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Connectin, an elastic protein of striated muscle

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Abstract

Connectin, also called titin, a giant elastic protein of striated muscle (≈ 3000 kDa) mainly consists of fibronectin type III and immunoglobulin C2 globular domains, the β-sheets of which are parallel to the main axis of the molecule. One connectin molecule runs through the I band and binds onto the myosin filament up to the M line starting from the Z line. It positions the myosin filament at the center of a sarcomere. Connectin is also responsible for resting tension generation. Biodiversity of the connectin family exists in invertebrate muscle.

Key words: Connectin; Titin; Elastic protein; Striated muscle

1. Introduction

In 1954 Natori observed that resting tension developed, when his skinned (cell membrane-removed) muscle fibers were stretched and the fibers returned to the resting state upon release. Therefore, Natori assumed the presence of an elastic structure in myofibrils [1]. Since then a number of scientists tried to substantiate the elastic structure without success (cf. reviews [2,3]) except for Sjöstrand (1962) who showed the appearance of gap filament between the separated myosin and actin filaments in extremely stretched muscle sarcomeres [4].

The present writer initiated his work stimulated by Natori and named the elastic protein in question connectin [5]. Two years later Wang defined it as the doublet band on an SDS gel electrophoresed and called the protein titin [6]. The giant protein did not attract attention for almost ten years except for meat scientists. Thanks to the entry of Podolsky [7–10] and Weber [11–13] into this field, their elegant work has greatly contributed to the general acceptance of this giant elastic protein. Tedious sequence work by Labeit and co-workers has partially elucidated the primary structure of connectin [14,15]. Thus, now, nobody doubts the presence of this gigantic muscle protein (cf. reviews [16–18]).

2. Preparation

Before the description of the preparation method, terminology is needed to be explained. The mother molecule, α-connectin (titin 1), ≈ 3000 kDa, always coexists with its proteolytic fragment, β-connectin (titin 2), ≈ 2000 kDa, in an SDS extract of rabbit, chicken and frog skeletal muscle (Fig. 1). When rabbit skeletal muscle myofibrils are stored for 12–24 h at 4°C, α-connectin is degraded into β-connectin and a 1200

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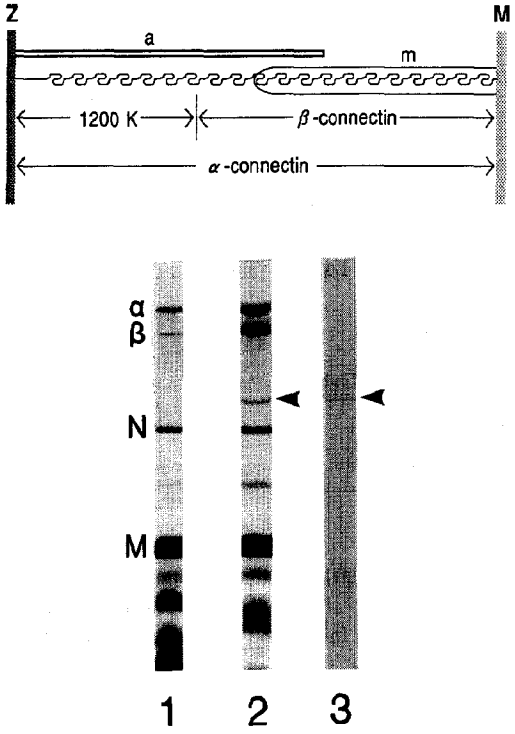

Fig. 1. Connectin filament in a half sarcomere and SDS gel electrophoresis patterns of connectin. 1, whole SDS extract of rabbit skeletal muscle; 2, myofibrils stored for 20 h at 4°C; 3, isolated 1200 kDa fragment (arrowhead). α, α-connectin; β, β-connectin; N, nebulin; M, myosin heavy chain. Z, Z line; a, actin filament; m, myosin filament; M, M line. Modified from ref. [23,24]. (Courtesy of N. Kanzawa and J. Suzuki.)

kDa fragment (Fig. 1). The latter is important in view of its elastic function (see below).

2.1. Isolation of connectin

β-connectin is soluble in 0.6 M KCl and is extractable together with myosin. After the precipitation of myosin by dialysis against 0.1 M phosphate buffer, pH 7.0, β-connectin can be easily purified by DEAE Sephadex and hydroxyapatite column chromatography. β-connectin was isolated from chicken or rabbit skeletal muscle independently by Kimura and Maruyama [19], Trinick et al. [20] and Wang et al. [21].

On the other hand, α-connectin was not isolated until 1989. First, it is difficult to get solubilized in salt solution and easy to be degraded and furthermore, it is not easy to be separated from its proteolytic products. Kimura and Maruyama [22] solublized a small amount of α-connectin (≈ 5% of the total amount) with 0.2 M phosphate buffer from washed myofibrils of rabbit skeletal muscle and separated it from β-connectin in the presence of 4 M urea by DEAE Toyopearl chromatography. Urea was removed after separation. Even now, without use of urea it is very hard to separate α-connectin from β-connectin [23].

The 1200 kDa fragment was prepared from aged myofibrils [24]. The final product containing α-connectin was dialyzed against 5 mM phosphate buffer and 1200 kDa fragment was separated by ultracentrifugation because α-connectin was sedimented at low ionic strength. Recently, an easy procedure for 1200 kDa fragment purification has been developed by Takahashi and coworkers [25].

2.2. Proteolysis of connectin

Connectin *in situ* is very sensitive to any proteolytic enzymes. Addition of only a few tenth of μg of trypsin per ml results in splitting of α-connectin to β-connectin. Purified β-connectin is degraded into 1700 kDa and 400 kDa fragments by trypsin or chymotrypsin, but remained as such for a while [26].

When washed myofibrils are kept at 4°C, connectin is slowly hydrolyzed into β-connectin and 1200 kDa fragment. Addition of 0.1% casein abolished the proteolysis of both connectin and nebulin. The *in situ* proteolysis of connectin was not inhibited by 1 mM DFP (diisofluorophosphate) or PMSF (phenylmethylsulfonyl fluoride) suggesting that serine protease was not involved. 1 mM leupeptin or 1 mM E64c, potent thiol proteinase inhibitor, nearly completely stopped proteolysis of α-connectin showing the action of thiol proteinase. Although added calpain effectively degraded α-connectin into β-connectin [27], addition of Ca^{2+} or EGTA did not affect markedly the spontaneous splitting of α-connectin *in situ*. At present it has not yet been able to identify the proteinase contained in myofibrils which is responsible for breakdown of connectin [28].

Table 1
Molecular mass of connectin (MDa)

$\alpha(T_1)$	$\beta(T_2)$	Method	Ref.
1		SDS PAGE	1979 [21]
2.8	2.1	SDS PAGE	1984 [30]
	2.7	sedimentation equilibrium	1984 [30]
2.4–2.6		sedimentation equilibrium	1988 [34]
3.6	2.7	SDS PAGE	1989 [32]
> 1		mRNA	1990 [15]
	2	dynamic light scattering	1993 [35]
2.8	2.4	SDS PAGE	1993 [33]

Recently, Astier et al. reported that trypsin, endoproteinase Arg-C or V8 proteinase easily degraded connectin in myofibrils and the proteolytic fragments of various sizes were set free into medium together with intact α-actinin [29].

3. Molecule

3.1. Molecular weight

Since the time of discovery, connectin was regarded as an extraordinary large protein. However, due to technical difficulty in determinations of MW of order of 10^6 or larger, the precise value has not yet been established (Table 1).

Wang and co-workers [6] first estimated the MM of connectin to be ≈ 1000 kDa based on the comparison of the electrophoretic mobility with those of myosin heavy chain oligomers. The value was later corrected to be ≈ 3000 kDa [30]. This is largely because during the oligomer formation dimers of myosin heavy chain tend to associate with each other to form tetramers, hexamers etc., unless care is taken [31]. It is necessary to use very fragile gels made of ≈ 2% polyacrylamide and even so the mobility is very slow. Therefore, it is only possible to estimate a rough value of MW [32,33]. Sedimentation equilibrium and dynamic light scattering measurements also gave a similar MW ($2–3 \times 10^6$) [29,34,35]. However, anybody was reluctant to accept such a large value, simply because there was a view that the size of any single peptide was below one million.

In 1990 Labeit and associates clearly showed that rabbit skeletal muscle mRNA is larger than 25 kb corresponding to a product of MW, 10^6, by Northern blot tests [14]. This observation has established the presence of a giant protein of MW larger than 10^6. The MM of α-connectin and β-connectin are now tentatively taken as 3000 and 2000 kDa, respectively. For the precise value of α-connectin, we have to wait for the complete cDNA sequencing.

3.2. Molecular shape

Rotary shadowed images of isolated β-connectin molecules showed entangled filaments, up to 1 μm long and 4 nm wide [20,21,30]. Weber and co-workers introduced an elegant technique where β-connectin molecules were oriented as parallel straight filaments on a mica by centrifugal force [12]. There was a globular head at one end of the filament and it was suggested as M line constituting proteins, 165 and 190 kDa, by immunoelectron micrography. These two proteins have recently been sequenced and characterized further [36].

The results of Nave et al. [12] were confirmed by Sonoda et al. [37] who estimated the lengths of α- and β-connectin to be ≈ 1.0 and 0.9 μm, respectively. It is of importance to note that some rotary shadowed images showed one filament, 4 nm wide, consisted of two filaments. Nave et al. [12] already pointed out the presence of laterally associated oligomers. The rotary shadowed image of isolated connectin also showed double-stranded structure according to Pierobon-Bormioli et al. [38]. It is thus possible that a single connectin filament is 2 nm wide.

A recent investigation showed that the lengths of 1200 kDa fragment, β-connectin and α-connectin were 0.35, 0.95 and 1.3 μm, respectively (J. Suzuki et al., to be published), as shown in Fig. 2.

3.3. Sequence

In 1990 Labeit and associates sequenced connectin cDNA fragments screened from rabbit skeletal muscle cDNA library using monoclonal antibodies to connectin [14]. Two species of motifs, I and II, fibronectin type III and immunoglobulin C2 globular domains, were aligned

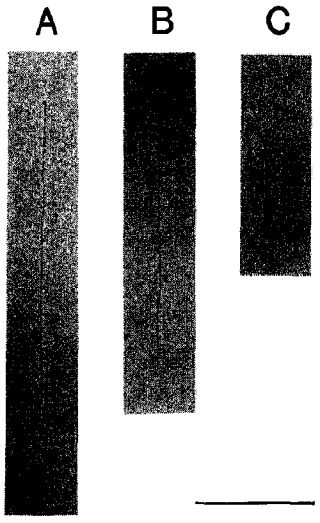

Fig. 2. Electron micrographs of rotary shadowed images of α-, β-connectin and 1200 kDa fragment. A α-connectin; B β-connectin; C 1200 kDa fragment. (———) 0.5 μm. (Courtesy of J. Suzuki.)

in tandem (Fig. 3). This is also the case with rabbit cardiac muscle connectin [39]. These repeated sequences had been already found in twitchin, the product of unc 22 gene of *C. elegans* [40].

In the C terminal region, there is a domain homologous to myosin light chain kinase (MLCK) (Fig. 3). This is also the case with twitchin [40]. In human cardiac connectin, there are 10 motif IIs with 8 interdomains following the MLCK domain. Interestingly, there are four KSP repeats in the fifth interdomain, serine of which can be phosphorylated by a neonatal muscle extract [41]. In chicken embryonic muscle connectin, four RSP repeats are present near the C terminus [42].

Labeit et al. [15] sequenced the portion of connectin on the myosin filament and showed that there are super-repeats of I–I–II–I–I–I–II–I–I–I–II (≈ 120 kDa). There are eleven super-repeats on the half A band (≈ 1300 kDa). It is thought that this super-repeat corresponds to the 43 nm periodicity of crossbridges on the myosin filament. In *C. elegans*, the repeat consists of I–I–II [40].

The sequence of the portion of connectin in the I band has been only partially known: II–I–II–II near the edge of the edge of the A band [43] and II–II–II–II–II–II–II near the N_2 line

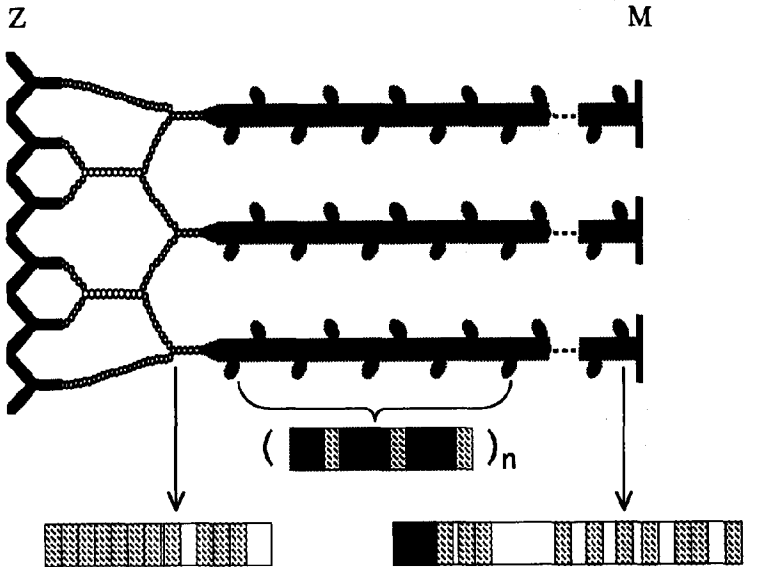

Fig. 3. Repeated motifs I and II in connectin molecule. Shadowed box, motif I; dotted box, motif II; black box, MLCK domain. Reconstructed from ref. [15,41,44].

region [44]. In the latter, there is a sequence homology to neurofilament H and M subunits. It appears that the elastic portion of connectin consists of mainly motif IIs (immunoglobulin C2).

3.4. Molecular structure

Since cDNA sequencing revealed that connectin molecule mainly consists of motifs I and II in tandem, an extremely long connectin molecule is a chain of ≈ 300 globular domains. The three-dimensional structure of immunoglobulin C2 domains (motif II) are shown to be a typical β-barrel consisting of seven β-strands [45]. Recently, a similar 3D structure of fibronectin type III domain (motif I) has been demonstrated [46]. The both domains are folded into two layers of antiparallel β-sheet (three and four β-strands, respectively) structure which enclose a hydrophobic interior. The dimension of motif I is 4.0 × 1.7 × 2.8 nm and that of motif II is approximately 4 × 2 × 2 nm. The both domains consist of ≈ 60% β-sheets. In good agreement with the abundance of β-sheets in the motifs I and II, connectin molecule and its proteolytic fragments, β-connectin and 1200 kDa fragment have been shown to consist of ≈ 60% β-sheet and 30% β-turn (Table 2). α-helices are not present at all.

Questions arise as to whether the β-sheets in each domain (motif I or II) are parallel to the mainaxis of the connectin molecule or not. In order to solve this problem infrared dichroism of the artificially oriented fibers made of isolated β-connectin or 1200 kDa fragment was measured [47,48]. The differences between the infrared spectra with polarization parallel and perpendicular to the fiber axis showed that the amide II and III bands were stronger and the amide A and I bands were weaker. These differences clearly indicated that the dichroic nature was clear with polarizations typical of antiparallel β-plated sheets aligned parallel to the fiber axis. This conclusion with β-connectin fibers was the same as the 1200 kDa fragment fibers. Hence the β-sheets in each motif are largely parallel to the main axis of connectin molecule. A model of a part of the molecular structure of connectin is depicted in Fig. 4. It is to be added that the amide III peak frequency (1235 and 1236 cm^{-1} for β-connectin and 1200 kDa fragment, respectively) was different from that (1254 cm^{-1}) of elastin. Therefore, β-spiral structure of elastin is not present in connectin.

Table 2
Secondary structure of connectin (%)

	α-connectin	β-connectin	1200 kDa fragment [a]
α-helix	0	0	0
β-sheet	61	60	55
β-turn	32	32	25
remainder	7	8	20

[a] Taken from ref. [23,24].

4. Localization

It was observed that a pair of fluorescent stripes were formed in a sarcomere and the positions of the stripes were movable on stretch, when glycerinated psoas fibers were treated with fluorescent dye-conjugated polyclonal antibodies to connectin [6]. Immunoelectron microscopy revealed that the stripes were symmetrical to the M

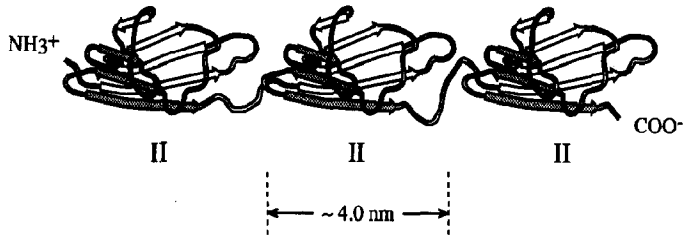

Fig. 4. Molecular structure of connectin in the I band region. Modified from ref. [48]. (Courtesy of Y. Nakauchi.)

line and suggested that the putative connectin filaments linked the Z line to the M line of the A band [49]. Furthermore, it was shown that the stripes in the I band were moved away from both the Z band and the edge of the A band upon stretch whearas the stripes in the A band did not move unless extremely stretched [50]. The portion of connectin near the A band is less extensible than that in other part of the I band [10]. The portion near the Z line (up to N_1 line, 0.1 μm from the Z line) is functionally stiff, although elastic upon extreme stretch [51].

4.1. Epitope mapping

A number of monoclonal antibodies to connectin have been prepared in several laboratories and the site of each epitope has been located in various positions within the sarcomeres of chicken and rabbit skeletal muscle [11,13,50,52]. Because of synchronous positioning of an epitope of connectin filament, a stripe due to antibody binding is formed in each half sarcomere. Fig. 5 summarizes the epitope map in a sarcomere, ranging from the Z line to the edge of the M line. This fact was first established by Weber's school with a use of a variety of monoclonal antibodies [11]. Some monoclonal antibodies recognizes only a single epitope within a long molecule, but several antibodies bind to two, three or more epitopes.

A monoclonal antibody, T33, binds to the epitope situated 55 nm from the center of the M line [13]. This antibody was shown to bind to the head portion of isolated β-connectin to which the M line proteins (165 and 190 kDa) bound [12]. Recently, it has been reported that monoclonal antibodies to 190 kDa protein (myomesin [53]) bind to the center of the M line and those to 165 kDa (M protein [54,55]) is situated 18 or 15 nm from the center of the M line [36]. Interestingly, both proteins contain the domain sequence of II–II–I–I–I–I–II–II–II–II–II [36,55]. Although it is

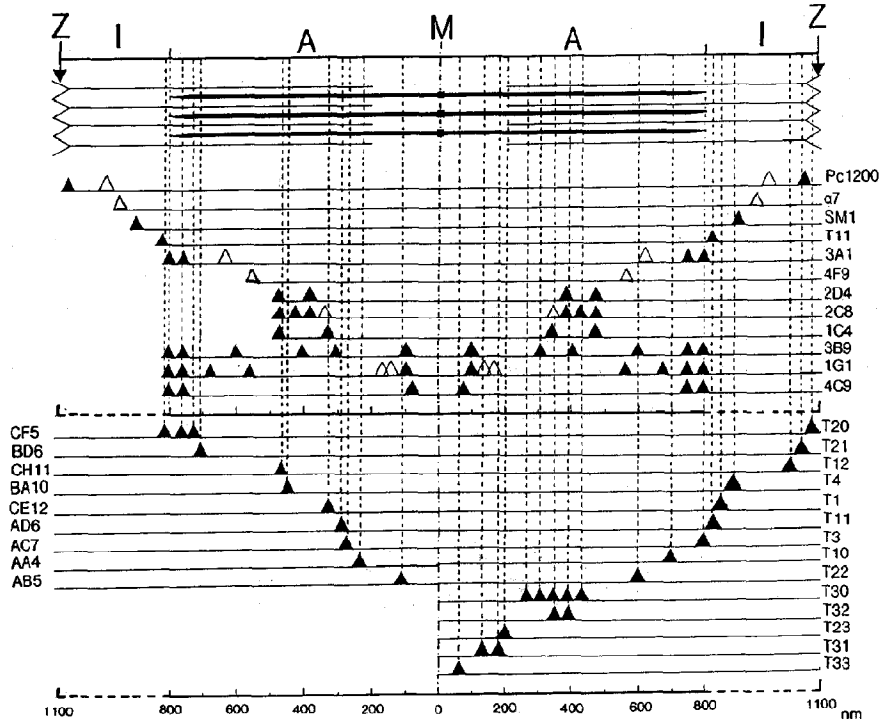

Fig. 5. Epitope maps of connectin in a sarcomere. A, A band; I, I band; M, M line; Z, Z line. Upper is based on the results of the author's laboratory cf. [50]. Lower left: ref. [52]; Lower right: ref. [11,13]. (Courtesy of Y. Kawamura.)

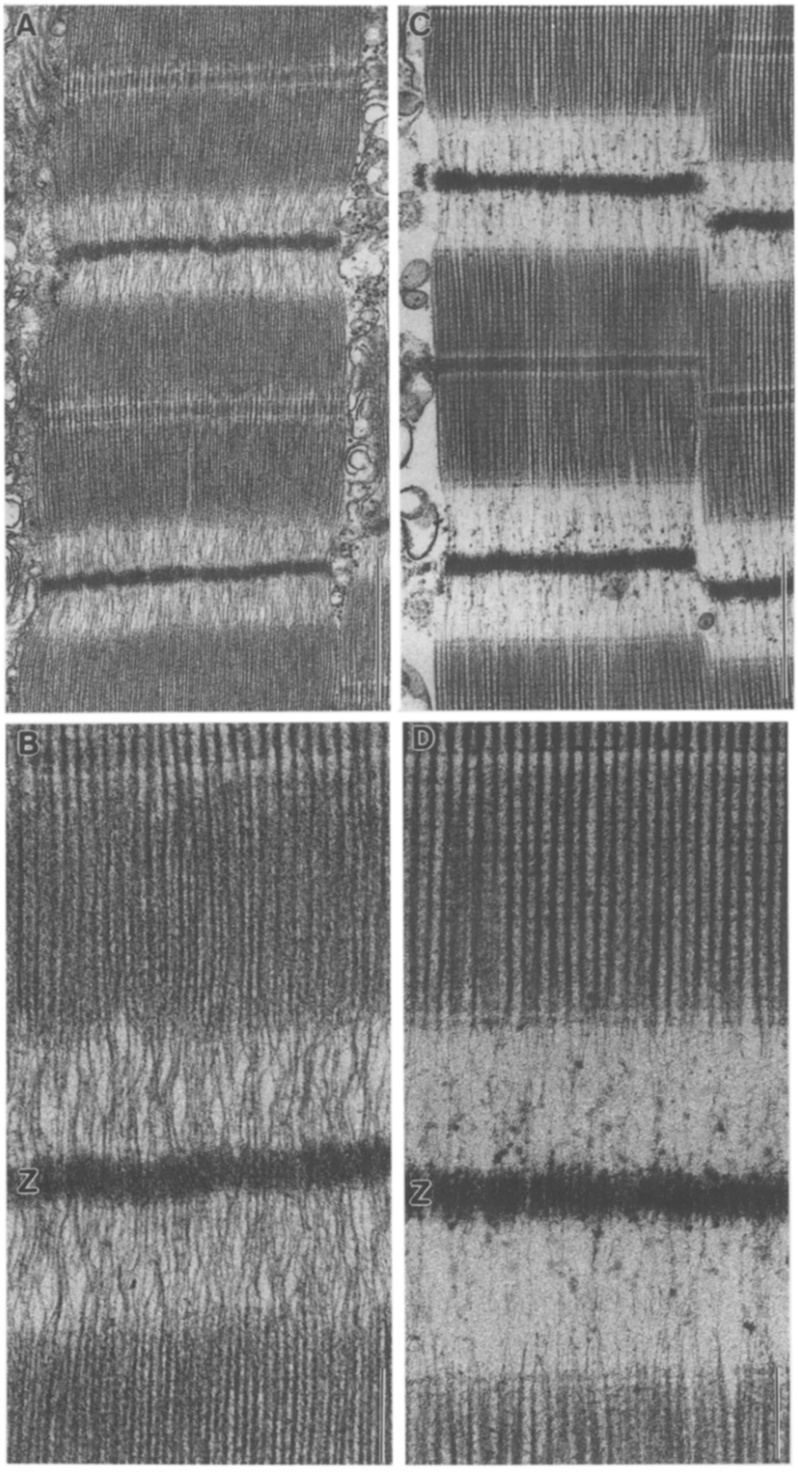

Fig. 6. Connectin in gelsolin-treated sarcomere of rabbit cardiac muscle. Freeze-substituted rabbit cardiac muscle sarcomeres. A and B, intact sarcomeres; C and D, gelsolin treated sarcomeres. (———) 1 μm (A, C); 0.2 μm (B, D). Taken from ref. [57]. (Courtesy of T. Funatsu.)

not definitely proved, it appears that the head domain of connectin associated with M protein and myomesin extends into the center of the M band [36].

It is of interest to note that a monoclonal antibody (T30) binds to five epitopes each 42 nm apart on the myosin filament where C-protein binds coincidently [13]. However, this is rather exceptional. In view of repeated presence of the motif II there could be an antibody common to all the II, the epitope of which is distributed through a whole molecule. Such an antibody has not been prepared.

4.2. Visualization of connectin filaments in a sarcomere

Connectin filaments are usually not seen in an electron micrograph of longitudinal section of a sarcomere because of the abundance of the actin filaments in the I band. Funatsu et al. [56] were first able to visualize connectin filaments by removing the actin filaments with the treatment with gelsolin, an actin filament-cutting protein. Very thin filaments appeared in the actin-free I band and they were identified as connectin by the binding of a monoclonal antibody specific to connectin [57]. When rabbit psoas muscle was used, nebulin released from an actin filament bound to a connectin filament [56]. Therefore, later, nebulin-free cardiac muscle was employed. As shown in Fig. 6, connectin filament, ≈ 4 nm wide, extends from the tip of a myosin filament to the I band and united and branched at the so-called N_2 line and then reached the Z line [57]. Perhaps due to lateral association during the preparation, it is not easy to follow each connectin filament from the one end to another in gelsolin treated sarcomeres. It should be mentioned that a 4 nm periodicity was recognized in visualized connectin filaments.

4.3. How many connectin filaments per half myosin filament?

As Wang et al. [6] first pointed out, connectin is a third abundant protein next to myosin and actin in vertebrate skeletal myofibrils. Knowing the contents of myosin (44%) and of connectin (10%) in myofibrillar proteins and assuming the MWs to be 0.5×10^6 and 3×10^6, respectively, and also that a myosin filament consists of 300 myosin monomers, calculation led to 12 connectin filaments per myosin filament or 6 connectin per half myosin filament. Recent estimation has shown 4.5 connectin in rabbit skeletal muscle and 3 connectin in rabbit cardiac muscle [58]. Uncertainty exists in the MW value of connectin.

Connectin must be bound onto the myosin filament so as to not interfere with the cross-bridge function. At the tip of the myosin filament the connectin filaments united to one filament up to the N_2 line in the I band. This portion was called "end filament" [59].

5. Function

The physiological function of connectin in myofibrils is its elasticity to position the myosin filament at the center of a sarcomere and to generate resting tension upon stretch of a sarcomere. The latter enables the stretched sarcomere return to the resting state upon release. The mechanism of elasticity is regarded to be ascribed to reversible folding and unfolding of the β-barrel domains linked in tandem but the details have not yet been worked out.

5.1. A band centering action

When glycerinated rabbit psoas fibers are isometrically contracted for a prolonged time, the A band moves to either side of the Z line. This is because during contraction, the force generated on each half of the myosin filament is proportional to the fraction of its cross-bridge containing length which overlaps with the actin filaments. However, when such fibers were put into a relaxing solution, the moved A band returns to the center of the sarcomere. Horowits and Podolsky [8,9] ascribed the A band centering action to the connectin filaments which support each myosin filament as spring from both sides. The participation of connectin was clearly shown by a

monoclonal antibody binding to it at the I bands of the A band-moved sarcomeres [10].

It is of interest that the A band movement does not occur in sarcomeres stretched to longer than 2.8 μm sarcomere length during prolonged contraction [9]. Evidently, at sarcomere lengths shorter than 2.8 μm, connectin filaments do not act as spring (do not generate tension to hold the myosin filament).

5.2. Passive tension generation

Active force is produced by the ATP-induced interactions of myosin and actin in striated muscle fibers. The muscle fibers also produce passive forces (passive tension) that resist stretch independent of the actin–myosin interactions. Resting tension controls the extent of motion caused by an opposing muscle during active shortening. The extent of passive tension of rabbit psoas muscle fibers at 3.5 μm sarcomere length is ≈ 70% of that of active tension (≈ 1 kg/cm^2) at 2.4 μm [60].

When skinned fibers are passively stretched, resting (passive) tension is generated at sarcomere lengths longer than ≈ 2.5 μm. Tension generation exponentially increases up to a certain sarcomere length. This tension generation was decreased in parallel with splitting of α-connectin to β-connectin by a mild trypsin treatment [56,61,62]. Degradation of connectin by ionic radiation also resulted in loss of passive tension generation [7]. Removal of actin filaments by gelsolin treatment did not affect the passive tension generation [56]. Hence the passive tension development upon stretch is ascribed to elasticity of connectin filaments.

Wang and co-workers investigated the process of resting tension generation at varied sarcomere length in six kinds of rabbit skeletal muscles [63]. Horowits also compared active and passive tension generations of rabbit psoas muscle fibers with those of rabbit soleus muscle fibers [60]. The passive tension is generated at longer sarcomere lengths in muscle fibers containing larger connectin molecules.

The tension-sarcomere length curve of a skinned psoas fiber shows a multiphasic shape [63,64]. (1) No tension rises from 2.2 μm (slack length) to 2.5–2.6 μm. In this range of sarcomere length, connectin filaments do not show any elasticity. (2) Then tension is generated exponentially up to 3.8–3.9 μm. The distance between the epitope of a monoclonal antibody in the I band and the Z line increases linearly as the sarcomere lengths is elongated from 2.5 to 3.8 μm. In this range of sarcomere length, a connectin filament is elongated from 0.45 μm to 1.1 μm. Since 0.1 μm near the Z line is inextensible [51], a connectin filament is approximately three-fold elongated. When released, tension rapidly drops with a little hysteresis. Mathematical analyses led to the proposal of a linear segmental extension model by Wang et al. [63,64]. Here each segment corresponds to the motifs II and/or I. (3) From 3.8 to 4.5 μm the peak tension remains constant. However, when released, the highly stretched fiber becomes slack around 3.0 μm for a while without tension generation. When restretched, a higher peak tension is generated at about 4.6 μm instead of 3.8 μm. This decrease in stiffness is explained by a net increase in the number of the extensible segment newly recruited from the bound portion of connectin on the myosin filament. It is known that the myosin-bound portion of connectin becomes elastic when separated from the myosin filament [65].

5.3. Interactions with other proteins

β-connectin has an affinity with myosin filaments to form a large aggregate [66]. Only rod portion (L-meromyosin) bound to connectin and S1 did not [67–69], although the binding of S-1 to connectin has recently been reported [69]. The motifs I and II are regarded to be myosin-binding sites, because myosin associated proteins, C-protein [70], myomesin [36,53], M protein [36,54,55], and 86 kDa protein [71] share the motifs I and II. It is to be noted that monoclonal antibodies to connectin do not crossreact with these proteins. C-protein binds to connectin [70,72]. There is also evidence that bacteria-expressed motifs I and II interact with myosin and C-protein [15]. Another myosin binding protein, AMP deaminase also binds to connectin [68,72].

β-connectin also binds to F-actin to form bundles [73]. However, β-connectin does not bind to myosin S-1 decorated actin filament, probably due to steric hindrance [73].

The binding of connectin to the M line constituting proteins (M protein, myomesin) was shown in an elegant manner by Weber and co-workers [12,36]. This is physiologically important to position the C terminus of the connectin filament near the center of the myosin filament.

On the other hand, the positioning of the N terminus of the connectin filament at the Z line is little known. The *in vitro* binding of connectin to α-actinin, the most abundant protein in the Z line has been shown [74]. The 1200 kDa fragment also binds to α-actinin [25].

5.4. Phosphorylation of connectin

In vivo phosphorylation of connectin was observed with frog skeletal muscle [75]. *In vivo* phosphorylation occurred mainly on serine residues of connectin [76]. Similarly, it was reported that, projectin (connectin family) of *Drosophila* was labelled, when flies were grown in the presence of radioactive inorganic phosphate [77]. There are four RSP motifs in the C terminal region of human cardiac connectin [41]. The serine residues of the peptide expressed in *E. coli* were phosphorylated by an extract from neonatal skeletal muscle [41]. However, the physiological significance of these phosphorylations remains obscure.

Connectin contains a region homologous to myosin light chain kinase (MLCK) in the C terminal region [14]. It is of interest whether this MLCK region is functionally active or not. Isolated β-connectin was observed to be autophosphorylated, but it did not phosphorylate the light chains of added myosin [78].

6. Biodiversity

In all the vertebrate skeletal muscles examined, 3000 kDa connectin is present, although the size difference exists, e.g. connectin in turtle skeletal muscle is the largest [79]. Connectin is also present in cardiac muscle and β-connectin is isolated from porcine cardiac muscle [80]. On the other hand, connectin is not expressed in vertebrate smooth muscle.

Table 3
Biodiversity of connectin family

Term	MM (MDa)	Muscle
connectin/titin	3	vertebrate striated
connectin	2–4	most invertebrate striated and smooth
twitchin	0.6–0.75	nematode, annelid bodywall molluscan adductor
projectin	1.2	arthropod striated
kettin	0.5–0.7	insect striated

It is of interest whether connectin-like elastic proteins are present in nonmuscle cells or not. Eilertsen and Keller reported that there were connectin-like doublet bands in chicken intestinal epithelial cell brush border cytoskeleton and the antibodies to them reacted with chicken breast muscle connectin [81]. They were localized in the brush border terminal web region, but not in microvilli. The partially purified preparation contained very long filaments of ≈ 1 μm in length. A connectin-like protein was detected in sea urchin egg cytomatrix, which was reactive with antibodies to chicken breast muscle connectin [82]. It was shown that the high molecular weight protein was concentrated at the cleavage furrow of the sea urchin egg.

In invertebrate muscle, the situation becomes complicated. Connectin family proteins are present not only in striated but also in smooth muscle. Several monoclonal antibodies to vertebrate connectin crossreact with invertebrate connectin family proteins. There are three types of connectin family differing in sizes in invertebrate muscle: connectin, ≈ 3000 kDa; projectin, ≈ 1200 kDa and twitchin, ≈ 700 kDa (Table 3). The latter two are also called mini-titin [83]. It appears that connectin and projectin or twitchin coexist in one type of muscle. In addition there is a Z line binding protein, kettin, 500–700 kDa, in insect flight and leg muscles [84].

6.1. Twitchin

Bennian et al. [40,85] sequenced the unc 22 gene of *C. elegans* and called the product, 753 kDa, twitchin. It consists of repeated motifs, I-I-II, and MLCK domain in the C terminal region. It is localized on the myosin filament of *C. elegans* bodywall muscle. Nave et al. isolated it from *C. elegans* and showed its filamentous structure, 0.24 μm long [86]. Twitchin is present in earthworm bodywall muscle [86] and in scallop striated and smooth muscles [87]. Vibert et al. characterized scallop twitchin in detail and showed its presence on the myosin filament [87].

6.2. Projectin

Projectin [88] is found in arthropod striated muscle. The MM is ≈ 1200 kDa [89]. Although Nave and Weber [83] estimated it as 700 kDa, its electrophoretic mobility was clearly slower than that of twitchin (753 kDa). cDNA coding projectin was partially sequenced and the presence of motifs I and II was shown [90,91]. Projectin was isolated from locust flight and crayfish claw muscles [83,89]. Projectin links the Z line to the myosin filament in locust and honeybee flight and crayfish stretcher muscles. However, in the giant sarcomere of crayfish claw muscle, projectin is present on the myosin filament as twitchin [92].

6.3. Connectin

First, invertebrate connectin seemed to be much smaller in size than vertebrate one and therefore, Nave and Weber [83] called it minititin. However, it turned out that there exists a high molecular weight connectin in most of invertebrate muscles, although the content is much less as compared to that of twitchin or projectin.

In the giant sarcomeres (10 μm sarcomere length at rest) of crayfish claw muscle, 3000 kDa connectin links the Z line to the myosin filament [92]. It seems that projectin supports the 3000 kDa filament on the long myosin filament (6 μm) [89,92]. In annelid bodywall muscle it appears that 4000 kDa connectin links myosin filament and dense body (Y. Kawamura et al., to be published).

7. Perspectives

The third filament, connectin (titin), next to actin and myosin filaments, has been established as an elastic structure of vertebrate striated muscle. There are, however, a number of problems to be cleared up.

(1) A whole sequence is soon expected to be established. Naturally, an exact value of MW will be determined.

(2) The molecular mechanism of tension generation during folding and unfolding of the β-barrel domains should be solved.

(3) How are the connectin filaments arranged on the myosin filament? Is the C terminus of connectin located at the center of the M line?

(4) How is the N terminal region of the connectin filament linked to the Z line? Does connectin constitute a part of the Z line structure? A mild proteolysis releases intact α-actinin from the Z line. Is the N terminal region of connectin the target of a proteinase?

(5) Why is β-connectin present in most of skeletal muscles? Is β-connectin split off from α-connectin by proteinase *in vivo*? Or natural β-connectin exists on the myosin filament as twitchin?

(6) What is the physiological significance of phosphorylation of connectin? Does MLCK domain functionally active?

(7) How long is the life time of this giant protein?

(8) Does biodiversity of connectin in invertebrate muscle give a hint of evolution of connectin?

(9) How is connectin incorporated into the sarcomere structure during myofibrillogenesis [93,94]?

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