Analysis of Myofibrillar Structure and Assembly Using Fluorescently Labeled Contractile Proteins

JOSEPH W. SANGER, BALRAJ MITTAL, and JEAN M. SANGER
Department of Anatomy/G3 and Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

ABSTRACT To study how contractile proteins become organized into sarcomeric units in striated muscle, we have exposed glycerinated myofibrils to fluorescently labeled actin, alpha-actinin, and tropomyosin. In this in vitro system, alpha-actinin bound to the Z-bands and the binding could not be saturated by prior addition of excess unlabeled alpha-actinin. Conditions known to prevent self-association of alpha-actinin, however, blocked the binding of fluorescently labeled alpha-actinin to Z-bands. When tropomyosin was removed from the myofibrils, alpha-actinin then added to the thin filaments as well as the Z-bands. Actin bound in a doublet pattern to the regions of the myosin filaments where there were free cross-bridges i.e., in that part of the A-band free of interdigitating native thin filaments but not in the center of the A-band which lacks cross-bridges. In the presence of 0.1–0.2 mM ATP, no actin binding occurred. When unlabeled alpha-actinin was added first to myofibrils and then labeled actin was added, fluorescence occurred not in a doublet pattern but along the entire length of the myofibril. Tropomyosin did not bind to myofibrils unless the existing tropomyosin was first removed, in which case it added to the thin filaments in the I-band. Tropomyosin did bind, however, to the exogenously added tropomyosin-free actin that localizes as a doublet in the A-band. These results indicate that the alpha-actinin present in Z-bands of myofibrils is fully complexed with actin, but can bind exogenous alpha-actinin and, if actin is added subsequently, the exogenous alpha-actinin in the Z-band will bind the newly formed fluorescent actin filaments. Myofibrillar actin filaments did not increase in length when G-actin was present under polymerizing conditions, nor did they bind any added tropomyosin. These observations are discussed in terms of the structure and in vivo assembly of myofibrils.

The myofibrils of cross-striated muscles are contractile complexes of thick myosin filaments, that interdigitate and interact with thin tropomyosin-coated actin filaments (16). These thin filaments are attached or embedded in a Z-band composed of alpha-actinin and several other proteins (4). Although thick and thin filaments can self-assemble in vitro from their constituent molecules, the synthetic filaments are not uniform in length, as they are in vivo (21, 23, 24). Furthermore, we do not know how newly synthesized thick and thin filaments become oriented to form functional contractile units in muscle cells. Studies that have attempted to identify which contractile proteins are synthesized first in developing muscle cells have concluded that there is coordinate synthesis of actin, myosin, tropomyosin, and alpha-actinin during myogenesis (5). Immunocytochemical (15) and electron microscopic data (7) suggest that thick and thin filaments are formed concurrently in the cytoplasm and subsequently become organized into interdigitating arrays that at first lack organized Z-bands. Several workers have suggested from structual studies that the first myofibrils, often found near the cell membrane (2, 8, 20), might serve as templates for the formation of additional myofibrils (7, 15). We have tried to explore this idea by following the incorporation of fluorescently labeled contractile proteins into glycerinated myofibrils from adult and embryonic animals. This aproach has lead to new information about the organization of proteins

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 MARCH 1984 825-833

in myofibrils. We suggest that new actin filaments may be incorporated into myofibrils in vivo after addition of alpha-actinin to the Z-bands.

MATERIALS AND METHODS

Preparation of Glycerinated Myofibrils and Cardiac Muscle Cells: Bundles of psoas muscle were dissected from a freshly killed rabbit and tied with thread to applicator sticks. The bundles were placed immediately in a solution of 50 parts glycerol and 50 parts standard salt (0.1 M KCl, 0.01 M phosphate buffer, 0.001 M $MgCl_2$, pH 7.0) at 4°C. After a day, the glycerol was replaced with a fresh 50% glycerol solution and the muscle stored in this solution at −20°C. Glycerinated bundles of trout muscle were a gift of Dr. F. A. Pepe, University of Pennsylvania.

Insect muscle was obtained from flight muscles in the thoraxes of the moth, *Hyalophora cecropia* (24). The thoraxes were placed in a glycerol solution (see above) over night (4°C) and then placed in a fresh solution of glycerol and stored (−20°C) until needed. Embryonic cardiac muscle from the chick was grown in culture on glass coverslips (28). Glycerination was accomplished by replacing the culture medium with cold 50% glycerol in standard salt and keeping the cells in this solution for 2 h at 4°C. The cells were washed with cold standard salt before being exposed to labeled proteins.

Myofibrils were prepared by washing small pieces (several millimeters) of either rabbit or fish or insect glycerinated muscle in standard salt for an hour at 4°C to remove the glycerol. The pieces were placed in a Sorvall Omni Mixer (Sorvall, Newtown, CT) in 10 ml of cold standard salt and blended for ~20 s or until the bundles were broken into myofibrils. After several rinses with standard salt, the myofibrils were used for labeling experiments or were stored in 50% glycerol at −20°C.

Myofibrils usually were stained while suspended in a small volume (100 μl) of standard salt. In experiments where myosin was removed from myofibrils to create myofibril ghosts, the myofibrils were treated with Hasselbach-Schneider extraction solution (0.6 M KCl, 10 mM $Na_4P_2O_7$, 1 mM $MgCl_2$, 0.1 M KH_2PO_4, pH 6.40 (13). Myofibrils in glycerol solution were added in one or two drops from a Pasteur pipette to a coverslip. They were spread over the surface of the coverslip with the pipette and the coverslip then placed in a 35-mm petri dish. Glycerol was removed by adding several changes of cold standard salt. The myosin then was extracted by adding 2 ml of cold Hasselbach-Schneider solution and incubating for 10 min at 4°C. After extensive washing with fresh Hasselbach-Schneider solution and then with cold standard salt solution, the myofibrils were stained on the coverslip with labeled protein, washed, and mounted on a glass slide for observation.

Preparation of Fluorescently-labeled Actin, Alpha-actinin, Tropomyosin, and Heavy Meromyosin (HMM)[1]: F-actin was prepared from acetone powder of rabbit skeletal muscle by standard procedures (22) (Fig. 1 *C*). Tropomyosin and alpha-actinin were prepared from fresh or frozen chicken gizzards (6, 30) (Fig. 1, *b* and *d*). Each protein was labeled with lissamine rhodamine sulfonyl chloride (LR) according to the method of Brandt-zaeg (3) for antibody labeling. LR was synthesized from lissamine rhodamine B (Polysciences, Inc., Warrington, PA) and phosphorous pentachloride (3) or was purchased from Molecular Probes (Junction City, OR). Unbound dye was removed from the labeled protein preparations by passing the protein-LR through a G-25 Sephadex column (Pharmacia, Inc., Piscataway, NJ). The labeled proteins subsequently were passed through a DE 52 column (Whatman Co., Clifton, NJ) to remove over- and under-labeled protein by eluting with a salt gradient (32). Alpha-actinin was stored for later use in a 50% glycerol-standard salt solution. Tropomyosin-LR was stored in a lyophilized form. Actin-LR was stored in the fibrous form as F-actin-LR and converted to monomer form by dialysizing against depolymerizing buffer (0.2 mM ATP, 2 mM Tris, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol and 0.005% NaN) before labeling experiments were carried out (22). HMM was prepared as previously reported (25–27) but was labeled with lissamine rhodamine sulfonyl chloride. The HMM-LR was stored in 25–50% glycerol in standard salt solution.

Addition of Labeled Proteins to Myofibrils and Muscle Bundles: Monomer actin-LR was added to myofibrils either in the presence of ATP or in the absence of ATP. In the former case, 1 vol of monomer actin-LR (0.5 mg/ml) present in depolymerizing buffer that contained 0.2 mM ATP (see above) was added to myofibrls bathed in an equal volume of standard salt. To prevent the myofibrils from contracting when the ATP-containing buffer was added, the myofibrils were attached to a glass coverslip by spreading a drop

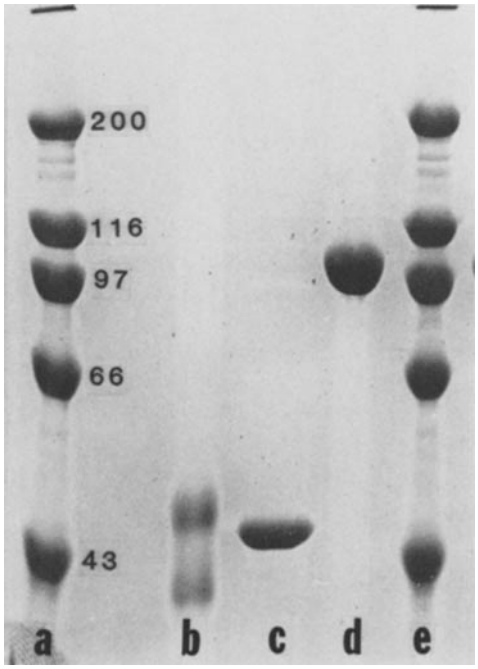

FIGURE 1 SDS polyacrylamide gel (10%) of proteins prior to labeling with lissamine rhodamine. (*b*) Tropomyosin, alpha, and beta chains; (*c*) actin; (*d*) alpha-actinin; (*a* and *e*) marker proteins: rabbit muscle heavy chain of myosin (*200* kdaltons), β glactosidase (*116* kdaltons), phosphorylase B (*97* kdaltons), BSA (*66* kdaltons), and egg albumin (*43* kdaltons).

of a myofibril suspension (in standard salt) over the coverslip with a Pasteur pipette. Unattached myofibrils were rinsed off with standard salt, and 100 μl of standard salt was added to the coverslip to bathe the myofibrils. The same volume of monomer-actin-LR in depolymerizing buffer was added, and the myofibril incubated for 30–60 min at 4°C. The coverslips were then rinsed thoroughly with standard salt to remove unbound actin, and mounted on a slide in 25 or 50% glycerol-standard salt.

In the second method, free ATP was removed from the monomer-actin LR solution before incubation with myofibrils. To do this, the solution was treated with Dowex-1 (Sigma Chemical Co., St. Louis, MO) for 10 min and the Dowex beads were then spun down leaving monomer actin-LR in a solution that was free of ATP. 100 μl of the actin solution was added to myofibrils in suspension in an equal volume of standard salt solution. The myofibrils were incubated with the protein for 30–60 min at 4°C, washed in several changes of standard salt, and mounted on a slide in 25–50% glycerol in standard salt.

Tropomyosin-LR and alpha-actinin-LR were each added to myofibrils at a concentration of 1 mg/ml for 1 h at 4°C. The myofibrils were washed and mounted as described for actin-LR. For some experiments, unlabeled monomer actin (1–2 mg/ml) or unlabeled alpha-actinin (2–5 mg/ml) were first added to the myofibrils for an hour at 4°C, and then the myofibrils were rinsed several times before the labeled protein was added. Processing was carried out as described for additon of a single labeled protein.

Small bundles of rabbit, trout, and moth muscles were teased from the glycerinated muscle. These small fibers were exposed to alpha-actinin-LR for periods of 1–2 h at 4°C, then washed with many changes of cold standard salt solution for 30 min to remove any unbound labeled protein. The bundles were fixed in 3% paraformaldehyde (29), dehydrated in an ethanol series, and embedded in Epon. 1-μm sections were cut with an LKB Ultramicrotome III and mounted on a glass slide, covered with Histoclad (Clay-Adams, New York, NY) and a coverslip.

Negative Staining: Samples of monomer actin-LR were polymerized under conditions identical to those used for incubation with myofibrils (see above) i.e., 1 vol of monomer actin-LR in depolymerizing buffer (either with or without ATP as described above) plus an equal volume of standard salt for 30–60 min at 4°C. A drop of sample was palced on a Formvar-coated grid, washed with 0.1 M KCl, and then stained with a 1% aqueous solution of uranyl acetate and examined with a Philps 201 electron microscope.

Microscopy: Myofibrils and the plastic embedded sections were examined with an Olympus Vanox Photomicroscope equipped for epifluorescence. The objectives used were Zeiss Planapochromats, × 63 and 100. Images

[1] *Abbreviations used in this paper:* HMM, heavy meromyosin; LR, lissamine rhodamine sulfonyl chloride.

were recorded using Kodax Tri-X film developed with Acufine for an ASA of 1,000. All photomicrographs in this article are printed at the same magnification.

RESULTS

Addition of Contractile Proteins to Control Myofibrils

The contractile proteins, alpha-actinin, actin, and tropomyosin (Fig. 1), all labeled with lissamine rhodamine (LR), each reacted with glycerinated myofibrils in a different way. Alpha-actinin-LR and tropomyosin-LR were added in standard salt solution to myofibrils that were suspended in standard salt. Alpha-actinin-LR bound to the Z-bands of rabbit psoas, fish and insect flight muscle myofibrils (Fig. 2, *a* and *b*), and to the Z-bands and intercalated discs of embryonic chick myocardial cells (Fig. 2 *c*). Cross sections of plastic embedded rabbit and insect myofibrils showed that the fluorescence was localized throughout the Z-band (Fig. 2, *f–i*). In contrast, tropomyosin-LR did not bind to any region of the various types of control myofibrils (Fig. 2, *d* and *e*).

Monomer actin-LR in depolymerizing buffer (see Materials and Methods) formed filaments of F-actin several microns long when diluted 1:1 with standard salt solution (Fig. 3).

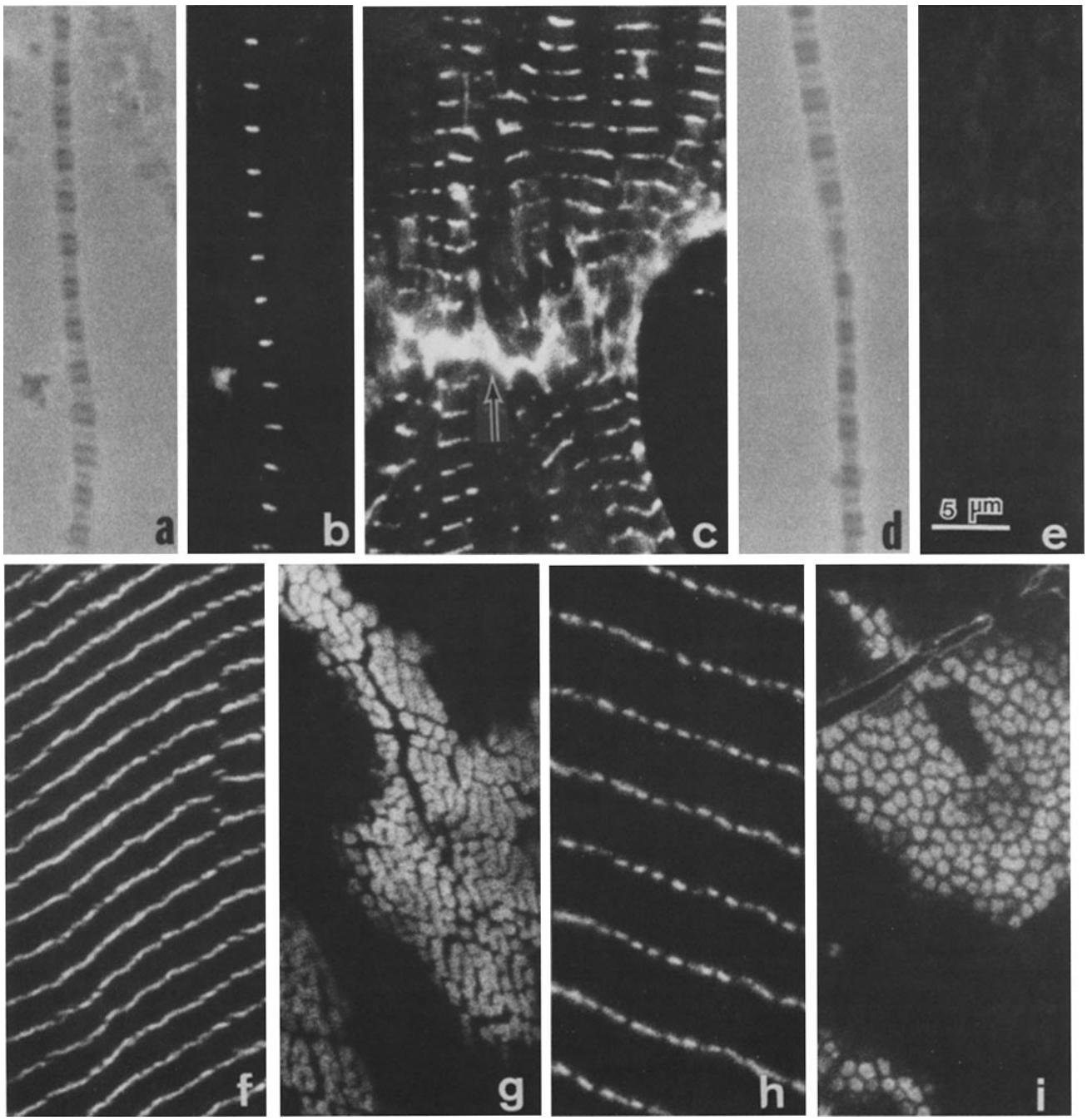

FIGURE 2 Phase and fluorescence (a and *b*) of rabbit psoas myofibril with Z-bands stained with alpha-actinin-LR. (c) Glycerinated embryonic chick cardiac muscle cell with stain in the Z-bands and intercalated disc (arrow). (*d* and e) Phase and fluorescence of rabbit psoas myofibril stained with tropomyosin-LR. No binding occurs in any of the bands of the myofibril (*f–i*) Longitudinal and cross sections of rabbit (*f* and *g*) and moth (*h* and *i*) myofibrils stained with alpha-actinin-LR. The entire disc stains with alpha-actinin in cross-section. × 2,500.

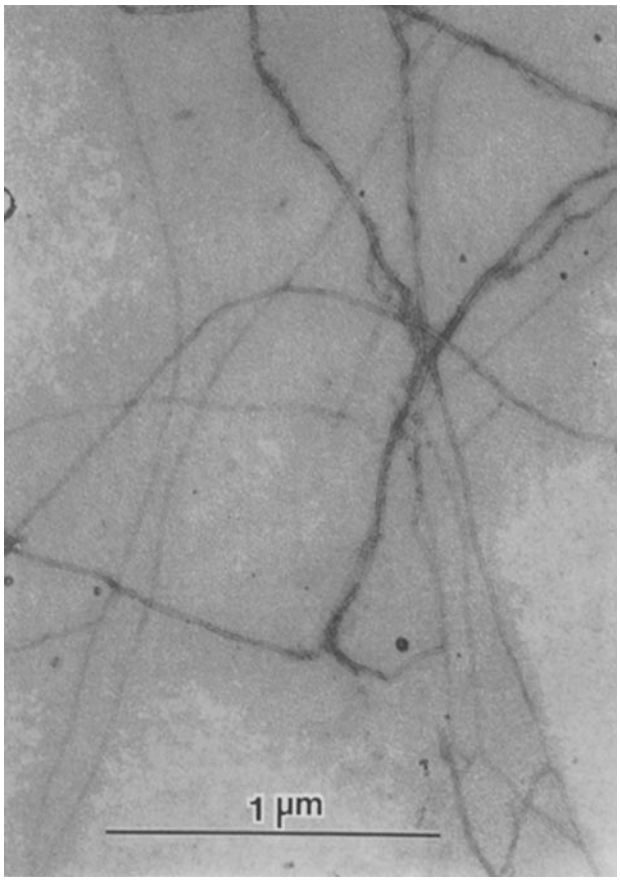

FIGURE 3 Field of actin filaments formed by mixing monomer actin-LR with an equal volume of standard salt after 30 min. Scale marker equals 1 μm.

This was true whether or not the ATP was removed from the depolymerizing buffer. When monomer actin-LR in depolymerizing buffer from which ATP had been removed (see Materials and Methods) was added to myofibrils that were in an equal volume of standard salt, actin binding occurrred in a bright doublet in the H-zone of the myofibrils (Fig. 4, *a* and *b*), and in the myofibrils of chick cardiac cells. Stretched myofibrils with wider H-zones bound actin-LR in doublets that were correspondingly wider. In asymmetrically contracted sarcomeres where the thin filaments completely overlapped all of the cross-bridges in one-half of a sarcomere, no actin-LR bound in that area. However, actin was bound in the other half of the sarcomere, resulting in a single band of fluorescence (Fig. 4, *c–e*). In the presence of 0.2 mM ATP, actin-LR did not bind to any region of the myofibril. Neither actin-LR nor alpha-actinin-LR bound to myofibrils and cells that had been previously fixed in 3% paraformaldehyde for 5 min.

Addition of Contractile Proteins to Treated Myofibrils

The high salt Hasselbach-Schneider solution (13, 16) is used to remove myosin from myofibrils (Fig. 5, *a* and *b*). When myofibrils with H-zone doublets of actin fluorescence were treated with this solution the actin fluorescence was eliminated. Because of its high ionic strength, Hasselbach-Schneider solution should also extract tropomyosin from muscle (22). This was confirmed by running gels of the supernatant of the extracted myofibrils (data not shown). If tropomyosin-LR was added to myofibrils that had been extracted with Hasselbach-Schneider solution, fluorescence was found along the actin filaments in the I-band (Fig. 5, *c* and *d*). No staining was observed in the Z-band (Fig. 5, *c* and *d*). HMM-LR stained myosin extracted myofibrils just as tropomyosin-LR did (Fig. 5, *a* and *b*). If alpha-actinin-LR was added to similarly extracted myofibrils, binding occurred in the Z-band as well as the rest of the I-band (Fig. 5, *e* and *f*).

Alpha-actinin will not self-associate in buffer containing 1 mM bicarbonate (19). when alpha-actinin-LR was added to myofibrils in 1 mM bicarbonate, there was no binding of the alpha-actinin-LR (Fig. 6, *a* and *b*). Indirect immunofluorescence staining with alpha-actinin antibody showed that alpha-actinin was still present in the Z-bands. After the bicarbonate-treated myofibrils were returned to standard salt, alpha-actinin-LR bound to the Z-bands and also to the rest of the I-band (Fig. 6, *c* and *d*). Gels of the supernatant from the bicarbonate-treated myofibrils indicated that tropomyosin was extracted from these myofibrils (Fig. 7). Tropomyosin-LR bound to the thin filaments in the I-bands but not to the Z-bands of these bicarbonate-treated myofibrils.

Addition of Contractile Proteins to Myofibrils Pretreated with Exogenous Contractile Proteins

When an excess of unlabeled alpha-actinin (3–5 mg/ml) was allowed to bind to myofibrils and then alpha-actinin-LR added, fluorescent localization occurred at the Z-bands just as when control myofibrils were used. If actin-LR was added to the myofibrils that had been pretreated with unlabeled alpha-actinin, fluorescent actin staining was found along the whole length of the myofibril (Fig. 8, *a* and *b*). When unlabeled actin was reacted with myofibrils followed by addition of tropomyosin-LR, the resultant fluorescent localization was in a doublet pattern in the H-zone (Fig. 8, *c* and *d*) just as when control myofibrils were exposed to actin-LR (Fig. 4).

DISCUSSION

Our results show that fluorescently labeled alpha-actinin will associate in vitro with native alpha-actinin in the Z-bands of isolated myofibrils, muscle bundles, and in intercalated discs of glycerinated cardiac muscles. Recently, Geiger has shown that fluorescently labeled alpha-actinin bound to stress fibers, attachment plaques, and cell junctions of detergent-treated nonmuscle cells (8). We have also demonstrated that neither labeled actin nor tropomyosin associated with its myofibrillar counterpart. Labeled tropomyosin did not bind to any region of control myofibrils, whereas actin bound in a doublet pattern to part of the A-bands. The actin binding was confined to the region of the A-band where there are free cross-bridges available on the myosin filaments. In the center of the thick filaments where cross-bridges are absent, there was no actin binding. Furthermore, no actin-LR bound in the overlap region of the A-band where thick filaments interdigitate with the native thin filaments. In contracted sarcomeres there is greater overlap of thick and thin filaments, and the amount of actin-LR that bound to the sarcomeres was diminished correspondingly (Fig. 4). The lack of actin-LR staining in the overlap region is not due to steric problems since HMM-LR readily binds to these areas (25, 26). The doublet staining pattern might have arisen if actin-LR polymerized from the ends of the thin filments; if this were the case, the binding

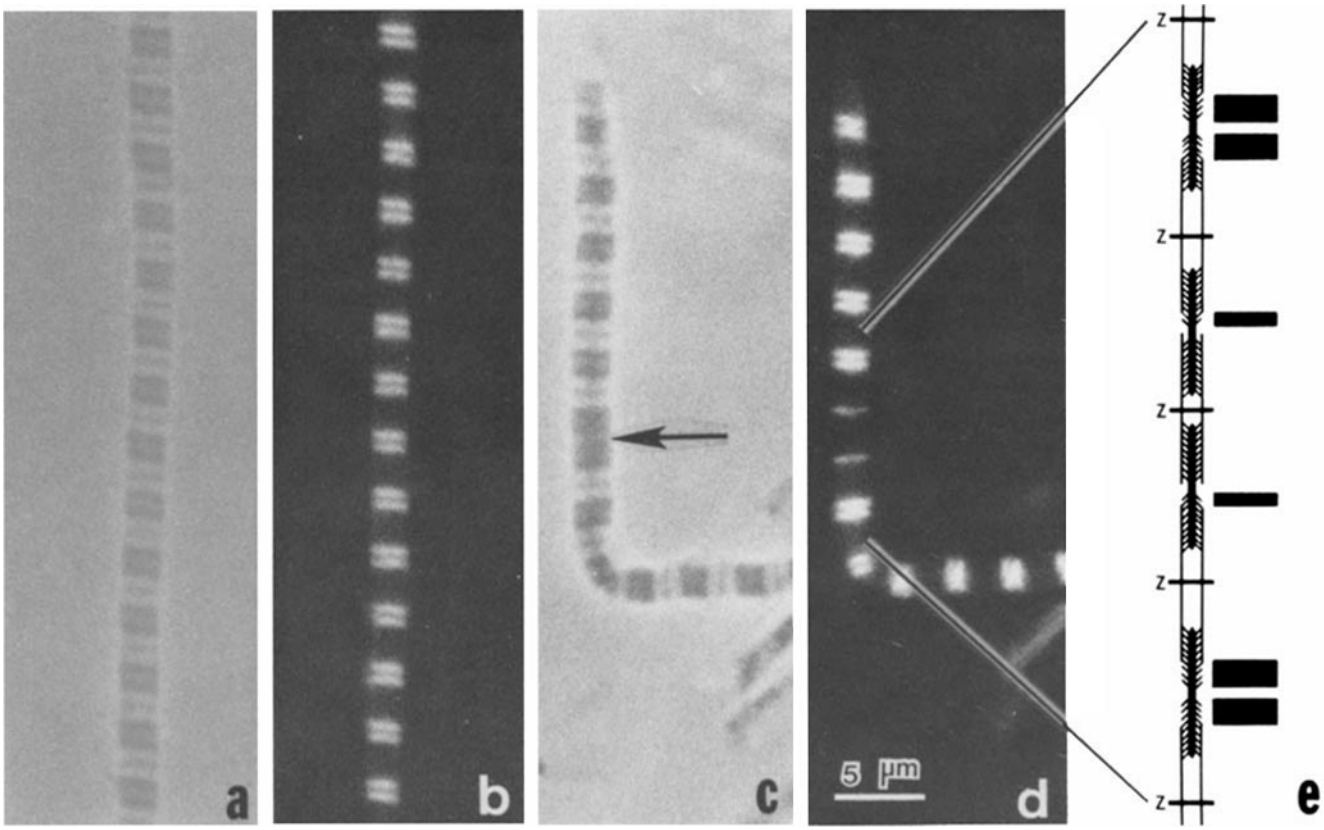

FIGURE 4 (*a–d*) Myofibrils stained with actin-LR. The phase dense A-bands (*a* and *c*) are slightly wider than the bands of fluorescence (*b* and *d*), indicating that actin-LR binds to the area of the myosin filaments free of interdigitating thin filaments. The two single bands of fluorescence in *d* result from the staining of two sarcomeres that contracted asymmetrically (arrow, *c*). The diagram in e shows schematically the relationship between the fluorescent staining (solid bars) and the position of thin and thick filaments and the thick filament cross-bridges. × 2,500.

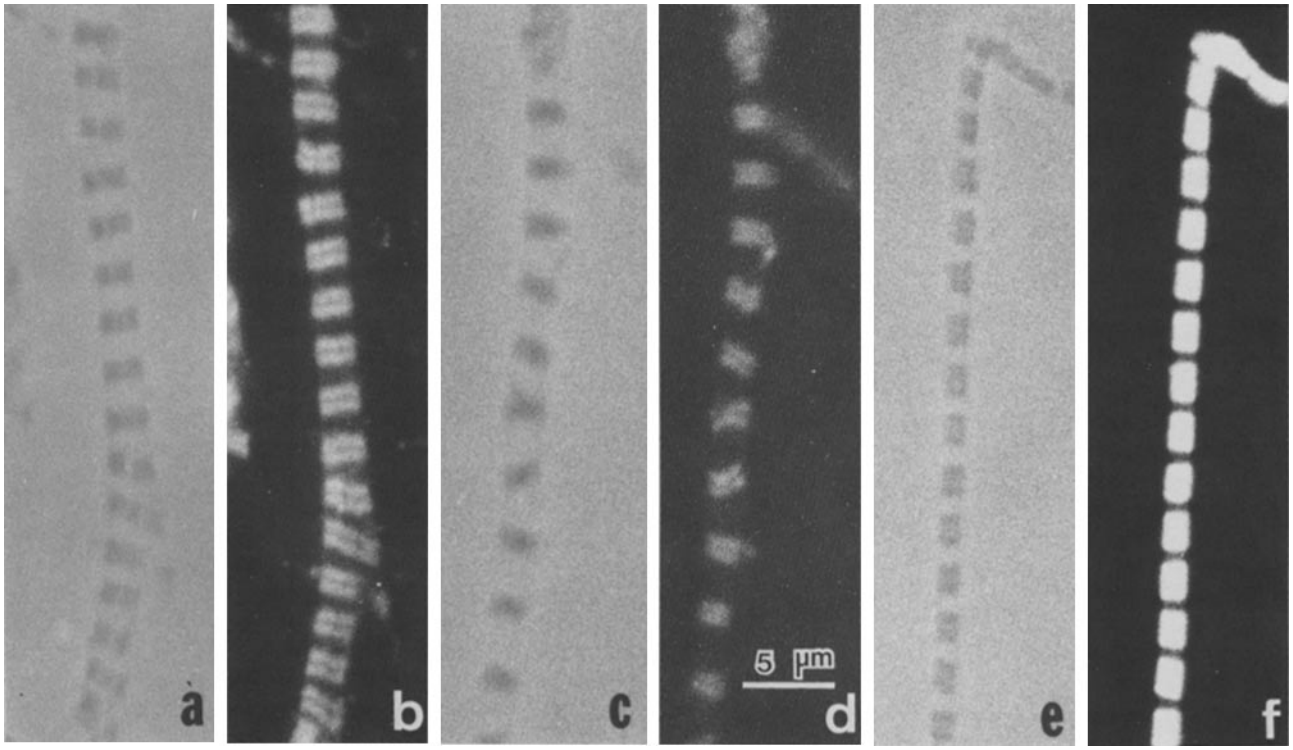

FIGURE 5 Myofibrils extracted with Hasselbach-Schneider solution to remove myosin and stained with various contractile proteins. With removal of the A-bands and exposure to added proteins, the I-bands appear as phase-dense bands (*a*, *c*, and e). The I-band minus the Z-band stains with HMM-LR (*b*) and tropomyosin-LR (*d*). Alpha-actinin-LR stains the entire I-band (*f*). × 2,500.

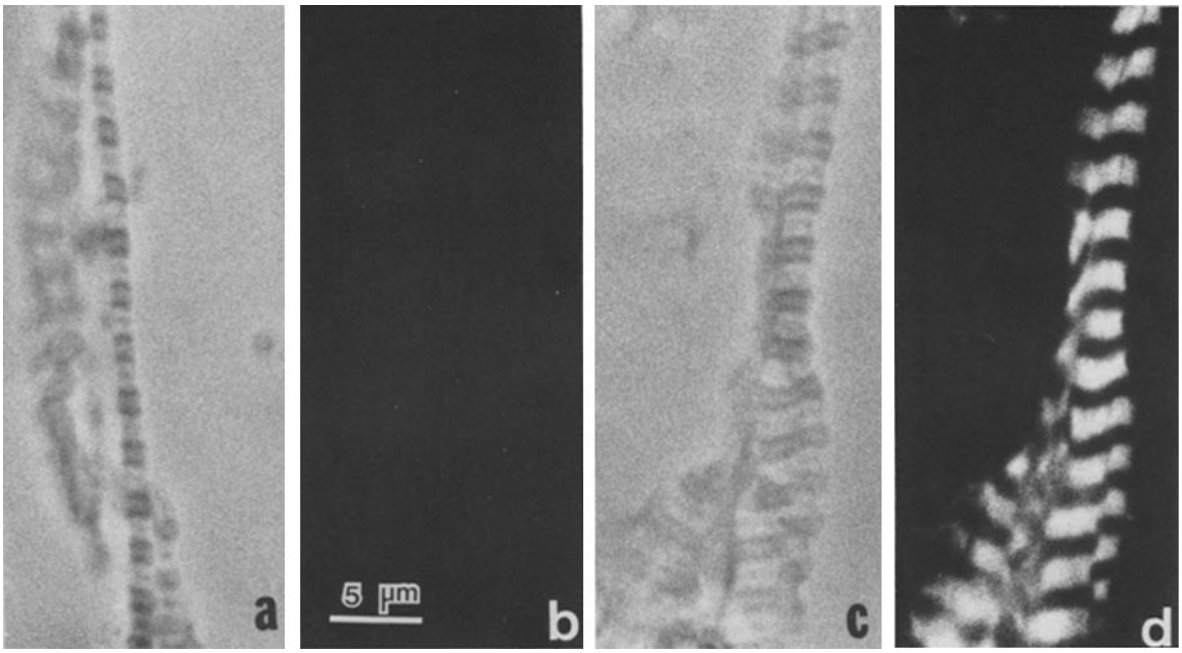

FIGURE 6 Myofibrils treated with 1 mM $NaHCO_3$. In this solution, myofibrils do not bind alpha-actinin-LR (*a* and *b*). If the myofibrils are returned to standard salt, alpha-actinin-LR binds to the entire I-band (*c* and *d*). × 2,500.

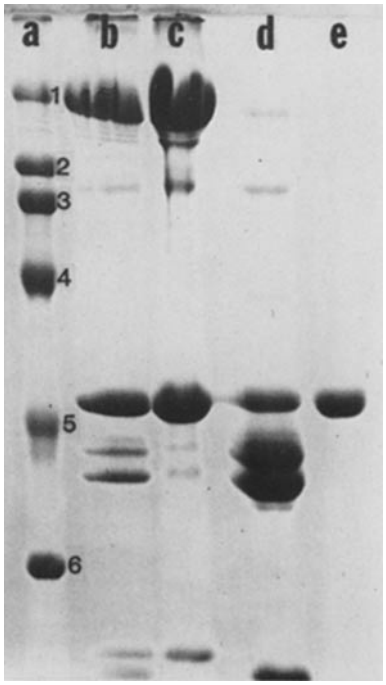

FIGURE 7 SDS polyacrylamide gel (7.5%) of myofibrils treated with 1 mM $NaHCO_3$ for 1 h. (a) markers: *1*, myosin heavy chain (200 kdaltons); *2*, β-galactosidase (116 kdaltons); *3*, phosphorylase B (97 kdaltons); *4*, BSA (66 kdaltons); *5*, egg albumin (43 kdaltons); *6*, carbonic anhydrase (29 kdaltons). (*b*) Rabbit myofibrils; (*c*) myofibrils after treatment with $NaHCO_3$; (*d*) extract from myofibrils treated with $NaHCO_3$; (*e*) actin.

should not have been inhibited by 0.2 mM ATP, as it was. ATP blocks actin-myosin interaction (16) and thus would have prevented actin-LR from binding to the free myosin cross-bridges of myofibrils, but ATP does not inhibit actin polymerization (21). Moreover, the doublet actin staining pattern was removed by Hasselbach-Schneider solution, a solution that removes myosin from myofibrils (16). These experiments indicate that actin-LR binding to myofibrils occurs where free myosin cross-bridges are available and does not result from polymerization at the ends of the native thin filaments.

There were only two conditions under which tropomyosin-LR would bind to myofibrils. If actin was added to myofibrils first, followed by addition of tropomyosin-LR, binding occurred as a doublet in the A-band (Fig. 8, *c* and *d*), which is the same pattern that was obtained when actin-LR alone was added to myofibrils. Tropomyosin has been shown to bind to actin in the ratio of one tropomyosin molecule per seven actin molecules (21). Since the exogenously added actin was free of any tropomyosin (Fig. 1 *c*), the tropomyosin-LR was able to bind to it. The fact that tropomyosin did not bind the native thin filaments of the myofibrils indicates that these actin filaments are fully saturated with tropomyosin. In the second case, tropomyosin-LR bound to the I-bands (but not the Z-band) of myofibril ghosts and of bicarbonate-treated myofibrils. This occurred because the high salt Hasselbach-Schneider solution that was used to extract myosin and make ghosts, also extracted tropomyosin from the myofibrils as did the 1 mM of bicarbonate solution. Thus, there were free tropomyosin binding sites available on the thin filaments to which tropomyosin-LR could add.

The addition of alpha-actinin-LR to myofibrils occurred not just at the periphery but throughout the entire thickness of the Z-band in both rabbit and insect myofibrils (Fig. 2, *g* and *i*). The binding was not saturatable as evidenced by the binding of alpha-actinin-LR to Z-bands after the myofibrils had been pretreated with unlabeled alpha-actinin. In vitro, alpha-actinin self-associates in 0.1 M KCl, but is prevented from self-association in 1 mM bicarbonate solution (19). In the same manner, we found that alpha-actinin-LR would not associate with the Z-bands in the presence of 1 mM sodium bicarbonate.

In addition to self-association, alpha-actinin also binds to actin (12). Jockusch and Isenberg (17) have recently reported that in this binding of alpha-actinin to tropomyosin-free fibrous actin, the alpha-actinin molecule behaves as a cross-linking molecule with two equivalent binding sites on the opposite ends of the elongated molecule. We found that alpha-actinin-LR did not bind to the thin filaments in I-band or in the A-band, but when we made myofibril ghosts, or when we

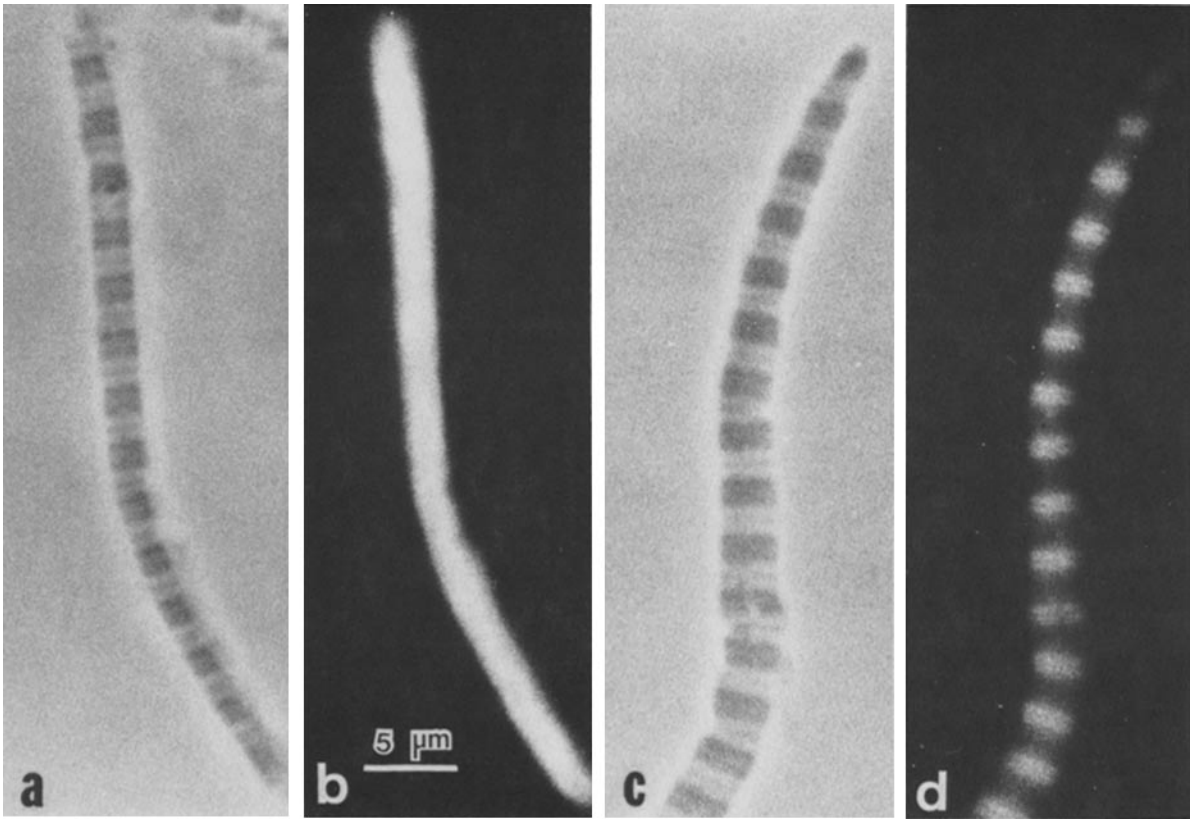

FIGURE 8 Myofibrils pretreated with unlabeled protein followed by labeled protein. (*a* and *b*) Myofibrils were first exposed to unlabeled alpha-actinin and then to actin-LR. (*c* and *d*) Myofibrils were exposed to unlabeled actin and then to tropomyosin-LR. Staining is in a doublet in the A-band, the same pattern seen when actin-LR is used to stain myofibrils (see Fig. 3). × 2,500.

treated myofibrils with 1 mM sodium bicarbonate, we removed tropomyosin, and under these conditions, alpha-actinin-LR bound not only to the Z-bands but also along the entire I-band. Therefore the native tropomyosin on the thin filaments in myofibrils prevents alpha-actinin from binding along the length of the thin filament.

Although actin binds to alpha-actinin in vitro (12, 17), we observed no binding of actin-LR to Z-bands in control myofibrils. If, however, myofibrils were pretreated with unlabeled alpha-actinin and then actin-LR added, actin-LR staining occurred along the entire length of the myofibril. This indicates that the native alpha-actinin in Z-bands was fully saturated with actin, but when exogenous alpha-actinin was added to the Z-bands, these Z-bands could then bind additional actin. Although, we have not shown that the fluorescent actin binding to these myofibrils is in the form of long filaments, an examination of actin-LR under identical conditions but without myofibrils present showed that actin filaments up to several microns long were formed (Fig. 4). We conclude that the exogenous alpha-actinin associates with the Z-bands and binds the actin filaments that formed in the standard salt-Tris incubation medium. In the same incubation medium, but without the presence of the added alpha-actinin, actin can bind only to the free myosin cross-bridges (Fig. 4) and the unbound actin filaments that formed are washed away when the myofibrils are rinsed.

Our results lead us to the following conclusions about myofibrillar structure: (*a*) Z-bands bind a full complement of thin actin filaments (labeled actin did not bind to Z-bands), but (*b*) can incorporate an indefinite amount of added alpha-actinin. (Labeled alpha-actinin bound to Z-bands and the binding could not be saturated by prior addition of unlabeled alpha-actinin.) (*c*) Exogenous alpha-actinin incorporated into Z-bands can now bind new actin filaments. (If alpha-actinin was added to myofibrils followed by labeled actin, fluorescence was seen along the whole length of the myofibril). (*d*) The thin filaments of myofibrils are maximally complexed with tropomyosin. (Labeled tropomyosin did not bind to myofibrils unless the native tropomyosin was first extracted with Hasselbach-Schneider solution or 1 mM bicarbonate. When actin lacking tropomyosin was first added to myofibrils, followed by labeled tropomyosin, fluorescence occurred in a doublet in the A-band, the site where actin binds to myofibrils.) (*e*) Tropomyosin prevents alpha-actin from binding to actin along the length of the thin filaments. (Labeled alpha-actinin did not bind thin filaments in the I-band unless tropomyosin was first extracted.) (*f*) The thin filaments are capped in some way that prevents additional growth when exogenous actin is present. (Labeled actin added to the A-band of myofibrils as a doublet that was removed when myosin was extracted. The doublet actin staining pattern is prevented when ATP is present in the medium.) (*g*) The thick filaments of rabbit myofibrils in rigor are fully bound to the interdigitating thin filaments. (No labeled actin bound in the overlap region of the A-band. When the extent of overlap increased in contracted sarcomeres, less actin bound to the A-band. In fully contracted muscle no actin bound to the thick filaments.)

These results have several implications for theories of muscle formation and growth. They suggest that in developing muscle, newly synthesized alpha-actinin would associate with forming Z-bands, and then could promote the attachment of

new actin filaments to the Z-band, thus causing myofibrils to increase in diameter. Subsequent interdigitation of myosin filaments would produce wider sarcomeres. Taniguchi and Ishikawa (31) showed that if myosin was added to myosin-extracted myofibrils in glycerinated cultured muscle cells, new thick filaments formed within the framework of actin filaments that projected from the Z-bands. The disposition of the filaments in the reconstructed myofibrils closely resembled their arrangement in vivo. In developing insect muscle, A-band length increases in sarcomeres that are already well ordered (1, 2), which shows that myosin can polymerize in vivo into an existing actin sarcomeric lattice.

On the ultrastructural level, it has not been possible to determine if there is a sequential assembly of sarcomeric components during myogenesis. Biochemical studies of developing muscle cells indicate that actin, myosin, tropomyosin, and alpha-actinin are all synthesized concurrently (6). Muscle filaments are first detected in the electron microscope as single filaments or in small bundles of interdigitating thick and thin filaments in cells that sometimes appear to lack Z-band material (7). Hayashi et al. (14) demonstrated that polymerization of actin in the presence of myosin filaments leads to the formation of bipolar arrays of thick and thin filaments. It may be that actin and myosin filaments first associate with one another and then alpha-actinin binds to the thin filaments at their ends, the only binding sites available if the actin is complexed with tropomyosin (12).

There have been speculations that myofibrils could serve as templates for a further increase in the number of myofibrils (15). Goldspink (10, 11) has extended this view by suggesting that there is a critical width for a Z-band beyond which it would be subjected to tearing forces during contraction (due to the mismatch in the square lattice of the thin filaments attached to the Z-band and the hexagonal lattice of the thin filaments interdigitating in the A-band) leading to longitudinally split myofibrils which are often seen in published micrographs (10). Our results show that alpha-actinin, when added to myofibrils, is incorporated thoughout the Z-band and then can promote actin filament binding. If this happens in vivo, it would lead to expanison of the Z-band as well as an increase in the number of thin filaments bound to a Z-band in a sarcomere. Association of myosin with the additional thin filaments would give rise to an increase in width of the myofibril.

The data we have obtained by exposing glycerinated muscle to labeled contractile proteins has added significance for studies of microinjection of fluorescent contractile proteins into living cells. Geiger (8) first demonstrated that in demembrarated nonmuscle cells, fluorescently labeled alpha-actinin associated with areas of the cells known to contain alpha-actinin. He pointed out that this indicated that a living cell was not needed for alpha-actinin incorporation. Our Z-band labeling with alpha-actinin-LR matches the Z-band labeling that Kreis and Birchmeier (18) obtained by injecting labeled alpha-actinin into living cultured myotubes. We also have injected the same alpha-actinin-LR used for staining glycerinated cardiac muscle cells into living cardiac and skeletal muscle cells. The patterns in the living and glycerinated cells are indistinguishable (Sanger, J., M. Pochapin, B. Mittal., and J. Sanger, manuscript in preparation), confirming Geiger's results with nonmuscle cells.

Recently, Glacy (9) has reported the results of microinjecting fluorescently labeled actin into living chick embryonic cardiac muscle cells. He observed labeled actin in the I-bands (but not the Z-bands) and in the M-lines as well. The labeled actin, however, did not stain any part of the overlap region of the A-band where actin is also present. We have never observed any binding of actin-LR to the I-bands or M-lines of our various skeletal and cardiac myofibrils. Presumably the different staining patterns of fluorescent actin in our cell models and the living cell is due to an active process of incorporation in the living cell.

Comparison of the staining of cell models and the microinjection of living cells with the same fluorescently labeled protein will make it possible to distinguish processes of self-assembly or passive binding from active processes of incorporation. In addition, labeling of glycerinated myofibrils can provide a sensitive control for the purity and nativeness of labeled contractile proteins since impure and denatured labeled proteins give rise to spurious staining of myofibrils.

This research was supported by grants from the National Institutes of Health (HL-15835 to the Pennsylvania Muscle Institute and GM 25653 to J. W. Sanger and J. M. Sanger).

Received for publication 20 June 1983, and in revised form 14 November 1983.

REFERENCES

1. Aronson, J. Sarcomere size in developing muscles of a tarsonemid mite. 1961. *J. Biophys. Biochem. Cytol.* 11:147–156.
2. Auber, J. La myofibrillogenese du muscle strie. I. Insectes. 1969. *J. Microsc. (Paris).* 8:197–232.
3. Brandtzaeg, P. Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic-exchange chromatography. 1973. *Scandanavian J. Immunol.* 2:273–290.
4. Chowrashi, P. K., and F. A. Pepe. The Z-band: 85,000 dalton amorphin and alpha-actinin and their relation to structure. 1982. *J. Cell Biol.* 94:565–573.
5. Devlin, R. B., and C. P. Emerson. Coordinate regulation of contractile protein synthesis during myoblast differentiation. 1978. *Cell.* 13:599–611.
6. Feramisco, J. R., and K. Burridge. A rapid purification of alpha-actinin, filamin, and a 130,000 dalton protein from smooth muscle. 1980. *J. Biol. Chem.* 255:1194–1199.
7. Fischman, D. A. Development of striated muscle. *In* The Structure and Function of Muscle. 1972. G. H. Bourne, editor. Academic Press, Inc., New York. 75–148.
8. Geiger, B. The association of rhodamine-labelled alpha-actinin with actin bundles in demembranated cells. 1981. *Cell Biology International Reports.* 5:627–634.
9. Glacy, S. D. Pattern and time course of rhodamine-actin incorporation in cardiac myocytes. 1983. *J. Cell Biol.* 96:1164–1167.
10. Goldspink, G. Changes in striated muscle fibres during contraction and growth with particular reference to myofibril splitting. 1971. *J. Cell Sci.* 9:123–138.
11. Goldspink, G. Growth of muscle. *In* Development and Specialization of Skeletal Muscle. 1980. D. F. Goldspink, editor. Cambridge University Press, Cambridge. 19–35.
12. Goll, D. E., A. Suzuki, J. Temple, and G. R. Holmes. 1972. Studies on purified alpha-actinin. I. Effect of temperature and tropomyosin on the alpha-actinin/F-actin interaction. *J. Mol. Biol.* 67:469–488.
13. Hasselbach, W., and G. Schneider. Der L-Myosin-und Aktingehalt des Kaninchenmuskels. 1951. *Biochem. Z.* 321:461–482.
14. Hayashi, T., R. B. Silver, W. Ip, M. L. Cayer, and D. S. Smith. Actin-myosin interaction. Self-assembly into a bipolar "contractile unit." 1977. *J. Mol. Biol.* 111:159–171.
15. Holtzer, H., J. M. Marshall, and H. Finck. An analysis of Myogenesis by the use of fluorescent antimyosin. 1957. *J. Biophys. Biochem. Cytol.* 3:705–724.
16. Huxley, H. E. Molecular basis of contraction in cross-striated muscles and relevance to motile mechanisms in other cells. *In* Muscle and Nonmuscle Motility. 1983. A. Stracher, editor. Academic Press, Inc., New York. 1–104.
17. Jockusch, B. M., and G. Isenberg. Interaction of alpha-actinin and vinculin with actin: opposite effects of filament network formation. 1981. *Proc. Natl. Acad. Sci. USA.* 78:3005–3009.
18. Kreis, T. E., and W. Birchmeier. Stress fiber sarcomeres of firbroblasts are contractile. 1980. *Cell.* 22:555–561.
19. Masaki, T., and O. Takaita. Some properties of chicken alpha-actinin. 1969. *J. Biochem. (Tokyo).* 66:637–643.
20. Nag, A. C., and M. Cheng. Differentiation of fibre types in an extraocular muscle of the rat. 1982. *J. Embryo. Exp. Morphol.* 71:171–191.
21. Oosawa, F. Macromolecular assembly of actin. *In* Muscle and Nonmuscle Motility. 1983. A. Stracher, editor. Academic Press, Inc., New York. 1:151–216.
22. Pardee, J. D., and J. A. Spudich. Purification of muscle actin. *Methods in Enzymol.* 1982. 85:271–289.
23. Pepe, F. A. Macromolecular assembly of myosin. *In* Muscle and Nonmuscle Motility. 1983. A. Stracher, editor. Academic Press, Inc., New York. 1:105–149.
24. Sanger, J. W. Formation of synthetic myosin filaments: influence of pH, ionic strength, cation substitution, dielectric constant and method of preparation. 1971. *Cytobiologie.* 4:450–466.
25. Sanger, J. W. Changing patterns of actin localization during cell division. 1975. *Proc. Natl. Acad. Sci. USA.* 72:1913–1916.
26. Sanger, J. W. The presence of actin during chromosomal movement. 1975. *Proc. Natl. Acad. Sci. USA.* 72:2451–2455.

27. Sanger, J. W. Intracellular localization of actin with fluorescently labelled heavy meromyosin. 1975. *Cell Tissue Res.* 161:432–444.
28. Sanger, J. W. Mitosis in beating cardiac myoblasts treated with cytochalasin-B. *J. Exp. Zool.* 201:463–469.
29. Sanger, J. W., J. M. Sanger, and B. M. Jockusch. Differences in the stress fibers between fibroblasts and epithelial cells. 1983. *J. Cell Biol.* 96:961–969.
30. Smillie, L. B. Preparation and identification of alpha- and B-tropomyosins. 1982. *Methods Enzymol.* 85:234–241.
31. Taniguchi, M., and H. Ishikawa. *In situ* reconstitution of myosin filaments within the myosin-extracted myofibril in cultured skeletal muscle cells. 1982. J. Cell Biol. 92:324–332.
32. Wang, Y. L., and D. L. Taylor. Preparation and characterization of a new molecular cytochemical probe: 5-iodoacetamido fluorescein-labeled actin. 1980. *J. Histochem. Cytochem.* 28:1198–1206.