Molecular Dissection of N2B Cardiac Titin's Extensibility

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ABSTRACT Titin is a giant filamentous polypeptide of multidomain construction spanning between the Z- and M-lines of the cardiac muscle sarcomere. Extension of the I-band segment of titin gives rise to a force that underlies part of the diastolic force of cardiac muscle. Titin's force arises from its extensible I-band region, which consists of two main segment types: serially linked immunoglobulin-like domains (tandem Ig segments) interrupted with a proline (P)-, glutamate (E)-, valine (V)-, and lysine (K)-rich segment called PEVK segment. In addition to these segments, the extensible region of cardiac titin also contains a unique 572-residue sequence that is part of the cardiac-specific N2B element. In this work, immunoelectron microscopy was used to study the molecular origin of the in vivo extensibility of the I-band region of cardiac titin. The extensibility of the tandem Ig segments, the PEVK segment, and that of the unique N2B sequence were studied, using novel antibodies against Ig domains that flank these segments. Results show that only the tandem Igs extend at sarcomere lengths (SLs) below ~2.0 μm, and that, at longer SLs, the PEVK and the unique sequence extend as well. At the longest SLs that may be reached under physiological conditions (~2.3 μm), the PEVK segment length is ~50 nm whereas the unique N2B sequence is ~80 nm long. Thus, the unique sequence provides additional extensibility to cardiac titins and this may eliminate the necessity for unfolding of Ig domains under physiological conditions. In summary, this work provides direct evidence that the three main molecular subdomains of N2B titin are all extensible and that their contribution to extensibility decreases in the order of tandem Igs, unique N2B sequence, and PEVK segment.

INTRODUCTION

When the myocardium is stretched, a passive force is generated that influences ventricular filling during diastole and ventricular emptying during systole. Many observations have indicated that, in the generation of passive force, a significant role is played by a unique protein called titin (also known as connectin). Titin is the largest protein known to date (a single polypeptide is ~3 million dalton) and it is the third-most abundant myofibrillar protein, making up about 10% of the total muscle protein mass. In addition to influencing ventricular filling, titin also helps to maintain sarcomeric integrity during contraction and has been implicated in myofibrillogenesis as a thick filament scaffold and cell-signaling molecule. (For reviews and original citations, see Gregorio et al., 1999; Labeit et al., 1997; Maruyama, 1997; Trinick, 1996; Wang, 1996.)

Titin's force arises from its extensible I-band region, which consists of two main segment types: 1) a segment type rich in proline (P), glutamate (E), valine (V) and lysine (K) residues (the so-called PEVK segment) and 2) serially linked immunoglobulin-like domains (tandem Ig segments) flanking this PEVK segment (Labeit and Kolmerer, 1995). In skeletal muscles, the PEVK and tandem Ig segments differ in length in different muscles (Labeit and Kolmerer, 1995). The extensible region of skeletal muscles also contains the N2A element (4 Ig domains and a 106-residue unique sequence). N2A titin has also been found in a human cardiac titin isoform (Labeit and Kolmerer, 1995). A different splice element known as the N2B element (3 Ig domains and a 572-residue unique sequence) is found exclusively in cardiac muscle and contains, within its central I-band region, a 163-residue PEVK segment and tandem Ig segments with 37 Ig domains (Labeit and Kolmerer, 1995). Myocardium of avian and small mammals (such as rat, mouse, and rabbit) expresses predominantly N2B titin, whereas large mammals coexpress N2B and N2A titins (Cazorla et al., 2000).

In slack sarcomeres, titin's extensible segment has a short end-to-end length as a result of thermally-induced bending motions that impact the flexible titin molecule and that result in a "contracted state" with a maximal entropy (Trombitás et al., 1995; Granzier et al., 1996; Linke et al., 1996). Straightening the extensible region by extending the sarcomere lowers the conformational entropy and results in a force, known as entropic force. Our work on human soleus muscle reveals that Ig domain unfolding is likely to be absent along the physiological sarcomere length range (Trombitás et al., 1998). Furthermore, when slack skeletal muscle fibers are stretched, extension of titin's I-band region does not occur uniformly, rather tandem Ig extension dominates initially and, at a sarcomere length (SL) of ~2.6 μm, PEVK segment extension becomes dominant (Linke et al., 1998a,b; Trombitás et al., 1998). This behavior can be reproduced by modeling the tandem-Ig segments (containing folded Ig-like domains) and the PEVK segment (acting

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largely as an unfolded polypeptide) as serially-linked worm-like chains (WLCs) with different bending rigidities (Trombitás et al., 1998).

In a recent study of rat cardiac titin, we found that the extensible region of titin extends beyond what can be explained by the contour length of the tandem Ig and PEVK segments (Helmes et al., 1999). This contour length adjustment was seen in sarcomeres that were longer than ~2.1 μm, and results suggested that contour length adjustment does not arise from Ig domain unfolding, but, surprisingly, from extension of the 572-residue unique N2B sequence. In this work we extended these novel findings by a direct study of the behavior of the tandem Ig, PEVK, and unique N2B sequence that together make up the extensible I-band region of titin. Immunoelectron microscopy with sequence-specific antibodies that flank the various segments provided conclusive evidence that the unique sequence of N2B titin is extensible. The I-band region of titin may be viewed as molecular spring that contains three extensible segments: the tandem Igs, the PEVK segment, and the unique sequence.

MATERIAL AND METHODS

Cardiac myocyte isolation

Both mouse and rabbit cardiac myocytes were used in this study. These species were selected because they express predominantly N2B cardiac titin, unlike large mammals that express also a titin isoform that contains the N2A element (Labeit and Kolmerer, 1995; Cazorla et al., 2000). The protocols conform to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health, 1985. Mouse myocytes were isolated from 10–12-week-old FVB-Taconic mice, using an isolation procedure adapted from Wolska and Solaro (1996). Briefly, the coronary arteries were perfused with oxygenated physiological saline solution (PSS) containing (in mmol/l): 133.5 NaCl, 4 KCl, 1.2 NaH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 11 glucose, pH 7.4. Solutions were preheated to achieve a perfusion temperature of 37°C. During perfusion, the heart was immersed in a tissue-organ bath kept at 37°C. The hearts were first perfused for 5 min with Ca^{2+}-free PSS containing 0.1% bovine serum albumin (BSA) at a 1.3 ml/min flow rate, and subsequently for 10–20 min with PSS containing 25 μmol/l $CaCl_2$, collagenase (317 U/mg, type II Worthington, Freehold, NJ) and hyaluronidase (1000 U/mg, Sigma, St. Louis, MO) using a 1.6 ml/min flow rate. Then the heart was washed with Ca^{2+}-free PSS containing 15 mmol/l 2,3-butanedione monoxime (BDM). Ventricles were cut into small pieces that were repeatedly drawn through a plastic pipette tip to release isolated cells. The majority of myocytes obtained this way had a normal, rod-like shape. The cells were skinned for 50-min with 1% Triton X-100 in relaxing solution containing, in mmol/l: 40 imidazole; 10 EGTA; 6.4 Mg-Acetate; 5.9 NaATP; 10 creatine-phosphate; 80 K-propionate; 1.0 DTT; pH 7.0 at 21°C. The detergent was removed through extensive rinsing with relaxing solution. To prevent degradation, all solutions contained protease inhibitors (PMSF: 0.5 mmol/l; leupeptin: 0.04 mmol/l and E64: 0.01 mmol/l).

For some of our studies, we also used rabbit myocytes. Adult male New Zealand white rabbits were used (weight ~3 kg). The rabbits were anesthetized by intramuscular injection of ketamine, xylazine, and atropine (35, 7.5, and 0.02 mg/ml, respectively) and exsanguinated by removing the heart. Myocytes were isolated from the left ventricle as explained earlier (Granzier and Irving, 1995).

Antibodies to titin

For raising titin domain-specific antibodies, human cardiac titin cDNA fragments were isolated by polymerase chain reaction from total cardiac cDNA. The following primer pairs were used (sequences were derived from EMBL data library accession X90568): x214: tttccatg GAA GGC ACT GGC CCA ATT TTC ATC AAA GAA; x215: tttggtacc-ta-GTC TGT GTC TTC CAG AAG CAC AAG CAG CTC; x216: tttccatg-GAG GAT GGC CCC ATG ATA CAT ACA CCT TTA; x217: tttggtacc-ta-CAC TGT CAC AGT TAG TGT GGC TGT ACA GCT. (Small letters denote 5′ mismatch tags for introducing cloning sites, start and stop codons; capitals are for codons/reverse codons from titin.)

The amplified cDNA fragments, x214–x215 and x216–x217 were subcloned into modified pET9D vectors, which expresses their insert sequences as fusions with N-terminal His_6-tags. The recombinant peptides were purified from the soluble fractions by nickel chelate affinity chromatography on NTA (Ni-NTA) resins as specified by the manufacturer (Quiagen, Chatsworth, CA). Antibodies to the respective peptides were raised in rabbits by Biogenes (Berlin, Germany) and the specific IgG fraction was isolated by affinity chromatography. Western blot analysis of extracts of cardiac and skeletal muscle was used to verify the specificity of the antibodies.

The Ig repeats N2B-I16–I17 are recognized by anti-x214–x215. Because this antibody binds N-terminal of the unique N2B sequence, it will be referred to as Un. The Ig repeats N2B-I18–I19 are recognized by anti-N2B x216–x217, and this antibody will be referred to as Uc (for C-terminal of the unique N2B sequence). The existing antititin antibodies I20/22, T12 and MIR were used as well. Antibody I20/I22 was to the nucleotide positions 13978–14826 of the human cardiac titin data library entry (GenBank accession no. X90568). This is an affinity-purified polyclonal antibody raised in rabbit (Linke et al., 1998a). T12 is a mouse monoclonal antibody (Fürst et al., 1988) mapped to I2/I3 (Sebestyén et al., 1995) and was purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). MIR is a polyclonal antibody raised in rabbit to the expressed Ig/FN3 repeats I45-I46-I47 (Freiburg et al., submitted for publication).

Immunoelectron microscopy

Cardiac myocytes were present in relaxing solution and were stretched (at ~0.25 μm/sarcomere/s) to different lengths, held in that state for about 5 min, and then fixed, immunolabeled, embedded, and processed for electron microscopy (EM) as explained in Trombitás et al., (1991) and Granzier et al., (1996). Briefly, the muscle samples were fixed in 3% formaldehyde/PBS solution for 20 min, then washed and blocked in 1% BSA/PBS solution for one hour. After dilution of antibodies to the appropriate concentrations (typically ~50 μg/ml), the samples were labeled with first the primary and then secondary antibodies, for 24 h each. The samples were fixed in glutaraldehyde and osmium-tetroxide solutions, and embedded in araldite. Sections were cut using a Leica microtome, the sections were stained with 2% potassium permanganate and lead citrate. EM negatives were taken using JEOL 1200 type electron microscope.

The Z-line to epitope distances were measured from EM negatives following high-resolution scanning (UMAX, UC-1260) and digital image processing using custom written macros for the image analysis program National Institutes of Health Image (v. 1.6, Wayne Rasband, National Institutes of Health). For spatial calibration, the electron microscope's (JEOL 1200) magnification was used.

When myocytes were labeled with only one antibody, a single epitope was typically detected. On occasion, however, additional weak and spotty epitopes were also seen that may result from the coexpression of N2B titin with a small amount of a larger N2A containing isoform. Alternatively, a low level of nonspecific staining may have occurred. In this work, we focused on the prominent epitopes that were consistently detected.

RESULTS

Experiments were performed to monitor the following titin epitopes (Fig. 1 *A*) by using antititin antibodies: Ig I2-I3

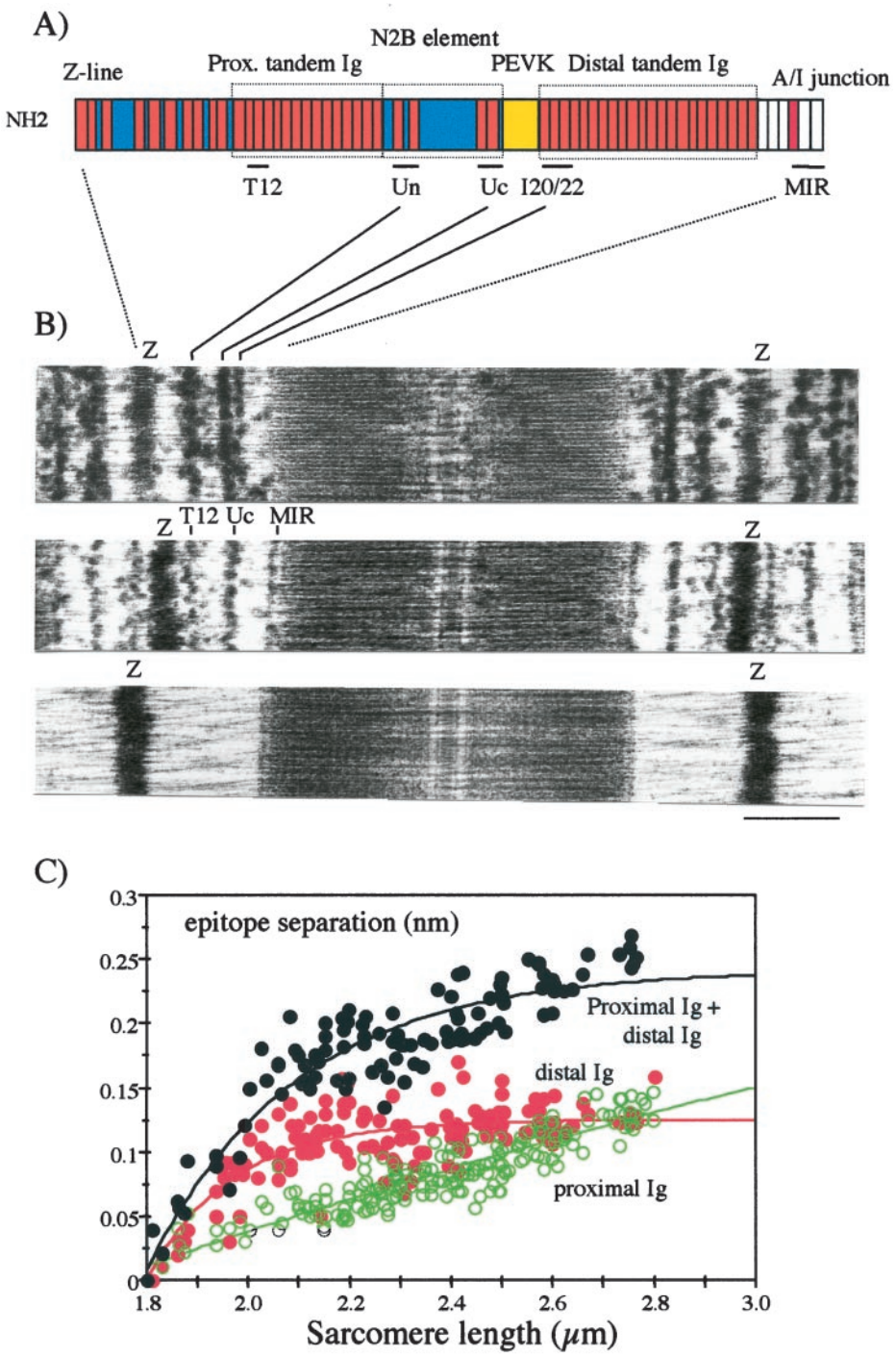

FIGURE 1 (*A*) Domain structure of I-band region of cardiac N2B titin (adapted from Labeit and Kolmerer, 1995) and location of binding sites of antibodies used in this work. (*Red*, Ig domains; *white*, fibronectin domains; *yellow*, PEVK segment; *blue*, unique sequence.) (*B*) *Top:* example of rabbit sarcomere labeled simultaneously with Un, Uc, and I20/22. Note that the unique sequence (Un-Uc) and the PEVK (Uc-I20/22) are both extended. *Middle:* rabbit sarcomere labeled with T12, Uc and MIR antibodies. *Bottom:* control rabbit sarcomere labeled with secondary antibody only. (Scale bare: 0.5 μm.). (*C*) Extension of tandem Igs versus sarcomere length. See text for further details. (Proximal tandem Ig: T12-Un; distal tandem Ig: I20/22-MIR. Results are from five mouse cells and nine rabbit cells. Rabbit and mouse data were indistinguishable and, for clarity sake of the graph, they are shown with the same symbols. Curves represent exponential fits to all data.)

(recognized by T12 antibody); Ig repeats N2B-I16-I17 (recognized by Un), Ig repeats N2B I18-I19 (recognized by Uc), Ig repeats I20-I22 (recognized by I20/22), and I45/46 (recognized by MIR). The antititin antibodies labeled either in titin's extensible I-band region (T12, Un, Uc, I20/22) or at the A/I junction (MIR). Consistent with earlier work (Trombitás et al., 1995) in sarcomeres that were stretched to lengths as long as 2.8 μm, T12 stayed at a constant ~100 nm from the center of the Z-line, whereas MIR maintained a fixed position at the edge of the A-band (data not shown). However, the positions of the Un, Uc, and I20/I22 epitopes, changed both with respect to the Z-line and the A/I junction, reflecting the extensibility of the various sub-segments within titin's extensible region. The following segment lengths were measured: T12 to Un, Un to Uc, Uc to I20/22, and I20/22 to MIR, and these distances were used as measurements of the length of the proximal tandem Ig, unique N2B sequence, PEVK, and distal tandem Ig, respectively (See Fig. 1, *A* and *B*). Measurements were made on cardiac myocytes isolated from mouse and rabbit. Results of the two species were indistinguishable (see below).

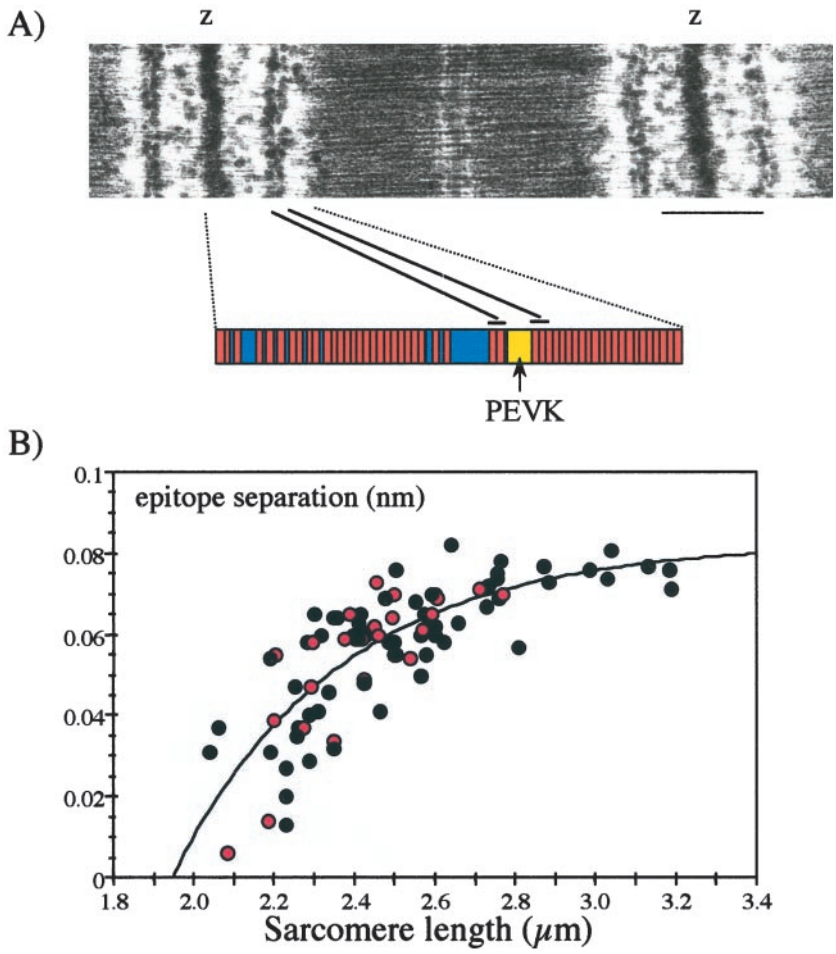

FIGURE 2 Extensible behavior of PEVK segment. (*A*) Example of sarcomere from rabbit double labeled with Uc and I20/22. Note the two I-band epitopes with a slight separation between them. (Scale bare: 0.5 μm.) (*B*) PEVK segment length (Uc-I20/22) versus sarcomere length. The PEVK segment extends in sarcomeres longer than ~2.0 μm and reaches a maximal extension of ~80 nm. (*Red symbols*, data from five mouse cells; *black symbols*, data from six rabbit cells. The curve represents the exponential fit to all data.)

We first studied the behavior of the tandem Ig segments. The data reveals that tandem Ig segments have a near zero end-to-end length in slack sarcomeres (~1.8 μm), i.e., the segments are in a contracted state. In response to sarcomere stretch, the tandem Ig segments extended. However, the extensible behavior of the two tandem Ig segments was not identical. The distal segment extended in a more-or-less step-like fashion and the proximal segment displayed continuous extension (Fig. 1 *C*). The combined extension of the two tandem Ig segments shows a relatively rapid extension between the slack sarcomere length (~1.85 μm) and ~2.1 μm and a more gradual extension at higher sarcomere stretch (Fig. 1 *C*).

The behavior of the PEVK segment is shown in Fig. 2. The PEVK segment extends in sarcomeres longer than ~2.0 μm (Fig. 2 *B*) and it reaches a length of 71 ± 7 nm at a sarcomere length of 2.8 μm (Table 1). This extension exceeds that which can be accommodated by the fully unfolded and maximally extended 163-residue PEVK segment (see Discussion). Because it is not known exactly where the antibodies bind in the antigen that was used for raising the antibodies, it is possible that the epitopes do not precisely demarcate the PEVK segment. Despite these uncertainties, the results clearly show that the PEVK segment is extensible and that its main extension takes place at a longer sarcomere length range than that of the tandem Igs. Thus, the extensibility of these two types of segments is very different.

Examples of sarcomeres labeled with antibodies that label the flanking regions of the unique N2B sequence are shown in Fig. 3 *A*. These examples provide direct evidence for our earlier conclusion (Helmes et al., 1999) that the unique sequence is extensible. The scattergram of results in Fig. 3 *B* reveals that the extension of the unique sequence starts at an SL of ~2.0 μm, and that it continues along the full SL range that was studied. A maximal length of 201 ± 23 nm is reached at an SL of 3.3 μm. Such long length can only be explained by a fully unfolded and nearly maximally extended sequence (see Discussion).

TABLE 1 Mean segment lengths

SL (μm)	Proximal tandem Ig (nm)	Distal tandem Ig (nm)	PEVK (nm)	Unique sequence (nm)
2.3	73 ± 12	103 ± 18	57 ± 13	79 ± 27
2.8	131 ± 10	123 ± 14	71 ± 7	165 ± 13

Proximal tandem Ig: T12-Un; Distal tandem Ig: I20/22-MIR (note that this segment includes three Fn domains as well); PEVK: Uc-I20/22; unique sequence: Un-Uc. Shown are the mean ± SD. The number of data points was typically ~20.

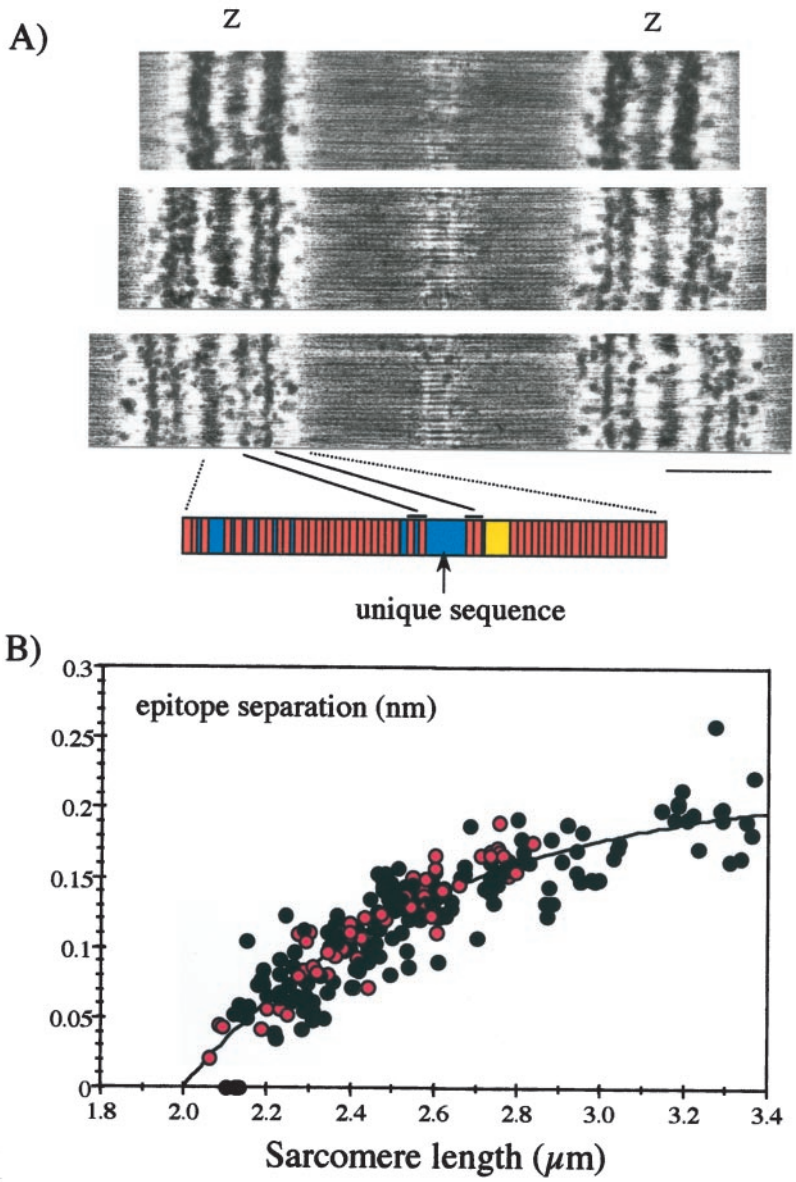

FIGURE 3 Extensible behavior of N2B unique sequence. (*A*) Examples of rabbit sarcomeres double labeled with Un and Uc. Note that the two epitopes in the middle and bottom sarcomeres are clearly separated, revealing the extensibility of the unique sequence. (Scale bare: 0.5 μm.) (*B*) End-to-end length of unique sequence (Un-Uc) versus sarcomere length. The unique sequence extends in sarcomeres longer than ~2.0 μm and reaches an extension of ~200 nm at an SL of 3.3 μm. (*Red symbols*, data from seven mouse cell; *black symbols*, data from eight rabbit cells. The curve represents the exponential fit to all data.)

To compare the extensible behavior of the tandem Igs, PEVK, and unique sequence, their fitted extension curves are shown superimposed in Fig. 4. The vertical line in Fig. 4 denotes the maximal SL that may be encountered under physiological conditions (see Discussion). Along the physiological SL range, all three segment types contribute to titin's extensibility. The SL range within which the main segment extension occurs varies for the three segment types; 0–70% of the maximal extension of tandem Ig, PEVK, and unique sequence occurs at an SL range of 1.8–2.0 μm, 2.0–2.2 μm, and 2.0–2.6 μm, respectively.

DISCUSSION

In this work, the molecular origin of N2B cardiac titin's extensibility was dissected by using immunoelectron microscopy with antibodies that demarcate the tandem Igs, PEVK, and unique N2B sequence. Results indicate that the various segments all extend, but that their main extension occurs at different SLs with tandem Igs extending from the slack SL onward, and PEVK and unique segment extension starting at a SL of ~2.0 μm. Considering that the end-diastolic SL range in small mammals may have an upper limit of ~2.3 μm (Grimm et al., 1980, 1991; MacKenna et al., 1996) extension of the tandem Igs and that of PEVK and unique sequence are likely to be physiologically relevant.

Tandem Igs

Single molecule mechanical experiments (Kellermayer et al., 1997; Rief et al., 1997; Tskhovrebova et al., 1997) have provided evidence that the persistence length of tandem Ig segments is relatively high. The conformational entropy of tandem Igs is therefore low, and tandem Igs are predicted to

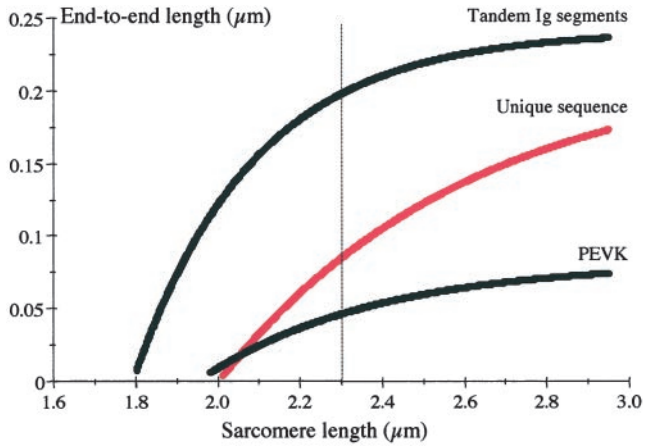

FIGURE 4 Comparison of extensible behavior of tandem Igs, unique sequence, and the PEVK segment. (Shown are the fitted curves of the data in Figs. 1–3). Tandem Ig segments contribute most to titin's extensibility. The unique sequence contributes less and the PEVK segment contributes the least. Vertical broken line indicates the maximal sarcomere length that may be reached under physiological conditions. See text for details.

extend at low force. The rapid extension of tandem Igs seen at SLs between slack and ~2.1 μm, an SL-range where myocytes develop relatively low levels of passive force (Helmes et al., 1999), is thus consistent with the mechanical properties of titin obtained at the single molecule level. Our work reveals, however, that the proximal and distal tandem Ig segments do not behave identically. The distal tandem Ig segment extends rapidly until an SL of ~2.1 μm is reached and extension is much slower at longer SLs. The proximal tandem Ig segment, in contrast, extends continuously over the full SL-range that was studied (Fig. 1 *C*). This continuous extension may be explained by Ig domain unfolding at long SLs. Previously, we estimated the mean spacing of folded domains in the fully extended proximal tandem Ig segment of human soleus muscle at 5.0 nm (this value includes an ~4.0-nm-long β-barrel and an ~1.0-nm-long linker sequence) (Trombitás et al., 1998). Thus, the maximal predicted length of the proximal tandem Ig segment while completely straight and all of its domains folded is ~75 nm, and that of the distal tandem Ig segment ~110 nm. The mean domain spacing of the proximal tandem Ig segment that we measured in this study (Table 2) is below 5.0 nm in sarcomeres shorter than 2.3 μm, whereas, at longer SLs, the domain spacing exceeds 5.0 nm (at an SL of 2.8 μm the value is 8.7 nm). These high values at long SLs can be explained by unfolding of some of the domains contained within the segment (because unfolding of one domain results in a length gain of ~30 nm, unfolding of two domains could explain the large mean domain spacing at 2.8-μm SL). In contrast, the distal tandem Ig has a mean domain spacing that is less than 5.0 nm in sarcomeres as long as 2.8 μm (Table 2) suggesting that domains in this region are more stable than those of the proximal tandem Ig. Studies by Greaser et al. (1996) and Witt et al. (1998) have shown that the domains from the proximal tandem Ig form a structurally distinct group of domains, characterized by, for example, fewer residues in one of the β-strands and a shorter linker sequence. It is possible that, because of these differences, the Ig domains from the proximal tandem Ig are less stable and, thus, more prone to force-induced unfolding than those from the distal tandem Ig.

TABLE 2 Mean domain/residue spacing

SL (μm)	Proximal tandem Ig (nm)	Distal tandem Ig (nm)	PEVK (Å)	Unique sequence (Å)
2.3	4.8	4.1	3.5	1.4
2.8	8.7	4.9	4.3	2.9

Assumptions: T12-Un: 15 domains; I20/22-MIR: 25 domains; Uc-I20/22: 163 residues; Un-Uc: residues. Shown are the mean ± SD. The number of data points was typically ~20.

In summary, in highly stretched sarcomeres, all domains in the distal tandem Ig are likely to remain folded, whereas unfolding of several Ig domains is likely to take place in the proximal tandem Ig segment. At physiological SLs, however, tandem Ig extension can be explained by the alignment of Ig domains without requiring domain unfolding.

PEVK segment

Extension of the PEVK starts at an SL of ~2.0 μm and continues until a maximal length of ~70 nm is reached at an SL of ~ 2.6 μm (Fig. 2 *B*). Considering the 163 PEVK residues (Labeit and Kolmerer, 1995) and the 3.8-Å maximal residue spacing of an unfolded peptide (Cantor and Schimmel, 1980), the predicted maximal length of the N2B PEVK is 62 nm. The longer maximal length that was measured may result from Uc and I20/22 epitopes that are not localized exactly at the edge of the PEVK (see Results).

The average PEVK residue spacing at 2.3-μm SL is 3.5 Å (Table 2), suggesting that, at end-diastole, the PEVK may be largely unfolded. The conclusion that the PEVK reaches an unfolded state at a relatively short SL is consistent with the preponderance of prolines and charged residues that make the formation of stable structures unlikely. Whether the PEVK refolds into secondary structures upon sarcomere shortening remains to be established. Refolding during release and unfolding during stretch would be energetically costly because it would result in hysteresis (force during stretch would be higher than during release). Our recent single-molecule mechanical experiments (Kellermayer et al., in preparation) suggest that, when the titin molecule is held at a short length for several minutes, noncovalent interactions take place between different regions of the PEVK polypeptide. Furthermore, these interactions are abolished in repeated stretch–release cycles, giving rise to hysteresis-free force-length loops. We speculate that, in vivo, the PEVK segment behaves analogously and that, under the repeated stretch–release conditions that occur in the beating heart, the PEVK behaves as a permanently unfolded polypeptide with a force-length curve during stretch (diastole) and release (systole) that is hysteresis-free.

Unique N2B sequence

The central I-band region of cardiac titins contains a nonrepetitive element referred to as the N2B element (Labeit and Kolmerer, 1995). In addition to the Ig repeats I16, I17, and I18, the N2B element codes also for a central 572-residue-large unique sequence. Previously, we compared our immuno-EM results obtained with an antibody that labels within this sequence to results obtained by others who used an antibody against I18, and concluded from this comparison that the unique sequence is extensible (Helmes et al., 1999). To test this novel conclusion, we used, in the present work, new antibodies that flank the unique sequence. Double labeling with both antibodies provided direct evidence that the unique sequence indeed extends (Fig. 3). At the upper limit of the end-diastolic SL range (2.3 μm), the unique sequence accounts for ~25% of the total extension of titin's extensible I-band region. In the absence of unique sequence extension, the fractional extension of the tandem Ig and PEVK segment (at a given SL) would be much elevated and, as a result, passive force would be expected to be much higher. This may be undesirable because the irreversible structural sarcomere damage that is known to occur in sarcomeres stretched to lengths longer than physiological (for example recruitment of titin from the A-band: see Trombitás et al., 1995; Granzier and Irving, 1995) would now occur at physiological SLs. Thus, the unique sequence allows sarcomeres to be extended to the upper limit of the physiological SL range while avoiding an extremely steep increase of passive force.

The structure of the 572-residue sequence is currently not known. Sequence-based predictions indicate a tendency for coiled-coil α-helical conformations in part of the sequence (Gautel et al., 1996). Recent work by Rief et al. (1999) has shown that the unfolding forces of α-helical coiled-coil structures are several-fold less than those of Ig domains, and this is consistent with our finding that the unique sequence undergoes large-scale extension at SLs where Ig domain unfolding is minor or absent. Comparison of the N2B sequence to other known proteins reveals no significant homologies, with a best match to tropomyosin (~20% identity). The N2B sequence comparison between human patients (Siu et al., 1999) and comparison between rabbit and human (Freiberg et al., submitted for publication) suggests that the N2B sequence has a lower conservation than the Ig repeats and the PEVK segment. Considering the importance of the unique N2B sequence, its structure and the molecular mechanism of its extensibility are worth studying in detail.

CONCLUSIONS

At the upper end of the physiological SL range, the unique N2B sequence contributes ~80 nm to the extensibility of titin's I-band region. In the absence of unique sequence extension, tandem Igs and the PEVK would have to be extended to a higher degree and force would be highly elevated. Thus, it is likely that Ig domain unfolding would now have to take place at relatively short SLs. We propose, therefore, that the extension of the unique N2B sequence eliminates the need for unfolding of Ig domains under physiological conditions.

Our work on the large human soleus titin isoform (Trombitás et al., 1998) and the present work on N2B cardiac titin suggest that, under physiological conditions, Ig domain unfolding is undesirable. Considering that refolding during release may be fast enough to take place under physiological conditions (Kellermayer et al., in preparation), unfolding during stretch and refolding during release may be undesirable because it would lead to energy loss due to hysteresis. Another possibility is that the unfolded Ig polypeptide is highly sensitive to proteolysis (it contains a sensitive proline-rich sequence) and that this is why unfolding has to be avoided under physiological conditions.

In conclusion, our work shows that the tandem Igs, unique sequence, and PEVK are all extensible. Thus, N2B cardiac titin is a three-element molecular spring. In comparison, skeletal muscle titin contains only the tandem Ig and PEVK segment as extensible elements and, thus, it is a two-element spring. Extension of the N2B unique sequence eliminates the need for Ig unfolding in cardiac N2B titin under physiological conditions. Whether it provides an additional reserve for extensibility that is relevant for pathological conditions remains to be established.

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