Connectin Filaments Link Thick Filaments and Z Lines in Frog Skeletal Muscle as Revealed by Immunoelectron Microscopy

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ABSTRACT In an earlier study connectin, an elastic protein of striated muscle, was found to be associated with "gap filaments" originating from the thick filaments in the myofibril, but it was not clear whether it extends to Z lines or not (Maruyama, K., H. Sawada, S. Kimura, K. Ohashi, H. Higuchi, and Y. Umazume, 1984, *J. Cell Biol.*, 99:1391–1397). In the present immunoelectron microscopic study using polyclonal antibodies against native connectin, we have concluded that the connectin structures are directly linked to Z lines from the thick (myosin) filaments in myofibrils of skinned fibers of frog skeletal muscle. There were five distinct antibody-binding stripes in each half of the A band and two stripes in the A-I junction region. Deposits of antibodies were recognized in I bands and Z lines. We suggest that connectin filaments run alongside the thick filaments, starting from a region ~0.15 μm from the center of the A band.

Connectin (also called titin) is a very long flexible protein of striated muscle (1–3) that is assumed to be responsible for passive tension generated upon stretch (4, 5).

Immunofluorescent studies have shown that connectin is mainly located in the A-I junction area in a sarcomere (6–9). In a recent paper (8) we showed that the connectin filaments are associated with the "gap filaments" that Sjöstrand described in 1962 (10). Trinick and associates (3) have demonstrated that "end filaments" extruding from the isolated native thick (myosin) filaments are morphologically indistinguishable from connectin filaments (11).

On the other hand, it was not clear whether the connectin filaments run through the entire I band to Z lines or not. Wang has presented the view that connectin filaments are linked to "nebulin meshwork" that is assumed to be connected with Z lines (12). Our previous study using immunoelectron microscopic observations was inconclusive with respect to this (8). Present reinvestigation has clearly shown that connectin structures are longitudinally located through the I band reaching the Z lines from the both sides of the thick filaments.

MATERIALS AND METHODS

Preparation of Skinned Fibers: Single muscle fibers were dissected from semitendinous muscle of the bullfrog (*Rana castesbeiana*), and mechanically skinned fibers were prepared in a relaxing solution (90 mM KCl, 5.2 mM $MgCl_2$, 4.3 mM ATP, 4.0 mM EGTA, and 10 mM PIPES, pH 7.0). Partial dissociation of thick filaments in skinned fibers was performed by the extraction with a solution of ionic strength of 0.35 (relaxing solution containing 290 mM KCl) for 10 min at 20°C at appropriate sarcomere lengths. Skinned fibers were fixed in situ for 10 min at 20°C with the relaxing solution or the solution of ionic strength of 0.35 containing 10% formalin.

Another type of skinned fiber was prepared at low ionic strength. Skinned fibers in the relaxing solution were incubated for 10 min at 5°C in a rigor solution (110 mM KCl, 1.2 mM $MgCl_2$, 4.0 mM EGTA, and 10 mM PIPES, pH 7.0). They were then immersed for 5 min at 20°C in 0.5 mM PIPES buffer, pH 7.0. Skinned fibers were fixed for 20 min at 20°C each time with 0.5 mM PIPES buffer containing formalin, the concentration of which was successively increased (0.5, 1.0, 2.0, 4.0, and 10%).

Specificity of Antiserum Against Connectin: The antiserum against connectin was the same as that used in our earlier immunofluorescence study (8). It was raised in a rabbit by injection of native connectin isolated from chicken skeletal muscle. Fig. 1 in that paper showed the specific reaction with connectin in an SDS extract of chicken breast muscle by an immunoblot method (8). Therefore, a similar immunoblot pattern is presented in the Fig. 1 here, using an SDS extract of frog skeletal muscle. The nitrocellulose sheets

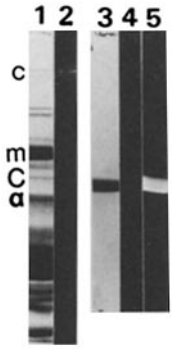


FIGURE 1 Immunofluorescent examination of cross-reactivity of antiserum against β-connectin from chicken breast muscle by immunoblot method. Lane *1*, Coomassie Blue-staining pattern of a total SDS extract of frog skeletal muscle. *c*, connectin; *m*, myosin; *C*, C protein; *α*, α-actinin. Lane *2*, corresponding immunofluorescent staining pattern with antiserum against chicken breast muscle connectin. Lane *3*, Coomassie Blue-staining pattern of α-actinin from frog skeletal muscle. Lane *4*, corresponding immunofluorescent staining pattern with antiserum against chicken breast muscle connectin. Lane *5*, corresponding immunofluorescent staining pattern with antiserum against chicken breast muscle α-actinin.

onto which peptides were electrophoretically transferred were treated with fourfold-diluted antiserum against connectin or α-actinin for 48 h at 4°C. Only connectin reacted with anti-connectin serum against chicken connectin (Fig. 1, lanes *1* and *2*). Fig. 1 also indicates that the antiserum did not react with α-actinin prepared from frog skeletal muscle according to Masaki and Takaiti (13) (Fig. 1, lanes *3* and *4*). The latter, however, cross-reacted with antiserum against α-actinin from chicken skeletal muscle (14) (Fig. 1, lanes *3* and *5*). It was very likely that the antiserum did not contain antibodies against C protein, since it did not cross-react with C protein from chicken skeletal muscle (donated from Professor T. Obinata, Chiba University).

Immunoelectron Microscopy: Skinned fibers fixed with formalin were washed three times at 10-min intervals with a solution containing 0.1 M NaCl and 10 mM phosphate buffer, pH 7.0 (PBS), at 20°C. The fibers were well washed with PBS for 12 h in the cold room and treated with 1% ovalbumin in PBS for 24 h in the cold room, then washed with PBS for 24 h. Then the fibers were incubated with antiserum against native connectin (8) diluted five times with PBS for 24–36 h in the cold room (4°C). For the control, nonimmune serum diluted five times with PBS was used. The treated fibers were washed with PBS for 24 h and incubated with fluorescein isothiocyanate–labeled anti-rabbit IgG antibodies (Miles-Yeda, Rehovot, Israel) diluted five times with PBS for 24–36 h, then washed with PBS for 24 h. The washed fibers were treated with 10% formalin in PBS for 5 min at room temperature, and washed with PBS for 1 h. The second antibodies labeling was carried out to allow comparison with the previous immunofluorescent microscopic observations (8). The fibers were then processed by standard procedures and Epon embedding.

Ultrathin (40–60 nm) sections were obtained on an LKB (LKB Producter AB, Bromma, Sweden) ultramicrotome with a diamond knife (Dupont Co., Wilmington, DE), stained with uranyl acetate for 30 min and lead citrate for 3 min, and observed in a JEM 100C electron microscope (Nihon-Denshi Co. Ltd., Tokyo).

Electron micrographs were taken at original magnifications of 2,000, 5,000, 10,000, and 20,000. These magnifications were checked with a calibration grid (No. 6002; 54,864 lines per inch; Ernest F. Fullam Inc., Latham,, NY) before the specimens were viewed. Prints were made at 3.5 times the original magnifications.

RESULTS

Fibers at Rest Length

Upon examination of sections of the skinned fibers treated with antiserum against connectin, the fine structures of myofibrils first appeared to be almost the same as those of intact fibers (see Fig. 4 of reference 8). However, it turned out that only a few layers of myofibrils at the periphery of a skinned fiber had bound with the antibodies, when low-magnification electron micrographs were carefully examined (Fig. 2*a*). Although the demembraned fibers were treated with the antiserum overnight, the diffusion of the antibodies into the myofibrils must have been very slow. The diffusion was much more limited than in the case of glycerinated muscle fibers used for the binding of antiserum against C protein (15).

In the first and second layers of myofibrils on the outer side of the antiserum-immersed fiber, there were several distinct stripes symmetrically arranged in each half of the A band (Fig. 2, *a* and *b*). The number and appearance of the five stripes in the A band did not vary in different sections, which suggests that the antigen sites are in register in adjacent filaments. The first stripe was present ~0.15 μm away from the center of the A band. Thus the two stripes are seen as the edges of a pseudo-H zone. Actually, the positions of the first stripe correspond to the edges of the central bare zone of the thick filaments in the A band (16, 17). The second stripe is located ~0.48–0.51 μm away from the center of the A band. Sometimes deposits of electron dense material were recognized between the first and second stripes, 0.3 μm from the center. More laterally, there were third and fourth distinct stripes, each 0.1 μm apart, in addition to the second stripe. The fifth stripe was located at the edge of the A band. Beyond the A band, there were two more stripes within 0.1 μm of the edge of the A band, although the regularity of their positions was not as constant as the position of stripes on the A band.

There were irregular deposits of electron-dense dots in the I band regions as well as in the Z lines (Fig. 2, *a* and *b*), which clearly indicates that the antigenic sites distribute all the way through Z lines. Thus, it is conclusively demonstrated that connectin structures occur throughout each half-sarcomere, from the A band center to the Z line.

Stretched Fibers

Frog semitendinous muscle fibers were stretched to ~5 μm. The maximal length was usually 8 μm, above which the fibers were torn. In the stretched fibers, the thick filaments are no longer in register and are pulled randomly to either side of Z lines (Fig. 3, *a* and *b*).

Antibody labeling was also random, and regular stripes were not observed (Fig. 3*a*). The apparent length of the myosin filaments due to the antibodies binding increased to ~1.8–2.2 μm, from the original 1.6 μm. Scattered labeling by the antibodies was noticed in the I bands. The Z lines also bound the antibodies.

Myofibrils Swollen at Low Ionic Strength

As is well known, myofibrils are remarkably swollen at low ionic strengths. It was expected that anti-connectin antibodies would easily diffuse into the inside of such swollen myofibrils. This was actually substantiated in the present investigation (Fig. 4).

Swollen myofibrils show a rather irregular appearance of both thick and thin filaments (Fig. 4*a*). The thick filaments became thinner and irregular. The thin filaments were wavy, and the boundary of the A and I bands became unclear. The Z line structure had evidently deteriorated.

The antiserum-treated swollen myofibrils showed remarkable labeling patterns all the way through whole fibers (Fig. 4, *b* and *c*). The A-I junction area was most electron dense, and the clear-cut stripes were observed on the A band, as in intact, rest-length myofibrils. There were seven stripes in each half of the A band and A-I junction region. The distance between the first stripes at the center was ~0.27–0.30 μm. The positions of each stripe from the center of the A band were 0.15, 0.5, 0.6, 0.7, 0.8, 0.85, and 0.9 μm, respectively. The fifth stripe was located at the tip of the thick filaments. The last stripe was frequently not detected. There were occasionally electron dense deposits between the first and second stripes, and only a few deposits were seen in the I band region, although clearly antibody binding was recognized in the deteriorated Z lines. The number of labeled sites and appearance

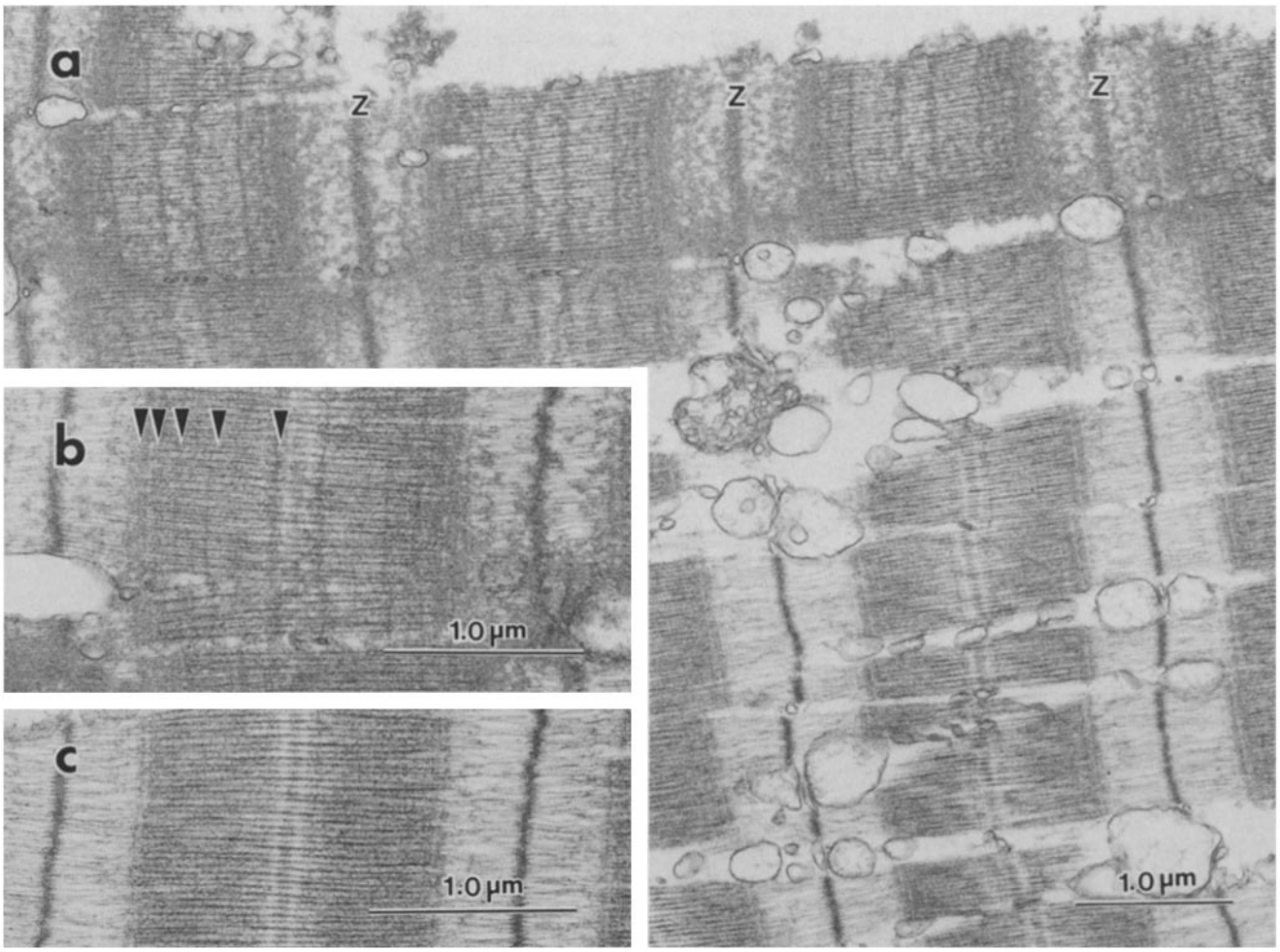


FIGURE 2 Immunoelectron micrographs of rest-length skinned fibers of frog skeletal muscle treated with antiserum against connectin. (*a* and *b*) Treated with anti-connectin serum; *c*, control. *Z*, Z line. Arrowheads show stripes due to the binding of anti-connectin antibodies.

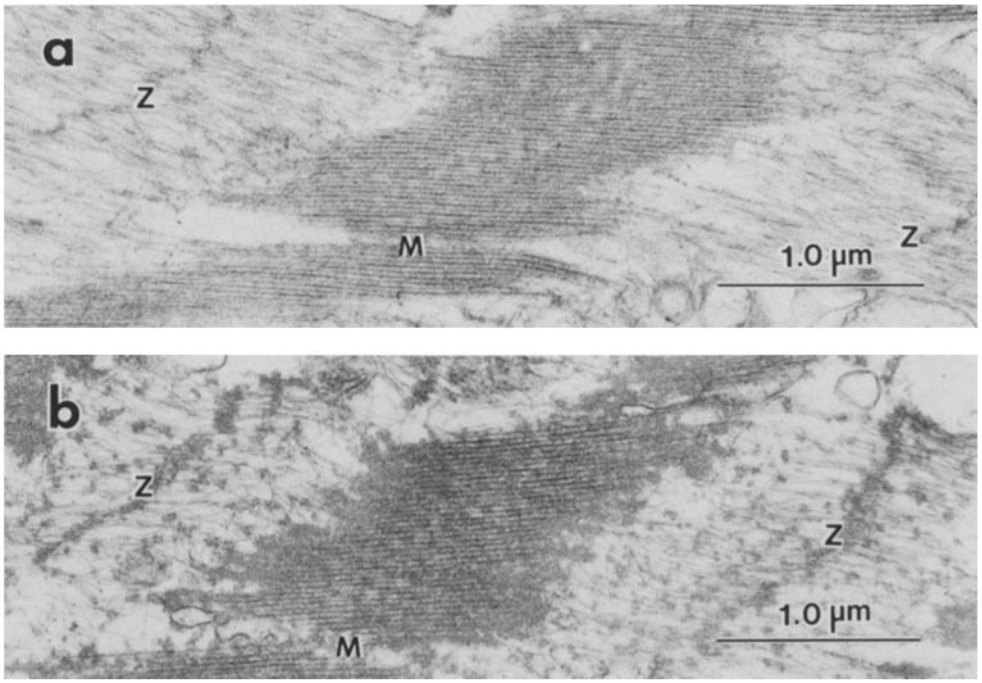
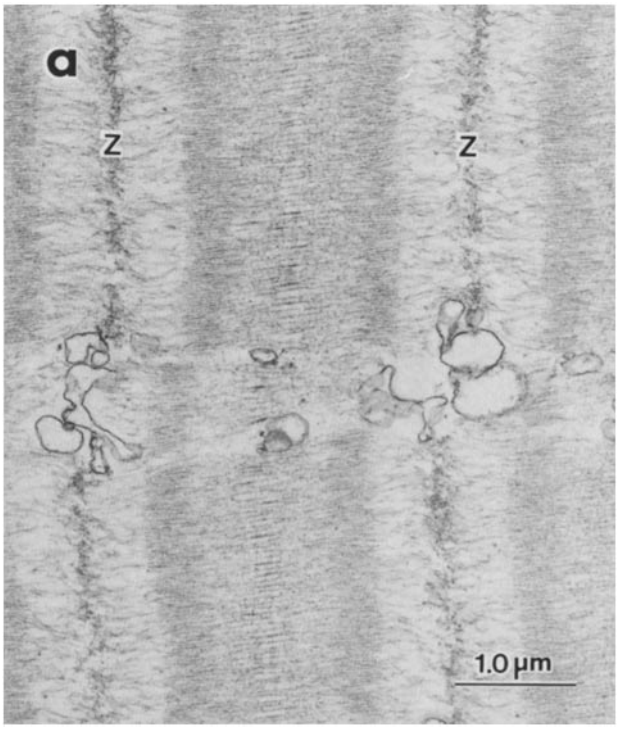
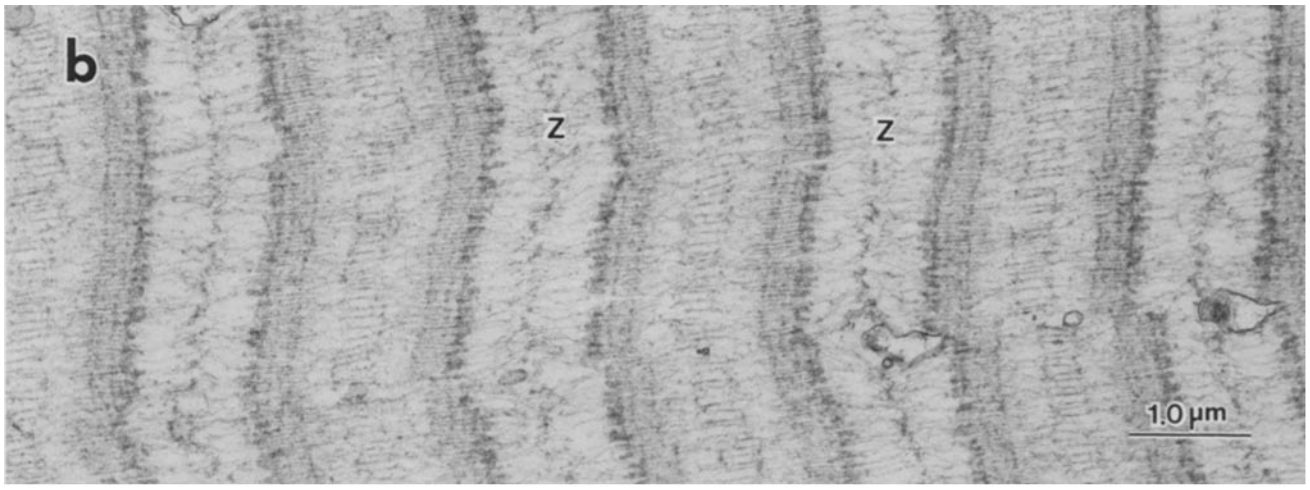


FIGURE 3 Immunoelectron micrographs of highly stretched skinned fibers of frog skeletal muscle treated with anti-connectin. (*a*) Control. (*b*) Treated with anti-connectin. *Z*, Z line. *M*, myosin filament.





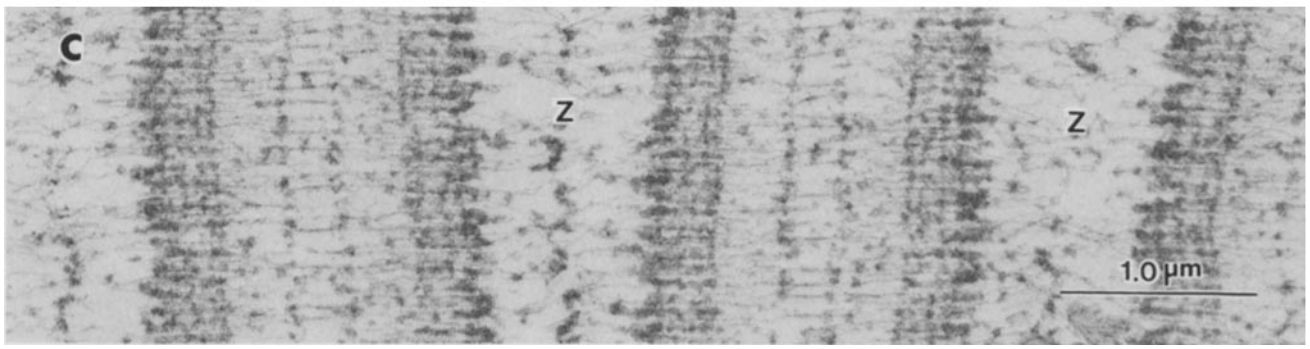


FIGURE 4 Immunoelectron micrographs of 0.5-mM PIPES-extracted skinned fibers of frog skeletal muscle treated with anti-connectin. (*a*) Control. (*b* and *c*) Treated with anti-connectin. *Z*, Z line.

of the antibody labeling were very similar to those in intact rest-length myofibrils except for the poor labeling in the I band.

Myofibrils Extracted with 0.29 M KCl

When stretched muscle fibers are extracted with 0.29 M KCl, generation of passive tension drops as myosin is dissolved from the both ends of the thick filaments (5). The electron micrograph of such KCl-extracted myofibrils shows that an electron-dense area, 0.4–0.5 μm long, remained around the center of the A band, although some longer thick filaments were still there (Fig. 5*a*). Upon close examination

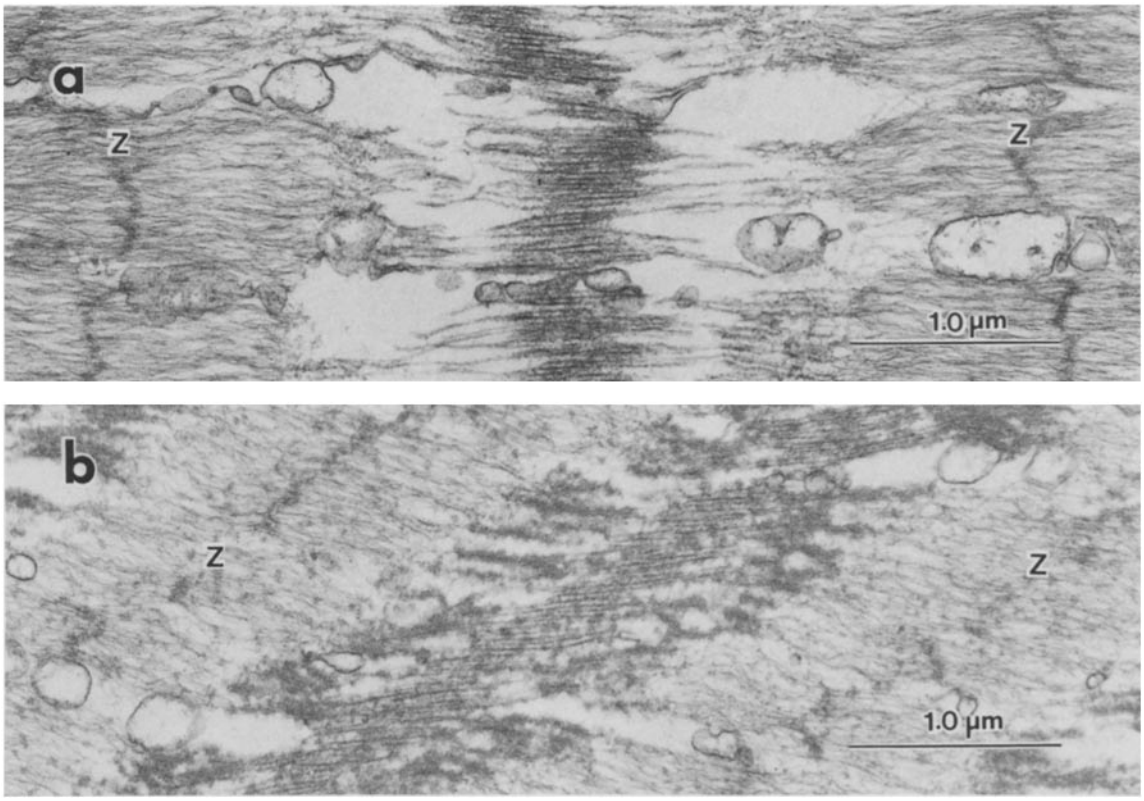


FIGURE 5 Immunoelectron micrographs of 0.29-M KCl-extracted skinned fibers of frog skeletal muscle treated with anti-connectin. (*a*) Control. (*b*) Treated with anti-connectin. *Z*, Z line.

of the remaining thick filaments, very thin filaments appeared to extend from their ends, and connectin-like filaments were seen between the thick filaments.

After treatment with anti-connectin antiserum, the remaining myosin filaments and their extensions were strongly labeled out to a total length of 1.9 to 2.1 μm (Fig. 5*b*). There were dense deposits of the antibodies at the level of the original ends of the thick filaments, although very few filaments remained in the control (Fig. 5, *a* and *b*). Periodic labeling was, however, not recognized.

The antibody deposits were not clearly observed in the I band region, although their presence in the Z lines was recognized.

DISCUSSION

So far, we have accumulated data on the various aspects of the elastic protein, connectin (also called titin): it is a very long, flexible, elastic protein of very high molecular weight (α-connectin, 2.8×10^6; β-connectin, 2.1×10^6) (1); the content is as high as 10% of the total myofibrillar structural proteins next to myosin and actin (6, 3); and connectin filaments extend from the myosin filaments to the I band region (3, 8).

Question arises whether the connectin filaments are directly linked to Z lines or not. Immunofluorescent studies have shown that connectin is mainly located at the A-I junction area and both sides of the A band (6–9). Wang has proposed that connectin is linked to nebulin, which is eventually connected to Z lines (12). On the other hand, we have claimed that connectin directly extends all the way to the Z lines (8). Until now, direct evidence was lacking for either view.

The present study has revealed that the connectin structure definitely extends from the myosin filaments to Z lines. Polyclonal antibodies against β-connectin that also react with α-connectin (8) were shown to bind to and to reveal distinct sites in each half of the A band and A-I junction, starting from the origin, ~0.15 μm away from the center of the A band. There was also antibody labeling in several sites in the I band and in the Z line. Since the length of a random coil of a peptide of 2.8×10^6 D can be theoretically stretched up to 3.7 μm, even if a connectin filament is assumed to be a double-stranded filament, it will be long enough to link the center of the A band to Z lines: The distance will be ~1.2 μm at rest and 3.5 μm at extreme stretch. One may speculate that the discontinuous labeling observed may be due to different epitope locations on the connectin filament. Distinct stripes observed in the present immunoelectron micrographs suggest that connectin filaments are oriented in register along the myosin filament. Recent observations using monoclonal antibodies against connectin (titin) have shown that only one band is labeled in each half of the sarcomere (18). The locations of the band differs depending on the monoclonal antibody species: A band, A-I junction area, or I band. This is most likely due to the probable specificity of the monoclonal antibody to a single epitope that is only exposed in one location.

Where are these connectin filaments bound on the myosin filament? The simplest assumption is that all the filaments are bound to the innermost site of the myosin filament, 0.15 μm away from its center, where H protein (19) and also C protein (20) probably are located. However, we cannot deny the possibility that connectin filaments are bound to myosin filament at the sites other than that mentioned above. Also,

it is possible one or two connectin filaments are present in the core of myosin filament and extend from the tip of the filament, as suggested by Locker (21) and Magid et al. (22). Evidently more work is needed to settle this problem.

The presence of desmin (skeletin) as an intermediate filament linking neighborhood Z lines longitudinally has been reported in chicken cardiac muscle (23). However, its presence in adult striated muscle has not yet been confirmed (24). Therefore, at the present time connectin filaments together with myosin filaments are considered to serve as the parallel elastic component of vertebrate striated muscle (compare reference 8). The role of connectin filaments in the generation of passive tension of stretched muscle fibers must be investigated from the view point of its location in myofibrils.

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