CHARACTERIZATION OF THE MYOSIN-PHOSPHORYLATING SYSTEM IN NORMAL MURINE ASTROCYTES AND DERIVATIVE SV40 WILD-TYPE AND *A*-MUTANT TRANSFORMANTS

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ABSTRACT

Myosin and myosin light-chain kinase have been isolated and characterized from small quantities of normal and SV40-transformed, murine astrocytic neuroglial cells in culture and from intact normal mouse brain. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the astrocyte myosins revealed a heavy chain of 200,000 daltons and two light chains of 20,000 and 15,000 daltons. These myosins are similar to other cytoplasmic myosins. The astrocyte 20,000-dalton light chain can be phosphorylated by an endogenous myosin light-chain kinase which has properties similar to those of the myosin light-chain kinase found in human platelets. No differences were detected in either the astrocyte myosins or myosin light-chain kinases between (*a*) the normal and transformed cells, (*b*) the transformed cells grown at the permissive and nonpermissive temperatures, or (*c*) the SV40 wild-type and *A*-mutant transformants.

KEY WORDS myosin · phosphorylation · astrocytes · SV40 transformation

Interest has been focused recently on the presence and role of cytoplasmic actin and myosin in nonmuscle cells. It would appear that cell motility, mitosis, and other cellular processes could be related to these contractile proteins (1, 12, 22, 29, 34), although this relationship has yet to be demonstrated. To date, the major part of such studies has been to localize cytoplasmic actin and myosin by using specific antibodies (19, 37) or fluorescently labeled heavy meromyosin (30, 31). These studies have demonstrated altered cellular distributions of these proteins during the different phases of the cell cycle (19, 30, 31, 37, 38), namely in interphase and mitosis, and in virally transformed cell lines grown at the permissive and nonpermissive temperatures. A secondary cell line of normal murine astrocytes and derivative lines transformed by SV40 wild-type and TS*A* virus, which have been described recently (5), is such a system and is used in this study.

A large group of temperature-sensitive (TS) SV40 mutants of gene *A* (as yet, the only early gene found [reference 9]) have now been generated and mapped (9, 17). Cells transformed by several representative TS*A* viruses have been found to be defective in many of the parameters of transformed growth when cultured at the nonper-

missive temperature (5, 21, 23). It has, therefore, been suggested that continuous activity of this gene *A* product is necessary to sustain at least part of the transformed cellular phenotype (5, 21, 36). However, the mechanism(s) by which these growth changes are mediated is not known. Differences in the distribution of actin and myosin have been demonstrated by specific antibody staining between normal and transformed cells in culture, and between a rat embryo cell line transformed by TS*A* SV40 (TS*A*28 [references 3, 19]) cultured nonpermissively, and the same line cultured permissively (27). Further, this line reacquires a normal morphology at the nonpermissive temperature (23, 27). These cellular systems are well suited for studies on the changes between the normal and virally transformed states. As such, it was of interest to characterize the contractile machinery of these states at the biochemical level. Therefore, actin, myosin, and the myosin phosphorylating system were examined.

Phosphorylation of myosin is an enzymatic reaction in which the terminal phosphate of adenosine 5′-triphosphate (ATP) is transferred to a specific amino acid residue of this protein, generally a serine residue (25). In myosin, the myosin light-chain kinase is the enzyme which transfers the phosphate group to specific light chains of the myosin molecule, those with molecular weights between 18,000 and 20,000 daltons. This covalently linked phosphate has been shown to influence the interaction of actin and myosin, that is, upon phosphorylation of the myosin light chains, the actin-activation of the myosin low salt ATPase activity is increased (2, 8, 33). Further, another group of enzymes, phosphatases, catalyze the reverse reaction, that is, the removal of the covalently linked phosphate from the phosphorylated light chain of myosin, and thereby return the myosin to the nonphosphorylated form. Such dephosphorylation has been shown to decrease the actin-activation of previously phosphorylated myosin (2, 8). It was, therefore, of interest to determine whether a phosphorylating system existed in these cells.

The purpose of the present study was to determine the following: (*a*) the feasibility of isolating myosin from small quantities of these cells, as in some cases < 1 ml of packed cells was available; (*b*) the type of myosin present and some of its properties; (*c*) the presence or absence of a myosin light-chain kinase and some of its substrates; and (*d*) the changes, if any, in any of these proteins in the various transformed states. In short, the myosin and myosin phosphorylating system of these cells were examined, as was the presence of stress fibers which stain with anti-actin antibodies.

MATERIALS AND METHODS

Cell Lines and Tissue

All the cell lines have been derived from National Institutes of Health (NIH) Swiss mouse brain as described previously (5). Normal mouse brain cells (astrocytes) are designated NMB and were grown from both frozen aliquots of cells previously reported, and from fresh lines initiated in a similar fashion. In this manner, enough of the normal astrocytic cells (0.7–2.0 ml packed cell volume) were obtained for analysis. These cells were harvested between passages 3 and 6 in culture. NMB cells transformed by the wild-type SV40 virus are designated SVWT-MB; cells transformed by a representative TS*A* mutant of SV40 (*A*239) are called *A*239-MB. The permissive temperature for these cells was 33°C, the nonpermissive, 40.8°C. Both transformed lines were used at an early passage (10 or fewer). Whole mouse brain, excluding the meningeal covering, was used in experiments on normal tissue.

Cell Growth and Harvest

The cells were grown in Dulbecco-Vogt (DV) medium (NIH media unit) and supplemented with penicillin, streptomycin, and fetal bovine serum (FBS) in a humidified atmosphere containing 6% CO_2. NMB cells were grown at 37°C with medium containing 20% FBS (DV20). *A*239-MB were grown both at 33°C (the permissive temperature) and at 40.8°C (the nonpermissive temperature) in DV10 or DV20. Reappearance of stress fibers in the TS*A* transformants may be expected by 4 days after the change to the nonpermissive temeprature (27). Serum concentration has no marked effect on the expression of these fibers (38).

Cells were harvested without trypsin, either with 5 mM EDTA in phosphate-buffered saline (PBS) followed by washing in cold PBS and collecting, or by scraping the cells directly into cold PBS, pelleting them (1,000 *g* for 5 min), and then storing them at −20°C.

Immunofluorescent Actin Staining

Cells growing on glass cover slips were prepared for immunofluorescent antibody staining by changing to a low serum-containing medium (DV-1) for at least 24-48 h. The cells were then fixed in either 10% formalin followed by acetone, or, in other experiments, in methanol for 10 min at room temperature. These fixed slides were stained for actin by indirect immunofluorescence with rabbit anti-chicken gizzard actin followed by goat anti-rabbit IgG coupled to fluorescein (14, 15, 18, 19, 27, 28). The percentage of cells with actin cables was

determined in a Zeiss Photomicroscope II with epifluorescence optics at × 630.

Protein Purification—Actomyosin, Myosin, Myosin Light Chains, Myosin Light-Chain Kinase

Cells were thawed and suspended in three volumes of extraction buffer (0.5 M KCl, 0.015 M Tris-HCl, 1 mM EDTA, pH 7.5) with 10 mM $Na_4P_2O_7$ and 10 mM dithiothreitol (DTT). The entire purification was done at 4°C, and deionized water was used throughout. The suspension was homogenized in a VirTis homogenizer (model 23, VirTis Co., Inc., Gardiner, N.Y.) for 2 min in 30-s pulses with 1 min of cooling between each pulse. This slurry was extracted with stirring for 30-45 min, and then centrifuged at 50,000 *g* for 20 min. The supernate was then dialyzed overnight against low salt (50 mM KCl, 2 mM Tris-HCl, 2.5 mM DTT, pH 7.5). The pH was lowered by addition of 0.4 M acetic acid to pH 6.3 for 1 h, and the resultant precipitate was collected by centrifugation. This precipitate was resuspended in the extraction buffer with 2.5 mM DTT at pH 7.5 and made 10 mM in $MgCl_2$ and Na_2ATP. It was then fractionated by the addition of saturated $(NH_4)_2SO_4$ (AS) which contained 10 mM EDTA. The 30–55% saturation fraction (actomyosin) was then suspended in the extraction buffer with 2.5 mM DTT and dialyzed against the same buffer. This fraction was made 10 mM with respect to $MgCl_2$ and Na_2ATP, and applied to a Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) column (either 0.9 × 50 or 1.5 × 90 cm) equilibrated and eluted with the extraction buffer containing 2.5 mM DTT at pH 7.5.

The elution profile of the astrocyte myosin after the Sepharose 4B column was determined by measuring the K_2EDTA-activated ATPase activity of each column fraction. Myosin light-chain kinase was detected by incubation of the column fractions with 0.005-0.010 mM human platelet myosin light chains, 0.2 mM Na_2ATP, 10 mM $MgCl_2$, 0.125 M KCl, and sufficient Na_4 γ-[^{32}P]ATP to give 50,000 dpm in 5 μl of the final mixture. These samples were then analyzed by the filtration method. Therefore, myosin and myosin light-chain kinase were obtained from the column fractions (Fig. 4).

Actomyosin was prepared from rabbit psoas muscle, human extensor carpi radialis, and human platelets as described above. Myosin light chains were prepared from these 30–55% AS fractions (actomyosin) according to the method of Perrie and Perry (24). The light chain-containing supernate from their procedure was exhaustively dialyzed against 20 mM NH_4HCO_3, 1 mM DTT at pH 7.5 and lyophilized. The light chains were then suspended in 10 mM KCl, 5 mM Tris-HCl at pH 7.5 when used for the phosphorylation assays.

Phosphorylation

Actomyosin (30–55% AS), containing an endogenous kinase, was phosphorylated by incubation with 4 mM $MgCl_2$, 2 mM Na_2ATP, γ-[^{32}P]ATP in 0.4 M KCl at pH 7.5 for 1 h at 25°C. The reaction was stopped by addition of 10 mM EDTA, and the excess ATP was removed by dialysis against the extraction buffer with 2.5 mM DTT. These samples were chromatographed on the Sepharose 4B column as described above.

Column-purified myosin was phosphorylated with column-purified myosin light-chain kinase (from the same column) in the presence of 0.4 M KCl, 25 mM Tris-HCl, 12.5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM Na_2ATP with sufficient Na_4 γ-[^{32}P]ATP (30 Ci/mmol) to give 200,000 dpm in 5 μl of the final incubation mixture at pH 7.5 for 1 h at 25°C. The reaction was stopped by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1 and 0.05% $Na_4P_2O_7$.

Myosin light chains from various sources were phosphorylated, as was the column-purified myosin, except that the KCl concentration was 0.125 M. Column fractions of phosphorylated samples were analyzed for radioactivity by counting 100 μl of each fraction in the ACS scintillation cocktail (Amersham/Searle Corp., Arlington Heights, Ill.).

The results of the phosphorylations were analyzed by either Millipore filtration (Millipore Corp., Bedford, Mass.) or on SDS-polyacrylamide gels. For the filter assays, the reactions were stopped by making the reaction mixture 10% in trichloroacetic acid (TCA) and 2% in $Na_4P_2O_7$. The samples were then heated to 90°C for 20 min. Each sample was then filtered through a Millipore HAWP 02500 filter and washed exhaustively with 5% TCA, 1% $Na_4P_2O_7$. The radioactivity was counted with a Packard 3375 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.), with ACS as the counting medium.

Phosphorylation of samples for electrophoresis on SDS-polyacrylamide gels was stopped by the addition of SDS and $Na_4P_2O_7$ to a final concentration of 1 and 0.05%, respectively. These samples were then dialyzed to equilibrium against 0.3, 0.2, 0.1 M NaCl, and against deionized water to remove the excess ATP.

The gels were then cut into 2-mm slices, crushed with a glass rod, and solubilized with 1 ml NCS:H_2O (9:1) (Amersham/Searle Corp.) for 2 h at 50°C, cooled and counted with 0.05% 2,5-diphenyloxazole (PPO) (Research Products International Corp., Elk Grove Village, Ill.) in toluene.

Analytical Methods

Proteins and their components were identified by electrophoresis in 1% SDS and 7.5 or 10% polyacrylamide gels prepared by the method of Fairbanks et al. (11). Lyophilized samples were prepared by solubilization in 1% SDS, 50 mM DTT, and boiled for 2 min. Protein concentration was estimated by the method of Lowry et al. (20), with bovine serum albumin as a standard. ATPase measurements were done at 37°C as described previ-

ously by Adelstein et al. (3) with the substitution of Tris-HCl for imidazole-HCl.

RESULTS

The prominent network of cytoplasmic fibrils (stress fibers) noted in the NMB cells (Fig. 1*a*; [reference 5]) stained brightly with fluorescent antibody to actin (Fig. 1*b*). This suggests an ordered contractile protein system in these cultured normal astrocytes. On the other hand, cells displaying transformed growth showed a diffusely staining actin matrix without the long, well-developed fibers (Fig. 1*c*). These results are similar to previous findings in mouse 3T3 and rat embryo lines and their transformants (27, 28, 38). These previous data also suggested a redistribution rather than a lower concentration of actin in the transformed cells.

The TS behavior of actin cable formation is shown in Table I. NMB cells contained stress fibers at both temperatures (93% cabled), whereas SVWT-MB cells showed the transformed (diffuse) pattern at both temperatures with stress fibers absent in 90% or more of these cells (1 and 10% cabled). However, whereas the culture of *A*239-MB cells clearly showed the transformed (diffuse) pattern at 33°C (4% cabled), it showed a significantly different percentage of cells containing stress fibers, intermediate between the normal (stress fiber-containing) and the transformed (diffuse) cultures at 40.8°C with 27% of the cells cabled.

Actomyosin and myosin were isolated from astrocytic cells of the following types: whole normal mouse brain, NMB in culture, SVWT-MB, and *A*239-MB cultures (grown at 33° and 40.8°C, the permissive and nonpermissive temperatures, respectively). As no significant differences in the parameters studied were found with any of these cells under the conditions used in these experiments, the results reported below for one cell type can be regarded as typical for all the other cell types, as shown in Table I. Actin was not isolated from these cells, but was seen on SDS-polyacrylamide gels of actomyosin (30–55% AS fraction), and associated with the initial peak of the myosin ATPase activity from the Sepharose 4B columns.

Fig. 2 shows the profiles of the K_2EDTA-activated ATPase activity and ^{32}P radioactivity of phosphorylated actomyosin from the *A*239-MB (33°C) cells chromatographed on a Sepharose 4B column. Two distinct peaks of ATPase activity and radioactivity can be seen. The initial peak is actomyosin, and the second is myosin, substantially free of actin. This is demonstrated by the SDS-polyacrylamide gels shown in Fig. 3. The electrophoretic pattern of the proteins in the initial peak from the column profile shown in Fig. 2 is shown in Fig. 3*a*. The major proteins present are the myosin heavy chain, actin, and the myosin light chains. The second peak eluted from the gel filtration column, shown in Fig. 3*b*, consists of the myosin heavy and light chains. Actin was not present in this fraction. For comparative purposes, Fig. 3*c* shows rabbit striated skeletal muscle myosin. These rabbit light chains have molecular weights of 25,000, 18,500, and 16,000 daltons. Therefore, the *A*239-MB myosin light chains have been assigned apparent molecular weights of 20,000 and 15,000 daltons, similar to the light chains from fibroblasts and platelets (3, 4). Further, the astrocyte myosin light chains co-electrophoresed with platelet myosin light chains.

That actin does migrate to the position labeled *A* in Fig. 3 is inferred from gel electrophoretograms that show it co-migrating with rabbit skeletal striated muscle actin prepared by the method of Spudich and Watt (35).

The myosin isolated in the second peak by the procedure described above represents a purification of approximately 150-fold from the low salt precipitate. The results of the myosin K_2EDTA- and Ca^{2+}-activated ATPase specific activities in high salt (0.5 M KCl) are shown in Table II. The values for the column-purified samples are the means of preparations from *A*239-MB (33°, 40.8°C), SVWT-MB (33°C), and NMB.

Samples of the *A*239-MB (40.8°C) column-purified myosin (Fig. 4) were lyophilized and electrophoresed on SDS-polyacrylamide gels. These gels were then sliced, eluted, and counted as described in Methods. One peak of ^{32}P radioactivity was seen in such gels (Fig. 5). The ^{32}P was found in a band corresponding to LC-1, the 20,000-dalton light chain of the astrocyte myosin. In similar experiments, the ^{32}P counts at the top of the gel were completely removed by incubation of the sample with RNase before electrophoresis.

The ^{32}P radioactivity incorporated into the proteins in Figs. 2 and 4 must be incorporated by an endogenous kinase, as no exogenous kinase was added to the 30–55% AS fraction of the astrocytes during the incubation with [^{32}P]ATP. The elution profile of the myosin light-chain kinase activity from the Sepharose 4B column is shown in Fig. 4. This kinase activity was detected by incubation of

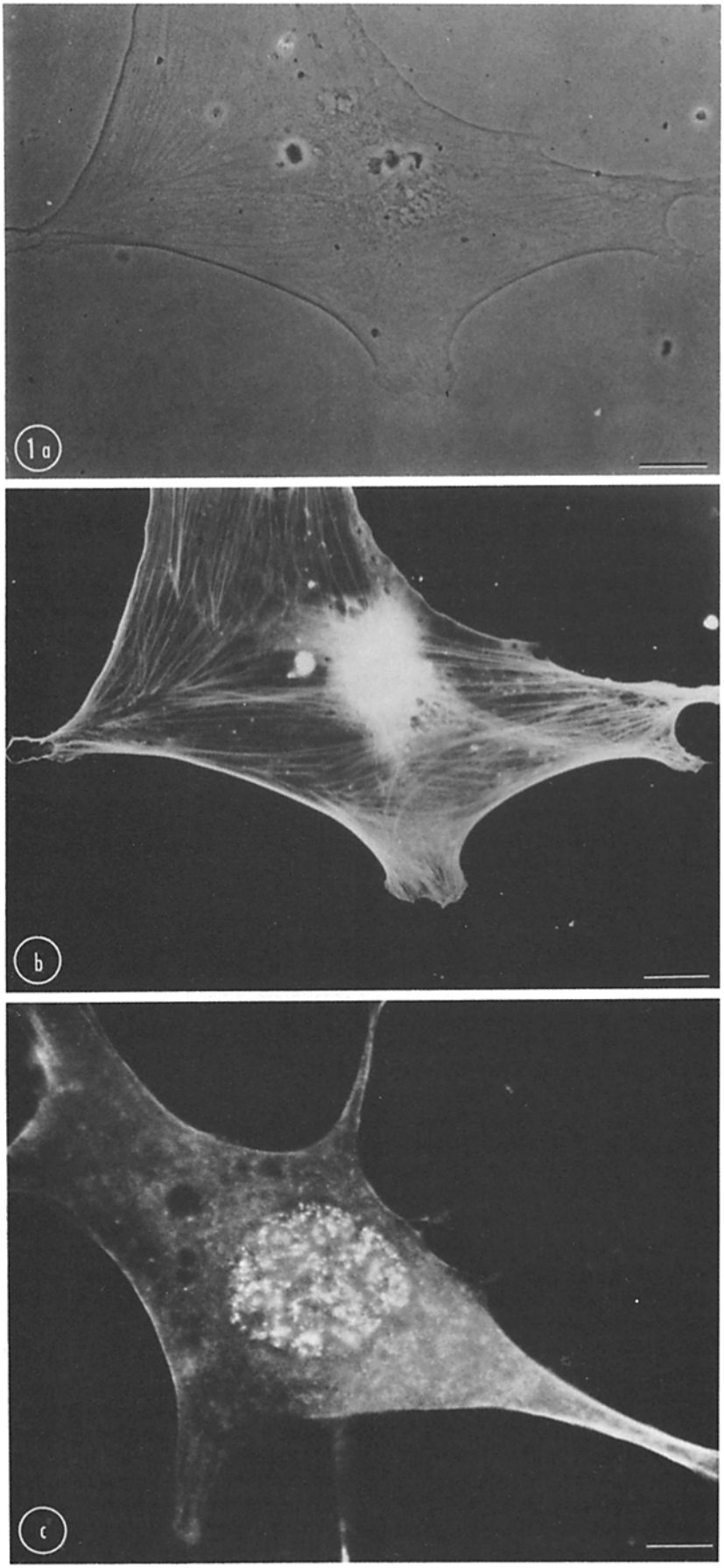
1a
b
c

TABLE I

Characterization of Myosin, Myosin Light-Chain Kinase, and Actin Cable Formation in Astrocytes

Cell type	Number of preparations	Amount of myosin	ATPase		Kinase presence	Cells containing stress fibers
			EDTA	Ca^{2+}		
		mg/g				%
NMB	2	0.97	0.126	0.105	+	93
SVWT-MB (33°C)	2	0.35	0.160	0.133	+	1
SVWT-MB (40.8°C)	1	0.23	0.149	0.119	+	10
*A*239 (33°C)	1	0.28	0.142	0.090	+	4
*A*239 (40.8°C)	1	0.48	0.167	0.139	+	27

The amount of myosin is expressed as milligrams of column-purified myosin per gram of starting material. The specific activities of the column-purified myosin are expressed as micromoles P_i per minute per milligram protein at 37°C. The percentage of cells containing stress fibers after 4 days in culture is indicated. Cells growing at low density at 33°C were changed to low serum medium (DV1) and kept at 33°C or shifted to 40.8°C. After 4 days the cells were formalin fixed, stained (see Methods), and scored blindly for actin cables.

the various column fractions with human platelet myosin light chains and [^{32}P]ATP under the phosphorylating conditions. Further, this kinase was shown not to phosphorylate light chains from rabbit skeletal or human skeletal muscle myosin under the conditions used, as can be seen in Table III.

Column-purified myosin (SVWT-MB, 33°C) that had not been previously phosphorylated did not phosphorylate itself when incubated with γ-[^{32}P]ATP under the phosphorylating conditions. This experiment was analyzed on the gels shown in Fig. 6. However, this same myosin when incubated under the identical phosphorylating conditions with the myosin light-chain kinase isolated from *A*239-MB (40.8°C) astrocytes (Fig. 4), did incorporate ^{32}P only into the 20,000-dalton light chain of the myosin. Fig. 6 clearly demonstrates that only with the addition of the *A*239-MB (40.8°C) myosin light-chain kinase did the SVWT-MB (33°C) myosin incorporate ^{32}P into the 20,000-dalton light chain.

DISCUSSION

Actin is present in the prominent stress fibers of NMB astrocytes, as in the fibers of cells previously studied from other sources (14, 15, 18, 19, 27, 28, 38). In agreement with earlier reports, this study shows that transformed cells (SVWT-MB, *A*239-MB, 33°C) also contain actin. However, the actin is present as a diffuse matrix. Nonpermissively grown cultures of TS*A*-transformed astrocytes (*A*239-MB, 40.8°C) show intermediate behavior (Fig. 1, Table I).

These results parallel the widely divergent growth phenotypes of these different cultures: NMB shows limited growth with doubling times of several days even in 20% serum; *A*239-MB, when grown at the nonpermissive temperature (40.8°C), shows an intermediate pattern with moderate growth in high serum (DV10), but little growth in low serum (DV2^1/$_2$). In addition, these cells are inhibited in colony formation on normal monolayers and in soft agar at 40.8°C; SVWT-MB and *A*239-MB (33°C) show transformed growth with rapid doubling times to high density even in low serum (DV2^1/$_2$) (5).

It is now apparent that actin and myosin are present in many, if not all, eukaryotic cells (7, 29). Therefore, it was expected that astrocytes, whether they be from whole brain, early NMB cultures, or established lines of virally transformed populations (SVWT-MB and *A*239-MB), would contain actin and myosin (Figs. 2, 3).

Judged by the K_2EDTA-activated ATPase measurements, the myosin from these various astrocyte populations was purified roughly 150-fold

FIGURE 1 (*a*) Normal astrocyte (NMB) fixed and then photographed with phase-contrast optics (see Methods). Bar, 12.5 μm. (*b*) Same cell as in (*a*) stained for actin, as described in Methods, and photographed with epifluorescent optics. Note the correspondence between the fibrils in (*a*) and the brightly fluorescing actin cables. Bar, 12.5 μm. (*c*) Transformed astrocyte (SVWT-MB) fixed and stained for actin as in (*b*). Note the diffuse staining as compared with the prominent actin cables in (*b*). Bar, 10.0 μm.

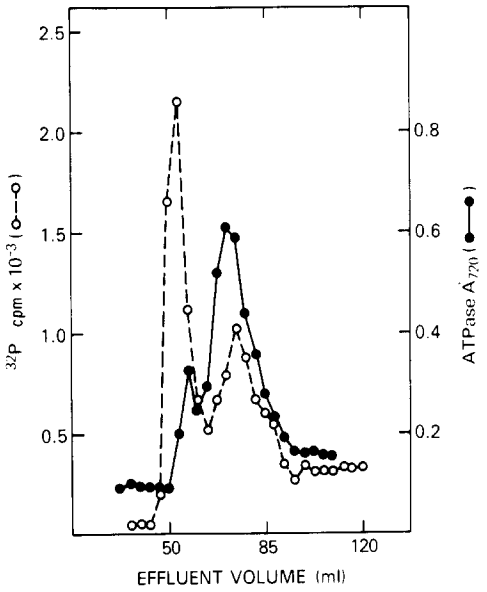

FIGURE 2 The elution profiles from Sepharose 4B chromatography of phosphorylated astrocyte (*A239-MB*, 33°C) actomyosin (35–55% AS fraction). A 2.1-ml phosphorylated sample was applied to the 1.5 × 90-cm column equilibrated and eluted at 17 ml/h with 0.5 M KCl–15 mM Tris-HCl (pH 7.5)-1 mM EDTA–2.5 mM DTT. Fractions of 2.7 ml were collected, of which 0.2 ml was used for the K_2EDTA ATPase assay (A_{720}) (●) and 0.05 ml for ^{32}P determination (○). The void and total volumes of the column were 40 and 145 ml, respectively.

from the ATPase in the low salt precipitate (Table II). The myosin ATPase values obtained are remarkably consistent (SE ±8%) though somewhat lower than the values obtained for other cytoplasmic myosins such as fibroblasts (0.43 [reference 3]) and brain (0.27 [reference 6]). These values (Table I) may reflect an inherently lower ATPase activity for myosin isolated from these particular cells. Alternately, they may reflect partial denaturation of the myosin secondary to the low concentration of protein after column chromatography. This, in turn, was due to the small amounts (often < 1 g) of starting material. Judged by SDS-polyacrylamide gel electrophoresis, the myosin was free of other contaminating proteins, and contained a heavy chain of 200,000 daltons as well as light chains of 20,000 and 15,000 daltons. These light chains are similar in molecular weight to those found for other nonmuscle myosins. Unlike

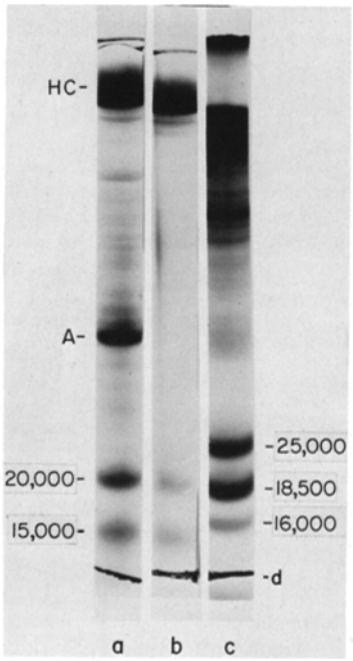

FIGURE 3 1% SDS–7.5% polyacrylamide gels of the initial and second peaks from the Sepharose 4B column shown in Fig. 2. Electrophoresis was carried out from top to bottom. The dye front is designated (*d*). All the samples were run at the same time. (*a*) The initial column peak shows the myosin heavy chain (*HC*), the light chains (*20,000* and *15,000*), and actin (*A*). (*b*) The second column peak shows pure myosin. (*c*) A standard of rabbit skeletal muscle myosin showing light chains of 25,000, 18,500, and 16,000 daltons.

TABLE II

Myosin ATPase Specific Activities and Protein Recovery

	K_2EDTA	Ca^{2+}	Protein recovered
Low salt precipitate	0.001	0.002	143
30-55% AS fraction	0.016	0.012	2.26
Column-purified	0.159 ± 0.013	0.131 ± 0.006	0.34

The specific activities are expressed as micromoles P_i per minute per milligram at 37°C. The column-purified myosins are given as the mean ± SE, $n=4$. The amount of myosin recovered at the various stages of purification is expressed as milligrams of protein per gram wet weight of starting material.

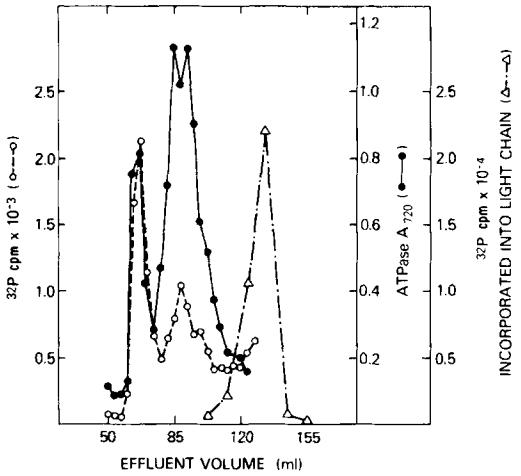

FIGURE 4 The elution profile from Sepharose 4B chromatography of phosphorylated astrocyte (*A*239-MB, 40.8°C) actomyosin (35–55% AS fraction). A 3.2-ml phosphorylated sample was applied to the 1.5 × 90-cm column equilibrated and eluted at 16 ml/h with 0.5 M KCl–15 mM Tris-HCl (pH 7.5)–1 mM EDTA–2.5 mM DTT. Fractions of 2.9 ml were collected, of which 0.2 ml was used for the K_2EDTA ATPase assay (A_{720}) (●), 0.05 ml for ^{32}P determination (○), and 0.2 ml was used in the kinase detection (△) assay utilizing human platelet myosin light chains as described in Methods. The void volume and salt boundary of the column were at 50 and 155 ml, respectively.

Burridge and Bray (7), we found no evidence for an additional 23,000-dalton light chain in our preparations.

Previous reports have demonstrated that myosin from both muscle and nonmuscle cells can be phosphorylated. In the case of white skeletal muscle myosin, the 18,500-dalton light chain is phosphorylated (13, 25), and in the case of platelets, the 20,000-dalton light chain is phosphorylated (2, 4). As in platelets and other nonmuscle myosins, the 20,000-dalton light chain is phosphorylated in astrocytes. Phosphorylation of muscle myosin and platelet myosin is catalyzed by specific kinases (10, 26). These kinases differ in their properties, particularly with respect to their divalent cation requirements. These data indicate that the platelet and astrocyte myosin light-chain kinases are a class of kinases, cytoplasmic kinases, separate and distinct from the striated muscle myosin light-chain kinase.

Although it had been demonstrated previously that the kinase isolated from platelets could phosphorylate other nonmuscle myosins, this cytoplasmic type of kinase had not been shown to exist in other nonmuscle cells. This study is the first to demonstrate that such a kinase does exist in dividing cells (32). Moreover, as shown in Table III, this kinase resembles the platelet kinase in substrate specificity; it can phosphorylate human platelet light chain, but does not phosphorylate human skeletal or rabbit skeletal muscle light

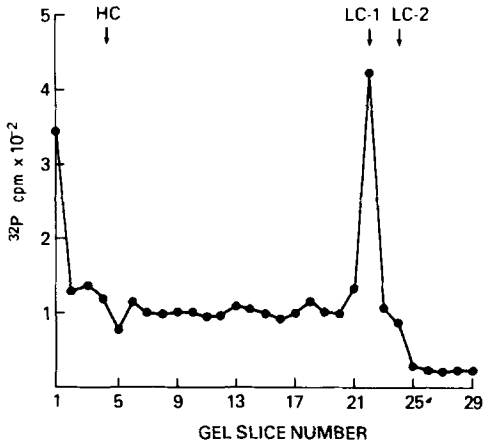

FIGURE 5 A profile of the ^{32}P incorporated counts from a 1% SDS–7.5% polyacrylamide gel of astrocyte myosin (*A*239-MB, 40.8°C). Electrophoresis was carried out from left to right. Astrocyte actomyosin (35–55% AS fraction), containing endogenous kinase, was incubated with Mg[^{32}P]ATP and then column chromatogrammed as described in Methods. The phosphorylated myosin peak from the column was then lyophilized and analyzed by SDS-polyacrylamide gel electrophoresis. The stained gel was then sectioned into 2-mm slices which were then eluted and counted in a scintillation counter. Note that *LC-1*, the 20,000-dalton light chain of the astrocyte myosin, has ^{32}P counts incorporated into it. *HC* is the myosin heavy chain, *LC-1* the 20,000-dalton light chain, and *LC-2* the 15,000-dalton light chain.

TABLE III

*Substrates for the Astrocyte Myosin Light-Chain Kinase**

Substrate (light chains)	−AMLCK‡	+AMLCK
None	–	1,742
Human platelet	315	26,352
Rabbit psoas	887	1,384
Human skeletal	281	1,017

* The results are expressed in ^{32}P counts per minute as analyzed by the millipore filtration method for phosphorylation (see Methods). The incubations were terminated after 10 min in the presence of Mg[^{32}P]ATP.

‡ Astrocyte myosin light-chain kinase (*A*239-MB, 40.8°C).

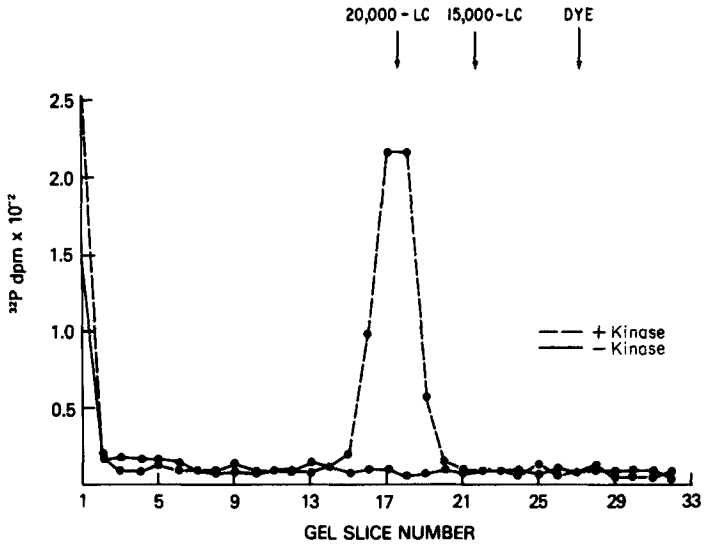

FIGURE 6 Profiles of the ^{32}P incorporated counts from 1% SDS–10% polyacrylamide gels of column-purified astrocyte (SVWT-MB, 33°C) myosin incubated with Mg[^{32}P]ATP in the presence and absence of column-purified myosin light-chain kinase (*A*239-MB, 40.8°C). Electrophoresis was carried out from left to right. Note that the 20,000-dalton light chain of the astrocyte myosin (*20,000-LC*) is phosphorylated only in the presence of the astrocyte myosin light-chain kinase. The 15,000-dalton light chain (*15,000-LC*) is not phosphorylated in either case.

chain as well. These results are probably a manifestation of the differences in the K_m of the kinase with the various substrates (33). Similarly, it is of interest to note that even though these astrocytic cells have a widely differing phenotype (SVWT-MB, 33°C, *A*239-MB, 40.8°C), albeit derived from the same primary source (NMB), the myosin light-chain kinase from one type can phosphorylate the myosin from the other (Fig. 6).

In summary, the results of this study reveal that the myosin phosphorylating system (i.e. myosin and the myosin light-chain kinase) of astrocytes of widely differing phenotypes can be isolated and purified from very small amounts of cells. Further, alterations in the distribution of the contractile proteins of the cell need not reflect changes in the basic biochemical interations they undergo. Whether changes in growth patterns or in cell division per se are reflected in changes in the contractile systems cannot be answered yet, but techniques for assessing these parameters are now available. In this regard, the phosphorylation of rat proliferative myoblast myosin, human platelet myosin, and smooth muscle myosin has been shown (in vitro) to increase the actin-activated ATPase activity (2, 8, 16, 33). Relating myosin phosphorylation to a physiological event such as cell division, cytokinesis, or secretion, is the ultimate aim of this work.

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