Titin: An Endosarcomeric Protein That Modulates Myocardial Stiffness in DCM

YIMING WU, MD, PhD,* SIEGFRIED LaBEIT, MD,† MARTIN M. LeWINTER, MD,‡ HENK GRANZIER, PhD*

Pullman, Washington; Mannheim, Germany; Burlington, Vermont

ABSTRACT

Titin is the third myofilament of the cardiac muscle sarcomere, with a single molecule spanning the half sarcomere. Titin contains a molecular spring segment that generates passive force in sarcomeres stretched above the slack length and restoring forces in sarcomeres shortened below this length. The roles of titin in heart disease remain to be established. In this work we review recent developments in the understanding of titin’s role in cardiac muscle. We focus on both short-term and long-term modulation of titin-based muscle stiffness, as well as on the recently discovered interplay between titin and actomyosin interaction, suggesting a possible role for titin in the Frank-Starling mechanism of the heart. Finally, we present evidence that suggests that titin plays a role in elevating passive stiffness of myocardium of dilated cardiomyopathy (DCM) hearts, via alterations in titin isoform expression.

Key Words: Titin, myocardium, diastole, phosphorylation, length-dependent activation, cardiomyopathy.

In addition to the thin and thick filaments, cardiac sarcomeres also contain a third filament system composed of titin. Titin is the largest protein known, with a single polypeptide containing ~30,000 amino acid residues. Titin constitutes ~10% of the myofibrillar protein mass of the myocardium, making it the third-most abundant muscle protein (after myosin and actin). Immunolabeling studies using epitope-mapped titin-specific antibodies have revealed that titin’s N-terminus spans the Z-disk of the sarcomere and that titins from adjacent sarcomeres overlap in the Z-disc, in an antiparallel fashion by ~100 nm. Similarly to the titin overlap in the Z-disc, titins from adjacent half-sarcomeres overlap in the M-line region of the sarcomere.

As a result, titin molecules form a filamentous system that is continuous along the full length of the sarcomere (Fig. 1A). Titin’s continuity along the sarcomere, and its early appearance during muscle development support the proposal that titin functions as a template for sarcomere formation.

Immunolabeling studies of titin in situ have revealed that the near Z-line and A-band segments of titin are inextensible because of the thin-filament and thick-filament binding properties of these segments, respectively, whereas the remaining segment of titin in the I-band is extensible (see Fig. 1A). The extensible I-band region of cardiac titin has a complex sequence with three distinct elements that extend along the physiologic sarcomere length (SL) range of the heart: (1) the PEVK segment (rich in proline [P], glutamic acid [E], valine [V], and lysine [K] residues), (2) serially linked immunoglobulin (Ig)-like domains that make up tandem Ig segments, and (3) the N2B element. Extension of these three sequence elements dominates at different sarcomere lengths, giving rise to a unique titin-based force–sarcomere length relation that could not be achieved with a homogenous segment.
Role Of Titin In Passive Force Generation Of Cardiac Myocyte

Because of titin’s multiple attachments points in the sarcomere, specific methods to extract only titin from the sarcomere are unavailable. The contribution of titin to passive force generated by cardiac myocytes can be studied, however, by extracting the thin and thick filaments (with relaxing solutions that contain high concentrations of KCl and KI, respectively) and removing thereby titins’ anchors in the sarcomere. \(^2\) To study the contribution of titin’s passive tension to the overall passive tension generated by isolated cardiac myocytes, one may also take advantage of the extreme proteolytic sensitivity of titin. \(^3\) Because of the high trypsin sensitivity of titin’s PEVK domain, a mild trypsin treatment can be used to degrade titin’s I-band region without significantly affecting other proteins. \(^3\) Trypsin is a proteolytic enzyme that splits peptide bonds on the carboxyl side of lysine and arginine.

Within the extensible region, the PEVK segment appears most prone to degradation, probably as a result of its disordered conformation (which promotes accessibility for trypsin) and its high lysine content. \(^5\) Even in absence of added trypsin, proteases present in the cell can result in cleavage of the full-length molecule (known as T1) into a large degradation product, known as T2. \(^13\) T2 is the product of proteolytic cleavage within the PEVK domain \(^15\) and comprises the portion of the molecule C-terminal to the PEVK domain. Fig. 1B shows an example of a force-sarcomere length relation of a cardiac myocyte measured before and after trypsin-based titin degradation. Results indicate that titin is the main source of passive force in the cardiac myocyte.

**Splice Isoform Diversity Of Titin**

Titin is encoded by a single gene that locates in both human and mouse on the long arm of chromosome 2. \(^16\)
From the titin gene, messenger RNAs (mRNAs) of up to 100 kb are transcribed. These mRNAs are translated into 27,000 to 33,000 residue giant polypeptides, whose N- and C-termini are anchored within the Z-disc and M-line lattice of the sarcomere, respectively. Differential splicing cascades in heart and skeletal muscles adjust the number and length of the expressed spring elements. As a result, titin’s I-band segment varies from ~800 kDa in the stiff cardiac muscle N2B isoform to up to ~1.5 MDa in the elastic soleus skeletal muscle. In the heart, titin transcripts are processed by two distinct splice routes that vary within their extensible I-band region: the so-called N2B titins (containing the N2B element) and N2BA titins (containing both the N2B and N2A elements); see also Fig. 2B). N2BA titins contain a much longer PEVK segment than do N2B titins (600-800 vs. 163 residues), as well as an additional tangential Ig segment. As a result of these sequence differences, N2BA titin has a higher molecular mass than N2B titin (~3.3 vs. 2.97 MDa) and has a slower mobility on SDS-PAGE gels.

Recent studies of myocardium isolated from various species indicate that the expression level of N2B and N2BA cardiac titins varies from predominantly N2B (small rodents) to predominantly N2BA (bovine atrium), with many species (including human) coexpressing both isoforms at intermediate levels. Interestingly, the coexpression ratio reveals transmural gradients with increasing N2B levels when comparing subendocardial to subepicardial regions. Mechanical experiments with single cardiac myocytes isolated from species that express predominantly N2B or N2BA titin have shown that the passive stiffness (slope of passive force–SL relation) is much steeper in N2B expressing cells that in N2BA cells (Fig. 3).

The higher passive stiffness of N2B expressing cardiac myocytes is likely to result from its shorter I-band segment that, at a given SL, results in an extensible segment with a fractional extension (end-to-end length divided by the maximal length) that is higher than in N2BA titin. The relation between fractional extension

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**Fig. 2.** (A) SDS-PAGE of left ventricular myocardium of rat, rabbit, bovine, and human. All samples contain a minor amount of T2 (degradation product of the full-length titin molecule, or T1) and a T1 band of similar mobility. In addition bovine and human (and, to a minor degree, rabbit) contain a second lower mobility T1 band. (Figure reproduced from Cazorla et al, Fig. 1, with permission). The top T1 band is a N2BA cardiac isoform and the bottom band T1 band is N2B titin (as determined by Western blotting with isoform-specific antibodies [see Freiburg et al]). (B) Sequence of extensible region of N2B and N2BA titin (based on Freiburg et al). Red rectangular boxes, immunoglobulin-like domains; yellow, PEVK; blue, unique sequences.
and passive stiffness can be evaluated by considering the molecular mechanism of passive force generation. A passive force model has emerged in which the Ig segments (containing folded Ig domains) and the PEVK segment (acting largely as an unfolded polypeptide) behave as serially linked entropic springs. In short sarcomeres these springs are in a high entropy state (PEVK and Ig segments are “contracted”) and upon sarcomere extension the springs straighten, lowering their conformational entropy and resulting in a passive force, known as entropic force. This passive (or entropic) force increases in a nonlinear fashion with the segment’s fractional extension (see Equation 1 in ref. 18). It follows from the higher fractional extension of N2B cells that at identical SLs passive forces are predicted to be higher in N2B than N2BA cells.

Using immunoelectron microscopy we were able to show that ventricular myocardium of large mammals co-expresses isoforms at the level of the half-sarcomere. Furthermore, when coexpressed, different isoforms extend independently of one another. Myocytes isolated from myocardium that coexpresses N2B and N2BA titin isoforms develop passive tension levels intermediate between that of N2B and N2BA pure cells. Such intermediate passive tension levels, in theory, could be achieved by varying the number of titin molecules per thick filament. However, this would also influence functions performed by the inextensible regions of titin. These regions are likely to play roles in construction and maintenance of Z-lines and M-lines and thick-filament length control (for a review, see ref. 5). Thus varying the number of titin molecules to achieve a certain passive force level may be undesirable, because other roles of titin would be affected as well. Consistent with this view are recent studies on myocardium isolated from species that express different cardiac titin isoforms that have shown that the stoichiometry of titin is constant (6 molecules per half thick filament). When passive force levels are tuned via variation in the coexpression ratio of isoforms, functions of the inextensible regions are not affected because these regions are the same in different isoforms. Any force level between that of isoform-pure sarcomeres may be obtained by varying the expression ratio (see Fig. 3) without affecting other functions of titin. Thus coexpression of isoforms at various ratios is an effective means for tuning of the passive properties of the cardiac sarcomere.

**Titin Phosphorylation And Passive Force Modulation**

Numerous studies have suggested that, in addition to its role in passive tension generation, titin also participates in cell signaling pathways. Titin contains a serine/threonine kinase domain located at the periphery of the M-line in the sarcomere. The titin kinase is thought to phosphorylate the Z-line-associated protein telethonin during myofibrillogenesis and may have additional roles in adult tissues. Titin is also targeted by kinases in muscle cells. Skeletal and cardiac muscle titins have been identified as phosphoproteins in situ, and titin phosphorylation by cdc2 kinase and ERK1 has been demonstrated in vitro. We have recently shown that PKA phosphorylates titin in cardiac myocytes and decreases passive tension. We also demonstrated that, as with the well-characterized myofibrillar PKA substrates MyBP-C and TnI, titin contains a PKA-responsive domain expressed only in cardiac muscle. Considering that the activation of PKA via β-adrenergic stimulation constitutes a major regulatory pathway in the heart and that this pathway may be altered in heart disease, these findings may have implications for the modulation of diastolic function in vivo.

**Titin’s Passive Force Influences Calcium Sensitivity Of Active Force**

We recently studied the effect of titin-based passive tension on length-dependent activation of cardiac myocytes to explore whether titin may play a role in the generation of systolic force. Length-dependent activation was studied by measuring the force-pCa relation at sarcomere lengths of 2.0 and 2.3 μm, determining at each length the pCa value that gives a force of 50% of maximal (pCa50) and calculating the
difference in pCa\textsubscript{50} at 2.3 and 2.0 \(\mu\text{m}\) (ApCa\textsubscript{50}). Increasing diastolic sarcomere length from 2.0 to 2.3 \(\mu\text{m}\) resulted in an increase in activation (at a given calcium level), as reflected by the positive ApCa\textsubscript{50} values. Passive tension at 2.3 \(\mu\text{m}\) SL was varied by adjusting the characteristics of the stretch imposed on the passive cell before activation. Interestingly, we found that passive tension increased length-dependent activation (Fig. 4A). Thus for maximal calcium sensitivity of skinned cardiac myocytes, a high level of titin-based passive tension is required.

To probe a possible mechanism for this effect, we studied whether titin-based passive tension influences the interfibril lattice spacing and performed small-angle X-ray diffraction on mouse left ventricular wall muscle. In these experiments, titin was degraded by using trypsin and the effect of titin degradation on interfibril lattice spacing was measured. Titin degradation was found to

\[ \Delta \text{pCa}_{50} \]

\[ \text{Z-line} \]

\[ \text{Thin filament} \]

\[ \text{Thick filament} \]

\[ F = \frac{F_T \times \sin \alpha}{F_L \times \cos \alpha} \]

**Fig. 4.** (A) Effect of passive tension on \(\Delta \text{pCa}_{50}\) (difference in pCa\textsubscript{50} at 2.3 and 2.0 \(\mu\text{m}\)) of mouse cardiac myocytes. Results indicate that passive tension significantly enhances the length-dependence of activation (\(\Delta \text{pCa}_{50}\)) with a \(\Delta \text{pCa}_{50}\) of \(\sim 0.25\) pCa units at the highest passive tensions employed. (B) Effect of degrading titin with trypsin on myofilament lattice spacing of skinned muscle. (Thin-thick filament spacing is \(d_{1,0}\) times 2/3.) Degrading titin significantly increased the lattice spacing, supporting that titin modulates the interfibril lattice spacing. (Figures 2C and 6A of Cazorla et al.,\textsuperscript{19} reproduced with permission.) (C) Model of how titin's force enhances activation. Near the Z-line titin binds to the thin filament and at the A/I junction to the thick filament tip. Thus the extensible region of titin is not parallel with the filaments and upon sarcomere stretch (bottom) titin's force (\(F\)) has a longitudinal and radial component (\(F_r\) and \(F_r\)). The radial force component compresses the myofilament lattice and thereby enhances the likelihood of crossbridge interaction with actin. The longitudinal force is postulated to increase crossbridge disorder by slightly increasing thick filament strain, which will also enhance crossbridge interaction.
significantly enhance lattice spacing (Fig. 4B), suggesting that titin-based passive tension modulates the myofilament lattice spacing.\textsuperscript{14}

It is now a widely held view that the reduction in interfilament lattice spacing that accompanies SL stretch enhances the probability of actomyosin interaction at a given calcium concentration and that this underlies length-dependent activation in cardiac muscle.\textsuperscript{30–32} The effect of titin on lattice spacing may thus explain the effect of titin’s passive tension on the length-dependence of calcium sensitivity. Other mechanisms cannot be excluded, however, and it is also possible that a role is played by an earlier proposed mechanism\textsuperscript{33,34} in which crossbridge disorder is enhanced by passive force-induced thick filament strain, which leads to an increased likelihood of actomyosin interaction and an increase in calcium sensitivity. Thus thick filament strain and interfilament spacing may be involved in linking titin to calcium sensitivity (both mechanisms are schematically indicated in Fig. 4C), and their relative importance and interrelationship remains to be established.

Differential Expression Of Titin Isoforms And Overall Behavior Of Muscle

Heart muscle also contains collagen as passive force generator; to elucidate the relevance of titin and the differential expression of cardiac titin isoforms to the overall properties of the myocardium, we determined the contribution of titin and collagen to the overall stiffness of cardiac muscle. We selected muscles that express predominantly N2B titin (mouse left ventricle), predominantly N2BA titin (cow left atrium), or that coexpress N2B and N2BA titins at similar levels (bovine left ventricle) (Fig. 5A). The total passive tension–SL relation was measured and the individual contributions of collagen and titin were determined by using KCl/KI extraction and trypsin degradation techniques.\textsuperscript{21}

The total passive tension differed greatly in the different muscle types (Fig. 5B), with tension being highest in mouse left ventricle (MLV), lowest in bovine left atrium (BLA), and intermediate in bovine left ventricle (BLV). We dissected the contribution of titin and collagen to overall passive tension and this revealed that collagen-based passive tensions (Fig. 5C) and titin-based tensions (Fig. 5D) significantly vary between the different muscle types. Furthermore, titin-based tension and collagen-based tension both decrease in the following order: MLV, BLV, and BLA. Thus the passive tension levels of collagen and titin are correlated, and their coordinated change is responsible for the large variation in passive muscle tension.

To determine the relative contribution of titin and collagen to overall passive tension of muscle, titin- and collagen-based tensions were calculated as fractions of the total muscle tension (Fig. 6). Near the maximal length of the sarcomere-length range with reversible passive properties (~2.3 \mu m for mouse and ~2.5 \mu m for bovine\textsuperscript{21}), titin and collagen contribute about equally to passive tension, whereas at shorter SLs titin’s contribution dominates. Thus in all investigated muscle types titin is a primary contributor to passive muscle stiffness and passive muscle stiffness can be modulated effectively via the expression of titin isoforms (inset Fig. 5D).

Titin’s passive tension is expected to play a role in determining the end-diastolic volume, and the high titin-based passive tension provided by N2B titin may allow rapid and stable determination of this volume at the high beat frequencies encountered in small rodents (where N2B titin dominates). Titin is also expected to play a role in centering the A-band within the sarcomere.\textsuperscript{35,36} As a result of, for example, microvariuation in the speed of calcium release in different parts of the sarcomere, the A-band may move off-center (i.e., shift) during systole, negatively affecting the efficiency of contraction. During diastole, titin-based passive tension will reset the A-band to its central location in the sarcomere, avoiding a progressive shift of the A-band with each systole. The rapid and frequent activation in small rodents may result in a more pronounced A-band shift, and high N2B-based passive tension will function to counter this by rapidly resetting the A-band during each diastole.

It is worthwhile to also consider titin’s contribution to restoring forces. The segment of titin near the Z-line binds to the thin filament (see Fig. 1A) and as a result it can withstand compressive forces. When sarcomeres shorten to below the slack length, the tip of the thick filament moves into the stiff region of titin near the Z-line, and titin’s extensible region extends in a direction that is opposite of that during stretching.\textsuperscript{15,57} The extensible region of titin functions thereby as a bidirectional molecular spring that in sarcomeres shortened to below the slack length (~1.9 \mu m) gives rise to restoring force (ie, a force that pushes the Z-lines away from each other) and in sarcomeres stretched above the slack length to passive force (ie, a force that pulls the Z-lines toward each other).\textsuperscript{15,57} Restoring and passive forces are symmetrical with respect to the slack length,\textsuperscript{15,57} and the measured passive forces in stretched sarcomeres suggest that the restoring forces should be highest in N2B expressing myocardium (mouse and rat) and lowest in N2BA expressing myocardium (bovine atrium). Considering that titin’s restoring force may contribute to the early diastolic suction force that aids ventricular filling\textsuperscript{30} and that the diastolic filling time is very short in small rodents, we hypothesize that high restoring forces are required in these species to achieve more rapid early diastolic filling.
It is likely that titin’s extensible region performs functions that go beyond passive force development and that any of these may be modulated via coexpressing isoforms. As reviewed previously, titin-based passive force influences active force development, and titin’s effect on active force may be regulated by coexpressing isoforms at varying ratios. Furthermore, titin’s extensible region is likely to contain isoform-specific binding sites for ligands. For example, a binding site for the protease P94 is found in the extensible region of N2BA titin only, and by varying the expression level of N2BA titin, the role of P94 in protein turnover may be modulated. In conclusion, coexpressing isoforms at the level of the half sarcomere allows for tuning of the passive properties of the sarcomere, but it may also perform additional functions.

Fig. 5. (A) SDS-PAGE of mouse left ventricle (MLV), bovine left ventricle (BLV), and bovine left atrium (BLA). MLV expresses N2B titin and BLA expresses N2BA titin. BLV expresses both N2B and N2BA titins. (T2 is a degradation product of T1.) (B-D) Average tension–SL relation of MLV, BLV, and BLA muscles (mean ± SD; n = 10)). (B) Total passive tension–SL relations. (C) Titin-based passive tension–SL relations. (D) Collagen-based passive tension–SL relations. (Methods for determining titin and collagen-based tension, see ref 21.) Inset of (D) shows the relation between titin-based stiffness and the N2B content (slope of passive tension–SL relation at SL 2.15 μm). A significant correlation (r² of slope of linear fit: 0.99) is apparent. Differential expression of titin isoforms is an effective means for modulating myocardial passive stiffness. (Results from Wu et al21 Figs. 1A, 3, and 6. Reproduced with permission.)
Titin And Cardiomyopathy

Several recent studies have reported that the amount of intact titin is reduced in myocardium of patients with dilated cardiomyopathy (DCM), and it has been suggested that titin could be involved in the altered ventricular compliance of the DCM heart.\textsuperscript{40-43} However, the reduced titin levels in DCM are expected to result in a reduction of diastolic stress, whereas passive stress is typically increased.\textsuperscript{40,44,45} As pointed out earlier,\textsuperscript{43} considering titin's high susceptibility to proteolysis and the possibility that elevated intracellular Ca\textsuperscript{2+} levels may be present in the failing heart, which may activate proteases that degrade titin, it is possible that normal levels of titin are present in the DCM heart, but that titin is subsequently degraded during preparation of the muscle sample for gel electrophoresis.

It is also worthwhile to consider that earlier studies carried out before the discovery of coexpressed titin isoforms in human myocardium assumed that there is only a single titin isoform. This may have resulted in the misidentification of the titin bands seen on gels. This issue is highlighted by the gel shown in Fig. 7 that compares samples from normal and DCM hearts. Assuming that there is only one titin isoform results in the conclusion that the control sample contains a large amount of intact titin (the top band) and that this intact titin is largely proteolysed in the DCM sample, giving rise to a large degradation product that migrates just below intact titin. However, based on our current level of understanding, the top band in the control sample can be identified as N2BA and the band below this as N2B titin, both of which are full-length molecules, giving rise to the conclusion that N2B titin is upregulated in the DCM sample. This upregulation is expected to result in el-

Fig. 6. SL dependence of contribution of titin (closed symbols) and collagen (open symbols) to total passive tension. In bovine atrium (blue filled circles) titin contributes >80% to total tension at SLs <2.4 \mu m whereas in mouse muscle (red filled circles) titin contributes >80% at SLs <2.1 \mu m. At longer SLs, titin's contribution decreases whereas that of collagen increases. (Results from Wu et al\textsuperscript{21} Fig. 5. Reproduced with permission.)

Fig. 7. SDS-PAGE analysis of human left ventricular (LV) myocardium of patient with DCM. Control: normal human myocardium (epicardial LV biopsy). Rat LV was coelectrophoresed as a reference. Human control myocardium expresses both N2B and N2BA titin isoforms. N2B titin is much more prominent in DCM than in the control. Note that the T2 band present in the DCM sample is barely visible, indicating that degradation was minimal.
Fig. 8. (A) SDS-PAGE of 4-week paced dog and of control dog. Tissue was obtained from the mid-layer of the free wall of the left ventricle. In the paced animal, N2B titin dominates, whereas in the control N2B and N2BA titin are found at similar levels. (B-D) Passive tension–SL relations of skinned muscle strips dissected from the mid-layer of the free wall of the left ventricle. (B) Total passive tension. (C) Collagen-based passive tension. (D) Titin-based passive tension. All tensions are higher in the paced animal.

(Methods for determining titin and collagen-based tension: see ref. 21.)

Elevated passive stiffness, and this is consistent with the elevated passive stiffness seen in DCM.

To further investigate changes in isoform coexpression in DCM, we used the pacing tachycardia heart failure model of dog. It was found that in myocardium of 4-week paced animals, N2B titin was upregulated (Fig. 8A). We also characterized the passive mechanical properties of skinned muscle strips prepared from the mid-wall region of the left ventricular free wall and determined the contribution of collagen and titin to total stiffness. This showed that total passive stiffness was markedly elevated in paced animals (Fig. 8B) and that this elevation is the result of increased stiffness of collagen (Fig. 8C) and titin (Fig. 8D). Thus collagen and titin change in a coordinated fashion, suggesting that both are important in determining the elevated diastolic stiffness of the myocardium of paced animals and that they play complementary roles. As for the increased titin-based stiffness in paced animals, because N2B titin is stiffer than the N2BA isoform, the observed stiffness increase is consistent with the elevated expression of N2B titin.

In conclusion our results indicate that shifts can occur in titin isoform expression and that the N2B/N2BA titin isoform content can be dramatically different in the normal versus DCM heart. Although it can not be excluded that titin degrades in end-stage heart failure, an earlier adaptation may be a switch in expression toward the stiff N2B isoform. This shift will result in elevated passive stiffness and may initially be beneficial, because it will counteract the increased myocardial strain during ventricular dilation.

References


