Elastic Filaments in Skeletal Muscle Revealed by Selective Removal of Thin Filaments with Plasma Gelsolin

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Abstract. Muscle needs an elastic framework to maintain its mechanical stability. Removal of thin filaments in rabbit skeletal muscle with plasma gelsolin has revealed the essential features of elastic filaments. The selective removal of thin filaments was confirmed by staining with phalloidin-rhodamine for fluorescence microscopy, examination of arrowhead formation with myosin subfragment 1 by electron microscopy, and analysis by SDS-PAGE. Thin section electron microscopy revealed the elastic fine filaments (~4 nm in diameter) connecting thick filaments and the Z line. After removal of thin filaments, both rigor stiffness and active tension generation were lost, but the resting tension remained. These observations indicate that the thin filament-free fibers maintain a framework composed of the serial connections of thick filaments, the elastic filaments, and the Z line, which gives passive elasticity to the contractile system of skeletal muscle. The resting tension that remained in the thin filament-free fibers was decreased by mild trypsin treatment. The only protein component that was digested in parallel with the decrease in the resting tension and the disappearance of the elastic filaments was α-connectin (also called titin 1), which was transformed from the α to the β form (from titin 1 to 2, respectively). Thus, we conclude that the main protein component of the elastic filaments is α-connectin (titin 1).

SEVERAL lines of evidence indicate that the mechanical stability of skeletal muscle is maintained by a filamentous structure other than thick (myosin) and thin (actin) filaments (Natori, 1954; Magid and Law, 1985; Higuchi and Umazume, 1985; Horowits et al., 1986). Many workers have observed the presence of extensible fine filaments in the gap region between thick and thin filaments of extremely stretched muscle fibers (Huxley and Peachey, 1961; Sjostrand, 1962; Locker and Leet, 1975) and fine filaments extending from the ends of isolated thick filaments (Trinick, 1981). Recently, models have been proposed in which such fine filaments are elastic and are responsible for the mechanical stability of muscle (Wang, 1985; Maruyama, 1986; Horowits and Podolsky, 1987). However, the overall features and physiological functions of this type of filament have not yet been clarified. We need to answer several questions. Although one end of these filaments appears to be attached to thick filaments, is the other end attached directly to the Z line or to thin filaments? To what extent are these filaments responsible for the passive elasticity of muscle fibers? Moreover, although the main component of the fine filaments has been suggested to be connectin (Maruyama, 1986) (also called titin; Wang, 1985), its precise localization and structure in the filaments remain to be clarified.

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We expected that if we could prepare muscle fibers from which the thin filaments had been selectively removed we would be able to directly examine the structure and function of fine filaments and might be able to characterize these filaments and their role in muscle structure and function. Many researchers have tried to remove thin filaments but failed to preserve the Z line (Wang and Greaser, 1985; Zimmer and Goldstein, 1987), which is essential for maintaining the integrity of fine filaments. On the other hand, there are several reports showing that gelsolin (Yin and Stossel, 1979), one of the actin-severing proteins, can be used as a molecular tool for removing actin filaments from various kinds of cells (Chaponnier et al., 1985; Cooper et al., 1987; Sanger et al., 1987). We have succeeded in preparing thin filament-free fibers from skeletal muscle by applying plasma gelsolin (Harris and Weeds, 1984): special care was taken to suppress proteolytic activity during sample preparation because the fine filaments were extremely sensitive to the proteolysis.

Materials and Methods

Solutions

We used the following solutions: EGTA rigor solution (0.17 M KCl, 1 mM $MgCl_2$, 10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)[1] (pH 7), 1

1. *Abbreviations used in this paper*: DFP, diisopropyl fluorophosphate; MOPS, 3-(*N*-morpholino)propanesulfonic acid; S-1, myosin subfragment 1.

mM EGTA, 2 mM diisopropyl fluorophosphate (DFP; Wako Pure Chemical Industries, Osaka, Japan) and 2 mM leupeptin (Boehringer Mannheim GmbH, Mannheim, FRG)); Ca rigor solution (0.17 M KCl, 1 mM $MgCl_2$, 10 mM MOPS (pH 7), 0.1 mM $CaCl_2$, 2 mM DFP, and 2 mM leupeptin); contracting solution (0.15 M KCl, 5 mM $MgCl_2$, 4 mM ATP, 10 mM MOPS (pH 7), 0.1 mM $CaCl_2$, 2 mM DFP, and 2 mM leupeptin); relaxing solution (0.15 M KCl, 5 mM $MgCl_2$, 4 mM ATP, 10 mM MOPS (pH 7), 1 mM EGTA, 2 mM DFP, and 2 mM leupeptin).

Preparation of Gelsolin

To prepare gelsolin, 800 ml of calf plasma was fractionated with $(NH_4)_2SO_4$. The 30–50% $(NH_4)_2SO_4$ fraction was dialyzed against 50 mM NaCl, 10 mM Tris-HCl (pH 8), 50 μM $CaCl_2$, 0.1 mM DFP, and 0.1 mM leupeptin and then adsorbed on an Affigel blue column (2.5 × 10 cm; Bio-Rad Laboratories, Richmond, CA). After washing with 0.12 M NaCl, 10 mM Tris-HCl (pH 8), 50 μM $CaCl_2$, 0.1 mM DFP, and 0.1 mM leupeptin, gelsolin was eluted with 0.35 M NaCl. The gelsolin fraction was loaded on a DE52 column (2.5 × 10 cm; Whatman, Maidstone Kent, England) equilibrated with 10 mM NaCl, 10 mM Tris-HCl (pH 8), 50 μM $CaCl_2$, 0.1 mM DFP, and 0.1 mM leupeptin. The gelsolin fraction was eluted with a linear gradient of 10–300 mM NaCl, dialyzed against 0.6 M NaCl, 1 mM Na-phosphate (pH 6), 50 μM $CaCl_2$, 0.1 mM DFP, and 0.1 mM leupeptin, and then loaded on a hydroxylapatite column (1.5 × 5 cm; Seikagaku Kogyo, Tokyo, Japan). Gelsolin was eluted with a linear gradient of 1–100 mM Na-phosphate and dialyzed against 0.2 M $(NH_4)_2SO_4$, 50 mM NaCl, 20 mM Tris-HCl (pH 7), 50 μM $CaCl_2$, 0.1 mM DFP, and 0.1 mM leupeptin. Finally, the gelsolin fraction was loaded on a phenyl-Sepharose CL4B column (1.5 × 8 cm; Pharmacia Fine Chemicals, Piscataway, NJ). Purified gelsolin was eluted with a linear gradient of 0.2–0 M $(NH_4)_2SO_4$, dialyzed against 0.5 mM $NaHCO_3$, concentrated with a DIAFLO ultrafiltration membrane filter (YM 10, 25 mm; Amicon Corp., Danvers, MA), and stored in liquid nitrogen. About 5 mg of gelsolin was obtained. All procedures were carried out at 4°C.

Muscle Fibers and Myofibrils

Rabbit psoas muscle fibers (3 mm diameter) were tied to a glass rod and incubated in 50% (vol/vol) glycerol, 0.5 mM $NaHCO_3$, 5 mM EGTA, and 1 mM leupeptin at 0°C overnight. Fibers were then stored in fresh solution at −20°C. Myofibrils were prepared by homogenizing the glycerinated fibers in EGTA rigor solution (Ishiwata and Funatsu, 1985).

Treatment of Myofibrils with Gelsolin

A drop of myofibril suspension was placed on a glass slide and covered with a coverslip. Two opposite sides of the coverslip were sealed with enamel, and the solution was exchanged by drawing the solution from one unsealed side of the coverslip with a piece of filter paper. Glass slides were placed on ice in a cold room during gelsolin treatment. Myofibrils were treated with 0.1 mg/ml of gelsolin in Ca rigor solution and then with gelsolin in contracting solution for 10 min each. 50–100 μl of gelsolin solution was used for each treatment. After gelsolin was washed out with relaxing solution, optical microscopic observation was performed at room temperature.

Identification of Actin Filaments with Phalloidin-Rhodamine

Localization of thin filaments in myofibrils and muscle fibers was examined by specific labeling of actin with phalloidin-rhodamine (Molecular Probes Inc., Eugene, OR; Wulf et al., 1979). Myofibrils and fibers were fixed with 3% formaldehyde in EGTA rigor solution (without DFP and leupeptin) for 30 min at room temperature and then labeled with 0.33 μM phalloidin-rhodamine in EGTA rigor solution (without DFP and leupeptin). Fixation was essential to detect actin in myofibrils because phalloidin-rhodamine stains only free ends of thin filaments and Z lines in unfixed myofibrils (Wilson et al., 1987). Phase-contrast and fluorescence micrographs were taken with an optical microscope (Fluophoto VFD-R; CF Plan DM 100× objective lens [1.25 NA]; Nikon Inc., Tokyo, Japan). The system camera (PFM; Nikon Inc.) and Tri-X films (Eastman Kodak Co., Rochester, NY) were used (Microdol-X developer diluted 1:3; Eastman Kodak Co.). Exposure times for phase-contrast and fluorescence micrographs were 1 and 30 s, respectively.

Treatment of Muscle Fibers with Gelsolin

For electron microscopy and SDS-PAGE, a bundle of glycerinated fibers (100–140 μm in diameter and 6–7 mm in length) composed of four fibers was tied at both ends to a platinum wire. For measurement of stiffness and tension, glycerinated single fibers were mechanically skinned (Natori, 1954) and tied to the hook of a tension transducer. In all cases, fibers were chemically skinned with 1% Triton X-100 in EGTA rigor solution for 30 min. After washing for 10 min in Ca rigor solution, the fibers were treated for 1 h with 0.5 mg/ml of gelsolin in Ca rigor solution. Fragmented thin filaments still bound to the fibers were further severed for 1 h more with 0.5 mg/ml of gelsolin in contracting solution and finally washed out for 1 h with relaxing solution. All procedures were carried out at 2°C.

Labeling of Thin Filaments in Muscle Fibers with Myosin Subfragment 1 (S-1)

Muscle fibers with or without gelsolin treatment were incubated with 2 mg/ml of S-1 for 1 h in EGTA rigor solution, and unbound S-1 was washed out with EGTA rigor solution for 1 h. All procedures were done at 2°C. S-1 was obtained by chymotryptic digestion of myosin prepared from rabbit back and leg white muscle (Weeds and Pope, 1977).

Digestion of Muscle Fibers with Trypsin

Muscle fibers were fully washed with several changes of relaxing solution (without DFP and leupeptin) for 2 h to remove protease inhibitors. They were then digested with 1.25 μg/ml of trypsin (T8253; Sigma Chemical Co., St. Louis, MO) in relaxing solution (without DFP and leupeptin) at 2°C. Digestion was terminated by putting fibers into relaxing solution (with 5 mM DFP), and fibers were immediately fixed for electron microscopy or dissolved for SDS-PAGE analysis.

Electron Microscopy

Muscle fibers were fixed with 1% formaldehyde in relaxing solution (without leupeptin; for S-1 labeling, EGTA rigor solution was used instead) for 30 min each at 2°C and then at 20°C and fixed with 2.5% glutaraldehyde, 0.5% tannic acid, and 0.1 M Na-phosphate (pH 7.2) for 1 h at 20°C. After washing with 0.1 M Na-phosphate (pH 7.2), they were postfixed with 1% OsO_4 in the same buffer for 1 h at 20°C, dehydrated with ethanol and acetone, and embedded in Epon 812. Thin sections were stained sequentially with saturated uranyl acetate and 2.6% lead citrate at 20°C. Electron micrographs were taken with an electron microscope (JEM 1200EX; operating at 100 kV; JEOL, Tokyo, Japan).

Optical Diffraction

Positive replicas of electron micrographs (15,000× direct magnification) were made on FG film (6,000× final magnification; Fuji Photo Film Co., Ltd., Tokyo, Japan); after suitable exposure, a specific area from which an optical diffraction pattern would be obtained was covered with a black piece of paper, and the surrounding area overexposed with intense light. The replica film thus obtained was mounted on a diffractometer (Klug and Berger, 1964), and optical diffraction patterns were made on Minicopy film (Fuji Photo Film Co., Ltd). The spacings of layer lines were estimated by assuming that the major meridional reflections corresponded to 14.3 nm (Huxley and Brown, 1967).

SDS-PAGE

Muscle fibers dissolved in 25 μl of lysis solution (5.5% SDS, 10 mM Tris-HCl (pH 6.8), 10% glycerol, and 1 mM DTT) were loaded on a 2–16% gradient polyacrylamide gel and electrophoresed (Somerville and Wang, 1981). Proteins were stained with Coomassie brilliant blue, and the absorbance at 560 nm of each band was measured with a chromatoscanner (CS-910; Shimadzu Corp., Kyoto, Japan). Since the volume of muscle fibers differed from preparation to preparation, we calibrated the amount of muscle proteins loaded by using myosin heavy chain as a standard; here, we confirmed with SDS-PAGE that myosin heavy chain was not extracted from fibers by the gelsolin treatment (cf. Fig. 1, lane *3*) and was not digested by trypsin under the conditions used here (cf. Fig. 8).

Measurement of Tension and Stiffness

Glycerinated single fibers were mechanically skinned to remove the elastic sarcolemma (Natori, 1954). The apparatus and methods for measuring active tension and sarcomere length of fibers were the same as those used by Horiuti et al. (1988). Measurement of resting tension and stiffness (1%

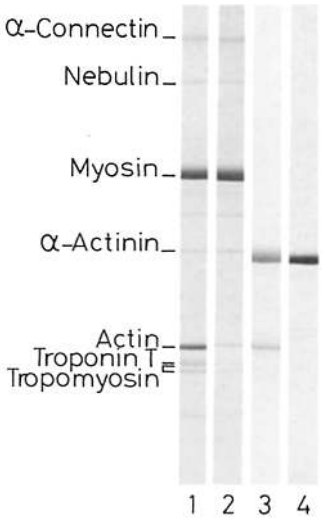

Figure 1. SDS-PAGE (2–16% gradient gel) patterns of untreated (lane *1*) and gelsolin-treated (lane *2*) glycerinated fibers of rabbit psoas muscle and of the extract of the gelsolin-treated fibers (lane *3*) and of purified plasma gelsolin (lane *4*). The proteins in lane *2* were from a bundle of muscle fibers treated with 0.5 mg/ml of gelsolin in Ca rigor solution and then in contracting solution for 1 h each. The proteins in lane *3* were from the gelsolin solution used for the treatment of hundreds of muscle fibers.

stretch within 2 ms) was performed as described previously (Higuchi and Umazume, 1985).

Results

Selective Removal of Thin Filaments: Analysis by SDS-PAGE

The selective removal of thin filaments from muscle fibers was checked by quantitative analysis of SDS-PAGE gels. The gelsolin treatment removed 90% of the components of thin filaments, such as actin, tropomyosin, and troponin, and 25% of the nebulin, which has recently been suggested to be a component of thin filaments (Wang and Wright, 1988). On the other hand, almost 100% of α-actinin and myosin (which are major components of Z lines and thick filaments, respectively) and 100% of α-connectin (titin 1) remained in the fibers (Fig. 1). After the gelsolin treatment, a small amount of gelsolin remained in the fibers: i.e., ~20% (wt/wt) of the amount of actin that remained.

Selective Removal of Thin Filaments: Optical Microscopic Observation

The selective removal of thin filaments was also checked by optical microscopy. We observed the structure of the gelsolin-treated myofibrils and determined the location of actin with phalloidin–rhodamine (Fig. 2, *a–c*). Fluorescence microscopy showed that thin filaments in the I band were removed after the gelsolin treatment under rigor conditions with Ca^{2+} (Ca rigor solution) since only the I band failed to stain with phalloidin–rhodamine after gelsolin treatment (Fig. 2 *b*). The tip part of thin filaments that overlapped with thick filaments appeared to be intact, judging from the facts that sliding movement of this part of the thin filaments was observed in contracting solution as previously reported in a different myofibril system (Yanagida et al., 1985) and that

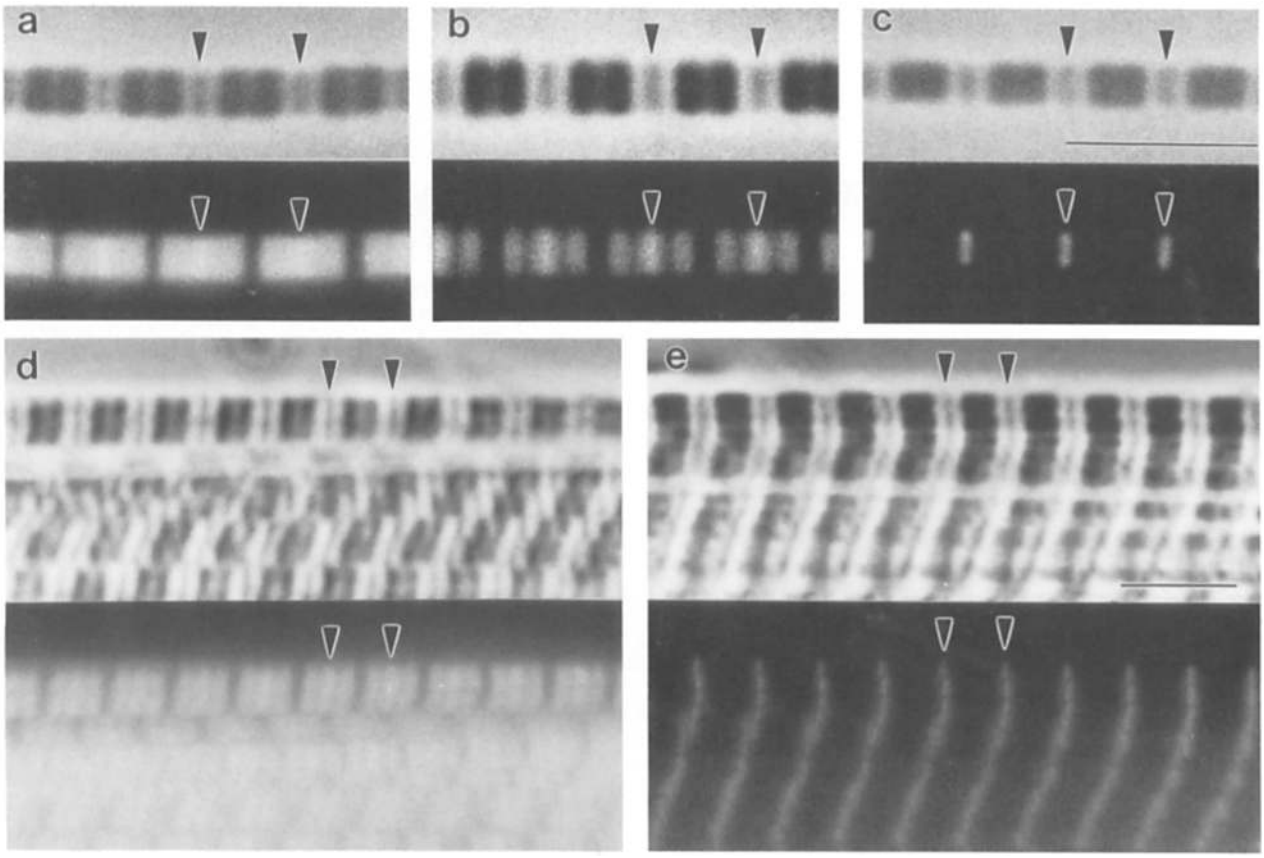

Figure 2. Optical micrographs of gelsolin-treated myofibrils and muscle fibers. (*a*) Untreated myofibril; (*b*) myofibril treated with 0.1 mg/ml of gelsolin in Ca rigor solution for 10 min; (*c*) myofibril treated with 0.1 mg/ml of gelsolin in Ca rigor solution and then with gelsolin in contraction solution for 10 min; (*d*) untreated muscle fibers; (*e*) fibers treated with 0.5 mg/ml of gelsolin in Ca rigor and contracting solution for 1 h each. After fixation with 3% formaldehyde for 30 min at room temperature, actin filaments were visualized by phalloidin–rhodamine staining. The upper and lower parts of each figure are phase-contrast and fluorescence micrographs, respectively. Arrowheads indicate the positions of Z lines. Bars, 5 μm.

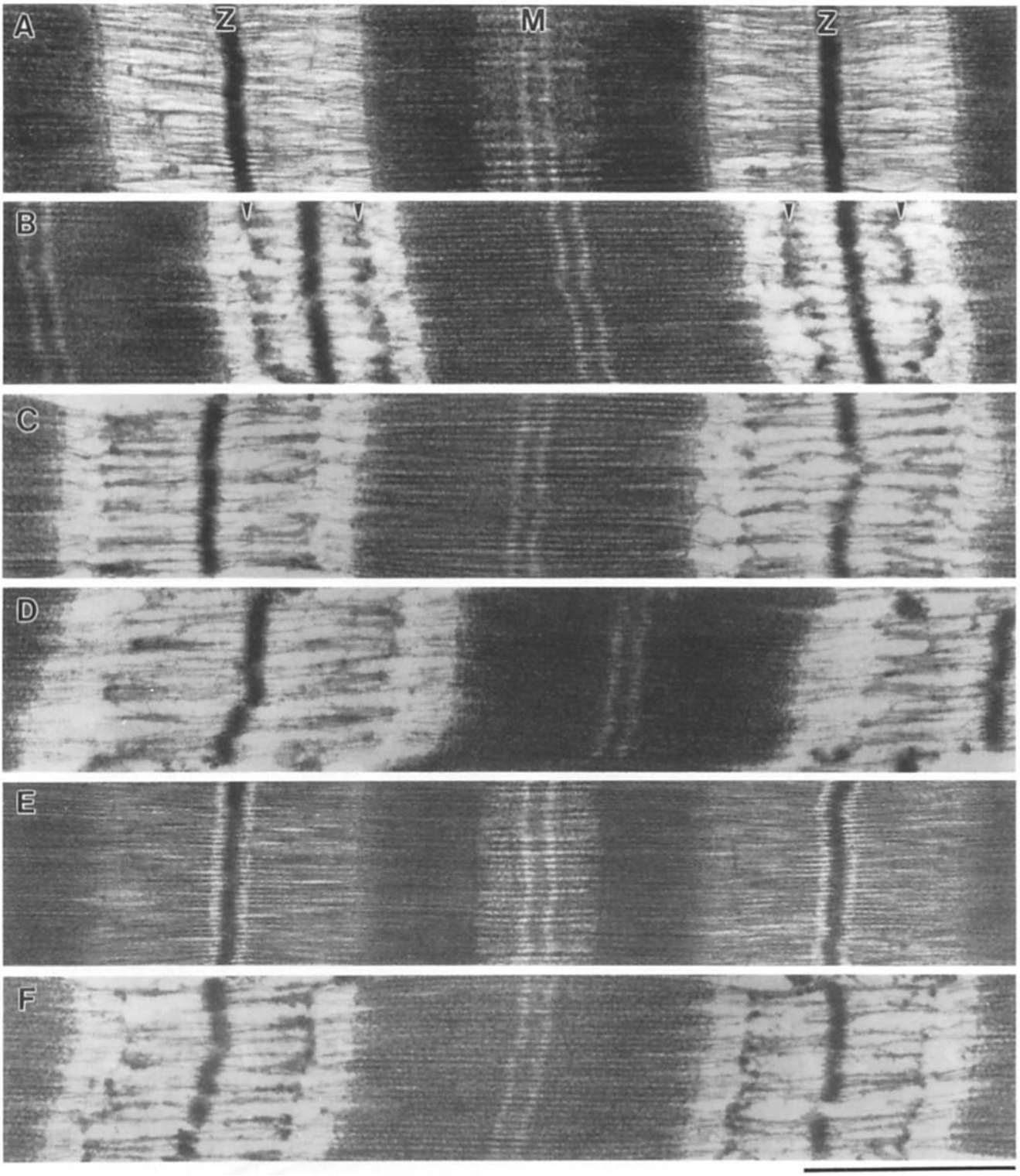

Figure 3. Electron micrographs showing the fine structure of thin filament-free fibers. (*A*) Control fiber; (*B–D*) gelsolin-treated fibers of different sarcomere lengths (2.7, 3, and 3.6 μm for *B–D*, respectively). Muscle fibers of each sarcomere length were treated with 0.5 mg/ml of gelsolin in Ca rigor and contracting solution at 2°C for 1 h each. (*E*) Muscle fiber decorated with S-1; fibers were incubated with 2 mg/ml of S-1 in EGTA rigor solution for 1 h at 2°C. (*F*) gelsolin-treated fibers incubated with S-1 as in *E*. *Z*, Z line; *M*, *M* line; *arrowheads*, N line. Bar, 1 μm.

fragments of thin filaments were observed around the overlap region in electron micrographs (data not shown). Cross-bridge formation under rigor conditions may protect thin filaments from being severed by gelsolin.

The thin filaments remaining in the overlap region were removed by further treatment with gelsolin in contracting solution. However, the Z line was still stained with phalloidin-rhodamine, suggesting that actin that is not dissociated by

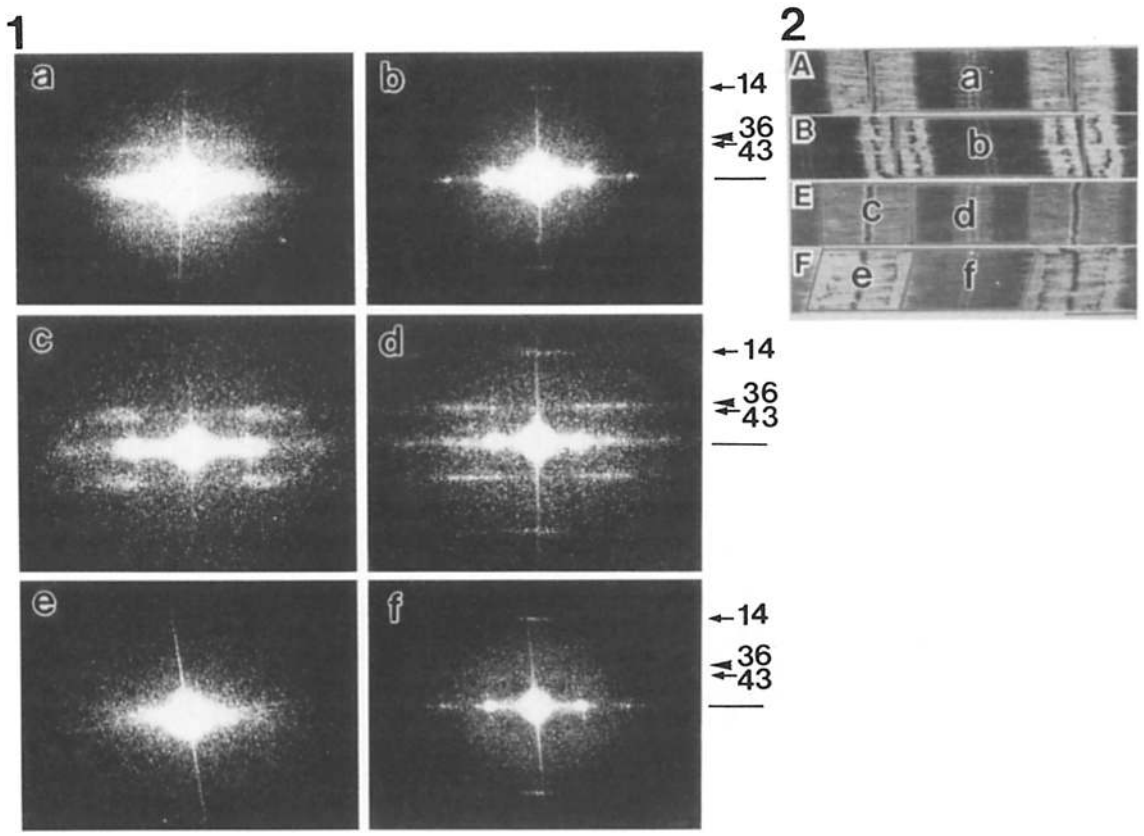

Figure 4. Optical diffraction patterns of electron micrographs shown in Fig. 3. (Part *1*) Optical diffraction patterns taken from the boxed regions in part *2*, in which *a–f* correspond to *a–f* in part *1*, respectively. Images are a single sarcomere of untreated (*a*) and gelsolin-treated (*b*) fibers; I-band region decorated with S-1 without (*c*) and after (*e*) gelsolin treatment; A-band region decorated with S-1 without (*d*) and after (*f*) the gelsolin treatment. The meridional reflections with a spacing of 14.3 nm originating from myosin filaments are indicated by the arrow with 14 (nm); the arrowhead with 36 (nm) and the arrow with 43 (nm) indicate layer lines originating from actin helices and myosin filaments, respectively; the horizontal thin lines indicate the equatorial axis. (Part *2*) A reduction of Fig. 3 showing the areas from which diffraction patterns were taken.

gelsolin treatment is present in the Z line (Fig. 2 *c*). The densities of the Z line and the A band observed by phase-contrast microscopy did not change (except for the disappearance of the H zone at the central part of the A band), suggesting that these structures are not disrupted by gelsolin treatment.

Next, the gelsolin-treated fibers were examined (Fig.2, *d* and *e*).The results were almost the same as those with myofibrils except that fibers required a higher concentration of gelsolin and longer treatment time to remove thin filaments to a similar degree.

Fine Structure of Thin Filament-free Fibers: Electron Microscopic Observation and Analysis of Optical Diffraction Patterns from Electron Micrographs

The fine structure of gelsolin-treated fibers was observed by electron microscopy (Fig. 3). Fig. 3, *B–F*, shows that thin filaments were removed by the gelsolin treatment. This was further confirmed by the optical diffraction patterns of electron micrographic images (Fig. 4). When the diffraction patterns were taken from a sarcomere (a region between Z lines), the layer lines at a spacing of 36 nm (originating from actin helices) disappeared after the gelsolin treatment, whereas the meridional reflection at 14 nm (originating from myosin filaments) did not change (Fig. 4, part *1*, *a* and *b*). Fig. 4, part 1, *c–f*, showed that the layer lines at 36 nm, which were intensified by the addition of S-1, disappeared after the gelsolin treatment not only in A bands (Fig. 4, part *1*, *d* and *f*) but also in I bands (Fig. 4, part *1*, *c* and *e*).

Fine filaments were apparent in the I bands after removal of thin filaments (Fig. 3, *B–D*). We conclude that these fine filaments are different from thin filaments for several reasons: (*a*) the fine filaments were more slender than thin filaments (the diameter of fine filaments observed at the tip end region of thick filaments was estimated to be ~4 nm by assuming that the diameter of thin filaments is 6 nm); (*b*) the fine filaments appear to be extensible unlike thin filaments (Fig. 3, *B–D*); (*c*) the fine filaments appear not to be decorated by S-1 (Fig. 3 *F* and Fig. 4, part *1*, *e*); and (*d*) the fine filaments were much more sensitive to proteolysis than thin filaments (Fig. 10).

The appearance of the fine filaments changes across one of the N lines (for the classification of N lines see Locker and Wild, 1984) in the mid I band; the filaments between the tip end of the thick filaments and this N line look thinnest and those between this N line and the Z line look a little thicker, although still thinner than thin filaments. At sarcomere lengths <~2.5 μm, the N line was merged into the A band, so that the thinner part of the fine filaments disappeared. As sarcomere length increased, both parts of the fine

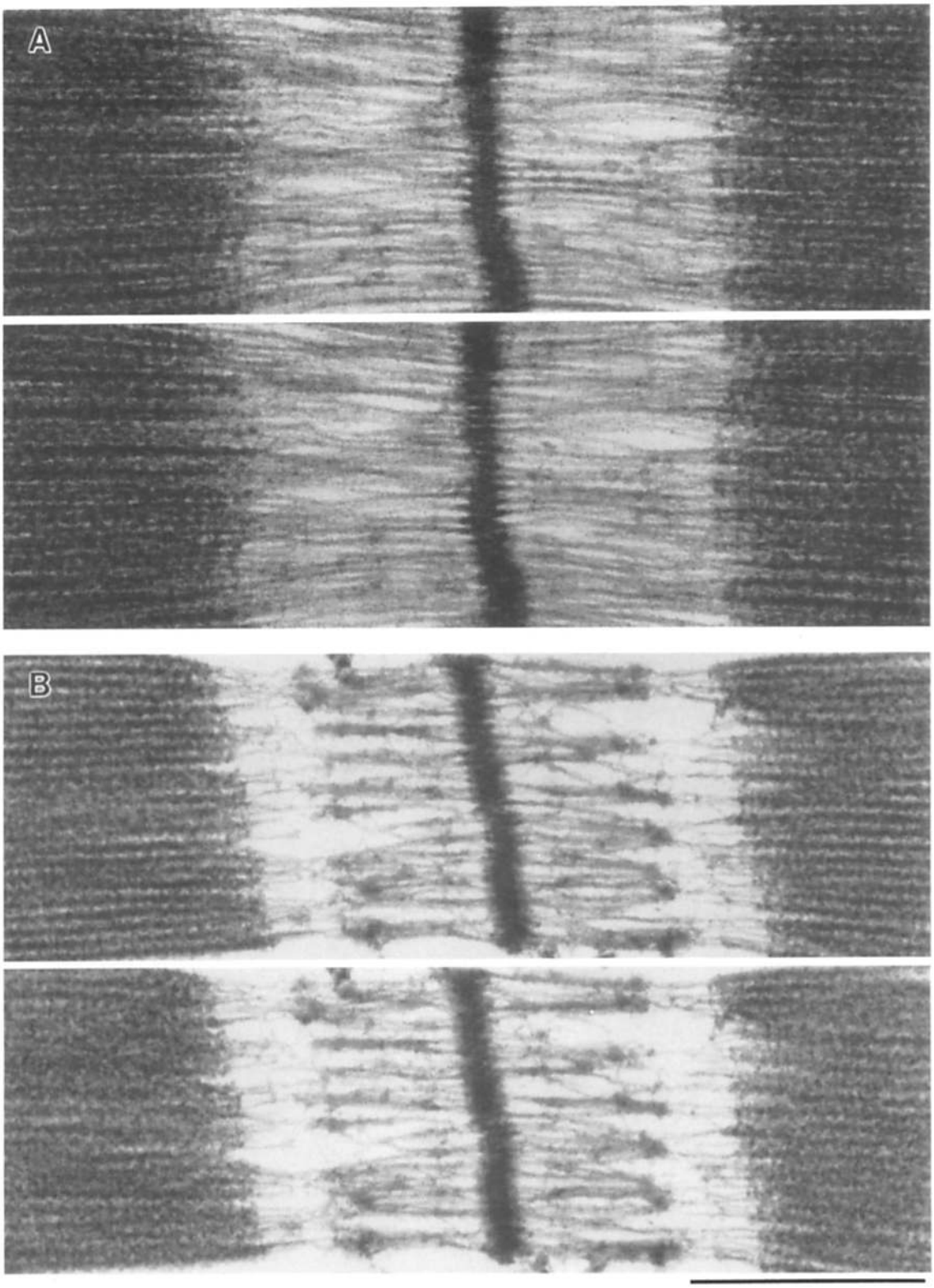

Figure 5. Stereo pairs of a region around the I band of untreated (*A*) and gelsolin-treated (*B*) muscle fibers (for the stereo view, observe from the side). Electron micrographs were taken at a direct magnification of 20,000× and a tilt angle of ±10 degrees. Bar, 0.5 μm.

filaments were stretched, but the structure of the thick filaments and the Z line was unchanged (Fig. 3, *B–D*). This indicates that the fine filaments are most elastic in the serial connections of thick filaments, the fine filaments, and the Z line. Thus, in the present work, we usually call these fine filaments the elastic filaments without regard to the difference in their appearance between the thick filaments and the Z line.

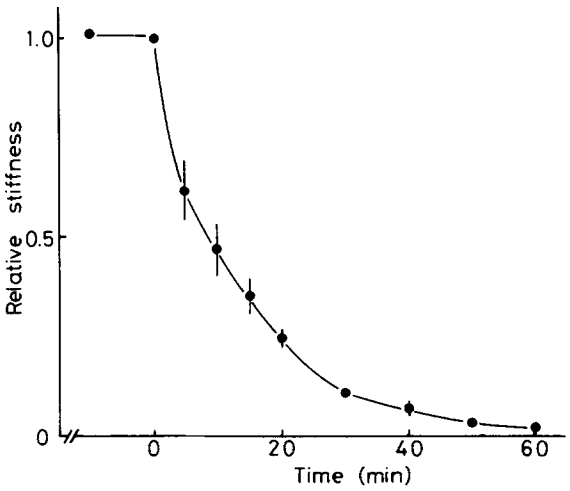

Figure 6. Change of rigor stiffness of fibers after the addition of gelsolin under rigor conditions. We prepared mechanically skinned fibers from glycerinated fibers, washed them for 30 min with 1% Triton X-100 in EGTA rigor solution and for 10 min with Ca rigor solution, and then measured their stiffness at a sarcomere length of 2.5 ± 0.1 μm before and after adding gelsolin (0.5 mg/ml final concentration in Ca rigor solution) at 2°C. The stiffness values were normalized against the value at time zero, when the gelsolin treatment started. The mean values ±SD of four independent experiments are shown.

It should be noted that the lateral distance between adjacent thick filaments became narrower upon lengthening of the sarcomere (Fig. 3, *B–D*). This observation suggests the presence of a network structure composed of the elastic filaments which contributes to the lateral elasticity of the sarcomeres.

We made stereo micrographs of gelsolin-treated fibers to precisely determine the location at which the elastic filaments attach. Fig. 5 shows that the elastic filaments connect thick filaments and the spine-like part of the Z line lattice, the same part to which thin filaments attach. The appearance of elastic filaments changes across the N line as was noted above in reference to Fig. 3. Moreover, the elastic filaments look entangled in the region between the Z line and the N line and are gathered around the N line and then dispersed to run to the tip end of thick filaments. We could not identify the elastic filaments in untreated fibers (Fig. 5 *A*).

Rigor Stiffness and Resting Tension of Thin Filament-free Fibers

After the addition of gelsolin, the rigor stiffness of muscle fibers decreased to nearly zero within an hour (Fig. 6). This was due to the severing of thin filaments in I bands (cf. Fig. 2 *b*). The fibers thus treated with gelsolin did not develop active tension (data not shown). On the other hand, the resting tension did not decrease at any sarcomere length up to 3.3 μm but rather increased slightly after the complete removal of thin filaments (Fig. 7). In other words, slack sarcomere length decreased by 0.1–0.2 μm. This result indicates that thin filaments do not contribute to the resting tension of muscle fibers.

Partial Digestion of Muscle Fibers with Trypsin

Fig. 8 shows that mild treatment with trypsin digests connectin (titin) from the α to the β form (from titin 1 to 2, respectively) and nebulin. Digestion of other proteins was scarcely detected because of the low concentration of trypsin used here. For example, we estimated that the fraction of myosin digested was at most 2%. Therefore, we could use myosin heavy chain as a standard for the calibration of the amount of protein loaded on SDS-PAGE gels. α-connectin (titin 1) was digested at a similar rate irrespective of the presence or absence of thin filaments. On the other hand, nebulin was digested four times faster upon removal of thin filaments.

The correlation between the degradation of proteins and the resting tension of muscle fibers was examined (Fig. 9). The resting tension decreased to 20% of its original value within 10 min after the addition of trypsin. The rate of decrease of resting tension did not depend on the presence or absence of thin filaments. The digestion of α-connectin (titin 1) occurred in parallel with the decrease in the resting tension. On the other hand, the digestion of nebulin occurred 3 times slower or 1.5 times faster than the decrease in the resting tension in the presence or the absence of thin filaments, respectively. The degradation rates of these proteins were not changed by prior mechanical skinning of the fibers.

The structure of fibers in which 80% of the α-connectin (titin 1) was digested was examined by electron microscopy (Fig. 10). A longitudinal section of untreated fibers showed that thin filaments were not severed by trypsin, although some kind of proteolytic products appeared ~60 nm from the center of the Z line (indicated in Fig. 10 *A*). In gelsolin-treated fibers, the structures of the Z lines, thick filaments, and the elastic filaments between the thick filaments and the N line were apparently unaffected (Fig. 10 *B*). The elastic filaments were preferentially digested between the Z line and the N line; the cut filaments seemed to be pulled back to the N line region (Fig. 10 *B*).

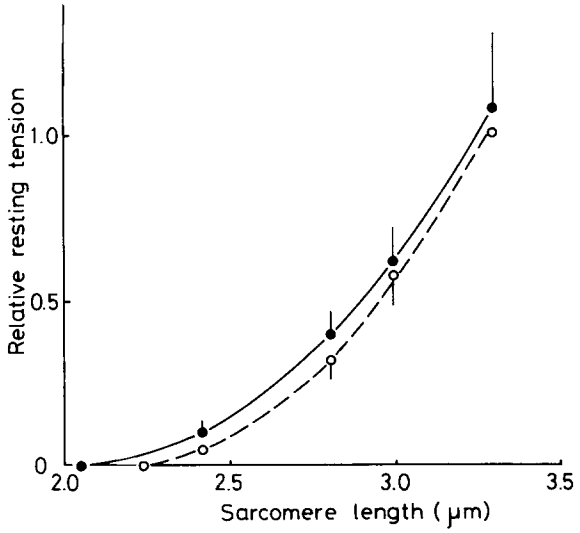

Figure 7. Relation between sarcomere length and the resting tension of mechanically skinned fibers. The resting tension was measured in relaxing solution before (○) and after (●) treating the fiber with 0.5 mg/ml of gelsolin in Ca rigor and contracting solution for 1 h each at 2°C. The tension was measured at stepwise, increasing sarcomere lengths after first setting the fiber at a slack length. The tension was normalized against that of untreated fibers obtained at a sarcomere length of 3.3 μm. The mean values ±SD of four independent experiments are shown.

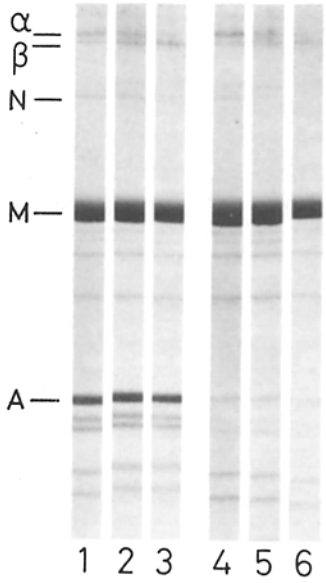

Figure 8. SDS-PAGE (2-16% gradient gel) patterns of muscle fibers digested with trypsin. Muscle fibers with (lanes *4–6*) or without (lanes *1–3*) gelsolin treatment were digested with 1.25 μg/ml of trypsin at 2°C for 0 (lanes *1* and *4*), 5 (lanes *2* and *5*), and 10 (lanes *3* and *6*) min. The digestion was terminated with 5 mM DFP. α, α-connectin (titin 1); β, β-connectin (titin 2); *N*, nebulin; *M*, myosin heavy chain; *A*, actin.

Discussion

Preparation of Thin Filament-free Fibers

We have succeeded in preparing thin filament-free fibers by using plasma gelsolin, one of the actin-severing proteins, as a molecular tool to selectively remove actin filaments. Other substances such as formamide (Wang and Greaser, 1985) and DNase I (Zimmer and Goldstein, 1987) have been used for the removal of thin filaments, but these agents concomitantly disrupted the Z line structure. In the case of gelsolin, not only the Z line structure but also other filamentous structures were maintained provided that care was taken to suppress any proteolytic activity contaminating the gelsolin preparation.

One might think that the gelsolin method is not practical because it seems to consume a lot of gelsolin, but this is not the case. As the severing activity of gelsolin does not significantly decrease after several cycles of freezing and thawing, we can use 0.5 mg/ml of gelsolin solution (200 μg total) repeatedly to obtain >100 thin filament-free fibers. We should mention here that a proteolytic fragment of gelsolin will be more useful for this approach because it does not require Ca^{2+} for its severing activity (Bryan and Hwo, 1986; Cooper et al., 1987), and therefore treatment will be possible under relaxing conditions.

It is well known that thick filament-free fibers (usually called ghost fibers) can be obtained by high salt treatment, but the usefulness of this model system is not yet fully developed mainly because the reconstitution of thick filaments by addition of exogenous myosin has not yet succeeded. On the other hand, it is probable that thin filaments can be reconstituted by adding exogenous actin and regulatory proteins to the thin filament-free fibers because the nucleus for actin polymerization is expected to remain at the Z line (for the fine structure of the Z line see Yamaguchi et al., 1985). In fact, we have succeeded in partial reconstitution of thin filaments and partial recovery of tension with Ca^{2+} sensitivity in myofibrils and glycerinated fibers from which contractility had been completely lost by the removal of thin filaments (Funatsu, T. and S. Ishiwata, manuscript in preparation).

Protein Composition of the Elastic Filaments

Recent immunological studies showed that single connectin (titin) molecules extend from thick filaments to the Z line (Maruyama et al., 1985; Furst et al., 1988); also, a physiological study suggested that connectin (titin) bears the resting tension of muscle fibers (Yoshioka et al., 1986). Here, we have shown quantitatively the correlation among the breakage of the elastic filaments, the degradation of α-connectin (titin 1), and the decrease in the resting tension. This leads to the conclusion that α-connectin (titin 1) is a major component of the elastic filaments. The degradation from the α to the β form (titin 1 to titin 2, respectively) occurs between the Z line and the N line (Fig. 9), which is consistent with the results of Furst et al. (1988).

Recently, the function and the localization of nebulin in muscle fibers were extensively studied by Wang and Wright (1988) by immunoelectron microscopy. They suggested that nebulin does not bear the elasticity of the fibers and associates with thin filaments. Our results are consistent with theirs. When fibers are digested with trypsin, there is no correlation between the degradation of nebulin and the decrease in the resting tension (Fig. 9), suggesting that nebulin does not bear the elasticity of fibers. Also, the degradation rate of nebulin increased about fourfold upon removal of thin filaments, suggesting that the proteolytic sites on nebulin are masked by the associated thin filaments.

We have examined the possibility that upon removal of thin filaments, a part of nebulin is entangled with the elastic filaments. We observed the fine filaments in rabbit cardiac muscle containing connectin (titin) but lacking nebulin (Wang and Wright, 1988). Compared with skeletal muscle, the fine filaments between the N line and the Z line looked thinner

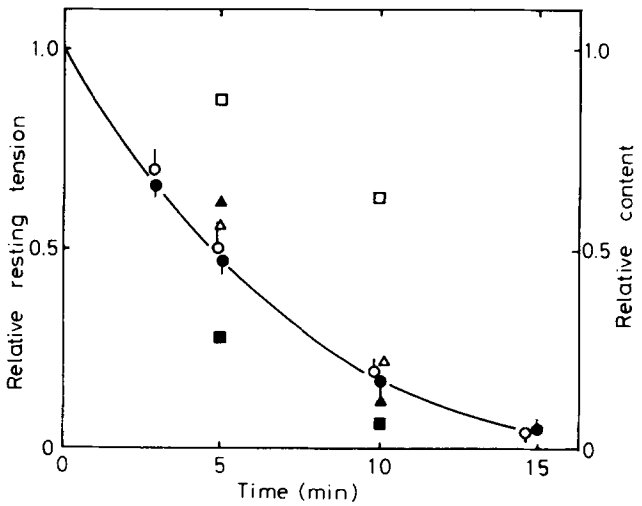

Figure 9. Decrease in resting tension and the relative content of α-connectin (titin 1) and nebulin resulting from tryptic digestion of gelsolin-treated or untreated muscle fibers. The decrease in the resting tension after the addition of 1.25 μg/ml of trypsin was measured at 2°C at a sarcomere length of 3.3 μm (● and ○). The mean values ±SD of three independent experiments are shown. The amounts of α-connectin (titin 1) (▲ and △) and nebulin (■ and □) present, relative to their amounts before tryptic digestion, were determined by densitometric measurement of protein bands in SDS-PAGE gels (Fig. 8). Closed and open symbols represent data from muscle fibers with and without gelsolin treatment, respectively.

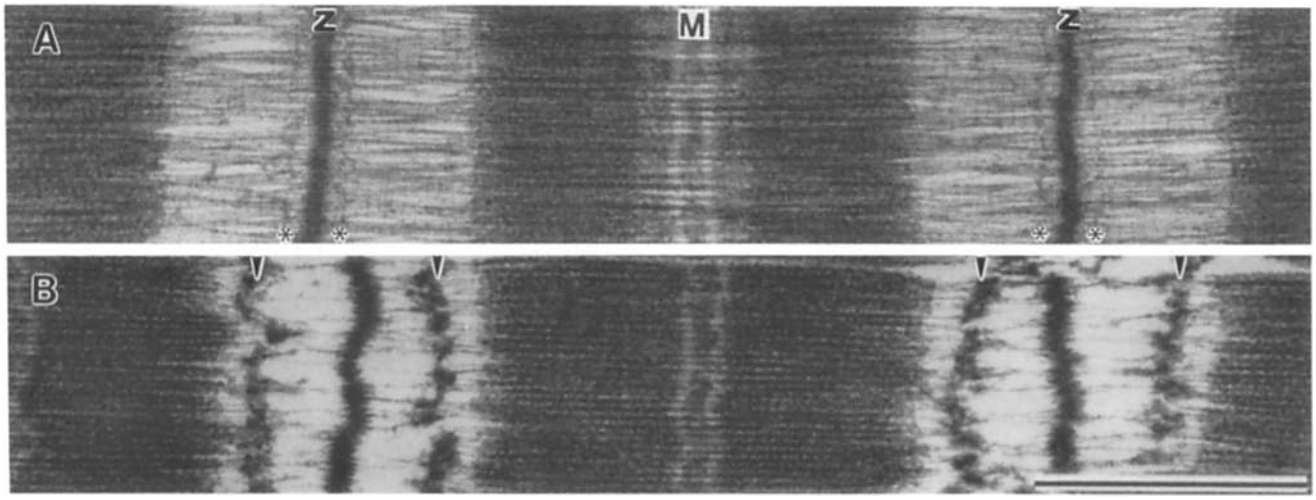

Figure 10. Electron micrographs of muscle fibers digested with trypsin. Untreated (*A*) or gelsolin-treated (*B*) muscle fibers were digested with 1.25 μg/ml of trypsin in relaxing solution (without DFP and leupeptin) at 2°C for 10 min. *Z*, Z line; *M*, M line; *arrowheads*, N line; *asterisks*, transverse striation located ~60 nm from the center of the Z line. Bar, 1 μm.

and the dense materials at the N line were not so prominent, suggesting that nebulin remaining in skeletal muscle associates with the connectin (titin) filaments between the N line and the Z line and that a part of nebulin is piled up at the N line (Funatsu, T., manuscript in preparation). In spite of these differences, the arrangement of the elastic filaments connecting the tip end of thick filaments and the Z line looked similar in both muscle types.

Three-dimensional Arrangement of the Elastic Filaments

The present work has shown that the elastic filaments connect thick filaments and the spine-like parts of the Z line, the same parts of the Z line to which thin filaments attach. On the other hand, it is known that thick filaments form a hexagonal lattice, but the spine-like parts of the Z line are arranged in a square lattice. In addition, there are twice as many spine-like parts as there are thick filaments (cf. Yamaguchi et al., 1985). How do the elastic filaments connect lattice points having different symmetry and different numbers? Three-dimensional rearrangement of the elastic filaments should occur somewhere around the middle of the I band to compensate for these differences.

It should be noted in this regard that the appearance of the elastic filaments changes across the N line (Figs. 3 and 5) and that the proteolytic products of the elastic filaments (Fig. 10 *B*) accumulate around the N line. Also, the elastic filaments cannot be identified in intact muscle fibers (Fig. 5 *A*), which is in contrast to flight muscle (cf. Trombitas et al., 1988).

Without structural information from intact muscle, it is difficult to evaluate to what extent the elastic filaments maintain their intact structure after the removal of thin filaments. However, we propose a model of the three-dimensional arrangement of the elastic filaments by assuming that the essential part of the arrangement is maintained in thin filament–free muscle fibers, taking into account the observations mentioned above and the following. Thick filaments have threefold rotational symmetry (Luther et al., 1981) and each thick filament contains 6–12 connectin (titin) molecules (the number has not been precisely determined; cf. Whiting et al., 1989). Given the fact that each thick filament has six nearest-neighbor thin filaments, we assume that the elastic filament extending from each end of the thick filament is composed of six protofilaments, each of which may correspond to a single connectin (titin) molecule. These protofilaments spread toward the six nearest-neighbor thin filaments; in other words, three protofilaments gather around each thin filament and are bundled at the position of the N line, where the three-dimensional rearrangement of the elastic filaments occurs. Thus, the N line may play a part in the elastic framework of muscle as has been suggested (Locker and Wild, 1984; Wang, 1985). Gathered protofilaments extend to the spine-like part of the Z line running along the thin filament. Although the elastic filaments may run very close to thin filaments, they do not associate tightly with thin filaments, if they associate with them at all, because the resting tension of fibers is not influenced by the removal of thin filaments. To confirm this model, we need to directly observe the protofilaments spreading from the end of thick filaments with higher-resolution electron microscopy.

Physiological Role of Elastic Filaments

The present work has demonstrated that the elastic filaments are responsible for the resting tension of muscle fibers (Figs. 9 and 10) because they are the most compliant components for stretch in the serial connections of thick filaments, the elastic filaments, and the Z line (Fig. 3). The elastic filaments keep the thick filaments at the center of sarcomeres even after the removal of thin filaments (Fig. 3). Besides, the elastic filaments appear to contribute to the lateral compression of the thick filament lattice (Fig. 3; cf. Higuchi and Umazume, 1986; Higuchi, 1987). These results indicate that the principal role of the elastic filaments is to give passive elasticity, both longitudinal and lateral, to sarcomeres and maintain their static and dynamic stability during relaxation and contraction by keeping thick filaments at symmetrical positions. This conclusion is in agreement with that of Horowits and Podolsky (1987) (Horowitz et al., 1986), who examined the positional stability of thick filaments during isometric contraction.

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