Connectin Filaments in Stretched Skinned Fibers of Frog Skeletal Muscle

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ABSTRACT Indirect immunofluorescence microscopy of highly stretched skinned frog semi-tendinous muscle fibers revealed that connectin, an elastic protein of muscle, is located in the gap between actin and myosin filaments and also in the region of myosin filaments except in their centers. Electron microscopic observations showed that there were easily recognizable filaments extending from the myosin filaments to the I band region and to Z lines in the myofibrils treated with antiserum against connectin. In thin sections prepared with tannic acid, very thin filaments connected myosin filaments to actin filaments. These filaments were also observed in myofibrils extracted with a modified Hasselbach–Schneider solution (0.6 M KCl, 0.1 M phosphate buffer, pH 6.5, 2 mM ATP, 2 mM MgCl₂, and 1 mM EGTA) and with 0.6 M KI. SDS PAGE revealed that connectin (also called titin) remained in extracted myofibrils. We suggest that connectin filaments play an important role in the generation of tension upon passive stretch. A scheme of the cytoskeletal structure of myofibrils of vertebrate skeletal muscle is presented on the basis of our present information of connectin and intermediate filaments.

It has long been assumed that some elastic component is responsible for the mechanical continuity and the passive tension existing in skeletal muscle. In 1954, Natori first demonstrated an elastic component in demembranated (skinned) frog skeletal muscle cells (14). When stretched beyond the overlap of actin and myosin filaments, skinned fibers generated passive tension, and the fibers returned to their original state upon release, suggesting the presence of a longitudinal elastic structure (14). We have extended this line of work, and proposed that an elastic network consisting of very thin filaments is responsible for the elasticity of myofibrils (12). We have named the protein composing these filaments connectin (12). Connectin consists of doublet bands in a SDS electrophoresis (27), the molecular weights of which are as large as 2.8 x 10⁶ and 2.1 x 10⁶, respectively (11). The smaller component (β-connectin) was isolated in a native form (5, 7) and shown to be a filamentous protein, 300–400 nm in length (11).

Recently, Wang and associates (28) have concluded that connectin nets (called the titin mesh in their terminology [27; cf. 10]) constitute simply matrix material, and that the longitudinal continuity and elasticity of the sarcomere are solely maintained by intermediate filaments (desmin fibers). Actually, Tokuyasu (23) has elegantly demonstrated the occurrence of longitudinally oriented desmin filaments in the interfibrillar spaces of chicken cardiac muscle.

In this article, we describe evidence for the presence of connectin filaments in sarcomeres of highly stretched skinned fibers of frog striated muscle.

MATERIALS AND METHODS

Preparation and Extraction of Skinned Fibers: Single muscle fibers were dissected from bullfrog semitendinous muscles, and skinned fibers were prepared in a relaxing solution (0.1 M KCl, 4 mM MgCl₂, 2 mM ATP, 2 mM EGTA, and 10 mM phosphate buffer, pH 6.8) as described previously (26). Skinned fibers were mechanically stretched, and the sarcomere lengths were measured by a laser light diffraction method (26). When extracted with a modified Hasselbach–Schneider solution (0.6 M KCl, 0.1 M phosphate buffer,
pH 6.5, 2 mM ATP, 2 mM MgCl2, and 1 mM EGTA) or with a KI solution (0.6 M KI, 0.06 M Na2SO4, and 10 mM phosphate buffer, pH 7.0), the medium was exchanged three times with an extraction interval of 1 min, and then 3-4 min extractions at 25°C were repeated five times. The fibers were finally extensively washed with a 0.1 M KCl solution. SDS-PAGE of proteins was performed by a modification of the Weber-Osborn method (30), using 3% polyacrylamide gels. Two to four skinned fibers ~3 cm long were dissolved in one drop of a SDS solution (10% SDS, 40 mM dithiothreitol, 10 mM EDTA, and 100 mM Tris-HCl, pH 8.0) and heated for 2 min at 100°C. Samples were applied onto gels 1.8 mm wide.

**Indirect Immunofluorescence Microscopy:** Antiserum against native connectin isolated from chicken breast muscle (5) was raised in a rabbit according to procedures described elsewhere (16). Goat fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibodies were purchased from Miles-Yeda Ltd. (Rohovot, Israel). A piece of freshly excised semitendinous muscle of a bullfrog was gently homogenized in a relaxing solution (60 mM KCl, 2.9 mM ATP, 3.4 mM MgCl2, 10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, and 6.7 mM PIPES buffer, pH 7.0) in an Ultra Turrax homogenizer (Janke & Kunkel and Co., GmbH, Staufen, Federal Republic of Germany). Isolated myofibrils were mounted on a slide glass, and a cover glass was applied and moved in one direction to stretch the myofibrils. To extract myosin, myofibrils were treated for 10 min at 20°C with a solution of ionic strength, 0.35 (290 mM KCl, 4.3 mM ATP, 5.2 mM MgCl2, 4 mM EGTA, and PIPES buffer, pH 7.0) and ionic strength, 0.6 (540 mM KCl containing other reagents as described above). After observations under a phase-contrast microscope, myofibrils were fixed in situ with 4% formalin in a relaxing solution for 10 min at room temperature. After being washed thoroughly with a solution consisting of 75 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2, and 10 mM EGTA, samples were treated with 15-fold diluted antiserum against connectin for 15 min at 20°C, and then washed with the same NaCl solution. Secondary staining with FITC-labeled anti-rabbit IgG antibodies was carried out in the same way. The specimens were observed under a fluorescent microscope (Fluophot, Nikon Ltd., Tokyo) and photographed on Kodak Tri-X film (Eastman Kodak Co., Rochester, NY).

**Electron Microscopy:** For electron microscopic examinations of anticonnectin-treated skinned fibers, stretched fibers were fixed in situ with 10% formalin in a solution containing 0.1 M NaCl and 10 mM phosphate buffer (PBS) pH 7.0, for 5-10 min at room temperature followed by washing with PBS. Then, fixed fibers were incubated with fivefold-diluted antiserum in PBS for 24 h at 4°C and washed with PBS for 12 h. Incubation was made with fivefold-diluted FITC-labeled anti-rabbit IgG antibodies for 24 h in a cold room. The fibers were treated with 3% glutaraldehyde in 0.12 M cacodylate buffer, pH 7.2, for 3-10 min at room temperature followed by fixation with 1% OsO4 in 0.12 M cacodylate buffer for 40 min at room temperature. Intact skinned fibers were fixed in a stretched state in a solution containing 1% glutaraldehyde, 1% glutaraldehyde, and 0.1 M cacodylate buffer, pH 7.4 (20). The specimen was stored in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for a day at 4°C. After brief washing in cacodylate buffer, specimens were postfixed with 2% OsO4 in 0.1 M cacodylate buffer for 2 h at 4°C. Tissue processing was completed by standard procedures for dehydration and Epon embedding. Thin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi Ltd. HU-12A electron microscope (Tokyo), operated at 100 kV.

**Immunoblotting of Connectin with Antiserum against Connectin:** A piece of freshly excised chicken breast muscle was solubilized with 10 vol of a hot solution containing 1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM dithiothreitol, and 0.004% bromphenol blue, and insoluble material was discarded by centrifugation at 10,000 g for 10 min. One-dimensional SDS gel electrophoresis was performed by the electrophoretic system of Fairbanks et al. (2) using a gradient gel of from 3% polyacrylamide and 0.3% N,N'-methylene-bis-acrylamide to 12% polyacrylamide and 0.06% N,N'-methylene-bis-acrylamide. After electrophoresis, peptides were transferred to nitrocellulose paper sheet according to Towbin et al. (24). The sheets were treated with tenfold diluted antiserum against connectin followed by the secondary reactions with goat FITC-labeled anti-rabbit IgG antibodies as described by Ohashi et al. (17).

**RESULTS**

**Immunofluorescent Staining of Stretched Myofibrils with Antiserum against Connectin**

The specificity of the anticonnectin antiserum used in this study was investigated using an immunoblot technique. Fig. 1 shows the indirect immunofluorescent staining pattern of an SDS extract of chicken breast muscle with anticonnectin serum. The anticonnectin antiserum stained only the connectin bands of the highest molecular weights among numerous muscle protein bands.

The antiserum against connectin from chicken breast muscle cross-reacted with connectin in frog skeletal muscle. Immunofluorescent observations showed the same staining patterns as in chicken breast muscle myofibrils (10; cf. 27). The A-I junction region of frog myofibrils was most strongly stained with the antiserum against connectin. The A band was appreciably fluorescent, the I band was faintly stained, and the Z lines were almost negative.

In stretched myofibrils (~4 μm in sarcomere length), the A-I junction region of low density was recognizable although the Z line was not seen (middle part of Fig. 2a). However, the Z lines were present in sarcomeres 3.5 μm long (left side of Fig. 2a). The density of the A-I junction region at the A band side was markedly increased after the treatment with the secondary antibodies against rabbit IgG (Fig. 2, a and b). After the antibody treatment, the apparent length of A band elongated to 2.5 from 1.6 μm under a phase-contrast microscope (Fig. 2b). The elongated A-I junction region including the edges of the A band was strongly fluorescent (Fig. 2c). The fluorescent area measured ~2.8 μm (Fig. 2c) indicating that the A-I junction region at the I band side was certainly fluorescent. The distance between the edges of the fluorescent region measured 1.2 μm. This observation suggests that connectin filaments are still concentrated in the A-I junction even when myosin and actin filaments are separated by a passive stretch. The I band was faintly fluorescent. There was not only any fluorescent staining but also any change in the myofibrillar structures at all when frog myofibrils were treated with nonimmune antiserum.

When stretched myofibrils (sarcomere length, 6 μm) were extracted with an ATP-containing solution (ionic strength, ~0.35), myosin was partly removed, and the remaining myosin was located along the middle portion of the A band (Fig. 2d). The Z lines became darker and wider. With the antibody treatment, the density of the area of the A band became somewhat enlarged (Fig. 2e). The fluorescent image showed that the strongly stained region was 4.0 μm long, and that the center where myosin still remained was much less fluorescent than both edges (Fig. 2f). This result shows that
connectin was still present at the A–I junction area when myosin was considerably removed. It is to be pointed out that the I band regions and Z lines became more fluorescent on partial removal of myosin.

On the other hand, when myosin was largely extracted with a solution of ionic strength 0.6, the fluorescent regions moved to the periphery of the Z lines (Fig. 2i). In Fig. 2g (sarcomere length, 3.6 μm), the dark region, 1.8 long, was the I–Z–I area. After the reaction with antibody, the dark area became slightly widened (2.0 μm). In the fluorescent microscope, this area measured 2.4 μm, both edges were strongly fluorescent, and the center (Z line) was dark. It appears that connectin nets were pulled away toward the Z line with the removal of myosin.

Protein Composition of Stretched Skinned Fibers after Extraction

Fig. 3a shows the SDS gel electrophoresis pattern of whole stretched skinned fibers of frog skeletal muscle in loose polyacrylamide gel (3%). The top band was connectin, and the large middle band was myosin. Actin and other proteins of lower molecular weights migrated to the bottom of the gels.

After extraction of the stretched fibers in situ with a modified Hasselbach–Schneider solution, some myosin always remained unextracted (Fig. 3b). This was also the case when 10 mM ATP, 6 M urea, or 2% Triton X-100 was added to the extracting solution. The residual myosin resisted further extraction with 0.6 M KI, although actin was completely solu-
the observations of Natori and his associates (15). This fact strongly suggests that myosin filaments are somehow pulled in the direction of the neighboring Z lines. In Fig. 5a, there are very thin filaments in the gap between myosin and actin filaments. The thin filaments attached to the tip or sides of myosin filaments, but it was not clear whether they bound to actin filaments or extended all the way to the Z line. These thin filaments are what Sjöstrand called "gap filaments" 20 years ago (21), as pointed out recently by Locker and Dainers (9). We regard these thin connecting filaments as connectin filaments (cf. 15). The width of the filaments was evidently wider than previously measured value, 2 nm (25). Connectin filaments isolated in native form were ~3-4 nm wide (11).

In skinned fibers from which myosin was extracted with a modified Hasselbach–Schneider solution, some thick filaments remained, and a few filaments extended through an entire sarcomere (Fig. 5b). The filaments seemed to be bound to remaining myosin filaments. There were not clear-cut evidence that the filaments were bound to actin filaments or extended directly to the Z line. Under these extraction conditions connectin is partly solubilized with the myosin (5, 10).

When myosin–extracted skinned fibers were further treated with 0.6 M KI, the I band disappeared, but longitudinal filaments were still visible between deteriorated Z lines (Fig. 5c). The filaments appeared to be connectin filaments associated with residual myosin filaments. Many densities in peripheral regions of widened Z lines were possibly artefacts caused by the accumulation of solubilized myosin, actin, connectin, and other proteins (3, 28). A number of very thin filaments were seen within the residual Z lines. We do not know whether these were desmin filaments (28), Z protein (17), or other proteins including connectin. Judging from the immunofluorescence observations of KI-extracted myofibrils, connectin filaments do not appear to exist in significant quantities in Z lines (10).

DISCUSSION

The present observations demonstrated the presence of very thin filaments connecting myosin filaments eventually to Z lines at the A–I junction area in extensively stretched myofibrils of frog skeletal muscle (Figs. 4a and 5a). We regard these connecting filaments as connectin (11) (also called titin; [27]) because (a) immunofluorescence microscopy localized connectin at the A–I junction (Fig. 2); (b) immunoelectron microscopical observations show the presence of electron-dense filaments from the A band to the I band (Fig. 4a); (c) connectin is a filamentous protein, 300–400 nm long and 3–4 nm wide (11, 19); and (d) connectin binds to both myosin and actin filaments in vitro (6). The findings indicate that the connectin filament is a candidate to be Sjöstrand’s "gap filament" (9, 21). In this connection a filament consisting of a 360,000-Mr protein bridges the edges of Z lines and the ends of myosin filaments in insect fibrillar flight muscle (18).

Recently, Higuchi and Umazume (4) have found that tension generated by passive stretch in skinned fibers of frog semitendinous muscle is progressively reduced in parallel with the disappearance of myosin from the both ends of myosin filaments in A bands during extraction of myosin with KCl solutions. It was postulated that the elastic component responsible for the generation of passive tension lies between Z lines and/or actin filaments and myosin filaments. It is not likely that the elastic filaments attach to the free ends of actin filaments, because the tips of actin filaments in stretched

**Figure 3**: Protein composition of stretched skinned fibers of frog skeletal muscle. A 3% polyacrylamide gel pattern of (a) intact fiber, (b) fiber extracted with a modified Hasselbach–Schneider solution, and (c) fibers extracted further with 0.6 M KI. c, connectin; n, nebulin; m, myosin; α, α-actinin; α, actin.
fibers are displaced considerably by the application of an electric field (26). If we assume that the elastic filaments are connectin filaments, the phenomena described above can be explained. On the other hand, the site of attachment of connectin filaments at the actin side is not conclusive. A number of connectin filaments originating from myosin filaments may fuse into a few filaments, which eventually extend to the Z line (Fig. 4a; see Fig. 6) or may link with another elastic component such as the N₂ line near the Z line, where nebulin is located (29).

In myofibrils extracted with Hasselbach–Schneider solution, some connectin-like filaments remained together with some myosin filaments (Fig. 5b). SDS PAGE showed that myosin remained in an amount similar to connectin (Fig. 3b). On further extraction with 0.6 M KI, the longitudinally oriented filaments were still present between the adjacent Z lines (Fig. 5c), although the amount of connectin was much more than that of myosin (Fig. 3c). It seems that there are a few connectin filaments associated with each other running through neighboring Z lines. The bulk of connectin filaments are pulled away near the Z lines, when myosin and actin are largely extracted away (Fig. 2f). In extensively stretched skinned fibers, actin was almost completely extracted with 0.6 M KI, although considerable myosin remained (Fig. 3c).
It is likely that some of filaments running through the Z lines might be in fact connectin filaments covered with myosin and other proteins in the KI extracted residues prepared by Wang and Ramirez-Mitchell (28).

Regarding the possible role of intermediate filaments in the continuity of a sarcomere (28), Tokuyasu has shown by immunoelectron microscopy that several intermediate filaments composed of desmin (skeletin) run longitudinally at the periphery of a sarcomere (22, 23), but are found in the cytoplasmic spaces between myofibrils. Therefore, the cytoskeletal structure of a sarcomere proposed by Wang and Ramirez-Mitchell is reasonable (Fig. 7 of reference 28). However, the intermediate filaments surrounding a myofibril cannot be involved with passive tension generation, because the intermediate filaments are not solubilized by Hasselbach-Schneider solution (3, 8). Furthermore, passive tension greatly drops on removal of myosin (4). It is possible that intermediate filaments are responsible for residual tension generation (<10% of the intact myofibrils).

In conclusion, elastic connectin filaments linking myosin filaments to actin filaments and/or Z lines play a predominant role in the tension generation of myofibrils stretched beyond the overlap of myosin and actin filaments. A schematic structure of a myofibril is presented in Fig. 6.

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REFERENCES

1. Brown, S. S., H. L. Malinoff, and M. S. Wicha. 1983. Connectin: cell surface protein that binds both laminin and actin. Proc. Nail Acad. Sci. USA. 80:5927-5930.
2. Fairbanks, G., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry. 10:2607-2617.
3. Granger, B. L., and E. Lazarides. 1978. The existence of an insoluble Z-disc scaffold in chicken skeletal muscle. Cell. 22:1233-1268.
4. Higuchi, H., and Y. Umazume. 1983. Location of the elastic component in skinned muscle fibers. Biophysic (Japan). 23:579.
5. Kimura, S., and K. Maruyama. 1983. Preparation of native connectin from chicken breast muscle. J. Biochem. 94:2083-2085.
6. Kimura, S., and K. Maruyama. 1983. Interaction of native connectin with myosin and actin. Biomed. Res. 4:607-610.
7. Kimura, S., H. Sawada, and K. Maruyama. 1982. Is there an elastic network structure in skeletal muscle? Biophysics (Japan). 22(Suppl. 1):53.
8. Lazarides, E., and B. D. Hubbell. 1976. Immunological characterization of the submit of the 100 A filaments from muscle cells. Proc. Nail Acad. Sci. USA. 73:4344-4348.
9. Locker, R. H., and G. J. Dairies. 1980. Gap filaments—the third set in the myofibril. In Fibrous Proteins, Scientific, Industrial and Medical Aspects, D. A. D. Part, and L. K. Creamer, editors. Academic Press, Inc., New York. 243-256.
10. Maruyama, K., S. Kimura, K. Obashi, and Y. Umazume. 1981. Connectin, an elastic protein of muscle: identification of "titin" with connectin. J. Biochem. 89:701-709.
11. Maruyama, K., S. Kimura, H. Yoshiodomi, H. Sawada, and M. Kikuchi. 1984. Molecular size and shape of connectin, an elastic protein of striated muscle. J. Biochem. 93:1423-1433.
12. Maruyama, K., S. Matsubara, R. Natori, Y. Nonomura, S. Kimura, K. Obashi, F. Murakami, S. Handa, and G. Eguchi. 1977. Connectin, an elastic protein of muscle: characterization and function. J. Biochem. 82:317-337.
13. Maruyama, K., N. Yamada, H. Ikeda, and S. Kimura. 1983. Connectin, one million dalton elastic protein of chicken breast muscle with a reference to dystrophic muscle. In Muscular Dysrophy, Biomedical Aspects, S. D. Baskin and E. Ozawa, editors. Japan Scientific Society Press, Tokyo/Springer-Verlag, Berlin. 201-208.
14. Natori, R. 1954. The property and contraction process of isolated myofibrils. Jikokai Med. 1:119-126.
15. Natori, Rb., Y. Umazume, R. Natori, and T. Yoshioka. 1980. The elastic structure of sarcomere. The relation of connectin filaments with thin and thick filaments. Jikokai Med. J. 27:83-97.
16. Obashi, K., T. Manaki, and K. Maruyama. 1977. I-protein, a new regulatory protein from vertebrate skeletal muscle. II. Localization. J. Biochem. 81:237-242.
17. Obashi, K., T. Mikawa, and K. Maruyama. 1982. Localization of Z-protein in isolated Z-disc sheets of chicken leg muscle. J. Cell Biol. 93:85-90.
18. Saide, J. D. 1981. Identification of a connecting filament protein in insect fibrillar flight muscle. J. Mol. Biol. 153:661-679.
19. Sawada, H., K. Maruyama, and S. K'mura. 1983. Electron microscopic observations of connectin filaments in KI-extracted residues of skeletal and cardiac muscles by the quick-freeze, deep-etch method. Biomed. Res. 4:603-606.
20. Simoneneu, N., and M. Simoneneu. 1976. Gallotyrlose and low molecular weight as monomers in electron microscopy. I. Procedure and evidence for the mantling effect. J. Cell Biol. 76:608-621.
21. Sjöstrand, F. S. 1962. The connections between A- and I-band filaments in striated frog muscle. J. Ultrastruct. Res. 7:225-246.
22. Tokuyasu, K. T. 1980. Immunochemistry on ultra thin frozen sections. J. Cell Biol. 96:562-570.
23. Tokuyasu, K. T. 1983. Visualization of longitudinally oriented intermediate filaments in frozen sections of chicken cardiac muscle by a new staining method. J. Cell Biol. 97:562-565.
24. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. Proc. Nail Acad. Sci. USA. 76:4350-4354.
25. Toyoda, N., and K. Maruyama. 1978. Fine structure of the connectin nets in cardiac myofibrils. J. Biochem. 84:239-241.
26. Umazume, Y., and S. Fujine. 1975. Electromechanical properties of extremely stretched skinned muscle fibers. Biophys. J. 15:163-180.
27. Wang, K., J. McColl, and A. Tu. 1979. Titin: major myofibrillar component of striated muscle. Proc. Nail Acad. Sci. USA. 76:3608-3702.
28. Wang, K., and R. Ramirez-Mitchell. 1983. A network of transverse and longitudinal intermediate filaments is associated with sarcomeres of adult vertebrate skeletal muscle. J. Cell Biol. 96:552-570.
29. Wang, K., and C. L. Williamson. 1980. Identification of an N2-line protein of striated muscle. Proc. Nail Acad. Sci. USA. 77:3254-3258.
30. Weber, K., and M. Osborn. 1965. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244:4406-4412.

FIGURE 6 Diagram of cytoskeletal structure of a myofibril. Connectin filaments are very thin filaments linking myosin filaments to Z lines. Desmin intermediate filaments longitudinally run to Z lines on the periphery of a sarcomere and also link Z lines of neighboring myofibrils.

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FIGURE 5 Longitudinal thin sections of stretched skinned fibers of frog skeletal muscle. Mordanted with tannic acid. (a) Intact myofibril. There were very thin filaments, presumably connectin, connecting myosin filaments to actin filaments and/or Z line. (b) Myofibrils extracted with a modified Hasselbach-Schneider solution. Note that myosin filaments were almost dissolved away leaving actin filament and few connectin filaments, running through a sarcomere. (c) Myofibrils extracted further with 0.6 M KI. Actin filaments were entirely extracted away leaving deteriorated Z line and few connectin filaments running through a sarcomere. × 52,000. Insets, (a) × 12,500; (b and c) × 25,000.