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Isolation and Characterization of Myosin from Cloned Mouse Fibroblasts

(muscle/SDS-acrylamide gel electrophoresis/actin binding/agarose filtration)

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ABSTRACT Myosin has been isolated from cloned mouse fibroblasts, line L-929. Fibroblast myosin: (*i*) binds to rabbit muscle actin and is dissociated from it by ATP, (*ii*) has an ATPase activity that is suppressed by Mg^{2+} in 0.6 M KCl and is activated by rabbit muscle actin in the presence of Mg^{2+} in 14 mM KCl, (*iii*) forms thin bipolar aggregates in 0.1 M KCl when viewed in the electron microscope, (*iv*) possesses a heavy chain with the same mobility as muscle myosin (molecular weight 200,000) in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In these respects, fibroblast myosin appears to be similar to muscle myosin in structure and function.

Since the work of Ishikawa *et al.* (1) on the presence of actin-like filaments in fibroblasts, the possible existence of a myosin counterpart in these cells has been raised. Recently, Yang and Perdue (2) succeeded in isolating and purifying actin from tertiary cultures of chick embryo cells and found it similar to muscle actin in its molecular properties. Furthermore, the the work of Bray (3) has indicated that cytoplasmic actin can be isolated from several different embryonic sources.

cAMP has a marked effect on the motility, adhesion, morphology, and growth of fibroblasts grown *in vitro* (4–9). It is possible that some of the effects of cAMP could be mediated through one or more of the "contractile" proteins (actin, myosin, and troponin–tropomyosin). To explore this hypothesis, we began by attempting to isolate a myosin-like protein from cloned fibroblasts.

Using a modification of a technique developed for the isolation of platelet myosin (10), we isolated myosin from cloned mouse fibroblasts grown *in vitro*. This protein has been characterized by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis, ATPase assay with and without muscle actin, actin binding, and electron microscopy.

MATERIALS AND METHODS

Cloned mouse (L-929) fibroblasts were grown either as monolayers or in spinner bottles. The cells were scraped off the sides of the bottles or sedimented from the suspended culture. They were washed three times in ten volumes of 0.9% NaCl–0.3% sodium citrate–5 mM dithiothreitol (S_2threitol)–1 mM EDTA, and sedimented each time at 13,000 × *g* for 15 min. The final wash was found to be essentially free of protein. The fibroblast pellet was stored at −15° for up to 2 weeks before use.

Abbreviations: S_2threitol, dithiothreitol; SDS, sodium dodecyl sulfate.

Purification procedures

All steps were performed at 2–4°; deionized water was used throughout.

Preparation of Actomyosin. The procedure used was similar to that outlined for the purification of platelet myosin by Adelstein *et al.* (10). 20 Grams of cells was mixed with 2.5 volumes of 0.6 M KCl–15 mM Tris·HCl (pH 7.5) (Buffer A)–10 mM S_2threitol and lysed with 3% *n*-butyl alcohol. After extraction for 75 min, the lysate was centrifuged for 15 min at 48,000 × *g* to yield the "extract supernatant" (Table 1). Fibroblast actomyosin was precipitated from this supernatant by dilution with 5 volumes of 2 mM $MgSO_4$ and lowering the pH to 6.4. The precipitate was collected by centrifugation at 27,000 × *g* for 15 min and dissolved in 10 ml of Buffer A–10 mM S_2threitol. This precipitation was performed a total of three times; the final precipitate was suspended in 8 ml of Buffer A–10 mM S_2threitol, and allowed to dissolve overnight. (Total volume = 12 ml).

The solution was then centrifuged at 100,000 × *g* for 3.5 hr to sediment the fibroblast actomyosin.

TABLE 1. *Fibroblast myosin: Purification and enzyme activity*

			Specific activity†		
	Protein (total mg)	Total EDTA-+K ATPase*	EDTA-+K AT-Pase	Ca-AT-Pase	Mg-ATPase
Extract supernatnat	1260	5.0	4	—	—
100,000 × *g* supernatant +Mg-ATP	178	0.9	5	21	11
Ammonium sulfate 0–25%	74	0.07	1	4	3
25–50%	26	0.75	29	34	7
50–70%	20	0.34	17	10	<1
Sepharose 4B (25–70%) fraction	0.60	0.26	430	500	<10

* Expressed in μmol of Pi released per min.
† Expressed in nmol of Pi released per mg of protein per min.

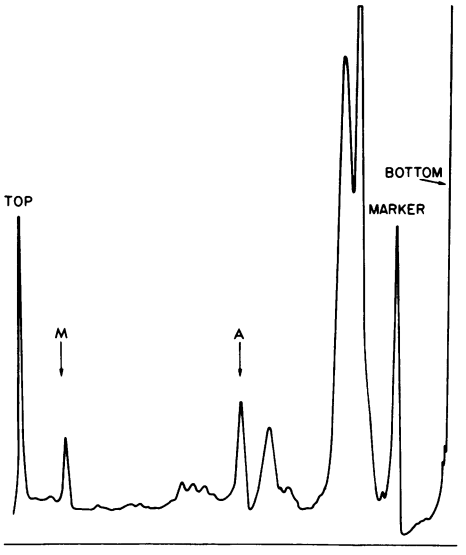


FIG. 1. 600-nm scan of 0.1% SDS-7.5% acrylamide gel of fibroblast actomyosin. Electrophoresis is from *left* to *right*. *M* indicates the peak electrophoresing with the same mobility as muscle myosin heavy chain and *A* the peak electrophoresing with actin.

Fractionation of Actin and Myosin. The 100,000 × *g* pellet, containing fibroblast actomyosin, was solubilized in 6 ml of Buffer A–10 mM ATP–10 mM $MgCl_2$–10 mM S_2threitol for 1 hr, then centrifuged at 100,000 × *g* for 45 min. The supernatant was dialyzed overnight against Buffer A–5 mM S_2threitol; before ammonium sulfate fractionation, the retentate was made 10 mM with respect to $MgCl_2$ and ATP. Originally, three fractions (see Table 1) were obtained with saturated ammonium sulfate—0–25%, 25–50%, and 50–70%—later, a 0–25% fraction was used for preparation of actin and the 25–60% fraction for myosin preparation.

Purification of Fibroblast Myosin. The 25–50% and 50–70% ammonium sulfate fractions were combined and chromatographed on two 2.5 × 82 cm columns of Sepharose 4B connected in series and equilibrated with Buffer A–5 mM S_2-threitol–1 mM ATP–1 mM $MgCl_2$. The resulting fractions were assayed for ATPase activity and were pooled for further study.

Biochemical Assays. ATPase was assayed (10) at 37° in 10 mM imidazole·HCl (pH 7.0), 2 mM ATP, 0.6 M KCl, and either 2 mM EDTA, 10 mM $CaCl_2$, or 5 mM $MgCl_2$. Phosphate production was measured for linearity. For actin activation, the following assay conditions were used: 3 mM imidazole·HCl, 2.7 mM $MgCl_2$, 1.0 mM ATP, 1.0 mM EGTA, 14 mM KCl. A 10-fold excess of rabbit skeletal muscle actin over fibroblast myosin (on a weight basis) was used. Protein concentration was estimated by the method of Lowry *et al.* (11), after precipitation with Cl_3CCOOH; bovine-serum albumin was used as a standard.

Electrophoresis. Polyacrylamide gel electrophoresis was performed as described (10). Instead of reduction and alkylation of sulfhydryl groups, the samples were dissolved in 1% SDS–1 mM sodium phosphate (pH 7.0)–50 mM S_2threitol and boiled for 5 min just before electrophoresis.

Binding of Fibroblast Myosin to Rabbit Muscle F-Actin. Fibroblast myosin was mixed with a 4.6-fold excess by weight of rabbit muscle F-actin in the presence of 25 mM imidazole·HCl (pH 7.0), 0.6 M KCl, 5 mM $MgCl_2$, and either 5 mM ATP or an equal volume of water. The samples were centrifuged for 60 min at 100,000 × *g*. The supernatant was assayed for ATPase activity and electrophoresed in SDS–polyacrylamide gels.

Materials

Rabbit skeletal muscle actin was prepared by the method of Spudich and Watt (12). Rabbit skeletal muscle myosin, a gift of Mr. L. Dobkin, was prepared by the method of Kielley and Bradley (13).

RESULTS

Isolation and purification

Two different methods were used to grow clone L-929 mouse fibroblasts. In the initial experiment, twelve 1-liter bottles of monolayer cells yielded 58 mg of protein in the extract supernatant. Actomyosin was prepared as described above, and a sample of the impure protein was electrophoresed in SDS–acrylamide gels. Fig. 1 shows a scan at 600 nm of such a gel stained with aniline blue-black. The position of two peaks, which have the same electrophoretic mobility as the heavy chains of muscle myosin and actin, are indicated. These initial experiments with monolayer cultures of fibroblasts suggested the presence of the two contractile proteins, actin and myosin. To obtain more cells we turned to spinner bottle cultures.

20 Grams of fibroblasts were harvested from 22 liters of spinner cultures. The purification procedure is summarized in Table 1, along with the yields of proteins and enzymic activity.

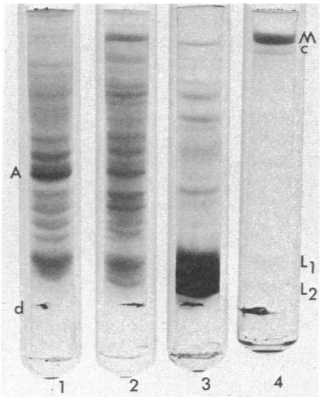
SDS–polyacrylamide gel electrophoresis of the ammonium sulfate fractions revealed that the 0–25% fraction contained a prominent band with the same mobility as rabbit muscle actin, and the 25–50% and 50–70% fractions contain a band with the same mobility as the heavy chain of muscle myosin (Fig. 2). The very dense band near the dye marker on the third gel is probably due to histones. These proteins can be markedly decreased in the preparation without any loss of myosin by ending the ammonium sulfate fractionation at 60% saturation. The specific activity of each of the fractions is indicated in Table 1.

TABLE 2. *Fibroblast myosin: Recovery of protein and enzymic activity*

	Protein (Total mg)	Total EDTA-+K ATPase*	Specific activity† of EDTA-+K ATPase
Extract supernatant	1250	5.0	4
Ammonium sulfate 25–60%	335	3.2	10
Sepharose 4B peak	5.2	2.2	430

* μmol of Pi released per min.

† nmol of Pi released per mg of protein per min.



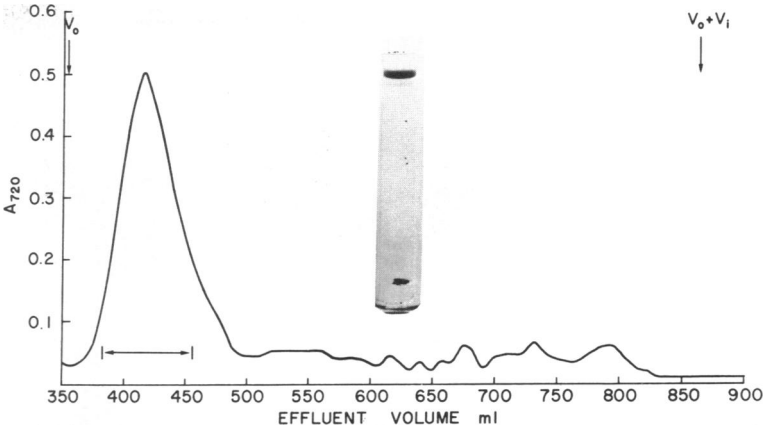


FIG. 2. (*left*) Purification of fibroblast myosin. 0.1% SDS–7.5% acrylamide gels. From *left* to *right;* gel *1*, 0–25% $(NH_4)_2SO_4$ fraction; gel *2*, 25–50% fraction; gel *3*, 50–70% fraction; gel *4*, fibroblast myosin after Sepharose 4B chromatography. *M* coelectrophoreses with muscle myosin heavy chain and *A* with muscle actin. The extent of dye migration is indicated by *d*. In gel *4*, *c* indicates the band due—possibly—to *c* protein and L_1 and L_2 are the light chains of myosin.

FIG. 3. (*right*) Profile of Sepharose 4B agarose filtration of 25–70% ammonium sulfate fraction of fibroblast myosin. The equilibration buffer was 0.6 M KCl–15 mM Tris·HCl (pH 7.5)–1 mM ATP–1 mM $MgCl_2$–5 mM S_2threitol. The two 2.5 × 82 cm columns connected in series were eluted with equilibration buffer. The ordinate indicates A_{720} of the ATPase assay. The flow rate was 20 ml/hr. The photograph is of a 0.1% SDS–7.5% acrylamide gel from the indicated pooled fraction. Migration is from *top* to *bottom*. The extent of bromphenol blue dye migration is marked near the gel bottom.

Agarose chromatography of the 25–70% ammonium sulfate fraction on two 2.5 × 82 cm columns connected in series yielded a single peak of ATPase activity, with the same elution properties as rabbit muscle myosin (Fig. 3). The appropriate fractions were pooled and concentrated in an Amicon Ultrafiltration cell, assayed for the ATPase activities shown in Table 1, and analyzed by SDS–acrylamide gel electrophoresis (Figs. 2 and 3). This purified material was used for further studies.

Characterization of fibroblast myosin

The agarose-purified material has several properties that suggest that it is myosin: (*a*) the intact molecule has the same R_F on Sepharose as muscle myosin, and the dissociated molecule has a heavy chain with the same mobility in an SDS–polyacrylamide gel as the heavy chain of muscle myosin (molecular weight 200,000); (*b*) the purified material has an ATPase activity that is stimulated by EDTA+K and Ca^{2+} and is suppressed by Mg^{2+} in 0.6 M KCl (Table 1).

The myosin was next tested for its ability to bind to rabbit skeletal muscle actin. After centrifugation of fibroblast myosin with rabbit muscle F-actin in the presence and absence of ATP, the supernatants were analyzed by gel electrophoresis and assay of ATPase activity to determine whether the protein was bound to actin and, thus, sedimented with it. When ATP was present in the initial mixture of fibroblast myosin and muscle actin, the supernatant retained both the myosin (Fig. 4) and the ATPase activity. When ATP was not included in the mixture, however, all the ATPase activity and the protein (as indicated by gel *2*, Fig. 4) was lost from the supernatant (a small portion of actin that did not sediment can be seen in both gels). This experiment indicates that fibroblast myosin binds to rabbit muscle actin, and that this complex is dissociated by ATP.

The activation of fibroblast myosin ATPase activity by rabbit muscle actin in the presence of Mg^{2+} at low ionic strength was determined under the conditions outlined in Table 3. A 9-fold activation was found with this preparation. In other preparations, the extent of activation varied from 3- to 4-fold.

An aliquot of the myosin was dialyzed in 0.1 M KCl (pH 7.0) and used for electron microscopy (performed by Dr. Thomas Pollard). In 0.1 M KCl the fibroblast myosin forms short (200-nm long), thin (10-nm wide) bipolar aggregates. These aggregates have a smooth central shaft and tufted ends similar in appearance to short muscle myosin filaments formed *in vitro* (Fig. 5). Treatment of fibroblast myosin with 5 mM $CaCl_2$ in 0.1 M KCl–10 mM imidazole·HCl (pH 7.0) promotes the formation of larger bipolar thick filaments, a result similar to that reported by V. Nachmias (19).

Modified procedure for increased myosin recovery

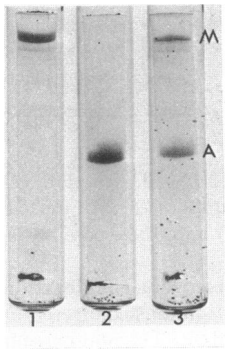
Inspection of Table 1 reveals that only 18% of the total EDTA+K ATPase activity was recovered in the 100,000 × *g* supernatant. Assay of the intermediate fractions showed that practically all of the missing ATPase activity remained in the supernatant after the first low salt (0.1 M KCl) precipitation at pH 6.4. Therefore, the above procedure was modified to omit the low salt precipitation. Ammonium sulfate fractionation was performed directly on the extract supernatant, and the appropriate fraction was chromatographed on Sepharose 4B, as described above. The myosin obtained

TABLE 3. *Activation of fibroblast myosin by rabbit skeletal muscle actin*

	Specific activity*
Fibroblast myosin	0.01
Fibroblast myosin + muscle actin	0.09

Reaction mixture: 3 mM imidazole·HCl, 2.7 mM $MgCl_2$, 1.0 mM ATP, 1.0 mM EGTA, 14.0 mM KCl, 0.30 mg/ml of actin, and 0.03 mg/ml of myosin.

* μmol of Pi per mg of myosin per min.



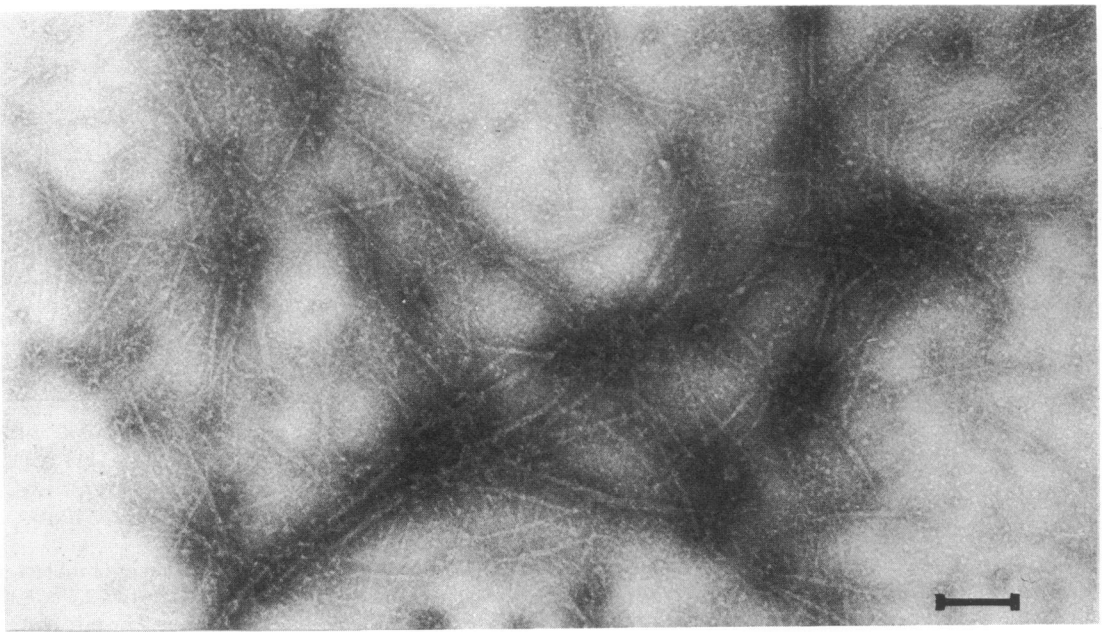


FIG. 4. (*left*) Supernatant fractions from the binding of rabbit skeletal muscle actin to fibroblast myosin. 0.1% SDS–7.5% acrylamide gels. From *left* to *right;* gel *1*, fibroblast myosin, for comparison; gel *2*, supernatant of incubation of fibroblast mysoin with actin in the absence of ATP; gel *3*, supernatant of incubation of fibroblast myosin with actin and 5 mM ATP. Some of the actin, *A*, remains in supernatant of gels *2* and *3* and can be used as an internal standard for the size of the sample applied to the gel. A small amount of Bio-Rad AG1-X2 used for destaining can be seen on the gels.

FIG. 5. (*right*) An electron micrograph of fibroblast myosin filaments formed by dialysis of purified fibroblast myosin against 0.1 M KCl–10 mM imidazole·HCl (pH 7.0). The fibroblast myosin forms short bipolar rods with filamentous substructure. The center of the rods is bare, but projections are found at the tapered ends. There is a tendency for these projections on several rods to clump together. Most of the rods are 150- to 250-nm long and about 10-nm wide. Negatively stained with 1% aqueous uranyl acetate. Magnification ×100,000. *Bar* represents 100 nm.

from this procedure had the same specific activity (see Table 2) as that obtained in the original procedure. The pattern on SDS–acrylamide gels was identical.

DISCUSSION

In this paper we have demonstrated for the first time that an established line of fibroblasts (clone L-929) contains a protein that has structural and enzymic properties similar to muscle myosin. The properties of this protein, including its ability to interact with muscle actin, suggest that it is best described by the name fibroblast myosin.

The method selected for isolation and purification of fibroblast myosin initially involved three steps: precipitation of the actomyosin complex at pH 6.4 at low salt concentration, fractionation of the actin and myosin by ammonium sulfate, and—finally—purification of the myosin on Sepharose 4B. We later found that an increased yield of myosin can be obtained without loss of specific activity by eliminating the low salt precipitation step (see Table 2). Since myosin is the major fibroblast protein of very high molecular weight (about 450,000), Sepharose chromatography proved to be the most critical step of all.

The loss of *total* EDTA+K ATPase shown in Table 1 after Sepharose chromatography was due to the mechanical difficulties of pooling and concentrating small amounts of protein from large volumes. We now consistently recover over 65% of the applied EDTA+K activity. In contrast to platelet myosin, which elutes from Sepharose 4B with two peaks of ATPase activity, fibroblast myosin eluted only as a single peak. This single peak, which has the same R_F as muscle myosin, indicates that unlike platelet myosin, fibroblast myosin has not undergone a partial proteolytic cleavage (10).

Careful inspection of the gel patterns after SDS–acrylamide electrophoresis shows a major band electrophoresing with a mobility similar to the heavy chain of muscle myosin (molecular weight 200,000), a light band just below the heavy band, and two light bands near the bottom of the gel (Figs. 2 and 3). The band just below the heavy chain band may be the recently indentified C protein (14), which is also found bound to muscle myosin. The absence of this band in the binding experiment supernatant is consistent with this possibility (Fig. 4). The lightly staining bands near the dye marker may represent the light chains of fibroblast myosin.

Smooth muscle myosin differs from skeletal muscle myosin in having a lower EDTA+K-stimulated ATPase activity in 0.6 M KCl and a smaller activation of ATPase activity by actin in 14 mM KCl (15). A specific activity of 0.4 μmol of Pi per mg per min for the purified myosin suggests that fibroblast myosin, like platelet myosin, is similar to smooth rather than skeletal muscle myosin. The relatively small (3- to 9-fold) stimulation of myosin ATPase activity by muscle actin is also consistent with this analogy. Whether this lower activation is an inherent property of this myosin or is due to a partial denaturation of the molecule during its preparation (e.g., the oxidation of a specific sulfhydryl group despite the presence of S_2threitol) cannot be assessed (16).

Studies by Bray (3) indicate that actin constitutes about 10% of the extractable protein from several different embryonic cells. Our results indicate that myosin constitutes at least 0.4% of the extractable protein from cloned fibroblasts (see Table 2). The presence of such significant amounts of these proteins in embryonic cells and fibroblasts suggests that they are related to basic cell functions. Cell division, cell morphology, and cell motility are processes that may be moderated by contractile proteins. Therefore, it is of great interest to understand how myosin and actin function in fibroblasts.

Recent work by Perrie *et al.* (17) and by Stull *et al.* (18) has shown that contractile proteins may be phosphorylated. Perrie *et al.* reported a light chain of myosin to be phosphorylated, and Stull *et al.* reported a similar finding with troponin I. In view of the effects of cAMP on fibroblast motility, growth, morphology, and adhesion, the question of whether control of fibroblast actomyosin can be mediated through a phosphorylated contractile protein becomes of great interest.

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