Titin Elasticity and Mechanism of Passive Force Development in Rat Cardiac Myocytes Probed by Thin-Filament Extraction

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ABSTRACT Titin (also known as connectin) is a giant filamentous protein whose elastic properties greatly contribute to the passive force in muscle. In the sarcomere, the elastic I-band segment of titin may interact with the thin filaments, possibly affecting the molecule's elastic behavior. Indeed, several studies have indicated that interactions between titin and actin occur in vitro and may occur in the sarcomere as well. To explore the properties of titin alone, one must first eliminate the modulating effect of the thin filaments by selectively removing them. In the present work, thin filaments were selectively removed from the cardiac myocyte by using a gelsolin fragment. Partial extraction left behind ~100-nm-long thin filaments protruding from the Z-line, whereas the rest of the I-band became devoid of thin filaments, exposing titin. By applying a much more extensive gelsolin treatment, we also removed the remaining short thin filaments near the Z-line. After extraction, the extensibility of titin was studied by using immunoelectron microscopy, and the passive force-sarcomere length relation was determined by using mechanical techniques. Titin's regional extensibility was not detectably affected by partial thin-filament extraction. Passive force, on the other hand, was reduced at sarcomere lengths longer than ~2.1 μm, with a 33 ± 9% reduction at 2.6 μm. After a complete extraction, the slack sarcomere length was reduced to ~1.7 μm. The segment of titin near the Z-line, which is otherwise inextensible, collapsed toward the Z-line in sarcomeres shorter than ~2.0 μm, but it was extended in sarcomeres longer than ~2.3 μm. Passive force became elevated at sarcomere lengths between ~1.7 and ~2.1 μm, but was reduced at sarcomere lengths of >2.3 μm. These changes can be accounted for by modeling titin as two wormlike chains in series, one of which increases its contour length by recruitment of the titin segment near the Z-line into the elastic pool.

INTRODUCTION

Titin is a giant sarcomeric protein that runs from the Z-line to the M-line (for recent reviews see Keller, 1995; Trinick, 1996; Labeit et al., 1997; Maruyama, 1997). The I-band segment of the molecule extends when slack sarcomeres are stretched or shortened, thereby functioning as a molecular spring that generates passive or restoring forces in skeletal and cardiac muscle (Horowits et al., 1986; Higuchi, 1992; Wang et al., 1993; Linke et al., 1994; Granzier and Irving, 1995; Granzier et al., 1996; Helmes et al., 1996). Recent data indicate that not all of the I-band is elastic, and that, in slack cardiac myocytes, the elastic segment has an end-to-end length that is considerably shorter than its contour length (Trombitás et al., 1995; Granzier et al., 1996). A passive force model was proposed in which thermal fluctuations impacting the segment give rise to an entropic elastic force at sarcomere lengths ranging from slack to ~2.0 μm, while at longer lengths subdomains of the elastic segment unfold (Granzier et al., 1996).

The molecular composition of the elastic segment can be determined from the sarcomeric binding sites of the sequence-specific anti-titin antibodies T12 and Ti-102 (Trombitás et al., 1995; Jin, 1995; Sebestyén et al., 1995). These studies revealed that the elastic segment includes 41 immunoglobulin-like (Ig) domains, the majority of which are located in two regions known as the tandem Ig, and five fibronectin-like (Fn) domains (Labeit and Kolmerer, 1995). Furthermore, the elastic segment of titin also contains the unique PEVK domain, which may constitute a low-stiffness region in the molecule (Labeit and Kolmerer, 1995; Linke et al., 1996; Gautel and Goulding, 1996; Tskhovrebova and Trinick, 1997).

Recent molecular mechanical experiments revealed that titin is a highly nonlinear spring, making it worthwhile to examine the detailed molecular mechanisms of titin elasticity and passive force development in muscle. A complicating factor is the presence of actin-titin interaction. Such interaction is strong in vitro and may also occur in situ (Trombitás and Granzier, 1997, and references therein). In this work we have investigated titin's elasticity and the possible interaction between titin and actin near the Z-line and elsewhere in the I-band. Studies were performed on cardiac myocytes, where nebulin is absent (Kruger et al., 1991), thus eliminating nebulin-titin interaction as an additional complicating factor.

Thin filaments were selectively removed by using gelsolin. Gelsolin is an actin-severing protein that has been successfully used to extract actin from vertebrate muscles (cardiac and skeletal) and insect flight muscle (Funatsu et al., 1990, 1993; Granzier and Wang, 1993; Fujita et al.,

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1996). Partial extraction left behind ~100-nm-long thin filaments extending from the Z-line. By applying a much more extensive gelsolin treatment, we removed even the short actin filaments near the Z-line. Partial extraction did not affect titin's regional extensibility, whereas passive force was reduced in sarcomeres longer than ~2.1 μm. Complete thin-filament extraction recruited the titin segment near the Z-line, which is otherwise inelastic, into the elastic pool, and passive forces at short sarcomere lengths were thereby enhanced.

Results were simulated by the wormlike chain (WLC) model of entropic elasticity, using properties of the molecule understood from our recent study of the mechanical behavior of single titin molecules (Kellermayer et al., 1997). That study indicated that a portion of the molecule may be permanently unfolded, giving it properties strikingly different from those of the rest of the molecule. The unfolded region, as seen in the present work, is likely to be the PEVK domain. The entire molecule behaves as a WLC, but the unfolded region has a persistence length (a measure of the chain's bending rigidity) of 2 nm, and the rest of the molecule (with native structure) has one of 15 nm (Higuchi et al., 1993). Modeling the elastic segment of cardiac titin as two WLCs with different persistence lengths in series correctly simulated the effect of thin-filament extraction on epitope movements and on passive force.

MATERIALS AND METHODS

Preparations

Single rat cardiac myocytes were isolated by the method of Granzier and Irving (1995). Cells were skinned and kept continuously in relaxing solution. To prevent titin degradation, solutions contained high concentrations of the protease inhibitors leupeptin, E64, and phenylmethylsulfonyl fluoride (for solution compositions see Granzier and Irving, 1995).

Mechanics

Cells were added to a temperature-controlled flow-through chamber. A single cell was glued at one end to a motor and at the other end to a force transducer. Sarcomere length was obtained by the method of Granzier and Irving (1995). To measure the passive force-sarcomere length relation, cells were slowly stretched (150 nm/s per sarcomere) from their slack sarcomere length to ~2.6 μm, followed by a release back to the slack length (cf. Granzier and Irving, 1995). Stretch-release protocols were interrupted by 10-min rest periods, resulting in high reproducibility of results. Identical stretch-release protocols were imposed on single cardiac myocytes before and after gelsolin treatment. In previous work we have shown that, as a result of gelsolin treatment, the slack sarcomere length decreases from 1.85 μm in the control cells to 1.81 μm in partially extracted cells and to 1.71 μm in completely extracted cells (Trombitás and Granzier, 1977). In the present work these sarcomere lengths were used to establish the zero-force level of our force-sarcomere length curves. Because passive force in collagenase-treated myocytes is borne by intermediate filaments (IFs) as well, IF-based force was measured after KCl/KI treatment (cf. Granzier and Irving, 1995). Over the sarcomere length range studied, IF-based forces were negligible (<5% of the total force).

Immunolabeling and electron microscopy

Cells were glued in the stretched state to the bottom of a minichamber that was used for immunolabeling, fixing, and embedding of the cells. For a detailed description of these methods, see Granzier et al. (1996).

Expression and purification of FX-45

The gelsolin clone FX-45 contains the N-terminal half (a.a. 1–406) of human gelsolin cDNA constructed in a PET3d expression vector (Yu et al., 1991). Expression and purification were as described by Trombitás and Granzier (1997).

FX-45 treatment of myocytes

Passive cells were first mechanically characterized and then extracted. Extraction was initiated by removing relaxing solution from the cell-containing chamber and quickly adding FX-45 (0.3 mg/ml) in relaxing solution. The extraction solution contained the protease inhibitors leupeptin (3.0 mM), diisopropylfluorophosphate (3.0 mM), E64 (0.75 mM), and PMSF (3.0 mM). After incubation for 10 min, FX-45 was removed from the chamber, and cells were extensively washed with relaxing solution and mechanically characterized. Cells were extracted for an additional 90 min, followed by washing with relaxing solution and mechanical characterization.

Calculations

To model titin epitope mobilities and passive force, titin was assumed to behave as two wormlike chains in series: the tandem Ig domain and the PEVK domain. For a wormlike chain, the external force (F) is related to the fractional extension (z/L) by the following relationship (Bustamante et al., 1994; Marko and Siggia, 1995):

$$\frac{FA}{k_BT} = \frac{z}{L} + \frac{1}{4(1-(z/L))^2} - \frac{1}{4} \tag{1}$$

where A is the persistence length, k_B is Boltzmann's constant, T is absolute temperature, z is the end-to-end length (extension), and L is the contour length. The persistence length (A) is a measure of the chain's bending rigidity, and the contour length (L) is equal to the end-to-end length (z) of the chain stretched with infinite force. Because the tandem Ig and the PEVK domains are assumed to be connected in series, they bear equivalent forces. Because of the equivalence of forces,

$$\frac{\mathrm{Ext_P}}{A_P} + \frac{1}{4A_P(1-\mathrm{Ext_P})^2} - \frac{1}{4A_P} = \frac{\mathrm{Ext_{Ig}}}{A_{Ig}} + \frac{1}{4A_{Ig}(1-\mathrm{Ext_{Ig}})^2} - \frac{1}{4A_{Ig}} \tag{2}$$

where $\mathrm{Ext_P}$ and $\mathrm{Ext_{Ig}}$ are the fractional extension (z/L) and A_P and A_{Ig} are the persistence lengths of the PEVK and the tandem Ig domains, respectively. Thus the relative fractional extension of the PEVK and tandem Ig domains is determined solely by the difference in their persistence lengths. Equation 2 allows the calculation of the fractional extension of one domain as a function of the fractional extension of the other by using A_P = 2 nm (Kellermayer et al., 1997) and A_{Ig} = 15 nm (Higuchi et al., 1993). Because

$$z = \mathrm{Ext}L \tag{3}$$

the end-to-end lengths of the domains can be calculated from their fractional extension (Ext) and their contour length (L). The contour length of the elastic segment between the T12 and PEVK domain was assumed to be ~90 nm (18 Ig-like domains with an interdomain distance of ~4 nm (Erickson, 1994) and an 18-nm-long unique sequence in the N2B domain).

The contour length of the elastic segment located between the PEVK domain and the A-band was assumed to be ~110 nm (28 Ig/Fn-like domains with an interdomain distance of 4 nm). Thus the total contour length of the native titin segment, excluding the PEVK domain, is estimated to be ~200 nm. The contour length of the titin segment recruited from near the Z-line in completely extracted cells was assumed to be 61 nm (see Results). Its persistence length was assumed to be identical to that of the tandem Ig domain. The contour length of the fully denatured PEVK domain is ~60 nm, calculated from its number of residues (~160 for N2B cardiac titin; Labeit and Kolmerer, 1995) and a maximal residue spacing of 0.38 nm (Erickson, 1994). The properties of titin's I-band domains in control and completely extracted cells are schematically indicated in Fig. 1 *A*. The fractional extensions of the PEVK and tandem Ig domains versus sarcomere lengths are shown in Fig. 1 *B*.

From the fractional extensions of the PEVK and tandem Ig domains, we calculated the corresponding sarcomere length (SL) as

$$SL = 2T12 + 2\mathrm{Ext}_{\mathrm{Ig}}L_{\mathrm{Ig}} + 2\mathrm{Ext}_{\mathrm{P}}L_{\mathrm{P}} + \mathrm{A-band} \quad (4)$$

where T12 is the distance from the center of the Z-line to the T12 epitope. This distance is 100 nm in unextracted cells, and 50 nm plus the end-to-end length of the recruited titin segment in completely extracted cells. For the completely extracted cells, L_{Ig} of the recruited segment was assumed to be 61 nm, and the T12 epitope distances from the Z-line were calculated as

$$\mathrm{Epi}_{\mathrm{T12}} = T12 + \mathrm{Ext}_{\mathrm{Ig}}L_{\mathrm{Ig}} \quad (5)$$

The 9D10 epitope distances from the Z-line were calculated in control cells, assuming that the center of the 9D10 epitope corresponds to the center of the PEVK domain (hence the $0.5L_{\mathrm{P}}$), as

$$\mathrm{Epi}_{\mathrm{9D10}} = T12 + \mathrm{Ext}_{\mathrm{Ig}}L_{\mathrm{Ig}} + 0.5\mathrm{Ext}_{\mathrm{P}}L_{\mathrm{P}} \quad (6)$$

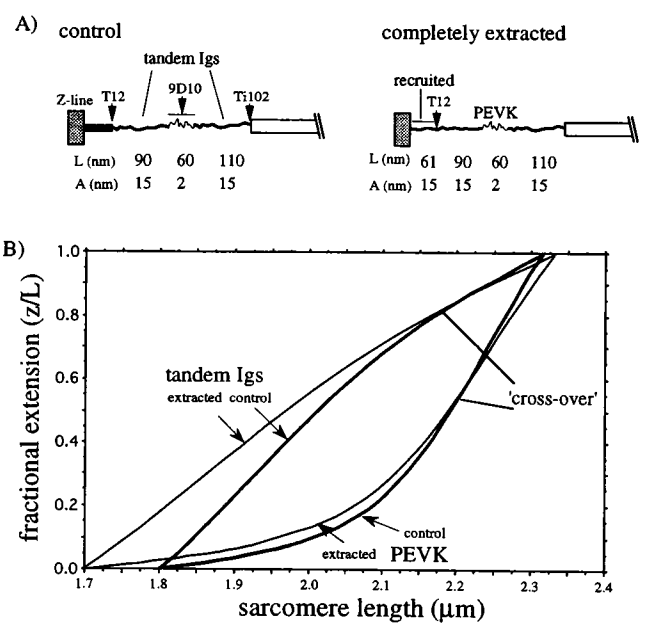

FIGURE 1 (*A*) Model of the elastic region of titin, with the tandem Ig and the PEVK domains behaving like WLCs. The estimated contour lengths (L) and persistence lengths (A) of the domains are indicated, along with the binding sites of the anti-titin antibodies that were used in this study. 9D10 is assumed to bind the full width of the PEVK domain (Trombitás et al., manuscript in preparation). Note that after a complete extraction, a segment of titin near the Z-line that is rigid in control myocytes behaves as a WLC with a 15-nm persistence length. (*B*) Fractional extensions versus sarcomere lengths of the tandem Ig and PEVK domains of control and completely extracted myocytes. Initially, the tandem Ig domains extend more rapidly with sarcomere stretch, whereas at sarcomere lengths greater than ~2.05 μm, the PEVK domain extends more rapidly. Note that the sarcomere length with zero fractional extension is 1.8 μm in control cells (Granzier et al., 1996) and 1.7 μm in completely extracted cells (this study). The fractional extension curves of control and completely extracted myocytes "cross over" at a sarcomere length of ~2.2 μm. (For simplicity's sake, the several Fn domains close to the A-band, as well as the N2B domain, are included in the tandem Ig segments.)

RESULTS

Differential thin-filament extraction by gelsolin

Cardiac myocytes were extracted with the gelsolin fragment FX-45 (0.3 mg/ml) in relaxing solution. Extraction for 10 min resulted in a partial removal of thin filaments, leaving behind ~100-nm-long thin filaments attached to the Z-line (Fig. 2, *B* and *C*). To remove these short thin filaments, the extraction time was increased to 100 min (Fig. 2 *D*). Darkly staining regions near the Z-line, presumably containing unextracted thin filaments, were also observed in cardiac trabeculae extracted with full-length gelsolin (Funatsu et al., 1993; Fujita et al., 1996). Thus the extraction-resistant nature of thin filaments near the Z-line observed here is not due to a unique aspect of our extraction method or preparation. Rather, the thin filaments have a unique property in this region.

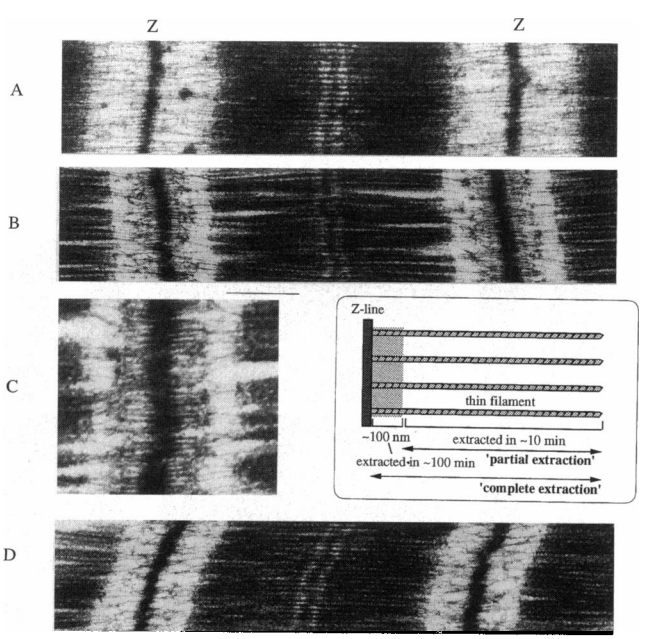

FIGURE 2 Electron micrographs of control and extracted sarcomeres. (*A*) Control. (*B* and *C*) Extracted with FX-45 for 10′. (*D*) Extracted for 100′. Note the dark, ~100-nm-wide region near the Z-line in *B* and *C*, which is absent in *D*. This darkly staining region contains short filaments with a diameter greater than that of filaments elsewhere in the I-band. In earlier work it was shown that these short filaments are thin filaments (Trombitás and Granzier, 1997). (*Inset*) Schematic diagram showing the effect of 10′ and 100′ extraction on the thin filament. Bar represents 0.5 μm, except for *C*, where bar denotes 0.16 μm.

The differential extraction property of the thin filament was used to extract thin filaments partially or completely (Fig. 2, *inset*). This made it possible to extract thin filaments 1) near the Z-line, where titin is known to be inextensible, and 2) just outside this region, where titin is extensible. The effect of the differential thin-filament removal on passive force and titin's extensibility was investigated. Gelsolin does not extract actin or α-actinin from the Z-line (Trombitás and Granzier, 1997), both of which are required to anchor titin to the Z-line (Ohtsuka et al., 1997; Sorimachi et al., 1997). Thus gelsolin-based thin-filament extraction can be used to study titin-actin interaction in the I-band without releasing titin from its Z-line anchors.

Titin epitopes followed with immunoelectron microscopy

The effect of thin-filament extraction on the extensibility of titin was studied by using anti-titin antibodies. Cells that were stretched and then labeled with both T12 and Ti-102 are shown in Fig. 3. In control cells, T12 labeled ~100 nm from the center of the Z-line and Ti-102 labeled at the A/I junction (Fig. 3 *A*). Results were similar for cells that had been partially extracted or fully extracted (Fig. 3, *B* and *C*), although, after full extraction, T12 labeling tended to be somewhat less regular. (For changes in epitope locations, see below.) Myocytes were also labeled with the anti-titin antibodies T12, 9D10, and Ti-102 simultaneously. As seen in earlier work (Trombitás et al., 1995), 9D10 labels between the T12 and Ti-102 epitopes (Fig. 4). Extracted cells labeled with 9D10 antibody contained titin filaments close to the A/I junction, which were extremely regular (Fig. 4, *B* and *C*). Such a high degree of regularity is typically absent from cells labeled with T12 and Ti-102 only (see Fig. 3, *B* and *C*), and may result from antibody-based cross-linking of titin filaments at the 9D10 binding site. Indeed, the IgM nature of 9D10 (Wang and Greaser, 1985) allows cross-linking to occur. The regularity of titin filaments near the A-band also revealed that virtually every thick filament extended into a titin filament (Fig. 4, *B* and *C*), indicating the absence of considerable titin degradation. Importantly, large numbers of titin filaments were seen only if the extraction took place in the presence of high concentrations of protease inhibitors (see Materials and Methods). Otherwise, I-bands were devoid of titin.

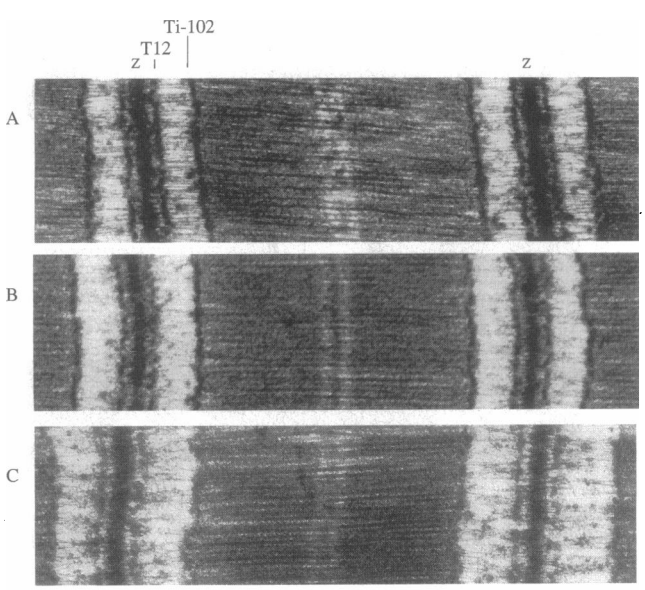

FIGURE 3 Electron micrograph of slightly stretched sarcomeres labeled with the anti-titin antibodies T12 and Ti-102. (*A*) Control cell. (*B*) Partially extracted cell. (*C*) Completely extracted cell. In all cells, T12 labels close to the Z-line, and Ti-102 labels at the edge of the A-band. Bar = 0.5 μm.

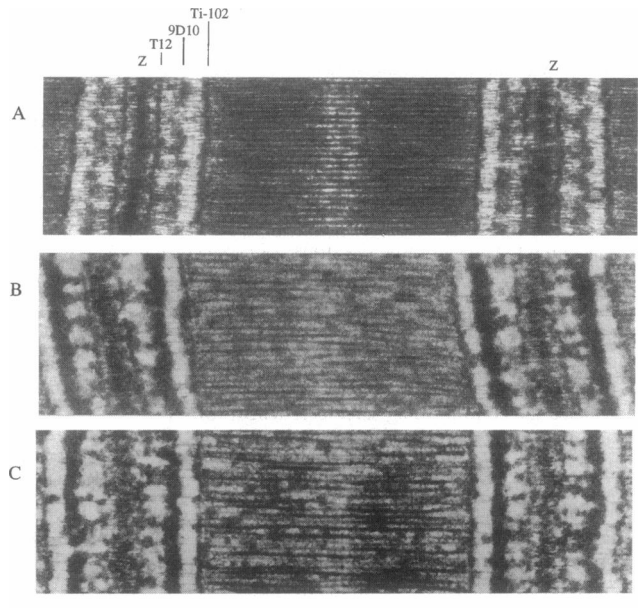

FIGURE 4 Electron micrographs of slightly stretched sarcomeres simultaneously labeled with the anti-titin antibodies T12, 9D10, and Ti-102. (*A*) Control cell. (*B*) Partially extracted cells. (*C*) Completely extracted cell. Note the regularly arranged fine filaments located between Ti-102 and 9D10 in the extracted cells. Bar = 0.5 μm.

Myocytes were also extracted while attached at one end only, allowing them to adjust their slack length during the extraction process. In partially extracted slack cells, thick filaments abutted the short thin filaments at the Z-line (Fig. 5 *A*). A narrow, darkly staining region was found ~100 nm from the Z-line (*arrow* in Fig. 5 *A*). The appearance of this region was most likely caused by an accumulation of the elastic titin segment of titin that runs from the T12 epitope (Fig. 5 *B*) to the tip of the thick filament. After complete thin-filament extraction, the slack sarcomere length dropped from ~1.85 μm to ~1.7 μm, and the thick filaments abutted the Z-line in unlabeled slack cells (Fig. 5 *C*). In unlabeled cells, the Z-lines appeared somewhat broadened and had "fuzzy" edges. T12 now labeled near the Z-line (Fig. 5 *D*), suggesting that in fully extracted cells the elastic titin segment accumulated near the Z-line. Thus, after the complete removal of thin filaments, titin collapsed toward

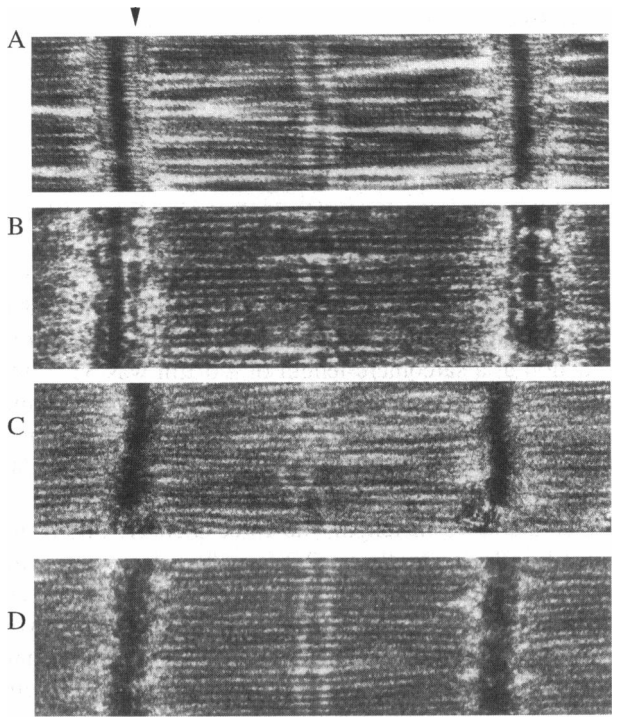

FIGURE 5 Electron micrographs of slack sarcomeres. (*A* and *B*) Partially extracted. (*C* and *D*) Completely extracted. (*B* and *D*) Labeled with T12. Arrow in *A* indicates the dark, narrow region ~100 nm from Z-line. See text for further details. Bar = 1.0 μm.

the Z-line, driving the observed reduction in slack sarcomere length.

In control cells, the distance between T12 and the Z-line varied only slightly with sarcomere length, the 9D10 epitope moved linearly away from the Z-line upon sarcomere stretch, and the Ti-102 epitope remained at the edge of the A-band at all sarcomere lengths (Fig. 6). There was no statistically significant difference in the T12, 9D10, and Ti-102 epitope locations between control and partially extracted cells (Fig. 6).

As a result of complete thin-filament extraction, the epitope locations changed significantly. Relative to the control, in sarcomeres shorter than ~2.0 μm the T12 epitope moved toward the Z-line, and in sarcomeres longer than ~2.3 μm the epitope moved away from the Z-line (Fig. 7 *A*). At a sarcomere length of 2.6 μm, for example, the T12 epitope was ~40 nm further from the Z-line than in control sarcomeres. At sarcomere lengths between ~2.0 μm and ~2.3 μm, the T12 epitope location was indistinguishable from that of control cells (Fig. 7 *A*). Results with the 9D10 antibody paralleled the T12 behavior. At sarcomere lengths longer than ~2.3 μm, the 9D10 epitope moved further away from the Z-line than in the control cells (Fig. 7, *B* and *C*). At a sarcomere length of 2.6 μm, for example, 9D10 was ~45 nm further away from the Z-line. This movement is similar to that of the T12 epitope at the same sarcomere length. The Ti-102 epitope, on the other hand, was unaffected by the 100-min extraction (Fig. 6 *C*). Thus the effect of complete

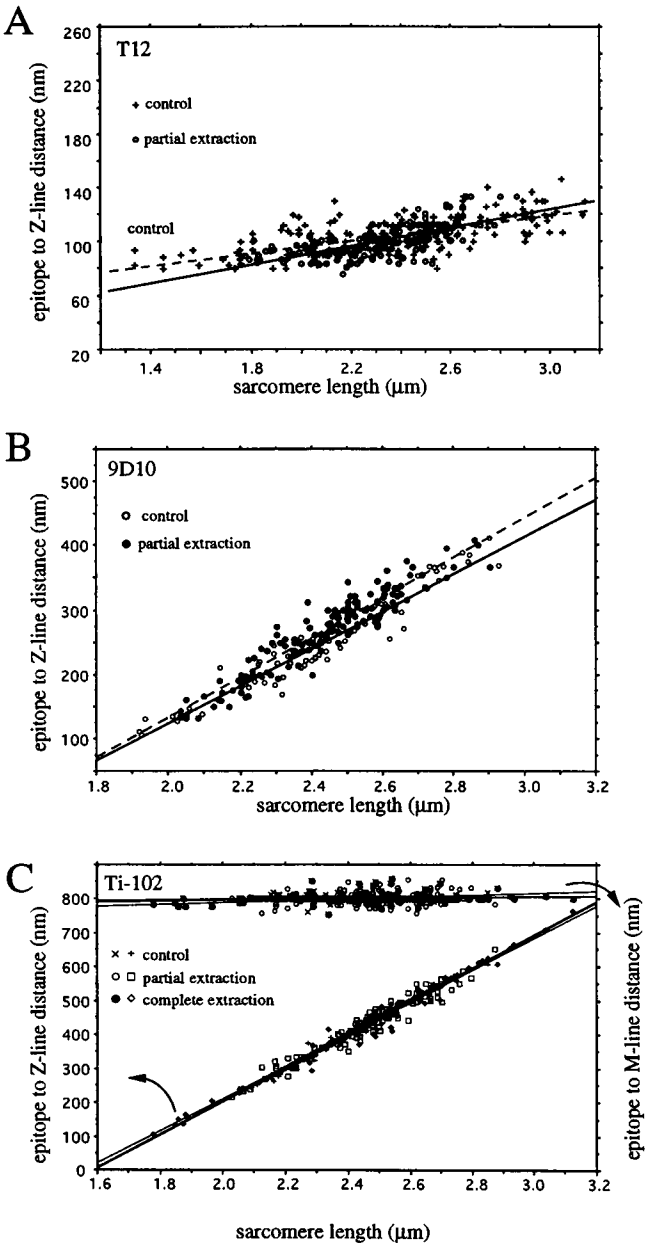

FIGURE 6 Sarcomere-length dependencies of the distances between (*A*) Z-line and T12 epitope, and (*B*) Z-line and 9D10 epitope. Results of control and partially extracted cells are superimposed. (*C*) Sarcomere-length dependencies of distances between the Z-line and Ti-102 epitope and between the edge of the A-band and the Ti-102 epitope. Superimposed are results from control and partially and completely extracted cells. Epitope locations are unaffected by extraction. Lines are linear regression fits.

thin-filament extraction on titin's extensibility is restricted to the I-band. The main effect appears to be a recruitment of the titin segment near the Z-line into the extensible titin pool.

To determine the sarcomere lengths at which the behavior of the T12 and 9D10 epitopes in completely extracted cells begins to deviate from that in control cells, results at sarcomere lengths greater than 2.3 μm were fit with linear regression lines (see Fig. 7). The lengths at which the lines in extracted and control cells crossed were determined. The

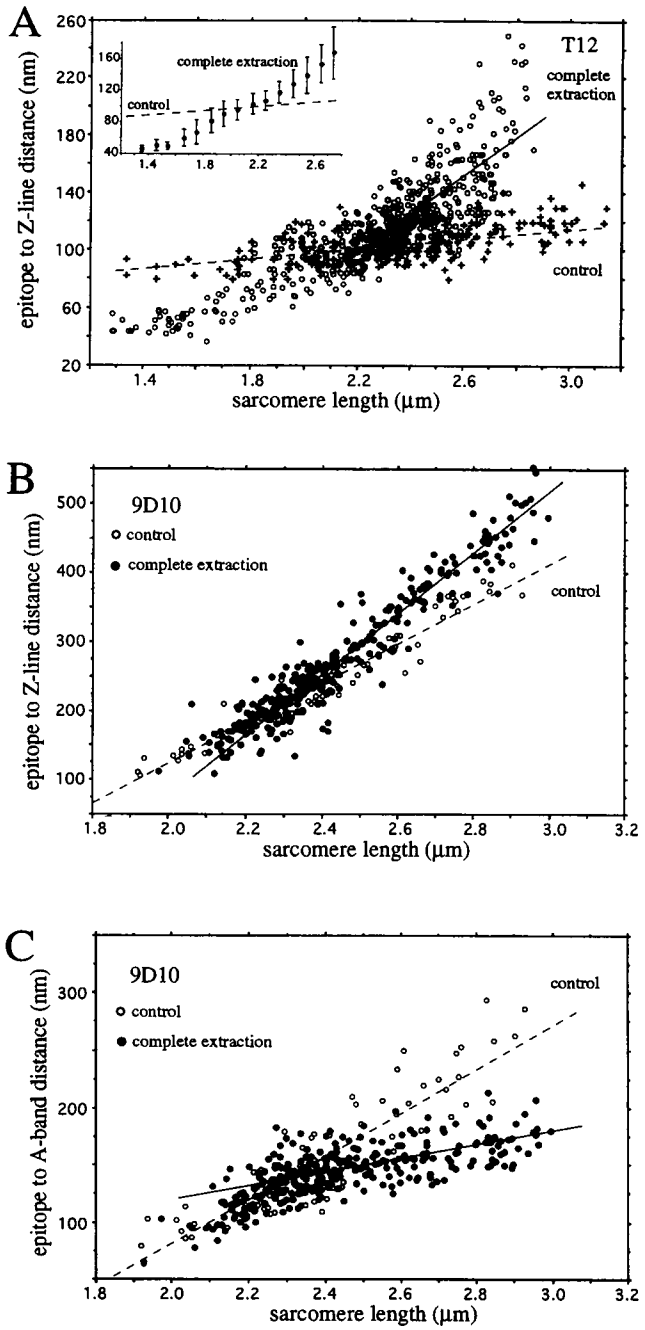

FIGURE 7 Sarcomere-length dependencies of epitope locations of control and completely extracted cells. (*A*) T12 epitope to Z-line distance. (*Inset*) T12 results for 0.1-μm-wide sarcomere length bins (mean ± SD). (*B*) 9D10 epitope to Z-line distance. (*C*) 9D10 epitope to A-band distance. The T12 epitope is mobile after extraction and, relative to the control, moves toward the Z-line in sarcomeres shorter than ~2.0 μm and away from the Z-line in sarcomeres longer than ~2.3 μm. In sarcomeres stretched beyond ~2.3 μm, the 9D10 epitope moved further away from the Z-line (*B*) and closer to the A-band (*C*). Straight lines represent linear regression fits to the results obtained at sarcomere lengths of >2.3 μm. Equations: *A* control: $y = 23x + 51$; *A* extracted: $y = 133x - 196$; *B* control: $y = 289x - 454$; *B* extracted: $y = 439x - 800$; *C* control: $190x - 299$; *C* extracted: $55x + 14$. (y = distance in nm; x = sarcomere length in μm.)

"cross-over" length for the T12 results (Fig. 7 *A*) was 2.25 μm. Results for 9D10 (Fig. 7, *B* and *C*) were 2.30 μm and 2.32 μm, respectively.

Passive force

Partial thin-filament extraction had no significant effect on passive force at sarcomere lengths between slack (1.85 μm) and ~2.1 μm. At longer lengths, however, force was reduced. A typical example is shown in Fig. 8 *A*, and the average results are displayed in Fig. 8 *B*. The average force reduction at a sarcomere length of 2.6 μm was 33 ± 9%.

After complete extraction, passive forces at sarcomere lengths between 1.7 and ~2.0 μm were higher than in the control cells (Fig. 8, *A* and *B*). The force was 167 ± 80% (mean ± SD) higher at a sarcomere length of 1.95 μm. With further increases in length, the force increase diminished, and at lengths longer than ~2.2 μm, the force was less than in the control. The average reduction at a sarcomere length of 2.6 μm was 46 ± 13% (mean ± SD). It is also worth highlighting the observation that at sarcomere lengths shorter than ~2.25 μm, force after a complete extraction

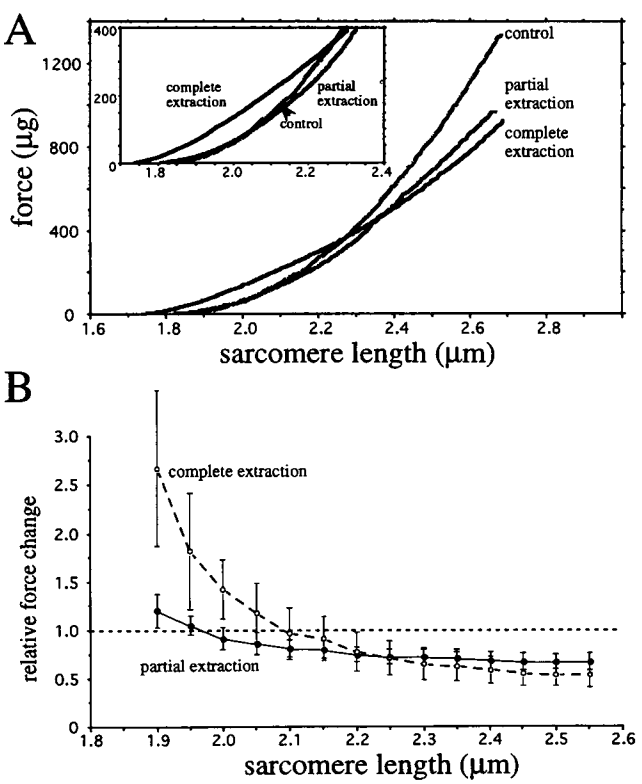

FIGURE 8 Effect of actin extraction on passive force-sarcomere length relation of a cardiac myocyte. (*A*) Results of single cell. At lengths greater than ~2.1 μm, the force after a partial extraction is less than in control. Below this length, extraction has little effect on force. At lengths shorter than ~2.25 μm, force after a complete extraction is higher than in control, whereas at longer lengths force is less. (*Inset*) An expanded version of the low force region. (*B*) Relative force change (force after extraction divided by control force) for partially and completely extracted cells. Mean ± SD; $n = 5$.

was higher than that after a partial extraction, whereas at longer lengths force was less (Fig. 8 *B*).

Modeling titin as serially linked wormlike chains

Findings were compared to the predictions of the wormlike chain (WLC) model in which the extensible segment of titin was assumed to behave as two WLCs in series: the tandem Ig domain and the PEVK domain (see Materials and Methods). Importantly, these domains have very different elastic properties, as indicated by their different persistence lengths. The model predicts (for calculations see Material and Methods) that tandem Ig extension dominates at short sarcomere lengths, PEVK extension at sarcomere lengths beyond ~2.05 μm, and that full extension of both WLCs occurs at a sarcomere length of ~2.3 μm (Fig. 1). The model was used to calculate the extensibility of titin between the Z-line and the 9D10 epitope in control cells. Because 9D10 labels the PEVK domain (Trombitás et al., manuscript in preparation), it was assumed that 1) half of the PEVK domain, 2) a tandem Ig domain, and 3) the inextensible Z-line to T12 segment were located between the center of the 9D10 epitope and the Z-line. (For calculation of epitope distances, see Materials and Methods.) Results revealed that the predicted Z-line to 9D10 epitope distances of control cells were indistinguishable from those measured (Fig. 9 *A*).

In completely extracted myocytes, the distance between the center of the Z-line and the T12 epitope was predicted. It was assumed that the recruited titin segment between the Z-line and T12 behaved as a WLC with the same persistence length as the native tandem Ig domains (15 nm). When the force in the partially extracted cells is equal to the force in the fully extracted cells, the fractional extensions of the recruited segment and the Ig domains have to be the same. This occurs at a sarcomere length of ~2.2 μm (Fig. 8 *B*), where the fractional extension is 84% (Fig. 1). At this sarcomere length, the extension of the recruited titin segment between the edge of the Z-line and T12 epitope is 51.7 ± 12.2 nm. (The value was determined from data in Fig. 7 *A*, and 50 nm was subtracted for the half Z-band width.) It follows that the contour length of the recruited segment is 61 nm. The predicted Z-line to T12 distance of completely extracted cells is shown in Fig. 9 *B*. At short sarcomere lengths, the predicted distances are slightly less than the mean measured values. This difference could result from a persistence length of titin near the Z-line that is higher than the assumed 15 nm. It is possible that a residual interaction with near-Z-line components increases titin's apparent persistence length. Overall, however, the predicted values are very close to those measured.

The entropic force-sarcomere length relation was also calculated, for both control and completely extracted myocytes. At sarcomere lengths shorter than 2.2 μm, the calculated forces were higher after extraction, and at longer sarcomere lengths forces were lower after extraction (Fig. 10 *A*). The relative force change was determined from these curves; the predicted and measured relative force changes are shown in Fig. 10 *B*. The WLC model was able to simulate the measurements well.

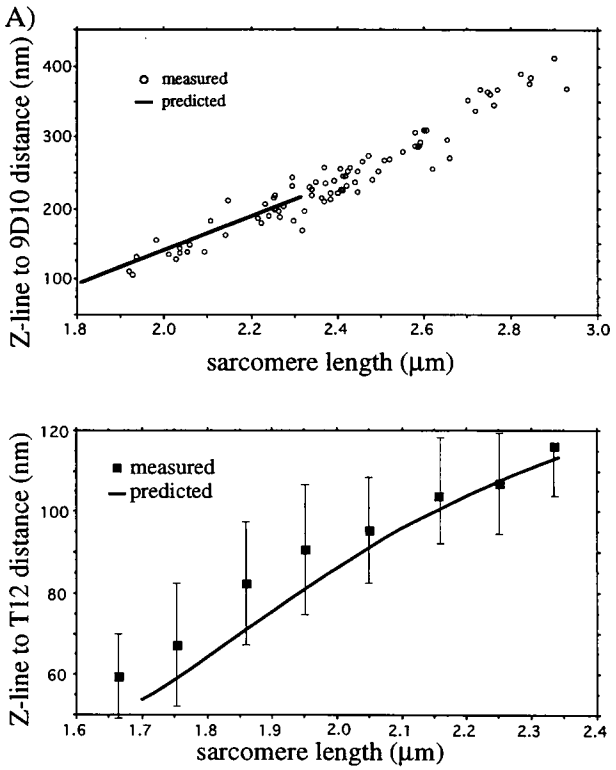

FIGURE 9 (*A*) Predicted and measured Z-line to 9D10 distances versus sarcomere length for control cells. The center of the 9D10 epitope was assumed to coincide with the center of the PEVK domain. The Z-line to T12 distances were measured in control cells and added to the calculated extensions of the tandem Ig domain and half of the PEVK domain between 9D10 and T12. Because the WLCs approach their contour length at a sarcomere length of ~2.3 μm, predicted data only extend to this length. The measurements shown are the same as those displayed in Fig. 6 *B*. See text for further details. (*B*) Predicted and measured Z-line to T12 distance versus sarcomere length of completely extracted myocytes. Predictions were based on the assumption that the now elastic titin segment between the Z-line and T12 behaves like a WLC with a persistence length of 15 nm, a contour length of 61 nm, and a zero extension ratio at a sarcomere length of 1.7 μm. Measurements are the same as in Fig. 7 *A*.

DISCUSSION

Titin-actin interaction was investigated in cardiac myocytes by extracting thin filaments with the gelsolin fragment FX-45. Results indicate that a weak interaction between titin and actin may occur in the elastic region of titin, and a strong interaction may occur near the Z-line. Abolishing the weak interaction reduces the passive force at long sarcomere lengths, whereas abolishing the strong interaction increases the passive force at shorter lengths. The results are consistent with the recruitment of a partially extended elastic domain near the Z-line into the elastic titin pool.

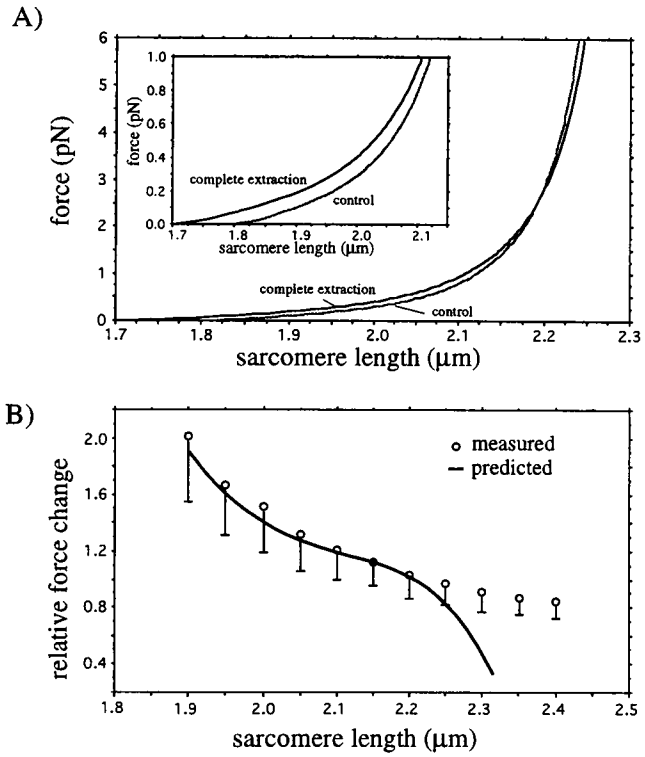

FIGURE 10 (*A*) Predicted entropic force versus sarcomere length for control and completely extracted myocytes. Predicted force after complete extraction is higher than in the control at sarcomere lengths less than ~2.2 μm, and less than in the control at lengths greater than ~2.2 μm. (*Inset*) An expanded version of the low force region. (*B*) Relative force change of completely extracted cells versus sarcomere length. Predicted relative force change is based on data shown in *A*, and the measured relative force change is from data in Fig. 8 *B* (force of completely extracted cells divided by force of partially extracted cells).

Partial thin-filament extraction

By limiting the extraction duration to 10 min, actin can be extracted solely from the region of the I-band where titin is extensible, providing a unique opportunity to study the effect of actin removal on titin's extensibility and force. Immunoelectron microscopy revealed that the location of the 9D10 epitope was essentially unaffected by thin-filament removal (Fig. 6), indicating that titin's regional extensibility was unaltered. In contrast, passive force at sarcomere lengths longer than ~2.1 μm was considerably reduced (Fig. 8). It is unlikely that this force reduction results from titin degradation, because high levels of protease inhibitors were included in the extraction buffer (see Materials and Methods), and electron microscopy revealed that, even after the lengthy labeling protocols, one strand of titin molecules per thick filament was typical (Fig. 4, *B* and *C*). In unextracted cells each of these titin strands may contain up to six molecules (Granzier and Irving, 1995). The number of titin molecules per strand could in theory be reduced because of degradation. However, significant titin degradation is unlikely to have occurred here, because this would have depressed the passive force equally at all sarcomere lengths. In contrast, the force reduction after limited actin extraction occurred at sarcomere lengths longer than ~2.1 μm only (Fig. 8).

An alternative mechanism of force reduction is the abolishment of interaction between actin and the elastic region of titin. This mechanism is supported by the in vitro actin-titin binding studies reported by several laboratories (Kimura et al., 1984; Maruyama et al., 1987; Jin, 1995; Li et al., 1995; Kellermayer and Granzier, 1996a,b). The reported interaction of a bacterially expressed PEVK fragment with F-actin (Gutierrez et al., 1997) indicates that actin-titin interaction may occur in the region of titin that behaves elastically in the sarcomere. The possibility that actin-titin interaction occurs in vivo, however, has not been widely considered. Undoubtedly, this is related to immunolabeling studies that suggest that titin and actin act independently in the sarcomere (Itoh et al., 1988; Fürst et al., 1988; Horowits et al., 1989; Trombitás et al., 1991, 1997; Wang et al., 1993). However, weak and reversible interaction between titin and actin is not precluded. Consistent with the possibility of a weak actin-titin interaction in the elastic region of titin is the freeze-fracture study by Funatsu et al. (1993), which indicated that titin and the thin filament constitute a composite filament in the I-band. According to binding and ultrastructural studies, a weak interaction between actin and titin in the cardiac myocyte is thus possible.

The fact that partial thin-filament extraction affected passive force but not titin's regional extensibility indicates that the whole elastic titin segment interacts uniformly with actin. Alternatively, only a subsegment of titin interacts with actin, with 9D10 labeling in the middle of the segment (explaining that the 9D10 epitope location is not altered by partial extraction). It is also possible that actin-titin interaction does affect regional titin's extensibility, but only on a faster time scale than that of our immunolabeling studies. Further experiments are required to explore these possibilities.

Complete thin-filament extraction

Extraction of the near-Z-line actin results in a reduction of the slack sarcomere length and an increase in passive force at short to intermediate sarcomere lengths (Figs. 5 and 8). Similar findings have been reported to occur after actin extraction from skeletal muscle fibers (Funatsu et al., 1990; Granzier and Wang, 1993). Funatsu et al. (1993) studied the effect of actin extraction on titin elasticity in cardiac trabeculae at sarcomere lengths between ~2.0 μm and ~2.4 μm. No significant effect of actin extraction on titin's extensibility was found. This is in agreement with our results, because titin extensibility was not affected in a similar sarcomere length range (Fig. 7 *A*). However, expanding the sarcomere-length range revealed that titin's extensibility is considerably altered in sarcomeres shorter than ~2.0 μm and longer than ~2.4 μm (Fig. 7).

Our study revealed that the titin segment near the Z-line, which is demarcated by the T12 epitope, was rendered

extensible after complete thin-filament extraction (Fig. 7). In control myocytes, the T12 epitope is mobile only after extreme stretch to lengths longer than ~3.0 μm (Trombitás and Granzier, unpublished observation). After complete thin-filament extraction, however, the T12 epitope became mobile in much shorter sarcomeres (Fig. 7 *A*). Thus it is likely that a strong interaction between actin and titin in control cells is responsible for rendering titin near the Z-line inextensible. The segment of titin between its N-terminus and the T12 epitope contains 12 Ig-like domains and several domains with unique sequences (Labeit and Kolmerer, 1995), and any of these subdomains may have strong actin-binding properties.

Titin as serially linked wormlike chains

Our recent mechanical studies of single titin molecules using force-measuring laser tweezers (Kellermayer et al., 1997) revealed that part of the molecule may be unfolded, behaving as a wormlike chain (WLC) with a 2-nm persistence length. This unfolded region is likely to include the PEVK segment, as its amino acid composition suggests that it is without stable secondary structure (Labeit and Kolmerer, 1995), and as immunolabeling of titin in situ and "molecular combing" of single titin molecules indicate that the PEVK extends easily upon stretch of the molecule (Linke et al., 1996; Gautel and Goulding, 1996; Tskhovrebova and Trinick, 1997; Trombitás, manuscript in preparation). Therefore the PEVK domain of cardiac titin was modeled as a WLC with a persistence length of 2 nm and a contour length of ~60 nm (see Materials and Methods). Because the persistence length of native titin is 15 nm (Higuchi et al., 1993), the native tandem Ig segments were modeled as a WLC with a persistence length of 15 nm and a total contour length of 200 nm.

The predicted entropic forces of the tandem Ig segment only and the tandem Ig in series with the PEVK segment are shown in Fig. 11. Force in the tandem Ig segment is low between a sarcomere length of ~1.85 μm and ~2.00 μm, but increases steeply at longer lengths (Fig. 11, *curve A*). Placing the PEVK segment in series with the tandem Ig segment (*curve A + B*) indicates that force is minimally affected at sarcomere lengths shorter than ~2.05 μm, whereas force is reduced at longer lengths (Fig. 11, *inset*). At sarcomere lengths up to those where native tandem Ig and the denatured PEVK approach their contour length (~2.25 μm), modeling the elastic segment of titin as two WLCs in series closely simulates the measured epitope movements in both control and extracted cells (Fig. 9). Furthermore, it also predicts the relative force change after thin-filament extraction near the Z-line, with a level of increase that closely resembles the measured level (Fig. 10).

In a recent work by Tskhovrebova et al. (1997), published while this article was being reviewed, considerably lower persistence lengths were reported for both native titin and the PEVK segment (4.8 nm and 0.15 nm, respectively).

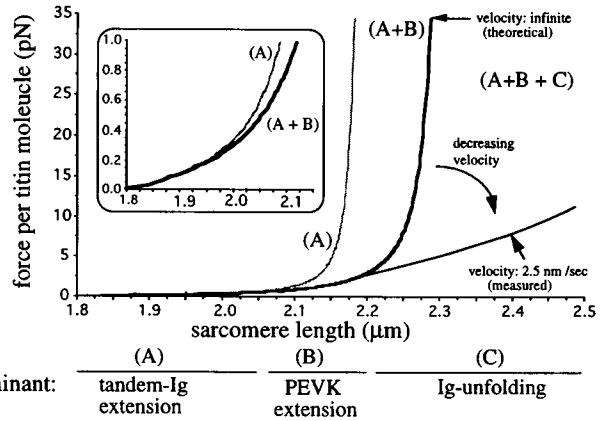

FIGURE 11 Predicted entropic force-sarcomere length relation of a single titin molecule in cardiac myocytes, based on the hypothesis that tandem Ig and PEVK domains behave like WLCs with different persistence lengths (see Materials and Methods for details). Curve A indicates the predicted entropic force of the native tandem Igs only, whereas A + B indicates the predicted entropic force of native tandem Igs in series with the PEVK domain. The inset shows that when the two WLCs are in series, sarcomere stretch from slack length to ~2.0 μm results in force that is mainly determined by extension of native tandem Igs, whereas at longer lengths, PEVK polypeptide extension determines the level of entropic force. Unfolding of the Ig-like domains may occur at lengths greater than ~2.2 μm. For infinitely high velocities, unfolding will be absent and force will increase according to curve A + B. Lower stretch velocities allow unfolding to occur, with increasing levels of unfolding as velocity decreases. Shown is the measured force of cardiac myocytes stretched with a velocity of 2.5 nm/s per half-sarcomere (data from Granzier et al., 1996, figure 7 *A*, scaled down to the level of the single titin molecule as described in Granzier and Irving, 1995). See text for additional details.

Predicted epitope movements are determined mainly by the difference in persistence lengths of tandem Ig and PEVK segments, and not by their absolute values (Materials and Methods), and modeling epitope movements, using these lower persistence lengths, simulated the data as well. On the other hand, entropic force varies with the magnitude of persistence lengths, and the predicted passive forces based on the work of Tskhovrebova et al. (1997) were much higher than measured. A detailed comparison between predictions based on the two studies (Kellermayer at al., 1997; Tskhovrebova et al., 1997) is in preparation (Trombitás et al., manuscript in preparation).

Ig/Fn unfolding

The predicted entropic force derived from extending native tandem Igs in series with the PEVK polypeptide rises steeply at sarcomere lengths longer than ~2.2 μm, as the WLCs approach their contour lengths (Fig. 11, *curve A + B*). Thus, in the absence of other processes occurring in the titin molecule, it would be impossible to extend sarcomeres much beyond a length of ~2.2 μm. However, at these lengths, unfolding of Ig/Fn-like domains is likely to take place, resulting in a decrease in the steepness of the force-length curve. Unfolding at sarcomere lengths longer than ~2.2 μm is suggested in our work by the continuous

movement of the T12 epitope away from the Z-line in fully extracted cells at sarcomere lengths greater than ~2.2 μm (Fig. 7 *A*). The work of Kellermayer et al. (1997) and Rief et al. (1997) indicated that, because of the kinetic nature of the Ig unfolding, the force level at which unfolding occurs varies with stretch velocity, with minimal unfolding at infinitely high velocity, and progressively increasing unfolding as velocity decreases (Fig. 11, *shaded area*). Fig. 11 shows the measured passive force-sarcomere length relation of rat cardiac myocytes that were slowly stretched. At sarcomere lengths beyond ~2.2 μm, the measured forces are much less than predicted from two serially linked WLCs only, indicating that under the experimental conditions, considerable Ig/Fn unfolding took place.

Mechanism of passive force development and the effect of thin-filament extraction

The three identified processes that determine the force-length relation make it possible to dissect the origin of the effect of thin filament extraction on force. Partial extraction results in a reduction of passive force at sarcomere lengths longer than ~2.1 μm (Fig. 8). As tandem Ig extension is dominant at shorter lengths, this process is apparently independent of actin. Only when the elastic segment is considerably extended does an interaction between actin and titin occur that is sufficient to affect passive force. The effect on force occurs during both PEVK polypeptide extension and Ig unfolding, and each could be modulated by actin. A weak actin-titin interaction may modulate the unfolding/refolding kinetics of titin and thereby affect passive force, while at the same time being insufficient to prevent titin from behaving elastically while the actin filament is stiff.

After complete thin filament extraction, force increases at short sarcomere lengths, where tandem Ig extension is the dominant feature that determines passive force (Figs. 8 and 11). The contour length of the elastic segment between the Z-line and the T12 epitope that is recruited after complete extraction is estimated to be 61 nm. The measured distance between the Z-line and the T12 epitope in control cells (50 nm) indicates that the fractional extension of the attached segment is ~84%. At short sarcomere lengths (< ~2.2 μm) this partially extended titin segment is allowed to contract, thereby pulling the T12 epitope toward the Z-line and increasing the passive force. (The passive force increases because the extension of the entire elastic titin segment increases.) Conversely, at long sarcomere lengths (> ~2.2 μm), the originally inextensible segment becomes further extended, thereby allowing the T12 and 9D10 epitopes to move away from the Z-line, reducing passive force. (Passive force drops because the extension of the entire elastic segment decreases.)

Physiological relevance

Each of the three processes that underlie the shape of the passive force-sarcomere length curve of titin (tandem-Ig extension, PEVK polypeptide extension, and Ig/Fn domain unfolding) are likely to be physiologically relevant. Extension of tandem Igs will occur both when sarcomeres shorten below slack and when they are stretched beyond slack (Helmes et al., 1996). At sarcomere lengths of ~1.7 μm and ~2.0 μm, Ig extension results in entropic forces of ~0.3 pN per titin molecule. When scaled up to the level of cardiac muscle (for calculations see Granzier and Irving, 1995), this value corresponds to ~0.5 mN/mm^2, or ~1% of its active twitch force (ter Keurs et al., 1983; Hancock et al., 1993). This level of passive force is expected to be relevant to ventricular filling during early diastole. Extension of the PEVK polypeptide allows sarcomeres to reach intermediate sarcomere lengths while avoiding the energetically costly hysteresis that results from Ig unfolding/refolding during repetitive loading of heart muscle (Kellermayer et al., 1997). Finally, the unfolding of Ig domains allows sarcomeres to be stretched to lengths longer than ~2.2 μm, and the velocity dependence of this unfolding could be relevant to adapting passive force as the beat frequency and the filling volume of the heart change.

Analysis of the three identified processes that underlie the shape of the passive force-sarcomere length curve (tandem-Ig extension, PEVK polypeptide extension, and Ig/Fn domain unfolding) has led us to establish that a weak interaction between the elastic segment of titin and actin is likely to affect PEVK extension and Ig unfolding. We propose that this weak interaction provides a physiological pathway for passive force modulation.

Note added in proof. Following acceptance of this article, a related study was published by Linke et al (1997). A detailed comparison of the two studies will be presented elsewhere.

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REFERENCES

Bustamante, C., J. Marko, E. Siggia, and S. Smith. 1994. Entropic elasticity of λ-phage DNA. *Science.* 265:1599–1600.

Erickson, H. 1994. Reversible unfolding of fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin in fibronectin. *Proc. Natl. Acad. Sci. USA.* 91:10114–10118.

Fujita, H., K. Yasuda, S. Niitsu, T. Funatsu, and S. Ishiwata. 1996. Structural and functional reconstitution of thin filaments in the contractile apparatus of cardiac muscle. *Biophys. J.* 71:2307–2318.

Funatsu, T., H. Higuchi, and S. Ishiwata. 1990. Elastic filaments in skeletal muscle revealed by selective removal of thin filaments with plasma gelsolin. *J. Cell Biol.* 110:53–62.

Funatsu, T., E. Kono, H. Higuchi, S. Kimura, S. Ishiwata, T. Yoshioka, K. Maruyama, S. Tsukita. 1993. Elastic filaments in situ in cardiac muscle:

deep-etch replica analysis in combination with selective removal of actin and myosin filaments. *J. Cell Biol.* 120:711–724.

Fürst, D., M. Osborn, M. Nave, and K. Weber. 1988. The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z-line extends close to the M-line. *J. Cell Biol.* 106: 1563–1572.

Gautel, M., and D. Goulding. 1996. A molecular map of titin/connectin elasticity reveals two different mechanisms acting in series. *FEBS Lett.* 385:11–14.

Granzier, H., M. Helmes, and K. Trombitás. 1996. Nonuniform elasticity of titin in cardiac myocytes: a study using immunoelectron microscopy and cellular mechanics. *Biophys. J.* 70:430–442.

Granzier, H., and T. Irving. 1995. Passive tension in cardiac muscle: contribution of collagen, titin, microtubules, and intermediate filaments. *Biophys. J.* 68:1027–1044.

Granzier, H. L. M., and K. Wang. 1993. Passive tension and stiffness of vertebrate skeletal muscle and insect flight muscle: the contribution of weak crossbridges and elastic filaments. *Biophys. J.* 65:2141–2159.

Gutierrez, G., A. van Heerden, J. Wright, and K. Wang. 1997. Titin elasticity: interaction and extensibility of PEVK segment of human fetal skeletal titin. *Biophys. J.* 72:A279 (Abstr.).

Hancock, W., D. Martyn, and L. Huntsman. 1993. Ca^{2+} and segment length dependence of isometric force kinetics in intact ferret cardiac muscle. *Circ. Res.* 73:603–611.

Helmes, M., K. Trombitás, and H. Granzier. 1996. Titin develops restoring force in rat cardiac myocytes. *Circ. Res.* 79:619–626.

Higuchi, H. 1992. Changes in contractile properties with selective digestion of connectin (titin) in skinned fibers of frog skeletal muscle. *J. Biochem.* 111:291–295.

Higuchi, H., N. Nakauchi, K. Maruyama, and S. Fujime. 1993. Characterization of β-connectin (titin 2) from striated muscle by dynamic light scattering. *Biophys. J.* 65:1906–1915.

Horowits, R., E. S. Kempner, M. E. Bisher, and R. Podolsky. 1986. A physiological role for titin and nebulin in skeletal muscle. *Nature.* 323:160–164.

Horowits, R., K. Maruyama, and R. Podolsky. 1989. Elastic behavior of connectin filaments during thick filament movement in activated skeletal muscle. *J. Cell Biol.* 109:2169–2176.

Itoh, Y., T. Suzuki, S. Kimura, K. Ohashi, H. Higuchi, H. Sawada, T. Shinizu, M. Shibata, and K. Maruyama. 1988. Extensible and less-extensible domains of connectin filaments in stretched vertebrate skeletal muscle sarcomeres as detected by immunofluorescence and immunoelectron microscopy using monoclonal antibodies. *J. Biochem.* 104: 504–508.

Jin, J.-P. 1995. Cloned rat cardiac titin class I and class II motifs: expression, purification, characterization, and interaction with F-actin. *J. Biol. Chem.* 270:6908–6916.

Keller, T. 1995. Structure and function of titin and nebulin. *Curr. Opin. Cell Biol.* 7:32–38.

Kellermayer, M., and H. Granzier. 1996a. Calcium-dependent inhibition of in vitro thin-filament motility by native titin. *FEBS Lett.* 380:281–286.

Kellermayer, M., and H. Granzier. 1996b. Elastic properties of single titin molecules made visible through fluorescent F-actin binding. *Biochem. Biophys. Res. Commun.* 221:491–497.

Kellermayer, M., S. Smith, H. Granzier, and C. Bustamante. 1997. Folding-unfolding transitions in single titin molecules characterized with laser tweezers. *Science.* 276:1112–1116.

Kimura, S., K. Maruyama, and Y. Huang. 1984. Interactions of muscle β-connectin with myosin, actin, and actomyosin at low ionic strength. *J. Biochem.* 96:499–506.

Kruger, M., J. Wright, and K. Wang. 1991. Nebulin as a length regulator of thin filaments of vertebrate skeletal muscles: correlation of thin filament length, nebulin size, and epitope profile. *J. Cell Biol.* 115: 97–107.

Labeit, S., and B. Kolmerer. 1995. Titins, giant proteins in charge of muscle ultrastructure and elasticity. *Science.* 270:293–296.

Labeit, S., B. Kolmerer, and W. Linke. 1997. The giant protein titin. Emerging roles in physiology and pathophysiology. *Circ. Res.* 80: 290–294.

Li, Q., J.-P. Jin, and H. Granzier. 1995. The effect of genetically expressed cardiac titin fragments on in vitro actin motility. *Biophys. J.* 69: 1508–1518.

Linke, W., M. Ivemeyer, M. Olivieri, B. Kolmerer, J. Rüegg, and S. Labeit. 1996. Towards a molecular understanding of the elasticity of titin. *J. Mol. Biol.* 261:62–71.

Linke, W., V. Popov, and G. Pollack. 1994. Passive and active tension in single cardiac myofibrils. *Biophys. J.* 67:782–792.

Linke, W., M. Ivemeyer, S. Labeit, H. Hinnsen, J. Rüegg, and M. Gautel. 1997. Actin-titin interaction in cardiac myofibrils: probing a physiological role. *Biophys. J.* 73:905–919.

Marko, J., and E. Siggia. 1995. Stretching DNA. *Macromolecules.* 28: 8759–8770.

Maruyama, K. 1997. Connectin/titin, giant elastic protein of muscle. *FASEB J.* 11:341–345.

Maruyama, K., T. Endo, H. Kume, Y. Kawamura, N. Kanzawa, Y. Nakauchi, S. Kimura, and K. Maruyama. 1993. A novel domain sequence of connectin localised at the I-band of skeletal muscle sarcomous: homology to neurofilament subunits. *Biochem. Biophys. Res. Commun.* 194:1288–1291.

Maruyama, K., D. Hu, T. Suzuki, and S. Kimura. 1987. Binding of actin filaments to connectin. *J. Biochem.* 101:1339–1346.

Ohtsuka, H., H. Yajima, K. Maruyama, and S. Kimura. 1997. Binding of the N-terminal 63 kDa portion of connectin/titin to α-actinin as revealed by the yeast two-hybrid system. *FEBS Lett.* 401:65–67.

Rief, M., M. Gautel, F. Oesterhelt, J. Fernandez, and H. Gaubb. 1997. Reversible unfolding of individual titin immunoglobulin domains by AFM. *Science.* 276:1109–1112.

Sebestyén, M., J. Wolff, and M. Greaser. 1995. Characterization of a novel 5.4 kb cDNA fragment coding for the N-terminal region of rabbit cardiac titin. *Biophys. J.* 68:A65.

Sorimachi, H., A. Freiburg, B. Kolmerer, S. Ishiura, G. Stier, C. Gregorio, D. Labeit, W. Linke, K. Suzuki, and S. Labeit. 1997. Tissue-specific expression and α-actinin binding properties of the Z-disc titin. Implications for the nature of vertebrate z-discs. *J. Mol. Biol.* 270:688–695.

ter Keurs, H. 1983. Calcium and contractility. *In* Cardiac Metabolism, A. J. Drake-Holland and M. Noble, editors. John Wiley and Sons, New York.

Trinick, J. 1996. Cytoskeleton: titin as scaffold and spring. *Curr. Biol.* 6:258–260.

Trombitás, K., P. Baatsen, M. Kellermayer, and G. Pollack. 1991. Nature and origin of gap filaments in striated muscle. *J. Cell Sci.* 100:809–814.

Trombitás, K., M. Greaser, and H. Pollack. 1997. Interaction between titin and thin filaments in intact cardiac muscle. *J. Muscle Res. Cell Motil.* 18:345–351.

Trombitás, K., and H. Granzier. 1997. Actin removal from cardiac myocytes shows that near the Z-line titin attaches to actin while under tension. *Am. J. Physiol. (Cell).* 42:C662–C670.

Trombitás, K., J.-P. Jin, and H. Granzier. 1995. The mechanically active domain of titin in cardiac muscle. *Circ Res.* 77:856–861.

Trombitás, K., and G. Pollack. 1993. Elastic properties of the titin filament in the Z-line region of vertebrate striated muscle. *J. Muscle Res. Cell Motil.* 14:416–422.

Tskhovrebova, L., and J. Trinick. 1997. Direct visualization of extensibility in isolated titin molecules. *J. Mol. Biol.* 265:100–106.

Tskhovrebova, L., J. Trinick, J. Sleep, and R. Simmons. 1997. Elasticity and unfolding of single molecules of the giant muscle protein of titin. *Nature.* 387:308–312.

Wang, K., R. McCarter, J. Wright, B. Jennate, and R. Ramirez-Mitchell. 1993. Viscoelasticity of the sarcomere matrix of skeletal muscles: the titin-myosin composite filament is a dual-range molecular spring. *Biophys. J.* 64:1161–1177.

Wang, S.-M., and M. Greaser. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J. Muscle Res. Cell Motil.* 6:293–312.

Yu, F., D. Zhou, and H. Yin. 1991. Chimeric and truncated gCap39 elucidate the requirements for actin filament severing and end capping by the gelsolin family of proteins. *J. Biol. Chem.* 266:19269–19275.