersed (5, 7). These thick filaments are very dynamic, and can rapidly relocate in response to the chemoattractant, cAMP (47). Living cells respond to cAMP by retracting pseudopods and rounding-up within 30 s of stimulation (12). During this time there is an increase in actin polymerization (14) and in the amount of myosin associated with the cytoskeleton (10). Cytoskeletons prepared from amoeba retain the ability to contract in the presence of Mg-ATP, as first shown by Yumura and Fukui (47). A more detailed study of *Dictyostelium* cytoskeletal contraction by Reines and Clarke implicated myosin II as the force producing molecule, and showed that contraction only occurred under conditions in which the myosin molecules were assembled into thick filaments (43).

Although such studies clearly demonstrate acto-myosin-mediated contraction, they are limited because only a few cells at a time can be monitored in the light microscope. Moreover, it is impossible to accurately determine rates of contraction, an aspect that is important for studying the effects of pH, ionic strength, nucleotides, and other agents on the contractile machinery. Likewise, identifying essential

components of the contractile apparatus or measuring subtle changes in regulation that occur during chemotaxis require a sensitive rate assay. Therefore, we have developed a stopped-flow spectroscopic assay for monitoring the contraction of *Dictyostelium* cytoskeletons that allows for reproducible rate measurements within 100 ms of mixing with ATP. Fractionation studies revealed that myosin II (conventional myosin) is essential for contraction, and that rabbit skeletal muscle myosin cannot substitute for amoeba myosin. In addition, cytoskeletons from a myosin null mutant did not contract, but were made functional by the addition of purified *Dictyostelium* myosin.

## Materials and Methods

### Cell Culture

*Dictyostelium* strain Ax3 was cultured at 20–22°C in HL-5 medium in Erlenmeyer flasks on a rotary shaker (200 rpm) (44). Myosin null mutants prepared using gene replacement plasmids and homologous transformation (31) were a gift of Dr. James Spudich. These cells were grown on 150-mm plastic petri dishes in HL-5 medium supplemented with penicillin and streptomycin.

### Preparation of Cytoskeletons

Amoebae at late logarithmic growth phase ( $5-8 \times 10^6$  cells/ml) were harvested by centrifugation (2,000 rpm, 4 min, 4°C). Confluent cultures of the myosin minus mutant were dislodged from the petri dishes with a rubber policeman and then collected by centrifugation. All of the subsequent manipulations were performed at 4°C. The cell pellet was resuspended in wash buffer (100 mM Pipes, pH 6.8, 2.5 mM EGTA, 1 mM MgCl<sub>2</sub>, 10 mM N-p-tosyl arginine methyl ester, and 20 mM benzamide), centrifuged, and then resuspended at a ratio of  $10^8$  cells per ml of lysis buffer (wash buffer supplemented with 0.5% Triton X-100 and 10 µg/ml leupeptin). The resulting cytoskeletons were sedimented, washed once in half the volume of lysis buffer and once in wash buffer. The final pellet was brought to a concentration of  $1-5 \times 10^8$  cytoskeletons per ml of wash buffer.

### Removal and Add-back of Myosin

To depolymerize myosin thick filaments, KCl was added to cytoskeletons to a final concentration of 0.2 M. ATP was then added to a final concentration of 1 mM to separate myosin monomers from actin filaments. Upon centrifugation, >90% of the myosin was released to the supernate (see Fig. 6). The cytoskeletons were washed once in wash buffer containing KCl and ATP, followed by two washes in standard wash buffer. This preparation is referred to as "stripped" cytoskeletons.

Purified myosin was added to stripped cytoskeletons in 0.2 M KCl in wash buffer to insure that the myosin was monomeric. In some cases, the preparation was dialyzed against the same buffer overnight to remove residual ATP from the stripped cytoskeletons. Unbound myosin was removed by centrifugation, and assembly was induced either by resuspending directly in wash buffer, or by resuspending in wash buffer with 0.2 M KCl and then dialyzing against wash buffer.

### Stopped-flow Assay

A SFA-11 stopped flow apparatus (Hi-Tech Scientific Company, Salisbury, England) was used for the assay, and the temperature of the apparatus and the cuvette holder was maintained at 20°C by water jacketing. Cytoskeletons at a concentration of  $2 \times 10^7$ /ml were placed in one of the mixing syringes and the appropriate buffer (with twice the final desired concentration of reagent) was placed in the other syringe. Upon mixing the concentration of cytoskeletons (and reagents) was diluted by one half. A volume of 3 ml of cytoskeletons was sufficient to exchange solutions within the tubing of the stopped flow device and allow for four to five assays. To understand the experiment shown in Fig. 2 it should be realized that successive assays are illustrated. Thus, the absorbance at time = 0 represents the conclusion of the previous mixing event. At the point indicated by the arrow, fresh cytoskeletons and buffer were introduced into the cuvette (at the same time expelling the previous batch of cytoskeletons). That is why, in experiments

where there is no contraction, the absorbance does not decrease by 50% due to dilution (i.e., both the previous batch and the new batch are at exactly the same concentration).

Contraction was measured as an increase in absorbance at 310 nm, using a spectrophotometer (model HP-8850; Hewlett Packard Co., Palo Alto, CA). Integration time was set at 100 ms, which allowed for 10 measurements per second. The contraction rate was calculated from data obtained during the first few seconds of the reaction using curve fitting software provided with the spectrophotometer.

### Measurement of Cell Area

Cytoskeletons were fixed for 10 min at room temperature with 2% glutaraldehyde in wash buffer and then placed on a glass slide. To prevent flattening the cytoskeletons, the coverslip was supported by pieces of broken coverslips and then fixed to the slide with a 1:1:1 mixture of lanolin, Vaseline, and paraffin. The cytoskeletons were examined with the 100× oil immersion lens of a phase-contrast microscope connected to a video monitor. The projected areas of individual cytoskeletons were measured by tracing the outline with the cursor of a computerized imaging system (Southern Micro Instruments, Inc., Atlanta, GA). To rapidly fix cytoskeletons for the time course shown in Fig. 3, the fixative (100 mM PIPES, 3% glutaraldehyde, 6% formaldehyde, pH 6.5) was cooled to  $\sim -18^\circ\text{C}$  in a salt-water/ice bath. One volume of this fixative was added to an equal volume of the contracting cytoskeletons, vortexed, and immediately placed into a  $-18^\circ\text{C}$  ice bath.

### Protein Purification

Actin was purified from an acetone powder of rabbit skeletal muscle using the method of Pardee and Spudich (36). Amoeba myosin was isolated from vegetative *Dictyostelium* amoebae as previously described (23). Rabbit skeletal muscle myosin, prepared according to the method of Mommaerts and Parrish (34), was a gift from Dr. Meg Titus. *Acanthamoeba* myosin II was a gift from Dr. Chandra Ganguly.

### ATPase Assay

Actin-activated ATPase activity was measured at 25°C using purified proteins. *Dictyostelium* myosin (6 µg) was mixed with rabbit skeletal muscle actin (20 µg) in 15 mM KCl, 5 mM MgCl<sub>2</sub>, 25 mM MES buffer (pH 7.0) in a final volume of 100 µL. The reaction was initiated by adding ATP to a final concentration of 1.0 mM. Phosphate was measured colorimetrically (27).

### Protein Determination

Protein concentrations were measured by the method of Bradford (2) as modified by Read and Northcote (41). BSA was used as standard.

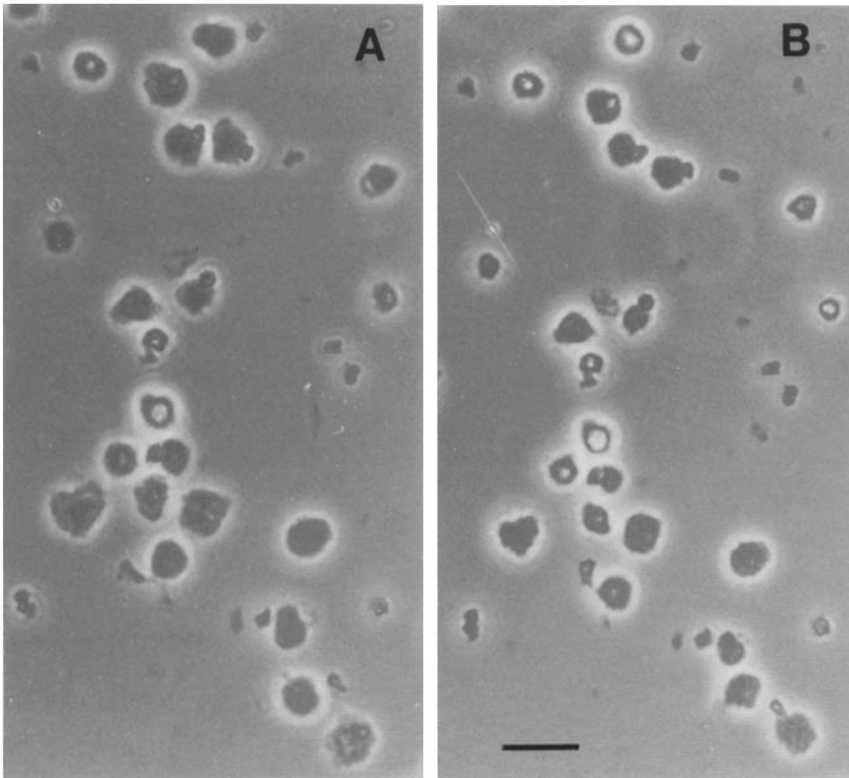
### SDS-PAGE

SDS-PAGE was according to the method of Laemmli (25) using a slab gel apparatus. Gels were stained with Coomassie brilliant blue (0.05% in 25% isopropyl alcohol, 10% acetic acid) and destained with 10% acetic acid. To quantitate the amount of myosin, gels were scanned with a densitometer, and care was taken to work within the linear range of absorbance (no more than 4 µg of myosin heavy chain).

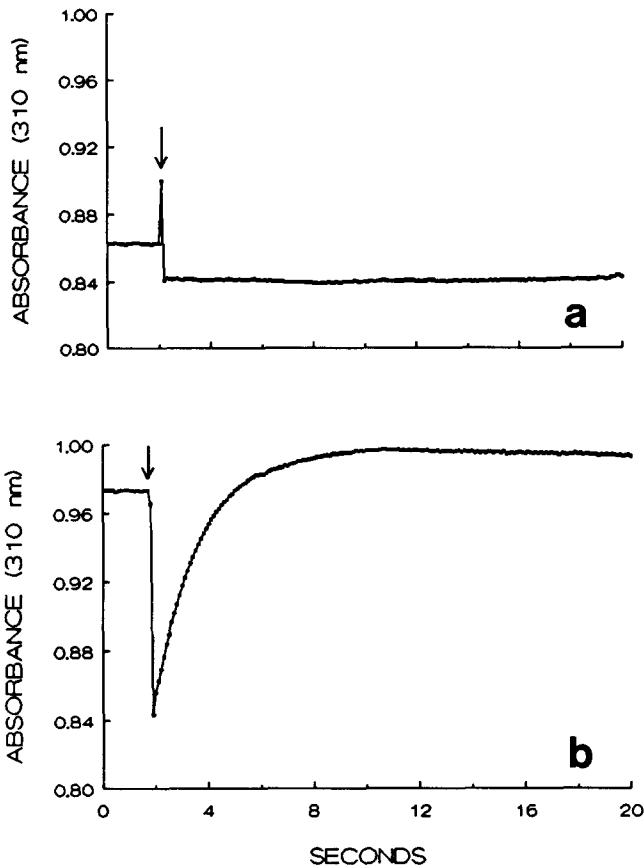
## Results

### ATP Causes Cytoskeletal Contraction

Contraction of cytoskeletons could be visualized in the phase-contrast microscope. Because the detergent treatment removed membrane components and many soluble proteins, the cytoskeletons appeared much less phase dense than intact cells. The isolated cytoskeletons retained the generally spherical shape of the vegetative cells, and exhibited a range of diameters (Fig. 1 a). To induce contraction, wash buffer containing 0.1 mM ATP was pulled under the coverslip. As the buffer reached the preparation, all of the cytoskeletons within the microscopic field of view contracted and the aver-



**Figure 1.** ATP contracts *Dictyostelium* cytoskeletons. Cytoskeletons suspended in wash buffer were placed on a glass slide, and covered with a coverslip. The same field of cytoskeletons is shown before (a) and after (b) perfusion with wash buffer containing 0.1 mM ATP. Bar, 10  $\mu$ m.

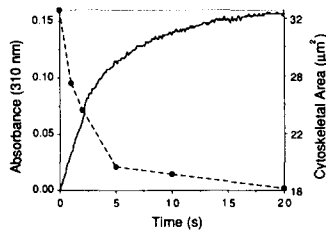


age diameter decreased by 25–30% (Fig. 1 b). After contraction cytoskeletons were more phase dense, presumably because the effective protein concentration increased as the cytoskeletal volume decreased. Indeed, measurement in a spectrophotometer showed that the absorbance of a population of cytoskeletons was higher following contraction. This observation was used to develop an assay to measure the rate of contraction of large numbers of cytoskeletons.

#### **Contraction Can Be Quantified by Stopped-Flow Analysis**

Cytoskeletons were placed in one syringe of the stopped-flow apparatus, and the appropriate test buffer was added to the second syringe. Rapid mixing of cytoskeletons with wash buffer alone had no effect on the  $A_{310}$  of the cytoskeletons (Fig. 2 a). In contrast, within 100 ms of mixing with ATP a rapid increase in absorbance was detected (Fig. 2 b). In 0.1

**Figure 2.** Cytoskeletal contraction can be measured in a stopped-flow device. Cytoskeletons were placed in one of the mixing syringes, and the appropriate buffer was placed in the second syringe. Upon mixing (arrow), cytoskeletons from the previous assay were expelled as fresh cytoskeletons and buffer entered the cuvette. Wash buffer caused a slight mixing artefact, but no increase in absorbance (a). In contrast, mixing with ATP caused a rapid increase in absorbance (b). The time for 50% completion of contraction, calculated from five determinations, was  $2.03 \pm 0.17$  s. Final concentrations were  $1 \times 10^7$  cytoskeletons per ml and 0.1 mM ATP in wash buffer.



**Figure 3.** Comparison of absorbance and size changes during contraction. ATP was mixed with cytoskeletons in a test tube at time = 0, and at the indicated intervals aliquots were fixed with  $-18^{\circ}\text{C}$  fixative. The areas of 50 cytoskeletons were measured for

each time point. Absorbance changes were measured by the standard method in the stopped flow device. The increase in absorbance was found to be strongly correlated with the decrease in diameter.

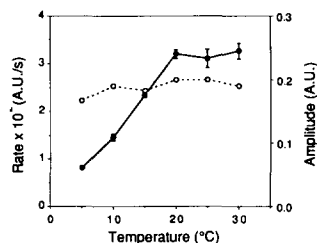
mM ATP the rate of this reaction was  $3.5 \times 10^{-2}$  A.U. per second, and the reaction was 50% complete in  $\sim 2$  s. With 0.5 mM ATP the rate increased (see Fig. 5) and the time for 50% completion dropped to 1 s.

Mechanical forces associated with mixing had no effect on the average cytoskeleton diameter, and no clumping of cytoskeletons was observed. To measure the actual contraction of individual cytoskeletons, samples were rapidly mixed with  $-18^{\circ}\text{C}$  fixative at intervals following the addition of Mg-ATP. The projected area of the fixed cytoskeletons was then measured in the microscope. As shown in Fig. 3, there was a very good correlation between the decrease in average cytoskeletal area and the increase in  $A_{310}$ . Thus, this spectroscopic assay was a valid method for determining the rate at which cytoskeletons contracted.

For reproducible rates it was important to use final cytoskeletal concentrations of  $1-3 \times 10^7$  cytoskeletons per ml. At concentrations greater than  $5 \times 10^7$ , light transmittance was reduced and the apparent rate decreased. At optimum cytoskeletal concentrations the rates were very reproducible. For example, with 6 independent preparations made over the course of three months the average contraction rates varied by no more than 10%. The actual decrease in cytoskeletal area, as measured microscopically, varied between 40 and 53%.

### Contraction Is Temperature Dependent

Between 5 and  $20^{\circ}\text{C}$  the rate of cytoskeletal contraction increased linearly. Temperatures greater than  $20^{\circ}\text{C}$  gave no further increase in rate (Fig. 4), and at  $40^{\circ}\text{C}$  contraction was completely inhibited. Despite the fact that the rates varied, the net change in absorbance (the amplitude) was quite similar. Microscopic measurements showed that between 5 and  $30^{\circ}\text{C}$  the average change in area was nearly identical. Thus, although rates differed, the cytoskeletons eventually attained



**Figure 4.** Contraction rate versus temperature. The temperature of the stopped flow device and cuvette was controlled by water jacketing. The rate of contraction increased with temperature up to about  $20^{\circ}\text{C}$  (closed symbols). The final extent of contraction (the amplitude; open symbols) was similar for all temperatures.

**Table I. Effect of Nucleotides on Cytoskeletal Contraction Rate and ATPase of Purified Dictyostelium Myosin**

Nucleotide	Rate $\times 10^2$ (A.U./s)	ATPase ( $\mu\text{mol}/\text{min}/\text{mg}$ )
ATP	$4.55 \pm 0.19$	0.168
CTP	$1.20 \pm 0.07$	0.090
ITP	$0.03 \pm 0.01$	0.052
GTP	0.00	0.026
ATP- $\gamma$ -S	0.00	0.037

Final concentrations of nucleotides were 0.5 mM. Rates given as the mean and standard deviation for three determinations. Actin activation was determined with purified amoebae myosin and rabbit skeletal muscle actin, as described in Materials and Methods.

the same degree of contraction and this was reflected in similar signal amplitudes.

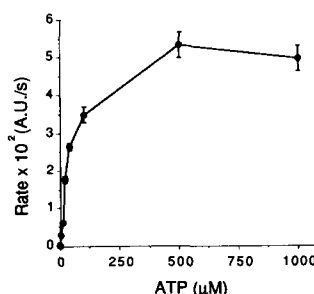
### Nucleotide Dependence

Cytoskeletal contraction was most rapid with ATP; other nucleotides were less effective in producing contraction. With CTP, for example, the rate was only 25 per cent of the ATP rate, while little or no contraction was seen with ITP, GTP, or ATP- $\gamma$ -S (Table I). This pattern of nucleotide effectiveness suggested that myosin II was involved in force production, since the actin-activated ATPase activity of purified myosin showed a similar nucleotide response (Table I). Interestingly, all of these nucleotides could be hydrolyzed to some extent by the myosin.

The rate of cytoskeletal contraction varied with the concentration of ATP (Fig. 5). Only 20–40  $\mu\text{M}$  ATP was required for the half maximal rate. Concentrations greater than 1 mM were actually inhibitory since both the contraction rate and per cent change in cell area decreased (Table II). This effect may in part be due to the fact that higher concentrations of ATP tended to release myosin from the cytoskeleton (Table II).

### Effects of Ionic Strength and pH on Contraction

Cytoskeletal contraction was inhibited by high salt concentrations. Contraction was fastest in the low ionic strength wash buffer, which contained no KCl. The addition of salt slowed the reaction, and above 200 mM KCl contraction was completely inhibited. In high salt the myosin molecules remained tightly associated with the cytoskeleton by an ATP-dependent linkage (see below). Exposure to high salt (up to 0.5 M KCl) did not irreversibly disrupt the cytoskeletal structure since full contractility was restored if the KCl was removed by dialysis (data not shown).



**Figure 5.** Cytoskeletal contraction rate versus ATP concentration. The rates of contraction were determined from the absorbance increases following mixing in the stopped-flow apparatus. The values represent the mean of three determinations. The concentration of ATP required for half-maximal contraction was  $\sim 40 \mu\text{M}$ .

**Table II. Effect of ATP Concentration on Rate of Contraction, Cytoskeletal Size, and Myosin Release**

ATP $\mu\text{M}$	Rate $\times 10^2$ A.U./s	Area $\mu\text{m}^2$	Myosin release %
0.0	0.0	30.1 $\pm$ 7.4	0.0
5	0.63 $\pm$ 0.28	20.7 $\pm$ 5.4	3.2
50	3.03 $\pm$ 0.15	15.6 $\pm$ 3.9	5.1
500	2.85 $\pm$ 0.06	16.8 $\pm$ 4.3	33.0
5000	2.15 $\pm$ 0.25	20.6 $\pm$ 3.6	75.6

The rate of contraction was calculated from the absorbance increase following mixing in the stopped-flow apparatus (mean of three determinations). Following this assay the cytoskeletons were recovered from the stop syringe, and for each concentration of ATP the area of 100 cytoskeletons was measured (results are given as mean and standard deviation). In separate experiments cytoskeletons were incubated for 15 min at 25°C with the indicated amount of ATP and then sedimented. The amount of myosin released was determined from scanning gels of the resulting supernatant fraction.

Contractility was pH dependent. Between pH 5–6, little or no contraction was observed and above pH 6 the rate increased approximately linearly and reached a maximum at about pH 8. The rates were somewhat reduced at higher pH values, but even at pH 10.0 the cytoskeletons still exhibited 50% of the maximal contraction rate. This is perhaps not too surprising, since the actin-activated ATPase of purified *Dictyostelium* myosin remained high in the same pH range (data not shown). In the range examined, changes in pH alone had no effect on the average cytoskeletal area.

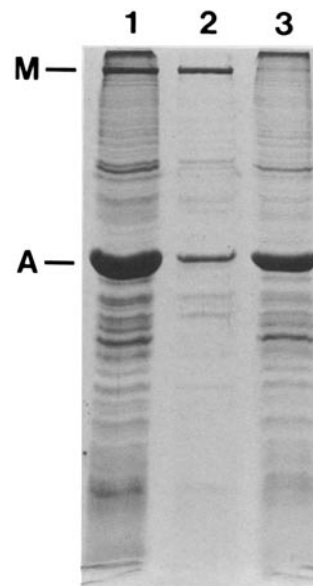
#### Nucleotides and Release of Myosin from the Cytoskeleton

Although treatment with high salt presumably depolymerized thick filaments, the myosin molecules remained attached to the cytoskeleton, as determined by SDS-PAGE (data not shown). If this linkage was due to myosin heads binding to actin filaments (rigor complexes) nucleotides would be expected to release myosin. To test this, cytoskeletons were incubated in wash buffer supplemented with 0.2 M KCl and 0.5 mM nucleotide, and then sedimented. SDS-PAGE revealed that with ATP, CTP, or ITP, over 70% of the myosin appeared in the supernatant fraction (Table III). Much less myosin was released with other nucleotides, and with GTP only 4% of the myosin was released.

**Table III. Effect of Salt and Nucleotides on Release of Myosin from Cytoskeletons**

Nucleotide	Myosin release (% of total)	
	WB	WB + 200 mM KCl
ATP	27	74
CTP	12	74
GTP	0	4
ITP	0	72
ATP- $\gamma$ -S	0	55
ADP	0	35
AMP	0	26

Cytoskeletons were incubated at 25°C with the indicated nucleotide (final concentration = 0.5 mM) in wash buffer (WB) or WB plus 0.2 M KCl. After 10 min the cytoskeletons were sedimented, and the amount of myosin released was determined by scanning gels of the supernate.



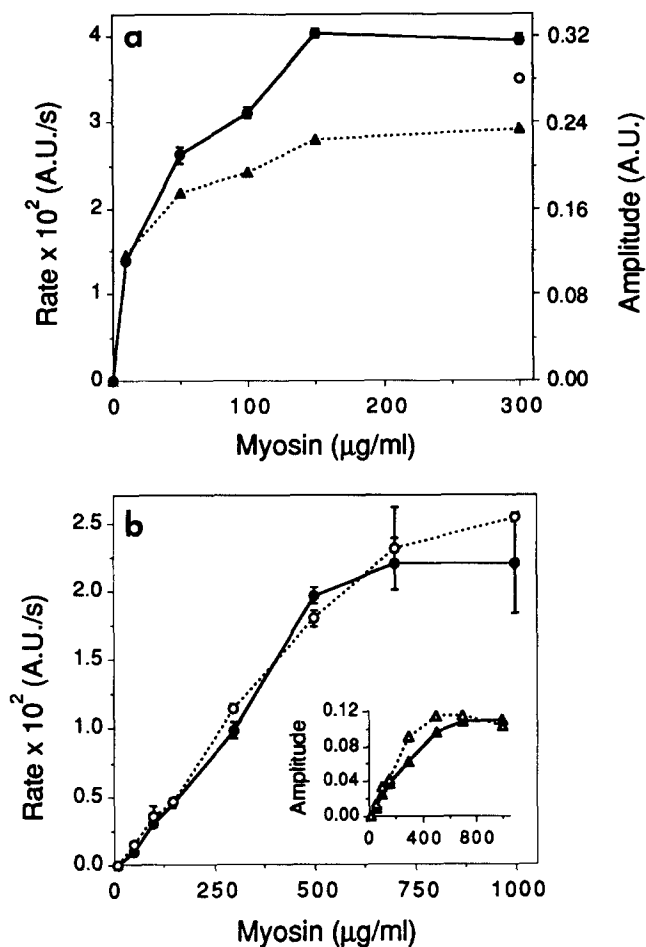
**Figure 6.** 12% SDS-PAGE showing removal of myosin from the cytoskeletons by high salt and ATP. (lane 1) Protein composition of isolated cytoskeletons. (lane 2) The supernatant fraction after extraction with 1 mM ATP, 0.2 M KCl in wash buffer contained nearly all of the myosin, a small percentage of the actin, and a few minor proteins. (lane 3) The cytoskeletal residue that sedimented after extraction was devoid of myosin but otherwise had a composition similar to the untreated cytoskeleton. M, myosin; A, actin.

The pattern of nucleotide release was influenced by ionic strength. In low salt only 27% of the myosin was released by 0.5 mM ATP and the only other nucleotide capable of releasing myosin was CTP (Table III). It is not known if the released myosin was monomeric or filamentous. The physical release of myosin was not a requirement for contraction since high rates were observed in 50  $\mu\text{M}$  ATP, even though only  $\sim$ 5% of the myosin was released (see Table II).

#### Myosin II Is Essential for Contraction

Based on the observations that contraction did not occur in high salt, and that ATP released myosin, it was possible to “strip” myosin from cytoskeletons. Fig. 6 shows the protein composition of the cytoskeletons prepared by the standard method (Fig. 6, lane 1). After extraction with 0.2 M KCl and 1 mM ATP, the cytoskeletons were centrifuged. Over 90% of the myosin was released to the supernate, along with some of the actin and small amounts of other proteins (Fig. 6, lane 2). The majority of the actin and the minor proteins sedimented (Fig. 6, lane 3). Microscopic examination revealed that such stripped cytoskeletons retained their basic structure, although they appeared less phase dense. When assayed in the stopped flow apparatus these “stripped” cytoskeletons were unable to contract, suggesting that myosin was essential for function. Indeed, if the supernatant and pellet were mixed together and the salt and ATP removed by dialysis, myosin rebound and the cytoskeletons regained the ability to contract (data not shown).

Although myosin II was assumed to be responsible for restoration of contractility, the possibility remained that some of the minor protein components present in the supernate were also involved. To test this, stripped cytoskeletons were washed free of myosin and other proteins and then incubated with highly purified *Dictyostelium* myosin. As shown in Fig. 7 (a and b, closed circles), myosin alone was sufficient to restore contractility. SDS-PAGE confirmed that myosin had bound to the cytoskeletons, and that the myosin binding sites could be saturated (data not shown). The rate of contraction increased as a function of myosin concentra-



**Figure 7.** Purified *Dictyostelium* myosin restores contractility to stripped wild type or myosin-minus mutant cytoskeletons. (a) Stripped wildtype cytoskeletons regained the ability to contract after binding myosin. The rate (●) increased with the amount of myosin, and appeared to saturate at about 150 μg/ml added myosin. The amplitude of the absorbance change (▲) also increased and reached a maximum. Also shown is a single point for mutant cytoskeleton contraction rate (○) following incubation with 300 μg/ml myosin. A second curve comparing the contractile rate for both types of cytoskeletons is shown in b. Here, the increase in rate of contraction as a function of myosin concentration was similar for both wild type (●) and the myosin minus mutant (○). The insert shows that the change in amplitude likewise was nearly identical for stripped wild type (▲) and mutant (△) contraction. Cytoskeletons were prepared and used directly in the case of the myosin-minus mutant while the wild type was first stripped of myosin as described in Materials and Methods. The indicated concentration of highly purified *Dictyostelium* myosin was added to aliquots of cytoskeletons ( $2 \times 10^8$  cytoskeletons/ml) in wash buffer containing 0.2 M KCl. The monomeric myosin was allowed to bind for 30 min, and unbound protein removed as described in Materials and Methods. Final concentrations in the assay were  $1 \times 10^7$  cytoskeletons/ml and 0.5 mM ATP. The specific activities of the added myosins (μmol/min/mg myosin) for graphs a and b were 0.2 and 0.06, respectively.

tion and eventually reached a plateau value. The net change in absorbance (amplitude) behaved in a similar manner, suggesting that the degree of cytoskeletal contraction depended on the amount of myosin bound. The specific activity of the *Dictyostelium* myosin also influenced the rate of con-

traction. For the experiment shown in Fig. 7 a, the actin-activated ATPase was rather high (0.2 μmol P<sub>i</sub>/min/mg myosin). The curve in Fig. 7 b used myosin with a specific activity of only 0.06, and it can be seen that higher concentrations of myosin were needed to attain the maximum rate of contraction. Purified rabbit skeletal muscle myosin also bound to the stripped cytoskeletons. However, these reconstituted cytoskeletons showed no contraction by either the spectrophotometric or microscopic assays, even when saturating amounts of myosin were bound (data not shown).

### Myosin-minus Mutant Cytoskeletal Contraction

Further evidence for a role of myosin in cytoskeletal contraction came from studies with a myosin-minus mutant. Triton cytoskeletons of mutant cells exhibited absolutely no contraction upon the addition of ATP. Since these cells had never seen myosin molecules or thick filaments, it was possible that the overall organization of the other cytoskeletal elements was grossly altered. However, if mutant cytoskeletons were incubated with purified *Dictyostelium* myosin, significant rates of contraction were observed. Fig. 7 a shows a single point in which myosin was added to mutant cytoskeletons at a concentration of 300 μg/ml. The rate was somewhat lower than that seen with stripped cytoskeletons. In another experiment increasing amounts of myosin were added to null mutant cytoskeletons or stripped wild type cytoskeletons. The rate curves were nearly identical (Fig. 7 b), suggesting that the essential cytoskeletal components were present and properly arrayed within the myosin-minus mutant. The inset in Fig. 7 b compares the amplitude of the absorbance change and suggests that wild type and myosin-minus mutant cytoskeletons contracted to similar extents.

### Discussion

Although it is possible to study cytoskeletal contraction by simple visual observation in a microscope (43, 47), this method is tedious and only allows for the analysis of small numbers of cells. More importantly, it is difficult to establish rates of contraction. To study factors that have subtle influences on contraction or to evaluate reconstitution studies, it is essential that actual rates be compared. We found that a small increase in absorbance accompanied the contraction of *Dictyostelium* cytoskeletons. Presumably this reflected an increase in the refractive index as a given amount of protein came to occupy a smaller volume. Regardless of the exact nature of the signal, it provided a way to measure the rate at which a population of cytoskeletons contracted. Since the reaction was complete within a few seconds, it was necessary to use a stopped-flow apparatus to quickly and uniformly mix the cytoskeletons with the appropriate test buffer. Although the sampling time of the spectrophotometer was rather slow (100 ms per time point), smooth curves showing changes in absorbance were obtained.

The spectrophotometric change was strongly correlated with contraction of the individual cytoskeletons. Fixative cooled to below 0°C was used to stop the reaction and prevent further change in size or shape. Although some contraction may have occurred before chemical fixation, the strong temperature dependence of the reaction (see Fig. 4) suggests that rapid cooling stopped movement very quickly. As shown in Fig. 3, there was a very good correspondence between the

time course for decrease in cytoskeletal size and the increase in absorbance. Thus, the rate of contraction for the population of cytoskeletons within the mixing cuvette could be determined simply by measuring the change in absorbance with respect to time.

Spectrophotometric assays have been used to monitor gelation and contraction of extracts from both *Acanthamoeba* (39) and *Dictyostelium* (15, 35). When warmed to room temperature, these extracts (which contained actin, myosin, and gelation factors) became turbid as gelation occurred, and this was associated with an increase in absorbance. Subsequent myosin-dependent contraction of the gel gave further changes in turbidity, but it was difficult to interpret these since the absorbance could rapidly shoot toward infinity or drop to nearly zero. In the assay described here, the curves were well-behaved and there was no gross change in the turbidity of the solution. It is unlikely that there was any polymerization or gelation of cytoskeletal components, and the major microscopic change was simply contraction of the cytoskeleton.

*Dictyostelium* cytoskeletons contracted much faster than those from tissue culture cells. Under optimal conditions, contraction was 50% complete in  $\sim 1$  s. In contrast, Masuda et al. (33) found that human fibroblast contraction required about 40 seconds for 50% completion. Part of this difference may reflect the requirement for calcium-calmodulin-dependent phosphorylation of the myosin light chain in tissue culture cells (20). As previously reported by Reines and Clarke (43) and confirmed by us, *Dictyostelium* cytoskeletal contraction did not require calcium, and significant amounts of myosin light chain phosphorylation were not observed. Although not determined for the present study, purified *Dictyostelium* myosin has  $\sim 0.24$  mol  $P_i$  per mol heavy chain and 0.1 mol  $P_i$  per mol light chain (24). However, the effects of altered phosphorylation levels on cytoskeletal contraction have not been examined. Compared to most nonmuscle myosins (40), purified *Dictyostelium* myosin normally has very high ATPase activity (0.2–0.3  $\mu\text{mol}/\text{min}/\text{mg}$  myosin), and this may contribute to the observed speed of contraction. It should also be noted that intermediate filaments, which may tend to counter contractile forces in fibroblasts, have not been identified in *Dictyostelium* amoeba.

The reaction was temperature dependent, increasing linearly between 5 and 20°C. Over this range the calculated  $Q_{10}$  was 2.3, reflecting the biochemical nature of the contraction (most enzymatic reactions have values between 2 and 3 [16]). Higher temperatures presumably altered the contractile proteins; above 20°C the rate leveled off (Fig. 4), and at 40°C contraction was completely inhibited. These ranges are physiologically relevant since *Dictyostelium* amoebae are fairly temperature sensitive, and cannot survive at temperatures above 24°C (44). The temperature curve also demonstrates that the extent of the reaction (i.e. the amplitude of the absorbance change) can reflect the degree of contraction. Although the rates of contraction decreased as the temperature was lowered, microscopic measurement indicated that eventually all cytoskeletons contracted to about the same extent. Likewise, the net change in absorbance (amplitude) for each point was similar over the same temperature range (Fig. 4). It should be noted that for other curves where rates varied (e.g., ATP curves and myosin add-back curves), the final extent of the absorbance change was not al-

ways identical for each point. Thus, it would be important to include the proper microscopic measurements to support any conclusions based on amplitude changes.

Cytoskeletal contraction requires that myosin be assembled into filaments (43) that presumably have high ATPase activity. Purified *Dictyostelium* myosin is quite sensitive to salt concentrations, and both the actin-activated ATPase activity (6) and the ability to form filaments (24) are abolished by salt concentrations greater than 75 mM. Interestingly, much higher concentrations of KCl were required to inhibit cytoskeletal contraction;  $\sim 130$  mM KCl was required to give 50% inhibition of contraction. Moreover, cytoskeletons exhibited substantial contraction at high pH values despite the fact that purified *Dictyostelium* myosin thick filaments are readily disassembled by pH greater than 7.5 (24). It may be that thick filaments are stabilized within the cytoskeleton. Actin is known to accelerate assembly of *Dictyostelium* myosin filaments (30) and high local concentrations in the cytoskeletal matrix may play an important role in stabilizing myosin filaments. It is also possible that other components of the cytoskeleton bind to and regulate the myosin filaments.

Is myosin release required for contraction? Masuda et al. (32) reported that following contraction of human fibroblasts over 70% of the myosin was released, and Broschat et al. (3) suggested that filament assembly and release accompanied contraction of isolated brush border. Reines and Clarke (43) also reported that  $\sim 20\%$  of the myosin was immediately released during contraction of *Dictyostelium* cytoskeletons, and that the majority of the myosin was released within 2 min. However, in their study the concentration of ATP was relatively high (1 mM), and we have confirmed that such concentrations do release significant amounts of myosin (Table II and Fig. 6). Very rapid contraction can be obtained using only 50  $\mu\text{M}$  ATP, despite the fact that only  $\sim 5\%$  of the myosin is released under these conditions (Table II). Thus, physical release of the myosin does not appear to be a prerequisite for effective contraction of *Dictyostelium* cytoskeletons.

In low salt only ATP and CTP released myosin (Table III). Although in this study it was not determined whether the myosin was released as intact filaments or as individual monomers, Reines and Clarke (43) reported that in low salt buffer ATP-released myosin was monomeric. The interactions of the myosin molecules within the cytoskeleton are probably fairly complex. In addition to myosin–myosin interactions within the thick filament, there are myosin to actin associations (“rigor complexes”), and perhaps interactions with other myosin binding proteins of the cytoskeleton. In high salt, filaments should be fully depolymerized, with nucleotide-induced release primarily affecting actin–myosin interactions. Not all nucleotides were equally effective, however, since the amount of myosin released ranged from 4% for GTP up to 74% for ATP and CTP (Table III). These differences, along with the fact that  $\sim 25\%$  of the myosin was not released with ATP may indicate that myosin is linked to components other than actin. It is known, for example, that a component of the erythrocyte cytoskeleton binds myosin and can influence its ATPase activity (37).

Actin is by far the largest single constituent of the cytoskeleton, and probably is responsible for maintaining the overall shape of the isolated complex. Indeed, when myosin is re-

moved the integrity of the cytoskeleton is maintained. Moreover, by adding back myosin it is possible to fully restore contractile function. The myosin molecules, which were added as monomers in high salt, presumably "lathered" around the framework of actin filaments forming "arrow-head" complexes, and then assembled into thick filaments as the salt concentration was lowered. Rather than causing some type of steric interference, it is interesting to note that as the myosin concentration was raised, the rate of contraction increased (Fig. 7). During contraction the myosin heads presumably are able to find appropriate interactions with actin filaments in the immediate vicinity of the thick filaments. Indeed, it has been shown that myosin molecules do have a great degree of rotational freedom, allowing for effective interactions with actin filaments of the correct polarity (42, 45). Despite the fact that function can be reconstituted, the reaction appears to be more than a simple "super precipitation" of actin and myosin because muscle myosin failed to generate contraction. This lack of response was rather surprising since both purified *Dictyostelium* and rabbit muscle actins are equally effective in activating the ATPase activity of rabbit muscle myosin (46). The muscle myosin used in this study had good actin-activated ATPase activity (0.4  $\mu\text{mol Pi/min/mg}$  myosin) and in a functional assay was able to restore contractility to "ghost" myofibrils (Kuczmariski, E. R., and Z. Yao, unpublished observations). One possible explanation is that skeletal muscle myosin tends to form longer thick filaments (1.0  $\mu\text{m}$  or greater [21], compared to 0.3-0.4  $\mu\text{m}$  for *Dictyostelium* thick filaments [24]). This cannot be the entire answer, however, since pig brain myosin (I), chicken gizzard myosin, and *Acanthamoeba* myosin II all form short filaments, yet none of these myosins could restore contractile function (our unpublished observations). Part of this may be due to the fact that light chain phosphorylation is required for stability of smooth muscle and brain myosin filaments. Further work will be required to determine the exact requirements for functional interaction with the cytoskeleton. It will also be important to study the function of different myosin types in living cells. Both normal and modified *Dictyostelium* myosins can be expressed in the null mutant (11) and contractile function is restored. The results reported here, however, suggest that muscle myosin might not function in the mutant cells.

Cytoskeletons prepared from the myosin-minus mutant did not contract. This is consistent with the observation that these cells do not undergo cytokinesis or cap cell surface receptors, and exhibit reduced cortical tension (38). Nonetheless, it was possible to produce a functional cytoskeleton by adding back purified *Dictyostelium* myosin. This suggests that the forces normally associated with bipolar myosin filaments in the living cell are not a prerequisite for functional organization of actin within the cytoskeleton.

The ability to remove and add back components of the cytoskeleton, in conjunction with a quantitative contraction assay, provide an important bridge between studies using highly purified proteins and those using intact cells. The approach should be applicable to other systems, and preliminary studies suggest that the stopped-flow assay can be used to study contraction of vertebrate nonmuscle cytoskeletons.

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## References

- Barylko, B., P. Tooth, and J. Kendrick-Jones. 1986. Proteolytic fragmentation of brain myosin and localisation of the heavy-chain phosphorylation site. *Eur. J. Biochem.* 158:271-282.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Broschat, K. O., R. P. Stidwill, and D. R. Burgess. 1983. Phosphorylation controls brush border motility by regulating myosin structure and association with the cytoskeleton. *Cell.* 35:561-571.
- Cande, W. Z. 1980. A permeabilized cell model for studying cytokinesis using mammalian tissue culture cells. *J. Cell Biol.* 87:326-335.
- Clarke, M., and A. Baron. 1987. Myosin filaments in cytoskeletons of *Dictyostelium* amoebae. *Cell Motil. Cytoskeleton.* 7:293-303.
- Clarke, M., and J. A. Spudich. 1974. Biochemical and structural studies of actomyosin-like proteins from non-muscle cells. *J. Mol. Biol.* 86:209-222.
- Condeelis, J. 1979. Isolation of concanavalin A caps during various stages of formation and their association with actin and myosin. *J. Cell Biol.* 80:751-758.
- de Lanerolle, P., R. S. Adelstein, J. R. Feramisco, and K. Burridge. 1981. Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in nonmuscle cells. *Proc. Natl. Acad. Sci. USA.* 78:4738-4742.
- Dedman, J. U., M. J. Welsh, and A. R. Means. 1978.  $\text{Ca}^{2+}$ -dependent regulator. Production and characterization of a monospecific antibody. *J. Biol. Chem.* 253:7515-7521.
- Dharmawardhane, S., V. Waren, A. L. Hall, and J. Condeelis. 1989. Changes in the association of actin-binding proteins with the actin cytoskeleton during chemotactic stimulation of *Dictyostelium discoideum*. *Cell Motil. Cytoskeleton.* 13:57-63.
- 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.
- 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.
- Greenberg-Giffard, R., J. A. Spudich, and A. Spudich. 1983.  $\text{Ca}^{2+}$  sensitive isolation of a cortical actin matrix from *Dictyostelium* amoebae. *J. Muscl. Res. Cell Motil.* 4:115-131.
- Hall, A. L., A. Schlein, and J. Condeelis. 1988. Relationship of pseudopod extension to chemotactic hormone-induced actin polymerization in amoeboid cells. *J. Cell. Biochem.* 37:285-299.
- Hellewell, S. B., and D. L. Taylor. 1979. The contractile basis of amoeboid movement. VI. The solation-contraction coupling hypothesis. *J. Cell Biol.* 83:633-648.
- Hoar, W. S. 1966. General and Comparative Physiology. Prentice Hall Inc., Englewood Cliffs, NJ.
- Hoffmann-Berling, H. 1954. Adenosintriphosphat als Betriebsstoff von Zellbewegungen. *Biochim. Biophys. Acta.* 14:182-194.
- Holzappel, G., J. Wehland, and K. Weber. 1983. Calcium control of actin-myosin based contraction in triton models of mouse 3T3 fibroblasts is mediated by the myosin light chain kinase (MLCK)-calmodulin complex. *Exp. Cell Res.* 148:117-126.
- Keller, T. C. S., and M. S. Mooseker. 1982.  $\text{Ca}^{2+}$ -calmodulin-dependent phosphorylation of myosin and its role in brush border contraction in vitro. *J. Cell Biol.* 95:943-959.
- Kitanishi-Yumura, T., and Y. Fukui. 1989. Actomyosin organization during cytokinesis: Reversible translocation and differential redistribution in *Dictyostelium*. *Cell Motil. Cytoskeleton.* 12:78-89.
- Koretz, J. F. 1982. Hybridization and reconstitution of thick-filament structure. *Methods Enzymol.* 85:20-55.
- Kreis, T. E., and W. Birchmeier. 1980. Stress fiber sarcomeres of fibroblasts are contractile. *Cell.* 22:555-561.
- Kuczmariski, E. R. 1986. Partial purification of two myosin heavy chain kinases from *Dictyostelium discoideum*. *J. Muscl. Res. Cell Motil.* 7:501-509.
- Kuczmariski, 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.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680-685.
- Langanger, G., M. Moeremans, G. Daneels, A. Sobieszek, M. De Brabander, and J. De Mey. 1986. The molecular organization of myosin in stress fibers of cultured cells. *J. Cell Biol.* 102:200-209.
- Lanzetta, P. A., L. J. Alvarez, P. S. Reinach, and O. A. Candia. 1979.



- An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.* 100:95-97.
28. Lazarides, E., and K. Burridge. 1974. Alpha-actinin: immunofluorescent localization of a muscle structural protein in nonmuscle cells. *Cell.* 6:289-298.
  29. Mabuchi, I., S. Tsukita, and T. Sawai. 1988. Cleavage furrow isolated from newt eggs: Contraction, organization of the actin filaments, and protein components of the furrow. *Proc. Natl. Acad. Sci. USA.* 85:5966-5970.
  30. Mahajan, R. K., K. T. Vaughn, J. A. Johns, and J. D. Pardee. 1989. Actin filaments mediate Dictyostelium myosin assembly in vitro. *Proc. Natl. Acad. Sci. USA.* 86:6161-6165.
  31. Manstein, D. J., M. A. Titus, A. DeLozanne, and J. A. Spudich. 1989. Gene replacement in Dictyostelium: generation of myosin null mutants. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:923-932.
  32. Masuda, H., K. Owaribe, and S. Hatano. 1983. Contraction of triton-treated culture cells: a calcium-sensitive contractile model. *Exp. Cell Res.* 143:79-90.
  33. Masuda, H., K. Owaribe, H. Hayashi, and S. Hatano. 1984. Ca<sup>2+</sup>-dependent contraction of human lung fibroblasts treated with triton X-100: a role of Ca<sup>2+</sup>-calmodulin-dependent phosphorylation of myosin 20,000-dalton light chain. *Cell Motil.* 4:315-331.
  34. Mommaerts, W. F. H. M., and R. G. Parrish. 1951. Studies on myosin: Preparation and criteria of purity. *J. Biol. Chem.* 188:545-552.
  35. Mornet, D., R. Bertrand, P. Pantel, E. Audemard, and R. Kassab. 1981. Structure of the actin-myosin interface. *Nature (Lond.).* 292:301-306.
  36. Pardee, J. D., and J. A. Spudich. 1982. Purification of muscle actin. S. P. Colowick and N. O. Kaplan, editors. *Methods Enzymol.* 85:164-181.
  37. Pasternack, G. R., and R. H. Racusen. 1989. Erythrocyte protein 4.1 binds and regulates myosin. *Proc. Natl. Acad. Sci. USA.* 86:9712-9716.
  38. Pasternak, C., J. A. Spudich, and E. L. Elson. 1989. Capping of surface receptors and concomitant cortical tension are generated by conventional myosin. *Nature (Lond.).* 341:549-551.
  39. Pollard, T. D. 1976. The role of actin in the temperature-dependent gelation and contraction of extracts of Acanthamoeba. *J. Cell Biol.* 68:579-601.
  40. Pollard, T. D., and R. R. Wehling. 1974. Actin and myosin and cell movement. *CRC Crit. Rev. Biochem.* 2:1-65.
  41. Read, S. M., and D. H. Northcote. 1981. Minimization of variation in the response to different proteins of the Coomassie blue G dye-binding assay for protein. *Anal. Biochem.* 116:53-64.
  42. Reedy, M. C., C. Beall, and E. Fyrberg. 1989. Formation of reverse rigor chevrons by myosin heads. *Nature (Lond.).* 339:481-483.
  43. 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.
  44. Spudich, J. A. 1982. Dictyostelium discoideum: Methods and perspectives for study of cell motility. *Methods Cell Biol.* 25:359-364.
  45. Toyoshima, Y. Y., C. Toyoshima, and J. A. Spudich. 1989. Bidirectional movement of actin filaments along tracks of myosin heads. *Nature (Lond.).* 341:154-156.
  46. Uyemura, D. G., S. S. Brown, and J. A. Spudich. 1978. Biochemical and structural characterization of actin from Dictyostelium discoideum. *J. Biol. Chem.* 253:9088-9096.
  47. Yumura, S., and Y. Fukui. 1985. Reversible cyclic AMP-dependent change in distribution of myosin thick filaments in Dictyostelium. *Nature (Lond.).* 314:194-196.