BHK21 MYOSIN: ISOLATION, BIOCHEMICAL CHARACTERIZATION AND INTRACELLULAR LOCALIZATION

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SUMMARY

Myosin has been isolated from baby hamster kidney cells (BHK21/C13) in high yield and characterized biochemically and immunologically. The subunit composition consists of 2 heavy chains, approximately 200,000 Daltons each, and 2 classes of light chains of approximately 16,000 and 20,000 Daltons. The myosin exhibits ATPase activity in the presence of K+-EDTA or Ca2+ but very little activity with Mg2+-ATP. The Mg2+-ATPase activity is stimulated only about 2-fold by skeletal actin, but a much larger activation is obtained in the presence of a protein kinase isolated from chicken gizzard. The increase in actin activation is accompanied by the phosphorylation of the 20,000-Dalton light chain. BHK21 myosin is insoluble at low ionic strength and forms typical bipolar thick filaments. A specific antiserum generated against this protein forms a single precipitin line with the antigen but does not crossreact with either skeletal or smooth muscle myosin. The antiserum also specifically stains stress fibres in BHK21 cells as shown by indirect immunofluorescence.

INTRODUCTION

Myosin-like proteins have been found in many non-muscle cells and tissues including Amoebae (Pollard & Korn, 1973), Dictyostelium (Clarke & Spudich, 1974), Physarum (Hatano & Tazawa, 1968; Adelman & Taylor, 1969; Nachmias, 1974), platelets (Bettex-Galland & Lüscher, 1959; Adelstein, Pollard & Kuehl, 1971), leukocytes (Stossel & Pollard, 1973), brain (Berl, Puszkin & Nicklas, 1973; Burridge & Bray, 1975), liver (Brandon, 1976), adrenal medulla (Johnson, McCubbin & Kay, 1977), starfish egg (Mabuchi, 1976) and cultured mammalian cells (Adelstein et al. 1972). In association with actin and other proteins, it has been suggested that these myosins are involved in various aspects of motility (Pollard & Weihting, 1974; Goldman, Pollard & Rosenbaum, 1976).

There is very little information available on the regulation of actomyosin-linked motile phenomena. A Ca2+-dependent regulation of contractility has been demonstrated in cell models and in isolated cytoplasm (Hatano, 1970; Hatano & Oosawa, 1971; Cohen & De Vries, 1973; Taylor, Condeelis, Moore & Allen, 1973; Izzard & Izzard, 1975; Mooseker, 1976; Taylor, Condeelis & Hammond, 1977). In addition Ca2+-sensitive actomyosin has been reconstituted from slime mould (Nachmias, 1975).

Even though myosin has been isolated from cultured cells and characterized both
with regard to its subunit composition and some of its ATPase properties (Adelstein et al. 1972; Ostlund & Pastan, 1976), very little is known about the regulation of its activity. This is due primarily to the low recovery of purified myosin from cell cultures. Therefore, we have developed a procedure which results in significantly higher yields of myosin than those obtained previously (Adelstein et al. 1972; Ostlund & Pastan, 1976). Baby hamster kidney cells (BHK21/C13) have been selected as the source of myosin because they grow rapidly to high densities and they have been utilized extensively in relating structure with function in various motile phenomena (Goldman et al. 1973; Goldman & Knipe, 1973).

The intracellular localization of myosin is another important aspect in determining its function. This has been attempted by indirect and direct immunofluorescence techniques (Weber & Groeschel-Stewart, 1974; Fujiwara & Pollard, 1976). However, very few attempts have been made to localize myosin within the same cell from which the myosin has been obtained. This is an important step in the intracellular localization of myosin due to the distinct possibility that there are multiple forms of non-muscle myosins (Burridge, 1976). Therefore, we have also initiated a study of the immunological properties of BHK21 myosin and its intracellular localization by immunofluorescence.

**MATERIALS AND METHODS**

**Cell culture**

Baby hamster kidney cells (BHK21/C13) were grown in Dulbecco's Modified Eagle's medium supplemented with 10% calf serum, 10% tryptose phosphate broth, 100 units/ml of penicillin and 100 µg/ml streptomycin. Stock cultures were maintained at 37 °C in a humidified atmosphere of 95% air-5% CO₂. Confluent cultures in 100-mm plastic Petri dishes (Falcon Plastics, Oxnard, Calif.) were transferred to other dishes or glass roller bottles following treatment with 0.25% trypsin-ethylenediaminetetra-acetate (EDTA) solution (Grand Island Biological Company). When confluent, the cells were harvested in 0.54 mM EDTA in phosphate-buffered saline (PBSa) (0.17 M NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), and washed 3 times in PBSa.

Colchicine (Sigma Chemical Co.) was used for some experiments at 10 µg/ml in the culture medium (Goldman, 1971).

**Preparation of myosin**

All steps were performed at 4 °C; deionized water was used throughout. The procedure used was similar to the one described by Spudich & Clarke (1974), for the isolation of Dictyostelium actomyosin. Thirty grammes (~30 ml) of packed cells were mixed with 5 vol. of a buffer containing 15 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2 mM dithiothreitol (DTT) and 30% sucrose (w/v). The suspension was homogenized several times in a motor-driven Potter Elvehjem homogenizer. After extraction for 1 h, KCl was added to a final concentration of 0.1 M and the cell debris was sedimented at 30000 g for 1 h. The resulting supernatant was dialysed overnight against 15 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.2 mM DTT, 0.1 M KCl. The next day, the actomyosin was collected by centrifugation at 30000 g for 1 h.

The actomyosin pellets were resuspended in 6–8 ml of 15 mM Tris-HCl, pH 7.5, 0.2 mM DTT, 2 mM EDTA, 0.6 M KI. This suspension was homogenized and stirred on ice for 15 min. Adenosine triphosphate (ATP) (Sigma grade, Sigma Chemical Co.) and MgCl₂ were added to final concentrations of 5 and 10 mM, respectively. The solution was clarified by centrifugation at 100000 g for 3 h. Samples were applied to a 95 x 2.6 cm column of Sepharose 4B (Pharmacia) in a discontinuous buffer system (Pollard, Thomas & Niederman, 1974). The
resin was pre-equilibrated with 2 column volumes of KCl buffer (0-6 m KCl, 15 mM Tris-HCl, pH 7-5, 2 mM EDTA, 0-2 mM DTT). Sixty-five millilitres of a buffer containing 0-6 M KCl, 15 mM Tris-HCl, pH 7-5, 5 mM ATP, 10 mM MgCl₂, 0-2 mM DTT (K₁ buffer) were applied just ahead of the sample. After application of the sample, 15 ml of K₁ buffer were allowed to flow into the column. The elution was carried out with KCl buffer. Fractions of 3·15 ml were collected at a flow rate of 20 ml/h. Absorptions were measured at 320, 278 and 260 nm in a Zeiss PM6 spectrophotometer. Blue Dextran (Sigma Chemical Co.) and ATP were used to determine the void volume and the final volume of the column, respectively. The fractions were concentrated by ultrafiltration, ammonium sulphate fractionation or precipitation after dialysis at low ionic strength (10 mM Tris-HCl, pH 7-5, 0-2 mM DTT).

**Measurement of adenosine triphosphatase (ATPase) activity**

All ATPase assays were carried out in a final volume of 2 ml containing 25 mM Tris-HCl, pH 7-5, and 2·5 mM ATP (Hartshorne & Mueller, 1969). The amount of inorganic phosphate released during the reaction was determined by the method of Fiske & Subbarow (1925). The K⁺-EDTA ATPases were assayed in 0-6 M KCl, 1 mM EDTA. Ca²⁺-ATPase was determined in 2·5 mM CaCl₂ and either 0-6 M KCl or 60 mM KCl. Mg²⁺-dependent actin activation was studied under the following conditions: 25 mM Tris-HCl, pH 7-5, 10 mM MgCl₂, 50 mM KCl, 2·5 mM ATP.

**Phosphorylation assay**

Phosphorylation of myosin was measured using a procedure similar to that described by Daniel & Adelstein (1976). The reaction mixture contained 0·3–0·5 mg of myosin in 25 mM Tris-HCl, pH 7-5, 10 mM MgCl₂, 50 mM KCl in a final volume of 2-0 ml. Following pre-incubation at 25 °C for 10 min the reaction was started by the addition of stock ATP solution (10 ml 50 mM ATP plus 100 μCi of γ-labelled ³²P-ATP; specific activity ~ 28 Ci/mmol) to a final concentration of 2·5 mM. After 10 min, the reaction was stopped by the addition of 2 ml 10% trichloroacetic acid, 2% sodium pyrophosphate. The samples were then heated to 90 °C for 20 min, cooled and filtered through Millipore filters. Each assay tube was washed with several volumes of 5% trichloroacetic acid, 1% sodium pyrophosphate and rinsed with distilled water. After transfer to scintillation vials, the filters were dried at 50 °C for 3 h. The scintillation fluid described by Fricke (Fricke, 1975) was added to the vials and the extent of ³²P incorporation was estimated in a nuclear Chicago Isocap 300 Scintillation Counter. The sites of phosphorylation were determined by SDS gel electrophoresis. Phosphorylation was carried out as described above through the heating step. The myosin was then dialysed exhaustively against 10 mM Tris-HCl, pH 7-5, and applied to polyacrylamide gels. Fifty microgrammes of protein were loaded on each of 12 gels. Following staining and destaining, the protein bands from the 12 gels were cut out and each component combined in a scintillation vial; 0·7 ml of 30% H₂O₂ was added and the samples were heated at 55 °C for 5 h. Scintillation liquid was added and the samples were counted.

**Protein determination**

Protein concentrations were determined either by the microbiuret method (Itzhaki & Gill, 1964) using bovine serum albumin as a standard or by the micro-Kjeldahl method assuming a 16% nitrogen content.

**Gel electrophoresis**

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis was performed either according to the procedure of Fairbanks, Steck & Wallach (1971) or on 7·5% polyacrylamide slab gels with 4·5% stacking gels (Laemmli, 1970). The samples were boiled for 5 min just before electrophoresis. Gels were scanned at 550 nm in a Beckman Model DU spectrophotometer equipped with a Gilford linear transport model 2410 and a Gilford 2000 recorder.
Preparation of other proteins

Skeletal muscle tropomyosin was prepared according to the technique of Hartshorne & Mueller (1969). The crude kinase was prepared from frozen chicken gizzards by the method of Ebashi et al. (1966), as modified by Aksoy, Williams, Sharkey & Hartshorne (1976).

Immunization procedures

Prior to immunization, New Zealand rabbits were bled and pre-immune serum was prepared. Two days later, purified BHK21 myosin (200 μg) was injected in complete Freund’s adjuvant (Difco) into the footpads and subcutaneously into the neck. Three weeks later, an additional 200 μg of myosin in incomplete Freund’s adjuvant were given by intramuscular injection. One week after the last injection, blood was collected from an ear vein, left to clot at 37 °C for 2 h and overnight at 4 °C. The serum was clarified by 2 centrifugations at 2000 g for 30 min. One-millilitre aliquots were frozen at −20 °C.

Immunoprecipitation

A crude BHK21 extract was prepared from 10 confluent dishes of cells and was tested to evaluate the specificity of the interaction between the antiserum and BHK21 myosin. Following a one-step glycerol extraction (Schloss, Milsted & Goldman, 1977), the cells were scraped in 20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM phenylmethylsulphonylfluoride (PMSF) (Sigma Chemical Co.) and collected by low-speed centrifugation. The cell pellet was then frozen at −20 °C. Upon thawing the pellet was resuspended in 20 mM Tris-HCl, pH 7.5, 0.6 M KCl, 0.5 % Nonidet P40 (Particle Data Laboratories, Ltd), 3 mM PMSF and sonicated. The suspension was clarified by centrifugation at 10000 g for 30 min and the resulting supernatant was used for the precipitin reaction as described by Levinson & Levine (1977); 0.2 ml aliquots of extract were incubated at 25 °C for 30 min with either 20 μl of the antiserum or 20 μl of preimmune serum. Goat anti-rabbit antiserum (Miles) was added at equivalence to the mixture and the double antibody mixture was left to react for 8 h at 4 °C. The extracts were then centrifuged at 800 g for 10 min and the resulting pellets were washed twice with 10 mM sodium phosphate buffer, pH 7.2, 150 mM NaCl, 0.5 % Nonidet P40, 0.5 % deoxycholate, once with distilled water and were finally dissolved in Laemmli gel sample buffer (Laemmli, 1970).

Double immunodiffusion analysis

Immunodiffusion was carried out in 1 % agar (Difco purified), dissolved in 0.6 M KCl, 10 mM potassium phosphate buffer, pH 7.4. Fifty microlitres of antiserum against BHK21 myosin were added to the centre well and 50 μl of test proteins were placed in the 6 outer wells. The protein concentrations were: BHK21 myosin, 1 mg/ml; smooth muscle myosin, 2 mg/ml; skeletal muscle myosin, 15 mg/ml. After incubating the dishes for 4 days at 4 °C, precipitin lines could be seen. The agar was washed in 0.6 M KCl, phosphate buffer, and rinsed in distilled water. After drying at 55 °C, the dishes were stained in 0.25 % Coomassie blue, 50 % methanol, 10 % acetic acid for 3 h and destained in 50 % methanol, 10 % acetic acid.

Indirect immunofluorescence

For immunofluorescence studies, cells grown to subconfluence on glass coverslips were rinsed in PBS (PBSa + 1 mM CaCl2, 0.5 mM MgCl2) to remove excess culture medium. The coverslips were then immersed in acetone at −20 °C for 1 min and air dried. Fifty microlitres of diluted serum (60 × dilution in PBS) were applied to each coverslip. After a 30-min incubation at 37 °C in a humidified chamber, the coverslips were washed carefully in PBS and then incubated for 30 min with goat anti-rabbit IgG (0.5 mg/ml) coupled with fluorescein (Miles). The stained coverslips were washed in several changes of PBS and finally in distilled H2O over a period of 1 h and mounted on glass slides in H2O.

Cells were also extracted by a rapid glycerination procedure prior to staining (Schloss et al.)
BHK21 myosin

Medium was removed and replaced by 50% glycerol in MSS (50 mM KCl, 15 mM MgCl₂, and 7 mM potassium phosphate, pH 7.0). The glycerol solution was drained after 2 min at room temperature and the coverslips were rinsed carefully in MSS. They were then incubated at 37 °C in a humidified chamber with 50 μl of serum diluted in MSS (60x dilution). Thirty minutes later, the coverslips were washed several times in MSS and then incubated for 30 min with fluorescein conjugated goat anti-rabbit IgG (Miles) diluted (0.5 mg/ml) in MSS. Following this staining procedure, the coverslips were washed in several changes of MSS and mounted on a microscope slide in the same buffer. The coverslips were viewed immediately in a Zeiss Photomicroscope III equipped with a III RS epifluorescence condenser containing a Zeiss FITC filter assembly and phase-contrast optics. The illumination was provided by an XBO 75-W Xenon lamp, powered with a DC power supply. Photomicrographs were taken on Plus-X film and developed in Diafine (Acufine, Inc., Chicago).

Electron microscopy

Purified BHK21 myosin was dialysed against 10 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM MgCl₂, in order to form synthetic thick filaments. Samples were negatively stained with 2% aqueous uranyl acetate on Formvar-carbon-coated copper grids. Electron micrographs were taken on a Philips 201C microscope.

RESULTS

Preparation of BHK21 myosin

The isolation of myosin is based on the extraction of actomyosin in a sucrose solution followed by precipitation at low ionic strength. The electrophoretic mobilities of the major polypeptides extracted under these conditions are shown in Fig. 1. High-speed centrifugation of the actomyosin in 5 mM ATP, 10 mM MgCl₂ results in a supernatant enriched in myosin. When this supernatant is chromatographed on Sepharose 4B in KCl buffer containing either Mg-ATP or Mg-pyrophosphate, most of the myosin is contaminated by actin and elutes in the void volume. However, when the KI discontinuous buffer system is employed, the myosin and the actin are readily separated. Fig. 2 shows a typical elution profile performed in the presence of KI. The first peak contains very little protein and has an A₂₈₀/A₂₆₀ ratio lower than 1.0. A second peak is characterized by an A₂₈₀/A₂₆₀ ratio of 1.4-1.7 and possesses most of the ATPase activity. The column fractions comprising the third peak contain mainly actin as determined by SDS polyacrylamide gel electrophoresis. The second Sepharose peak is composed of myosin characterized by 3 major bands (Fig. 1) which comigrate with purified chicken gizzard myosin. The heavy chain is about 200000 Daltons and there are 2 classes of light chains with molecular weights of 16000 and 20000 Daltons. Gel scans (Fig. 4) demonstrate that this preparation consists of 95-98% myosin. The band seen below the myosin heavy chain probably represents a breakdown product because this band is not present in all myosin preparations and its percentage relative to the heavy chain increases upon storage. The molar ratios which would best account for the densities observed on these scans are 1:1:1 for the heavy chain and the 2 light chains.

The total protein content of 30 g of cells (wet weight) is about 1.8 g. From this starting material we have been able to recover between 5 and 8 mg of myosin following column chromatography. We estimate our yield of myosin to be between 30 and 45% of the total cell myosin, as judged from comparisons of the gel scans (Figs. 3, 4).
The estimate of myosin in the total cell protein is ~ 1% and is based on the area under the peak comigrating with the purified myosin heavy chain.

Fig. 1. Purification of fibroblast myosin. 5-6% polyacrylamide - 1% SDS gels: a, 20 µg of whole BHK homogenate; b, 30 µg of BHK actomyosin after sucrose extraction and dialysis at low ionic strength; c, 27 µg of high-speed supernatant enriched for myosin following centrifugation of actomyosin in dissociating conditions (5 mM ATP, 10 mM MgCl₂); and d, 20 µg of purified BHK21 myosin. The symbols A, M, L₁ and L₂ are used to indicate the bands with the mobility of actin, myosin heavy chain and myosin light chains, respectively.

Fig. 2. Column chromatography profile. Sepharose 4B chromatography of a high-speed supernatant enriched for BHK myosin. Twenty-five milligrams of protein were applied to a 95 x 26 cm column as described in Materials and methods; 315-ml fractions were collected at a flow rate of 20 ml/h. Absorptions were measured at 320, 278 and 260 nm and absorption values corrected for light scattering. The second peak corresponds to the purified myosin. Void and total column volumes are indicated (V₀ and Vₜ) and correspond to the blue dextran and ATP peaks, respectively.

**ATPase activities**

The ATPase activities of BHK₂₁ myosin utilizing different assay conditions are presented in Table 1. These figures represent the means of values obtained from 5 different myosin preparations. The K⁺-EDTA ATPase activity is on the order of 0.65 µmol P₀/mg myosin/min which is lower than the activity of skeletal muscle myosin (Seidel, 1969) and is similar to that measured for smooth muscle myosin (Driska, 1976). The Ca²⁺-ATPase activity is stimulated by increasing the ionic strength to 0.6 M KCl. This effect is not seen with skeletal muscle myosin but has been reported in smooth muscle systems (Needham & Cawkwell, 1956).

The data on Mg²⁺-ATPase activity are summarized in Table 2. The addition of skeletal muscle actin up to a molar ratio of 20:1 (actin:myosin), results in about a 2-fold activation of the Mg²⁺-ATPase activity. In the presence of a protein kinase
isolated from chicken gizzard (Aksoy et al. 1976), the Mg\(^{2+}\)-ATPase activity of the BHK21 myosin is increased 6- to 8-fold by skeletal muscle actin. This activation occurs only in the presence of Ca\(^{2+}\), as indicated by the assays done in the absence of Ca\(^{2+}\) using EGTA (see Table 2). As a control for any possible influence of tropo-
Table 1. Myosin ATPase activities

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<th>1 mM EDTA</th>
<th>2.5 mM CaCl₂</th>
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<tr>
<td>0.6 M KCl</td>
<td>0.65</td>
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<tr>
<td>0.6 M KCl</td>
<td>0.41</td>
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Following purification on Sepharose 4B, myosin was assayed for its ATPase activities. The specific activity, μmol Pi released/mg of myosin/min, was measured in 25 mM Tris-HCl, pH 7.5, 2.5 mM ATP at 37 °C. The myosin concentration was 0.05 mg/ml.

Table 2. Actin activation of myosin at low ionic strength and related enzymic activities

<table>
<thead>
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<th>Specific activity</th>
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<tr>
<td>BHK21 myosin</td>
<td>2.9</td>
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<tr>
<td>BHK21 myosin + muscle actin</td>
<td>6.1</td>
</tr>
<tr>
<td>BHK21 myosin + muscle actin + kinase</td>
<td>21.5</td>
</tr>
<tr>
<td>BHK21 myosin + muscle actin + kinase + EGTA</td>
<td>7.0</td>
</tr>
<tr>
<td>BHK21 myosin + muscle actin + muscle tropomyosin</td>
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The effects of skeletal muscle actin, skeletal muscle tropomyosin and gizzard kinase on BHK21 myosin were measured in 25 mM Tris-HCl, pH 7.5, 2.5 mM ATP, 10 mM MgCl₂, 50 mM KCl at 25 °C. EGTA (1 mM) was added for assays done in the absence of Ca²⁺. Proteins were used at the following concentrations: myosin, 0.15 mg/ml; actin, 0.30 mg/ml; tropomyosin, 0.075 mg/ml. The specific activity is expressed in nmol of Pᵢ released/mg of myosin/min.

Fig. 5. Localization of ³²P in BHK21 myosin. Histogram of ³²P distribution in BHK21 myosin following phosphorylation in the presence of chicken kinase as determined by SDS gel electrophoresis.

In the presence of γ-labelled ³²P-ATP the chicken kinase preparation phosphorylates BHK21 myosin, and about 1.8 mol Pᵢ are incorporated per mol of myosin. In the absence of kinase or in the absence of Ca²⁺, no incorporation of phosphate can be detected. The site of phosphate incorporation has been determined following SDS polyacrylamide electrophoresis. As indicated in Fig. 5, only the 20000-Dalton light chain of myosin shows any significant incorporation. The level of incorporation therefore corresponds to 0.9 mol of phosphate per 20000-Dalton light chain.
Fig. 6. Electron micrograph of BHK21 myosin thick filaments. Fractions containing the purified myosin were pooled and dialysed several hours against 10 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM MgCl₂. Short (240–300 nm) bipolar filaments were observed in these negative stained preparations. × 88 500.

Thick filament formation

When BHK21 myosin is dialysed against 10 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM MgCl₂, short bipolar filaments are formed and can be seen following negative staining (Fig. 6). No actin filaments have been observed in these preparations. The
Fig. 7. Immunoprecipitin analysis. SDS polyacrylamide slab gel (7.5%) with stacking gel (4.5%): A, precipitate resulting from the interaction between the serum prior to immunization and whole BHK21 cell extract; B, BHK21 myosin standard (5 µg); and C, precipitate obtained after incubation of whole BHK21 extract with immune serum. The symbols A, M, IgG are used to indicate the bands with the mobilities of actin, myosin heavy chain and immunoglobulin G, respectively.

Fig. 8. Immunodiffusion analysis. The antiserum (AS) formed a single precipitin band with fibroblast myosin, wells (a) and (b), and did not crossreact with chicken gizzard smooth muscle myosin, wells (c) and (d), or with chicken skeletal muscle myosin, wells (e) and (f).
length of the thick filaments varies between 240 and 300 nm (mean = 274 nm, standard deviation = 14 nm, n = 100).

**Immunological properties of BHK21 myosin**

Figure 8 shows a double immunodiffusion plate containing purified BHK21 myosin, smooth muscle myosin, skeletal muscle myosin and a rabbit antiserum prepared against purified BHK21 myosin. From the pattern observed, it is evident that the antiserum reacts only with BHK21 myosin. Therefore, despite similarities in their biochemical properties (e.g., SDS polyacrylamide gel profiles, ATPase activities and light-chain phosphorylation), BHK21 myosin and smooth muscle myosin are immunologically distinct. Although no actin is seen on the SDS gels or by electron microscopy, actin was also tested for its interaction with the antiserum against BHK21 myosin and no precipitin line could be detected (Yerna & Goldman, unpublished observations).

BHK21 cells display intense fluorescence when prepared for indirect immunofluorescence with the myosin antiserum. Fluorescent stress fibres are visible only in the more extensively spread cells (Figs. 9, 11). The majority of cells do not display obvious stress fibres and the cytoplasm appears uniformly fluorescent (Fig. 13). Cells stained with the preimmune serum followed by fluorescein-labelled goat anti-rabbit IgG display no obvious fluorescence.

In order to facilitate the localization of myosin within stress fibres, colchicine is added to the culture medium. This alkaloid causes the cells to assume a more extensively spread configuration within 12–24 h (Goldman, 1971) and therefore stress fibres are easily resolved in all of the cells (Goldman, Yerna & Schloss, 1976). An additional benefit of colchicine treatment is derived from the fact that cytoplasmic microtubules are no longer present and that 10-nm filaments are localized in a juxtanuclear cap. These cells therefore provide an excellent control, as microfilament bundles are the only fibrous structures retaining their normal intracellular organization and distribution (Goldman et al. 1973; Goldman, Lazarides, Pollack & Weber, 1975). Combined phase-contrast and epifluorescence observations of colchicine-treated cells prepared according to the myosin antiserum staining procedure demonstrate the identity of phase-dense and fluorescent stress fibres (Figs. 9–12). Many fluorescent stress fibres reveal alternating fluorescent and non-fluorescent regions which in some cases are coincident with alterations in the phase density seen along stress fibres (Figs. 14, 15).

While the myosin antiserum can be shown to interact with purified myosin by double immunodiffusion analysis and to yield an immunofluorescence pattern similar to that seen by others (Weber & Groeschel-Stewart, 1974; Fujiwara & Pollard, 1976), it is important to demonstrate that it does not interact with other components in the cell. Such interactions might occur due to changes in cellular proteins brought about by conventional immunocytochemical procedures which have employed fixation by formaldehyde-acetone or acetone alone. These fixation techniques have been utilized in the localization of many contractile and cytoskeletal proteins and may lead to denaturation or loss of structure (Forer, Kalnins & Zimmerman, 1976).

We have initiated attempts to alleviate this problem by developing a cell lysis
Figs. 9–12. Light micrographs of acetone-extracted BHK21 cells stained with myosin antibody. Figs. 9, 10 show the same colchicine-treated cell which has been processed for simultaneous observation of immunofluorescence and phase-contrast images as described in Materials and methods. The nucleus (n) of these cells and the juxanuclear 10-nm filament cap (c) do not stain, x 500. Figs. 11, 12 show a higher magnification (x 1250) of a BHK21 cell plated in colchicine. There is a 1:1 correspondence between fluorescent stress fibres and phase-dense fibres.
Figs. 13-17. Light micrographs of BHK21 cells stained for myosin localization. Fig. 13 presents a field of BHK21 cells grown in normal medium and then extracted with acetone prior to staining for myosin localization. Some of these cells display stress fibres while others are not well enough spread to allow visualization of stress fibres. ×500. Figs. 14, 15, portion of an extensively spread BHK21 cell following treatment with colchicine. This cell has been extracted with acetone and stained for myosin localization. The stress fibres display a periodicity (arrows) which can be observed in phase-contrast and epifluorescence optics. ×1200. Fig. 16, a glycerol-extracted BHK21 cell stained for myosin localization. ×500. Fig. 17, higher magnification of a BHK21 cell prepared as in Fig. 16. A periodicity (arrows) similar to that seen in acetone-treated cells is observed. ×1250.
procedure which preserves stress fibre structure as well as many of the native properties of BHK21 myosin. The cell lysis procedure involves rapid glycerination (Schloss et al. 1977) followed by treatment with the immune serum and fluorescein-conjugated goat anti-rabbit IgG. Under these conditions most of the actin-like and myosin-like proteins are retained in the lysed cell (Goldman, Schloss & Starger, 1976). Cells treated in this fashion yield a staining pattern which is similar to that seen in acetone-treated cells (Figs. 16, 17). A further control for this type of cell preparation involves determining whether or not the myosin retained within glycerinated BHK21 cells interacts with the myosin antiserum. The gel profile of protein extracted from glycerinated cells and precipitated with the antiserum obtained against BHK21 myosin is presented in Fig. 7. Myosin and actin are the only apparent cell proteins which interact specifically with the antiserum. The immunoprecipitin test was carried out under non-dissociating conditions (without Mg$^{2+}$-ATP). Therefore the antiserum probably precipitated myosin as an actomyosin complex. It should also be re-emphasized that no interaction was detected between purified actin and the antiserum by immunodiffusion. The other minor bands seen on the gel may be attributed to cellular proteins trapped during complex formation as shown by substituting preimmune serum in the precipitin reaction (Fig. 7).

**DISCUSSION**

Myosin has been purified from BHK21 cells and found to be free of actin and nucleic acids. The yield of myosin obtained by this method is significantly higher than that achieved previously (Adelstein et al. 1972; Ostlund & Pastan, 1976). This is an important factor in facilitating the biochemical characterization of the protein. The technique has also been used successfully on other hamster cell lines (Yerna & Goldman, unpublished observations) and chick embryo fibroblasts (Milsted & Goldman, unpublished observations). The myosin obtained in these cases appears to be identical to BHK21 myosin as determined by SDS polyacrylamide gel electrophoresis.

The ATPase activities of BHK21 myosin are similar to those reported by other workers for fibroblast myosin (Adelstein et al. 1972; Ostlund & Pastan, 1976), and in general are similar to smooth muscle myosin (Needham & Cawkwell, 1956; Driska, 1976). The small degree of actin activation that is found is similar to that encountered with smooth muscle myosin (Bûrany, Bûrany, Gaetjens & Bailin, 1966; Hartshorne et al. 1973). It is significant that phosphorylation of both smooth muscle myosin and BHK21 myosin enhances considerably the level of actin-activated ATPase activity, which has also been reported by Adelstein & Conti (1975) using platelet proteins. However, in the latter case Ca$^{2+}$-sensitive phosphorylation was not obtained (Adelstein & Conti, 1975). In subsequent studies using a 'hybrid system' of the platelet kinase and fibroblast myosin these latter workers reported only a low degree of phosphate incorporation in the 20000-Dalton light chain of the myosin (about 0.15 mol of phosphate per mol of myosin). The effect of phosphorylation on the Mg$^{2+}$-dependent actin activation was not investigated (Daniel &
Adelstein, 1976). Our results with chicken gizzard kinase and fibroblast myosin indicate a much higher level of incorporation (approximately 1-8 mol of phosphate per mol of myosin) accompanied by a 6- to 8-fold stimulation of the actin activation of BHK21 myosin.

In skeletal muscle the regulatory effect of Ca$^{2+}$ is mediated via the interactions of the thin filament proteins: actin, troponin and tropomyosin (Weber & Murray, 1973). In molluscs, the regulatory system is a part of the myosin molecule itself (Szent-Györgyi, Szentkirályi & Kendrick-Jones, 1973; Lehman & Szent-Györgyi, 1975). In the case of smooth muscle it is thought that regulation involves phosphorylation of the 20000-Dalton light chain of myosin by a kinase (Górecka, Aksoy & Hartshorne, 1976) which occurs only in the presence of Ca$^{2+}$. In the absence of Ca$^{2+}$, a phosphatase removes the phosphate groups and the myosin is not activated by actin (Górecka et al. 1976). In cultured mammalian cells, the nature of the control system remains unknown. However, the results of this study indicate that the phosphorylation of the 20000-Dalton light chain could be involved in regulating the interaction of cytoplasmic actin and myosin in BHK21 cells. Attempts are being made to isolate an endogenous kinase from BHK21 cells.

BHK21 myosin forms 240-300-nm bipolar thick filaments at low ionic strength, which is a common property of muscle (Huxley, 1963; Sobieszek & Small, 1973) and non-muscle myosins (Adelstein et al. 1971; 1972; Clarke & Spudich, 1974; Nachmias, 1974). These synthetic thick filaments are shorter than skeletal or smooth muscle thick filaments formed under similar conditions and are about the same size as other fibroblast myosin thick filaments (Adelstein et al. 1972).

The antiserum produced against BHK21 myosin is specific as judged by immunodiffusion. It does not crossreact with smooth muscle or skeletal muscle myosin. This result confirms the report published by Willingham, Ostlund & Pastan (1974) which demonstrated the lack of cross-reactivity between a goat antibody directed against L-929 cell myosin and muscle myosins.

In order to localize a particular intracellular component by immunofluorescence, the antigen used to produce the antibody should be as pure as possible. As described in the Results section, we estimated the purity of the myosin used as the antigen to be greater than 95%. Considering that small amounts of protein were used to elicit the antibody (less than 500 μg), the chances of obtaining antiserum against other cellular components are minimal.

Utilizing this antiserum for indirect immunofluorescence, glycerol-extracted or acetone-treated cells display similar fluorescent stress fibre patterns. In both cases, the distribution of the fluorescent stress fibres resembles those obtained with actin antibody and fluorescein-labelled sub-fragment-1 (Goldman et al. 1975; Schloss et al. 1977). In glycerinated BHK21 cells, myosin is retained in its ‘native’ state with regard to antigenicity as determined by the immunoprecipitation test. Furthermore, actomyosin extracted from glycerol-treated BHK21 cells has ATPase activities similar to those obtained for actomyosin prepared from cells not treated with glycerol (Yerna & Goldman, unpublished observations). These results provide further support that myosin is probably an integral component of stress fibres.
In spite of the above observations, we cannot rule out completely the possibility that components of the immunofluorescence images may be produced by non-specific interactions. This is suggested by the immunoprecipitin assay in which there is some reaction between the antiserum and cellular components not containing myosin. However, by using (1) a highly purified antigen, (2) the localization of the antigen in the same cell type from which it was obtained, (3) identical localization of the antibody in cells prepared by acetone or glycerol treatment, (4) the absence of pre-immune serum staining, and (5) the demonstration of specific antigen-antibody interaction utilizing both immunodiffusion and immunoprecipitation, we feel that the probability of non-specific interactions has been greatly reduced.

Other approaches using immunofluorescence have been used in the localization of myosin within cultured cells. For example, an antiserum directed against chicken gizzard smooth muscle myosin has been used to determine the distribution of myosin in 3T3 fibroblasts (Weber & Groeschel-Stewart, 1974). However, the results of Willingham et al. (1974) and the results of this study show that antibodies made against fibroblast myosin fail to cross-react with smooth muscle myosin and that antibodies generated against smooth muscle myosin do not cross-react with fibroblast myosin (Willingham et al. 1974; Yerna & Goldman, unpublished observation). In a more recent study, Fujiwara & Pollard (1976) have shown cross-reactivity between a serum prepared against platelet myosin and HeLa cell extracts and have used directly conjugated antibody to localize myosin in the stress fibres of ENSON cells, another human cell line. In light of these findings concerning the available immunodiffusion and immunofluorescence data, the localization of an antibody directed against BHK21 myosin within the stress fibres of BHK21 cells is an important achievement in confirming the presence of myosin within these structures which are known to represent the microfilament bundles seen in ultrastructural investigations (Goldman et al. 1975).

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REFERENCES


BHK21 myosin


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