Molecular Mechanics of Cardiac Titin’s PEVK and N2B Spring Elements*

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Titin is a giant elastic protein that is responsible for the majority of passive force generated by the myocardium. Titin’s force is derived from its extensible I-band region, which, in the cardiac isoform, comprises three main extensible elements: tandem Ig segments, the PEVK domain, and the N2B unique sequence (N2B-Us). Using atomic force microscopy, we characterized the single molecule force-extension curves of the PEVK and N2B-Us spring elements, which together are responsible for physiological levels of passive force in moderately to highly stretched myocardium. Stretch-release force-extension curves of both the PEVK domain and N2B-Us displayed little hysteresis: the stretch and release data nearly overlapped. The force-extension curves closely followed worm-like chain behavior. Histograms of persistence length (measure of chain bending rigidity) indicated that the single molecule persistence lengths are ~1.4 and ~0.65 nm for the PEVK domain and N2B-Us, respectively. Using these mechanical characteristics and those determined earlier for the tandem Ig segment (assuming folded Ig domains), we modeled the cardiac titin extensible region in the sarcomere and calculated the extension of the various spring elements and the forces generated by titin, both as a function of sarcomere length. In the physiological sarcomere length range, predicted values and those obtained experimentally were indistinguishable.

Titin forms a striated muscle-specific myofilament that develops passive force in response to sarcomere stretch (for a recent review with original citations, see Ref. 1). Titin’s force is generated by serially linked and mechanically distinct spring elements (2). Tandem Ig segments (tandemly arranged Ig-like domains) and the PEVK domain (rich in proline, glutamate, valine, and lysine residues) are spring elements found in both cardiac and skeletal muscle titins (3, 4) that vary in length in different isoforms of titin. For example, in human, the PEVK domain varies from 188 residues in the cardiac-specific N2B isoform to 2181 residues in skeletal soleus muscle (2). The cardiac-specific N2B unique sequence (N2B-Us)3 forms a third spring element in cardiac titins and provides extensibility at the upper range of physiological sarcomere lengths in the heart (5–7). The three-spring system of cardiac titin results in a unique force-extension curve that underlies the majority of the physiological passive tensions of the myocardium, and the variable-length tandem Ig and PEVK elements allows passive tension to be adjusted so that it matches the mechanical demands placed on normal and diseased myocardium (8, 9).

Immunoelectron microscopy has shown that in slack sarcomeres (no external force), the tandem Ig segments are in a “contracted” state. When the sarcomeres are stretched, the segments greatly extend (4) due to unbending of linkers between folded Ig domains (4) and possibly to limited domain unfolding (10). The tandem Ig segments exhibit worm-like chain (WLC) behavior with a persistence length (measure of chain bending rigidity) that is relatively long (13.5 nm based on a recent single molecule study) (11), explaining why tandem Ig segment extension dominates in moderately stretched sarcomeres where passive force is low (4). The PEVK domain extends at relatively high forces (4), and this process is likely to result from straightening of random coil and polyproline II helices that comprise this element (12). Single molecule (13) and immunolabeling (4) studies of skeletal muscle titin suggest that the PEVK domain acts as a WLC with a persistence length of ~2 nm, whereas recent single molecule work on the cardiac PEVK domain ("N2B isoform") revealed persistence lengths that range from 0.4 to 2.5 nm (14). As for cardiac-specific N2B-Us, immunoelectron microscopy has well established that this sequence extends in the cardiac sarcomere (5–7), forming the cardiac titin third spring element. The molecular mechanical characteristics of this spring have not yet, however, been determined.

Here we investigated the extensibility of the cardiac titin PEVK element and N2B-Us using an atomic force microscope (AFM) specialized for stretching single molecules. Both the PEVK domain and N2B-Us displayed little force hysteresis during stretch-release cycles, and their force-extension curves followed WLC behavior. Persistence length histograms revealed multiple peaks, with the main peak at the longest length occurring at ~1.4 and ~0.65 nm for the PEVK domain and N2B-Us, respectively. Assuming that these peaks reflect the persistence length of the single molecule allowed us to

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3 The abbreviations used are: N2B-Us, N2B unique sequence; WLC, worm-like chain; AFM, atomic force microscopy/microscope; pN, pico-newton(s); PP, polyproline.
predict, within experimental error, the measured extensible behavior of the titin spring elements in the cardiac sarcomere as well as the passive force-sarcomere length relation of cardiac myocytes. We conclude that the serially linked WLC model with mechanically distinct springs using parameters established in this work closely describes the in vivo behavior of cardiac titin.

**EXPERIMENTAL PROCEDURES**

**Protein Fragments**—Cardiac titin cDNA fragments (see Fig. 1) were amplified by PCR using primer pairs derived from the human cardiac titin N2B isoform (GenBank Accession number X90568). Fragments were cloned into modified pET vectors, expressed as poly-Ig fragments, and purified using the soluble fraction on Ni2+-nitrilotriacetic acid columns with a His-Bind purification kit (Novagen, Madison, WI). Fragment boundaries from the human cardiac titin N2B isoform corresponded to 191–98 (residues 5237–5959), the PEVK domain with flanking Ig domains (residues 4337–4713), and N2B-Us (residues 3671–4242). We also used the skeletal muscle-specific fragment I65–70, with residues 7005–7572 (human soleus skeletal muscle titin, GenBank Accession number X90569) as the fragment boundaries. Purified proteins were dialyzed into assay buffer (25 mM imidazole HCl (pH 7.4), 0.2 mM KCl, 4 mM MgCl2, 1 mM EGTA, 0.01% NaN3, and 5 mM dioctadecylammonium bromide, quick-frozen, and stored at –80 °C until later use.

**Mechanical Manipulation of Titin**—Molecules were stretched using a molecular force probe (Asylum Research, Santa Barbara, CA), an AFM specialized for stretching molecules. The molecular force probe was mounted on a custom-built, low profile, inverted light microscope containing a motorized stage for sample microinjection and a computer controlled load system. We used the domain numbering system of Bang et al. (13) and Trinick and Labeit (18) wherever necessary.

**Calculations of Extension and Force**—The elastic region of cardiac titin (N2B isoform) in the sarcomere was modeled as three serially linked WLCs with different contour and persistence lengths: the tandem Ig segments, the PEVK segment, and N2B-Us. The contour length of the tandem Ig segments (proximal + distal segments) was calculated by adding their combined number of amino acids (termination of the N2B element were included) multiplied by a domain spacing of 4.5 nm (17) in a completely extended chain (40 domains × 4.5 nm = 180 nm) (Note that this assumes that all domains are folded. For calculations that involve unfolding, see below.) The persistence length of the native tandem Ig segment was set as 13.5 nm based on the recent study by Ding et al. (41) and Trinick and Labeit (18). The contour length of the PEVK domain and N2B-Us were obtained from their number of amino acid residues times the maximal residue spacing in an unfolded polypeptide (0.38 nm). The number of PEVK residues in human titin N2B was determined assuming that the C-terminal end of the flanking Ig domain I27 is given by the amino acid sequence TVTV and the N-terminal end of the flanking Ig 184 by PLKF, resulting in 188 PEVK residues and a PEVK contour length of 71.4 nm (188 × 0.38 nm). The number of N2B-Us residues is 572 in human, giving rise to a 217.4-nm contour length. For calculations of extensibility of rat cardiac titin (for Fig. 6), we took into account the slightly shorter N2B-Us and PEVK sequences in this species and used 199.8 and 70.7 nm as contour lengths. The persistence lengths of the PEVK domain and N2B-Us were set at 1.4 nm, respectively. For details regarding the composition of the tandem Ig segments, the PEVK domain, and N2B-Us, see Ref. 18 and GenBank Accession number X90568.)

Because the tandem Ig, PEVK, and N2B-Us segments are connected in series, they bear equal forces. Therefore, for a given force, the extension of each segment (z2g + PEVK, and z2N2B-Us) can be calculated. We may then calculate for that force the total extension of the titin elastic segment (z2g + zPEVK + zN2B-Us). By adding the total length of non-extensible sarcomeric components (1800 nm) (see Ref. 19), the sarcomere length can be calculated (z2g + zPEVK + zN2B-Us) × 2 + 1800 nm, and the predicted force-sarcomere length relation constructed.

**Persistence Length of PEVK Domain-containing Polyproline (PP) Helices**—The above calculations assume that the PEVK domain comprises solely random coil structure. However, it is likely that some of the residues form PP helices (see “Discussion”). Therefore, we calculated the apparent persistence length of the PEVK segment (cardiac N2B isoform) assuming that the PP residues are either type II or I or A search for PPXX, PXXXP, and PXXPPP motifs (cf. Ref. 12) suggested that 7 of the total 188 PEVK residues (or 3.7%) are part of polyproline helices. (Note that this is slightly higher than the PEVK domain, in which 7.8% of the residues are predicted to be part of the PP motifs, but much less than the 57% of the residues in the 28-residue fetal titin fragment used by Ma et al. (12).) The 0.31- and 0.19-nm translations per residue for PPPII and PPII helices, respectively, allow the contour lengths of 7-residue PPII and PPII helices to be calculated. We assumed that the persistence lengths of PPII and PPII helices are 1000 and 0.1 nm, respectively (these values are extremes, with 1000 and 0.1 nm representing ultra-high and ultra-low stiffness, respectively), and that there is no conversion between the two PP states under our experimental loading conditions (cf. Li et al. (14)). The persistence length of the remaining 197 PEVK residues that form a random coil was set at 1.4 nm (see “Results”), and the contour length at 68 nm (179 × 0.38 nm). We then calculated the force-extension curve of the 179-residue random coil region linked with either the 7-residue PPII or PPII helix (following the methods explained above). The apparent persistence length of the PP helix-containing PEVK segment was obtained by fitting the curve with the WLC model (for results, see “Discussion”).

We also calculated the effect of unfolding of the PPII helix on the force-extension curve of the PEVK domain. Before unfolding, we assumed that these 7 residues had contour and persistence lengths of 2.17 nm (7 × 0.31 nm) and 1000 nm, respectively. After conversion of the PPII helix to a random coil, we assumed that the values were 2.66 nm (7 × 0.38 nm) and 1.4 nm, respectively. We then calculated the overall contour and persistence lengths of the PEVK domain and used these values to calculate the WLC force of the PEVK element before and after unfolding.

**Probability of Domain Unfolding**—The above-described calculations of extension and force in the sarcomere assume that Ig domain unfolding does not occur during sarcomere stretch. To ascertain whether this is a valid assumption, we calculated the probability of domain unfolding

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1 We used the domain numbering system of Bang et al. (41) throughout this work, but made references to the previous nomenclature of Labeit and Kolmerer (18) wherever necessary.

2 M. Greaser, manuscript in preparation.
by superimposing domain unfolding/refolding kinetics on the WLC force-extension curve (see above). To model domain unfolding/refolding, Monte-Carlo simulations were carried out based on previously used simulation algorithms (13, 20). For a given force ($F$), the number of unfolded/refolded domains was calculated according to the following equation: $dN = N_0 dF/(k_b T)$, where $dN$ is the change in number of folded domains during the $dt$ polling interval and $N_0$ is the number of available folded domains. The attempt frequency ($\alpha_0$) was $10^9$ (21), and $k_b T = 4.14$ pNnm. The activation energies ($E_a$) for unfolding and refolding and the widths ($\Delta x$) of the unfolding and refolding potentials were user-adjustable. In all simulations, we assumed for refolding $E_r = 82$ pNnm (corresponding to a zero-force refolding rate of $2.5 \times 10^{-5}$s$^{-1}$) and $\Delta x = 2.3$ nm (based on measurements for I91) (22). The $\Delta x$ unfolding values were obtained from previous work, and unfolding activation energies were calculated from published unfolding rate constants at zero force ($\alpha_0$): native titin, $\Delta x = 0.3$ nm and $\alpha_0 = 3.3 \times 10^{-5}$ s$^{-1}$ (16); I91 (formerly I127), $\Delta x = 0.25$ nm and $\alpha_0 = 6 \times 10^{-8}$ s$^{-1}$ (22); I92 (or I28), $\Delta x = 0.25$ nm (23) and $\alpha_0 = 2.5 \times 10^{-6} s^{-1}$; I91–98, $\Delta x = 0.25$ nm and $\alpha_0 = 6.1 \times 10^{-5} s^{-1}$; and I65–70, $\Delta x = 0.35$ nm and $\alpha_0 = 1 \times 10^{-5} s^{-1}$. Following each calculation step, the domain unfolding process was permitted or prohibited depending on a comparison of $dN$ with a number generated randomly between 0 and 1. Unfolding events decreased the number of remaining folded domains and the length of the folded segment by 4.5 nm and increased the length of the unfolded segment by 30 nm. Refolding events increased the number of folded domains and the length of the folded segment by 4.5 nm and decreased the length of the unfolded segment by 30 nm. Calculations were carried out using a stretch-release protocol with a constant velocity of 100 nm/s (to match the velocity used during force measurements on cardiac cells; see below) and a stretch amplitude determined by the preset maximal allowable force. This maximal force was selected at 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, and 70 pN. Simulations were repeated 1000 times, and the total number of domains that unfolded ($x$) was determined. The probability of unfolding was then defined as $x/1000$. Calculations and simulations were conducted using Object Pascal (Metrowerks CodeWarrior Version 10) on a Power Macintosh G3 computer.

**Passive Force**—We used our previously determined passive tension-sarcomere length relation measured with a stretch velocity of 100 nm/s on rat cardiac myocytes (rat expresses primarily cardiac titin N2B) (5). We converted passive tensions (force/mm$^2$ of cross-sectional area of myocyte) to force per titin molecule, taking into account that only 82% of the cell’s cross-section contains myofibrils (5). Furthermore, the average area in the cross-section of the myofibril occupied by a single thick filament was calculated as $d_2^2 \times 2$ (25) ($d_2$ lattice spacing of rat slack myocardium is 43 nm; see Ref. 26), and we assumed that there are 6 titin molecules/half-thick filament (27, 28). Accordingly, there are $2.31 \times 10^8$ titin molecules/mm$^2$ of cell cross-sectional area, and this value was used to convert measured passive tensions (5) to passive force per single titin molecule (see Fig. 6D).

**Extensions**—To obtain the measured extensions of tandem Ig segments, the PEVK domain, and N2B-Us, we used the results of our earlier immunoelectron microscopic studies on N2B-expressing cardiac myocytes (6, 29). Because the C-terminal end of the distal tandem Ig segment was determined with the MIR antibody, which does not exactly label the C-terminal end (6), we adjusted the measurements assuming that a native domain (Ig or fibronectin-type 3) contributes 4.5 nm to the contour length. The measured extension-sarcomere length data were segregated in sarcomere length bins of 0.05 mm, and mean values ± S.E. were then calculated (see Fig. 6C).

**Structure Predictions**—We used the following programs: PELE and GOR4,4 nnPredect,5 and PONDR (Predictor of Natural Disordered Proteins).6

### RESULTS

To study the mechanical properties of the cardiac PEVK domain (N2B isoform) and N2B-Us, we used recombinant fragments containing the 572-residue N2B-Us or the 188-residue PEVK domain along with its flanking Ig domains I27 and I84 (Fig. 1). Force-extension curves were measured using an AFM (molecular force probe) specialized for stretching individual molecules (Fig. 2A). Because our goal was to determine the persistence lengths of single PEVK and N2B-Us molecules, we optimized the likelihood of stretching single molecules using protein concentrations that were so low that, during continuous probing of the surface with the AFM, only few contacts were made. Furthermore, we also studied poly-Ig fragments (165–70 and 191–98 from the proximal and distal tandem Ig segments, respectively) because they display force sawteeth, with a spacing that is likely to be regular when pulling single molecules and irregular when pulling multiple molecules in parallel (30), and explored how stretching multiple molecules affects the persistence length.

An example of a stretch-release curve of I91–98 is shown in Fig. 2B. The stretch curve displays sawtooth-like force peaks, indicating that repetitive structural transitions occurred during stretching, with each sudden force drop representing the unfolding of a β-barrel Ig domain. At a stretch rate of 0.5 μm/s, the mean unfolding force was ~230 pN, consistent with the reported high stability of Ig domains (31). The curve in Fig. 2B is from an experiment in which the force peaks were regularly spaced (average spacing was ~28 nm), whereas, on occasion, irregularly spaced peaks were obtained, an example of which is shown in Fig. 2C. We determined the persistence length of unfolded Ig domains by analyzing the release data of stretch-release curves. The data were segregated into two groups: (i) data containing stretch curves with regularly spaced force sawteeth (as in Fig. 2B) and (ii) data containing stretch curves with irregularly spaced force sawteeth (as in Fig. 2C). We found that the persistence length distribution from data with regularly spaced force sawteeth had a major peak at ~0.6 nm (Fig. 2D, shaded bars). The distribution obtained from curves with irregular force peaks was shifted to shorter lengths and had a major peak at ~0.35 nm (Fig. 2D, white bars). Our interpretation is that the “irregular” curves are derived mainly from doublets (2 molecules connected in parallel) because such an arrangement would double the force of the release curve and twice the persistence length (minor deviations could result if the 2 molecules interacted). Consistent with our earlier work (13), multimodal persistence length histograms allow the single molecule persistence length to be identified from the peak in the histogram at the longest length. Thus, we used this approach to study the PEVK domain and N2B-Us.

Examples of stretch-release curves obtained with the PEVK...
fragment are shown in Fig. 3. Because this fragment contains the PEVK domain and its flanking Ig domains (I27 and I84), we limited the stretch amplitude such that the curves were typically in the 0–100-pN force regime, reducing the likelihood of Ig domain unfolding. The obtained force curves during stretching largely overlapped with those measured during release; and thus, hysteresis was small. On occasion, the initial part of the stretch curve was irregular, with small sudden reductions in force (as in Fig. 3D). Because these features were not always observed, they possibly resulted from processes between the molecule and the substrate/cantilever tip (for example, “unsticking”). It seems unlikely that the force reductions were due to unfolding of PPII helices (the PEVK domain consists of short PPII helices interspersed between the random coil; see “Discussion”) because their length gain upon unfolding is small (~0.7 Å/residue). Calculations (see “Experimental Procedures”) showed that even if all PPII helices were to simultaneously unfold in the force range used in this work, the ensuing force reduction would be below the resolution of the AFM (~5 pN).

Fitting the PEVK domain release curves with the WLC equation (results shown as black lines in Fig. 3) revealed that the persistence lengths of the curves varied. Fig. 3 shows examples of curves with persistence lengths of ~0.7–0.8 nm (A and B) and ~1.4 nm (C and D). The persistence length histogram of all results is shown in Fig. 4A. The distribution was multimodal, with peaks at ~1.4, ~0.8, and ~0.45 nm. We interpret our findings by assuming that the persistence length peak at ~1.4 nm reflects the persistence length of the single molecule and

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**Fig. 2.** A, schematic of force-measuring AFM. A molecule is attached between the tip of the cantilever and a glass microscope slide. Extension of the molecule by retraction of the piezoelectric positioner generates force in the molecule, deflecting the cantilever. Cantilever deflection is measured, from which the force generated in the molecule can be calculated using the experimentally determined cantilever stiffness (see “Experimental Procedures”). B and C, examples of stretch (red)-release (blue) curves of I91–98, with the WLC fit to the release curve (black lines). B shows an example of a stretch curve with regularly spaced (~28 nm) force peaks, and C shows an example of a curve with irregularly spaced force peaks. D, persistence length distribution obtained from WLC fits. The distribution of curves with regularly (shaded bars) and irregularly (white bars) spaced sawteeth had major peaks at ~0.6 and ~0.35 nm, respectively. The results in D are pooled results from I91–98 and I65–70 fragments. Their individual persistence length distributions were indistinguishable.
that the peaks at $0.8$ and $0.45$ nm reflect the persistence lengths of doublets and triplets, respectively (see also “Discussion”). The red lines in Fig. 4A indicate the persistence length histogram determined in a recent study by Li et al. (14) using the engineered polypeptide (I91-PEVK)$_3$ (immunoglobulin domain I91 (previously referred to as I27) coupled to the PEVK domain of the cardiac N2B isoform and serially linked three times). Except for persistence lengths shorter than $0.5$ nm, the results of the two studies are similar.

The contour length distribution obtained from the WLC fits is shown in Fig. 4B. Because the cantilever tip attached to the protein at random locations, a broad contour length distribution with a maximal length of $70$ nm (predicted PEVK contour length; see “Experimental Procedures”) was expected. Although a few of the values were $>70$ nm (they may have resulted from multimolecular complexes), the sharp drop-off in data at $60-70$ nm is consistent with the maximal contour length of the PEVK sequence.

Examples of force-extension curves obtained for N2B-Us are shown in Fig. 5. As was seen for the PEVK fragment, the force-extension curve measured during stretching (blue) largely overlapped with the curve measured during release (red). In earlier work, we proposed that N2B-Us may be responsible for passive force hysteresis in cardiac myocytes (5), and the present work does not fully exclude this possibility, as in some of the curves, a limited degree of hysteresis appeared to be present (see, for example, Fig. 5A). However, relative to the force noise, hysteresis was small and difficult to quantify, and a more conclusive evaluation of the level of hysteresis has to await future work with lower force-noise techniques.

The release curves of N2B-Us could be fit well with the WLC model (black lines superimposed on the release curves in Fig. 5). Histograms of the derived persistence and contour lengths are shown in Fig. 4 (C and D, respectively). Consistent with N2B-Us (predicted contour length of 217 nm; see “Experimental Procedures”), the contour length distribution (Fig. 4D) has

![Figure 3: Examples of PEVK domain force-extension curves.](image-url)

Note that the stretch (blue) and release (red) curves largely overlap. The WLC was fit to the release curve and is shown in black, with the persistence length (A) indicated. A and B show examples in which the curve has a relatively short persistence length, and C and D are examples with a long persistence length.
few data points at lengths >220 nm. The persistence length histogram has a broad distribution, with major peaks at ~0.65 and ~0.3 nm (Fig. 4C). Fig. 4C also shows our PEVK results superimposed (broken red lines), clearly showing that the two fragment types are very different. We interpret the multimodal persistence length distribution of N2B-Us by assuming that the persistence length peak at 0.65 nm reflects the persistence length of the single molecule and that the peak at ~0.3 nm reflects that of doublets.

To determine the validity of the deduced single molecule characteristics in the context of the sarcomere, we modeled the extensible region of cardiac titin (N2B isoform) as three serially linked WLCs: tandem Ig segments, the PEVK domain, and N2B-Us (Fig. 6A). To ascertain whether unfolding of Ig domains has to be taken into account when modeling the extensible region of titin, we first studied the probability of domain unfolding as a function of force in simulated stretch-release cycles. Because unfolding characteristics (unfolding rate at zero force and width of the unfolding potential) have not been determined for all of the Ig domains found in the extensible region of cardiac titin, we assumed that all domains have characteristics like those determined for 1) native titin, 2) I91–98, 3) I65–70, 4) I92, and 5) I91 (for details, see “Experimental Procedures”). The results indicate that in repeated stretch-release cycles, the probability of domain unfolding is highest if all domains behave like I91 (Fig. 6B, curve 5). However, because all other curves have a much lower probability than the I91 curve (Fig. 6B, curves 1–4), it is likely that I91 is an atypical domain and that most other domains in the titin extensible region have a lower unfolding probability. Even if all domains were to behave like I91 (which is unlikely), the probability of unfolding of a single domain at the upper range of the

Fig. 4. A and B, persistence and contour length histograms, respectively, of the PEVK domain. Note the multimodal distribution of persistence lengths in A, with peaks at ~1.4, ~0.8, and ~0.45 nm. Inset, examples of curves with short and long persistence lengths. Red lines in A are the PEVK domain persistence lengths of Li et al. (14) obtained using the polyprotein (I27-PEVK). Note that except for lengths shorter than ~0.5 nm, the results of the two studies overlap. C and D, persistence and contour length histograms, respectively, of N2B-Us. Note the bimodal distribution of persistence lengths in C, with peaks at ~0.65 and ~0.3 nm. Red lines in C and D represent the PEVK domain results of A and B, respectively.
physiological force regime (~10 pN) is ~0.1, indicating that of all titin molecules in the sarcomere, ~10% may have a single domain unfolded. We conclude that our simulation suggests that at physiological force levels, domain unfolding is not a likely event and that it is justified to simulate the extensible region of cardiac titin with tandem Ig segments containing folded domains.

We calculated the extension of each spring element that makes up the titin extensible region (Fig. 6A) as a function of sarcomere length and compared results with those experimentally determined in a previous immunoelectron microscopic study (see “Experimental Procedures” for details). This revealed that in sarcomeres longer than ~2.5 μm, the measured tandem Ig segment extension exceeds predicted values, whereas the measured N2B-Us extension is slightly less than predicted (Fig. 6C). However, in the physiological sarcomere length range (~1.8–2.4 μm), predictions and measurements are similar.

We also used the serially linked WLC model of the cardiac titin extensible region to predict the force of a single titin molecule in the cardiac sarcomere. The results obtained were compared with the measured passive force-sarcomere length relation of cardiac myocytes (expressing primarily cardiac N2B titin) scaled down to the single molecule level (see “Experimental Procedures” for details). The results show that the predicted passive forces are within experimental error of those measured (Fig. 6D).

**DISCUSSION**

Force developed by the cardiac titin extensible region underlies the majority of the tension developed by the myocardium during the filling phase of the heart (8). In the contracting myocardium, titin’s force may modulate actomyosin interaction by a titin-based alteration of the distance between myosin heads and actin (32). Novel ligands have been identified that link titin to membrane channels, protein turnover, and gene expression (1). Considering titin’s wide range of functions, it is important to understand the mechanism of force development by titin. Here we studied mechanical properties of the PEVK domain and N2B-Us, spring elements that determine the physi-
iological levels of passive tension in moderately to highly stretched myocardium (6).

Persistence Length Histograms—Titin molecules have a tendency to aggregate (33). Therefore, in AFM-based mechanical experiments, the data may be derived from single molecules and multimolecular complexes. In earlier work with skeletal muscle titin (13), we presented evidence that when performing a large number of experiments, a persistence length histogram can be constructed that is multimodal, with the peak at the longest length reflecting the persistence length of the single molecule. This notion was tested here in experiments in which we stretch-released poly-Ig fragments and determined the persistence lengths of unfolded Ig domains from the release part of the cycle. When plotting results in a histogram (Fig. 2D), a bimodal distribution was obtained with peaks at $-0.6$ and $-0.35$ nm. The peak at the long length was derived from experiments in which regularly spaced sawteeth were present during stretching, suggesting that single molecules were stretched (regularly spaced peaks are generally viewed as a single molecule “fingerprint”) (14). The persistence length peak at the short length was from curves with irregularly spaced sawteeth, suggesting that they were derived from multimers. The finding that the short length ($-0.35$) is close to half of the long length ($-0.6$) indicates that the predominant multimer is a doublet. (The short length is not exactly half of the long length, and this may be due, for example, interaction between the molecules that make up the doublets that stiffens the composite chain.) Thus, the experiments with poly-Ig fragments support the notion that the persistence length histogram allows the single molecule to be identified.

Persistence Length of the PEVK Domain—In agreement with a recent study of a polyprotein with three repeats of the cardiac PEVK domain coupled to I91 (127 in older nomenclature) (14), stretch-release cycles of the PEVK domain display little or no hysteresis, and forces can be fit well with the WLC model (Fig. 3). Thus, the PEVK domain behaves as an entropic spring with a random coil structure. The PEVK domain persistence length histogram shows a multimodal distribution with peaks at $-1.4$, $-0.8$, and $-0.45$ nm, consistent with being derived from singlets, doubles, and triplets, respectively. The persistence length distribution at lengths longer than $-0.5$ nm is similar to that reported by Li et al. (14) using the above-described polyprotein (Fig. 4A). However, in the work of Li et al., few data points were found at lengths $<0.5$ nm, whereas we found a large peak at $-0.45$ nm (Fig. 4A, red lines). It is possible that the protein fragment that we used has a higher tendency for aggregation than the polyprotein used by Li et al. Alternatively, in the polyprotein (14), the I91 module was included to provide a single molecule fingerprint (using its unfolding force of $-200$ pN), and this may have largely eliminated those curves that were derived from triplets from the analysis (because they were judged not to have the correct fingerprint).

Our interpretation of the PEVK domain persistence length distribution differs from that of Li et al. (14), who assumed that all results of their study were derived from single molecules, but that the persistence length of the single molecule could vary greatly (from 0.4 to 2.5 nm). This interpretation is based on the recent work with a 28-amino acid PEVK fragment from human fetal skeletal muscle titin (12). Using CD and NMR techniques, Ma et al. (12) provided evidence that this fetal...
PEVK fragment contains both random coil and PPII helix structure. Li et al. (14) proposed that the polyproline helix conformation can be either type II or I and that differences in the persistence length of the two types underlie the wide persistence length range of the cardiac PEVK domain. PPII helices are likely to have the longest persistence length because they have a more elongated structure (in the PPII helix, the axial translation per residue is 1.2 Å larger than in the PPII helix). Thus, a PEVK molecule with its PP helices in the type II conformation may have a longer overall persistence length than a molecule with type I helices. A search for PXPP, PXXPP, and XPXXPP motifs (cf. Ref. 12) suggests that 7 of the total 188 human PEVK (N2B isoform) residues are part of polyproline helices. We calculated the persistence length of the PEVK domain assuming that the helices are either type II or I (for details, see “Experimental Procedures”) and obtained values of 1.47 and 1.27 nm, respectively. The difference in values is much less than the range of values observed in our study or that of Li et al. (14) (0.4–2.4 nm). The explanation of the results requires not only extremely different persistence lengths for type I and II helices (see “Experimental Procedures”), but also that nearly 50% of all PEVK residues are part of PP helices. It is also worth noting that PPII helices have a unique CD spectrum with a strong positive band at 215 nm (34) and that this signature feature is not present in the reported CD spectrum of the fetal PEVK fragment (which is unusually rich in PP structure; see “Experimental Procedures”) studied by Ma et al. (12); instead, the CD spectrum provides evidence for only PPII helices. Thus, although PP helices may have important functions (such as mediating protein-protein interactions) (12, 33, 35), they may not explain the broad persistence length distribution that we measured for the PEVK domain (Fig. 4A). Instead, we propose that this persistence length histogram is based on the presence of singlets, doublets, and triplets and that, consequently, the persistence length of the single molecule is ~1.4 nm.

**Persistence Length of N2B-Us**—The cardiac-specific N2B-Us extends greatly toward the upper limit of the physiological sarcomere length range and determines the upper range of physiological forces (6). Here we characterized the mechanical properties of N2B-Us using single molecule techniques. Stretch curves revealed, on occasion, abrupt force reductions (as in Fig. 5B), but they were not seen consistently (they were absent in ~95% of the curves) and may not be a typical feature of the molecule. To determine whether the absence of force peaks in most of the curves may be due to limitations of our AFM technique, we also stretched spectrin, a protein that has been shown to display low amplitude (~20–30 pN) force peaks during stretching (36). Employing methods identical to those used for N2B-Us revealed sawteeth with an average peak force of 21.1 ± 1.6 pN (n = 17). We conclude that it is likely that force peaks are absent from N2B-Us curves, not because of some experimental artifact, but because no stable structures are formed that can withstand detectable levels of force before unfolding during stretching. The absence of consistent force peaks in the N2B-Us stretch curves and the finding that the stretch-release cycles were largely reversible and followed WLC behavior suggest that, mechanically, N2B-Us behaves like a random coil.

The secondary structure composition of N2B-Us has not yet been experimentally determined. Secondary structure predictors (see “Experimental Procedures”) suggest that a few residues (~10% of the total) participate in β-strand formation, approximately one-third of the residues form α-helical structures, and approximately two-thirds of the residues may be unstructured. Results using disorder predictors (see “Experimental Procedures”) support the conclusion that the majority of the residues (60–80%) may be unstructured. Comparison with earlier work by Fernandez and co-workers (30) on the α-helix protein calmodulin indicates that the predicted α-helical content of N2B-Us is not expected to give rise to force peaks. Steered molecular dynamics simulation of calmodulin showed that stretching of its α-helix causes sequential breakage of intrahelix hydrogen bonds that requires little force, and this was confirmed by AFM of calmodulin, which displayed pure entropic spring behavior without detectable force peaks (30). (Spectrin-like proteins may be an exception because they form regularly repeating units of triple-helical anti-parallel coiled-coils (37) that give rise to just detectable force peaks.) Thus, although α-helical structures may perform important functions at low force (for example, they may mediate protein-protein interactions), they appear to be not intended to withstand high mechanical forces. In conclusion, the structure predicted for N2B-Us (limited α-helix and largely disordered) is consistent with the lack of force transitions during stretching and the overall WLC behavior observed by AFM.

The persistence length histogram of N2B-Us is multimodal, with peaks at ~0.65 and ~0.3 nm (Fig. 5C), and this may be explained by a single molecule persistence length of 0.65 nm and the presence of doublets with a persistence length of 0.3 nm. The tendency to form doublets may be due to, for example, electrostatic interactions between individual molecules or α-helices of different molecules that form coiled-coil structures. It is interesting to note that the single molecule persistence length of N2B-Us is similar to that of unfolded Ig domains, but much shorter than that of the PEVK domain (all values based on force measurements in the ~0–100-pN regime). Thus, the long persistence length of the PEVK domain may be unusual, possibly due to the high proline content of the PEVK domain, whereas the values of N2B-Us and unfolded Ig domains may be more typical of the average protein. Finally, it is worth noting that like other recently discovered proteins with unfolded regions that are essential for their function (24, 38–40), cardiac titin is a prime example of a protein for which the unfolded state is functionally important.

**Serially Linked WLC Model**—The extensible region of cardiac titin contains three mechanically distinct springs that are serially linked: tandem Ig segments, N2B-Us, and the PEVK domain (Fig. 6A). Using the above-deduced molecular characteristics of the PEVK domain and N2B-Us and those determined earlier for the tandem Ig segment with folded Ig domains (see “Experimental Procedures”), we calculated the extension of the various spring elements and predicted the forces generated by titin, both as a function of sarcomere length. In the physiological sarcomere length range (1.8–2.4 μm), predicted values were within experimental error of measured values (Fig. 6, C and D), suggesting that the model parameters used in the simulations approximate closely those in situ. Because the model calculations assume that unfolding of Ig domains is absent (see “Experimental Procedures”), these findings also indicate no or limited unfolding. Only at sarcomere lengths greater than ~2.5 μm does the measured tandem Ig segment extension exceed that predicted (Fig. 6C); and thus, beyond the physiological sarcomere length range, Ig unfolding is likely to take place (calculations reveal that unfolding of less than two domains explains the results). The absence of large-scale unfolding at physiological sarcomere lengths is supported by the calculated probability of domain unfolding in stretch-release cycles with progressively increasing maximal force (Fig. 6B). By having Ig domains in their folded state, the tandem Ig segments attain a long persistence length (~15 nm), allowing them to extend under low force (Fig. 6, compare C and...
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D) and to set the sarcomere length at which extension of the PEVK domain and N2B-Us starts to dominate. Thus, their stable tertiary structure allows tandem Ig segments to function as a “molecular leash.”

In summary, AFM studies revealed that the PEVK domain and N2B-Us both behave as entropic springs, but with different persistence lengths. Persistence length histograms allow the single molecule persistence lengths to be determined; and using the obtained PEVK domain and N2B-Us values, a model of the extensible region of cardiac titin can be constructed that simulates very closely the complex extension of titin in the sarcomere and the unique passive force-sarcomere length relation of cardiac myocytes.

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