Invited Review

Stretching molecular springs:
elasticity of titin filaments in vertebrate striated muscle

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Summary. Titin, the giant protein of striated muscle, provides a continuous link between the Z-disk and the M-line of a sarcomere. The elastic I-band section of titin comprises two main structural elements, stretches of immunoglobulin-like domains and a unique sequence, the PEVK segment. Both elements contribute to the extensibility and passive force development of nonactivated muscle. Extensibility of the titin segments in skeletal muscle has been determined by immuno-fluorescence/immunoelectron microscopy of sarcomeres stained with sequence-assigned titin antibodies. The force developed upon stretch of titin has been measured on isolated molecules or recombinant titin fragments with the help of optical tweezers and the atomic force microscope. Force has also been measured in single isolated myofibrils. The force-extension relation of titin could be readily fitted with models of biopolymer elasticity. For physiologically relevant extensions, the elasticity of the titin segments was largely explainable by an entropic-spring mechanism. The modelling explains why during stretch of titin, the Ig-domain regions (with folded modules) extend before the PEVK domain.

In cardiac muscle, I-band titin is expressed in different isoforms, termed N2-A and N2-B. The N2-A isoform resembles that of skeletal muscle, whereas N2-B titin is shorter and is distinguished by cardiac-specific Ig-motifs and nonmodular sequences within the central I-band section. Examination of N2-B titin extensibility revealed that this isoform extends by recruiting three distinct elastic elements: poly-Ig regions and the PEVK domain at lower stretch and, in addition, a unique 572-residue sequence insertion at higher physiological stretch. Extension of all three elements allows cardiac titin to stretch fully reversibly at physiological sarcomere lengths, without the need to unfold individual Ig domains. However, unfolding of a very small number of Ig domains remains a possibility.

Key words: Connectin, Skeletal muscle, Cardiac muscle, Passive tension, Wormlike chain

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Introduction: elastic filaments revealed

Since the sliding filament theory of muscle contraction was first proposed in the early 1950s (Huxley and Hanson, 1954; Huxley and Niedergerke, 1954), the structural unit of muscle, the sarcomere, has been understood to consist of two interdigitating filament systems sliding past one another during shortening: the thick and thin filaments. While this two-filament view of a sarcomere is still found today in many textbooks, investigators in the muscle contraction field have become increasingly aware of the presence of a "third" sarcomeric filament system essential for the mechanical functioning of muscle. The presence of some kind of third filament was already recognized some 45 years ago, when it was shown that upon extraction of actin and myosin, the sarcomere does not fall apart; it remains held together by a "ghost-like" set of longitudinal elastic filaments (Huxley and Hanson, 1954). The existence of such a filament set was reinforced when fine filaments, 2-4 nm in diameter, were found to span the gap between thick and thin filaments in sarcomeres stretched beyond overlap (Carlsen et al., 1961; Huxley and Peachey, 1961; Sjöstrand, 1962). But because of the small diameter of these filaments, and their proximity to the larger thick and thin filaments, firm conclusions about their disposition and function were difficult to reach.

Many models for this third filament have been suggested, which, in turn, has given rise to an often confusing array of names: S-filaments, linking thin filament tips to one another across the center of the sarcomere (Hanson and Huxley, 1956); gap filaments, linking thick filaments to thin filaments at their tips (Sjöstrand, 1962); T-filaments, linking Z-line to Z-line, external to thick filaments (McNeill and Hoyle, 1967); core filaments, linking Z-line to Z-line through a thick filament core (Guba et al., 1968); connecting C-filaments, linking Z-lines and thick filaments in insect flight muscle (Garamvolgyi, 1966; White and Thorson, 1973; Trombitas and Tigyi-Sebes, 1974); and (again) gap filaments, linking the tips of two adjacent thick filaments through the Z-line (Locker and Daines, 1980). The difficulty in deciding among these models has

resided partly in the interpretational limits of electron micrographs, but mainly in uncertainty as to which protein was involved in constituting these structures.

Evidence for the presence of the third filament increased considerably following the discovery of a major new myofibrillar protein first described as connectin (Maruyama et al., 1977) and later named titin (Wang et al., 1979). One reason for the protein's late discovery is its huge size, which precludes entry into ordinary SDS polyacrylamide gels: titin was estimated to have a molecular mass of about 3 MDa (Maruyama et al., 1984; Kurzban and Wang, 1988) and is the largest polypeptide so far described. Titin molecules have a string-like appearance (Maruyama et al., 1984; Trinick et al., 1984; Wang et al., 1984) and are ~1 μm long (Nave et al., 1989). Ultimate evidence for the existence of such a giant single polypeptide chain was provided when the full-length sequence of human skeletal and cardiac titin was reported (Labeit and Kolmerer, 1995). It appears that titin is, after myosin and actin, the third most abundant protein of vertebrate striated muscle, making up approximately 10% of the combined muscle protein content. A human adult with 80 kg body weight may contain almost half a kilogram of titin. A current view of the arrangement of titin filaments in the sarcomere and of the titin domain architecture is schematically shown in Fig. 1.

Structural arrangement and functional properties of titin

Titin filaments span a half-sarcomere from the Z-line to the M-line (Wang et al., 1984; Maruyama et al., 1985; Fürst et al., 1988; Itoh et al., 1988; Whiting et al., 1989). The A-band part of the molecule is functionally stiff

(Fürst et al., 1988; Trombitas et al., 1991; Wang et al., 1991; Higuchi et al., 1992), most likely because it is firmly bound to thick filament proteins, such as myosin, myosin-binding protein-C (C-Protein), M-protein and myomesin (Fürst et al., 1989; Labeit et al., 1992; Houmeida et al., 1995; Obermann et al., 1996, 1997). A-band titin features a "super repeat" pattern (Fig. 1) consisting of both immunoglobulin-like (Ig) and fibronectin type-3-like (FN3) modules (Labeit et al., 1990, 1992), which fold into globular domains (Politou et al., 1994, 1996; Improta et al., 1996; Muhle-Goll et al., 1998). It is thought that A-band titin is a structural polypeptide important for the regular assembly of thick filaments (see reviews by Trinick, 1996; Gregorio et al., 1999). Moreover, titin contains, near its COOH-terminal end at the M-line, a kinase domain (Labeit et al., 1992), which may be active during muscle development (Mayans et al., 1998). Hence, titin may also be involved in intracellular signal transduction pathways (Labeit et al., 1997).

The I-band section of titin is elastic along most of its length, except near the Z-line (Fürst et al., 1988; Trombitas and Pollack, 1993; Trombitas et al., 1995), where the molecule associates with actin (Linke et al., 1997; Trombitas and Granzier, 1997). Within the Z-line itself, titin provides binding sites for other myofibrillar proteins such as α-actinin (Ohtsuka et al., 1997; Sorimachi et al., 1997; Young et al., 1998) and T-cap/telethonin (Gregorio et al., 1998; Mues et al., 1998). The elastic I-band titin consists of two main structural elements (Labeit and Kolmerer, 1995), stretches of tandemly arranged Ig domains which flank a unique sequence rich in proline (P), glutamate (E), valine (V) and lysine (K) residues, the PEVK segment (Fig. 1). Both elements are expressed in muscle type-specific length variants, which results in the characteristic size of
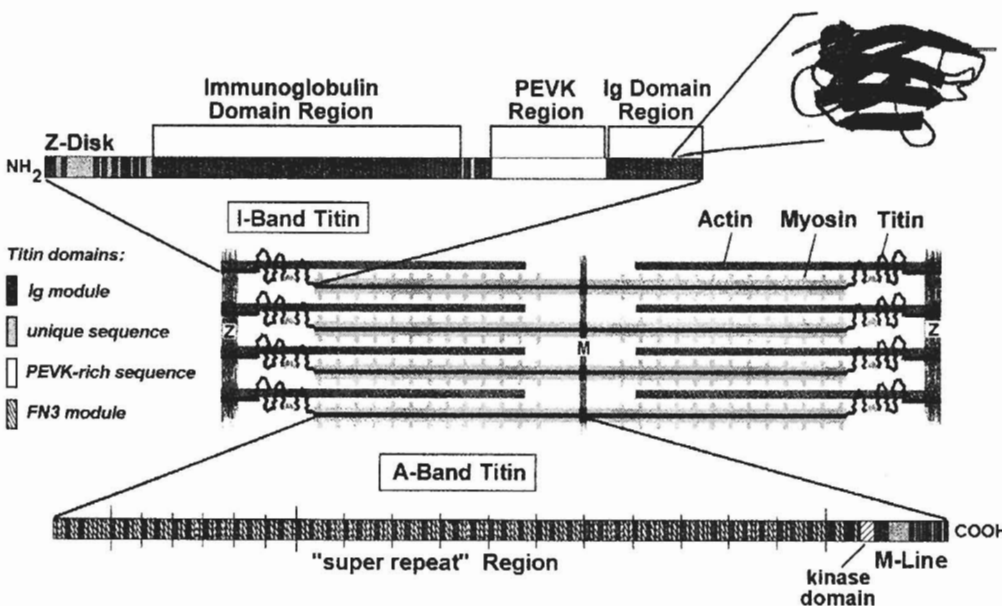
Fig. 1. Three-filament model of the sarcomere and domain architecture of titin according to Labeit and Kolmerer (1995). Shown is the structure of human soleus titin. The I-band and A-band sections of titin are indicated. A-band titin consists of two motif types, immunoglobulin-like (Ig) modules and fibronectin type-3-like (FN3) domains; both types are arranged in super-repeat patterns. M-line titin and Z-disk titin contain no fibronectin modules but are composed of Ig domains interspersed with unique sequences. The main section of I-band titin is made up of two structurally distinct regions, stretches of tandem-Ig modules that adopt a stable ß-sheet fold (Politou et al., 1995), separated by a unique sequence, the PEVK domain. Also shown is the position of the titin kinase domain.

a given titin isoform. For example, human cardiac titin has a molecular mass of ~3 MDa, human soleus titin of ~3.7 MDa (Labeit and Kolmerer, 1995). These differences in the structure of I-band titin give rise to characteristic elastic properties of the sarcomeres in different muscles. An example is shown in Fig. 2: when single myofibrils of either cardiac or skeletal (psoas) muscle are stretched and the passive forces measured, it appears that the length-tension relation is much steeper in cardiac than in skeletal myofibrils (Bartoo et al., 1993; Linke et al., 1994, 1996). Since no extramyofibrillar structures are present (for a review on the cytoskeleton in muscle cells, see Stromer, 1998), the differences are brought about by the intrinsic mechanical properties of the sarcomeric elastic elements, most likely the titin filaments. The elastic properties of titin will be the focus of this review.

Early concepts of titin elasticity

Already before the primary structure of I-band titin became known, a number of studies had indicated that the molecule's elastic region is responsible for several phenomena: (a) for the development of resting tension upon stretch of nonactivated skeletal muscle (Magid and Law, 1985; Funatsu et al., 1990; Wang et al., 1991, 1993; Granzier and Wang, 1993) and cardiac muscle (Funatsu et al., 1993; Linke et al., 1994; Granzier and Irving, 1995); (b) for restoring sarcomeres to their initial length after physiological stretch (Wang et al., 1991, 1993; Trombitas et al., 1993); and (c) for keeping thick filaments at the center of the sarcomere during active contraction (Horowits et al., 1986, 1989; Horowits and Podolsky, 1988). It was also suggested that titin may be responsible in part for the opposing forces that restore the length of cardiac-muscle sarcomeres after isotonic contraction (Helmes et al., 1996).

In initial models of titin elasticity it was assumed that the extension properties of the Ig and/or FN3 repeats could represent the molecular basis of this elasticity. It was proposed that during stretch, the domains composed of seven strands of antiparallel ß-sheets (Pfuhl and Pastore, 1995; Politou et al., 1995) may unfold, which might account for the generation of passive tension (Soteriou et al., 1993; Erickson, 1994). Further, with Ig and FN3 domains also present in the titin A-band section (Labeit et al., 1990, 1992), it was suggested that at higher forces, the A-band domains might be recruited to contribute to elasticity (Wang et al., 1991, 1993). An alternative hypothesis to explain titin elasticity was put forth, proposing that the interdomain linker sequences between I-band titin modules might be extensible (Politou et al., 1995). During stretch of these "hinge" regions, the exposure of hydrophobic residues to water was thought to be relevant for the onset of passive tension. In short, the hypotheses proposed until 1995 assumed that rapid and reversible conformational transitions within the Ig and FN3 domains may represent the molecular basis of titin elasticity.

After the discovery of the PEVK domain (Fig. 1), investigators began to look at different possibilities to explain the nature of titin elasticity. Labeit and Kolmerer (1995) argued that the PEVK region could be a compliant spring. Because the Ig modules fold into thermodynamically stable domains (Politou et al., 1995), it was reasoned that the poly-Ig regions might represent stiff components. With the primary structure of I-band titin in hand, the concepts were now testable.

Recently employed methods to study titin elasticity

Two main experimental approaches have since been taken to study the nature of titin elasticity. One approach consists in the use of single molecules to determine the mechanical properties of titin *in vitro*. A variety of elegant methods has been employed, including a "molecular combing" technique where titin is stretched with meniscus force (Tskhovrebova and Trinick, 1997) and micromechanical manipulation of molecules by optical tweezers (Kellermayer et al., 1997; Tskhovrebova et al., 1997) or the atomic force microscope (Rief et al., 1997, 1998). Very recently, the single-molecule studies have been complemented nicely by theoretical work, in which important features of titin elasticity are predicted by mathematical modelling (e.g., Carrion-Vazquez et al., 1999; Zhang et al., 1999).

In another experimental approach, the mechanism of titin elasticity was probed in preparations in which the orderly structure of the sarcomere is preserved. Soon after the full titin sequences became available, stretched sarcomeres were immunostained with sequence-assigned I-band titin antibodies to investigate the extensibility of individual titin regions (Gautel and Goulding, 1996; Linke et al., 1996). Subsequently the nature of titin elasticity was investigated in the context of the sarcomere by Trombitas et al. (1998) and Linke et al. (1998a,b) for skeletal muscle, and Granzier et al. (1997), Helmes et al. (1999), and Linke et al. (1999) for cardiac muscle. The main results of these studies are summarized below.

Molecular basis of titin elasticity in skeletal muscle

Differential extensibility of I-band titin

Studies by Gautel and Goulding (1996) and Linke et al. (1996) made it clear that both the Ig-domain regions and the PEVK segment contribute to the extensibility of skeletal-muscle titin. Linke et al. (1996) also demonstrated that low stretch forces extend titin poly-Ig chains, whereas much higher forces are needed to extend the PEVK domain. Further, unfolding of Ig domains was considered to be unlikely, because the Ig-domain regions almost ceased to extend at modest stretch. By contrast, the PEVK region seemed capable of unravelling at high force to a fully extended polypeptide chain—a conclusion later supported in an EM study on isolated titin molecules (Tskhovrebova and Trinick, 1997). These

results implied that the poly-Ig segments apparently act as compliant springs and the PEVK region as a higher-stiffness element. A first clue as to why the structurally distinct titin segments exhibit such differential extensibility, then came from the results of single-molecule studies on titin.

Micromechanical measurements on single titin molecules

These studies suggested that, at stretch forces similar to those occurring during normal muscle function, the elasticity of titin may be explained by an "entropic spring" mechanism (Kellermayer et al., 1997; Rief et al., 1997; Tskhovrebova et al., 1997). The entropic-chain characteristics of the molecule would result in a nonlinear force response to stretch, similar to the well-known nonlinear rise in passive force seen during stretch of myofibrils (Fig. 2b). At low forces, the force-extension relation of titin could be well fitted with a wormlike chain (WLC) model of entropic elasticity. An

Fig. 2. Mechanics of the single myofibril (Linke et al., 1994). **a.** Electron micrograph (top panel) and phase-contrast images (lower two panels) of isolated single cardiac myofibril. The lower panels illustrate stretch of a specimen mounted in a setup used to measure passive tension with a sensitive force transducer (resolution, ~5 nN). **b.** Comparison of passive length-tension curves recorded from nonactivated single cardiac and psoas myofibrils. Cardiac sarcomeres are much stiffer than skeletal sarcomeres.
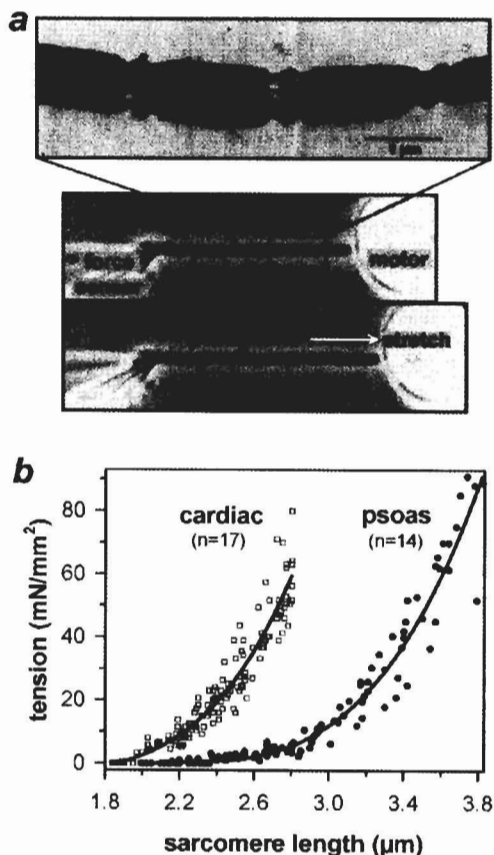
entropic chain undergoes thermally-induced bending movements that tend to shorten its end-to-end length. Stretching such a chain reduces its conformational entropy and thus, requires an external force. Taken simply, the stiffness of a WLC may be parameterized by its contour length and a persistence length, which is a measure of the chain's bending rigidity (also see the section "Application of polymer elasticity theory"). The larger the persistence length, the more rigid the polymer and the smaller the external forces required for stretching out (straightening) the molecule. In the single-molecule studies on titin it was proposed that the persistence length of the Ig-domain region might be relatively large, whereas that of the PEVK segment might be much smaller; this would result in the differential extension behavior of titin.

The *in-vitro* studies on titin also indicated that the molecule's Ig domains are capable of unfolding, at least at high external forces. Whether Ig-domain unfolding was a mechanism relevant to the physiological function of titin, remained a matter of dispute. To address this issue and to probe the entropic-spring concept, it was necessary to examine whether the elastic behavior of titin is similar in the single isolated molecule and in the sarcomere, where titin filaments are arranged in a three-dimensional network. In addition, *in vivo*, titin filaments are functionally elastic only along part of their length, and their extensible I-band section has a heterogeneous structure consisting of poly-Ig regions and the PEVK domain (Fig. 1). Therefore, it was desirable to "dissect" I-band titin into the structurally distinct regions and measure the elastic properties of each segment in the environment of the sarcomere.

Refined model of titin-segment extension

The initial concept of sequential titin extension suggested by Linke et al. (1996) was based on observations made on mechanically manipulated single myofibrils. The specimens can be immunolabeled with sequence-assigned antibodies to I-band titin (Fig. 3a) and visualized in the fluorescence mode of an inverted microscope by using a fluorophore-conjugated secondary antibody (Fig. 3b). Because the position of titin epitopes in the I-band varies with myofibril stretch, the method can be applied to analyze the extension behavior of titin segments under defined experimental conditions. Alternatively, electron microscopical methods have been employed to study the extensibility of titin regions with higher resolution (Linke et al., 1998a,b; Trombitas et al., 1998). Fig. 3c shows examples of electron micrographs of rat psoas sarcomeres immunostained with titin antibodies flanking both the Ig-domain region nearest to the Z-line (proximal poly-Ig region) and the PEVK segment.

A summary of results of such immunostaining experiments is shown in Fig. 4a (Linke et al., 1998a,b). The results indicate that (a) the contribution of the Z-line-T12 segment to the extensibility of I-band titin is

negligable; (b) the segment between the T12 and N2-A epitopes, which consists solely of Ig-domains (Fig. 3a), elongates at low to modest sarcomere stretch, but only little above ~2.6 μm sarcomere length (SL); and (c) the segment between the N2-A and I20/I22 epitopes, which contains the PEVK segment, extends substantially only above ~2.5 μm SL and contributes a lot to the extensibility of titin at higher stretch.

To obtain an estimate of the extension capacity of individual titin segments, it is useful to determine the relative (or fractional) extension of each segment. This is done by relating the end-to-end length of a titin segment to the contour length predicted from the segment's sequence structure. For psoas titin, the proximal Ig-domain region is predicted to have a contour length of 225 nm (50 folded domains; 4.5 nm maximal domain spacing); the PEVK domain a contour length of ~475 nm (1,400 residues; 0.34 nm maximal residue spacing), and the distal Ig-domain region a contour length of ~115 nm (22 domains plus a few extra modules at the A/I junction; 4.5 nm maximal domain spacing). Fig. 4b then demonstrates the relationship between fractional extension and SL, using the data shown in Fig. 4a (Linke et al., 1998a,b). This analysis reveals that (a) extension of the Ig-domain regions precedes that of the PEVK segment and (b) the end-to-end length of all titin segments stays below the predicted contour length over a physiologically relevant range of SLs. Similar conclusions were drawn by Trombitas et al. (1998) in a study on the extensibility of human soleus titin.

Force-extension relations of titin segments

To define the elastic properties of titin segments, the force developed on stretch of titin can be related to the relative extension of a given titin segment. For rat psoas titin, the force per molecule has been estimated to be in the range of 5-10 pN at 3.0 μm SL and 20-40 pN at 3.5 μm SL (Linke et al., 1994, 1998b; also see Wang et al., 1993). These numbers are based on the assumption that 3-6 titin molecules exist per half thick filament (Whiting et al., 1989; Higuchi et al., 1993; Maruyama, 1994). A plot of force per titin strand versus relative titin segment extension is shown in Fig. 5 (thick shaded curves). It appears that straightening out the (proximal) Ig-segment requires very little force, at least up to 80-85% relative extension—corresponding to 2.6-2.7 μm SL. In other words, the Ig-domain region behaves more like a leash than a spring (Erickson, 1997). Above 90% extension, the Ig-segment curve begins to rise dramatically, indicating the region's low extensibility on stretch beyond 2.8 μm SL. Titin now elongates mainly by extension of the PEVK domain. Because the PEVK force-extension curve exhibits a relatively steep slope over the whole range of extensions, elongation of the PEVK segment will be substantial only at moderate to high stretch forces. These results, independently reported by Trombitas et al. (1998) and Linke et al. (1998a,b), confirmed and extended earlier observations of
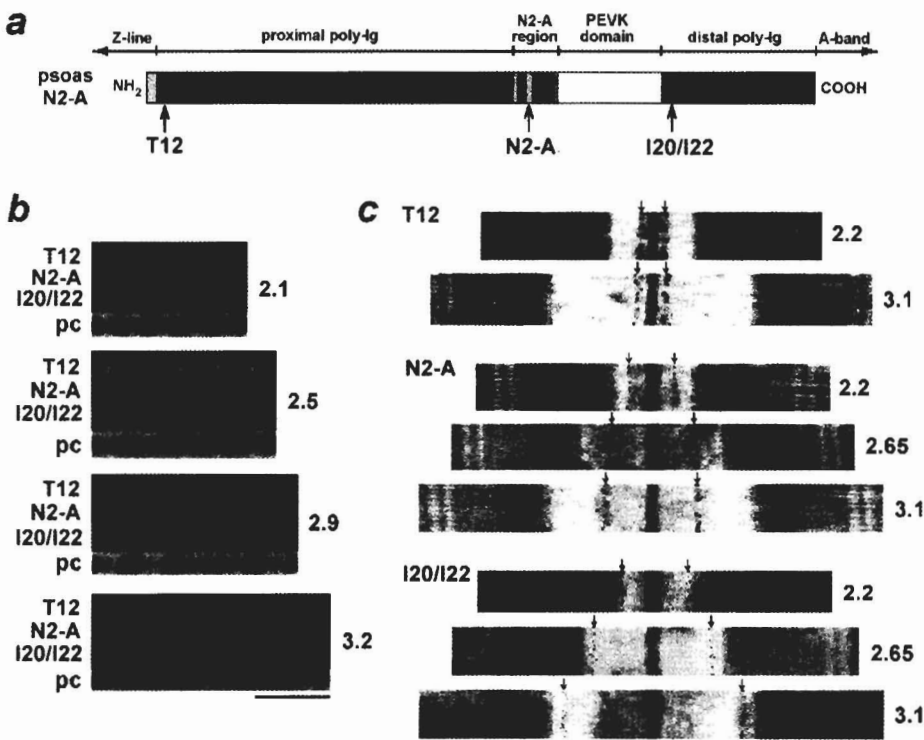
Fig. 3. Measurements of extensibility of structurally distinct segments within I-band titin from skeletal muscle. **a.** Schematically shown is the domain structure of the elastic I-band section of the N2-A isoform in psoas muscle (Labeit and Kolmerer, 1995). Arrows indicate the epitope locations of sequence-assigned titin antibodies used to measure extension of both the proximal Ig-domain region (Linke et al., 1998b) and the PEVK segment (Linke et al., 1998a). **b.** Immunofluorescence images of single psoas myofibrils stretched to different SLs (the actual degree of stretch (μm units) is indicated on the right) and labeled with the respective anti-titin antibodies and fluorophore-conjugated secondary antibody. pc: phase-contrast images. Scale bar: 5 μm **c.** Immunoelectron micrographs of psoas-muscle sarcomeres at different degrees of stretch (SL indicated on the right) and stained with T12, N2-A or I20/I22 titin antibodies. The nanogold particles indicate the respective epitope positions (arrows). Scale bar: 0.5 μm.

sequential titin-segment extension.

Application of polymer elasticity theory

Because the single-molecule experiments had suggested that titin may be an entropic spring showing WLC behavior (Kellermayer et al., 1997; Rief et al., 1997; Tskhovrebova et al., 1997), it appears to be a valid approach to fit the experimentally obtained force-extension data for the titin segments with a standard WLC model of entropic elasticity. According to this model (Bustamante et al., 1994; Marko and Siggia, 1995), the external force (f) is related to the fractional extension (z/L) of the chain by

$$f = \left(\frac{\kappa_B T}{A}\right)\left[\frac{1}{4(1 - zL)^2} - \frac{1}{4} + \frac{z}{L}\right] \qquad (1)$$

where A is the persistence length, k_B is the Boltzmann constant, T is absolute temperature (~300 K in the experiments described), z is the end-to-end length, and L is the chain's contour length. Entropic compliance results when $L \gg A$, due to the numerous configurations the polymer may adopt.

Ig-segment fits

For the proximal Ig-domain region of rat psoas muscle (Linke et al., 1998b), a best-fit curve based on Eq. 1 is shown in Fig. 5 (curve named "WLC model with A=21 nm", because a persistence length of ~21 nm was calculated). Interestingly, this curve followed the experimental data curve only up to forces of ~35 pN. Systematic deviations between fits and data occurred at larger forces and may indicate the onset of structural alterations in the molecule, presumably large-scale unfolding of Ig modules. On the other hand, the excellent fit results at forces <35 pN suggested that unfolding is unlikely to take place at physiologically relevant stretch forces, or at SLs below ~3.4 μm in rat psoas muscle. It followed that the proximal poly-Ig region may behave as a WLC over the entire physiological SL range. This finding is consistent with
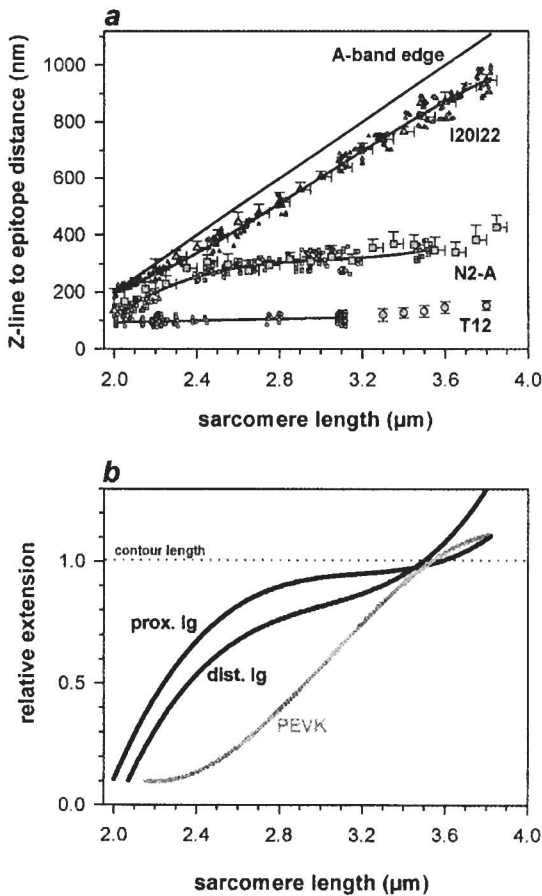
Fig. 4. Extensibility of I-band titin segments in rat psoas muscle (Linke et al., 1998a,b). **a.** Summary of results of immunolabeling experiments. Data points for the Z-line center to epitope spacing at different SLs, determined by immunoelectron microscopy, are shown for the titin antibodies, T12 (small circles), N2-A (small squares), and I20/I22 (small triangles). The larger shaded circles and error bars indicate the mean distances from the Z-line (in 0.1 μm SL bins) and standard deviations for the same antibody epitopes, measured by immunofluorescence microscopy. Data sets were fitted as follows: T12 by linear regression, N2-A and I20/I22 by third-order regressions. **b.** Extension of a given titin segment versus SL, relative to each segment's contour length predicted from the sequence structure (Labeit and Kolmerer, 1995). Predictions were as follows: proximal Ig-domain region, 225 nm; PEVK domain, 475 nm; and distal Ig-domain segment, 115 nm. The curves were calculated from the fit data in a.
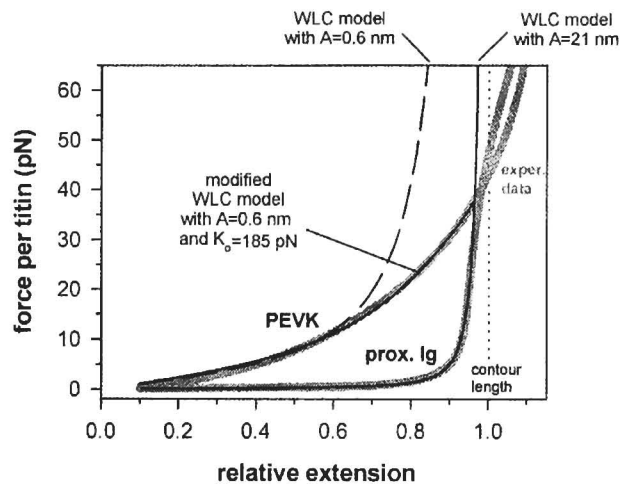
Fig. 5. Fits of the experimentally determined force-extension curves for the proximal Ig-domain region and the PEVK segment of rat psoas muscle according to polymer-elasticity theory (Linke et al., 1998a,b). Force per titin was deduced from the tension data shown in Fig. 2b and plotted versus relative extension of the respective titin segment (thick shaded curves). These data curves were then tried to be simulated with a standard WLC model of entropic elasticity (thin black line for prox. Ig curve; dashed line for PEVK curve). The PEVK data were also fitted with a modified WLC model of entropic-enthalpic elasticity, which revealed a much better fit above 60% relative extension. For further details, see text.

that of Trombitas et al. (1998), who fitted the extension data of the proximal Ig-domain region from human soleus titin to the WLC model. In addition, these investigators reported that WLC behavior can also be ascribed to the distal Ig-domain region. To sum up, (a) a WLC model can correctly describe the elastic properties of the poly-Ig region of skeletal-muscle titin; (b) WLC behavior may be found over the entire range of physiological SLs; and (c) individual Ig domains are unlikely to unfold in great numbers at physiologically relevant stretch forces.

PEVK-domain fits

The standard WLC model (Eq. 1) was also used to fit the PEVK force-extension data. For rat psoas titin (Fig. 5, dashed curve), the model described the experimental data curve reasonably well for forces below ~12 pN or relative extensions below 60% (corresponding to SLs up to ~3.0 μm); a best fit returned a persistence length of ~0.6 nm (Linke et al., 1998a). This value is approximately 35 times smaller than the persistence length calculated for the proximal Ig-domain region (21 nm). Similarly, the persistence length of the titin regions made up of globular domains (Ig, FN3) was previously suggested to be ~25 times larger than that of the PEVK segment (Tskhovrebova et al., 1997). In another study, Trombitas et al. (1998) fitted the soleus PEVK-extension data to the standard WLC model and concluded that, due to its relatively low bending rigidity (persistence length), the PEVK domain is a much stiffer spring than the Ig-domain region. The results of these studies thus provide a framework to explain the differential extension behavior of titin.

Whereas Eq. 1 was successfully used to describe PEVK-titin elasticity over the whole range of physiological extension in human soleus muscle (Trombitas et al., 1998), the WLC model did not sufficiently explain the high-extension data for the

PEVK domain of rat psoas titin (Fig. 5, dashed curve; Linke et al., 1998a). In search of a possible theoretical explanation for this observation, the data were tried to be fitted with other polymer elasticity models. Much better results for the moderate and high force regimes were obtained with entropic-enthalpic models, particularly with a modified WLC model (Wang et al., 1997). This model uses similar parameters to the standard WLC model (Eq. 1) to describe entropic elasticity, but also defines a stretch modulus to incorporate enthalpic stretching—that is, a polymer's intrinsic resistance to longitudinal strain. Details on the modeling procedure are described (Linke et al., 1998a). With the modified WLC model, the PEVK fit at lower forces was comparable to that obtained with Eq. 1, but the data curve was particularly well reproducible for forces >12 pN (Fig. 5, curve named "modified WLC model..."); a best fit returned a persistence length of ~0.6 nm and a stretch modulus of 185 pN. This result may imply that the PEVK region is no pure entropic spring.

In this regard, it is pointed out that myofilaments are not ideal elastic structures but exhibit distinct viscoelastic properties, such as stress relaxation and hysteresis, already at relatively low stretch (e.g., Wang et al., 1993; Linke et al, 1998b). These properties cannot be explained by assuming a titin spring that is purely entropic in nature. Rather, factors other than those recognized so far may contribute to titin elasticity. Then, it is indeed not unlikely that the stiffness of some titin segment, such as the PEVK domain, may be based in part on enthalpic contributions to elasticity. Such contributions have been proposed to originate in electrostatic and perhaps hydrophobic interactions within the PEVK segment and/or result from elastic anisotropy (Linke et al., 1998a). Further studies are needed to establish whether some structuring of the PEVK segment could be a pre-requisite for the viscoelasticity of titin and/or wether stress relaxation and hysteresis might be related to unfolding of a small
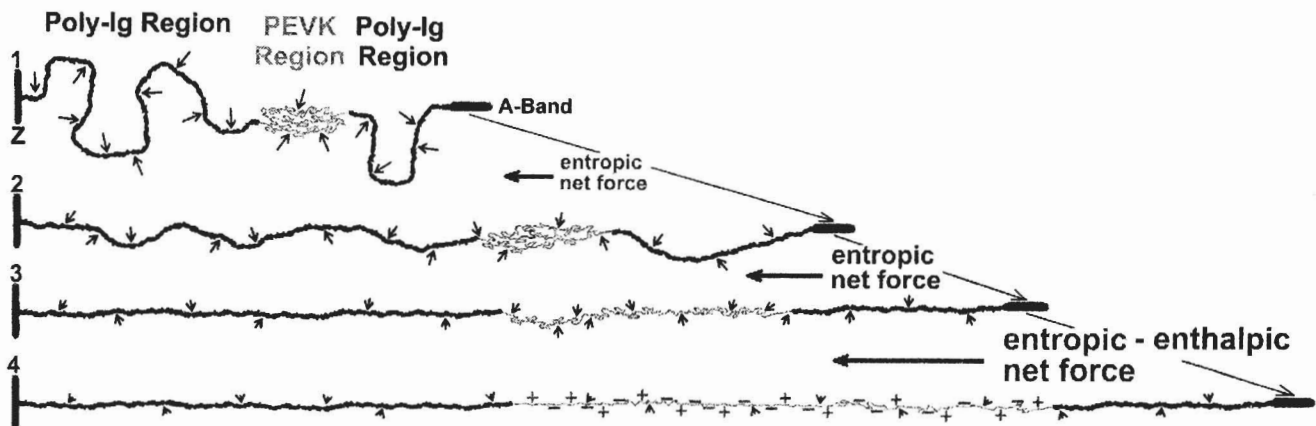

Fig. 6. Model of sequential extension of skeletal-muscle titin. The scheme shows the elastic I-band section of psoas titin at different degrees of stretch. Arrows indicate thermally-induced fluctuations of the polymer chain, giving rise to entropic elasticity; the + and - symbols refer to the finding that electrostatic interactions may contribute to the enthalpic component of PEVK-segment elasticity (Linke et al., 1998a).

number of Ig domains.

Sequential titin extension model

Fundamental questions about the nature of titin elasticity can now be answered. A summary is given by the model shown in Fig. 6. At low stretch (stages 1-2), the poly-Ig regions of titin elongate by straightening out, thus conferring much of the extensibility to titin while developing a very small entropic net force. At modest stretch (stages 2-3), the Ig-domain regions are almost maximally straightened, whereas the PEVK domain unravels and now contributes most of the extensibility to titin; the sequential extension is explainable by the entropic-spring behavior of the segments and the relative difference in bending rigidity between Ig-domain regions and the PEVK domain. At high stretch (stages 3-4), enthalpic compliance of the PEVK-titin might become increasingly important; the developed net force is of entropic-enthalpic nature.

Validity of the approach

Some potential limitations resulting from the application of polymer elasticity theory to the current analysis should be discussed. For valid application of WLC theory, one must assume independent mechanical behavior of individual titin strands—a situation not obvious in the sarcomere, which contains many parallel filaments. For example, titin may interact weakly with other filaments, which could affect passive force transiently. The presence of titin-actin interactions has been demonstrated in vitro (e.g., Maruyama et al., 1987; Soteriou et al., 1993; Jin, 1995; Kellermayer and Granzier, 1996), but not in the elastic I-band region of intact skeletal sarcomeres. Preliminary biochemical evidence has been published indicating interaction

between the PEVK segment and actin (Gutierrez et al., 1998). Clearly, filament-packing constraints in the I-band could hinder the random motion of titin molecules, thereby modifying entropic force. Currently it is unknown whether steric hindrance must indeed be considered. Finally, it is pointed out that the entropic-spring concept for titin has not yet been proven experimentally. It will be interesting to see whether the hypothesis is confirmed, for instance, by measuring the temperature dependence of force development on titin stretch.

Cardiac titin elasticity

Structure of cardiac I-band titin

Cardiac titin exists in different length isoforms expressed in the molecule's I-band region (Labeit and Kolmerer, 1995; Labeit et al., 1997). Both isoforms, termed N2-A and N2-B, comprise stretches of Ig-like modules separated by the PEVK domain. It is not unlikely that the structurally distinct segments of the N2-A isoform may have comparable elastic properties in cardiac and skeletal muscles. Therefore, the conclusions made in the analysis described in the previous sections may also apply to the cardiac N2-A isoform. On the other hand, significant structural differences are found between the cardiac N2-A and N2-B isoforms. The proximal Ig-domain region of N2-B titin is much shorter than that of the N2-A isoform, and also the N2-B-PEVK segment is very short (only 163 residues in humans, cf Fig. 7a). In addition, the central part of N2-B titin contains isoform-specific Ig motifs and nonmodular sequences, notably a 572-residue insertion (Fig. 7a). These structural differences give rise to a number of important functional differences between the isoforms.
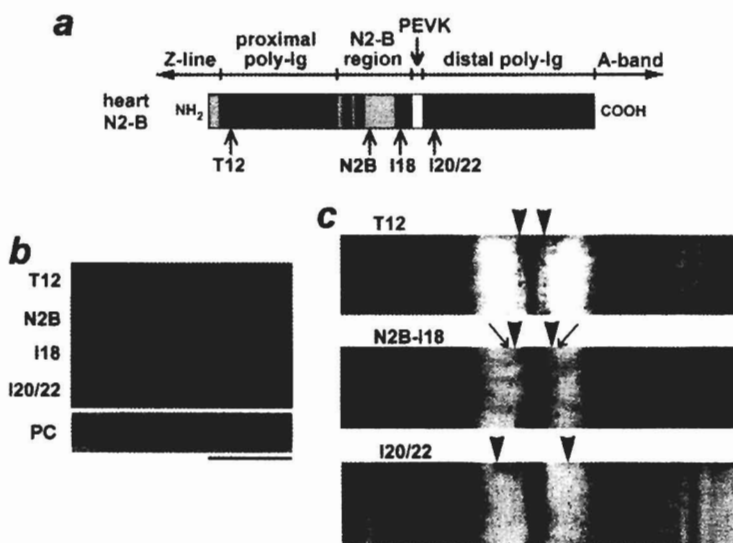
Fig. 7. Extensibility measurements of I-band titin segments in cardiac muscle (Linke et al., 1999). **a.** Schematically shown is the domain structure of the elastic I-band section of the cardiac-specific N2-B titin isoform (Labeit and Kolmerer, 1995). The N2-B titin is co-expressed at the sarcomere level with an N2-A isoform, whose domain structure is not shown. Note the short PEVK segment (163 residues) and the 572 residue-long sequence insertion in the middle N2-B region. Arrows indicate the epitope locations of sequence-assigned titin antibodies used to measure extension of the proximal Ig-domain region, the unique sequence insertion, and the PEVK segment. **b.** Immunofluorescence images of stretched single rabbit cardiac myofibrils labeled with primary antibody and fluorophore-conjugated secondary antibody at 2.7 μm SL. pc, phase-contrast image. Scale bar: 5 μm. **c.** Immunoelectron micrographs of stretched rabbit cardiac sarcomeres stained at 2.35 μm SL with the titin antibodies, T12 or I20/22, or double-stained with both N2B and I18 antibodies. Arrowheads in top and bottom panel indicate the respective epitope positions (nanogold particles). In the middle panel, the secondary antibody for N2B was conjugated to 10 nm gold particles (arrowheads), and that for I18 to 15 nm particles (arrows). Scale bar: 0.5 μm.

Physiological extension of cardiac titin

From a physiological point of view, it is important to uncover the function of titin in the heart, whose elastic properties in diastole are highly relevant for the muscle's overall mechanical performance. Earlier it was shown that the titin filaments may be the principal determinants of resting cardiac-muscle stiffness over most of the physiological SL range, up to 2.1 (Granzier and Irving, 1995) or 2.2 μm (Linke et al., 1994). For comparison, the maximum SL reached in situ is probably 2.3 to 2.4 μm (Allen and Kentish, 1985; Rodriguez et al., 1992). Insights into the function of cardiac I-band titin have been obtained mainly in mechanical and immuno-labeling studies on isolated cells (Trombitas et al., 1995; Granzier et al., 1996, 1997; Helmes et al., 1999) and single myofibrils (Linke et al., 1996, 1997, 1999). It was proposed that in unstretched cardiac sarcomeres, the elastic section of titin is in a contracted state (Trombitas et al., 1995; Granzier et al., 1996). The small passive forces developing upon low stretch to ~2.0 μm SL were suggested to be entropic in nature and to arise from straightening of I-band titin. With further stretch and exponential rise of tension, the globular domains of titin were thought to unfold. In a later report, the SL for the onset of unfolding was proposed to be ~2.2 μm (Granzier et al., 1997). Similarly, it was concluded in a study on stretched single myofibrils that the Ig domains in cardiac titin may begin to unfold above 2.2-2.3 μm SL (Linke et al., 1996). Some arguments against large-scale Ig-domain unfolding were put forth by Gautel et al. (1996). These predictions were made based on the hypothesis that titin contains two structurally distinct extensible elements, poly-Ig chains and the PEVK domain (Labeit and Kolmerer, 1995; Linke et al., 1996; Granzier et al., 1997). A question arising out of these results and the knowledge of the primary structure of cardiac titin was whether the relatively short N2-B titin isoform must unfold at least some of its Ig domains to accommodate high physiological stretch (Linke and Granzier, 1998). Alternatively, it was conceivable that some other extensible element might exist in N2-B titin, perhaps the long sequence insertion within the central I-band region (Fig. 7a). These issues have been dealt with quite recently (Helmes et al., 1999; Linke et al., 1999).

Extensibility of the N2-B unique sequence insertion

The extension capacity of the Ig-domain regions, the PEVK segment, and the unique sequence insertion in N2-B titin was studied by following the epitope mobility of sequence-assigned antibodies (Fig. 7a) in stretched cardiac-muscle sarcomeres (Helmes et al., 1999; Linke et al., 1999). Both immunofluorescence microscopical and immuno-EM methods were employed. Typical images of immunostained rabbit cardiac sarcomeres are shown in Fig. 7b and 7c, respectively. In the immunofluorescence images, it is obvious that, at least at high stretch (2.7 μm SL), all antibodies stain different

positions on N2-B titin (Fig. 7b). By using immunoelectron microscopy, the distance of a given epitope from the center of the Z-disk was established at shorter SLs. Separation of the epitopes flanking the unique N2-B sequence was clearly detectable in sarcomeres stretched to 2.35 μm (Fig. 7c; cf. Linke et al., 1999). Thus, the N2-B unique sequence apparently contributes to the extension of cardiac I-band titin. Extensibility of the N2-B insertion was reported also by Helmes et al. (1999), who measured the mobility of the N2B antibody epitope in rat cardiomyocytes and compared it with the previously published mobility of the I18 epitope in rabbit cardiac cells (Gautel et al., 1996).

A summary of the results obtained by Linke et al.
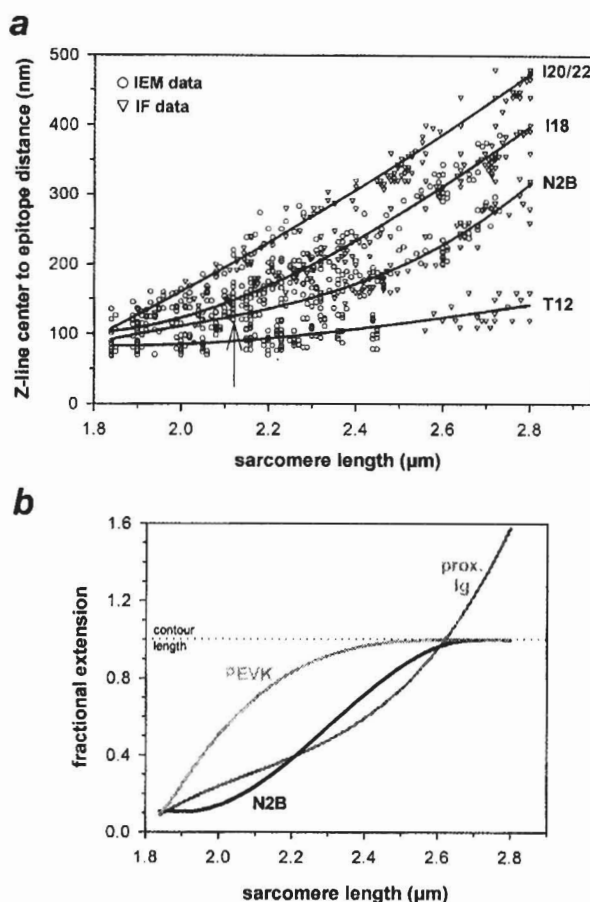
Fig. 8. Extensibility of I-band segments of the N2-B titin isoform in rabbit cardiac muscle. **a.** Summary of results of immunofluorescence (IF) and immunoelectron microscopical (IEM) measurements at SLs ranging from 1.84 to 2.8 μm. Data sets for each antibody type were fitted by third-order regressions. The arrow indicates the onset of statistically significant separation between N2B and I18 curves (Linke et al., 1999). **b.** Extension of the three structurally distinct I-band titin segments, relative to each segment's predicted contour length, plotted versus SL. Contour length predictions were as follows: T12-N2B segment (prox. Ig): 110 nm, assuming folded Ig modules; N2B-I18 segment (N2B): 87 nm, assuming a partially folded structure; I18-I20/22 segment (PEVK): 75 nm, assuming a completely unfolded polypeptide. The curves were calculated from the fit data in a.

(1999) is shown in Fig. 8a. The unique sequence flanked by the I18 and the N2B antibody was confirmed by statistical analysis to begin to lengthen significantly at ~2.15 μm SL (arrow in Fig. 8a). At high physiological stretch of 2.3 to 2.4 μm SL, the sequence insertion extended up to ~60 nm. Thus, the N2-B titin isoform recruits three molecular spring elements: the PEVK segment, the Ig-regions and the long unique sequence insertion.

The distances between the fit curves reveal the extension of a given titin segment. The extensions were then related to the contour length of each segment predicted from the available sequence data for cardiac N2-B titin (Labeit and Kolmerer, 1995). Plots of relative extension versus SL are shown in Fig. 8b for the titin segments flanked by T12 and N2B (proximal Ig-domain region), N2B and I18 (unique sequence) and I18 and I20/22 (PEVK domain), respectively (Linke et al., 1999). Both the PEVK domain and the unique N2-B sequence reached a plateau in their extension curves, whereas the proximal Ig-segment extended beyond its predicted contour length at SLs >2.6 μm. Because the latter can be interpreted as large-scale unfolding of Ig domains, it is most likely that such event will not take place during physiological stretch. Possibly, the unique N2-B sequence acts as some kind of stretchable buffer element, whose extension helps prevent potentially catastrophic events such as unfolding of many Ig domains in the heart's normal working range.

N2-B titin extension model

A model for extension of the titin N2-B isoform in cardiac muscle (Fig. 9) was proposed from the results discussed above by Linke et al. (1999) and in a similar form by Helmes et al. (1999). Low sarcomere extension is brought about by elongation of tandem-Ig segments and the PEVK domain (Fig. 9, stages 1-2). Above 2.15 μm SL, the 572-residue unique insertion also begins to elongate substantially (Fig. 9, stages 2-3) and may compensate for the relatively short length of the PEVK segment, whose extensibility would soon be exhausted. Elasticity of the unique N2-B sequence may also help the shorter N2-B titin isoform to adjust its range of extension to that of the longer N2-A isoform, since both
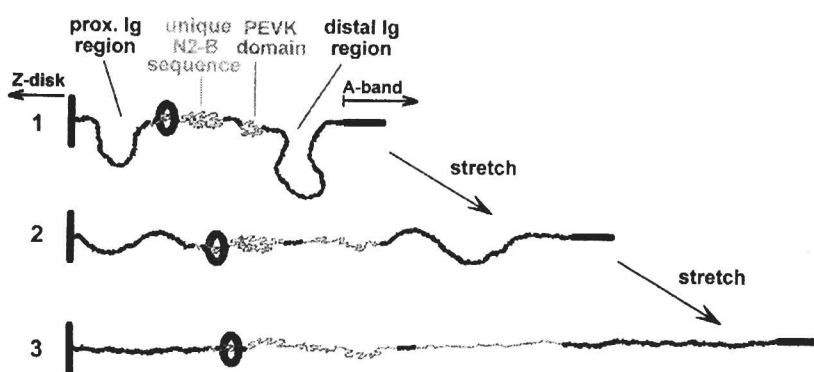
isoforms are co-expressed at the sarcomere level (Linke et al., 1996). During all three stages of extension (Fig. 9; up to 2.35 μm SL in stage 3), elongation of poly-Ig segments will be brought about mainly by straightening, rather than by domain unfolding. With maximum SLs in the working cardiac muscle presumably no longer than 2.3-2.4 μm, cardiac titin will be able to stretch reversibly even to long physiological SLs without requiring Ig domains to unfold.

Cardiac titin segments as serially linked entropic springs?

The elastic properties of cardiac titin have been modeled according to polymer-elasticity theory by assuming two different WLC elements, the Ig-domain segments and the PEVK domain, in series (Granzier et al., 1997). With the knowledge that a third type of elastic element exists in the cardiac N2-B isoform, it became obvious that the WLC modeling for cardiac titin needed modification. Therefore, it was suggested that the Ig-domain segments and the PEVK domain do act as WLCs, but that the N2-B unique sequence behaves as an adjustable spring element (Helmes et al., 1999). The intrinsic (visco)elastic properties of this sequence insertion were proposed to minimize hysteresis in successive stretch-release cycles. It will be interesting to follow up on this idea and test whether the unique insertion is the only candidate for an adjustable spring-like element. A more general complication with the WLC analysis applied to cardiac titin is that recent results from the laboratory of C.C. Gregorio reported in Linke et al. (1999) raise the possibility that part of the central N2-B-titin region is involved in protein/protein interactions. The oval in Fig. 9 schematically indicates the position on N2-B titin implicated in these interactions. Thus, cardiac titin may not be allowed to move freely in the sarcomere, which could affect the elastic properties of the protein chain. At this point it is unsettled how this possible association will change the (hypothesized) WLC behavior of cardiac N2-B titin.

Conclusions and future directions

The main aspects of this review may be summarized

prox. Ig **unique PEVK** **distal Ig**
region **N2-B domain** **region**
sequence

Z-disk
A-band
stretch
stretch

1
2
3

Fig. 9. Extension model for the N2-B titin isoform of rabbit cardiac muscle. The elastic I-band region is illustrated at three different stages of extension within a physiologically relevant SL range. Upon low stretch (first to second stage, 1.95 to 2.15 μm SL), proximal and distal Ig-segments, as well as the PEVK domain, begin to extend. On further stretch, the unique sequence insertion also elongates (second to third stage, 2.15 to 2.35 μm SL). This may help prevent potentially catastrophic events such as unfolding of many Ig-domains at high physiological stretch. The elastic behavior of cardiac titin might be constrained by protein/protein interactions involving specific domains of the N2-B region (indicated by the oval; Linke et al., 1999).

as follows. In skeletal muscle, I-band titin represents a highly nonlinear spring composed of two elastic elements in series, the poly-Ig chains and the PEVK domain; these segments extend sequentially. The poly-Ig regions may behave as entropic springs over the entire range of physiological sarcomere lengths. PEVK-titin elasticity appears to be mainly entropic in nature, but enthalpic contributions are also likely. Modelling the elastic properties of titin according to polymer elasticity theory explains why during stretch, the Ig-domain regions extend (straighten out) before the PEVK domain. Cardiac titin contains, within its N2-B isoform, a unique sequence insertion, which extends toward the high end of the physiological sarcomere-length range. Thus, cardiac titin is a molecular spring consisting of three distinct elastic elements: the unique N2-B sequence, the PEVK domain, and stretches of Ig modules.

Although our understanding of the nature of titin elasticity has advanced greatly over the past few years, many issues still await further examination. For example, does the PEVK domain adopt a folded conformation or is it permanently unfolded? Could a small number of titin Ig domains nevertheless unfold in normally stretched muscle? Does the elastic titin interact with other sarcomeric proteins? Could such interactions affect the elasticity of titin? Or, more generally, could the elasticity be affected because of steric hindrance due to filament-packing constraints? To answer these questions, more single-molecule/myofibril work, perhaps in combination with knock-out techniques, may be needed. Also, an intriguing task remains the experimental proof of the proposed wormlike-chain behavior of titin. And last but not least, it shall be interesting to study whether the mechanical properties of titin may be altered under certain pathological conditions, particularly in the heart.

References

Allen D.G. and Kentish J.C. (1985). The cellular basis of the length-tension relation in cardiac muscle. J. Mol. Cell. Cardiol. 17, 821-840.

Bartoo M.L., Popov V.I., Fearn L.A. and Pollack G.H. (1993). Active tension generation in isolated skeletal myofibrils. J. Muscle Res. Cell Motil. 14, 498-510.

Bustamante C., Marko J.F., Siggia E.D. and Smith S. (1994). Entropic elasticity of λ-phage DNA. Science 265, 1599-1600.

Carlsen F., Knappeis G.G. and Buchthal F. (1961). Ultrastructure of the resting and contracted striated muscle fiber at different degrees of stretch. J. Biophys. Biochem. Cytol. 11, 95-117.

Carrion-Vazquez M., Oberhauser A.F., Fowler S.B., Marszalek P.E., Broedel S.E., Clarke J. and Fernandez J.M. (1999). Mechanical and chemical unfolding of a single protein: a comparison. Proc. Natl. Acad. Sci. USA 96, 3694-3699.

Erickson H.P. (1994). Reversible unfolding of fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin and fibronectin. Proc. Natl. Acad. Sci. USA 91, 10114-10118.

Erickson H.P. (1997). Stretching single protein molecules: titin is a weird spring. Science 276, 1090-1092.

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

Funatsu T., Kono E., Higuchi H., Kimura S., Ishiwata S., Yoshioka T., Maruyama K. and Tsukita S. (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.O., Osborn M., Nave R. and Weber K. (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.

Fürst D.O., Nave R., Osborn M. and Weber K. (1989). Repetitive titin epitopes with a 42 nm spacing coincide in relative position with known A band striations also identified by major myosin-associated proteins; an immunoelectron microscopical study on myofibrils. J. Cell Sci. 94, 119-125.

Garamvölgyi N. (1966). Elongation of the primary myofilaments in highly stretched insect flight muscle fibrils. Biochem. Biophys. Acta 1, 89-100.

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

Gautel M., Lehtonen E. and Pietruschka F. (1996). Assembly of the cardiac I-band region of titin/connectin: expression of the cardiac-specific regions and their relation to the elastic segments. J. Muscle Res. Cell Motil. 17, 449-461.

Granzier H.L. and Irving T.C. (1995). Passive tension in cardiac muscle: Contribution of collagen, titin, microtubules, and intermediate filaments. Biophys. J. 68, 1027-1044.

Granzier H.L. and Wang K. (1993). Passive tension and stiffness of vertebrate skeletal and insect flight muscles: The contribution of weak cross-bridges and elastic filaments. Biophys. J. 65, 2141-2159.

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

Granzier H., Kellermayer M., Helmes M. and Trombitas K. (1997). Titin elasticity and mechanism of passive force development in rat cardiac myocytes probed by thin-filament extraction. Biophys. J. 73, 2043-2053.

Gregorio C.C., Granzier H., Sorimachi H. and Labeit S. (1999). Muscle assembly: a titanic achievement? Curr. Opin. Cell Biol. 11, 18-25.

Gregorio C.C., Trombitas K., Centner T., Kolmerer B., Stier G., Kunke K., Suzuki H., Obermayr F., Herrmann B., Granzier H., Sorimachi H. and Labeit S. (1998). The NH2 terminus of titin spans the Z disc; its interaction with a novel 19 kD ligand (T-cap) is required for sarcomeric integrity. J. Cell Biol. 143, 1013-1027.

Guba F., Harsanyi V. and Vadja E. (1968). Ultrastructure of myofibrils after selective protein extraction. Acta Biochim. Biophys. Acad. Sci. Hung. 3, 441-448.

Gutierrez G., van Heerden A. and Wang K. (1998). Interactions and conformational studies of PEVK segment of human fetal skeletal

muscle titin. Biophys. J. 74, A349.

Hanson J. and Huxley H.E. (1956). The structural basis of contraction in striated muscle. Symp. Soc. Exp. Biol. 9, 228-264.

Helmes M., Trombitas K. and Granzier H. (1996). Titin develops restoring force in rat cardiac myocytes. Circ. Res. 79, 619-626.

Helmes M., Trombitas K., Centner T., Kellermayer M., Labeit S., Linke W.A. and Granzier H. (1999). Mechanically driven contour-length adjustment in rat cardiac titin's unique N2B sequence: Titin is an adjustable spring. Circ. Res. 84, 1339-1352.

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

Higuchi H., Suzuki T., Kimura S., Yoshioka T., Maruyama K. and Umazume Y. (1992). Localization and elasticity of connectin (titin) filaments in skinned frog muscle fibres subjected to partial depolymerization of thick filaments. J. Muscle Res. Cell Motil. 13, 285-294.

Horowits R. and Podolsky R.J. (1988). Thick filament movement and isometric tension in activated skeletal muscle. Biophys. J. 54, 165-171.

Horowits R., Kempner E.S., Bisher M.E. and Podolsky R.J. (1986). A physiological role for titin and nebulin in skeletal muscle. Nature 323, 160-164.

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

Houmeida A., Holt J., Tskhovrebova L. and Trinick J. (1995). Studies of the interaction between titin and myosin. J. Cell Biol. 131, 1471-1481.

Huxley A.F. and Niedergerke R. (1954). Structural changes in muscle during contraction. Nature 173, 147-149.

Huxley A.F. and Peachey L.D. (1961). The maximum length for contraction in vertebrate striated muscle. J. Physiol. (London) 156, 150-165.

Huxley H.E. and Hanson J. (1954). Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature 173, 149-152.

Improta S., Politou A. and Pastore A. (1996). Immunoglobulin-like modules from I-band titin: extensible components of muscle elasticity. Structure 4, 323-337.

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

Jin J.-P. (1995). Cloned rat cardiac titin class I and class II motifs. J. Biol. Chem. 270, 6908-6916.

Kellermayer M.S. and Granzier H.L. (1996). Calcium-dependent inhibition of thin-filament motility by native titin. FEBS Lett. 380, 281-286.

Kellermayer M.S.Z., Smith S.B., Granzier H.L. and Bustamante C. (1997). Folding-unfolding transitions in single titin molecules characterized with laser tweezers. Science 276, 1112-1116.

Kurzban G.P. and Wang K. (1988). Giant polypeptides of skeletal muscle titin: sedimentation equilibrium in guanidine hydrochloride. Biochem. Biophys. Res. Commun. 150, 1155-1161.

Labeit S. and Kolmerer B. (1995). Titins, giant proteins in charge of muscle ultrastructure and elasticity. Science 270, 293-296.

Labeit S., Barlow D., Gautel M., Gibson T., Holt J., Hsieh C.-L., Francke U., Leonard K., Wardale J., Whiting A. and Trinick J. (1990). A regular pattern of two types of 100-residue motif in the sequence of titin. Nature 345, 273-276.

Labeit S., Gautel M., Lakey A. and Trinick J. (1992). Towards a molecular understanding of titin. EMBO J. 11, 1711-1716.

Labeit S., Kolmerer B. and Linke W.A. (1997). The giant protein titin. Emerging roles in physiology and pathophysiology. Circ. Res. 80, 290-294.

Linke W.A. and Granzier H. (1998). A spring tale: New facts on titin elasticity. Biophys. J. 75, 2613-2614.

Linke W.A., Popov V.I. and Pollack G.H. (1994). Passive and active tension in single cardiac myofibrils. Biophys. J. 67, 782-792.

Linke W.A., Ivemeyer M., Olivieri N., Kolmerer B., Rüegg J.C. and Labeit S. (1996). Towards a molecular understanding of the elasticity of titin. J. Mol. Biol. 261, 62-71.

Linke W.A., Ivemeyer M., Labeit S., Hinssen H., Rüegg J.C. and Gautel M. (1997). Actin-titin interaction in cardiac myofibrils: Probing a physiological role. Biophys. J. 73, 905-919.

Linke W.A., Ivemeyer M., Mundel P., Stockmeier M.R. and Kolmerer B. (1998a). Nature of PEVK-titin elasticity in skeletal muscle. Proc. Natl. Acad. Sci. USA 95, 8052-8057.

Linke W.A., Stockmeier M.R., Ivemeyer M., Hosser H. and Mundel P. (1998b). Characterizing titin's I-band Ig domain region as an entropic spring. J. Cell Sci. 111, 1567-1574.

Linke W.A., Rudy D.E., Centner T., Gautel M., Witt C., Labeit S. and Gregorio C.C. (1999). I-band titin in cardiac muscle is a three-element molecular spring and is critical for maintaining thin filament structure. J. Cell Biol. 146, 1-14.

Locker R.H. and Daines G.J. (1980). Gap filaments—the third set in the myofibril. In: Fibrous proteins: Scientific, industrial, and medical aspects. Vol. 2. Perry D.A.D. and Creamer L.K. (eds). Academic Press. New York. pp 43-45.

Magid A. and Law D.J. (1985). Myofibrils bear most of the resting tension in frog skeletal muscle. Science 230, 1280-1282.

Marko J.F. and Siggia E.D. (1995). Stretching DNA. Macromolecules 28, 8759-8770.

Maruyama K. (1994). Connectin, an elastic protein of striated muscle. Biophys. Chem. 50, 73-85.

Maruyama K., Matsubara S., Natori R., Nonomura Y., Kimura S., Ohashi K., Murakami F., Handa S. and Eguchi G. (1977). Connectin, an elastic protein of muscle: characterization and function. J. Biochem. (Tokyo) 82, 317-337.

Maruyama K., Kimura S., Yoshidomi H., Sawada H. and Kikuchi M. (1984). Molecular size and shape of ß-connectin, an elastic protein of striated muscle. J. Biochem. (Tokyo) 95, 1423-1493.

Maruyama K., Yoshioka T., Higuchi H., Ohashi K., Kimura S. and Natori R. (1985). Connectin filaments link thick filaments and Z lines in frog skeletal muscle as revealed by immunoelectron microscopy. J. Cell Biol. 101, 2167-2172.

Maruyama K., Hu D.H., Suzuki T. and Kimura S. (1987). Binding of actin filaments to connectin. J. Biochem. (Tokyo) 101, 1339-1346.

Mayans O., van der Ven P.F., Wilm M., Mues A., Young P., Fürst D.O., Wilmanns M. and Gautel M. (1998). Structural basis for activation of the titin kinase domain during myofibrillogenesis. Nature 395, 863-869.

McNeill P.A. and Hoyle G. (1967). Evidence for superthin filaments. Am. Zool. 7, 483-498.

Mues A., van der Ven P.F.M., Young P., Fürst D.O. and Gautel M.

(1998). Two immunoglobulin-like domains of the Z-disc portion of titin interact in a conformation-dependent way with telethonin. FEBS Lett. 428, 111-114.

Muhle-Goll C., Pastore A. and Nilges M. (1998). The 3D structure of a type I module from titin: a prototype of intracellular fibronectin type III domains. Structure 6, 1291-1302.

Nave R., Fürst D.O. and Weber K. (1989). Visualization of the polarity of isolated titin molecules: a single globular head on a long thin rod as the M Band anchoring domain? J. Cell Biol. 109, 2177-2187.

Obermann W.M.J., Gautel M., Steiner F., van der Ven P.F.M., Weber K. and Fürst D.O. (1996). The structure of the sarcomeric M band: localization of defined domains of myomesin, M protein, and the 250 kD carboxy terminal region of titin by immunoelectron microscopy. J. Cell Biol. 134, 1441-1453.

Obermann W.M.J., Gautel M., Weber K. and Fürst D.O. (1997). Molecular structure of the sarcomeric M band: mapping of titin and myosin binding domains in myomesin and the identification of a potential regulatory phosphorylation site in myomesin. EMBO J. 16, 211-220.

Ohtsuka H., Yajima H., Maruyama K. and Kimura S. (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.

Pfuhl M. and Pastore A. (1995). Tertiary structure of an immunoglobulin-like domain from the giant muscle protein titin: a new member of the I set. Structure 3, 391-401.

Politou A.S., Gautel M., Pfuhl M., Labeit S. and Pastore A. (1994). Immunoglobulin-type domains of titin: Same fold, different stability? Biochem. 33, 4730-4737.

Politou A.S., Thomas D.J. and Pastore A. (1995). The folding and stability of titin immunoglobulin-like modules, with implications for the mechanism of elasticity. Biophys. J. 69, 2601-2610.

Politou A.S., Gautel M., Improta S., Vangelista L. and Pastore A. (1996). The elastic I-band region of titin is assembled in a "modular" fashion by weakly interacting Ig-like domains. J. Mol. Biol. 255, 604-616.

Rief M., Gautel M., Oesterhelt F., Fernandez J.M. and Gaub H.E. (1997). Reversible unfolding of individual titin immunoglobulin domains by AFM. Science 276, 1109-1112.

Rief M., Gautel M., Schemmel A. and Gaub H.E. (1998). The mechanical stability of immunoglobulin and fibronectin III domains in the muscle protein titin measured by atomic force microscopy. Biophys. J. 75, 3008-3014.

Rodriguez E.K., Hunter W.C., Royce M.J., Leppo M.K., Doulas A.S. and Weisman H.F. (1992). A method to reconstruct myocardial sarcomere lengths and orientations at transmural sites in beating canine hearts. Am. J. Physiol. 263, H293-H306.

Sjöstrand F. (1962). The connections between A- and I-band filaments in striated frog muscle. J. Ultrastruct. Res. 7, 225-246.

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

Soteriou A., Gamage M. and Trinick J. (1993). A survey of the interactions made by titin. J. Cell Sci. 104, 119-123.

Stromer M.H. (1998). The cytoskeleton in skeletal, cardiac and smooth muscle cells. Histol. Histopathol. 13, 283-291.

Trinick J. (1996). Cytoskeleton: Titin as a scaffold and spring. Curr. Biol.

6, 258-260.

Trinick J., Knight P. and Whiting A. (1984). Purification and properties of native titin. J. Mol. Biol. 180, 331-356.

Trombitas K. and Granzier H. (1997). Actin removal from cardiac myocytes shows that near the Z-line titin attaches to actin while under tension. Am. J. Physiol. 273, C662-C670.

Trombitas K. and Pollack G.H. (1993). Elastic properties of connecting filaments along the sarcomere. Adv. Exp. Med. Biol. 332, 71-79.

Trombitas K. and Tigyi-Sebes A. (1974). Direct evidence for connecting C filaments in flight muscle of honey bee. Acta Biochim. Biophys. Acad. Sci. Hung. 9, 243-253.

Trombitas K., Baatsen P.H.W.W., Kellermayer M.S.Z. and Pollack G.H. (1991). Nature and origin of gap filaments in striated muscle. J. Cell Sci. 100, 809-814.

Trombitas K., Pollack G.H., Wright J. and Wang K. (1993). Elastic properties of titin filaments demonstrated using a "freeze-break" technique. Cell Motil. Cytoskeleton 24, 274-283.

Trombitas K., Jin J.-P. and Granzier H. (1995). The mechanically active domain of titin in cardiac muscle. Circ. Res. 77, 856-861.

Trombitas K., Greaser M., Labeit S., Jin J.-P., Kellermayer M., Helmes M. and Granzier H. (1998). Titin extensibility in situ: Entropic elasticity of permanently folded and permanently unfolded molecular segments. J. Cell Biol. 140, 853-859.

Tskhovrebova L. and Trinick J. (1997). Direct visualization of extensibility in isolated titin molecules. J. Mol. Biol. 265, 100-106.

Tskhovrebova L., Trinick J., Sleep J.A. and Simmons R.M. (1997). Elasticity and unfolding of single molecules of the giant muscle protein titin. Nature 387, 308-312.

Wang K., McCarter R., Wright J., Beverly J. and Ramirez-Mitchell R. (1991). Regulation of skeletal muscle stiffness and elasticity by titin isoforms: A test of the segmental extension model of resting tension. Proc. Natl. Acad. Sci. USA 88, 7101-7105.

Wang K., McCarter R., Wright R., Beverly J. and Ramirez-Mitchell R. (1993). Viscoelasticity of the sarcomere matrix of skeletal muscles. The titin-myosin composite filament is a dual-stage molecular spring. Biophys. J. 64, 1161-1177.

Wang K., McClure J. and Tu A. (1979). Titin: major myofibrillar component of striated muscle. Proc. Natl. Acad. Sci. USA 76, 3698-3702.

Wang K., Ramirez-Mitchell R. and Palter D. (1984). Titin is an extraordinary long, flexible, and slender myofibrillar protein. Proc. Natl. Acad. Sci. USA 81, 3685-3689.

Wang M.D., Yin H., Landick R., Gelles J. and Block S.M. (1997). Stretching DNA with optical tweezers. Biophys. J. 72, 1335-1346.

White D.C.S. and Thorson J. (1973). The kinetics of muscle contraction. Prog. Biophys. Mol. Biol. 27, 173-255.

Whiting A., Wardale J. and Trinick J. (1989). Does titin regulate the length of muscle thick filaments? J. Mol. Biol. 205, 263-268.

Young P., Ferguson C., Banuelos S. and Gautel M. (1998). Molecular structure of the sarcomeric Z-disk: Two types of interaction lead to an asymmetrical sorting of α-actinin. EMBO J. 17, 1614-1624.

Zhang B., Xu G. and Evans J.S. (1999). A kinetic molecular model of the reversible unfolding and refolding of titin under force extension. Biophys. J. 77, 1306-1315.