

# Localization of Cytoplasmic and Skeletal Myosins in Developing Muscle Cells by Double-label Immunofluorescence

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**ABSTRACT** Antibodies to a cytoplasmic myosin, rat lymphoma myosin, and to rat skeletal myosin were prepared in rabbits and shown to be specific for their corresponding antigens. The two antibodies did not cross-react. The skeletal myosin antibody was directly labeled with rhodamine, and the cytoplasmic myosin antibody was detected by indirect immunofluorescence with fluorescein-labeled goat anti-rabbit antibody.

The two antibodies were used to examine developing rat muscle cultures for the presence and location of the antigens. The antibody to cytoplasmic myosin reacted with multinucleated myotubes and with all the mononucleated cells in the culture. The antibody to skeletal myosin reacted with myotubes and with a small fraction of the mononucleated cells. In the myotubes, the cytoplasmic myosin appeared to be localized primarily in two structures: fine stress fibers, often visible also by phase microscopy and present predominantly in the ends of the cells, and in a submembranous rim all along the cell's border. In addition, a diffuse fluorescence within the cells was observed. The skeletal myosin was localized in the central part of the myotubes in sarcomeres or in fibers without periodicities and was excluded from the ends of the myotubes.

When the same cells were doubly stained with the two antibodies, the complementary distribution of the two isozymes was very clear. There was also a narrow region of overlap of staining, with cytoplasmic myosin present in some stress fibers that appeared to be continuous with fibrous elements containing skeletal myosin.

Myotubes that rounded up with cytochalasin B or with trypsin displayed a diffuse distribution of both isozymes. When these cells were allowed to respread into extended configurations, the location of the two myosins was essentially the same as in untreated cells.

The ability of myotubes to adhere to the surface and to move in culture may be related to the presence of cytoplasmic myosin. Our results show that in myotubes and myoblasts the two isozymes differ sufficiently to be localized in distinct regions of the cell and to be sorted out into different structures, even after the cytoplasmic contents have been reshuffled. The cell can, by some unknown mechanism, distinguish the two myosins.

Many tissues contain isozymes, which perform the same enzymatic functions but differ quantitatively in their properties and *in vitro* may hybridize to form enzymes with different subunits (20). In the case of skeletal and cardiac muscles, isozymes of sarcomeric myosin have been shown to be present in a single cell (11, 19, 31, 34), although differences in location within the

cell have not been studied extensively. Most nonmuscle cells contain another myosin isozyme that has come to be called cytoplasmic myosin (27). This isozyme shares many of the properties of the myosins found in the differentiated cells of skeletal, cardiac, or smooth muscle cells, such as interaction with actin, ATPase activity, formation of filaments, and overall

size and shape of the molecule and its subunits, but it differs quantitatively in many of these characteristics. By a variety of circumstantial pieces of evidence, such as the myosin being in the right place at the right time, this isozyme of myosin appears to be related to forms of contractility such as fibroblast or amoeboid motility, cytoplasmic streaming, capping of surface receptors, phagocytosis, and cytokinesis (4, 10, 12, 13, 22, 35, 37). In the case of cytokinesis, more direct evidence has been provided for a role of cytoplasmic myosin. Mabuchi and Okuno (18) showed that an antibody specific to starfish egg myosin would prevent cleavage in the eggs.

During the development of a myotube from precursor cells there are several striking events: fusion of the mononucleated cells to form elongated syncytia, appearance of sarcomeres containing skeletal myosin, actin, and associated proteins, accompanied by spontaneous linear contractions of the myotubes (2, 5, 36). The emergence of the apparatus for and the visual evidence of muscle contractility is sufficiently impressive that it is easy to overlook other motile properties of the myotube. Myotubes adhere to substrates, and time-lapse cinematography shows that they also move in culture (reference 2 and I. Konigsberg and H. Holtzer, personal communications). Endocytosis by myotubes has also been reported (6). If it is true that cytoplasmic myosin is necessary for such functions, then this isozyme should be present in the myotubes.

There are, in fact, two pieces of evidence suggesting that myotubes may contain cytoplasmic myosin, but neither is conclusive. First, ultrastructural observations have shown the presence of not only microtubules and intermediate filaments in myotubes but also microfilaments not associated with sarcomeres (8, 14, 15). Because microfilament bundles have been shown to be associated with cytoplasmic myosin in fibroblasts (10, 28), this might also be true for myotubes. Second, it has been observed that cytoplasmic myosin metabolically labeled in presumptive myoblasts persists at least into the early myotube stage (30).

We wanted not only to detect the possible presence of cytoplasmic myosin but also to compare its distribution with that of the skeletal myosin. To localize two different myosin types in a single myotube presents several difficulties. Cytoplasmic myosins cannot be reliably detected by electron microscopy, and the lack of pure populations of myotubes poses serious problems for a biochemical approach; small amounts of contamination with fibroblasts could make the assignment of cytoplasmic myosin to the myotube quite unconvincing.

We have, therefore, used double-label immunofluorescence, the best available technique. We have employed this method to demonstrate that cytoplasmic and skeletal myosins do indeed coexist in myotubes and also in some myoblasts. Some unexpected features of the distribution of the two myosins are observed. The results show that the cell can distinguish between the two closely related types of myosin and, in general, segregates them into different structures. The distributions also suggest that cytoplasmic myosin may have more than one function in myotubes.

Some of these results were presented at the annual meeting of the American Society for Cell Biology in November, 1979 (7).

## MATERIALS AND METHODS

### *Muscle Cultures*

Muscle cultures derived from Wistar rats were grown according to the method of Yaffe (40), with some modifications. Leg and lower back muscles of 1–2-day-

old rats were excised, minced, freed of visible connective tissue, and then immersed in at least 10 vol of 0.5 mg/ml collagenase (CLS, Worthington Biochemical Corp., Freehold, N. J.) in balanced salt solution. After 60 min at 37° C, the cells were spun down, washed once in medium, and then pipetted repeatedly to break up aggregates. The resulting suspension was filtered through two layers of lens tissue and counted in a hemacytometer. Preparations were routinely 80–90% viable as judged by trypan blue exclusion. Cells were plated out at a density of  $4\text{--}6 \times 10^5$  cells/ml on collagen-coated coverslips or tissue culture plastic. The medium consisted of four parts Dulbecco's Modified Eagle's Minimal Essential Medium (DMEM), one part Medium 199, 10% horse serum, 1% penicillin-streptomycin, and 20  $\mu\text{g}/\text{ml}$  of amphotericin B, all from Grand Island Biological Co. (GIBCO), Grand Island, N. Y., and 1% chicken embryo extract, a generous gift from Dr. H. Holtzer.

In most cultures, steps were taken to increase the proportion of myogenic relative to nonmyogenic cells. Here, after the initial 40 h of culture, cells were trypsinized briefly and then incubated in noncoated tissue culture dishes for 30–60 min. The less adherent myogenic cells were collected in the supernate (39). These cells were then replated on collagen-coated surfaces either immediately or after receiving 5,000 rads of x irradiation. This radiation treatment has been shown to effectively inhibit further cell division while leaving muscle differentiation largely unaffected (9). In either case, rapid fusion ensued within 5–10 h. Cultures were fed at 49-h intervals.

### *Fibroblast Cultures*

Rat fibroblasts were obtained either from the second passage of muscle cultures or from the adhering cells in the selective transfer step. The cells were maintained on uncoated surfaces in "fibroblast medium" consisting of 90% DMEM and 10% fetal calf serum, plus antibiotic-antimycotic as described above.

### *Rat Lymphoma Cell Line*

The Fisher-Dunning rat lymphoma cell line, obtained from Dr. Ronald Herberman of the National Institutes of Health, was grown in ascites form in F344 rats (Microbiological Associates, Walkersville, Md). Each animal was inoculated intraperitoneally with 50–100 million cells. After 5–7 d, the animals were killed, and their peritoneal cavity was injected with 9 ml of fibroblast medium. The abdomen was then opened and the cells were collected. The cells were washed twice in medium if they were to be used for transfer to other animals or in phosphate-buffered saline (PBS) if harvested for preparative use. Cells used exclusively for harvest were also grown in Wistar rats that had been given 600 rads of x irradiation. The final yield in either case was 0.5–1.5 ml of packed cells per animal.

### *Lymphoma Myosin Purification*

Native lymphoma myosin was purified by modification of previous methods (13, 21). All procedures were performed at 4° C. Cells, either frozen, for antigen preparation, or fresh, for enzyme studies, were mixed with two vol of cold extraction solution consisting of 0.34 M sucrose, 1 mM EDTA, 0.5 mM ATP, 5 mM dithiothreitol (DTT), 0.4 mM PMSF, 100,000 IU aprotinin, and 10 mM imidazole, pH 7.0. They were homogenized for 5 min in a Potter-Elvehjem motor-driven homogenizer with a Teflon pestle. For efficient disruption of fresh cells, an additional 50 passes in a Dounce homogenizer (Kontes Co., Vineland, N. J.) were required.

The resulting homogenate was spun at 100,000 g for 1 h to remove organelles and unbroken cells. The supernate, which had passed through glass wool to remove lipid, was then adjusted to 10 mM in MgATP and to 30% in neutralized ammonium sulfate. After gentle stirring for 30 min, the sample was spun at 40,000 g for 20 min and the precipitate was discarded.

The supernate was adjusted to 60% in ammonium sulfate, then stirred and spun as described above. The second precipitate was solubilized in 0.6 M KCl, 1 mM EDTA, 1 mM DTT, proteolytic inhibitors as described above, and 10 mM imidazole, pH 7.5. The solution was clarified by centrifugation at 40,000 g for 30 min and dialyzed for 6–12 h against 50 mM NaCl, 1 mM  $\text{MgCl}_2$ , 1 mM EGTA, and 10 mM imidazole, pH 6.8. The resulting precipitate was solubilized in 0.6 M KI, (Darco, decolorized with activated carbon immediately before use to remove free iodine) 5 mM DTT, 1 mM EGTA, 2.5 mM  $\text{MgPP}_i$ , proteolytic inhibitors as noted above, and 10 mM imidazole, pH 7.0. It was stirred gently for 15 min and centrifuged at 40,000 g for an additional 15 min to clarify it fully. The sample was then applied to a  $2.5 \times 90\text{-cm}$  column of 4% agarose (Bio-Gel, Bio-Rad Laboratories, Richmond, Calif., A-15 m) equilibrated with 0.6 M KCl, 0.2 mM EDTA, 0.2 mM DTT, and 10 mM imidazole, pH 7.0. Fractions eluting behind the void with high  $\text{K}^+$ , EDTA ATPase activity were pooled and then concentrated by dialysis vs. dry Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) or by negative pressure dialysis.

Lymphoma myosin heavy chain was purified by a preparative gel electrophoresis method. Here a low-salt precipitate was made directly from the extract supernate without previous fractionation. The crude actomyosin was boiled in sample buffer and applied to preparative slab gels (see below).

### Skeletal Myosin Purification

Back and hind leg muscle was obtained from Wistar rats. Early skeletal myosin purifications were carried out by a DEAE-Sephadex column method (23), with the addition of 10 mM ATP during the ammonium sulphate steps (25). Because of persistent proteolysis during the long dialysis, a more rapid procedure was adopted in which the reprecipitated myosin was chromatographed on 5% agarose using the column buffer described in the lymphoma myosin preparation.

### ATPase Assays

High-salt activity was measured at 37°C in 0.6 M KCl, 0.2 mM DTT, 1 mM ATP, 10 mM imidazole, pH 7.0, and either 5 mM CaCl<sub>2</sub> or 2 mM EDTA for K<sup>+</sup>, Ca<sup>2+</sup> or K<sup>+</sup>, EDTA ATPase activities, respectively. Inorganic phosphate was measured as described previously (21).

### SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in SDS was performed according to the method of Laemmli (17). For gradient gels, 7 μM flavin mononucleotide was substituted for ammonium persulfate, and the gel was polymerized with fluorescent light for 30 min. Gels were fixed and stained in 25% methanol, 10% acetic acid, and 0.25% Coomassie Brilliant Blue. The slab gels were scanned at 550 nm on a Transidyne scanner (Vibrodyn, Inc., Dayton, Ohio).

Preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 4.5-mm-thick 5% slab gels. One sample was applied to the entire gel and electrophoresed at 20 mA for 16–20 h. The myosin heavy chain was then visualized by immersing the unfixed gel in 50 mM KCl for 10–20 s. The band was then excised and ground with a glass rod. The attendant polypeptide was then eluted electrophoretically and collected in a dialysis bag as described by Tuszynski et al. (38).

### Antibodies

**ANTI-RAT LYMPHOMA MYOSIN:** Two rabbits (1 and 2) were injected subcutaneously in the back, axilla, and groin with 800 μg of electrophoretically purified lymphoma myosin heavy chain in complete Freund's adjuvant. They were boosted at the same sites 4 and 12 wk thereafter with 500 and 100 μg of the same antigen in incomplete Freund's adjuvant. The third rabbit was given a primary inoculation of 200 μg of electrophoretically purified heavy chain injected intrasplenically at surgery. This rabbit was boosted 4 and 12 wk later with 200 and 100 μg of column-purified myosin, respectively. Finally, 2 wk after the last native boost, this rabbit was injected with 250 μg of electrophoretically purified heavy chain. Boosts were given (a) subcutaneously and in footpads, and (b) intravenously, in incomplete Freund's adjuvant and 20 mM NaPP<sub>6</sub>, pH 8.0, respectively. All animals were bled 7–14 d after the last boost. The serum was stored at –20°C.

**ANTI-RAT SKELETAL MUSCLE MYOSIN:** 4 mg of DEAE-Sephadex-purified myosin in complete Freund's adjuvant was injected subcutaneously as described above. The rabbit was then boosted at the same sites at 3 and 6 wk with 1 and 0.5 mg, respectively, of the same antigen in incomplete Freund's adjuvant. The animal was bled as described above.

### Purification of IgG Fractions

The IgG used in the indirect immunofluorescence and immunochemical studies was purified by ammonium sulfate fractionation. The serum was brought to 50% saturation, and the precipitate was collected, redissolved in PBS, and fractionated twice more with 44% ammonium sulfate. The resulting IgG was brought to ~20 mg/ml, dialyzed against PBS, and stored at –20°C in 0.02% sodium azide.

### Direct Labeling of IgG

IgG to be directly labeled was purified and conjugated by the method of Cebra and Goldstein (3) with tetramethylrhodamine isothiocyanate (TMRITC, BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.). Labeled IgG was sterilized by filtration and stored in PBS with 0.02% azide at 4°C. The conjugates had a dye to protein ratio of 2.1.

### Staphylococcal Protein A Immunoprecipitation

Staphylococcal protein A-antibody absorbant (Staph) was prepared according to the method of Kessler (16) and stored at –70°C. Before use, the Staph was thawed, brought to 3% in SDS and 10% in 2-mercaptoethanol, and heated to 100°C for 30 min. The Staph was spun at 5,000 g for 10 min, the supernate was discarded, and the procedure was repeated. The absorbant was resuspended in 0.4 M NaCl, 5 mM EDTA, 50 mM Tris, pH 8.0, 0.5% Triton-X 100, and 0.02% sodium azide. The cells were incubated at 4°C for 15 min, washed twice in this buffer containing 0.05% Triton-X 100 (HiNet 0.05), and resuspended to 10% (vol/vol).

Immunoprecipitations and washes were carried out at 4°C in polypropylene centrifuge tubes; all spins were for 2 min in an Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, N. Y.) (8,000 g). The extract to be tested was incubated for 1 h with 0.1 vol of absorbant, the Staph was removed, and the desired IgG was added. After incubation for 3 h, a second volume of Staph (previously determined to be sufficient to bind the IgG present) was added and the mixture was incubated for 1 h. The Staph A (IgG, antibody-antigen) complex was spun down and washed three times in HiNet 0.05 with 3 M urea. The final Staph pellet was resuspended in 50–150 μl of Laemmli sample buffer and boiled for 5 min. The stripped Staph was removed by centrifugation for 10 min at 8,000 g. The supernate, containing IgG, antigen, and antibody, was either concentrated by lyophilization or used directly for SDS-PAGE.

### Cell Fixation and Antibody Staining

Cultures grown on glass coverslips were fixed either (a) at room temperature in 2% formaldehyde in PBS followed by 100% methanol or (b) in –20°C 100% methanol, airdried, rehydrated, and postfixed in 10% formalin in PBS at room temperature. The coverslips were then washed in PBS with 0.05% Triton X-100 (PBST). The cells were stained with 1 mg/ml IgG in PBST, washed for 1–3 h in PBST and, for indirect immunofluorescence, incubated with 0.5 mg/ml fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG (fGAR, N. L. Cappel Laboratories, Inc., Cochranville, Pa., lot 11290). All incubations were carried out at 37°C for 30 min in a humid chamber. After washing as described above, the coverslips were mounted in 90% glycerol–10% PBS, sealed with nail polish, and stored at 4°C in the dark until used.

For double staining, cultures were first indirectly labeled, as described above, except that the primary antibody and the fGAR were adjusted to 2 mg/ml in normal goat IgG. Unoccupied anti-rabbit IgG sites were then blocked by incubating the coverslips in 5 mg/ml normal rabbit IgG. The cultures were next directly labeled with 0.5 mg/ml TMRITC-conjugated rabbit anti-skeletal myosin (SkM) in 2.5 mg/ml normal rabbit IgG. Washing and mounting were performed as described above.

### Fluorescence Microscopy

Cells were examined under epifluorescent illumination in an Olympus microscope equipped with Zeiss ×40 and ×63 oil immersion lenses (NA 1.0 and 1.4, respectively). It was equipped with the following filter combinations: for FITC, the excitation filter was 455–490 BP blue and the barrier was a combination of LP 500 and BP 525; TMRITC excitation was with a BP 436/12 filter and the barrier was an LP 590. Exposures of 30–60 s were made on Tri-X pan film. The film was developed in HC-110 (MinMax, Harbor City, Calif.) dilution A (Kodak), with 1% factor 8 for an effective ASA of 1,000.

### Respreading

Cells in 72-h cultures were rounded up with 5 μg/ml of cytochalasin B (32) in 0.5% dimethyl sulfoxide in muscle medium described above. Cultures were reversed by washing out the drug-containing medium after 6 h and replacing it with fresh normal medium.

Myotubes in 42-h cultures were rounded up with 0.25% trypsin (GIBCO). After the cells had come off from the dish, the reaction was stopped with horse serum and the cells were washed once with normal medium. They were then plated out on collagen-coated surfaces and allowed to respread.

### Chemicals

Aprotinin was obtained from FBA Pharmaceuticals, Inc., N. Y. and cytochalasin B from Aldrich Chemical Co., Inc., Milwaukee, Wis.

## RESULTS

### Lymphoma Myosin Purification

An electrophoretic summary of the native myosin purification is presented in Fig. 1. As can be seen, the 30% ammonium sulfate cut (lane 3) removes a good deal of actin. The myosin is then precipitated at 60% ammonium sulfate (lane 4). The salted-out protein is then resolubilized in high salt. The precipitate obtained after subsequent dialysis against low salt is shown in lanes 5 and 6; it is clearly enriched for myosin. After treatment with KI, this material is then applied to the A15m gel filtration column. The fractions eluting with high  $K^+$ , EDTA ATPase are pooled and constitute the purified myosin, as shown in two loadings in lanes 8 and 9. Scanning densitometry of the sample shown in lane 9 indicates that the myosin heavy chain and the three low molecular weight polypeptides of  $\sim 17,000$ , 19,000, and 20,000 daltons constitute 97% of the protein. If the 20,000-dalton protein is regarded as a contaminant, the material is 95% pure.

The lymphoma myosin elutes from the column at the same volume as chicken or rat muscle myosin. The purified protein has a  $K^+$ , EDTA ATPase of  $0.9 \mu\text{mol P}_i/\text{min}/\text{mg}$ , and a  $K^+$ ,  $\text{Ca}^{2+}$  ATPase of  $0.45 \mu\text{mol P}_i/\text{min}/\text{mg}$ . These activities are in good agreement with results obtained from other cytoplasmic myosins (27).

### Antibodies

**ANTILYMPHOMA MYOSIN:** We screened our antibodies by indirect immunofluorescence staining of rat fibroblast cultures. We observed characteristic stress fiber patterns with immune but not preimmune sera. However, the positive antisera gave no detectable precipitin band when tested by double diffusion in agar against crude or purified lymphoma myosin.

The antisera were, therefore, tested immunochemically with the sensitive Staph A immunoprecipitation technique as previously described. An SDS gel of a Staph A immunoprecipitation is shown in Fig. 2. The gel demonstrates that the anti-rat lymphoma myosin (anti-rLM) specifically precipitates the lymphoma myosin heavy chain from a complex sucrose extract of lymphoma cells when mixed with Staph A. No polypeptides are precipitated by the preimmune IgG. When anti-rSkM was used, no precipitation was observed, but there was some degradation of the lymphoma myosin band presumably caused by impurities (data not shown).

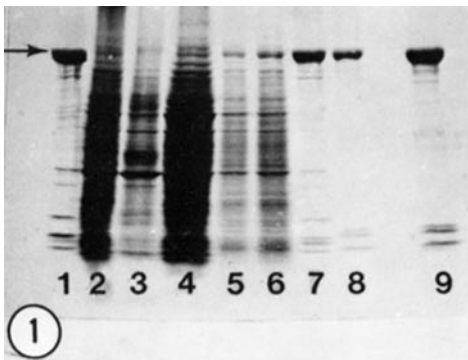


FIGURE 1 Lymphoma myosin purification. Lanes 1 and 7, chicken skeletal myosin standard; lane 2, sucrose extract; lane 3, 0-30% ammonium sulfate precipitate; lane 4, 30-60% ammonium sulfate precipitate, lanes 5 and 6, two loadings of material applied to A15m column; lanes 8 and 9, two loadings of purified lymphoma myosin. Arrow indicates position of myosin heavy chain.

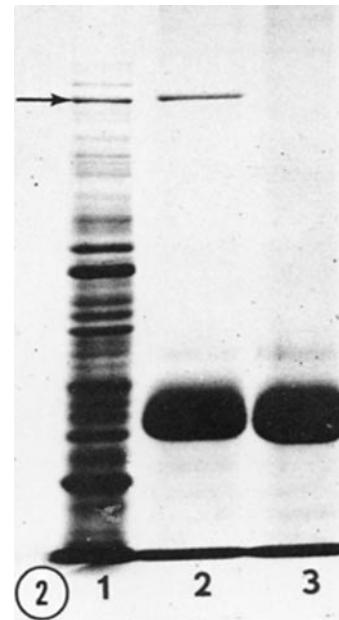


FIGURE 2 Staph A immunoprecipitate of rLM. Lane 1, lymphoma sucrose extract; lane 2, immunoprecipitate from this extract with anti-rLM (note that only this IgG precipitates one band); lane 3, as in 2, but with preimmune IgG. Arrow indicates the position of the myosin heavy chain. A 7% SDS polyacrylamide gel.

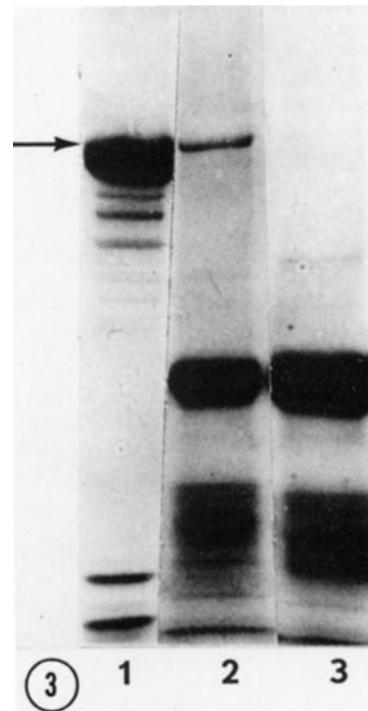


FIGURE 3 Staph A immunoprecipitate of rSkM. Lane 1, reprecipitated rat skeletal myosin; lane 2, immunoprecipitate with anti-rSkM; lane 3, as in 2, except with anti-rLM. This is a 12% SDS gel, so the heavy chains of the IgG do not migrate as far into the gel. Note that anti-rLM does not precipitate skeletal myosin.

**ANTISKELETAL MYOSIN:** Fig. 3 shows Staph A immunoprecipitates from a crude, once-reprecipitated sample of rat skeletal muscle myosin. The gel demonstrates that anti-rSkM specifically reacts with skeletal myosin heavy chain and that anti-rLM does not.

## Single Staining

We first tested our anticytoplasmic myosin IgG against rat fibroblast cultures and observed two patterns of staining. Fig. 4*a* shows the most common: typical stress fibers, usually with an irregular periodicity. Less often, a sheetlike arrangement of staining, with periodic gaps was seen, as shown in Fig. 4*b*. By altering the focus, this was seen to be close to the membrane. Such staining has also been observed in human fibroblasts stained with antiplatelet myosin (41).

We then examined rat muscle cultures. In addition to staining in all the mononucleated cells, there was striking evidence for cytoplasmic myosin in the myotubes. Fig. 4*c* shows a myotube on the right and a fibroblast on the left stained with anti-rat lymphoma myosins. Both show clear stress fiber patterns.

We also occasionally observed the sheetlike staining seen in the fibroblasts in myotubes; Fig. 4*d* is an example. It was not clear how these myotubes differed from those showing stress fibers. The sheetlike staining gave periodicities regular enough

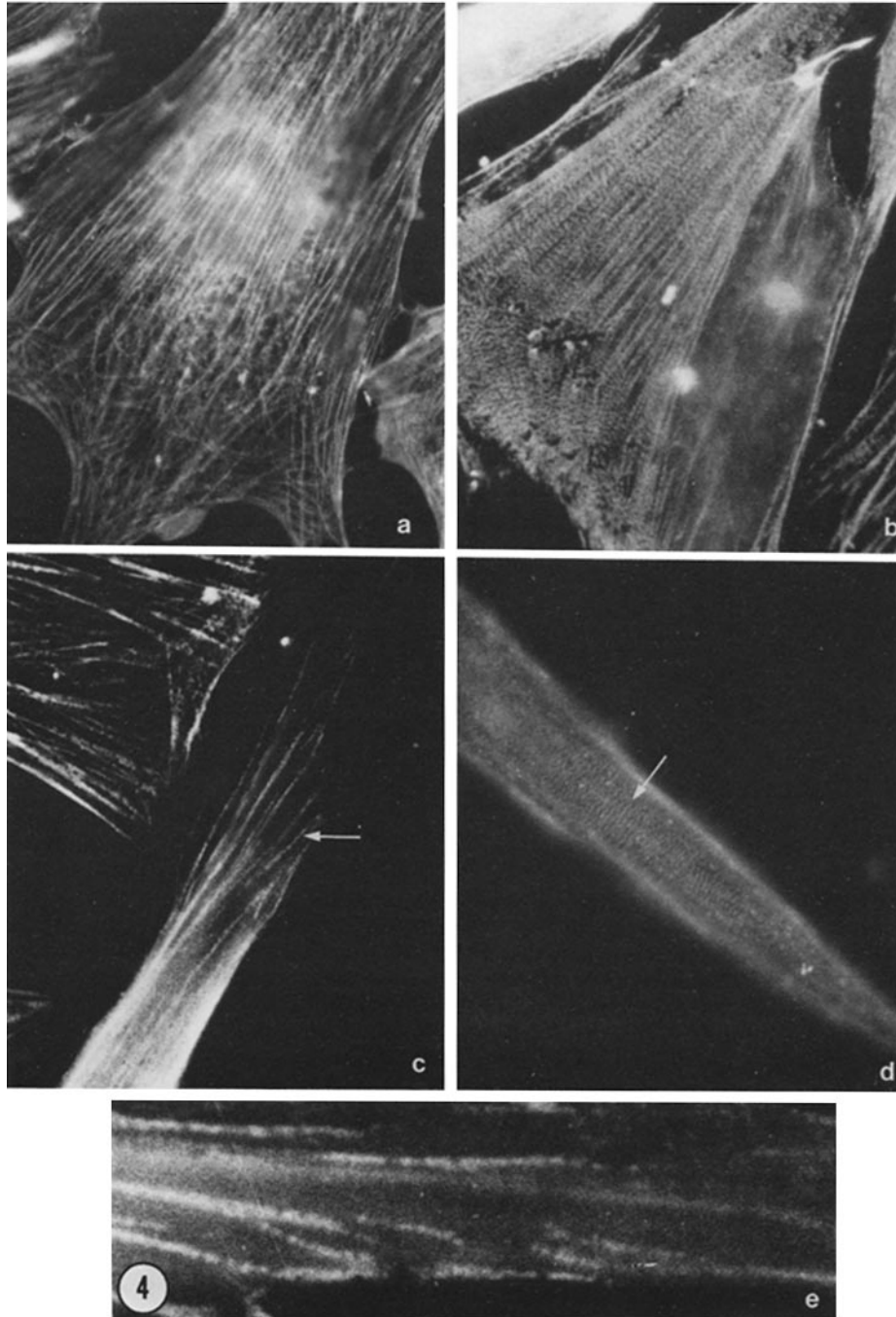


FIGURE 4 Antilymphoma myosin staining of fibroblasts and myotubes. (a) Fibroblast stained with anti-LM; no stress fiber-type pattern.  $\times 880$ . (b) A fibroblast with sheetlike periodic staining. The periodicity in the regular regions is  $1.1 \mu\text{m}$  (average); the width of each fluorescent spot is  $0.5 \mu\text{m}$ .  $\times 560$ . (c) Myotube (right) and fibroblast (left) stained with anti-rLM. Note stress fibers in both. Periodicities (arrow) are shown more clearly in e. (d) Myotube displaying sheetlike staining with periodicities of  $0.81 \mu\text{m}$ ; the width of each spot is  $0.48\text{--}0.5 \mu\text{m}$ .  $\times 1,050$ . (e) Enlargement of myotube end in c to show the irregular periodicities. The fluorescent spots are  $0.16\text{--}0.26 \mu\text{m}$  in diameter.  $\times 9,450$ .

to measure. The width of the fluorescent spots in Figs. 4 *b* and *d* are 0.48–0.51  $\mu\text{m}$ , the overall periodicities are 1  $\mu\text{m}$  in the fibroblast and 0.81  $\mu\text{m}$  in the myotubes. The periodicities in the myotube stress fibers were irregular (see Fig. 4 *e*); the fluorescent spots varied from 0.16 to 0.3  $\mu\text{m}$  in diameter.

By contrast, when we stained with the anti-rat skeletal myosin, the reaction was limited almost entirely to myotubes, whereas almost all of the mononucleated cells were unreactive (see below). The periodicities also differed. For example, Fig. 5 shows a clear illustration of sarcomeres within a myotube. The width of the fluorescent spots is 1.8  $\mu\text{m}$  and the overall periodicity is 2.9  $\mu\text{m}$ , both very different from periodicities resulting from the presence of cytoplasmic myosin. The clear sarcomeres of Fig. 5 are not always visible in rat myotubes that do not have the precise, early alignment characteristic of developing chick myotubes, which are more often studied.

### Controls for Immunofluorescence

In addition to the immunochemical test for specificity, we used the cultures to test our antisera. The preimmune IgGs were unreactive; no staining was seen with the identical conditions for labeling and photography that were used for the immune sera. If cultures were reacted with anti-rLM that had been preabsorbed with native lymphoma myosin, the staining was blocked (Fig. 6 *a* and *b*). Similarly, when cultures were labeled with anti-rSkM preabsorbed with SDS-purified rat skeletal myosin heavy chain, the staining was blocked (Fig. 6 *c* and *d*). But when cultures were treated with anti-rLM preabsorbed with SDS-purified rat skeletal myosin heavy chains, the staining was unaffected (Fig. 6 *e*). The staining was also unaffected when myotubes were reacted with anti-SkM preabsorbed

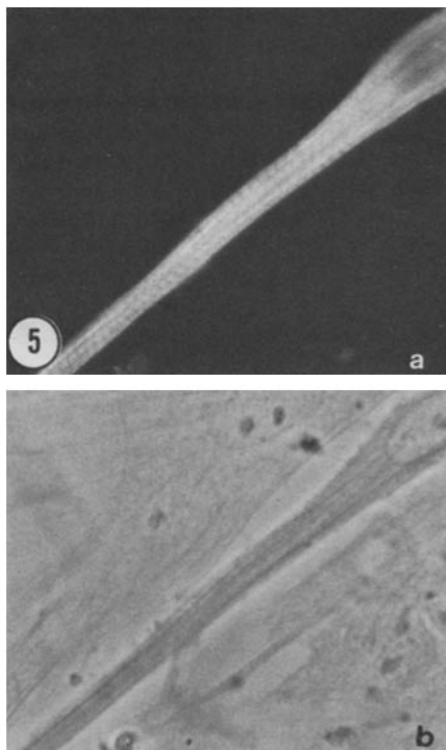


FIGURE 5 (a) Anti-rSkM staining of rat muscle culture. Compare the sarcomere staining with the phase image of *b* to see the lack of reactivity of the mononucleated cells. The sarcomeres have an overall periodicity of 2.9  $\mu\text{m}$ ; the fluorescent spots measure 1.8  $\mu\text{m}$ .  $\times 550$ .

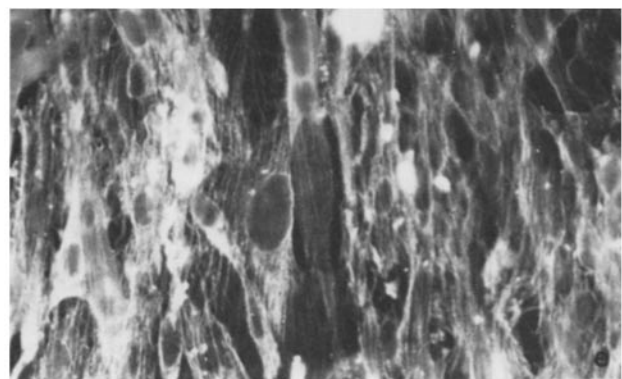
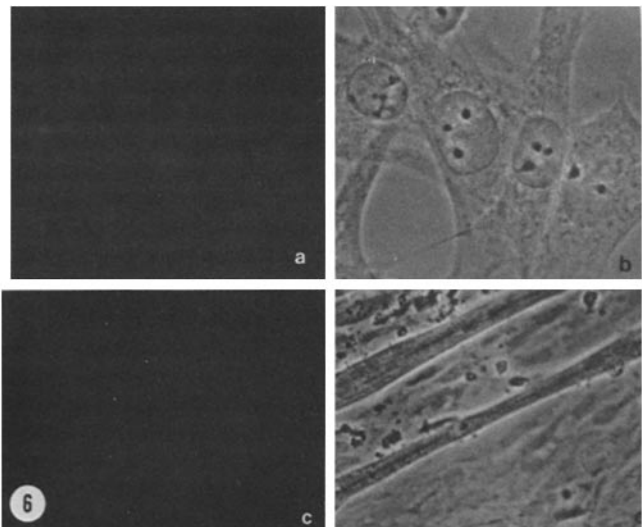


FIGURE 6 Immunofluorescence controls. (a and b) Fluorescent and phase images of fibroblasts stained with anti-rLM preabsorbed with native lymphoma myosin.  $\times 651$ . (c and d) Fluorescence and phase images of muscle culture staining with anti-rSkM preabsorbed with SDS-purified rat skeletal myosin heavy chain.  $\times 192$ . (e) Fluorescence image of muscle culture stained with anti-rLM preabsorbed with SDS-purified rat skeletal myosin. Cf. a.  $\times 630$ . (f) Fluorescence image of muscle culture stained with anti-rSkM preabsorbed with rat lymphoma myosin. Cf. 5 a.  $\times 400$ .

with SDS-purified rat lymphoma myosin heavy chain (Fig. 6 *f*). When myotubes were first stained with unlabeled anti-rSkM and then with TMRITC-anti-SkM, no fluorescence was seen.

Finally, anti-rSkM stained adult myofibrils and the whole central region of fibers in cryostat sections of adult muscle. The anti-rLM did not stain adult myofibrils and stained only a few peripheral perinuclear regions in cross sections of adult muscle

(data not shown). At the level of resolution of the light microscope, we could not determine whether this staining was inside the muscle fiber or was caused by cells outside the fiber, such as satellite cells or endothelial cells.

These controls convinced us that it should be possible to stain a culture with both antisera and to detect the presence of the two isozymes without interference. When this was done, the different locations of the two antigens in the myotube were shown most clearly, as can be seen in Fig. 7. A comparison of Fig. 7*a* and *b* shows that skeletal myosin is predominantly located in the central part of the myotube, whereas the cytoplasmic myosin extends farther into the end. Phase microscopy of the same field (Fig. 7*c*) shows that stress fiber elements correspond to the regions of staining resulting from the cytoplasmic myosin. The phase micrograph also shows that the cytoplasmic myosin staining does not quite extend to the plasmalemma at the end of the myotube but is present in a submembranous rim along the sides of the plasmalemma.

Fig. 8 shows a second example of a doubly staining myotube, again, the complementary localization at the end of the cell is evident. The lower myotube in this figure also illustrates the membrane-associated staining of the anti-rLM. A very similar picture emerged when we examined the few mononucleated

cells that reacted with both antibodies. The cell in Fig. 9*a* and *b* displays a striking example of anti-rLM membrane staining, as well as the overall complementary localization of the antigens. We also determined that the plasmalemma-associated antigen was submembranous: cells fixed only in formaldehyde and, thus, not permeable to antibody did not stain.

To see whether the complementary localization might be caused merely by the position at which new myoblasts entered the myotube, bringing perhaps cytoplasmic myosin with them, we rounded up myotubes with cytochalasin B or trypsin and observed the distribution during the rounded state and after respreading. In the early stages of rounding up after cytochalasin B, the stress fibers were observed to condense into brightly stained granules. Later, we saw a diffuse distribution of both antigens (Fig. 10*a* and *b*). When the cells were collected, replated, and allowed to respread, the cytoplasmic and skeletal myosins began to segregate as soon as the cell changed from the rounded state, regardless of whether cytochalasin B or trypsin was used (Fig. 10*c-f*). Cytoplasmic myosin entered the protrusions (arrow in Fig. 10*d*), whereas the skeletal myosin did not. The stress fibers containing cytoplasmic myosin reappeared (Fig. 10*f* and *g*). The complementary distribution appeared equivalent to that seen before the rounding up.

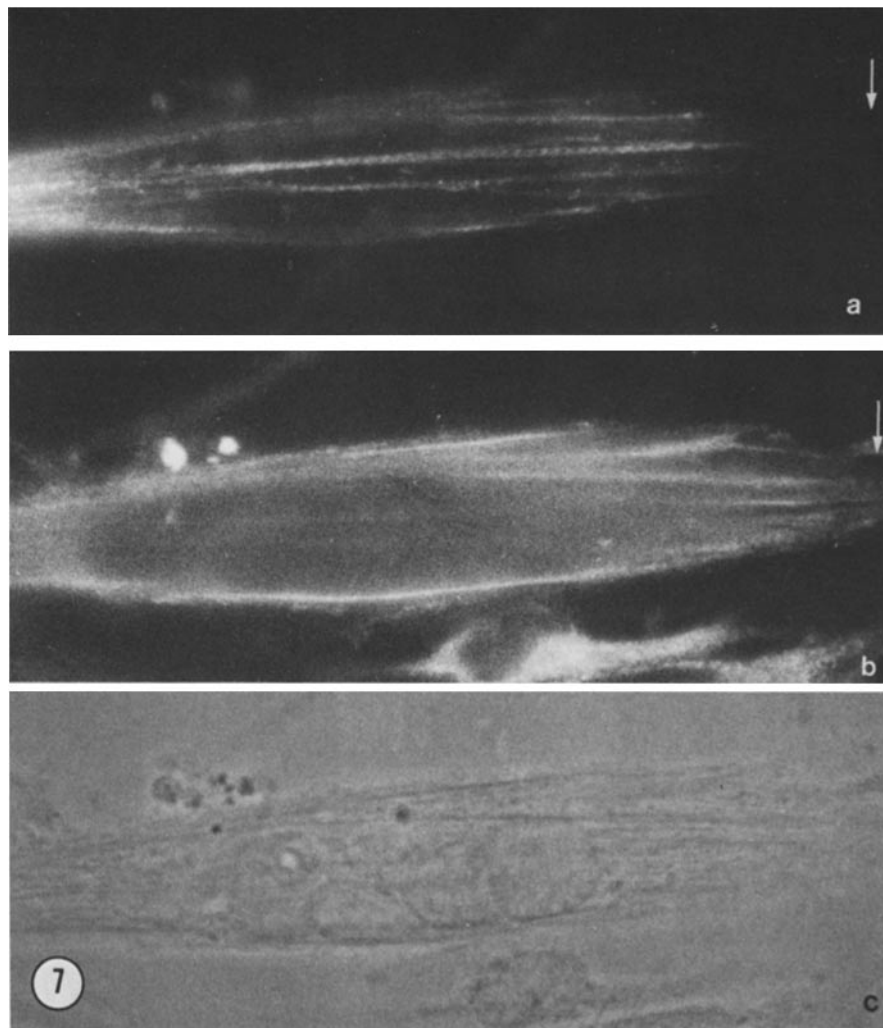


FIGURE 7 Double staining of rat muscle culture. End of myotube under conditions displaying the anti-rSkM staining. (*a* and *b*) The anti-rLM staining (*c*) With phase microscopy. The arrows indicate the position of the end of the cell in the fluorescence micrographs. The fluorescence micrographs are taken at close to the same focal plane.  $\times 1,200$ .

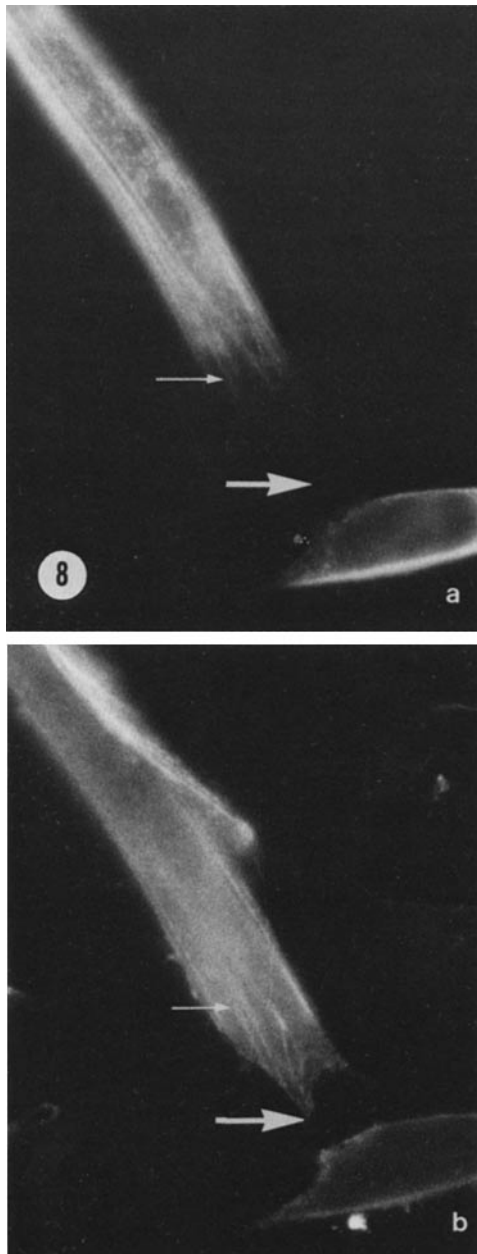


FIGURE 8 Double staining of rat myotube. (a) Myotube stained with anti-rSkM. (b) Myotube stained with anti-rLM. Note the overall complementarity of the fibers staining for cytoplasmic and skeletal myosin. The small arrow indicates the position of a fibrous structure that seems to stain with both antibodies, one continuous with the other. The large arrows indicate the position of the end of the myotube. Cytoplasmic myosin continues to the end of a cell and is also present in a protrusion on the upper surface.  $\times 750$ .

To see whether the ends of myotubes contained other components of the cytoskeleton, we used an antibody to 10-nm filament protein to stain myotubes. We observed a complementary staining similar to that seen with the myosins, but the antibody did not stain stress fibers. Instead, a very intense staining of the whole end of the myotube was observed (Fig. 11). At the lower titers, through-focusing of this region revealed a tangled, netlike arrangement radically different from the stress fiber patterns.

#### DISCUSSION

Our findings show that two distinct isozyms of myosin, cytoplasmic and skeletal, coexist in myotubes and in a small

proportion of the mononucleated cells, the definitive myoblasts. In both cell types, the myosins occupy largely distinct regions of the cytoplasm, and periodicities seen in the fine stress fibers or sheets stained with antibody to cytoplasmic myosin are different from those seen in central fibrous structures stained with antibody to skeletal myosin. It appears that the myosins do not randomly intermingle in the developing muscle tissue. There is also cytoplasmic myosin near the membrane and diffuse fluorescence in the cells; much of this is undoubtedly caused by overlapping structures in the relatively thick myotubes. Whether there is soluble myosin is not clear.

Because the identification and localization of the two myosins rests on antibody staining, the characterization of the antibodies is crucial. The precipitations of only the antigen from a heterogeneous mixture using the Staph A procedure with immune but not preimmune sera demonstrates that both anti-cytoplasmic myosin (anti-CM) and anti-SkM are specific for the respective myosins. The immunofluorescence absorption controls confirm this conclusion. The cross-absorption experiments also show that antibody to cytoplasmic myosin did not interact with skeletal myosin, and that antibody to skeletal myosin did not complex with cytoplasmic myosin. Separate immunofluorescence studies with each antibody also show no evidence of cross-reactivity—that is, no staining of fibroblasts was seen with the anti-SkM antibody, and no staining of sarcomeres or of adult myofibrils was seen with the anti-CM antibody. Using adult muscle, anti-SkM stained the fibrils in the interior of the cells, whereas anti-CM stained only the myofiber borders. It is clear, therefore, that the two antibodies are directed against distinct and noncross-reacting antigenic sites on the two myosins.

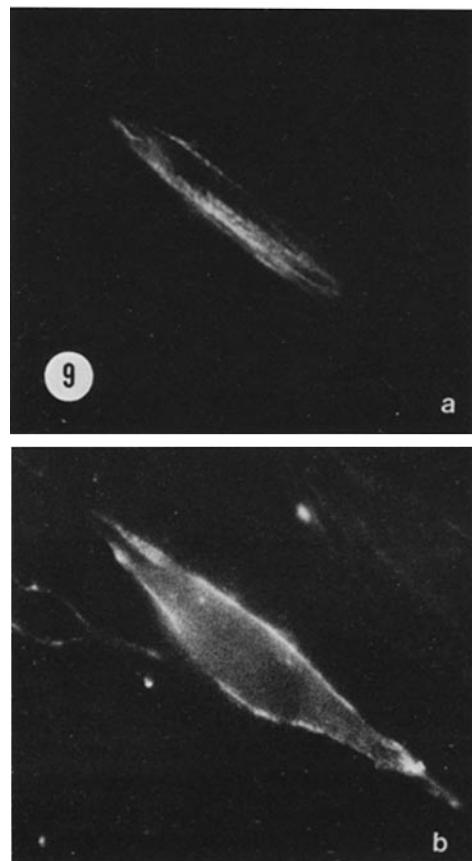


FIGURE 9 Double staining of rat myoblast. (a) Anti-rSkM. (b) Anti-rLM. Note the prominent submembranous staining of the anti-rLM and the sarcomere pattern of the anti-rSkM.  $\times 1,000$ .



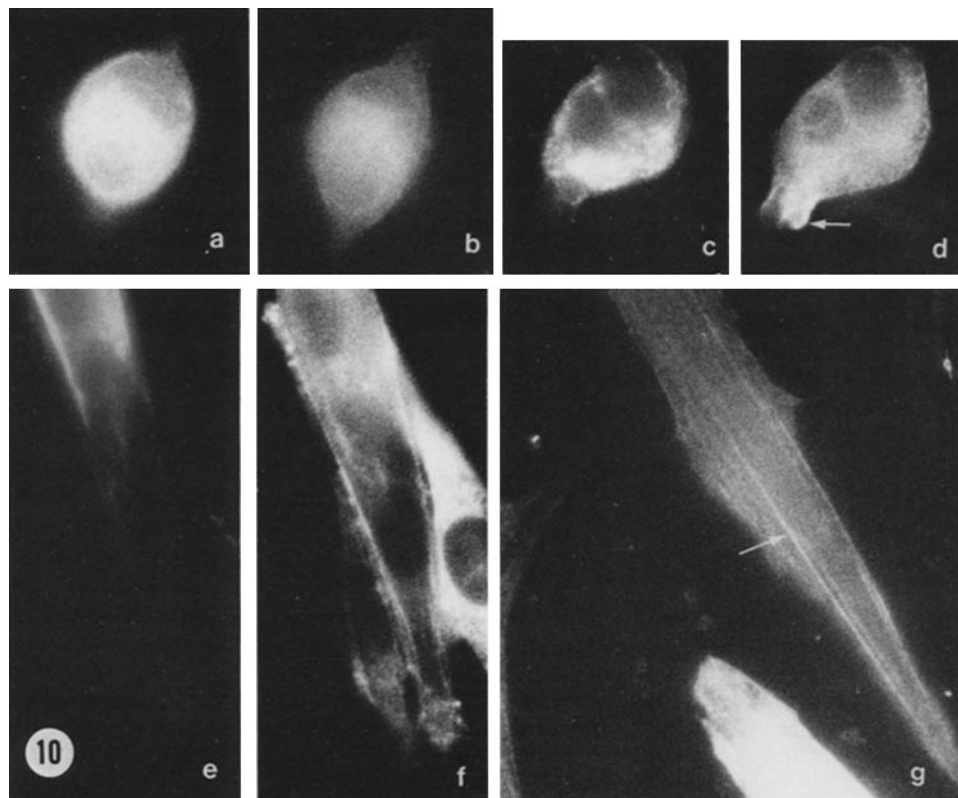


FIGURE 10 Respreading experiment. (a and b) Anti-rSkM and anti-rLM staining of myotube treated with 5  $\mu\text{g}/\text{ml}$  of cytochalasin B for 5.5 h. (c and d) Staining for skeletal and cytoplasmic myosin, respectively, of myotube allowed to respread for 60 min after trypsinization. Arrow shows area where CM and SkM have started to segregate. (e and f) Double staining of myotube 30 min after removal of cytochalasin B. Note the reappearance of cytoplasmic myosin containing stress fibers in f and the segregation of CM and SkM. (g) End of myotube stained with anti-rLM. Stress fibers are prominent. There was no skeletal myosin staining in the field. This myotube had recovered from trypsinization. a–f,  $\times 850$ ; g,  $\times 950$ .

Therefore, the fluorescent staining seen in myotubes treated with antibody to the cytoplasmic or skeletal myosin can be taken to reflect the localization of the antigen within the limits imposed by light microscopy and by the fixation procedures used. We found the cytoplasmic myosin to be localized in the myotubes in stress fiberlike elements predominantly at the ends of the cells, on the cytoplasmic side of the membrane, and diffusely throughout the cell. In contrast, the skeletal myosin was restricted to formed sarcomerelike elements and to fibers found predominantly in the center of the cell.

The location of the cytoplasmic myosin suggests several possible functions. First, the fine stress fibers in which it appears are present mainly in the basal cytoplasm and predominantly at the ends of the myotubes or the myoblasts, suggesting a role in adhesion of the cells to the substratum. This idea is strengthened by the observation that during a contraction in culture, the ends of the myotube often are the only parts of the cell that remain attached and do not move. Also, in very dense cultures, the myotube stress fibers are largely restricted to areas where there are no mononucleated cells under the myotube, i.e., where the cell contacts the substratum. In fibroblasts, there is evidence that stress fibers are involved in adhesion (26, 39) because cells that do not show these components are poorly adherent. The sheetlike pattern, also seen in fibroblasts (41), may be a separate entity or derive from stress fibers near the membrane.

Could these stress fibers or the diffuse “myosin” also be involved in the motility of the myotube? Adhesion and motility are closely related because cells must adhere to progress. Our observation of myotube cultures show that the ends of myo-

tubes occur in two configurations, spread (Figs. 7 and 8) and narrow (Figs. 10 and 11). The latter are reminiscent of the fibroblast “tails” and the former are somewhat similar to the ruffled borders of fibroblasts. Their existence suggests that previously reported movement of myotubes in culture (2) should be reexamined to see whether they are able to move forward in the direction of the spread regions and to retract the narrow terminal regions. A connection between cell movement and fusion has been made by the finding that cytochalasin B, which rounds up myotubes so that they no longer adhere or presumably move, prevents fusion in low-density cultures (32, 33).

The rim, or submembranous, cytoplasmic myosin may have quite a different function. The myosin is not on the outside of the plasmalemma because cells with intact membranes did not stain for the anti-CM. It is known that rat L6 myotubes can cap concanavalin A (1) and that myosin cocaps with this marker in lymphocytes (35). It is to be noted that the membrane-related myosin persists in aging cultures, whereas the stress fibers decrease in prominence. It is possible that the membrane myosin plays a role in the movement of surface receptors in developing myotubes; double-label techniques are suitable for testing this possibility.

One very interesting feature to emerge from the double-label preparations is the observation that in the region of overlap, some stress fibers appear to be continuous with fibrous structures containing skeletal myosin (Figs. 7 and 8). This region is at the ends of the myotubes, where the sarcomeres are actively elongating. Electron microscopy of myotubes has provided evidence for a network of filaments, including microtubules

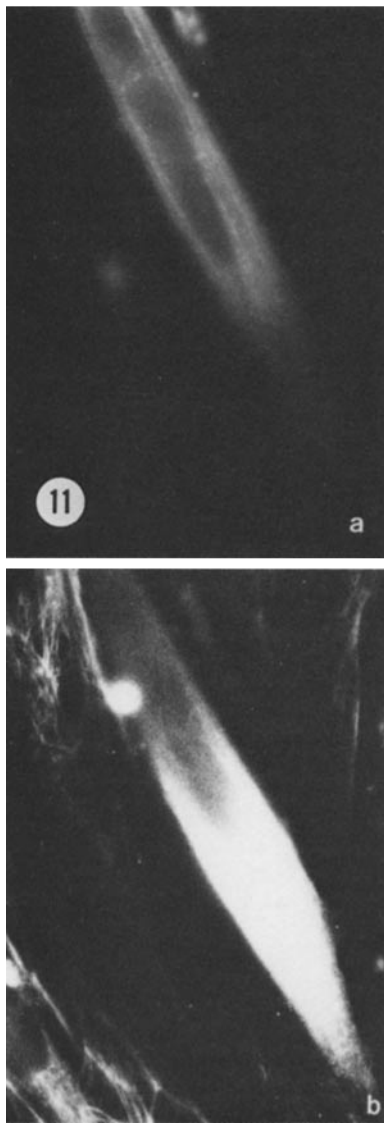


FIGURE 11 Rat myotube stained with (a) anti-rSkM and (b) anti-55K. The staining of the end of the myotube is so intense that details are blocked out; note the overall complementary staining as compared with a.  $\times 850$ .

and 10-nm filaments in myotubes (8, 14, 24). The localization of 10-nm filaments at myotube ends shows that cytoplasmic myosin is not unique in showing a complementary but overlapping distribution with sarcomeric myosin. This arrangement suggests that the cytoskeletal elements may play a role in providing a framework for the assembly of the growing sarcomere or perhaps for the support of incomplete sarcomeres. A similar suggestion has been made for developing cardiac muscle cells (29). We cannot rule out a zone of copolymerization of the two isozymes in this region, but our results show that the isozymes are mainly distinguished by the cell. This could be because each is part of a larger structure, a myofibril or a stress fiber, yet the existence of the diffuse and submembranous myosin suggests that the cytoplasmic and skeletal myosins differ enough to be distinguished, even when they are relatively free of other interacting molecules. It may be that skeletal myosin is rapidly assembled into myofibrils as soon as it is synthesized and so is never, in fact, free to interact with the cytoplasmic myosin which is more soluble in vitro and appears

more diffusely organized in vivo. It will be interesting to determine whether myotubes can synthesize cytoplasmic myosin or whether they harbor it as a donation from the myoblasts. It is not possible to determine this directly without homogeneous myotube cultures, which are not now available, but it will be very indicative if cytoplasmic myosin is found to be present in adult muscle fibers.

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