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Minireview

Understanding the functions of titin and nebulin

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Individual molecules of the giant muscle proteins titin and nebulin span large distances in the sarcomere. Approximately one-third of the titin molecule forms elastic filaments linking the ends of thick filaments to the Z-line. The remainder of the molecule is probably bound to the thick filament where it may regulate assembly of myosin and the other thick filament proteins. This region also contains a sequence similar to catalytic domains in protein kinases. Nebulin appears to be associated with thin filaments and may regulate actin assembly.

Titin; Nebulin; Protein-ruler; Twitchin

1. INTRODUCTION

Titin (also known as connectin) and nebulin are components of striated muscle myofibrils and are two of the largest polypeptides yet found (~3×10^6 Da [1,2] and ~8×10^5 Da [3,4] respectively). The products of unrelated genes, both molecules are highly extended structures approximately 1 μm long that run approximately parallel to the muscle fibre axis in situ. Individual titin molecules span between the Z-line and the M-line (Fig. 1) [5,6]. The I-band sections make elastic connections between the ends of thick filaments and the Z-line [5–7], forming a third type of sarcomere filament additional to thick and thin filaments. These connections centre the thick filaments between neighbouring Z-lines [8,9], and they are the main route of mechanical continuity through relaxed muscle fibres [10]. The A-band region of titin is likely to be an integral part of the thick filament [5,6]. Nebulin, on the other hand, is likely to be associated with the thin filament and individual molecules probably span the length of each filament [11]. These locations led to proposals that titin and nebulin act as templates for exact assembly of thick and thin filaments, respectively [6,11]. This review decribes recent progress in the study of both proteins.

2. TITIN

2.1. *Similarity of sequence to extracellular proteins*

Neither titin nor nebulin has yet been completely sequenced but partial sequences of both are available.

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Three sections of cDNA encoding approximately 10,000 amino acids of A-band titin have been completed and these probably constitute ~30% of the whole molecule [12,13]. The derived sequences consist almost entirely of repetition of two types of motif termed class I and class II. These motifs each contain roughly 100 amino acids and are respectively similar to type III fibronectin and C-2 immunoglobulins. Both motifs are therefore likely to fold to form separate globular domains. A linear array of ~100 residue domains was also predicted from the beaded appearance visible in titin by electron microscopy [6,14]. Bazan has suggested that both these domain types derive from a single ancestral gene [15].

Both domain types are common building blocks found, often together, in many other proteins, but until recently they were thought to be exclusively extracellular. Titin is one of a growing group of muscle proteins now known to have them. They were first described in twitchin which is a large A-band protein in *C. elegans*, identified by a mutant with a twitching phenotype [16]. Twitchin is probably the same as proteins known as mini-titin [17], P800 [18] or projectin [19,20] in other species. Most estimates of its molecular weight are ~800 kDa, but Hu et al. [21] quote a value of ~1200 kDa for crayfish projectin. Other muscle proteins composed of class I and II motifs are C-protein [22], skelemin [23], a protein of 87 kDa [22] and smooth muscle myosin light chain kinase [24]. A common feature uniting this muscle group is that they all probably interact with myosin [12]. There is also a preliminary report of titin (and nebulin) in brush-border [25].

2.2. *Super-repeat pattern of domains*

Titin cDNAs were identified in first instance by spe-

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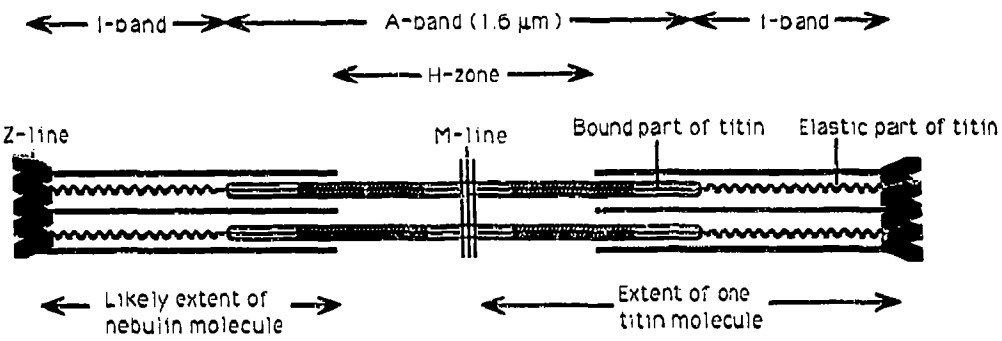

Fig. 1. Diagram showing the location of titin in the sarcomere. Also shown is the extent of nebulin, individual molecules of which probably span entire thin filaments. The C or constant packing region of the thick filament is shown stippled.

cific monoclonal antibodies [12,13]. Since these particular antibodies label the A-band in situ, the sequences are from this region of the molecule. Throughout most of the sequences so far determined, the class I and II domains are arranged in a regular eleven domain pattern or super-repeat, I-I-I-II-I-I-I-II-I-I-II, [13]. Local sequences at comparable positions in different super-repeats are more similar than comparable regions of the same domain class within a super-repeat. Since the interdomain spacing is known from electron microscopy to be ~4 nm [6,14], the super-repeat should span ~44 nm in situ (assuming that the titin molecule is not extensively wound around the thick filament) [13]. This is very close to the 43 nm repeat distance of the helix that describes the myosin geometry in thick filaments. More direct evidence of a 43 nm periodicity in A-band titin comes from some monoclonal antibodies which bind in this region of the molecule repetitively and with this spacing [26].

Interaction of titin with myosin has been reported [27] and was confirmed using both proteolytic fragments of native titin [28] and constructs expressed from cDNAs [13] in solid-phase assays. The latter studies both suggest that binding is to the light meromyosin region of the tail of myosin which forms the thick filament shaft. They also indicate binding to C-protein and X-protein (also known as slow C-protein). C- and X-protein are ~140 kDa components in the so-called C or constant packing region of thick filaments (Fig. 1) and have no known function. They are bound at multiple sites spaced 43 nm apart and their presence at this interval was difficult to explain. Myosin heads emerge from the filament at roughly one third of this distance (14.3 nm), and it was not understood what distinguishes one of the three 14.3 nm periods (Fig. 2). The evidence of a 43 nm periodicity in titin and the data indicating binding to C-and X-protein show how, in principle, assembly of this region of the thick filament may be specified.

2.3. *Kinase-like domain*

Titin sequences derived from monoclonal antibodies that label near the end of the thick filament and near the M-line show the super-repeat pattern breaks down away from the constant packing region [13]. There is not yet the intervening data to show where it is lost in the lateral part of the filament, but a cDNA identified by an antibody that labels ~100 nm from the middle of the filament reveals a different arrangement. Here the pattern of class I and II domains changes and there is also found a sequence of ~200 residues that is similar to the catalytic domains of protein kinases, the strongest similarity being to smooth muscle myosin light chain kinase (smMLCK) [24]. Such a catalytic-like domain was also found near the C-terminus of twitchin by Benian et al. [16], and these workers were the first to point out that flanking the catalytic domain in the smMLCK sequence are also class I and II motifs. When visualised by electron microscopy, isolated titin molecules have a string-like appearance and are about 900 nm long [29]. At one end is a globular head which is located in the M-line in situ [29] and which contains the C-terminus [13]. The site of the kinase-like domain is approximately 100 nm away from the head [13].

The similarity of these regions of titin and twitchin to smMLCK is striking, but also puzzling. The conservation (> 40%), despite the large evolutionary distance between *C. elegans* and vertebrates, indicates conserved functions. However, neither twitchin nor titin have yet been shown capable of phosphorylating myosin light chains in vitro. Moreover, even if this were to be demonstrated it is unclear how this reaction would function in vivo, since titin, twitchin and myosin are largely immobile in myofibrils. It has been shown that titin and twitchin can be both phosphorylated in vivo [30,31] and autophosphorylated in vitro [31,32]. However, the significance of these observations, particularly the autophosphorylation, is unclear, since the likely linear arrangement of the molecules in vivo would preclude their coiling up.

3. NEBULIN

Much less is known about nebulin than titin, since it has not yet been possible to isolate the native protein.

Most of the data available thus far have stemmed from antibodies produced to denatured preparations. Such antibodies are raised using nebulin purified in SDS and they have mainly been used in labelling studies [5,11,33–35]. They have also been used to identify cDNA clones [3] and a 560 amino acid partial sequence has been reported from one of these [36]. This sequence consists entirely of repetition of a motif consisting of approximately 35 amino acids. Analogous to the titin super-repeat, there is also a super-repeat consisting, in this case, of seven of the 35-residue motifs ($7 \times 35 = 245$ residues). Local sequences at comparable positions in different super-repeats are more similar then between 35- residue motifs within a super-repeat. There is a preliminary report that 80% of the entire nebulin molecule (which comprises roughly 8000 residues) has been sequenced and consists entirely of this $7 \times 35 = 245$ pattern [37]. Database searches did not reveal significant similarities between the nebulin sequence and known proteins [36]. However, the seven-fold periodicity reinforces the previous suggestion, based on antibody localisation, that nebulin is associated with thin filaments since the ratio of actin:tropomyosin:troponin in thin filaments is 7:1:1.

3.1. *Arrangement of nebulin in thin filaments*

The pattern of conserved residues in the 35-residue motif is suggestive of α-helical structure and the α-helix breakers P and G are rare in the middle of the motif [36]. However, in the published partial sequence there is no obvious heptad pattern of hydrophobic residues indicative of the coiled-coil α-helical packing common in fibrous proteins. The 7-fold character of the super-repeat suggests that successive discrete α-helical domains in the nebulin molecule bind to successive actin subunits in the thin filament. The nebulin molecule would then follow the long-pitch helical structure of the filament similar to tropomyosin (Fig. 3). This would require an axial translation/residue in nebulin only slightly greater than the value of 0.15 nm for a continuous α-helix. Such an arrangement would also be compatible with the estimated chain weight of nebulin of $\sim 8 \times 10^5$, since a completely α- helical molecule of this size would span ~1 μm, comparable to the length of the vertebrate skeletal muscle thin filament.

These considerations and the 2-fold screw symmetry of the filament resulted in a model in which pairs of single α-helical nebulin molecules staggered by 2.75 nm independently span each filament (Fig. 3) [36]. This configuration would also explain why nebulin was not detected in native thin filaments in 3D reconstructions from electron micrographs [38,39], since the diameter of the molecule would be below the ~4 nm resolution of the reconstructions. Binding experiments are also consistent with interaction between nebulin and actin in thin filaments. Although it is not possible to isolate the native protein, information about its interactions has been obtained using fragments expressed from cDNAs. Jin and Wang [40] report studies with constructs containing between 2 and 15 of the 35-residue repeat-motifs. Except for the small two-module construct, all the fragments bound to actin.

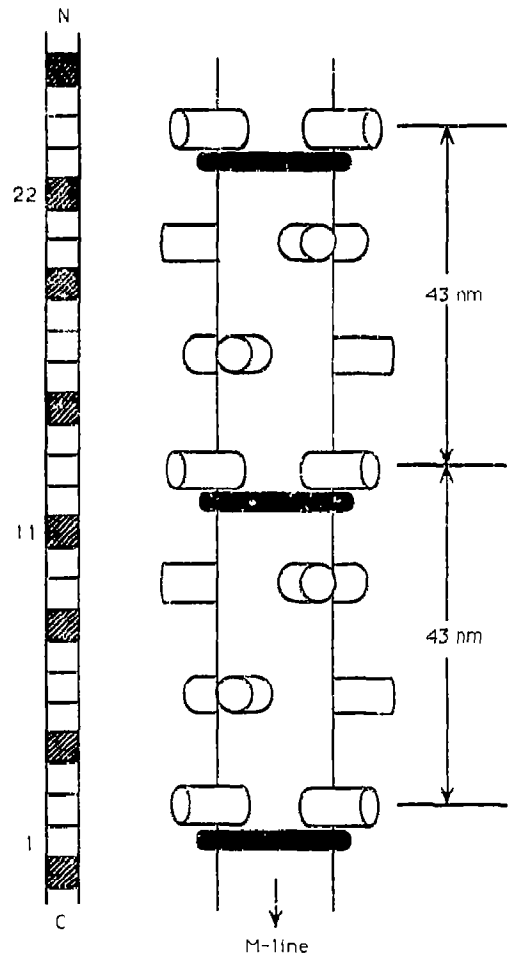

Fig. 2. Diagram showing the way in which the 11-domain super-repeat in titin is likely to span 43 nm in the thick filament. Clear blocks in the super-repeat represent class I (type III fibronectin-like) domains and hatched blocks class II (C-2 Ig-like) domains. The cylindrical projections on the filament each represent the heads of one myosin molecule. The disposition of C-protein (shown in black) is not known but it is shown here as a collar around the filament shaft. The location of titin on the thick filament, and the stagger of the super-repeat vs the myosin helical repeats, are also not known, although titin may be on the outside of the thick filament shaft [6].

Although the data suggest a nebulin molecule consisting of a series of 35-residue actin binding domains, there are reasons for believing that the interaction with the thin filament is more complex. The seven-subunit repeat of actin in thin filaments is defined by the regulatory proteins troponin and tropomyosin and there is no seven-fold character to filaments composed solely of actin. This suggests some type of interaction between nebulin and the regulatory proteins. There is also high (~70%) conservation between the 245-residue super-re-

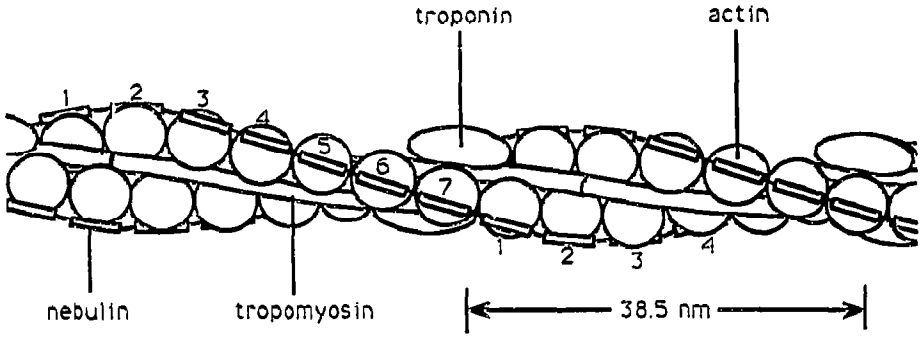

Fig. 3. Diagram showing the way in which successive nebulin domains formed by the 35-residue motif are proposed to interact with actin subunits in thin filaments. Numbers against one of the molecules indicate the super-repeat. It should be noted that there is very little information as to the radial position of nebulin on the filament, and that the location is depicted at high radius here only for simplicity. Adapted from [47].

peats and very high (98%) conservation between 300 residues common to both rabbit and human nebulin molecules [36]. This indicates that nebulin makes interactions throughout the 38.5 nm filament repeat. Since the actin binding site is likely to consist of only a few residues and to be conserved, and since troponin does not span 38.5 nm in the filament, interaction with tropomyosin was predicted [36]. Binding of the nebulin constructs to troponin and tropomyosin was not observed in solid-phase assays, but interaction of the constructs with actin was inhibited by these proteins [40].

3.2. *Protein-ruler hypothesis of nebulin function*

A plausible function for nebulin molecules spanning entire thin filaments is to regulate the length of the filament [11,36]. The precision with which the thin filament is assembled in vivo has been difficult to understand, since the sharpness of the edges of the H-zone in electron micrographs indicates the presence of exact numbers of actin, tropomyosin and troponin subunits (Fig. 1). These molecules are many times smaller that the filament length and it was unclear how assembly could be terminated exactly. A nebulin molecule acting as a giant template or 'protein-ruler' explains how, in principle, this may be achieved, since assembly may simply stop when the end of the molecule is reached.

A corollary of this hypothesis is that in muscles where thin filaments are exactly specified but have different lengths, the size of the nebulin molecule should vary in proportion. This prediction was borne out by analysis of chicken, rabbit and beef muscles on SDS-polyacrylamide gels [36]. These muscles have thin filaments that are 1.05, 1.1 and 1.3 μm long, respectively, and the mobility of the nebulin band on gels was seen to vary roughly in inverse proportion to these values. This correlation was confirmed by Kruger et al. using a similar size range but different muscles [41]. It is interesting to note that the converse of this argument is also true: in cardiac muscle, which does not have nebulin, the thin filament lengths are not exactly specified and vary by ~30% [42]. The gel analyses therefore indicate that nebulin is a family of proteins varying from perhaps 700 to 900 kDa, in filaments 1.05 to 1.3 μm long [41]. Interspecies size variations indicating titin isoforms have also been reported [43,44] and it has been proposed that differences in the elastic properties of these are responsible for the wide variations in resting stiffness of different striated muscles [45,46]. Kruger et al. [41] also reported that muscle labelled with three different nebulin monoclonal antibodies showed transverse striations with a periodicity of about 40 nm. This suggests that there are repeating features in the nebulin molecule with the thin filament periodicity of 38.5 nm. This in turn is compatible with the proposal that the 245-residue super-repeat in the molecule spans 38.5 nm.

4. CONCLUSIONS

The available data therefore suggest that nebulin and A-band region of titin are closely associated with the thin and thick filaments respectively. The evidence that nebulin regulates thin filament length is suggestive but not yet conclusive. Whether titin also acts as a protein-ruler to control exact length in thick filaments remains an open question. This and the other functions of titin, such as elasticity and enzymic activity, remain exciting challenges to be explored.

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