Novex-3, the tiny titin of muscle

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Abstract The giant multi-functional striated muscle protein titin is the third most abundant muscle protein after myosin and actin. Titin plays a pivotal role in myocardial passive stiffness, structural integrity and stress-initiated signaling pathways. The complete sequence of the human titin gene contains three isoform-specific mutually exclusive exons [termed novel exons (novex)] coding for the I-band sequence, named novex-1 (exon 45), novex-2 (exon 46) and novex-3 (exon 48). Transcripts containing either the novex-1 or novex-2 exons code for the novex-1 and novex-2 titin isoforms. The novex-3 transcript contains a stop codon and polyA tail signal, resulting in an unusually small (~700 kDa) isoform, referred to as novex-3 titin. This ‘tiny titin’ isoform extends from the Z-disc (N-terminus) to novex-3 (C-terminus) and is expressed in all striated muscles. Biochemical analysis of novex-3 titin in cardiomyocytes shows that obscurin, a vertebrate muscle protein, binds to novex-3 titin. The novex-3/obscurin complex localizes to the Z-disc region and may regulate calcium, and SH3- and GTPase-associated myofibrillar signaling pathways. Therefore, novex-3 titin could be involved in stress-initiated sarcomeric restructuring.

Keywords Titin · TTN sequence · Novex-3 titin · Obscurin

Introduction

Titin, the giant filamentous protein, is encoded by the TTN gene that is located on chromosome 2q31 (Bang et al. 2001). The human titin gene contains 363 exons, encoding 38,138 residues with a maximum molecular mass of 4200 kDa (Bang et al. 2001). Titin is composed of immunoglobulin (Ig) and fibronectin type III (FN3) domains (Labeit and Kolmerer 1995). It extends from the Z-disc (N-terminus) to the M-band region (C-terminus) of the sarcomere; hence a single titin molecule spans half the length of the sarcomere in both skeletal and cardiac muscle (Furst et al. 1988). Z1/Z2, the most N-terminal domains of titin’s Z-disc binding region, act as a mechanosensor (Linke 2008). Titin’s extensible I-band region has a molecular spring function (Granzier and Irving 1995). The A-band region of titin is bound to myosin via FN3 domains and is functionally inextensible (Tskhovrebova and Trinick 2004; Linke 2008). Titin’s M-band region contains multiple structural and signaling complexes (Witt et al. 2005; Musa et al. 2006). Moreover, recent findings show that titin integrates and probably coordinates diverse signaling pathways, especially in cardiomyocytes (Granzier and Labeit 2004; Kruger and Linke 2011; LeWinter and Granzier 2013).

Titin isoforms

Titin is found in cardiac and skeletal muscle. Differential mRNA splicing events occur mainly in the I-band region, resulting in different isoform classes of titin. The N2A (~3.7 MDa) isoform is present in skeletal muscle and its spring region varies in size in different muscle types (Fig. 1a). There are three major classes of cardiac titin isoforms: the adult N2BA (3.3 MDa) and N2B (2.97 MDa) (Fig. 1b) and the fetal cardiac titin (FCT) (3.5–3.6 MDa) (Labeit and

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Fig. 1 Titin isoforms in skeletal and cardiac muscle. Adult striated muscles express three full-length titin isoforms (**a**, **b**). **c** Analysis of the complete gene sequence of titin revealed three novex isoforms, with the novex-1 and novex-2 isoforms being nearly identical to the cardiac N2B isoform (Bang et al. 2001; Linke 2008; Roberts et al. 2015) and novex-3, the 'tiny titin' being an unusually small (625 kDa) titin isoform that

extends from the Z-disk to novex-3. All novex isoforms are expressed in cardiac and skeletal muscle (Bang et al. 2001). Figure is based on Fig 3. in Bang et al. (2001), with permission. *PEVK* Rich in proline (P), glutamic acid (E), valine (V) and lysine (K) amino acid residues, *N2A*, *N2B* titin isoforms, *Ig* immunoglobulin

Kolmerer 1995; Freiburg et al. 2000; Bang et al. 2001; Lahmers et al. 2004; Opitz et al. 2004; Greaser et al. 2005). The adult cardiac titin isoforms contain constitutively expressed proximal and distal tandem Ig regions, an N2B unique sequence (N2B-us) and a PEVK domain [rich in proline (P), glutamic acid (E), valine (V) and lysine (K) amino acid residues]. The FCT and N2BA isoforms also have a middle tandem Ig-domain region (Bang et al. 2001; Lahmers et al. 2004). N2B is known as the stiffer titin isoform, as it is shorter and more force is needed to stretch it (Trombitás et al. 2000). In humans, the N2BA:N2B expression ratio is approximately 40:60 (Neagoe et al. 2002). Interestingly, an isoform-switching occurs during the perinatal period from a high to low N2BA:N2B ratio, resulting in a higher myofibrillar passive tension in adults (Lahmers et al. 2004; Opitz et al. 2004; Kruger et al. 2006; Linke 2008). However, this titin expression ratio can change in cardiac diseases. Ischemic cardiomyopathy patients express a higher N2BA:N2B ratio that is associated with decreased myocardial stiffness (Neagoe et al. 2002). The N2BA:N2B ratio is also elevated in dilated cardiomyopathy (DCM) accompanied by decreased titin-based passive tension (Nagueh et al. 2004; Makarenko et al. 2004). A possible reason for the isoform shift in heart diseases is a compensatory mechanism as an adaptation to the increased passive stiffness due to fibrosis (Nagueh et al. 2004). The RNA binding motif protein 20 (RBM20) regulates titin splicing. Humans with RBM20 missense mutations and rats with the RBM20 gene deleted have cardiomyopathy with fibrosis, as well as arrhythmia with sudden cardiac death (Guo et al.

2012). Methawasin et al. inactivated RBM20 for the first time in a mouse model, by deleting the RNA recognition motif (RRM) domain. Their findings show that diastolic stiffness is reduced at cardiomyocyte and left ventricular levels after the mutation of RBM20 (Methawasin et al. 2014.) Furthermore, loss of RBM20 ameliorates diastolic dysfunction and improves exercise tolerance in a transverse aortic constriction–deoxycorticosterone acetate mouse model (Methawasin et al. 2016; Hinze et al. 2016).

Additionally, analysis of titin's gene sequence revealed full-length titin variants, namely novex-1 and novex-2, and the smaller novex-3 (Fig. 1c). Real-time PCR studies show that these isoforms are expressed in the myocardium and in skeletal muscle (Bang et al. 2001).

Novex isoforms

Determination of titin's human gene sequence revealed three novel exons 5' of the PEVK and N2B spring elements, referred to as titin novex-1, -2 and -3. These three exons are 375, 576 and 6398 bp long (Bang et al. 2001), respectively, and are expressed in striated muscles (Bang et al. 2001; Granzier et al. 2007). However, real-time PCR studies suggest that novex-1, -2 and -3 transcripts are less abundant in skeletal muscle than in the myocardium (Bang et al. 2001).

Novex-1 (exon 45) and novex-2 (exon 46) inclusion produces the novex-1 and novex-2 titin isoforms (Fig. 1c). They each add an additional unique Ig domain and are nearly

identical to the cardiac N2B isoform (Bang et al. 2001; Linke 2008; Roberts et al. 2015).

The C-terminal region of ‘tiny titin’, encoded by the novex-3 exon, has characteristics not found in the full-length titin isoforms. The novex-3 titin isoform contains six Ig domains (I18–I23) that are specific to this isoform and conserved among species (Bang et al. 2001). Novex-3 titin was shown to be expressed in human cardiac and skeletal muscle, but not in smooth muscle or other non-muscle tissue (Bang et al. 2001). In silico analysis of titin’s entire I-band sequence from human, mouse and chicken samples show a unique 450-amino acid segment between Ig domains I20 and I21 specific to novex-3, as well as a segment of N2A between Ig domains I80 and I81 from longer titin isoforms; these segments are predicted to have regions with coiled-coil potentials, suggesting that they are evolutionarily conserved features of titin (Granzier et al. 2007). Furthermore, dimer formation has the highest probability for novex-3 and N2A segments (Granzier et al. 2007). Novex-3 includes a stop codon, which results in an unusually small, predicted 625-kDa novex-3 titin isoform (Fig. 1c). This isoform spans from the Z-disk domains to novex-3 domains in the proximal I-band region of the sarcomere; however, unlike the larger titin isoforms, it is too short to reach the A-band (Bang et al. 2001). Analysis of chicken, mouse, cow, frog and human titin revealed identities in a range of 39 to 48% of novex-3 (Brown et al. 2006). Moreover, the *Xenopus* titin novex-3 (Xtn3) ortholog is expressed in the embryo in all somites and primary myocytes but not in embryonic secondary myocytes, adult heart or skeletal muscle (Brown et al. 2006).

PCR (Bang et al. 2001; Granzier et al. 2007), Western blot, yeast two-hybrid screening (Y2H), immunoelectron and immunofluorescence microscopy studies were performed in the search for possible functions and potential ligands of novex-3 titin (Bang et al. 2001). Anti-Z1-Z2 antibodies verify that the N-terminus of novex-3 titin includes the N-terminal region of conventional titin. The C-terminus has the six novex-3-specific Ig domains. The 3’ end of the novex-3 RNA contains the stop codon polyadenylation signals and lacks any PEVK region-encoding exons (Bang et al. 2001). Thus, the novex-3 exon encodes a C-terminus leading to a truncated titin isoform and functions as an alternative 3’ end. In skeletal muscle, anti-novex-3 antibodies react with a band that migrates ~700 kDa, just below nebulin (Bang et al. 2001).

Obscurin

Yeast two-hybrid screening revealed that novex-3 interacts with the giant myofibrillar protein obscurin (Bang et al. 2001). Obscurin is the third giant protein in vertebrate muscle after titin and nebulin, encoded by the OBSCN gene on

chromosome 1q42. Like titin, obscurin has multiple isoforms: its molecular weight in humans was initially identified as 721 kDa with expression in skeletal and cardiac muscle (Young et al. 2001). Western blot studies show isoform size variability in rabbit skeletal muscle, ranging from 680 to 750 kDa (Prado et al. 2005). Makarenko et al. found that obscurin appears as a strong band at 900 kDa in human heart samples; however there is also a less obvious second band at a lower molecular weight (Makarenko et al. 2004). Moreover, Bang et al. showed that the antibody raised to obscurin’s N-terminal domains gives a reaction at 750 kDa in adult rat left ventricles (Bang et al. 2001). The variation in obscurin isoforms was reviewed by Ackermann et al. (2014). Obscurin is constructed from Ig and FN3 domains. Interestingly, most of the Ig domains are homologous to the tandem immunoglobulin domains of titin’s I-band region. Within its C-terminal region, obscurin contains IQ, dbl homology (DH), pleckstrin homology (PH) and src homology 3 (SH3) domains. The IQ motif is a functional domain interacting with calmodulin, and this might be part of a calcium sensor system. The DH domains (Rho-GEF domains) catalyze guanosine-5’-diphosphate (GDP)/guanosine-5’-triphosphate (GTP) exchange in small Rho GTPases, leading to multiple cellular responses as cytoskeletal reorganization or cell cycle control. DH motifs are linked to a PH domain, indicating that this domain plays a role in the function of DH motifs. The SH3 domains usually participate in signaling pathways of multicomponent protein complexes (Young et al. 2001).

An obscurin fragment (Ob48-49) binds to the Z9 and Z10 domains of titin in transfection and in vitro binding assays (Young et al. 2001). However, in mature myofibrils obscurin localizes in the M-band. Analysis of the localization of obscurin revealed that the Z-disk connection generally occurs in cells where actin is in a non-parallel, irregular pattern. The M-band interaction is observed in cells where myofibrils show parallel bundles in the cell center, such as that found in mature cardiomyocytes. These findings suggest that the interaction between titin and obscurin may be transient and developmentally regulated (Young et al. 2001). Additionally, immunoelectron microscopy measurements confirm that the antibody directed to F6/I7 epitopes (N-terminal of obscurin’s tandem Ig segments) stains both the Z-disk and M-band in adult rat cardiac and skeletal muscle (Bang et al. 2001). Similarly, Z-disk-associated staining is detected in immunofluorescence studies; however M-band-associated staining is also found in some preparations by using anti-F6/I7 antibody. In contrast, the obscurin I48/I49/F50 epitopes (located at the C-terminus of obscurin’s tandem Ig segment) stain the I-band region close to the Z-disk (Bang et al. 2001). Moreover, Bang et al. expressed the obscurin fragment I48/I49/F50 as a green fluorescent protein in live rat cardiac myocytes, and they observed that the fragment specifically targets to the Z-disk region in more than 80% of transfected cells (Bang et al. 2001).

The novex-3 titin isoform specifically interacts with obscurin. Glutathion-S-transferase (GST) fusion protein pull-down assays using obscurin I48/I49/F50 domains pull down in vitro transcribed/translated exon 48. Mapping of the interaction site in novex-3 was accomplished with a series of deletion constructs with obscurin I48/I49/F50-GST-pulldowns; a region of novex-3 just before Ig domain I21 interacts with this region of obscurin (Bang et al. 2001).

Possible functions of novex-3 titin

To date, it is unclear why very differently sized titin classes are expressed from the same TTN gene. Since it is unlikely that novex-3 titin can reach from the Z-disk to the A-band, a direct role in passive tension generation is unlikely. A possible role for novex-3 has been suggested based on the stoichiometry of six titin molecules per half thick filament, or three titin molecules per thin filament (Granzier and Irving 1995). Considering that the thick filament has a threefold symmetry, six titins can be easily accommodated. However, the thin filament has a twofold symmetry and having three titins per thin filament presents a paradox (i.e. either 2 or 4 molecules per thin filament are expected), referred to as the sarcomere symmetry paradox (Liversage et al. 2001). It has been hypothesized that the co-expression of novex-3 titin and full-length titins may adjust the titin filament system to both three- and twofold symmetries of thick and thin filaments, respectively (Bang et al. 2001). Is there enough novex-3 titin expressed to play such an adjustment role? Based on the analysis of Western blot studies using Z1/Z2 antibody, the novex-3 protein level is approximately one-tenth that of full-length titin (Bang et al. 2001). This 1:10 ratio is not sufficient to bring the three titin molecules per thin filament up to four for all thin filaments—only one of three thin filaments would get the desired number. It seems likely that there must be a different function for novex-3 titin.

Results from immunoelectron and immunofluorescence microscopy studies suggest that novex-3 titin might also play a role in myofibrillar signaling. This might involve obscurin, as it interacts with novex-3 titin; it has been found that the novex-3/obscurin complex extends when the sarcomere is stretched (Bang et al. 2001). This raises the possibility that both novex-3 and obscurin have elastic properties that may be conferred by the tandem Ig segments of both proteins. The coiled-coil potential suggests that the C-terminus of novex-3 might multimerize, which could be important in how novex-3 titin is integrated into the I-band lattice and possibly how it interacts with obscurin (Granzier et al. 2007).

The Z-band region of full-length titin and novex-3 titin both interact with a plethora of proteins, including small-ankyrin-1 isoform (sAnk-1) (Kontogianni-Konstantopoulos and Bloch 2003), telothonin (T-cap) (Gregorio et al. 1998),

cardiac-restricted ankyrin repeat protein (CARP) (Zou et al. 1997; Miller et al. 2003) or muscle LIM protein (MLP) (Arber et al. 1994; Hoshijima 2006). The novex-3 titin/obscurin complex appears to be another Z-disk-associated protein complex and might have an important role in Ca^{2+} , SH3 and GTPase associated signaling pathways. The novex-3 titin/obscurin complex could be involved in stretch-initiated sarcomeric restructuring that occurs during muscle development and cardiac diseases, which merits further research (Bang et al. 2001).

Recent studies have identified TTN as a major human disease gene (Herman et al. 2012). Truncations and missense mutations in TTN result in cardiac disease (DCM) and skeletal muscle (myofibrillar myopathies) disease (Gerull et al. 2002; Herman et al. 2012; Ceyhan-Birsoy et al. 2013; Chauveau et al. 2014). Estimates suggest that TTN mutations are responsible for 25% of the DCM cases (Herman et al. 2012). TTN truncating variants (TTNtv) are found along full-length titin in DCM patients; however in the most severe forms of DCM, truncations tend to reside in the A-band (Herman et al. 2012; Roberts et al. 2015). Some TTNtv are also found in novex exons, but so far they have only been found in healthy individuals (Roberts et al. 2015) and are not associated with DCM. Although at present there is lack of evidence for pathogenicity of novex-3 mutations, upon closer examination and with the rapidly expanding number of patients who are sequenced we cannot exclude that novex-3 titin mutations might play a role in the pathogenesis of some cardiomyopathies. Clearly, further work is needed on ‘tiny titin.’

Compliance with ethical standards

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Conflict of interest Dalma Kellermayer declares that she has no conflicts of interest. John E. Smith declares that he has no conflicts of interest. Henk Granzier declares that he has no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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