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Local Variability in the Mechanics of Titin's Tandem Ig Segments*

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The functionally elastic, I-band part of the myofibrillar protein titin (connectin) contains differentially expressed arrays of serially linked immunoglobulin (Ig)-like domains, the length and composition of which vary among the titin isoforms. The biological rationale of the differential expression as well as the contribution of the Ig domain mechanical characteristics to the overall mechanical behavior of titin are not exactly known. The paper briefly reviews the relevant works that have addressed the Ig-domain mechanics problems and presents the authors' experimental approach to studying the mechanical behavior of Ig domains. The mechanics of an eight domain segment from the differentially expressed tandem Ig region of titin (I55-62) was studied with an atomic force microscope specially used for stretching single molecules, and the results were compared to known mechanical properties of other domains and segments.

Keywords
titin
immunoglobulin domain
elasticity
single-molecule force spectroscopy
unfolding

INTRODUCTION

The myofibrillar protein titin (first known as connectin) forms the third filament system in the vertebrate striated muscle.[1–3] Individual titin molecules span half of the muscle sarcomere from the Z- to the M-line. The I-band part of titin is functionally elastic and is responsible for the generation of passive muscle force. This region contains two arrays of serially linked immunoglobulin (Ig)-like domains: the proximal (toward the Z-line) and the distal (towards the A-band) tandem Ig segments, both comprising Ig domains with a 7-stranded ß-sheet structure.[4] The two tandem Ig regions flank a segment rich in proline (P), glutamate (E), valine (V) and lysine (K) residues (PEVK segment) and, depending on the titin isoform, the N2A and/or N2B unique sequences. The above elements differ both in structure and mechanical properties and extend sequentially during stretch.[5–7]

Titin is expressed in muscle-specific isoforms containing I-band regions of different length and structure.[8] The expression of titin isoforms is achieved by differential splicing of the mRNA transcribed from a single gene.[9,10] The tandem Ig segments are divided into constitutively and differentially expressed regions (Figure 1). The constitutively expressed region is present in all isoforms and comprises the first 15 N-terminal domains of the proximal segment and the entire distal tandem Ig segment. The differentially expressed region, by contrast, has a length that varies from isoform to isoform; the greatest difference in the lengths of the differentially spliced tandem Ig segments is found between the N2-B cardiac and soleus-muscle titin, which contain 0 and 53 Ig domains, respectively (see Figure 1).

The biological rationale of the expression of segments having different length and composition is not ex-

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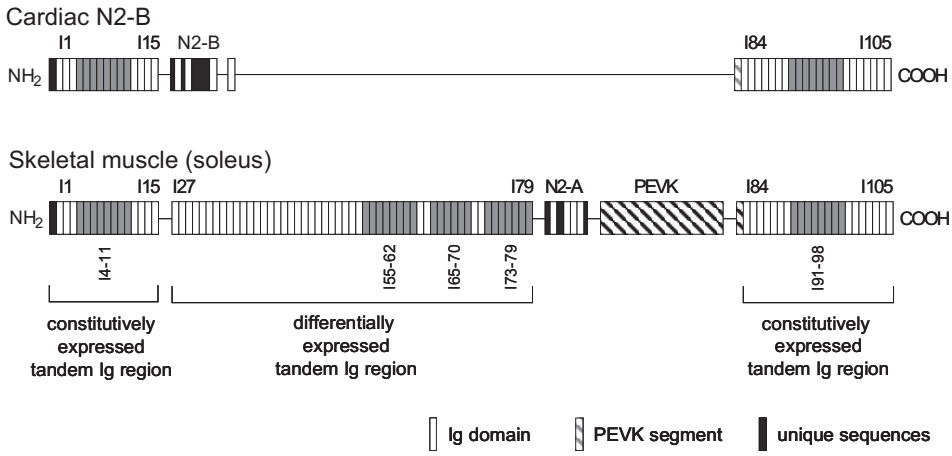

Figure 1. Domain structure of the I-band part of two titin isoforms, skeletal (soleus) and cardiac N2-B. Ig domains studied in the present and previous AFM experiments are marked grey.

actly known. It is also unknown why differential splicing affects only the proximal tandem Ig segment. Different length of the differentially expressed segments leads to different relative extensibilities of the I-band parts of titin, which was suggested to determine the different mechanical properties of titin isoforms.[11] It is yet unclear whether the extra elements included by differential splicing are simple appendages, or whether their composition and structure are also important. In the present paper, the relevant works that have addressed the Ig-domain mechanics problems are briefly reviewed and experimental approach to studying the mechanical behavior of Ig domains is presented.

The mechanism of titin's extensibility in the I-band and that of passive force generation is considered to be mainly the entropic elasticity of the tandem Ig regions and that of the PEVK domain.[7] Whether unfolding of Ig domains contributes significantly to titin's extensibility under physiological conditions is still debated. Immunoelectron microscopy studies by Trombitás *et al.*[7] indicated that unfolding of domains is likely to be absent at physiological sarcomere lengths. Another study,[12] however, suggested that unfolding of a few domains may occur during the sarcomere stretch. The possibility of domain unfolding at physiologically relevant force ranges is also supported by single-molecule mechanics experiments on titin,[13–15] which revealed the probabilistic nature of the unfolding events.

Identification of the modular structure of titin opened the way to characterizing the molecule's mechanical properties through dissection of the molecule into functionally and structurally relevant elements. Progress in molecular biology techniques provided the background for expressing arbitrarily chosen recombinant molecular segments.

Rief *et al.*[16] compared the mechanical stability of domain constructs containing Ig domains, situated in the I-band part of titin to those containing fibronectin (Fn) and Ig domains situated in the A-band. They found that Fn domains exhibit 20 % smaller unfolding forces than Ig domains. The mechanical stability of a tandem Ig segment from titin's differentially expressed region (I65-70) was compared and found to be lower than that of another segment (I91-98) from the constitutively expressed region.[17]

The strategy of studying the mechanical stability of individual domains has been the use of a multi-domain construct consisting of repeats of the same domain. The first domain studied by this method was the I27 (I91 in the new nomenclature),[9] because of its known structure[18] and stability.[19] Soon, the I27 module became the most extensively studied domain in molecular mechanics manipulation experiments and simulations.

The mechanical manipulation studies of titin domains and domain series suggested that the mechanical properties of different domains and regions along titin are heterogeneous. Based on these single-molecule studies, it is likely that the order of the unfolding of domains during the stretch of the molecule is not random but is rather determined by the mechanical responsivity of the domains. However, the full magnitude and pattern of the variations in the mechanical properties, as well as the exact order of the domain unfolding events along titin is unknown.

Here, we studied the mechanical behavior of an eight domain segment (octamer) from the differentially expressed tandem Ig region of titin (I55-62, nomenclature according to Bang *et al.*)[9] and compared it to the known mechanical properties of other domains and segments. Our approach differs from that of constructing a homopolymer from a single domain in that it examines the mechanics of a domain segment. Although in this case the mechanical fingerprints of the individual domains cannot be identified, the strategy has the advantage of studying a segment naturally occurring in titin. It was recently shown that the unfolding of protein domains is af-

fected by the adjacent domains;[20] therefore the above approach may yield results that are closer to physiologically relevant conditions. From the study of other Ig segments from titin's differentially expressed region, a more complete picture of its mechanical behavior may emerge. The single-domain approach, though it could provide a high resolution mapping of the mechanical stability along the differentially expressed region, would require the cloning, expression and single-molecule force spectroscopy analysis of 53 different homopolymers.

In the present work, it was found that the unfolding forces for the I55-62 differentially expressed octamer vary widely: the majority of the constituent domains are stable with unfolding forces comparable to those of constitutively expressed domains, and a small part of constituent domains display low unfolding forces comparable to those of differentially expressed Ig domains studied before.

EXPERIMENTAL

Cloning, Expression and Purification of the I55-62 Tandem Ig Segment

Human skeletal muscle cDNA library was a generous gift of Dr. Siegfried Labeit. The nucleotide sequence boundaries of the I55-62 tandem Ig segment were determined based on GenBank accession No. X90569 (version X90569.1),[8] and are as follows: 8227-10491 (aa: 2743-3497). The segment was cloned into a pET-28a vector (Novagen) between NheI and XhoI sites introduced independently with PCR by using specific oligonucleotides. To the C-terminus of the recombinant proteins, two vicinal cysteines were added to facilitate the specific binding of the protein to a gold substrate. Proteins were expressed soluble in *E. coli* (BL21-(DE3)pLysS). His_6-tagged (on N-terminus) proteins were purified on Ni^{2+}-NTA columns under native conditions following the manufacturer's instructions (Qiagen) and further purified on a Sephadex G-25 column. Concentrations were determined with Bradford reagent (Sigma). As evidenced by the gel profile (Figure 2), protein samples were of high purity and devoid of degradation products.

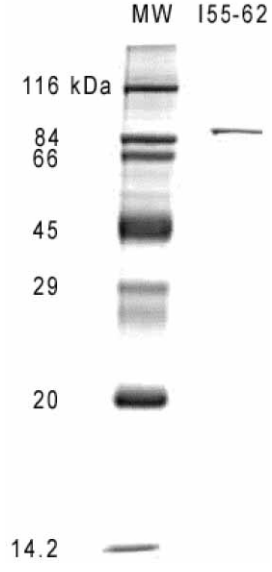

Figure 2. SDS gel electrophoresis profile of the purified I55-62 protein construct.

Single-molecule Force Spectroscopy

The I55-62 domain constructs were mechanically stretched by using an atomic force microscope (AFM) intended for stretching molecules (MFP1D, Asylum Research, Santa Barbara, CA). The AFM was mounted on a custom-built, low-profile, inverted light microscope. I55-62 domain constructs, diluted in AB buffer (25 mmol dm^{-3} imidazole-HCl, pH = 8.0, 0.2 mol dm^{-3} KCl, 4 mmol dm^{-3} $MgCl_2$, 1 mmol dm^{-3} EGTA) were allowed to bind to the surface of chromium-stabilized, freshly evaporated gold coverslips for 20 minutes. The gold surface was used to facilitate the specific binding of one end of the molecule. Unbound molecules were removed by extensive washing with AB buffer. The cantilever approach to the surface was monitored by following the amplitude of the thermally-driven cantilever oscillations.

Force *versus* displacement curves were collected in repeated stretch and release cycles at a constant rate (≈500 nm/s). Although unfolding forces are more strongly influenced by the loading rate (rate of force increase) than the stretch rate, stretch-rate-dependent measurements are commonly used.[21] The cantilever base was allowed to approach the surface until a predetermined cantilever bending was reached and was then retracted. Force values were calculated from the bending and the stiffness of the cantilever. Cantilever stiffness (κ) was determined by calibration with the thermal method.[22] The force *versus* displacement curves were corrected for several factors to obtain force *versus* molecular end-to-end length:[23] i) the zero-length, zero-force data point was obtained from the force response that corresponded to the cantilever tip reaching (or departing from) the substrate surface; ii) forces (F) were corrected for the baseline slope obtained from the force response of the displaced but unloaded cantilever; iii) the end-to-end length (z) of the tethered molecule was calculated by correcting the cantilever base displacement (s) with cantilever bending as

$$z = s - \frac{F}{\kappa}. \qquad (1)$$

Analysis of Force Data

Unfolding forces of the domains were determined as the maximum force value measured for each of the sawtooth peaks, since these peaks correspond to the unfolding of individual domains (see Figure 3b). Selection of the curves to be analyzed was done according to the principles and criteria given by Best *et al.*[21]

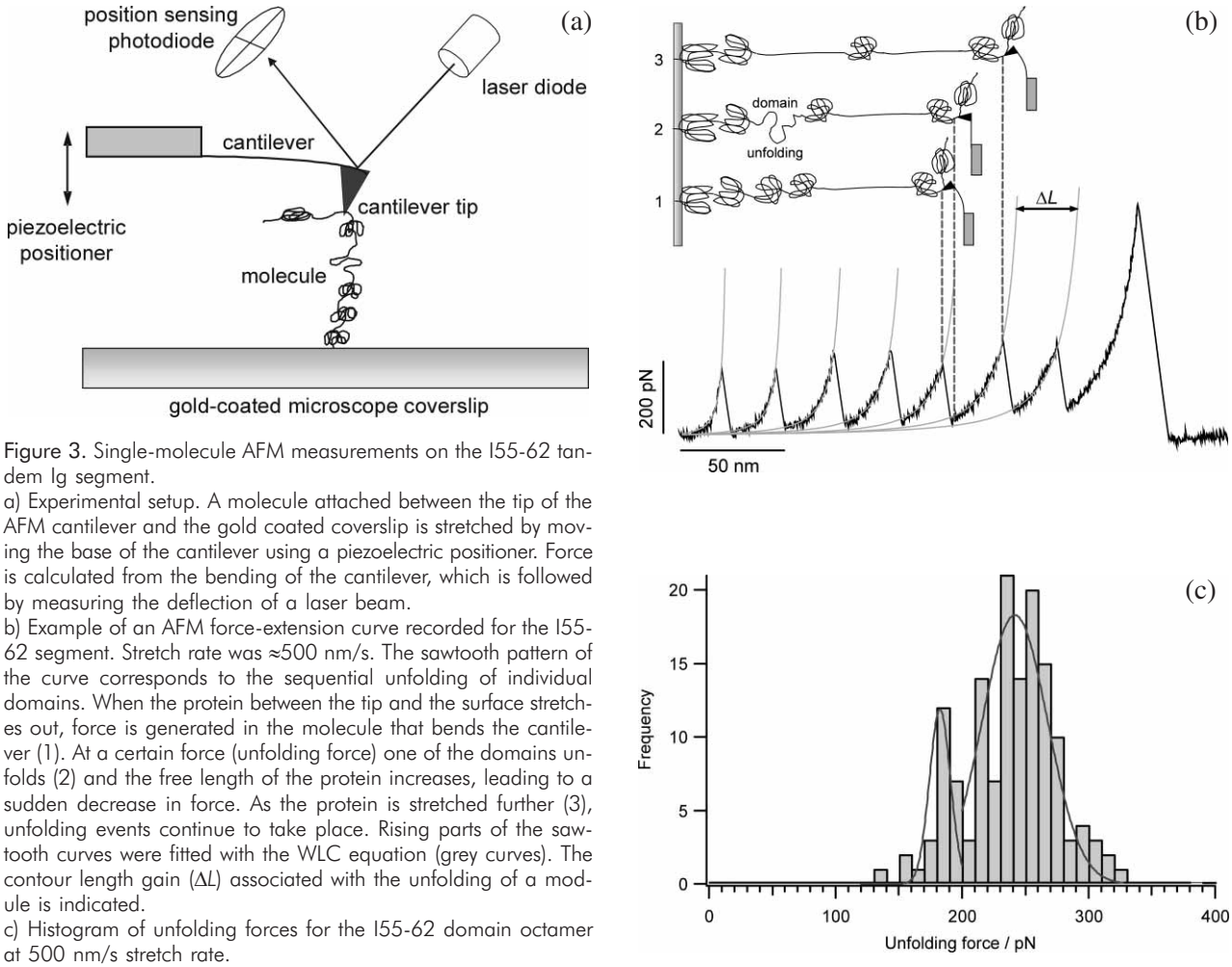

Figure 3. Single-molecule AFM measurements on the I55-62 tandem Ig segment.
a) Experimental setup. A molecule attached between the tip of the AFM cantilever and the gold coated coverslip is stretched by moving the base of the cantilever using a piezoelectric positioner. Force is calculated from the bending of the cantilever, which is followed by measuring the deflection of a laser beam.
b) Example of an AFM force-extension curve recorded for the I55-62 segment. Stretch rate was ≈500 nm/s. The sawtooth pattern of the curve corresponds to the sequential unfolding of individual domains. When the protein between the tip and the surface stretches out, force is generated in the molecule that bends the cantilever (1). At a certain force (unfolding force) one of the domains unfolds (2) and the free length of the protein increases, leading to a sudden decrease in force. As the protein is stretched further (3), unfolding events continue to take place. Rising parts of the sawtooth curves were fitted with the WLC equation (grey curves). The contour length gain (ΔL) associated with the unfolding of a module is indicated.
c) Histogram of unfolding forces for the I55-62 domain octamer at 500 nm/s stretch rate.

To estimate the contour length-gain associated with the unfolding events, the rising halves of consecutive force peaks were fitted with the wormlike chain (WLC) equation:

$$\frac{FA}{k_B T} = \frac{z}{L} + \frac{1}{4\left(1 - z/L\right)^2} - \frac{1}{4}, \qquad (2)$$

where F is the force acting on the molecule, z is the end-to-end distance, L is the contour length, A is the persistence length of the molecule (the measure of its bending rigidity), T is temperature, and k_B is Boltzmann's constant.[24] The use of the WLC model for a partly unfolded multidomain construct is an approximation, because only the unfolded segments can be treated as random polymer chains. However, the method can be used to determine the contour-length gain, and is expected to yield a better approximation than the simple determination of peak-to-peak distances. Fitting was performed using a non-linear least squares fit (Marquardt-Levenberg) (Igor Pro software, Wavemetrics).

RESULTS AND DISCUSSION

To study the mechanical properties of the differentially expressed tandem Ig region of titin, we cloned and expressed an eight-domain-long segment (octamer) spanning from the I55 to the I62 Ig domain. This octamer is part of the differentially expressed tandem Ig region of skeletal muscle titins, but is completely or partly missing from cardiac titins (see Figure 1).

Force-extension curves of the octamers were measured using an atomic force microscope specially intended for stretching single molecules. The experimental setup is shown in Figure 3a. When a protein is picked up and stretched, the resulting force-extension curve displays the characteristic appearance of a sawtooth pattern (Figure 3b). A steep increase in force is observed when the protein between the tip and the surface stretches out, and then a sudden drop in the force appears when one of the domains unfolds and adds its contour length to the free length of the protein. Further extension results in repetition of the above steps until all of the stretched domains unfold. Thus, each sawtooth peak on the curve corresponds to the unfolding of a single domain.

The contour-length gain associated with the domain unfolding events could be determined by fitting consecutive peaks with the wormlike chain model. The fitting yields a contour length value for each step, the differences of which give the contour length gain (Figure 3b).

The obtained average contour length gain was 29.8 ± 3.5 nm. This value is somewhat lower than that expected based on the average number of residues per domain for the I55-62 octamer (94), but exceeds that observed for the I27 (I91) Ig-domain polyprotein.[25]

The peak (unfolding) force was determined for each unfolding event. The ascending unfolding forces within a single curve would indicate a varying mechanical stability of the domains within the construct, and their hierarchical unfolding. A strong hierarchy was observed in the case of the I91-98 octamer,[26] but a weak hierarchy for the I4-11 octamer[27]. In our case, a relatively strong increasing tendency in the height of the force peaks was observed, suggesting that the mechanical stability of the domains within the I55-62 fragment is not homogenous.

To assess variations of the unfolding forces, a histogram of the measured peak forces was constructed. The histogram showed a relatively wide distribution of unfolding forces (Figure 3c) with values ranging from 150 to 320 pN and a mean of 237 ± 35 pN (mean ± SD, n = 143) at a stretch rate of 500 nm/s. The histogram shows an apparently multimodal distribution, with peaks at 182 pN and 241 pN. The multimodality of the histogram suggests that the domains of the I55-62 fragment may be segregated into distinct groups according to their mechanical stabilities. Further experiments using polyproteins of individual constituent domains of the I55-62 fragment may resolve the exact mechanical hierarchy within the fragment.

Since the stretch rate used in our work (≈500 nm/s) was commonly applied in previous studies, our results can be compared with the results obtained for other Ig segments. Literature data on different Ig domains and domain segments from titin's I-band region, together with our results are summarized in Table I. Localization of the represented domains is shown in Figure 1.

The mean unfolding forces for the I55-62 domain octamer were similar to those of the previously studied domains and domain series from the constitutively expressed tandem Ig region (I91-98 and domains within), and were the highest among the differentially expressed domain segments investigated to date (see Table I). The magnitude of forces required to unfold the individual domains measures the mechanical stability of the domains at a given stretch rate.[28] Accordingly, the I55-62 segment contains domains that display the highest mechanical stability at a stretch rate of ≈500 nm/s among the Ig domains and domain constructs from the differentially expressed tandem Ig region of titin studied until now. Our results do not permit extrapolation to determine the zero-force (spontaneous) domain unfolding rate. Further measurements at different stretch and loading rates will help establish the spontaneous unfolding rate.

Variations in the structure and length of the I-band portions of titin isoforms are thought to be a mechanism for modulating the mechanical properties, such as stiffness of different muscle types.[11] This mechanism was proposed to act based on the different entropic elasticity of the I-band regions of titin, which would not require differences in the stability of constitutively and differentially expressed domains. The biological rationale of including domains of smaller mechanical resistance in skeletal muscle titins is not yet understood. The experimental results suggest that under mechanical stress titin domains are not likely to unfold in a random order. There rather seems to be a hierarchy of the mechanical responsivity in titin's I-band region, which is expected to determine the order of unfolding. According to available data, the weakest segment of the extensible part of titin is found in the differentially expressed region. Domains with even lower unfolding forces (I1,[27] I4-11[29]) have been found near the N-terminal end of the proximal tandem Ig region, but this part of titin is strongly associated with the thin filaments and therefore does not participate in determining functional sarcomeric elasticity. Watanabe *et al.*[17] suggested that the differentially expressed domain constructs which unfold at lower forces may provide a safety mechanism that prevents the unfolding of the constitutively expressed modules. Our observations, on the other hand, suggest that the differentially expressed domains are not systematically weaker than the constitutively expressed ones, but they rather display a wide range of mechanical stability. Whether the weakest links in the series of domains in the extensible region of titin

TABLE I. Literature data on the mean unfolding forces of Ig domains and domain segments from the I-band region of titin

Domain / Domain segment[(a)]	Unfolding force[(b)] pN	Reference
I98 (I34)	281 ± 44	Li *et al.*[29]
I96 (I32)	298 ± 24	Li *et al.*[29]
I92 (I28)	264 ± 49	Marszalek *et al.*[31]
	257 ± 27	Li *et al.*[26]
I91 (I27)	210 ± 27	Marszalek *et al.*[31]
	204 ± 26	Li *et al.*[26]
I91-98 (I27-34)	≈ 200–250	Li *et al.*[26]
	237	Rief *et al.*[16]
	≈ 225	Watanabe *et al.*[17]
I73-79 (Sk47-53)	210	Rief *et al.*[16]
I65-70	≈ 185	Watanabe *et al.*[17]
I55-62	237 ± 35	present work
I4-11	≈ 160	Li *et al.*[29]
I5	155 ± 33	Li *et al.*[29]
I4	171 ± 26	Li *et al.*[29]
I1	127 ± 18	Li *et al.*[27]

(a) Domain numbering is given according to Bang *et al.*,[9] numbering according to older nomenclature[8] is shown in brackets.
(b) Mean ± SD.

are indeed found in the differentially expressed region may be revealed only on the basis of a complete mechanical stability map of titin's globular domains. A systematic exploration of domain stability along titin is therefore warranted.

It is also conceivable that the unfolding forces measured at AFM stretch rates do not give relevant estimates on the behavior and stability of the domains under physiological conditions. A detailed study of the I91 domain by Williams *et al.*[30] showed that, owing to the existence of an unfolding intermediate, the I91 domain might under physiological conditions withstand higher forces than previously predicted. Thus, simple extrapolation of AFM force data to physiologically relevant forces and loading rates is not straightforward, and reliable conclusions may only be drawn based on studies of the native as well as different mutant domains.

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SAŽETAK

Lokalna promjenljivost (nestalnost) mehaničkih svojstava tandemskih Ig/segmenata titina

László Grama, Attila Nagy, Clara Scholl, Tamas Huber i Miklós S. Z. Kellermayer

Funkcionalno elastična I-vrpca miofibrilarnoga proteina titina (konektina) sadrži različito izražene vrste serijski povezanih domena sličnih imunoglobulinu (Ig) čija se duljina i sastav razlikuju u pojedinim titinskim izoformama. Biološki razlozi diferencijalne ekspresije kao i doprinos mehaničkih svojstava domena koje su slične Ig ponašanju titina nisu u cjelosti poznati. U ovom su članku pregledno prikazani dosadašnji relevantni radovi koji se bave problemima mehaničkih svojstava Ig-domena te autorski eksperimentalni pristupi u istraživanju toga problema. U radu su također prikazani rezultati istraživanja mehaničkih svojstava diferencijalno eksprimiranoga tandemskoga Ig-područja titina (155-62), koji se sastoji od osam domena, pomoću mikroskopa atomske snage razlučivanja specijaliziranoga za rastezanje pojedinačne molekule. Ovim postupkom utvrđena mehanička svojstva ispitivanoga dijela titinske molekule uspoređena su s poznatim mehaničkim svojstvima drugih domena i segmenata.