Elastic Filaments in Situ in Cardiac Muscle: Deep-Etch Replica Analysis in Combination with Selective Removal of Actin and Myosin Filaments

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Abstract. To clarify the full picture of the connectin (titin) filament network in situ, we selectively removed actin and myosin filaments from cardiac muscle fibers by gelsolin and potassium acetate treatment, respectively, and observed the residual elastic filament network by deep-etch replica electron microscopy. In the A bands, elastic filaments of uniform diameter (6-7 nm) projecting from the M line ran parallel, and extended into the I bands. At the junction line in the I bands, which may correspond to the N2 line in skeletal muscle, individual elastic filaments branched into two or more thinner strands, which repeatedly joined and branched to reach the Z line. Considering that cardiac muscle lacks nebulin, it is very likely that these elastic filaments were composed predominantly of connectin molecules; indeed, anti-connectin monoclonal antibody specifically stained these elastic filaments. Further, striations of ~4 nm, characteristic of isolated connectin molecules, were also observed in the elastic filaments. Taking recent analyses of the structure of isolated connectin molecules into consideration, we concluded that individual connectin molecules stretched between the M and Z lines and that each elastic filament consisted of laterally-associated connectin molecules. Close comparison of these images with the replica images of intact and S1-decorated sarcomeres led us to conclude that, in intact sarcomeres, the elastic filaments were laterally associated with myosin and actin filaments in the A and I bands, respectively. Interestingly, it was shown that the elastic property of connectin filaments was not restricted by their lateral association with actin filaments in intact sarcomeres. Finally, we have proposed a new structural model of the cardiac muscle sarcomere that includes connectin filaments.

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T is widely accepted that the myofibrils in striated muscle consist predominantly of two types of filaments, thick (myosin) and thin (actin). In addition, intensive physiological analyses of muscular contraction have strongly suggested the presence of a third type of filament, tentatively named "elastic filaments," inside myofibrils. These elastic filaments are assumed to be responsible for the so-called "resting tension" of relaxed skeletal muscles (Funatsu et al., 1990), for keeping thick filaments at the center of the sarcomere on relaxation, for maintaining the tension output of the sarcomere despite thick-filament movement on contraction (Horowits and Podolsky, 1988; Horowits et al., 1989), and

for recovering sarcomeres to the initial state after extreme stretching and release (Maruyama et al., 1989). Actually, possible morphological counterparts of these elastic filaments at the electron microscopic level have been described as "gap filaments" found between I and A bands in extremely stretched muscle fibers (Sjöstrand, 1962; Locker and Leet, 1976; Maruyama et al., 1985; Trombitás et al., 1991), as "third filaments," found in myofibrils after the removal of myosin and actin filaments with potassium iodide (dos Remedios and Gilmour, 1978), and as "end filaments," found at the tips of isolated myosin filaments (Trinick, 1981). However, mainly due to the failure to directly visualize these elastic filaments in situ, their actual existence in myofibrils is still a matter of debate.

The connectin (also called titin) molecule has recently been identified and characterized as a possible constituent of the elastic filaments (for reviews see Wang, 1985; Maruyama

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et al., 1986; Trinick, 1991). The mother molecule, α-connectin, a huge molecule of ∼3,000 kD, has recently been isolated from rabbit skeletal muscle (Kimura and Maruyama, 1989). By low-angle rotary shadowing EM, its proteolytic fragment, β-connectin (∼2,000 kD), was reported to look like a very thin rod, ∼0.9 μm long, with a single globular head, consisting of M-line constituents, at one end (Nave et al., 1989). Immunoelectron microscopic studies, using antibodies specific for various portions of the rod-like connectin (or titin) molecules, have suggested that a single connectin molecule extends from the Z line to the M line in a half-sarcomere (Fürst et al., 1988; Itoh et al., 1988a; Whiting et al., 1989; Nave et al., 1989). These biochemical and immunohistochemical data strongly favor the view of the existence of elastic filaments throughout myofibrils in situ.

This being the background situation, we believed that for further evidence it would be necessary, again, to directly visualize the elastic filaments which could consist predominantly of connectin. In a previous study, we developed a technique for the selective removal of actin filaments with plasma gelsolin, and by applying this technique to skeletal muscle we succeeded in clearly visualizing the elastic filaments in the actin filament-free I band by thin-section EM (Funatsu et al., 1990). However, as stated above, this type of elastic filament has not been identified in thin-section electron microscopic images of intact striated muscle sarcomeres. How can we explain this discrepancy? In the present study, we attempted to address this question, using cardiac muscle fibers, which lack nebulin (Locker and Wild, 1986; Hu et al., 1986; Itoh et al., 1988b; Wang and Wright, 1988; Kruger et al., 1991). To avoid the possible induction of artifacts by chemical fixation, which could occur in the case of delicate structures such as elastic filaments, we used a rapid freezing method with liquid helium. To obtain three-dimensional information on elastic filaments at high resolution, we used a deep-etch replica technique. Further, to clarify whether elastic filaments were present at the A bands, we used a technique for the selective removal of myosin filaments. Combining these methods, we carefully compared the replica images of intact and S1-decorated sarcomeres with those of actin filament-free I bands and/or thick filament-free A bands. Finally, these observations allowed us to propose a model for the arrangement of connectin filaments in myofibrils in situ.

Materials and Methods

Muscle Fibers

Rabbit papillary muscle (2 × 5 mm), carefully dissected out of the heart, was tied to a glass rod and incubated in 50% (vol/vol) glycerol containing 0.5 mM NaHCO₃, 5 mM EGTA, and 1 mM leupeptin at 0°C overnight. Fibers were then stored in the fresh solution at −20°C for >1 wk.

Proteins

Plasma gelsolin was purified as described previously (Funatsu et al., 1990). Myosin subfragment 1 (S1)[1] was prepared from rabbit leg and back white muscle according to the method of Weeds and Taylor (1975).

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1. *Abbreviation used in this paper*: S1, myosin subfragment 1.

Antibodies

SM1, a monoclonal antibody against connectin, was kindly donated by Dr. Teruo Shimizu (Teikyo University). It was obtained from a BALB/c mouse immunized with a crude human myosin preparation (Shimizu et al., 1988). Anti-nebulin antiserum was prepared as described previously (Maruyama et al., 1989). Secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG; Cappel, West Chester, PA) were purchased.

Selective Removal of Actin and Myosin Filaments

A bundle of glycerinated muscle fibers (∼150 μm diam and ∼3 mm long) was tied at both ends to a platinum wire (0.3 mm φ). Actin filaments were removed with gelsolin, as described previously (Funatsu et al., 1990). Briefly, muscle fibers were chemically skinned with 1% Triton X-100 in EGTA rigor solution (0.17 M KCl, 1 mM MgCl₂, 10 mM 3-[N-morpholino] propanesulfonic acid [MOPS; pH 7.0], 1 mM EGTA, 2 mM diisopropyl fluorophosphate [DFP], and 2 mM leupeptin) for 30 min. Samples were treated with 0.5 mg/ml of gelsolin in Ca rigor solution (0.1 mM CaCl₂ was substituted for 1 mM EGTA in EGTA rigor solution) and then in contracting solution (0.15 M KCl, 5 mM MgCl₂, 4 mM ATP, 10 mM MOPS, pH 7.0, 0.1 mM CaCl₂, 2 mM DFP, and 2 mM leupeptin) for 1 h each. They were finally washed for 1 h with relaxing solution (1 mM EGTA was substituted for 0.1 mM CaCl₂ in contracting solution).

To remove myosin filaments, the actin filament-free muscle fibers were further treated with a high salt solution containing 1 M potassium acetate, 5 mM MgSO₄, 4 mM ATP, 4 mM EGTA, 20 mM MOPS, pH 7.0, 2 mM DFP, and 2 mM leupeptin for 30 min, and then washed for 30 min with relaxing solution. To leave the elastic filaments intact, 2 mM DFP and 2 mM leupeptin must be added to all the solutions used and all the procedures must be carried out at 2°C.

S1-Decoration

Cardiac muscle was chemically skinned with 1% Triton X-100 in EGTA rigor solution for 30 min. The samples were incubated with 2 mg/ml of S1 in EGTA rigor solution, and then washed for 1 h with EGTA rigor solution.

SDS–Gel Electrophoresis and Immunoblotting

Muscle fibers were dissolved in 25 μl of SDS solution (7.5% SDS, 10% glycerol, 1 mM DTT, and 10 mM Tris-HCl, pH 6.8) and heated for 3 min at 90°C. SDS gel electrophoresis was carried out according to the method of Laemmli (1970), using a 3–13% polyacrylamide gradient gel; protein bands were visualized with Coomassie brilliant blue R. For immunoblotting, freshly excised rabbit papillary muscle fibers were gently homogenized in 2 vols of solution containing 10% SDS, 40 mM DTT, 10 mM EDTA, and 100 mM Tris-HCl, pH 8.0. The sample was boiled for 3 min and clarified by centrifugation at 15,000 g for 20 min. The supernatant was electrophoresed, using a 2–8% gradient of polyacrylamide gels, according to the method of Laemmli (1970). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (Towbin et al., 1979) and then incubated with anti-connectin mAb (SM1) or anti-nebulin antiserum. Bound antibodies were detected after treatment with HRP-conjugated anti-mouse or anti-rabbit IgG (Cappel, West Chester, PA).

Rapid Freezing

A bundle of muscle fibers mounted on a holder was rapidly frozen by being placed against a pure copper block that was cooled to 4°K with liquid helium by using a freezing apparatus, RF-23 (Eiko Engineering, Ibaraki, Japan; Tsukita and Yano, 1985; Usukura, J., E. Yamada, H. Akahori, and H. Takahashi. 1981. *J. Electron Microsc.* 30:214 [Abstr.]). Specimens thus frozen were stored in liquid nitrogen until use.

Freeze Substitution

The frozen samples were freeze substituted for 2 d with acetone containing 4% OsO₄ at −80°C (Tsukita and Yano, 1985; Tsukita et al., 1988). They were transferred to a freezer maintained at −20°C, kept there for 1 h, and were then stored at 4°C for 2 h. After being washed with acetone, the samples were stained en bloc for 2 h at room temperature in ethanol containing 5% uranyl acetate. They were washed in ethanol, then in propylene oxide, and embedded in POLY/BED 812 (Polyscience, Warrington, PA). Thin sections were stained sequentially with 4% uranyl acetate and 0.4% lead citrate for 3 min each at 25°C.

Deep-Etch, Rotary-Shadow Replica EM

The deep-etch replica method used in this study has been previously described in detail (Tsukita et al., 1986, 1988). Rapidly frozen samples were mounted on an ultramicrotome (ULTRACUT, Reichert-Jung Optische Werke AG, Wien, Austria) equipped with a low temperature sectioning system (FC4E), and they were then fractured with a glass knife at $-150°C$. The samples were placed in a holder, and covered with a cooled cap; they were then transferred to a freeze-etch device (BAF 400D; Balzers Union Aktiengesellschaft, Liechtenstein) in a nitrogen atmosphere. When a vacuum of 2×10^{-6} torr was achieved at $-150°C$, the sample was warmed to $-100°C$ and the cooled cap covering the sample was removed. The sample was etched for 7 min at $-100°C$, followed by rotary shadowing with platinum-carbon and carbon at angles of $25°$ and $65°$, respectively. The sample was then removed from the freeze-etch device, immersed in methanol at $-20°C$ overnight, and then dissolved in household bleach. Replicas floating off the sample were washed three times with distilled water and collected on formvar-filmed grids. Stereo pair electron micrographs were taken on a JEM-1200EX electron microscope (JEOL, Tokyo, Japan), operated at 100 kV, by tilting the specimen stage at $\pm10°$. Electron microscopic negatives (Fuji Electron Microscopic Film FG; Fuji Photo Film, Japan) were reversed onto Kodak fine grain positive films (Eastman Kodak Co., Rochester, NY), and were then printed as negative images. A 1.6-μm-wide A band (Huxley, 1963) was used as an internal standard of length.

Immunoelectron Microscopy

Muscle fibers were fixed in a fixative containing 3.8% formaldehyde, 80 mM KCl, 5 mM MgSO$_4$, 4 mM ATP, 4 mM EGTA, 2 mM DFP, and 10 mM piperazine-N,N'-bis(2-ethane) sulfonic Acid (Pipes; pH 7.0) for 6 h. All the procedures were carried out at 2°C unless otherwise stated. After the samples were washed in PBS (0.1 M NaCl, 1 mM DFP, 1 mM leupeptin, and 10 mM sodium phosphate, pH 7.0) for 15 min, reactive aldehyde groups were blocked by incubating the samples sequentially in PBS containing 20 mM glycine for 30 min and in PBS containing 1% egg albumin for 30 min. The samples were washed with PBS for 15 min and then exposed to anti-connectin mAb (SM1) in PBS for 24 h. As a control experiment, some fibers were incubated in PBS without primary antibody. After being washed with PBS for 12 h, the samples were reacted with anti-mouse IgG in PBS for 12 h. They were then washed with PBS for 12 h and with 0.1 M sodium cacodylate buffer, pH 7.2, for 10 min, and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 h at 25°C and then overnight at 4°C. Samples were postfixed with 1% OsO$_4$ in the same buffer for 2 h at 2°C, washed with distilled water, stained with 0.5% uranyl acetate for 2 h at 25°C, dehydrated, and stained sequentially with 4% uranyl acetate and 0.4% lead citrate for 3 min each at 25°C.

Results

Elastic Filaments in Actin Filament-free I Bands of Cardiac Muscle

The selective removal of actin filaments from skeletal muscle was applied to rabbit cardiac muscle. As shown in Fig. 1, cardiac muscle fibers lack nebulin. When cardiac muscle cells were treated with plasma gelsolin, actin and tropomyosin were selectively and largely removed, indicating that this technique for the selective removal of actin filaments with gelsolin works also well in cardiac muscle fibers (Fig. 1 A).

First, the actin filament-free cardiac muscle fibers were rapidly frozen with liquid helium, freeze substituted, and observed by thin-section EM (Fig. 2). Actin filaments were completely removed, leaving myosin filaments, M lines, and Z lines intact, and residual elastic filaments were clearly visualized in the actin filament-free I bands. The actin filament-free I band appeared to be divided into two zones by the residual elastic filament network: A band-side and Z line-side zones (Fig. 2, C and D). The A band-side zone was characterized by straightly stretched elastic filaments with a uniform diameter, while the elastic filaments in the Z line-side
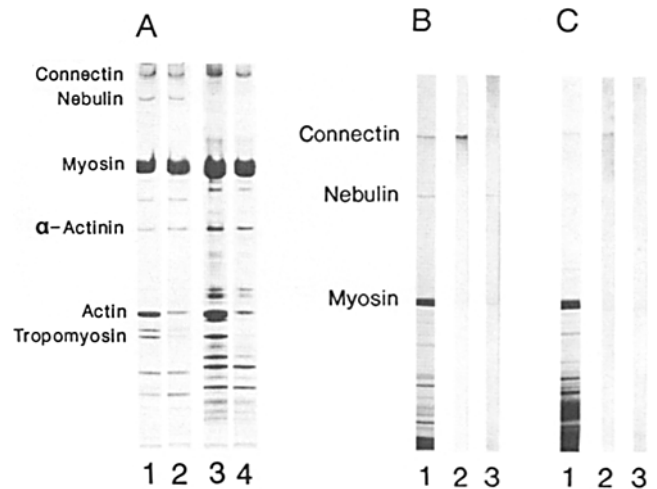

Figure 1. SDS-PAGE patterns and immunoblot test of skeletal and cardiac muscle fibers. (*A*), 3–13% polyacrylamide gradient gels stained with Coomassie brilliant blue. (Lane *1*) Intact rabbit skeletal muscle; (lane *2*) skeletal muscle treated with 0.5 mg/ml of gelsolin in Ca rigor solution and then in contracting solution at 2°C for 1 h each; (lane *3*) intact rabbit cardiac muscle; and (lane *4*) cardiac muscle treated with gelsolin. (*B* and *C*) Immunoblot tests of the reaction of anti-connectin and anti-nebulin antibodies with rabbit psoas (*B*) and papillary muscle (*C*) proteins. (Lane *1*) Coomassie brilliant blue–stained gel pattern of whole muscle proteins (2–8% polyacrylamide gradient gels); (lane *2*) treated with anti-connectin monoclonal antibody (SM1) and (lane *3*) treated with anti-nebulin polyclonal antibodies.

zone appeared to be variable in their diameter and direction. The boundary line of these two zones is tentatively called "a junction line" here. This junction line was not so clear in intact I band, but was sometimes detectable (Fig. 2 *B*).

Next, to obtain three-dimensional images of these elastic filaments at high resolution, the I bands of actin filament-free cardiac muscle fibers were analyzed by the deep-etch replica method (Figs. 3 and 4). The junction line and A band–side/Z line–side zones were also clearly observed in the actin filament-free I bands in the deep-etch replica images. Close stereoscopic inspection allowed us to pursue individual elastic filaments. In the A band–side zone, a single elastic filament of uniform diameter was shown to project from the tip of each thick filament and to extend in a straight line toward the junction line. The diameter of the elastic filament was $\sim$6–7 nm, assuming that the width of the backbone of the thick filament was 17.6 nm (Knight and Trinick, 1984) and that the thickness of the platinum replica was $\sim$1 nm. At the level of the junction line, two or three adjoining elastic filaments were laterally associated with each other and each filament appeared to branch off into two (or more) thinner strands. In the Z line–side zone, these thinner strands repeatedly joined and branched toward the Z line, finally being integrated into the meshwork of the Z line. Occasionally, a striation of $\sim$4 nm was observed on the surface of the elastic filaments. When the actin filament-free cardiac muscle fibers were stretched, the Z line–side zone, rather than the A band–side zone, appeared to be preferentially extended (Figs. 3 and 4).
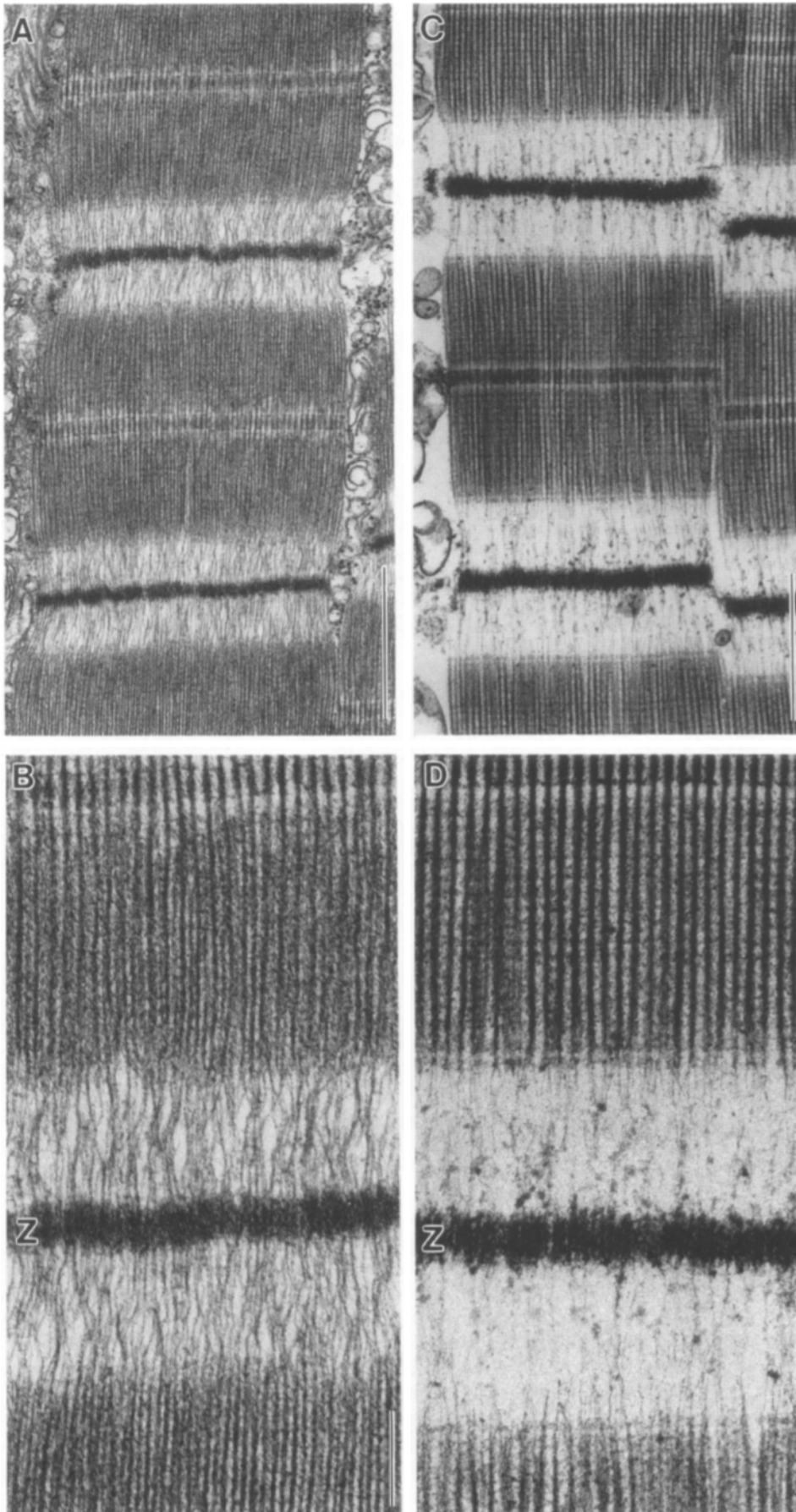
Figure 2. Thin sections of freeze-substituted cardiac muscle cells. (*A* and *B*) Intact muscle; and (*C* and *D*) muscle treated with 0.5 mg/ml of gelsolin in Ca rigor solution and then in contracting solution at 2°C for 1 h each. Z, Z line. (*A* and *C*) Lower magnifications; (*B* and *D*) higher magnifications. Bars: (*A* and *C*) 1 μm; (*B* and *D*) 0.2 μm.
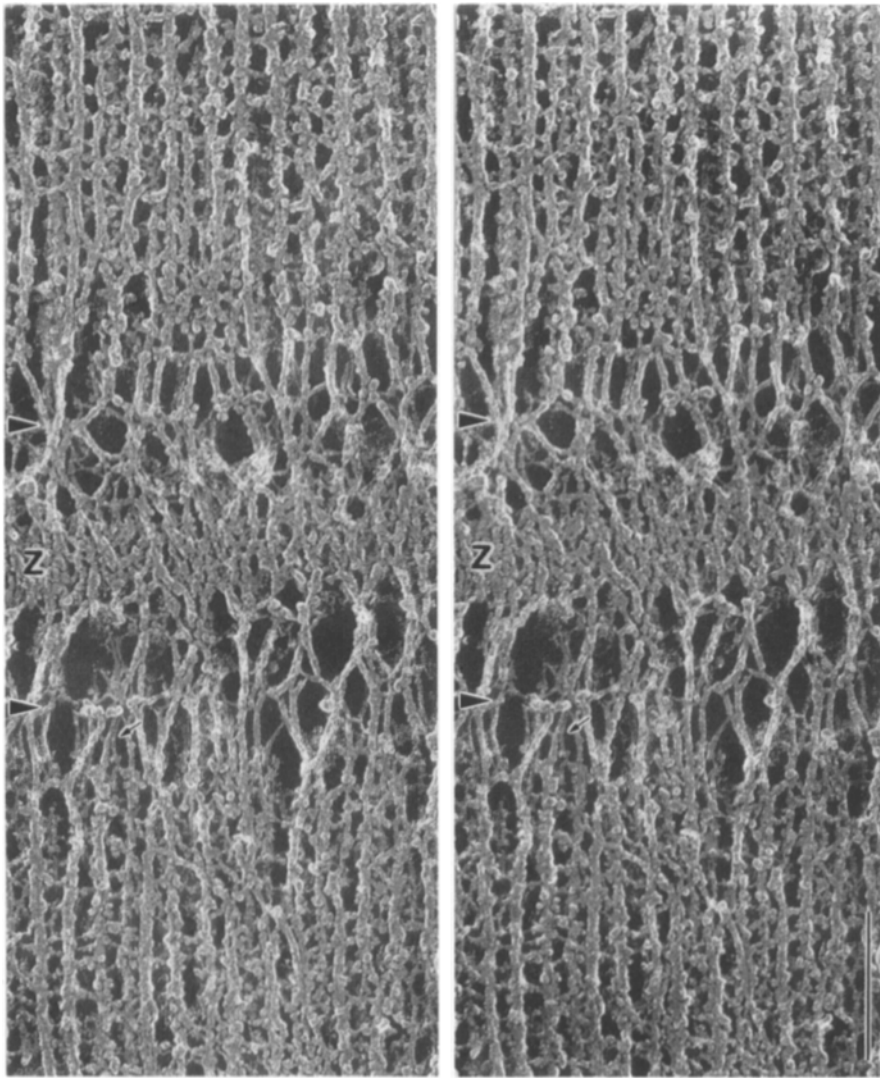
Figure 3. Stereo pair micrographs of freeze-etch replica of gelsolin-treated cardiac muscle at a sarcomere length of 2.1 μm. Arrowheads indicate the junction line of connectin filaments. An arrow indicates ∼4-nm connectin filament striation. Z, Z line. Bar, 0.2 μm.

Elastic Filaments in Intact and S1-decorated I Bands of Cardiac Muscle

We attempted to identify the elastic filaments in intact I bands. For this purpose, we first carefully observed an intact I band by deep-etch replica EM (Fig. 5). We were then led to the following conclusions: (*a*) judging from the striations of ∼5.5 nm on their surface (Heuser and Kirschner, 1980; Heuser and Cooke, 1983) and their diameter of ∼10 nm, almost all of the longitudinally oriented filaments in intact I bands can be identified as so-called thin filaments consisting predominantly of actin; (*b*) the tips of the thick filaments could not be identified, probably due to the elastic filaments projecting from each tip. When individual thick filaments were traced toward the Z line, they appeared to continue and merge into thin filaments; and (*c*) very thin strands cross-linking neighboring thin filaments, which may correspond to the "lateral struts" described previously (Trombitás et al., 1988), were clearly observed, frequently around the junction line and the Z line.

Next, the S1-decorated I bands in the deep-etch replica images were analyzed (Fig. 6). All the longitudinally oriented filaments in the I bands were fully decorated with S1 and showed a rope-like double helix appearance (35-nm half

pitch) with striations of ∼5.5 nm on their surface. This indicates that all the longitudinally oriented filaments visualized in the intact I bands were so-called thin filaments consisting predominantly of actin. Lateral struts were also clearly observed in S1-decorated I bands (*small arrows* in Fig. 6). Close inspection revealed that the thick filaments appeared to merge into rope-like S1-decorated thin filaments, probably through elastic filaments projecting from their tips (*large arrow* in Fig. 6). On the other hand, when actin filament-free I bands were treated with S1 under the same conditions, the elastic fibers showed no change in morphology (data not shown). Taking these results together, we were led to the conclusion that, in intact I bands, the so-called thin filament was a complex of the elastic filament and the actin filament with tropomyosin and troponin, and that, in S1-treated I bands, the elastic filaments were associated with S1, together with actin filaments. This interpretation was conclusively confirmed by the images shown in Fig. 7. In this experiment, the S1-decorated cardiac muscle fibers were mechanically stretched under the condition of rigor. As a result, in the I band, rope-like S1-actin filament complexes were broken off, leaving thin strands stretching between breaking ends. Judging from their diameter and the ∼4-nm striations on their surface, these thin strands seemed to be identical to the elas-

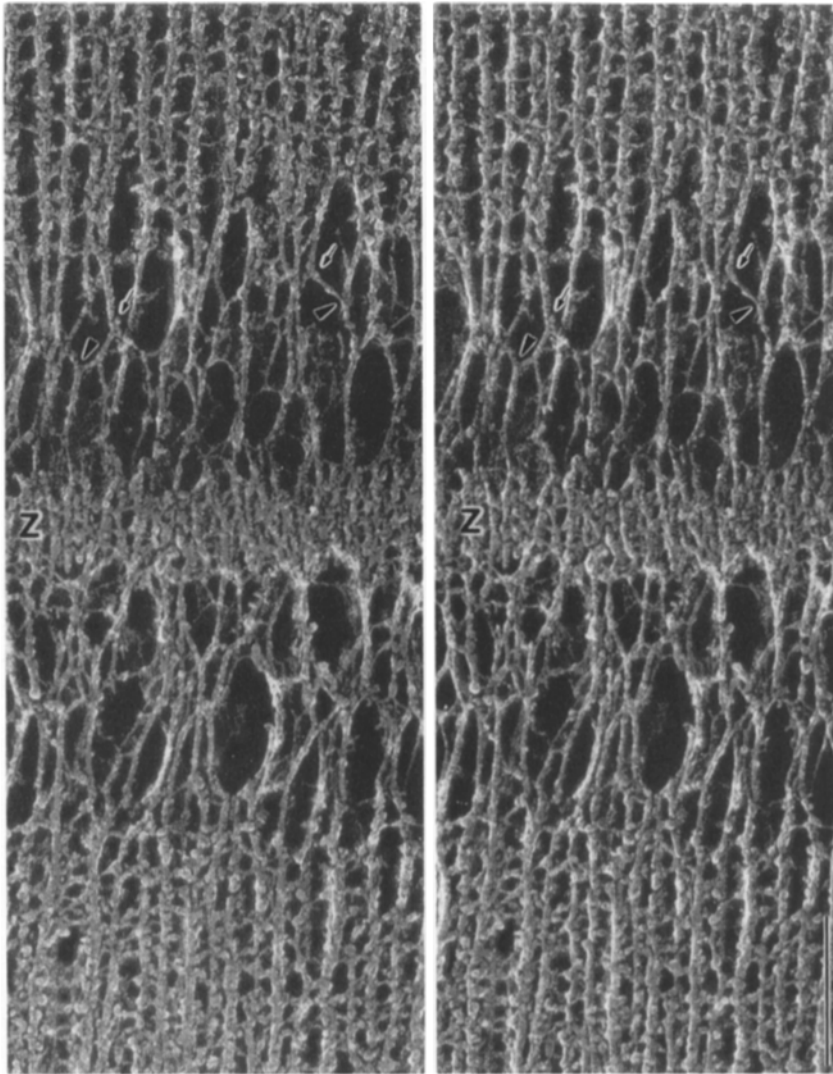*Figure 4.* Stereo pair micrographs of freeze-etch replica of gelsolin-treated cardiac muscle at a sarcomere length of 2.5 μm. Z, Z line. Bar, 0.2 μm.

tic filaments visualized in the actin filament-free I bands. This image, then, not only favored the above interpretation, but also suggested the elastic properties of the elastic filaments laterally associated with actin filaments (so called thin filaments).

Elastic Properties of Elastic Filaments

It is reasonable to regard the elastic filaments visualized in this study as connectin (titin) filaments. Therefore, we attempted to mark the elastic filaments with anti-connectin mAb (SM1). Treatment of intact cardiac muscle with SM1 resulted in the formation of a single electron-dense stripe in each I band (Fig. 8 *A*). These stripes were also formed in gelsolin-treated cardiac muscle fibers at positions similar to those in the I band, indicating that the SM1 epitope was located on the elastic filaments around the level of the junction line and that dislocation of this epitope in the longitudinal direction was not induced by gelsolin treatment (Fig. 8 *B*).

To study the elastic properties of elastic filaments, we analyzed the position of the SM1 epitope under various sarcomere lengths, both in intact and in actin filament-free I bands (Fig. 9). The distances between the Z line and the epitope (ZE distance) and between the edge of the A band and the epitope (AE distance) were measured. When these distances were plotted as a function of the distance between the Z line and the edge of the A band (ZA distance) (see Horowits et al., 1989), both ZE and AE distances increased linearly with the ZA distance (Fig. 9 *B*). As shown in Table I, comparison of the slope and intercept in intact and actin filament-free I bands clearly indicated that actin filaments with tropomyosin and troponin had no influence on the elastic properties of elastic filaments. In both intact and actin filament-free I bands, the ZE part of the elastic filaments appeared to be 2.1–2.4 times as extensible as the AE part, which was consistent with the deep-etch replica observation (Figs. 3 and 4).

This type of elastic property should be accompanied by some structural changes in elastic filaments. As shown in Figs. 3 and 4, elastic filaments showed ∼4-nm striations on their surface. Therefore, we measured the spacing of this striation in actin filament-free I bands under various sarcomere lengths (Fig 10). The striation spacing varied from ∼4 to ∼5 nm in proportion to the ZA distance. The reason is not clear why the striation spacing increases by ∼20% while the ZA distance increases 100%, but it may be partly due to the fact that elastic filaments are slack in short sarcomeres. There was no significant difference in this change of spacing between the A band–side and Z line–side zones.
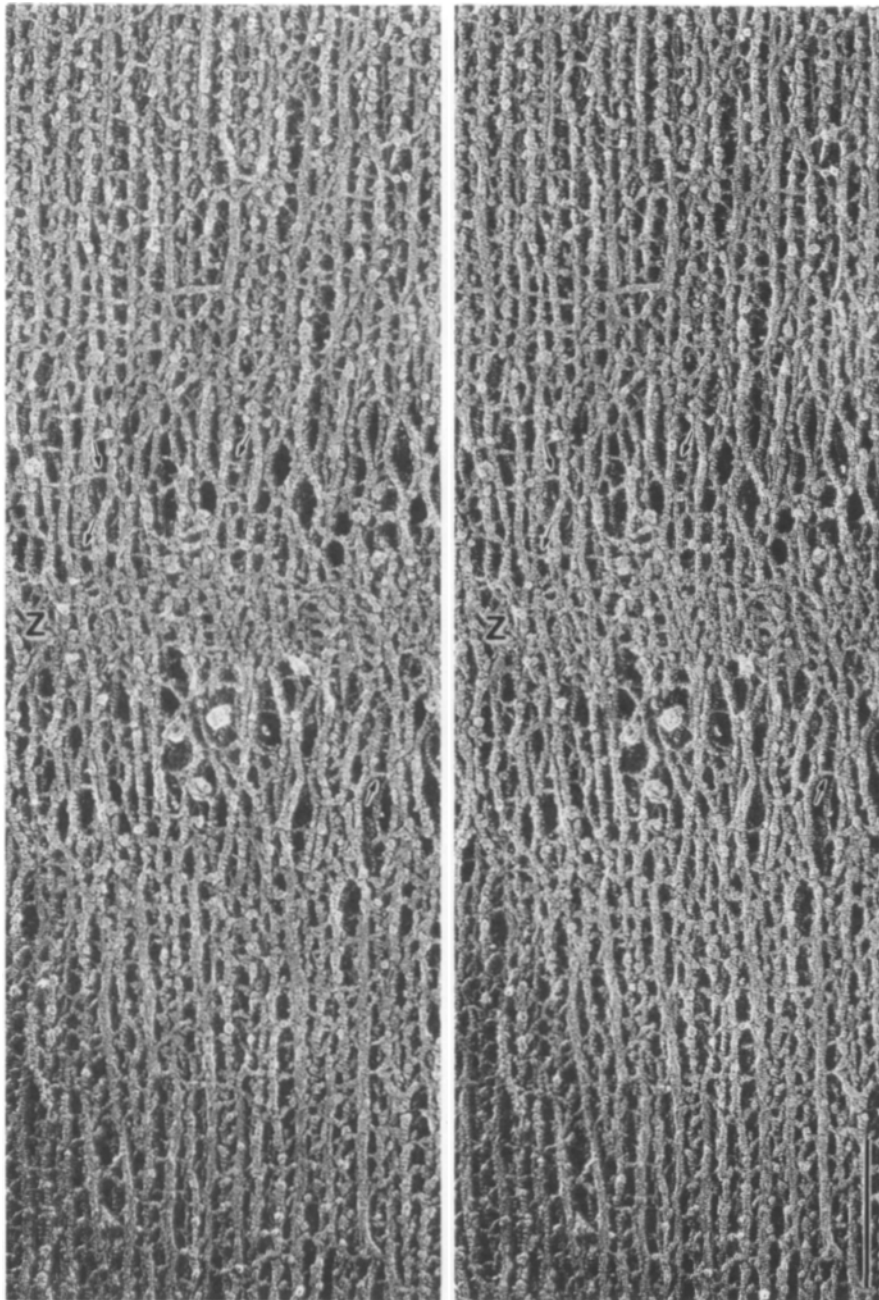
Figure 5. Stereo pair micrographs of freeze-etch replica of cardiac muscle sarcomere without gelsolin treatment at a sarcomere length of 2.2 μm. Arrows indicate lateral bridges connecting thin filaments. Z, Z line. Bar, 0.2 μm.

Elastic Filaments in Myosin and Actin Filament-free Cardiac Muscle

As shown in deep-etch replica images of actin filament-free I bands (Figs. 3 and 4), it is clear that single elastic filaments projected directly from the tips of individual thick filaments.

In the A band of these gelsolin-treated cardiac muscle fibers, there were no longitudinally oriented filaments other than the so-called thick filaments. However, it has already been shown that the elastic filament binds onto the thick filament up to the edge of the M line (Fürst et al., 1988). To visualize

Table I. Linear Regression Parameters of the Position of the SM1 Epitope as a Function of the Distance from the Z line to the A–I Junction

	Control		Gelsolin-treated	
	Slope	Intercept	Slope	Intercept
		nm		*nm*
Epitope to Z line	0.67 ± 0.02	−34 ± 1	0.70 ± 0.02	−32 ± 1
Epitope to A band	0.32 ± 0.02	35 ± 1	0.30 ± 0.02	32 ± 1

Dependence of the position of the SM1 epitope on the distance from the Z line to the A–I junction (Fig. 9) was analyzed by linear regression. Data are the fitted values ± expected standard errors ($n = 55$).
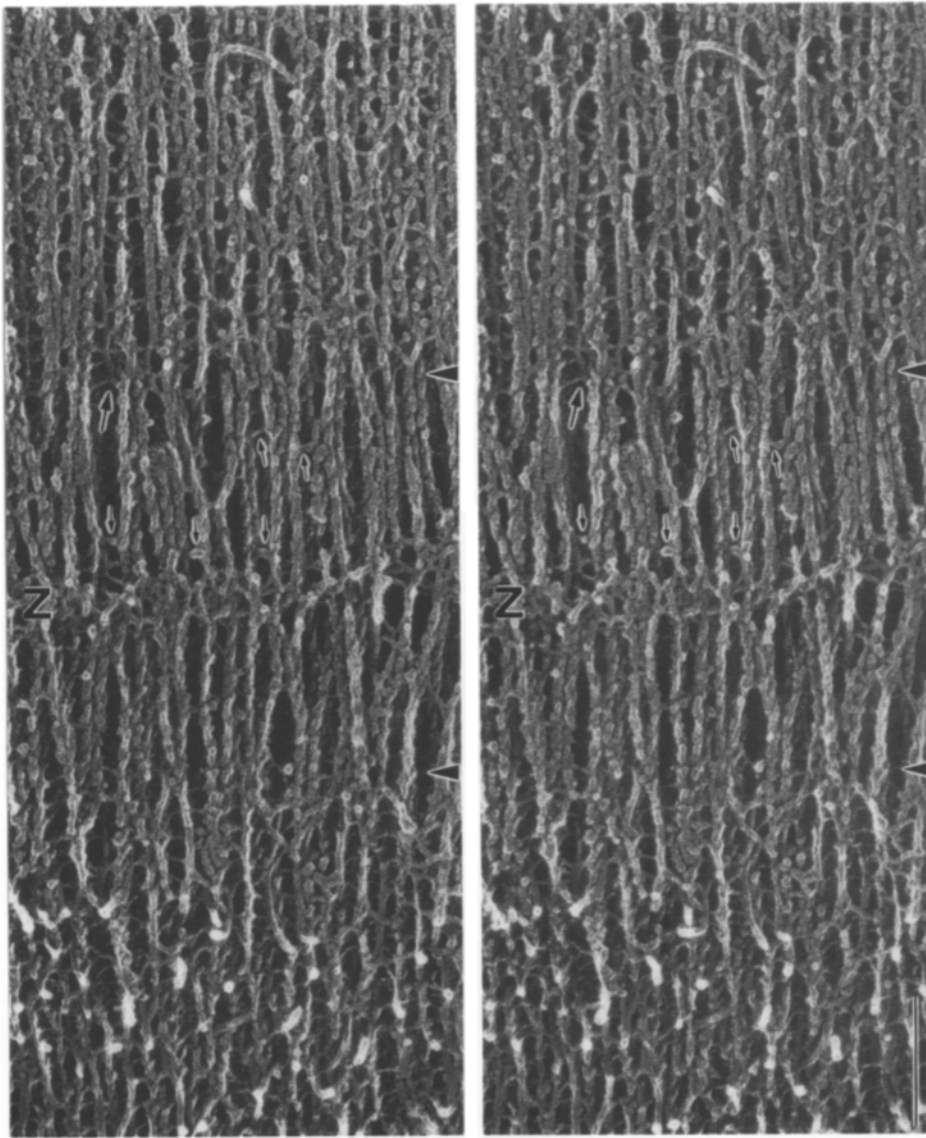
Figure 6. Stereo pair micrographs of freeze-etch replica of cardiac muscle sarcomere decorated with S1. Arrowheads indicate positions of ends of thick filaments; small arrows indicate lateral bridges connecting thin filaments; and large arrow indicates elastic filament projecting from the end of a thick filament. Z, Z line. Bar, 0.2 μm.

elastic filaments in the A band, we attempted to selectively remove myosin filaments from the actin filament-free cardiac muscle fibers. When a high concentration of potassium chloride was used to completely remove myosin filaments, all the elastic filaments appeared to be collapsed onto the Z line (Higuchi `et al., 1992). Although intermediate potassium chloride concentrations can control A band length from 1.6 to 0.3 μm (Ishiwata et al., 1985; Higuchi and Ishiwata, 1985; Higuchi et al., 1992), this extraction leaves some myosin molecules on elastic filaments to make the ultrastructural observation difficult. Instead of potassium chloride, we used a high concentration of potassium acetate, since we found that this induced the depolymerization of myosin filaments from both tips, leaving the central part of the myosin filament (0.4 μm long) intact. When gelsolin-treated cardiac muscle fibers were further treated with a high concentration of potassium acetate, SDS-PAGE analyses revealed that myosin was largely removed, leaving the connectin unextracted (Fig. 11).

The potassium acetate-treated actin filament-free cardiac muscle fibers were rapidly frozen and observed by freeze substitution EM (Fig. 12 *A*). Interestingly, elastic filaments were also observed in A band and appeared to stretch from the Z line toward the M line. In deep-etch replica images, these elastic filaments in the A bands were stereoscopically visualized at high resolution (Fig. 12 *B*). The elastic filaments in the A band–side zone of the I bands, which were characterized by a straight appearance and uniform diameter, continued deeply into the A band in a straight line with rare branching, and reached the M line. Lateral struts were observed between these A band elastic filaments. ∼4-nm striations were detected on the surface of these elastic filaments in the A bands similarly to those detected in the I bands.

Discussion

In the present study, we succeeded in selectively removing both actin and myosin filaments from cardiac muscle fibers and in directly visualizing the residual elastic filaments stretching between the M and Z lines by deep-etch replica EM (Fig. 12). To evaluate potential artifacts carried by the freeze-fracture/deep–etch method, ultrastructure was also
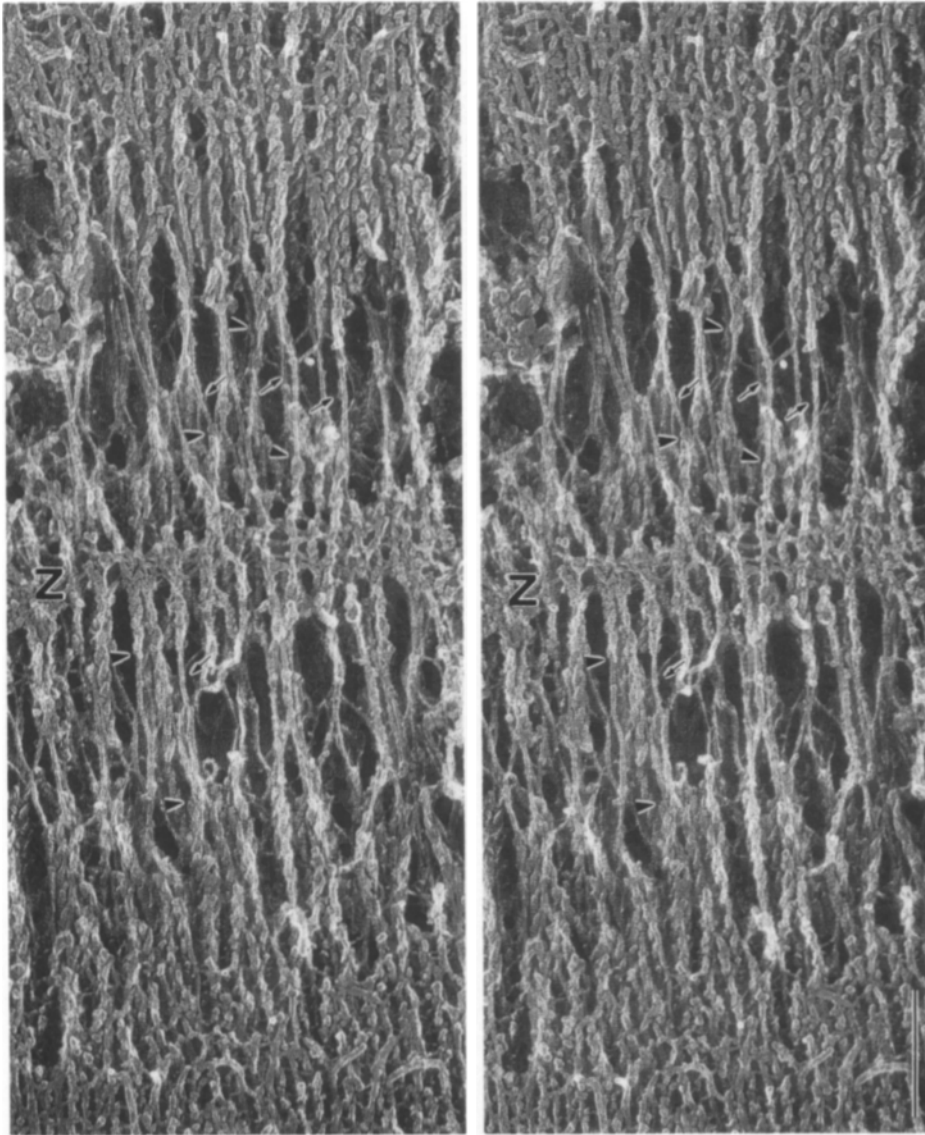
Figure 7. Stereo pair micrographs of freeze-etch replica of cardiac muscle sarcomere in which thin filaments decorated with S1 were torn off by stretching. Arrowheads indicate the ends of thin filaments cut by stretching; arrows indicate elastic filaments. Z, Z line. Bar, 0.2 μm.

examined with thin sections of freeze substituted or conventionally chemically fixed specimens. All these methods consistently showed images of the elastic filaments in situ. In the A band, the elastic filaments projecting from the M line which had a uniform diameter, run parallel, with rare branching, and extended as such to the junction line in the I band. At this line, individual elastic filaments branched off into two (or more) thinner strands, which repeatedly joined and branched to ultimately reach the Z line.

Recently, Nave et al. (1989) clearly visualized the molecular structure of the extractable form of titin (TII$_A$ and TII$_B$; β-connectin) by low-angle rotary-shadowing EM with novel specimen orientation methods. They found that the monomeric molecules had a single globular head at one end of a long and very thin rod ($\sim$900 nm long) of uniform diameter; these molecules were shown to be laterally associated with each other in a parallel manner to form dimers, tetramers, or higher oligomers. Nave et al. (1989) concluded that the globular head was composed of M band constituents and that the two distinct ends of the titin (connectin) molecule at-

tached to Z and M band material, respectively. Their images and their conclusion led us to the following interpretation regarding the molecular organization of the elastic filaments we visualized in the myosin and actin filament-free sarcomeres. Taking into consideration our finding of the epitope of anti-connectin mAb (SM1) on the elastic filaments in actin filament-free I bands (see Fig. 8) and our knowledge that cardiac muscle lacks nebulin, we believed it was evident that the elastic filaments we observed in this study were composed predominantly of connectin molecules. Connectin molecules stretched from the M to Z line, and, at the region between the M line and the junction, two (or more) connectin molecules appeared to be bundled in a parallel manner to form individual elastic filaments of uniform diameter, while at the level of the junction line this bundle appeared to break up into thinner strands consisting of one (or more) connectin molecule. This interpretation is highly consistent with the results obtained previously from immunoelectron microscopic studies that used antibodies specific for various portions of connectin (titin) molecules (Fürst et al., 1988; Itoh
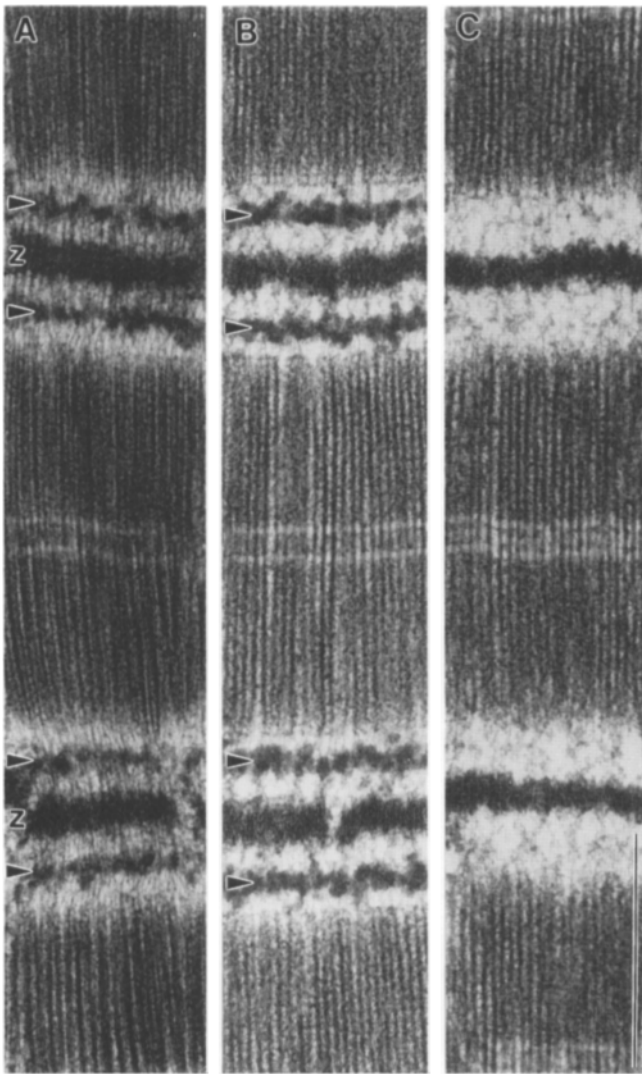
Figure 8. Immunoelectron micrographs of cardiac muscle sarcomeres. Papillary muscle fibers without or with gelsolin treatment were reacted with antibodies, as described in Materials and Methods. (*A*) Sarcomeres reacted with anti-connectin mAb (SM1) without gelsolin treatment; (*B*) gelsolin-treated sarcomeres reacted with SM1; and (*C*) gelsolin-treated sarcomeres reacted with second antibody only. Arrowheads indicated stripes due to antibody binding. Bar, 1 μm.

et al., 1988a; Whiting et al., 1989). Interestingly, the ∼4-nm striations on the surface of elastic filaments in the actin and myosin filament-free sarcomeres were also detected in negative staining and low-angle rotary-shadowing images of purified titin (connectin) molecules (Trinick et al., 1984; Wang et al., 1984). In light of this evidence, we hereafter refer to the elastic filaments as "connectin filaments."

Of course, in the myosin and actin filament-free sarcomeres, some structures are likely to have formed by collapse of connectin molecules that were more widely separated before myosin and actin extraction. Questions have then naturally arisen as to why such well-defined connectin filaments have so far not been visualized in intact striated muscles in situ and how connectin filaments are arranged in intact sar-

comeres. In some earlier studies, a small part of the filament was thought to have been observed in situ in extremely stretched muscle fibers as "gap filaments" (Sjöstrand, 1962; Locker and Leet, 1976; Maruyama et al., 1985; Trombitás et al., 1991). Also in the deep-etch replica images of intact sarcomeres, connectin filaments were hardly detected. It is now known that some flexible structures are apt to be collapsed artifactually during the process of deep etching. Therefore, it appears to be reasonable to speculate that in intact sarcomere the deep etching causes the artifactual collapse of all flexible connectin filaments onto actin and myosin filaments. However, careful comparison of the deep-etch replica images of gelsolin- and potassium acetate-treated sarcomeres with those of intact sarcomeres and/or S1-decorated intact sarcomeres did not favor this speculation, and led us to propose a model for the arrangement of connectin filaments (molecules) in an intact sarcomere (Fig. 13 *A*).

In the A bands, connectin filaments were barely visualized in actin filament-free muscle sarcomeres, and they could not be detected unless myosin filaments were removed by potassium acetate treatment. Lateral struts observed in the A band of gelsolin and potassium acetate-treated muscle might be morphological counterparts for unraveled connectin molecules or unidentified proteins. Further, connectin filaments were clearly observed to directly project from the tips of individual thick filaments. It is thus reasonable to conclude that, in the A bands, connectin filaments show an intimate spatial relationship with myosin filaments. Are the connectin filaments integrated inside the myosin filaments or not? Immunoelectron microscopic observations with anti-connectin (titin) antibodies have revealed that epitopes located in the A band were easily recognized in intact sarcomeres, favoring the view that the connectin filaments are not integrated inside the myosin filaments (Maruyama et al., 1985; Fürst et al., 1988; Whiting et al., 1989).

No connectin filaments were detected in the S1-decorated I bands. This observation compels us to speculate that connectin filaments are associated with actin filaments so intimately that they are cocovered by S1, together with actin filaments. This interpretation was conclusively confirmed by the deep-etch replica images of the mechanically torn S1-decorated I band (see Fig. 7). It should be noted that connectin filaments (β-connectin) bind to actin filaments in vitro (Maruyama et al., 1987). Taking all these lines of evidence together, we can conclude that the so-called thin filaments in the I band and the thick filaments in the A band, that have been visualized to date by electron microscopy in intact muscle, include connectin filaments as a major constituent.

As shown in Fig. 9 *B, a*, in intact cardiac muscle, the portion of connectin filaments between the Z line and SM1 epitope was 2.1–2.4 times as extensible as that between the SM1 epitope and the edge of the A band. Similar results were previously obtained in intact skeletal muscle (Horowits et al., 1989), indicating that cardiac and skeletal connectin filaments and their networks show similar elastic properties. Interestingly, these elastic properties were not affected by the removal of actin filaments by gelsolin treatment (see Fig. 9 *B, b*). We have previously shown that the resting tension of skeletal muscle does not change by the gelsolin treatment (Funatsu et al., 1990). These results suggest that, at the I bands, connectin filaments are laterally associated with actin
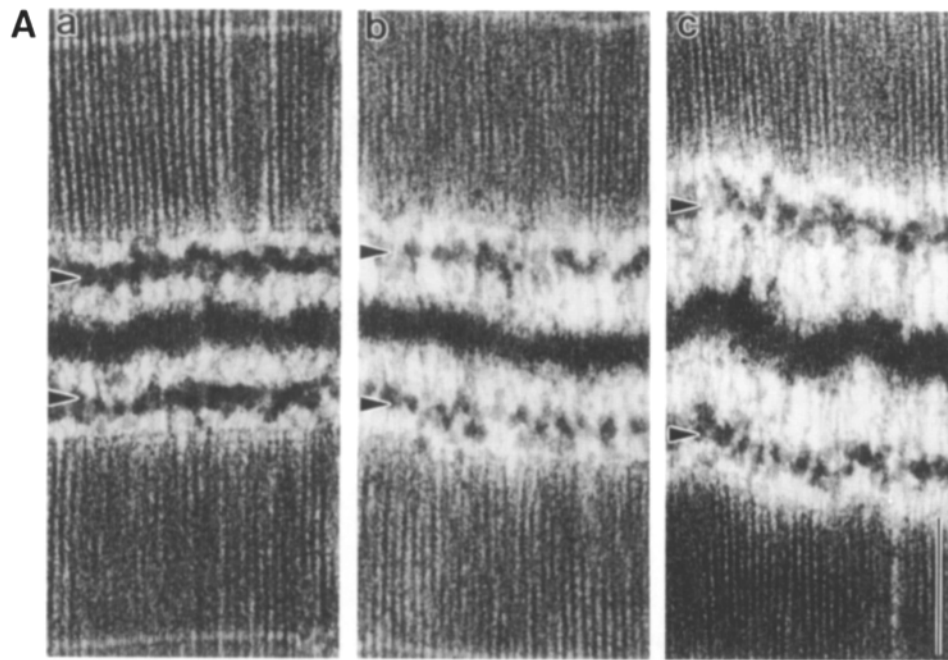
720

Figure 9. Changes in position of the epitopes of anti-connectin mAb (SM1) in cardiac muscle sarcomeres upon stretching. (A) Immunoelectron micrographs of gelsolin-treated and SM1-labeled muscle. Arrows indicate stripes due to antibody binding. Sarcomere length: (a) 2.3, (b) 2.5, and (c) 2.8 μm. (B) Position of SM1 epitopes without (a) or with (b) gelsolin treatment. Distances from the Z line to the epitopes (○) and from the epitopes to the edge of the A band (□) were plotted against the distance from the center of the Z line to the end of a thick filament. The lines were fitted to the data by linear regression.
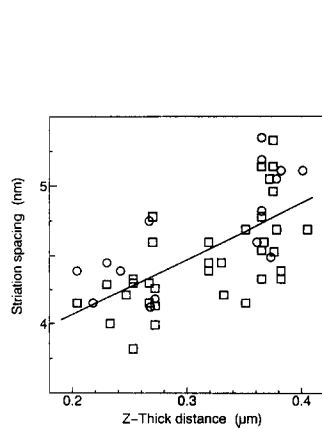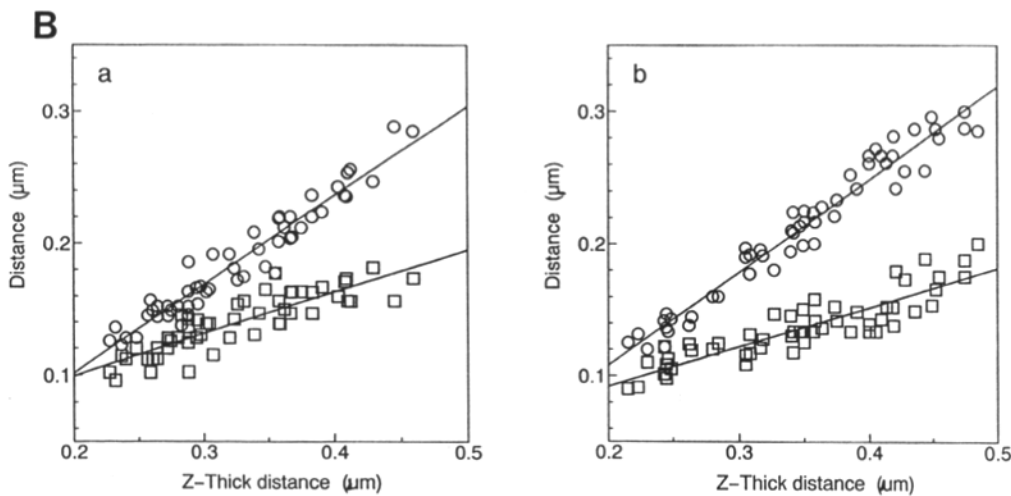
Figure 10. Changes in striation spacing of a connectin filament upon stretching of the sarcomere. Spacing periodicity on the surface of the connectin filament was plotted as a function of the distance from the center of the Z line to the end of the thick filaments. (○) Striation of connectin filament from the Z line to the junction line; (□) striation of connectin filament from the junction line to the thick filaments. The lines were fitted to the data by linear regression.
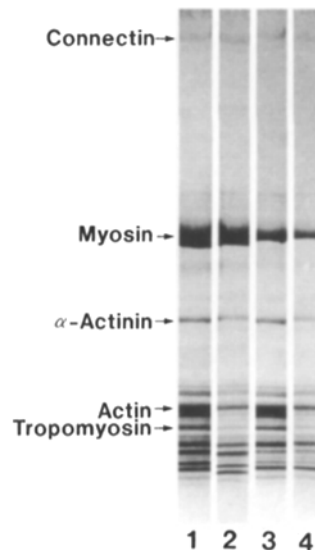
Figure 11. SDS-PAGE patterns of cardiac muscle fibers from which thin and thick filaments were removed; 3–13% polyacrylamide gels. (Lane 1) Intact muscle; (lane 2) gelsolin-treated muscle; (lane 3) muscle treated with a high salt solution containing potassium acetate; and (lane 4) muscle treated with gelsolin and then with potassium acetate.
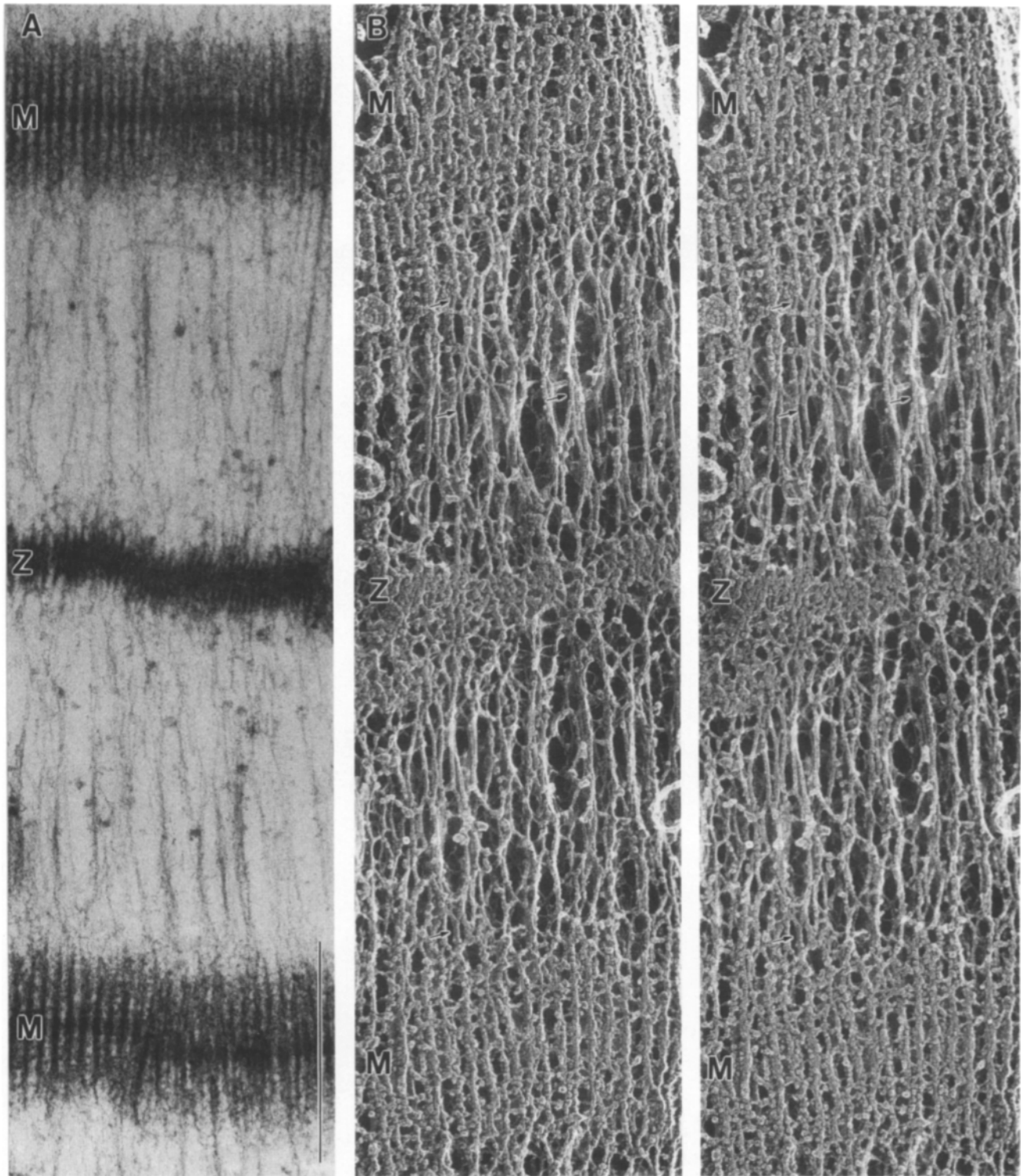
Figure 12. Cardiac muscle sarcomeres from which thin and thick filaments were removed. Muscles were treated with gelsolin and then with a high salt solution containing potassium acetate. (*A*) Thin section of freeze-substituted muscle. Z, Z line; *M*, M line. (*B*) Stereo pairs of deep-etch replica. Arrows indicate ∿4-nm striation on the surface of connectin filaments. Bar, 0.5 μm.

filaments weakly or transiently in such a manner that actin filaments by no means interfere with the elasticity of connectin filaments. In sharp contrast, it is likely that in A bands, the elasticity of connectin filaments is highly restricted by laterally associated myosin filaments (Itoh et al., 1988a). Most recently, Higuchi et al. (1992) have shown that the con-

nectin filaments freed from myosin filaments by KCl treatment, as well as those in the I band, were elastic.

The repeated β-sheet structures inside the connectin molecule are thought to account for its elastic properties (Maruyama et al., 1986). In our deep-etch replica images, connectin filaments showed ∿4-nm striations on their surface. This
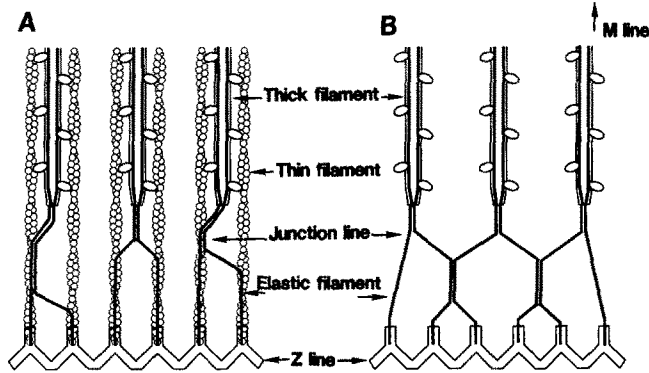
Figure 13. A model of the three-dimensional network of connectin filaments in the I band. (A) Possible model of the connection of thick filaments and the Z line by connectin filaments in situ. The connectin filament is divided into two thinner strands at the junction line and the strands are connected to the Z line. In intact muscle, connectin filaments may be weakly and randomly associated with thin filaments, so that only some portions of connectin bridging the thin filaments laterally are distinguishable from thin filaments. (B) After extraction of thin filaments, most of the connectin filaments between the junction line and the Z line would be associated with one another.

striation may correspond to the repeated β-sheet structures and/or to a repeated 100-residue motif previously identified in the connectin sequence (Benian et al., 1989; Labeit et al., 1990). Interestingly, in the I band, this striation was found to change in spacing, depending on the degree of stretching (see Fig. 10). In the A band, this ∼4-nm striation was observed on the surface of connectin filaments after potassium acetate treatment (see Fig. 12). The rod of the myosin filament has been reported to have a 4.2-nm striation, with 28-amino acid residues (Parry, 1981; McLachlan et al., 1982). Considering that connectin filaments are laterally associated with the rods of myosin filaments, this accordance of their striation periodicity might be important for this association. Furthermore, it is tempting to speculate that connectin filaments might determine the length of thick filaments as a "molecular template" (Whiting et al., 1989).

Finally, we should discuss the junction line identified in the gelsolin-treated cardiac muscle fibers. We observed the junction line where connectin filaments projecting from the tips of thick filaments were branched into two or more thinner strands. The location of this junction line corresponds to the N2 line which was first identified and thought to represent the location of radial structures in skeletal muscle (Locker and Wild, 1984). Wang and Wright (1988) endorsed their idea and concluded that the N2 line may also be a site of accumulation of damaged nebulin. In a previous study of gelsolin-treated skeletal muscle, we confirmed that enhancement of the density of the N2 line depended on nebulin proteolysis (see Fig. 10 B of Funatsu et al., 1990). In cardiac muscle cells, however, nebulin is not expressed. Thus, the N2 line in skeletal muscle fibers might be defined as the line where connectin filaments branch off, as in cardiac muscle fibers. Branching of connectin filaments at the N2 line could be responsible for the lateral compression of the thick filament lattice that occurs when myofibrils are stretched (Higuchi, 1987). At the junction line, we found that two or three adjoining connectin filaments projecting from the tips

of thick filaments were laterally bundled. This junction line may correspond to the ends of the end-filaments seen by negative staining in separated filaments (Trinick, 1981).

Our study is the first to visualize the connectin filament network inside the sarcomere while maintaining its morphological integrity. Based on comparison of this image with that of the intact and S1-decorated sarcomere, we proposed a model of the arrangement of connectin filaments in situ; this requires extensive alternations of the conventional model of sarcomere structure, which has been regarded as consisting predominantly of actin and myosin filaments. However, several important problems remain to be clarified. How many connectin molecules are laterally associated to form individual elastic filaments in the A and the I bands? How are the three different types of filaments, actin, myosin, and connectin, integrated into sarcomere structure during development? Further analyses of these problems will lead us to a better understanding of the physiological functions of connectin molecules.

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