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REVIEW

Amyloid Properties of Titin

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Abstract—This review considers data on structural and functional features of titin, on the role of this protein in determination of mechanical properties of sarcomeres, and on specific features of regulation of the stiffness and elasticity of its molecules, amyloid aggregation of this protein *in vitro*, and possibilities of formation of intramolecular amyloid structure *in vivo*. Molecular mechanisms are described of protection of titin against aggregation in muscle cells. Based on the data analysis, it is supposed that titin and the formed by it elastic filaments have features of amyloid.

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The story of amyloid studies started in 1639 when Nicolaus Fontanus described a strongly increased human spleen containing large white inclusions, which seemed to be amyloid deposits [1]. After 150 years, in 1789, Antoine Portal for the first time described liver amyloidosis [2]. In 1854, Rudolf Virchow studied inclusions in a “waxy” liver and found structures that were stained with iodine similarly to starch grains in plants. These structures Virchow termed “amyloid” from the Latin “amylum” or Greek “amylon” that means starch. However, in 1859 Carl Friedreich and August Kekule showed that these inclusions did not have a carbohydrate component but contained proteins. These data became a basis for studies on amyloids as protein derivatives [3, 4].

It is generally thought that amyloids are protein aggregates with a cross-β-structure capable of binding with the dyes Thioflavin T and Congo Red, possessing apple-green birefringence in polarized light, and resistant to action of solvents and proteases [5-8]. By now, more than thirty proteins/peptides are known that form amyloids found in humans in various diseases [8, 9]. Amyloids have been shown to play a central role in the pathogenesis of Alzheimer’s and Parkinson’s diseases, type II diabetes, prion diseases, and systemic amyloidoses [6, 10-12]. Amyloid deposits have been found in the intima and media of vessels under aorta amyloidosis and in striated muscles in myocardites, myositis, and cardiomyopathies [13-15]. In particular, in blood vessels amyloid aggregates have been found formed by serum A protein and its fragments, which are accumulated in the intima and media of arterioles, under endothelium of venules [13]. In aorta amyloidoses, amyloids of medin are found [14]. In the cardiac muscle amyloids are found formed by such proteins as transthyretin, light and heavy immunoglobulin chains, serum amyloid-A, apolipoprotein, apolipoprotein AIV, fibrinogen α-chain, and atrial natriuretic factor, which contribute to development of “amyloid cardiomyopathy” or “cardiac amyloidoses” [15, 16]. Amyloid deposits containing Aβ-peptide are found in skeletal muscle in myositis [8]. However, at present, the paradigm of amyloids as negative formations for the cell, which cause development only of pathologic processes in living systems, is under revision. There are works that have demonstrated that amyloids can also play a positive role in the organism. Thus, in prokaryotes functional amyloids have been found of such proteins as curli in *E. coli* [17], tafi in *Salmonella* spp. [18], and chaplins in *Streptomyces coelicolor* [19]. Amyloids of these proteins were shown to participate in cell adhesion and formation of biological films, and chaplin amyloids are involved in production of aerial hyphae and dissipation of spores [20]. Functional amyloids are also found in eukaryotes: on the surface of spores and fruiting bodies of some fungi amyloid aggregates form dense hydrophobic monolayers [21], in the silk moth *Antheraea polyphemus* amyloids

Abbreviations: Aβ-peptide, amyloid β-peptide; FnIII, fibronectin III-like titin domain; Ig, immunoglobulin-like titin domain.
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have a protective function of the chorion (egg envelope) [22]; amyloids of spidroin are components of spider silk [23]. There are also known functional amyloid fibrils in mammals that are formed in melanosomes from proteolytic fragments of protein Pmel17 [24].

Recent studies have shown that many proteins under certain conditions can form amyloids *in vitro*. The concept of an "amylome" appears, which denotes the universe of proteins that are capable of producing amyloid-like aggregates/fibrils [25]. These proteins also include muscle ones: myosin subfragment-1 [26], myosin-binding proteins of the titin family (C-, X-, H-proteins) [27, 28], and titin [29]. Can amyloid aggregation of titin and other muscle proteins occur *in vivo*, and what role might these aggregates play in the cell? These questions require further investigations. Nevertheless, recent studies allow us to state that on folding of unfolded, titin domains misfolded conformations can be produced, which the authors have named "intramolecular amyloid" [30].

This review presents data on structural and functional features of titin, the role of this protein in determination of mechanical properties of sarcomeres, specific features of regulation of the stiffness and elasticity of its molecules, amyloid aggregation of this protein *in vitro*, and possibilities of formation of intramolecular amyloid structures *in vivo*. Molecular mechanisms are described of titin protection against aggregation of its molecules in muscle cells. Based on analysis of the data, it is supposed that titin and the formed by it elastic filaments have features of amyloid.

STRUCTURE–FUNCTIONAL FEATURES OF TITIN AND ITS DOMAINS

Titin (connectin) is a protein with the highest molecular weight among known proteins: the molecular weight of titin isoforms reaches ~3.0-3.7 MDa in striated and ~2 MDa in smooth muscles [31, 32]. Titin has a multidomain structure, and its gene can encode a protein with the following structure: a protein kinase domain, 152 Ig-domains, 132 FnIII-domains, 31 PEVK domains, 7 Z-repeats, and 33 unidentified domains (based on the UniProt database).

In sarcomeres of cardiac and skeletal muscles, the amount of titin is third after the amounts of actin and myosin. Titin molecules with the length of about 1 μm and diameter of 3-4 nm [33-36] occupy a half of the sarcomere from the M-line to the Z-disc and form the third type of so-called elastic filaments [37]. In the sarcomere A-zone, titin is bound with myosin (thick) filaments [38]. In the sarcomere I-band, some regions of the titin molecule can interact with actin (thin) filaments [39-49]. However, the major part of the titin molecule can pass easily in this zone connecting the ends of myosin filaments with the Z-disc. It is supposed that for each myosin filament, there are six titin molecules [50], with the N-ends overlapped in the Z-line and the C-ends overlapped in the sarcomere M-line [37]. It has been shown that the structure of this giant polypeptide is different in various regions of the sarcomere and contributes to their architecture and functioning. The major part (to 90%) of the titin molecule consists of repeated immunoglobulin-like (Ig) and fibronectin III-like (FnIII) domains with β-folded structure [37]. In addition to these domains, titin contains unique sequences: the kinase domain near the sarcomere M-line, N2A, N2B, and PEVK-elements in the sarcomere I-band, as well as phosphorylated regions in the sarcomere M-, I-, and Z-zones [37, 51-55]. Titin localization in all sarcomere zones, its elastic features, and interactions with many proteins promote the polyfunctionality of this protein. It has been shown that titin: (i) is a carcass for assembly of myosin filaments and sarcomere [56-60]; (ii) contributes to maintaining a highly ordered sarcomere structure and, therefore, to the muscle contractile function [61, 62]; (iii) participates in triggering and regulation of the actin–myosin interaction mediated through the binding with proteins of thin filaments [41, 63] and through changes in the ATPase activity of myosin [36, 64, 65]. It is supposed that titin, in complex with signaling proteins united by titin in the network, acts as a sensor of stretching and tension, participating in intracellular signalization, in particular, in the regulation of expression of genes of muscle proteins and of protein metabolism in the sarcomere [52, 54, 66-77].

As differentiated from the sarcomere A-zone where titin is firmly bound to myosin filaments, in the I-zone the elastic part of its molecule can develop a passive tension under stretching [78] and restoring force under sarcomere contraction [79-82]. Biophysical studies have shown that this part of the titin molecule behaves as a "nonlinear entropy spring", which straightens under the influence of a force from 20 to 30 pN and demonstrates elastic resistance under compression with the force of 2.5 pN [83]. Using atomic force microscopy or modeling the molecular dynamics resulted in the higher values of the force required for unfolding of individual titin domains or of their tandem sequences (table).

The table shows that the Z1Z2-repeats of titin are the most resistant to stretching. In the stretchable I-band, the Ig domains of titin are unfolding in the range of forces of ~150-330 pN [86, 88]. In the A-part of the titin molecule, which is tightly bound to the myosin filament and does not change its length under natural conditions, the FnIII domains are unfolding at force from 100 to 200 pN [87, 89]. It should be noted that such values of the force required for unfolding the amyloid structure were obtained also for some amyloid fibrils: 115 pN for amyloids of the human prion protein (huPrP90-231) [90], 250 pN for amyloids of glucagon [91] (this value was obtained using atomic force microscopy), 522 pN for amyloids of Aβ-peptide [90] (the values were calculated

Force values required for unfolding of individual titin domains or of tandem sequences of its domains

Region of titin molecule	Force required for unfolding of the region, pN	References
	Z-disc	
Z1Z2-domains*	750	[84]
	I-zone	
Domains I27-I34**	237/150-330	[85, 86]
Tandem of I27 (8 domains)**	204	[86]
Tandem of I28 (8 domains)**	257	[86]
Tandem of I32 (8 domains)**	298	[86]
Tandem of I34 (8 domains)**	281	[86]
Tandem of 3 domains I27, N2B and 3 domains I27**	200	[86]
Tandem of four identical copies of I55-I56-domains**	156-222	[87]
Tandem of four identical copies of I57-I58-domains**	200	[85]
I91-I98-domains***	225	[88]
Ig domains Sk47-Sk53 specific for skeletal muscles**	210	[87]
	Border of the I- and A-band	
I48-I54-domains**	200	[87]
	A-band	
A60-A65-domains**	180	[87]
15 domains Fn3**	113	[87]

* The method of molecular dynamic simulations was used.
** Measured using atomic force microscopy.
*** Measured using a modified method of atomic force microscopy termed "molecular force probe".

using molecular dynamics simulations). Obviously, the method of molecular dynamics simulations gives overestimated values of the force, and therefore, comparing these values with others obtained using atomic force microscopy is not correct. The analogy found between titin and amyloids seems to be a consequence of a high content in them of the β-folded structure, which can be unfolded by nearly the same force. Thus, this parameter cannot be used as a basis for comparing mechanical properties of amyloid aggregates and titin molecules. Such comparisons should be based on specific structural features of the protein that determine its mechanical features or the protein itself and of its aggregates. In this connection, titin is interesting because its elasticity and resiliency mediate mechanical features of sarcomeres, cells, and the muscle as a whole.

ROLE OF TITIN IN MECHANICAL PROPERTIES OF MUSCLE CELLS. REGULATION OF TITIN MOLECULE STIFFNESS IN SARCOMERES. FUNCTIONAL ROLE OF IDENTITY OF AMINO ACID SEQUENCE OF TITIN DOMAINS

There is now no doubt that titin plays a role in mediating the elasticity of muscle cells, in particular, cardiomyocytes. The coexpression of N2B- and N2BA-isoforms of titin differing in the length of the stretchable I-part and the change in their ratio under the influence of external or internal factors (during ontogenesis, development of pathological processes, hibernation, microgravity (for references see [92]) are considered as one of molecular mechanisms of changes in the stiffness of cardiomyocytes and cardiac muscle as a whole. The titin N2BA-isoform (molecular weight ~3300 kDa) has the longer, more elastic and correspondingly less stiff I-part of the molecule; the N2B-isoform (molecular weight ~3000 kDa) of titin has the shorter, less elastic, and correspondingly stiffer I-part (Fig. 1). The content of the titin N2B-isoform is shown to directly correlate with an increase in passive tension on stretching of cardiac muscle myofibrils [93-96]. The regulation of cardiomyocyte stiffness on the level of ratio of long and short titin isoforms can be easily followed in the ontogenesis of mammals [96-98]. During the early postnatal period, the ratio of titin isoforms changes along with an increase in the pump function of the heart: the fraction of the shorter (stiffer) isoforms of this protein increases [93, 96, 97]. In adult animals, there is a correlation between heart rate and the fraction of the short N2B-isoform of titin in the left ventricle myocardium. In particular, in small animals with heart rate of 140-650 beats/min (rabbit, hamster,

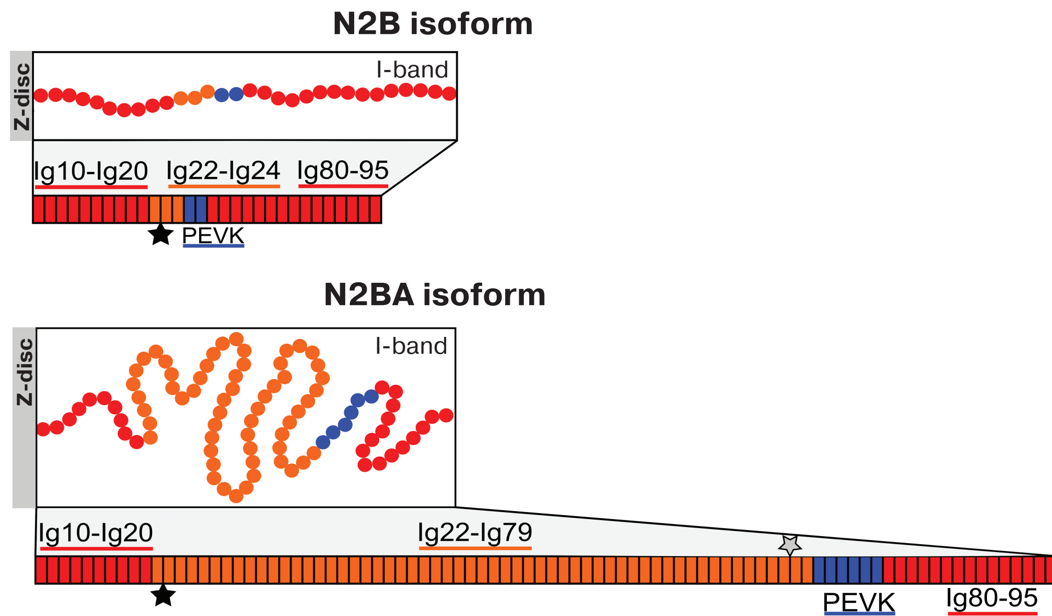

Fig. 1. Schematic picture of the domain structure of titin cardiac isoforms in the sarcomere I-band. The sequence of the titin domain structure is presented according to the UniProtKB database – Q8WZ42 (TITIN_HUMAN). Both isoforms contain identical sequences: the proximal sequence Ig10-Ig20 and the distal sequence Ig80-95. As differentiated from the shorter and stiffer N2B-isoform, the N2BA-isoform of titin has the longer middle region consisting of immunoglobulin-like domains Ig22-Ig79, the majority of which is a variable (differently spliced in N2BA-isovariants) region (Ig25-79). The PEVK sequence in the N2B-isoform is shorter. Both isoforms contain the unique N2-B sequence consisting of three Ig-like domains and a unique sequence containing 572 a.a. that is located after Ig22. In the N2BA-isoform there is also the N2A sequence consisting of four Ig-like domains and a region of unique sequence containing 106 a.a. (located before the PEVK region); in the scheme, their localization is shown by asterisks (N2B – black, N2A – gray).

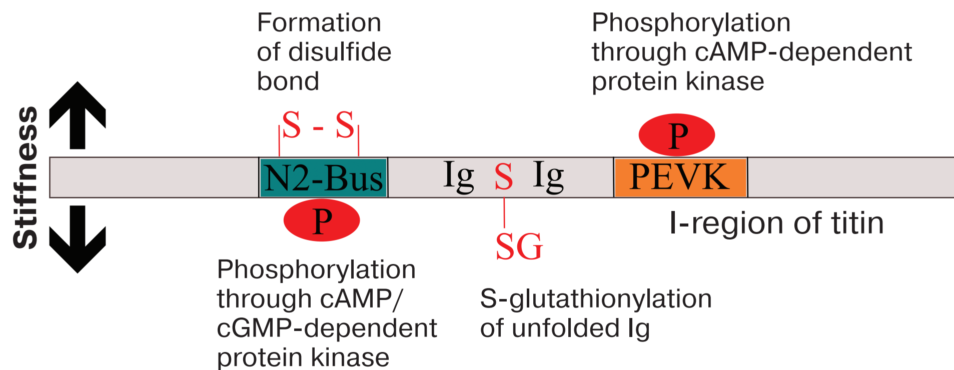

Fig. 2. Posttranslational mechanisms of changes in the stiffness of titin. Generation of disulfide bonds in the N2B region and phosphorylation of PEVK by cAMP-dependent protein kinase contributes to the stiffness of the molecule. On the contrary, phosphorylation through cAMP/cGMP-dependent protein kinase of the N2-B-sequence and S-glutathionylation of unfolded Ig-domains of titin reduces stiffness of titin molecules.

mouse, rat), the content of the stiff N2B-titin isoform in the left ventricle is 80-94%. In big animals with heart rate of 60-80 beats/min (sheep, pig, goat, cow), the content of the short titin isoform decreases to 41-76% [99]. It is supposed that the predominance of this titin isoform promotes an increase in the rate of the myocardium active contraction during the early systolic shortening and for the faster relaxation of the heart [99]. Thus, variability in the length of the resilient–elastic region of the titin isoforms in the I-band of the sarcomere is an important component of the molecular mechanism involved in the regulation of mechanical and contractile properties of cardiac muscle. Variability in the length of the extensible region of the titin molecule is characteristic also for its N2A-isoform in skeletal muscles. In particular, alternative splicing leads to generation of the titin N2A-isoform with shorter I-part of the molecule in the *m. psoas* (molecular weight ~3300 kDa) and longer I-part

of the molecule in the *m. soleus* (molecular weight ~3700 kDa) [100]. The functional significance of such variability in the length of the elastic I-part of the titin N2A-isoform in skeletal muscle isoforms remains unknown.

Stiffness and elasticity of titin molecules are regulated not only through changes in their length in the sarcomere I-zone, but also through posttranslational modifications [101] (Fig. 2). It has been shown that phosphorylation of the N2-B region by cGMP- or cAMP-dependent protein kinases decreases the stiffness of the titin molecule, whereas phosphorylation of the PEVK-sequence (enriched with residues of proline, glutamic acid, valine, and lysine) by cAMP-dependent protein kinase increases its stiffness [102, 103]. Stiffness of titin molecules can be induced by oxidative stress emerging in myocardium infarction, obesity, or diabetes mellitus, which makes worse the diastolic function of the left ventricle [104]. This is associated with generation of disulfide bonds in the titin N2-B sequence, which enhances the stiffness of the molecule. It has been also shown that the enhanced stiffness of titin can be compensated due to reversible S-glutathionylation of cysteines in the unfolded (due to increased load on the sarcomere) Ig-domains [104].

Mechanical properties of titin can be regulated more finely through variations in the identity of the amino acid sequence of its domains. This hypothesis was proposed based on data about the different aggregability *in vitro* of titin domains with different degree of amino acid sequence identity [105] and on our calculations of the Ig-domains of the variable region of the I-band of the N2B- and N2BA-isoforms of titin (Fig. 1). Data obtained using the dynamic light scattering method revealed that the aggregation rate *in vitro* of titin domains with higher identity of the amino acid sequence was higher than that of titin domains with lower identity of the amino acid sequence [105]. Using the BLAST program, we found that the domain of a variable region expressed only in the N2BA-isoform has higher identity of amino acid sequence (~32%, calculated for 26 domains) than that of other domains of the I-band of the N2B- and N2BA-titin isoforms (~23-25%, calculated for 30 domains). Up to now, there is no exact idea about the number of molecules of this giant protein and about their arrangement in the sarcomere I-band. However, considering data on titin aggregation *in vitro* [29, 30, 106, 107], including its amyloid aggregation [29], the binding *in vivo* of closely localized domains of one or several molecules of this protein cannot be neglected. In this case, it may be supposed that the domains of the variable region of the N2BA-isoform will be more prone to aggregation than other domains. This will lead to increase in the stiffness of the aggregated region of the N2BA isoform of titin, which can directly influence changes in the mechanical and contractile properties of muscle cells.

AGGREGATION PROPERTIES OF TITIN *in vitro* AND *in vivo*. MECHANISMS OF CELL PROTECTION AGAINST TITIN AGGREGATION

It is unknown whether the above-mentioned aggregation of titin can occur *in vivo*. However, studies on the *in vitro* aggregation of short amino acid sequences of some titin domains performed in 2005 [105] and other proteins [106] led to the conclusion that the capability of aggregating increases if the identity of the amino acid sequence of the domains is more than 30-40% [105]. These studies were preconditioned by the earlier works, which revealed the ability of neighboring identical domains of titin to form *in vitro* misfolded structures [107]. Recent studies published in *Nature Communications* in 2015 showed that, independently of the amino acid identity, misfolded conformations are produced during the folding of unfolded titin domains [30]. Molecular simulations allowed the authors to suppose that a significant part of these misfolded conformations can be an intramolecular amyloid [30]. Note that the higher identity in the amino acid sequence was favorable for production of more stable forms [30]. The authors of this study supposed that multidomain proteins including titin during evolution could undergo changes reducing the identity of the amino acid sequence of their domains for preventing or decreasing the probability of formation *in vivo* of resistant protein aggregates, including amyloid aggregates [30].

Nevertheless, the possibility of titin molecules to form aggregates *in vivo* in the sarcomere I-band cannot be excluded. It has been shown that during sarcomere extension, the titin domains can unfold, uncovering latent hydrophobic regions, and this can lead to aggregation of the protein and disturbance of its functions [108, 109]. The aggregation of unfolded Ig-domains in the I-band can result in an abnormally high stiffness of titin molecules and, consequently, of myocytes [110]. Interaction of this part of titin molecule with small heat shock proteins (sHsps) capable of suppressing aggregation of many proteins is one of the mechanisms preventing Ig-domain aggregation in the sarcomere. In particular, it has been shown that Hsp27 and αB-crystallin do not interact with distal Ig-domains localized near the sarcomere A-band, but they bind to Ig-domains of the titin molecule extensible part, which is localized between the Z-disc and PEVK [110]. It should be noted that these Ig-domains, as discriminated from PEVK and N2-B-sequences, have a higher tendency for aggregating under conditions of partial denaturation [110]. How can these differences be explained? It is known that proteins with unordered structure are resistant to aggregation due to different factors: a high total charge and a low hydrophobicity, a small number of amyloidogenic regions, and a high content of proline residues [105, 111]. The PEVK region of titin is enriched with proline and has a comparatively high total charge [34]. The N2-B-sequence also contains many

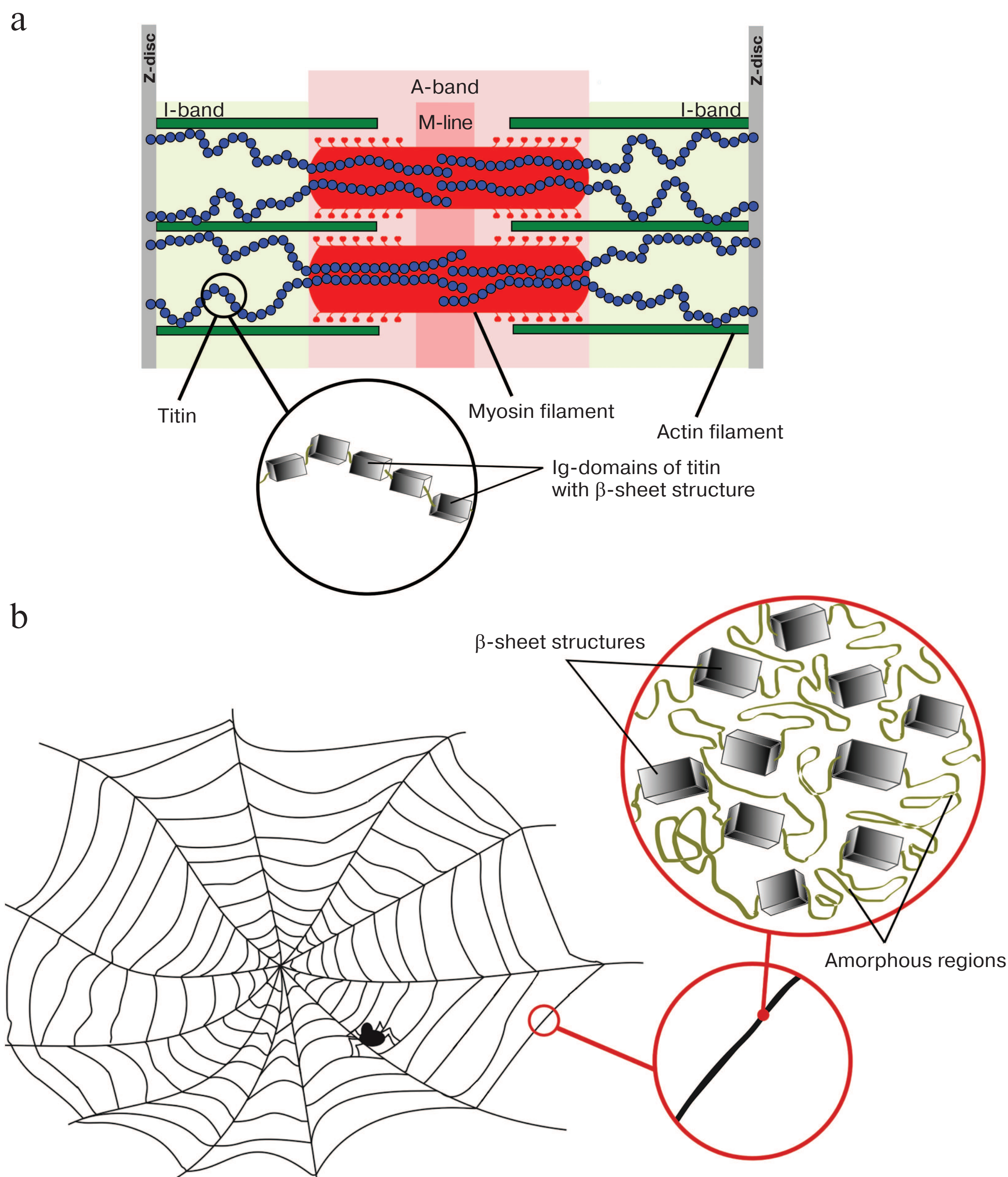

Fig. 3. Morphological similarity of titin molecule (a) and functional amyloids of spidroin – the protein of spider silk (b).

proline residues and higher total charge than the typical Ig-domain of titin [110]. Thus, the presence in the titin molecule of PEVK and N2-B-sequences decreases the probability of *in vivo* aggregation of this part of the protein. However, under conditions of muscle overextension, which occurs during intensive physical exercises and in pathologies (e.g. at ischemia when the death of myocytes can lead to overextension of the adjacent cells), the probability of unfolding and, consequently, of aggregation of titin Ig-domains increases [86]. Although the unfolded Ig-domains of titin are folding repeatedly *in vitro* in the absence of small heat shock proteins [108], this process can be difficult *in vivo* under a sufficiently tight localization of proteins in the sarcomere even if the above-men-

tioned proteins are present. The higher identity of the amino acid sequence of the variable region of the N2BA-isoform increases the probability of titin aggregation *in vivo*, which undoubtedly will lead to a significant increase in the stiffness of titin filaments. In turn, this will have negative consequences for mechanical properties and contractility of myocytes and the muscle as a whole. Increased proteolysis of titin in the sarcomere can protect against these changes. However, this pathway does not exclude accumulation of titin fragments in cells and formation of amyloid aggregates by them in the cytosol. In this case, cell autophagy is the last stage of the organism's protection against uncontrolled aggregation of cytoskeletal proteins of the sarcomere [110, 112, 113].

Can titin aggregates accumulate *in vivo*? Our studies *in vitro* have shown that such possibility exists. In particular, it has been shown that under conditions close to physiological, smooth-muscle titin can form amyloid aggregates within short time intervals (tens of minutes) [29]. Circular dichroism did not reveal structural rearrangements of the type α-helix transition into β-structure, which are characteristic for other amyloidogenic proteins [114, 115]. A pronounced cytotoxic effect of titin amyloid aggregations was detected on smooth muscle cells of bovine aorta culture, and this effect was accompanied by disorganization of the actin cytoskeleton [29]. These data not only demonstrated that titin amyloid aggregation can occur *in vivo*, but also suggest that this protein should be involved in the development of muscle amyloidoses.

POSSIBLE FUNCTIONAL ROLE OF TITIN AGGREGATION IN SARCOMERES

It is generally believed that amyloids play a negative role in living cells. Due to their stiffness, amyloid fibrils can mechanically tear the cell membrane; therefore, the accumulation of amyloid aggregates, which are resistant to proteolytic degradation, leads to cell death [116, 117] and development of a pathologic process [118]. However, it should be noted that a high stiffness is also inherent in functional amyloids, e.g. amyloids of spidroin, a protein participating in formation of solid elastic threads of the spider silk. Functional amyloids of spidroin and the titin molecule are similar in morphology (Fig. 3). The silk protein is enriched with a β-folded structure (up to 40-50% of the total volume of a silk fibril [119]), whereas the remaining part is filled with less ordered, possibly amorphous structures [120]. In addition to the β-folded structure, the titin molecule has about 50% amorphous structures that has been shown by circular dichroism [29] and short α-helical regions, e.g. in the PEVK sequence [40] – in the kinase domain [121].

Thin elastic threads of titin molecules form the intracellular cytoskeletal extensible carcass, which determines mechanical properties of muscle tissue. Perhaps the aggregation of titin molecules in the sarcomere I-band, including "formation of intramolecular amyloid structures", can play a functional role – to contribute to increasing muscle stiffness. In turn, this can play a protective role counteracting overextension of sarcomeres having unfavorable consequences for the muscle. Changes in the mechanosensory role of titin in the case of aggregation of its molecule also cannot be excluded.

In conclusion, based on analysis of data on the properties of titin, we pay special attention to the following facts: titin forms the intracellular cytoskeletal elastic carcass that determines mechanical properties of sarcomeres and muscle as a whole; titin forms amyloid aggregates *in vitro*; on folding of unfolded domains in the titin molecule, amyloid-like structures can be produced; titin has a morphological similarity with functional amyloids of spidroin. Based on these data, can we state that titin is a molecular amyloid? Up to now, we cannot answer this question in the affirmative. However, it is clear that during evolution one of the most unique structures of living nature has been created that combines features of amyloid and elastic protein participating not only in formation of sarcomere and maintenance of its structure, but also in the fine regulation of the actin–myosin interaction and intracellular signalization.

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