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Association of titin and myosin heavy chain in developing skeletal muscle

(myogenesis/cytoskeleton/assembly *in vivo*)

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ABSTRACT **To understand molecular interactions that organize developing myofibrils, we examined the biosynthesis and interaction of titin and myosin heavy chain in cultures of developing muscle. Use of pulse-labeling, immunoprecipitation, and a reversible cross-linking procedure demonstrates that within minutes of synthesis, titin and myosin heavy chain can be chemically cross-linked into very large, detergent-resistant complexes retaining many features of intact myotubes. These complexes, predominantly of titin and myosin, occur very early in myofibrillogenesis as well as later. These data suggest that synthesis and assembly of titin and myosin are temporally and spatially coordinated in nascent myofibrils and support the hypothesis that titin molecules help to organize sarcomere formation.**

Titin, unique to striated muscle, is an abundant myofibril protein of $\approx 2.8 \times 10^6$ kDa (1–3) that stretches from the Z line to the M line proteins of the muscle (4). Titin is the major component of the "third" or "gap" filament system of the myofibril (1, 2, 5).

Studies suggest that titin also functions when the myofibril forms. Titin is periodic when the first periodic arrays of myosin form in skeletal (6, 7) and cardiac (8) cells. Titin may be required for such arrays, since it is one of the few proteins unique to and ubiquitous in striated muscle. Does titin interact directly with myosin *in situ*? The two proteins are in proximity in myofibrils, and titin interacts with isolated thick filaments. Proteolytic fragments of titin interact with purified myosin (9).

Little is known about the synthesis or assembly of titin in muscle. Its huge size presents the question of how titin is oriented in the developing sarcomere. One approach to this question is to use reversible cross-linking agents to detect titin-associated proteins during development *in vitro*. We showed that titin associates with the cytoskeleton during and after synthesis (10). Here, we show that precipitation of cross-linked muscle cytoskeletons with an antibody to titin recovers both titin and myosin heavy chain, suggesting an association between these two proteins *in situ*. Furthermore, the association of titin and myosin detected by cross-linking is closely associated in time with the synthesis of these molecules. It is possible that the interaction of these two proteins is critical in organizing myofibrillogenesis.

MATERIALS AND METHODS

Cell Culture. Primary cultures of chicken leg myoblasts, synchronized by a cycle of divalent cation depletion and repletion, were prepared from day 12 embryos (11).

Radiolabeling and Extraction of Cultures. Muscle cell cultures were labeled with [^{35}S]methionine in methionine-deficient medium (10). After labeling, cultures either were extracted immediately or were chased by adding complete medium supplemented with 2 mM unlabeled methionine and incubating at 37°C for various times before extraction. Extractions used 0.5% Triton X-100 in extraction buffer (100 mM KCl/10 mM Pipes, pH 6.8/300 mM sucrose/2 mM $MgCl_2$/1 mM EGTA) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 100 mM leupeptin; ref. 11).

Immunoprecipitation. Titin was precipitated by using a mouse monoclonal antibody (mAb), AMF-1, as described (10). Muscle-specific myosin heavy chain (hereafter myosin) was precipitated with mAb MF-20 (12), a gift of D. Fischman (Cornell University, New York). Vimentin was precipitated with mAb AMF-17b (11). Rabbit anti-*a*-actinin mAb was from ICN. Immunoprecipitations were performed on samples containing SDS and 2-mercaptoethanol (2-ME) as described (10, 13). After separating immunoprecipitates by PAGE, gels were analyzed for radioactivity as described (10). Immunoprecipitated protein was measured by scanning densitometry at 600 nm before fluorography.

Chemical Cross-Linking of Muscle Cultures. After extraction, cytoskeletons were cross-linked for 25 min on ice with 0.75 mM ethylene glycol (bis-succinimidyl succinate) (EGS) (Pierce), dissolved in dimethyl sulfoxide, and added to the cross-linking buffer (extraction buffer minus Triton X-100 and leupeptin) just before use.

Lysine or glycine (10 mM final concentration) stopped the reaction, DNase I (Worthington; 50 g/ml final concentration) was added, and after 10 min on ice, the cross-linking buffer was removed. The remaining structures were harvested by scraping into SDS extraction buffer (2% SDS/1% 2-ME/10 mM Tris, pH 7.4/2 mM EDTA). Samples were heated at 70°C for 10 min, centrifuged at 10,000 × *g* for 30 sec, and prepared for immunoprecipitation as described above. To solubilize cross-linked cytoskeletons from older cultures (>4 days after repletion), it was necessary to boil samples for 1 min instead of heating at 70°C. A fraction of titin molecules is destroyed during this treatment.

To cleave the cross-linker, cross-linked samples were incubated with 1 M hydroxylamine (NH_2OH) in 1 M NaOH/50 mM sodium phosphate, pH 8.5, for 6 hr at 37°C. Samples were precipitated with cold 10% trichloroacetic acid (final concentration), centrifuged, and dissolved in SDS sample buffer (neutralized with 1 M Tris base). The samples then were either subjected to electrophoresis or prepared for reimmunoprecipitation as described above.

Other cross-linkers also tested were: bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone, which is base cleavable; disuccinimidyl tartarate, which is periodate cleavable; and dithio-

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Abbreviations: mAb, monoclonal antibody; EGS, ethylene glycol bis(succinimidyl succinate); 2-ME, 2-mercaptoethanol.

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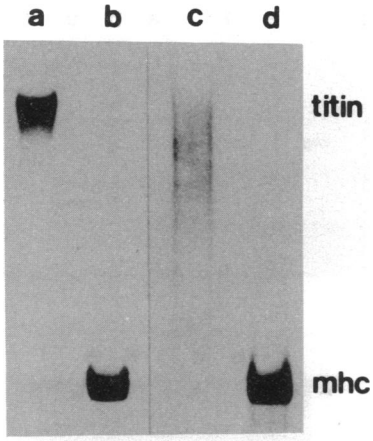

FIG. 1. Immunoprecipitation of titin and myosin from cultured muscle. Titin (lanes a and c) and myosin (lanes b and d) were precipitated from [^{35}S]methionine-labeled muscle cultures, and the samples were divided into equal parts. Half were untreated (lanes a and b); the others were treated with NH_2OH, which reverses the cross-linking (lanes c and d). Myosin is resistant to NH_2OH treatment, but titin is partially degraded. mhc, Myosin heavy chain.

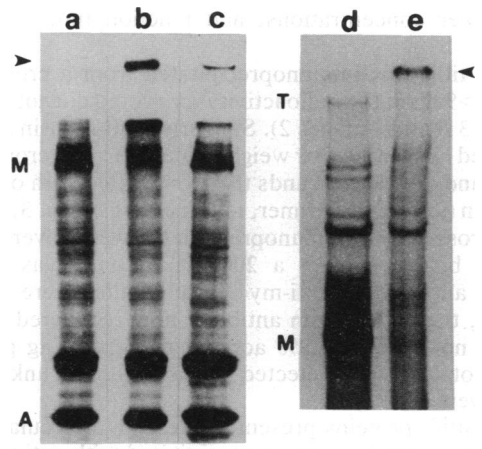

FIG. 2. Chemical cross-linking of myotube cytoskeletons with EGS. Muscle cultures were labeled for 16 hr with [^{35}S]methionine and extracted with 0.5% Triton X-100. The cytoskeletons were cross-linked with 0.75 mM EGS and analyzed on 9% acrylamide gels (lanes a–c) or 3.2% acrylamide/1% agarose gels (lanes d and e). Contents in lanes: a and d, control cytoskeleton; b and e, cross-linked cytoskeleton; c, cross-linked cytoskeleton treated with NH_2OH to reverse cross-linking. Titin, myosin, and actin are marked by T, M, and A. Arrowheads mark the top of the gels. Many proteins are not affected by cross-linking.

bis(succinimidylpropionate), which is thiol cleavable. The 2-ME in the immunoprecipitation protocol prohibited the use of the latter reagent.

Immunofluorescence. Cultures were fixed and stained as described (10). The antibodies used were those described above for immunoprecipitation, except that the antibody used to stain muscle-specific myosin was MF-18.

RESULTS

Titin and Myosin Can Be Chemically Cross-Linked *in Situ*. Titin becomes resistant to Triton extraction during translation (10). To determine whether this resistance reflects specific interactions with other proteins, we used a chemical cross-linking protocol. This procedure permeabilizes labeled cultures with Triton X-100 and uses the cross-linking reagent EGS to covalently link adjacent primary amino groups. After cross-linking, the cytoskeleton was dispersed in SDS and 2-ME, and the cross-linked adducts were recovered by immunoprecipitation. The cross-links could be broken ("reversed") by incubation with 1 M NH_2OH (pH 8.5). The proteins present in the cross-linked complexes could then be analyzed by electrophoresis before and after reversal of cross-linking.

Titin was partially degraded by this reversal step (Fig. 1) and was difficult to identify on a 3.2% gel. Because the titin fragments generated by NH_2OH were still very large, most accumulated as discrete bands between the stacking and separating gels when analyzed on Laemmli gels (see Fig. 3*A*, lane 2). Thus, most cross-linking experiments were analyzed by using this latter system. The titin epitope recognized by AMF-1 was not degraded, allowing identification of NH_2OH-treated titin; ≈50% of the cpm in a metabolically labeled population of titin molecules could be precipitated after NH_2OH treatment. Destruction of titin during reversal was more severe when base- or periodate-cleavable cross-linkers were used (data not shown). The lability of titin has been observed (1, 10).

Figs. 2 and 3 show the results of cross-linking muscle cultures labeled with [^{35}S]methionine for 16 hr. Comparing the cross-linked culture to an untreated sample revealed that virtually all of the myosin disappeared; an increased band of radioactivity appeared that did not enter the gel (Fig. 2, lanes a, b, d, and e). Most other abundant proteins appeared to be unchanged, even the polymeric proteins actin and vimentin. Similar results were obtained with several cross-linkers,

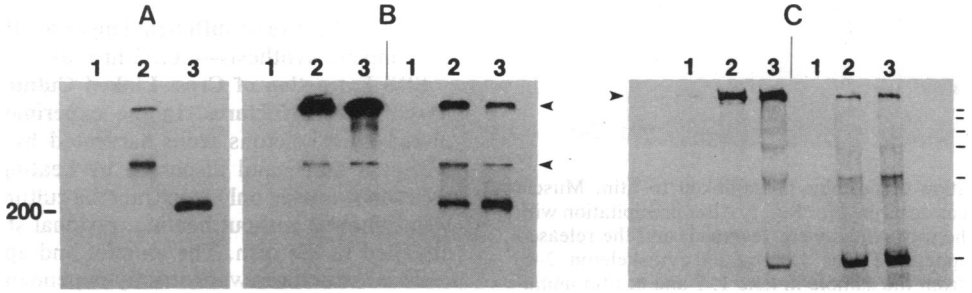

FIG. 3. Titin antibodies recover a 200-kDa protein from cross-linked cytoskeletons. Muscle cultures were prepared as in Fig. 2 and precipitated with anti-titin mAb (lanes 2), anti-myosin mAb (lanes 3), or nonspecific mouse IgG (lanes 1). The immunoprecipitates were run on 9% acrylamide gels (*A* and *B*) or 3.2% acrylamide/1% agarose gels (*C*) before (*B* and *C Left*) or after (*B* and *C Right*) reversal. (*A*) Immunoprecipitates from uncross-linked but "reversed" cytoskeleton (cross-linked cytoskeleton treated with NH_2OH to break the cross-links). Arrowheads on *B* mark the tops of the stacking and separating gels. Arrowhead on *C* marks the top of the gel. Molecular weights of cross-linked myosin standards are marked ($M_r \times 10^{-5}$) on the right side of *C*; from top to bottom the values are 12, 10, 8, 6, 4, and 2. Reversal of the anti-titin precipitate from cross-linked samples releases a 200-kDa protein (*B* and *C*, lanes 2). Bands at the top of the gel in lanes 2 and 3 of *C Right* reflect incomplete reversal of cross-links.

cross-linker concentrations, and reaction times (data not shown).

When titin was immunoprecipitated from a cross-linked sample, >90% of the radioactivity recovered did not enter the gel (Fig. 3 *B* and *C*, lanes 2). Similarly, anti-myosin antibody recovered high molecular weight material from a cross-linked sample and a series of bands that comigrated with oligomers of myosin (i.e., dimer, trimer, tetramer, etc.) (Fig. 3, lanes 3). When cross-linked immunoprecipitates were reversed with NH_2OH before PAGE, a 200-kDa protein was present, whether anti-titin or anti-myosin antibodies were used. In addition, the anti-myosin antibody now recovered material that did not enter the 9% acrylamide separating gel. Few other proteins were detected when the cross-linked complexes were reversed.

To identify proteins present in the high molecular weight complexes, complexes were precipitated with anti-titin antibody, treated with NH_2OH to reverse cross-linking, and reprecipitated with either the same or different antibodies. Reprecipitation of the reversed products with antibodies to titin, myosin, vimentin, or *a*-actinin recovered the proteins shown in Fig. 4. Reprecipitation with anti-titin mAb confirmed the presence of titin in the complexes. Reprecipitation with anti-myosin mAb showed that the 200-kDa protein released by reversal is muscle-specific myosin (Fig. 4, lane 11). Up to 80% of the muscle-specific myosin present in a culture could be recovered with anti-titin antibody after cross-linking. Reprecipitation of the reversed products with anti-vimentin or anti-*a*-actinin recovered no detectable protein, suggesting that these proteins are absent from the cross-linked complexes (Fig. 4, lanes 5, 6, 12, and 13).

Titin and Myosin Can Be Cross-Linked Within Minutes of Their Syntheses. To examine when titin and myosin become close enough after synthesis to be cross-linked, cultures were pulse-labeled for 6 min or 30 min, extracted, cross-linked, and subjected to precipitation with anti-titin or anti-myosin antibodies. For myosin, 40% of the myosin completed in a 6-min pulse was recovered with anti-titin antibody (Fig. 5 *Upper*). This increases to >65% after a 15-min chase. For newly synthesized titin, ≈20% of the radioactivity in titin was recovered with anti-myosin antibody after a 30-min pulse and cross-linking (Fig. 5 *Lower*). This value increased slightly to

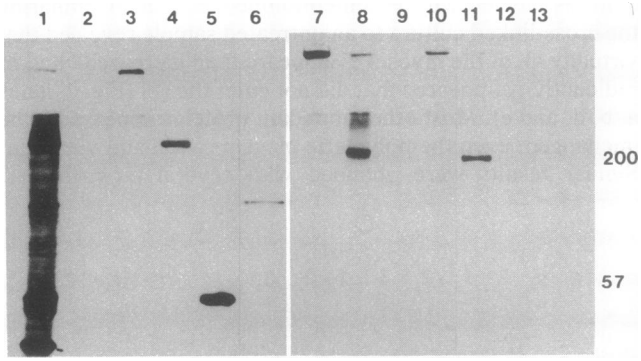

FIG. 4. Identification of proteins cross-linked to titin. Muscle cultures were treated as described for Fig. 2. After precipitation with anti-titin antibody, the cross-links were reversed, and the released proteins were reprecipitated. Lanes: 1, untreated cytoskeleton; 2–6, immunoprecipitates from the sample in lane 1; 7 and 8, titin immunoprecipitates from a cross-linked cytoskeleton (lane 7) whose cross-linking was reversed (lane 8); 9–13, proteins reprecipitated from the sample in lane 8. Antibodies used were nonspecific mouse IgG (lanes 2 and 9), titin mAb (lanes 3, 7, 8, and 10), myosin mAb (lanes 4 and 11), vimentin mAb (lanes 5 and 12), and *a*-actinin mAb (lanes 6 and 13). The anti-myosin mAb precipitates the 200-kDa band recovered with anti-titin mAb from cross-linked complexes. Neither vimentin nor *a*-actinin was recovered with anti-titin mAb from cross-linked complexes.

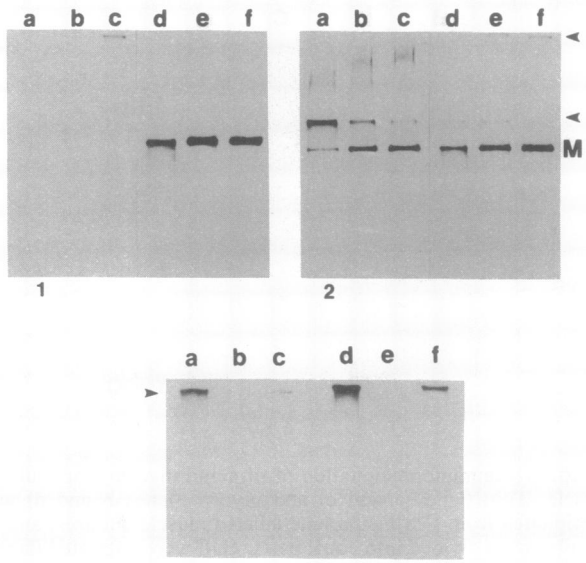

FIG. 5. Titin and myosin can be cross-linked within minutes of synthesis. (*Upper*) Muscle cultures were pulse-labeled with [^{35}S]methionine for 6 min and then either were extracted and cross-linked with EGS immediately (lanes a and d) or were chased with unlabeled methionine for 15 min (lanes b and e) or for 90 min (lanes c and f) before being extracted and cross-linked. Titin and myosin in the cross-linked cytoskeletons were immunoprecipitated. After cross-linking was reversed, the precipitates were analyzed on 9% acrylamide gels. Lanes: a–c, anti-titin precipitates; d–f, anti-myosin precipitates. Samples were from untreated cytoskeletons (*Upper Left*) or from cross-linked cytoskeletons whose cross-linking was reversed before PAGE (*Upper Right*). Note that even after a 6-min labeling period, anti-titin mAb recovers myosin (*Right*, lane a). Arrowheads mark the stacking and separating gels. The position of myosin is marked by M. (*Lower*) Muscle cultures were pulse-labeled with [^{35}S]methionine and extracted, and cytoskeletons were cross-linked as described above except that cells were labeled for 30 min and samples were chased for 2 hr. Cytoskeletons were precipitated with anti-titin or anti-myosin mAb. The anti-myosin precipitates were treated with NH_2OH to reverse cross-linking and were reprecipitated with anti-titin mAb. Samples were separated on 9% acrylamide gels; samples from cultures that were pulsed-labeled with no chase are in lanes a–c, while those from cultures chased before extraction and immunoprecipitation are in lanes d–f. Lanes: a and d, anti-titin immunoprecipitates from uncross-linked cultures to show the amount of immunoprecipitable titin present in the cultures; c and f, titin recovered after reversal of cross-linking of the anti-myosin precipitate from the cross-linked cytoskeletons; b and e, identical samples to those in c and f except that nonspecific mouse IgG was used during the reimmunoprecipitation step. Note that after cross-linking, titin synthesized during the pulse-labeling period can be recovered with anti-myosin antibody (lane c).

25% after a chase of sufficient length to allow all labeled titin to complete synthesis—i.e., 2 hr (10).

SDS Extraction of Cross-Linked Cultures Yields Residual Myofibrillar Structures. In the experiments above, cross-linked cytoskeletons were harvested by scraping into 2% SDS/1% 2-ME and dispersed by heating. When the SDS solution was used only to extract the cultures and the solution was removed without heating, residual structures remained attached to the dish. The amount and appearance of these residual structures were strictly dependent on the age of the cultures. In young cultures (<2 days after repletion), the structures, barely visible with phase-contrast microscopy, were composed of fine fibrillar networks. In older cultures with abundant sarcomeres, distinct periodic arrays were easily observed (see Figs. 7 and 8). PAGE of the proteins that resisted extraction by SDS revealed that, in young cultures, the high molecular weight cross-linked complexes compose >95% of the protein that resists extraction (Fig. 6 *Upper*) but

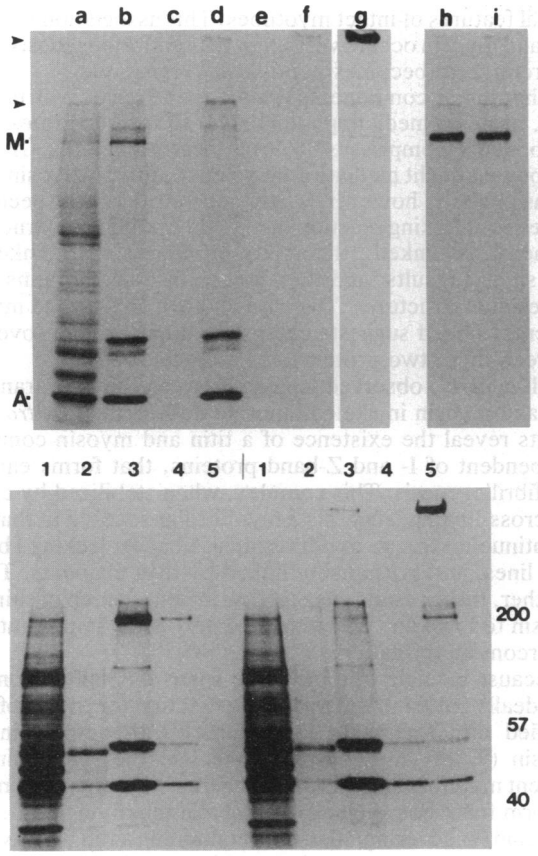

FIG. 6. SDS extraction of cross-linked cytoskeletons. (*Upper*) [^{35}S]Methionine was added to young muscle cultures 6 hr after repletion, and the cultures were labeled for 16 hr. The cultures were then extracted with 0.5% Triton X-100 and incubated with either EGS to cross-link or vehicle dimethyl sulfoxide as control. After 30 min on ice, the cultures were extracted with 2% SDS/1% 2-ME. After 5 min at room temperature, this solution was removed, and the dishes were rinsed twice with the SDS/2-ME. The various fractions were analyzed on 9% acrylamide gels. Lanes: a, Triton-extractable fraction; b, SDS-extractable fraction from uncross-linked culture; c, SDS-resistant fraction from uncross-linked culture; d, SDS-extractable fraction from cross-linked culture; e, SDS rinse fraction after SDS extraction of cross-linked culture; f, SDS-resistant fraction from cross-linked culture; g, lane f exposed to film 10 times longer. The SDS-resistant fraction shown in lane g was solubilized by heating and subjected to precipitation with anti-titin and anti-myosin mAbs. These immunoprecipitates are shown in lanes h and i, respectively, after reversal of cross-linking. Note that only the cross-linked complexes containing titin and myosin resist extraction with SDS. (*Lower*) An experiment identical to the one described above except with older cultures (7 days after repletion). (*Lower Left*) Samples from an uncross-linked culture. (*Lower Right*) Samples from a cross-linked culture. Lanes: 1, Triton-extractable fraction; 2, Triton rinse fraction; 3, SDS/2-ME extraction; 4, SDS/2-ME rinse fraction; 5, SDS-resistant fraction. Note that the major difference between the SDS-extractable fractions (lanes 3) of the cross-linked and uncross-linked samples is the almost complete absence of extractable myosin in the cross-linked sample.

only <5% of the protein present in the Triton-resistant cytoskeleton before cross-linking. In older cultures, the amount of titin and myosin increased (in absolute amounts as well as amounts relative to other proteins), and the SDS-resistant protein increased to 20% of the total cytoskeletal protein; 85% of this SDS-resistant protein was cross-linked (Fig. 6 *Lower*).

Immunoprecipitates of the SDS-resistant fraction are shown in Fig. 6. Almost all (>90%) of the protein present in this fraction could be recovered with either anti-titin or anti-myosin mAb. Reversing these precipitates revealed that

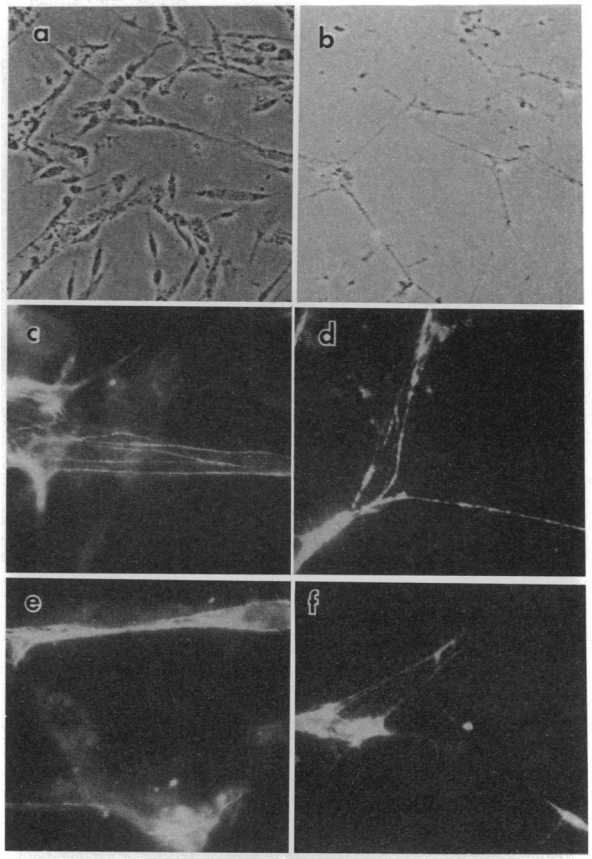

FIG. 7. Residual structures (from young cultures) containing titin and myosin resist SDS extraction after cross-linking with EGS. Muscle cultures (36 hr after repletion) were either fixed immediately with ethanol (*a*, *c*, and *e*) or extracted with Triton, cross-linked with EGS, extracted with 2% SDS/1% 2-ME, and then fixed with cold ethanol (*b*, *d*, and *f*). After fixation the cultures were stained for myosin (*c* and *d*) or titin (*e* and *f*). *A* and *B* show control cultures and cross-linked SDS-extracted cultures, respectively, by phase-contrast microscopy. See Fig. 6 *Upper*, lanes g, h, and i, for the protein composition of the structures observed in *b*, *d*, and *f*.

each mAb recovered the same amount of myosin. Thus, all of the SDS-resistant myosin is cross-linked (directly or via an intermediary) to either adjacent titin or myosin. Extracting with SDS removed proteins that were not cross-linked or were cross-linked only as small oligomers (e.g., myosin dimers, trimers, etc.) from the residual structures.

The location of titin and myosin in the residual structures was examined by immunofluorescence (Figs. 7 and 8). In young cultures, both antigens were found in the fine fibrils. On some fibers, these proteins were aperiodic or continuous, particularly myosin. In contrast, in older, more developed cultures, the periodicity of these cross-linked proteins was readily apparent. The location of titin and myosin within these residual structures was that observed in control myofibrils, except for a slight decrease in the width of A band staining by anti-myosin. When residual structures are stained with mAb specific for vimentin, abundant in myotubes of this age, little staining occurred, showing that this protein was extracted from cross-linked myotube cytoskeletons by SDS and 2-ME.

DISCUSSION

While cultured muscles develop, titin synthesis is closely related temporally with its assembly. We had shown that newly made titin rapidly forms contacts with the cytoskeleton; labeling nascent titin molecules with [^{3}H]puromycin

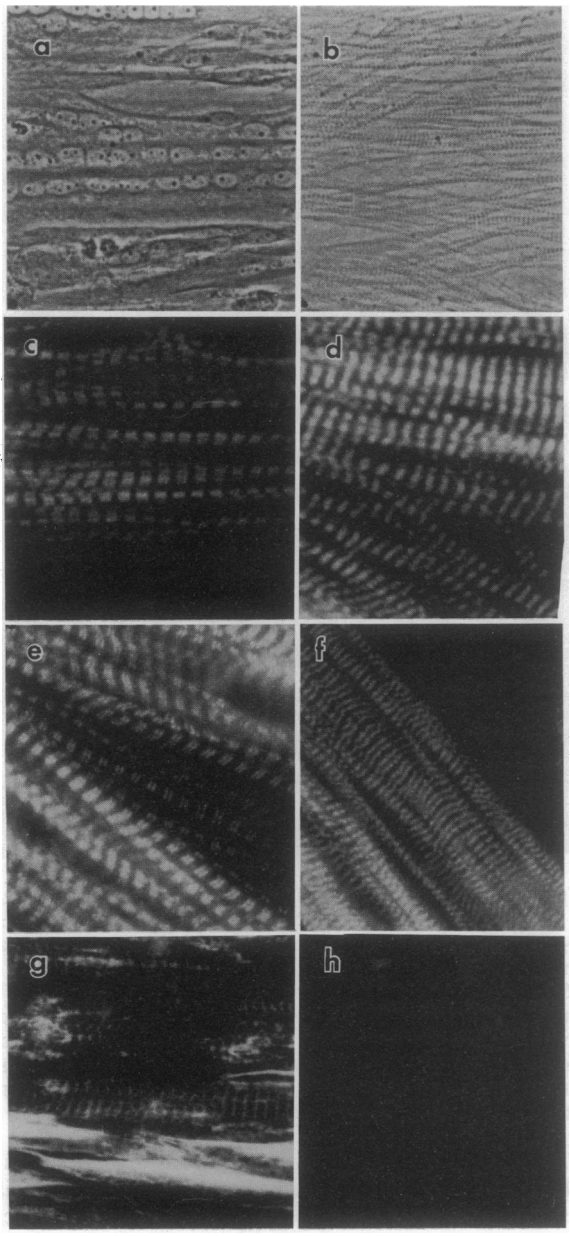

FIG. 8. Residual structures (from older cultures) containing titin and myosin resist SDS extraction after cross-linking with EGS. Muscle cultures (6 days after repletion) were either fixed directly in cold ethanol (*a*, *c*, *e*, and *g*) or extracted with Triton, cross-linked with EGS, extracted with SDS/2-ME, and then fixed with cold ethanol (*b*, *d*, *f*, and *h*). After fixation the cultures were stained for myosin (*c* and *d*), titin (*e* and *f*), or vimentin (*g* and *h*). *a* and *b* show control cultures and cross-linked SDS-extracted cultures, respectively, by phase contrast microscopy. Whereas titin and myosin remain after extraction of cross-linked cultures with SDS, vimentin is largely extracted (panels *g* and *h*). Note the loss of nuclear structure in the SDS-extracted cultures. See Fig. 6 *Lower*, lanes 5, for the protein composition of the structures observed in *b*, *d*, *f*, and *h*.

showed that these contacts form during translation (10). Results here implicate the site of this initial contact as either preexisting titin or myosin molecules. Once this contact is made, chemical cross-linking can stabilize a cytoskeletal structure containing largely titin and myosin with morphological features of intact myotubes. This association between titin and myosin occurs very early in myofibrillogenesis, even before myosin becomes periodically registered.

Other minor components remain in the residual structures (e.g., proteins mediating attachment to the substrate); such components comprise <10% of these structures. A third component might mediate attachment of titin to myosin. This seems unlikely, however, when one considers the specificity of the cross-linking reaction (most other polymeric structures are not cross-linked), the ability of various cross-linkers to give similar results, and the absence of other proteins from the residual structures. The ease with which titin and myosin are cross-linked suggests extensive direct contact (overlap) between these two proteins.

Hill *et al.* (7) observed linkage between the appearance of titin and myosin in skeletal muscle developing *in vitro*. Our results reveal the existence of a titin and myosin complex, independent of I- and Z-band proteins, that forms early in myofibrillogenesis. This complex, when stabilized by chemical cross-linking, suggests a myofibrillar scaffold in that it is a continuous structure with residual A bands, lacking I bands or Z lines, and presumably linked by titin filaments. Taken together, these results suggest that the interaction of titin and myosin to form an "organizing center" is an important step in sarcomere formation.

Because a single titin molecule spans the half sarcomere, it is ideally suited to an organizing function for the myofibril. Purified titin fragments can interact with both actin and myosin (9, 14) and thus may mediate the remodeling of nascent myofibrils (15) into sarcomeres. Domains of titin may perform functions such as binding and modifying (e.g., bundling and/or severing) filaments containing actin and myosin. The location of such domains could determine thick and thin filament organization within the sarcomere.

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