Cellular and Molecular Biology
of Muscle Development, pages 237-246
© 1989 Alan R. Liss, Inc.

ORGANIZATION OF MYOSIN IN LIVING MUSCLE AND NON-MUSCLE CELLS [1]

N.M. McKenna, C.S. Johnson, M.E. Konkel, and Y.-L. Wang

Worcester Foundation for Experimental Biology
Shrewsbury, Massachusetts 01545

ABSTRACT Fluorescent analogue cytochemistry was used to study the dynamic organization of myosin in living muscle and non-muscle cells. Fluorescently labeled skeletal muscle myosin dispersed after microinjection and became localized in the A-bands of cultured myotubes. Similarly, smooth muscle myosin became localized in beads along stress fibers and in a submembranous sheath of cultured fibroblasts. Myosin near the edge of non-muscle cells was capable of rapid reorganization. Fluorescently labeled regulatory light chain in fibroblasts showed a pattern of incorporation similar to that of myosin, suggesting that it can readily associate with endogenous myosin heavy chains. The dynamic behavior of myosin and its light chains provides a high degree of flexibility for the regulation of myosin-actin interactions.

INTRODUCTION

Although the biochemical and physical properties of many contractile proteins have been well characterized, the interactions of these proteins in living cells are poorly understood. Perhaps the best characterized system is the sarcomere of skeletal muscle, but even in this case, little is known about the intracellular dynamics.

We have attempted to approach these questions by labeling purified proteins with fluorescent probes and then microinjecting them into living muscle and non-muscle

[1]This work was supported by grants from MDA, NSF DCB-8796359, and NIH GM-32476.

cells. Fluorescently labeled proteins such as alpha-actinin and actin become localized into physiological structures, and their subsequent distributions can be used to follow structural dynamics during cellular activities such as locomotion, cell division and myogenesis (1,2).

In this paper, we describe several experiments that focus on aspects of myosin in living cells. We first examine the localization of microinjected skeletal muscle myosin in cultured chick myotubes. We then describe observations on the incorporation and reorganization of myosin in living non-muscle cells. Lastly, we discuss some experiments examining the role of the 20K dalton regulatory light chain (LC20) of myosin. Our experiments suggest that the activities of myosin in living cells may potentially be controlled at several different levels.

METHODS

Skeletal muscle myosin was isolated from rabbit back and hind leg muscles and labeled with iodoacetamido-fluorescein (IAF) or fluorescein isothiocyanate (FITC). The conjugate was cycled through low and high salts and dialyzed against an injection buffer of 450 mM KCl and 2.0 mM Pipes, pH 6.95. Smooth muscle myosin was isolated from turkey gizzards and labeled with iodoacetamidotetramethyl-rhodamine (IATR). LC20 was purified from turkey gizzards using a modified method of Hathaway and Haeberle (3). Thiophosphorylated LC20 was prepared by incubating crude actomyosin with ATP-γ-S. Purified LC20 was labeled with fluorescein isothiocyanate (FITC). Muscle and non-muscle cell cultures were prepared as described previously (4-7). Injection and observation procedures, as well as the procedures for image processing and analysis have also been described elsewhere (4-7).

RESULTS AND DISCUSSION

Microinjection of Myosin into Myotubes

The relatively low solubility of skeletal muscle myosin under physiological ionic conditions raises the question of whether myosin is able to diffuse within the cell and become associated with contractile structures. Following microinjection into living myotubes, fluorescently labeled

skeletal muscle myosin dispersed within 30 min. It became localized along myofibrils in 1.59 µm wide (se=0.03) bands which displayed a regular periodicity of 2.23 µm (se=0.02; Fig. 1a). In favorable images, each band was observed to be a doublet of narrower, 0.72 µm (se=0.02) bands. Images obtained after coinjecting with IATR-labeled alpha-actinin showed that microinjected myosin was located between the alpha-actinin containing Z-lines (Fig. 1) and thus colocalized with the A-bands of myofibrils.

The length of the fluorescent A-band in microinjected myotubes was very similar to that reported by others (8). The doublet appearance may be due to the preferential localization of the fluorescent label at the head of the myosin molecule. However, it is also possible that steric hindrance or the presence of accessory proteins may limit the access of myosin to the central region of the thick filament. Data on myosin exchange in cardiac tissue also indicate that exchange rate is higher at the ends of myosin filaments under some conditions (9).

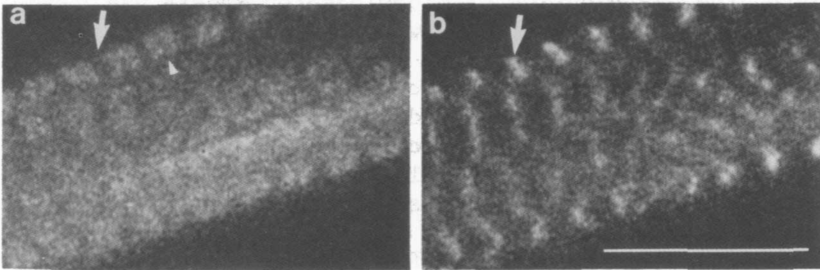

Figure 1. Microinjected IAF-labeled skeletal muscle myosin (a) and IATR-labeled alpha-actinin (b) in a cultured myotube (7 d in culture). Comparison of the two images indicates that the fluorescent myosin is localized in the A-bands. A doublet pattern can be discerned in some myosin bands (arrowhead, a). Arrows indicate the corresponding locations of a Z-line in the two images. Bar, 10 µm.

These data indicate that microinjected myosin, despite its low solubility in the cytoplasm, can diffuse throughout myotubes and become incorporated into appropriate structures such as the thick filaments. Therefore, the

incorporation of myosin molecules does not have to be coupled to translation, although such coupling may take place for endogenous molecules. Two possible mechanisms may be involved in the incorporation process. Either myofibrils may contain unoccupied sites for the association of additional myosin molecules, or myosin molecules may undergo exchange on and off myofibrils. Such exchange has been indicated both in vitro and in vivo in recent experiments, including fluorescence energy transfer (10), laser photobleaching (11), and immunogold labeling (9).

Microinjection of Myosin into Non-muscle Cells

Smooth muscle myosin, which is similar in many respects to non-muscle myosin, has also been fluorescently labeled and microinjected into chick fibroblasts, 3T3 and IMR33 cells. Microinjected myosin became localized in small beads which either arranged periodically along fibers (arrow, Fig. 2a) or formed a submembranous sheath (arrowhead, Fig. 2a). When microinjected cells were fixed and stained with indirect immunofluorescence for endogenous myosin, the distribution of microinjected myosin was observed to correlate closely with that of endogenous myosin (Fig. 2). In addition, no change in the pattern of distribution of endogenous myosin was detected in injected cells as compared to uninjected cells. Therefore, as in the case of muscle cells, injected myosin molecules became incorporated into physiological structures.

Skeletal muscle myosin also became localized in structures in non-muscle cells (Fig. 3). When IAF-labeled skeletal muscle myosin and IATR-labeled smooth muscle myosin were coinjected into a single 3T3 cell, the two myosins showed similar distributions (not shown). Therefore, myosin-containing structures of fibroblasts seemed capable of incorporating skeletal muscle myosin. Similarly, isoforms of many other proteins, including actin (5) and alpha-actinin (7), also colocalize after microinjection. The incorporation of skeletal muscle myosin most likely involves the formation of hybrid filaments with endogenous non-muscle myosin molecules. Such hybrids have been described previously in vitro (12). Given the significant differences in the biochemistry and regulation of these two myosins (13), one might expect disruptions to the cell upon the incorporation of skeletal muscle myosin molecules. However, since the amount of skeletal muscle

myosin injected is likely to be a small percentage of the myosin in the cell, such hybrid filaments are not expected to have a significant effect.

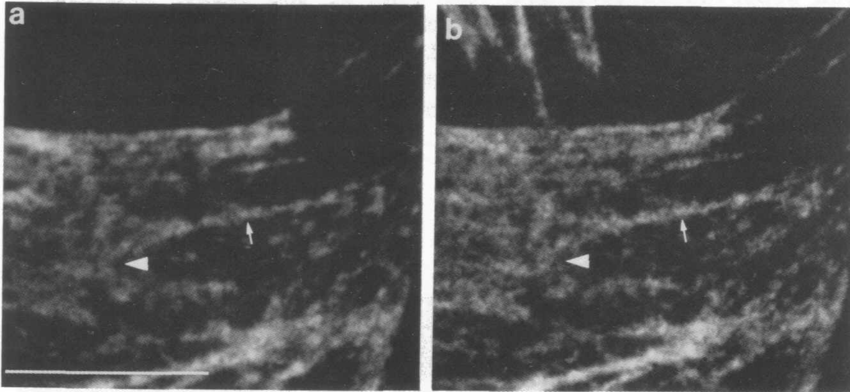

Figure 2. The images of microinjected IATR-labeled smooth muscle myosin (a) and fluorescein anti-platelet myosin immunofluorescence (b) are essentially identical. Myosin is present as small beads along fiber structures (arrows) and in a submembranous sheath (arrowheads). Bar, 10 µm.

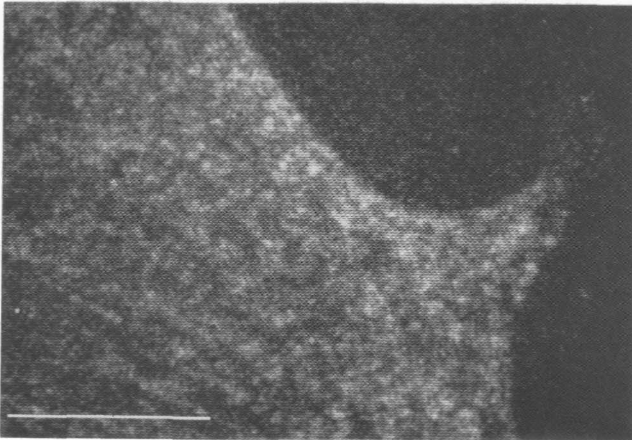

Figure 3. IAF-labeled skeletal muscle myosin microinjected into a 3T3 cell. The distribution is similar to that observed with smooth muscle myosin. Bar, 10 µm.

Since myosin may be involved in the dynamic reorganizations of cytoskeleton, we examined the changes in distribution of myosin-containing structures in individual living cells using time-lapse recording. Myosin beads have been observed to form near the edge of the cell. Translocation of beads, both individually and as part of a group, was also detected near the periphery (Fig. 4). Movement of individual bead may be in any direction relative to the edge of the cell, while mass movements of beads were most often centripetal.

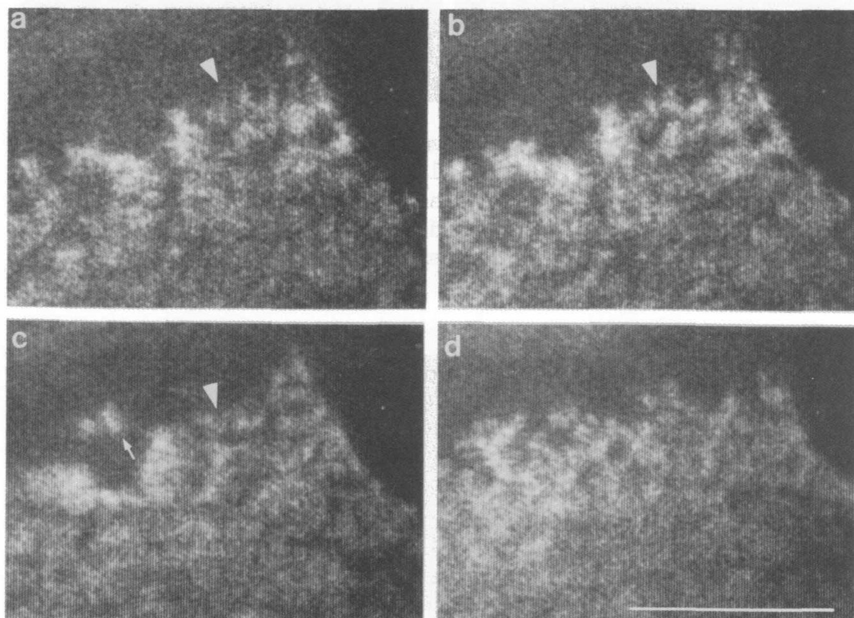

Figure 4. Time-lapse sequence of a chick fibroblast microinjected with IATR-labeled smooth muscle myosin. Myosin is observed in beads approximately 0.5 µm in diameter. In addition to changes in the distribution of these beads (arrowheads), formation of new beads is also detected (c, arrow). Images are recorded at 0 min (a), 2 min (b), 10 min (c), 27 min (d). Bar, 10 µm.

The significance of the formation and rearrangement of myosin in non-muscle cells is unclear at present. The appearance of new myosin beads usually takes place near the edge of the cell and may be membrane dependent. It is interesting to note that both new sarcomeres in muscle cells and new stress fibers in fibroblasts also develop near the membrane (4,8). The movement of myosin beads may be closely related to the reorganization of stress fibers (4), the translocation of actin and alpha-actinin along some stress fibers (14), and the centripetal movement of membrane components observed on motile cells. Given the size of these beads, it is likely that force generation and actin-myosin interactions are involved.

Microinjection of Myosin Light Chains

Myosin is comprised of two pairs of light chains as well as two heavy chains. The phosphorylation of the regulatory light chain (LC20) of smooth muscle and non-muscle myosin affects the formation of myosin filaments and the activity of the actin-activated myosin ATPase (13). However, it is not clear whether LC20 associate with the heavy chains as stable complexes in the cell, or they exist as independent entities. In order to study this question, we examine the ability of LC20 to associate with endogenous structures after microinjection.

We purified LC20 from smooth muscle, labeled it with fluorescein isothiocyanate (FITC) and microinjected it into 3T3 and IMR33 cells. Microinjected LC20 dispersed readily after microinjection, and became associated with discrete structures within 60 min. When microinjected cells were fixed and stained with indirect immunofluorescence for endogenous myosin, the distribution of fluorescent LC20 correlated closely with that of myosin (Fig. 5). Both were localized along stress fibers and in a submembranous sheath (Fig. 5).

These results indicate that LC20 is able to associate with endogenous heavy chains in living cells. Thus either there are heavy chains without associated LC20, or LC20 may undergo continuous exchange among different heavy chains. In either case, one may conclude that heavy and light chains exist as independent entities. The independence of heavy and light chains is also implied by their different turnover rates (15). This property may be particularly significant in smooth muscle and non-muscle cells because

of the involvement of LC20 in regulation. Changes in the level of phosphorylation of myosin in cellular structures may occur not just through the direct action of light chain kinase and phosphatase, but also through the exchange of phosphorylated or unphosphorylated LC20. Thus the site of phosphorylation-dephosphorylation may be separated from the heavy chain molecule, and one light chain molecule may be able to affect multiple heavy chains.

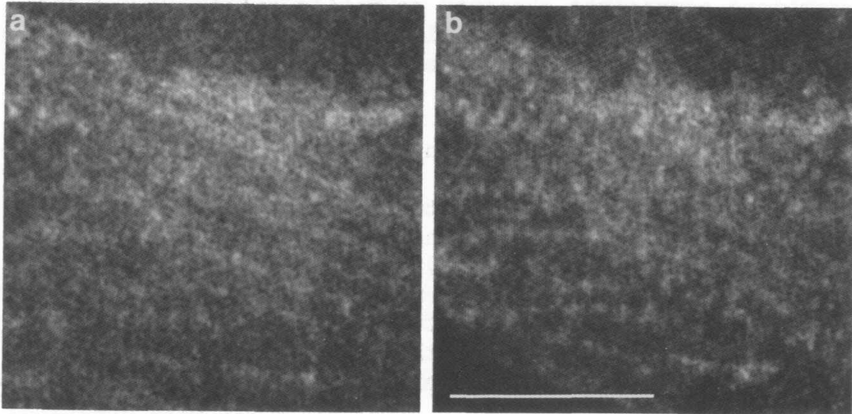

Figure 5. Images of FITC-labeled LC20 (a) and rhodamine anti-platelet myosin immunofluorescence (b) in a 3T3 cell. LC20 and myosin immunofluorescence colocalize in beads along stress fibers. Bar, 10 µm.

Since the phosphorylation of LC20 plays a key role in regulation, we have initiated experiments to examine the microinjection of thiophosphorylated LC20. 3T3 or IMR33 cells microinjected with FITC-labeled thiophosphorylated or unphosphorylated light chains were fixed, extracted and stained with anti-platelet myosin antibody and rhodamine secondary antibody. The distribution of both thiophosphorylated and unphosphorylated light chains appeared to be the same as the distribution of myosin (Figs. 5,6). In addition, no response reminiscent of contraction was detected upon the microinjection of thiophosphorylated LC20. These results indicate that the distribution of both microinjected unphosphorylated and thiophosphorylated LC20 mimic the distribution of endogenous myosin in non-muscle

cells. The simplest explanation is that both myosins with phosphorylated and unphosphorylated LC20 may coexist in the same structure. Alternatively, it is also possible that much of the LC20 present in the cells is phosphorylated, and that the microinjected unphosphorylated LC20 became phosphorylated soon after microinjection. This also explains the lack of cellular effect of microinjecting thiophosphorylated LC20. It would be important to determine unequivocally the distribution of unphosphorylated and phosphorylated LC20, either by blocking the phosphorylation site of LC20 before microinjection, or by using specific antibodies.

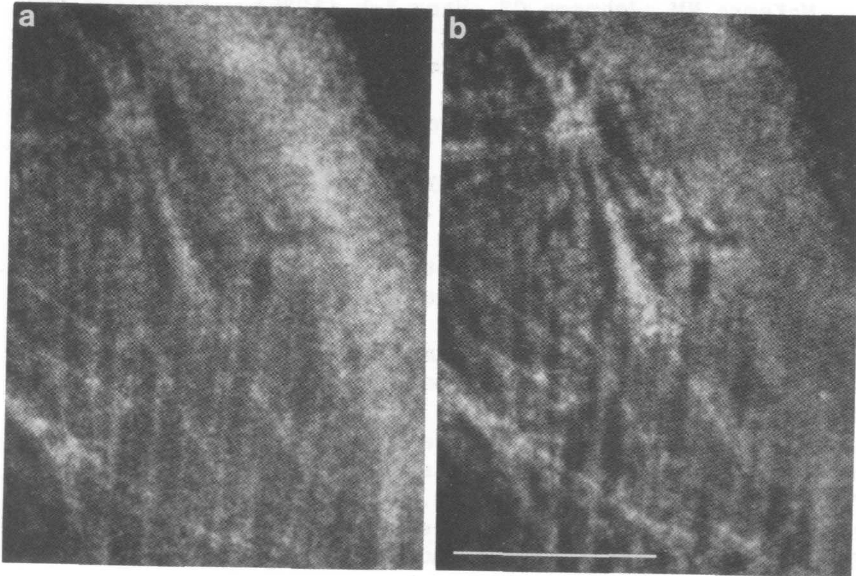

Figure 6. Images of FITC-labeled thiophosphorylated LC20 (a) and rhodamine anti-platelet myosin antibody immunofluorescence (b) in an IMR33 cell. LC20 and myosin have very similar distributions along stress fibers and in a broad band near the edge of the cell. Bar, 10 μm.

REFERENCES

1. Wang Y-L, Heiple JM, Taylor DL (1982). Fluorescent

analog cytochemistry of contractile proteins. Met Cell Biol 25:1.
2. Wang K, Feramisco JR, Ash JF (1982). Fluorescent localization of contractile proteins in tissue culture cells. Met Enzymol 85:514.
3. Hathaway DR, Haeberle JR (1983). Selective purification of the 20,000-Da light chains of smooth muscle myosin. Anal Biochem 135:27.
4. Wang YL (1984). Reorganization of actin filament bundles in living fibroblasts. J Cell Biol 99:1478.
5. McKenna NM, Meigs JB, Wang Y-L (1985). Identical distribution of fluorescently labeled brain and muscle actins in living cardiac fibroblasts and myocytes. J Cell Biol 100:292.
6. McKenna NM, Johnson CS, Wang Y-L (1986). Formation and alignment of Z-lines in living chick myotubes microinjected with rhodamine-labeled alpha-actinin. J Cell Biol 103:2163.
7. McKenna NM, Meigs JB, Wang Y-L (1985). Exchangeability of alpha-actinin in living cardiac fibroblasts and muscle cells. J Cell Biol 101:2223.
8. Fischman DA (1970). The synthesis and assembly of myofibrils in embryonic muscle. Curr Top Dev Biol 5:235.
9. Wenderoth MP, Eisenberg BR (1987). Incorporation of nascent myosin heavy chains into the thick filament of cardiac myocytes in thyroid-treated rabbits. J Cell Biol 105:2771.
10. Saad AD, Pardee JD, Fischman DA (1986). Dynamic exchange of myosin molecules between thick filaments. Proc Natl Acad Sci USA 83:9483.
11. Johnson CS, McKenna NM, Wang Y-L (1988). Association of microinjected myosin and its subfragments with myofibrils in living muscle cells. Submitted.
12. Pepe FA (1983). Macromolecular assembly of myosin. In Stracher A (ed): 'Muscle and Nonmuscle motility' New York: Academic Press, p 105.
13. Adelstein RS, Eisenberg E (1980). Regulation and kinetics of the actin-myosin-ATP interaction. Ann Rev Biochem 49:921.
14. McKenna NM, Wang Y-L (1986). Possible translocation of actin and alpha-actinin along stress fibers. Exp Cell Res 167:95.
15. Zak RA, Martin F, Prior G, Rabinowitz M (1977). Comparison of turnover of several myofibrillar proteins and critical evaluation of double isotope method. J Biol Chem 252:3430.