Modular Motif, Structural Folds and Affinity Profiles of the PEVK Segment of Human Fetal Skeletal Muscle Titin*

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The extension of the PEVK segment of the giant elastic protein titin is a key event in the elastic response of striated muscle to passive stretch. PEVK behaves mechanically as an entropic spring and is thought to be a random coil. cDNA sequencing of human fetal skeletal PEVK reveals a modular motif with tandem repeats of modules averaging 28 residues and with superrepeats of seven modules. Conformational studies of bacterially expressed 53-kDa fragment (TP1) by circular dichroism suggest that this soluble protein contains substantial polyproline II (PPII) type left-handed helices. Urea and thermal titrations cause gradual and reversible decrease in PPII content. The absence of sharp melting in urea and thermal titrations suggests that there is no long range cooperativity among the PPII helices. Studies with solid phase and surface plasmon resonance assays indicate that TP1 interacts with actin and some but not all cloned nebulin fragments with high affinity. Interestingly, Ca²⁺/calmodulin and Ca²⁺/S100 abolish nebulin/PEVK interaction. We suggest that in aqueous solution, PEVK is an open and flexible chain with stable structural folds of the polyproline II type. PEVK region of titin may be involved in interfilament association with thin filaments in a calcium/calmodulin-sensitive manner. This adhesion may modulate titin extensibility and elasticity.

The monumental sequencing work of Labeit and Kolmerer (1) has revealed the complete domain organization of the giant elastic protein titin. The bulk of cardiac titin consists of pre-repeats, and segments). In addition to these well characterized domains, a novel motif consisting of mainly four amino acid residues, Pro, Glu, Val, and Lys, is discovered in the elastic region of titin. The length of this PEVK segment varies from 183 residues in the human cardiac titin to 2174 residues in human soleus skeletal muscle. Differential splicing of the titin transcripts in the PEVK region as well as in an adjacent tandem Ig segment near the A band produce these size isoforms of titin (1, 2). Since the selective expression of titin size isoforms imparts distinct elasticity to skeletal and cardiac muscles, with the muscle expressing longer titin being more compliant (3), the observed length variation of PEVK and tandem Ig segment in titin isoforms immediately suggests the possibility that either or both segments may be the elastic elements. Labeit and Kolmerer (1) speculated that the PEVK region, with a predicted nonfolded polypeptide, is the key elastic element of titin. The concept of reversible unfolding and folding of Ig domains was considered unlikely, on the ground of the thermodynamic stability of Ig domains (4, 5).

Recent works on the elasticity of single titin molecules (6), single myofibrils (7), and single fibers (8, 9) revealed that, stretched modestly, sarcomere elasticity can be explained by the straightening of tandem Ig segment (without unfolding), followed by the extension of a permanently unfolded PEVK segment (10).

To further evaluate molecular theories of titin elasticity, studies of the conformation and stability of different classes of titin domains are essential. The systematic NMR studies of expressed single Ig and fibronectin domains by Pastore and coworkers (11) have provided insightful structural details and a possible model of force generation. In contrast, no molecular characteristics of the PEVK region have yet been described. We report the initial sequence and molecular analysis of a prolinc-rich region that we identified independently by screening expression libraries of human fetal skeletal muscle with anti-titin antibodies. One 2.5-kb clone was found to consist of tandem repeats of a fundamental module that averages 28 residues, mostly of PEVK residues. This sequence is classified as a PEVK segment by its high homology with human soleus PEVK. Conformational studies of a PEVK fragment (designated as TP1, containing 16 PEVK modules, 468 amino acid residues) indicate that PEVK is an open and flexible chain with stable structural folds, perhaps of left-handed polyproline II helices, but contains little, if any, α-helix and β-sheet types of secondary structure. Protein interaction studies with solid phase assays reveal modest micromolar range interactions between TP1 and nebulin and actin. Our data suggest that the PEVK region may serve multiple functions. It may serve as an entropic spring of a chain of structural folds. The PEVK region also may be a site of interaction with other myofilament proteins to form interfilament connectivity in the sarcomere.

1 The abbreviations used are: kb, kilobase pair; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; PPII, polyproline II; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; N-hydroxysulfosuccinimide.

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Experimental Procedures

Cloning and Sequencing of Human Fetal Skeletal Muscle PEVK—A λgt11 human fetal skeletal muscle library was screened by Western blot with a goat anti-rabbit skeletal muscle titin (goat 812) that has been absorbed with E. coli blots and supplemented with a mixture of monoclonal antibodies (RT10, -11, -13, and -15) (8). One clone, 5-1-2, containing a 2.5-kb open reading frame (designated as hT11; GenBank accession number AF 321609) was subcloned into Bluescript II and sequenced by rapid DNA sequencing (35). Digestion and ligation. Nested set deletions were produced by exonIII digestion, and each clone was sequenced by Sequence/TF polymerase/ universal and reverse primer with the dideoxy method. The 5′-deaza reaction was run to clean up digestion. The DNA sequence and deduced amino acid sequence were analyzed by MacVector and MAAC for homology and repeats (12).

Expression and Purification of PEVK Fragment PT1—A 1.4-kb open reading frame derived from hT11 was subcloned into a pET3d plasmid by digesting Bluescript clone with Hinfl and ligated to PET 3d, essentially as described in (12). The expressed protein, TP1 (51,467,469 residues), was purified from a 4-litter culture of BL21(DE3)pLysS host cells transformed with pET3d Hinfl plasmid that was incubated at 37 °C for 3.5 h upon isopropyl-1-thio-β-galactopyranoside induction (0.4 mM isopropyl-β-D-galactopyranoside) at 25 °C for 12 h. The bacteria were collected by centrifugation at 3800 × g for 10 min and lysed in a French press cell (3 × 1500 p.s.i.) in 45 ml of lysis buffer (10 mM NaPi, 1 mM EDTA, 0.1 mM DTT, 10 μg/ml leupeptin, 1 mg/ml aprotinin, 0.015% aqueous solution of D-10-camphosulfonate in the far UV region and with a detector set at 205 nm and the far UV region. A quartz cuvette (Hellma, Plainview, NY) with a 0.01-cm light path was used. For urea titration at 20 °C, TP1 (22 mg/ml) or polypropylene (2 mg/ml) was dialyzed against various concentrations of urea (2, 4, 6, and 8 M) in the same buffer. Concentrated urea solutions were prepared in water by using the density data of Kanahara (8) and then dialyzed against water and 10 M-concentrated buffer. Thermal denaturation of TP1 (2.2 mg/ml) and polypropylene (0.25 mg/ml) was monitored by following the change in CD at 205 nm (TP1) or 201 nm (polypropylene) from 20 to 90 °C at a rate of 50 °C/hr using a JASCO model FTC-348W Peltier type thermoelectric control system and a demountable 0.01-cm path length rectangular cuvette.

ELISA Protein Binding Assays—Purified TP1 (220 μg/ml in 20 mM sodium phosphate, 1 mM EDTA, 150 mM NaCl, pH 7.0) was absorbed onto microtiter plates overnight at 4 °C, washed once with TBS-T (10 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween 20), and blocked with 0.02% (v/v) bovine serum albumin in TBS-T for 1 h at 37 °C, followed by incubation with actin and nebulin fragments (NA3, NA4, NA10, NA10D66 in buffer 1 (10 mM imidazole, 150 mM NaCl, 2 mM MgCl2, 2 mM MgCl2, pH 7.0) at a concentration ranging from 0.7 to 50 μg/ml of TP1. After washing three times with TBS-T, mouse monoclonal antibodies against actin (JL20, NA3 (N109), NA4 (N103), NC17 (N107), NDS (N113), and ND66 (N113) were incubated for 1 h at 37 °C in 0.2% bovine serum albumin-TBS-T. Plates were washed three times with TBS-T and then incubated with peroxidase-conjugated rabbit anti-mouse antibody for 1 h at 37 °C in bovine serum albumin-TBS-T, followed by five washes with TBS-T. Color was developed for 20 min at 25 °C by adding ABTS-H2O2 substrate, 10 μl of H2O2, 10 μl of 100 mM citrate buffer, pH 4.2. Absorbance at 405 nm was measured using an ELISA microtiter plate reader (12, 16).

Surface Plasmon Resonance Assays—Surface plasmon resonance assays were done using a real time biosensor, IAsys Manual System (Affinity Sensors, Cambridge, UK). TP1 was attached to carboxymethylated dextran on the cuvette surface via EDC/NHS. The cuvette was activated with 0.4 M EDC, 0.1 M NHS in water for 8 min, washed twice with phosphate-buffered saline with 0.05% Tween 20, followed by a wash with 10 mM acetate buffer pH 5.0 for 3 min, and incubated with PT1 (0.22 mg/ml in 10 mM acetate buffer, pH 5.0) for 10 min. The cuvette was washed twice with phosphate-buffered saline with 0.05% Tween 20, followed by 1 mM ethylenediamine for 3 min to block excess activated carboxylic groups. The cuvette was then washed with acetate buffer (3×), 10 mM HCl, acetate buffer (3×), and finally PBST (3×). A blank cuvette from the same lot was processed in parallel, without TP1. The use of ethylenediamine to block excess carboxyls is essential for the study of the interaction of TP1 with the highly basic nebulin fragments. Polyproline, polyethyleneimine, or polyarginine were used as control substances to produce unacceptably high binding of basic proteins to the control blank cuvette even at high ionic strength (200 mM KCl). The introduction of basic groups to the dextran led to a gel layer that showed no significant electrostatic binding of NA4 (pl = 9.24) at and above 100 mM KCl (with response <50 arc seconds).

The binding of nebulin fragments (NA3, NA4, NA29, NC17, and ND66), actin, troponin, tropomyosin, calmodulin (bovine brain), and S100a (Sigma) to immobilized TP1 was done at 1 μM in 100 mM KCl, 1 mM CaCl2, 2 mM MgCl2, 10 mM imidazole, pH 7.0, at 25 °C. For some solutions, EGT (1 mM) was present to lower pCa to 8.0. Protein binding to the gel layer alters the refractive index profile occurring within the evanescent field and changes the measured resonance angle of the intensity peak (in arc seconds). The change in arc seconds is assumed to be proportional to the amount of bound substance (17). The association kinetics was followed for 10–15 min prior to washing with the interaction buffer to follow the time course of dissociation. At the end of each experiment, the cuvette was washed with 10 mM HCl for 1 min and buffer I (3×) to regenerate the cuvette. All of the data reported here were obtained on the same cuvette within a 2-day period to facilitate comparison. Resonance angles of the test cuvette were measured in parallel and subtracted from the corresponding values obtained with the TP1 cuvette.

Immunogold Localization—Localization of epitopes of monoclonal antibodies RT11, RT13, and RT15 in mechanically split rabbit psoas muscle fibers was done as described previously (18). Briefly, the split muscle fibers from rabbit psoas muscle in relaxing buffer were hand-
Modular PEVK of Titin

RESULTS

Tandem Repeats of a ~28-Residue PEVK Module in the PEVK Segment of Titin—Sequence analysis of a 2.5-kb cDNA clone (hfT11) was obtained by screening a human fetal muscle cDNA library with anti-titin antibodies revealed an open reading frame (designated as hfT11) of 786 residues that is enriched in prolines, glutamates, valines, and lysines (25% Pro, 16% Glu, 15% Val, 16% Lys) (GenBank accession number AF 321609). A search for internal sequence homology by matrix plots with MacVector indicates substantial internal repeats (Fig. 2A). The consensus sequence for the PEVK modules is shown below. Prolines are in green, valines are in black, basic residues (Lys, Arg) are in blue, and acidic residues (Asp, Glu) are in red. The enclosed region, TP1 (468 residues, 15% Val, 16% Lys) was expressed as a soluble protein in reasonable yield. Due to its low UV absorption (a single tyrosine residue at position 155), little homology with the modules from HRT14–27, but each module has ~25–35 residues, and three show a PE at the beginning of the module. These four modules are classified as nonrepeats. This nonrepeat region is characterized by clusters of acidic residues (e.g. HRT4) and basic residues (KRRKK in HRT8). The matrix plot of hfT11 against human soleus PEVK indicates that hfT11 open reading frame sequence shares significant homology with the C-terminal side of the PEVK region of human soleus titin (Fig. 2A).

The PEVK region of human fetal skeletal muscle titin sequenced so far clearly indicates three levels of motifs: repeats of ~28 residues, superrepeats of seven modules, and nonrepeat. The same research for internal repeats of human soleus PEVK (residues 0–2174, corresponding to 5618–7792 of EMBL X90569) revealed the same types of sequence motifs. The same research for internal repeats of human soleus PEVK (residues 0–2174, corresponding to 5618–7792 of EMBL X90569) revealed the same types of sequence motifs. The matrix plot in Fig. 2B indicates significant repeats between residues 300 and 1840 of the PEVK segment of human soleus titin. Indeed, similar 28-residue repeats are abundant, and seven-module superrepeats are also present. The balance of the sequence is nonrepeat and contains clusters of acidic residues (see Refs. 2 and 19). In summary, PEVK of titin appears to be modular and consists mainly of tandem repeats of a fundamental 28-residue module interspersed in highly charged nonrepeat regions.

Conformation of PEVK and Polyproline II (PPII) Helix—The presence of the modular structure of PEVK, previously unrecognized, raised the question of whether PEVK may have stable structural folds. To explore this possibility, we expressed in Escherichia coli a 469-residue fragment (from 128–597, HRT5 to HRT21, designated as TP1) that includes both the nonrepeat and part of the superrepeat of PEVK. To avoid possible interference by expression tags in protein interaction studies, a nonfusion protein was produced. As shown in Fig. 3, TP1 was expressed as a soluble protein in reasonable yield. Due to its low UV absorption (a single tyrosine residue at position 155),
purification and expression of TP1 were monitored by SDS gel electrophoresis and Western blotting with a monoclonal anti-titin, RT11. TP1 displayed unusually low SDS gel mobility and migrated with an apparent mass of 86 kDa. Moreover, TP1 was also difficult to transfer electrophoretically, and most TP1 stayed behind in the gel (Fig. 3A, post-transfer gel pattern). The preferential transfer of smaller degradation products of TP1 gave a smear in Western blots that significantly underestimated the purity of TP1 (Fig. 3A, RT11 Western blot). The low mobility may reflect either the lower than normal SDS binding or an extended conformation or stiffer SDS peptide complex for proline-rich peptides. For example, a proline-rich protein, calphotin (20), displays a similar low motility.

Purified TP1 is very soluble in a wide range of aqueous buffers and ionic strengths. The possible presence of secondary structure in TP1 was investigated by circular dichroism. CD spectra of TP1 display negative ellipticity with a minimum at 201 nm and a shoulder near 220 nm (Fig. 4D). At first impression, these are characteristic spectra of polypeptides generally classified as random coils. Indeed, calculations for secondary structure content with several software programs indicate negligible α-helix and β-sheet structure (data not shown). However, further CD studies of TP1 subjected to urea and thermal titration raised doubts about this interpretation and led us to search for evidence for the presence of other stable structural features. Given the proline-rich content of TP1, we considered the possible presence of PPII. Such a left-handed helix, containing three trans-proline residues per turn, is found in polyproline in aqueous solution (21, 25) and is also present as short stretches in many globular proteins (22).

A detailed comparison of CD spectra of polyproline and TP1 strongly supports this notion. As shown in Fig. 4, A and B, CD spectra of polyproline show the characteristic strong negative band at 205 nm and a weak positive band at 229 nm of PPII helices (21). Upon heating from 2 to 70 °C, both bands undergo incremental decrease in magnitude (Fig. 4A, inset), indicating a loss of PPII helical content at higher temperature. This series of CD spectra (Fig. 4A) displays an isodicchoic point at 215 nm, indicating an equilibrium of two major populations of conformations. Another unique characteristic of PPII is its response to urea and guanidinium chloride treatment (23). These chaptocropic agents that commonly cause unfolding and loss of most secondary and tertiary structures in fact increase the helical content of PPII (23). As shown in Fig. 4B, urea treatment up to 8 M causes an increase in magnitude of bands at 205 and 229 nm, confirming an earlier report of the enhancement effect of urea on PPII content (23). The presence of an isodicchoic point at 218 nm suggests again two populations of equilibrating conformations. High concentrations of urea progressively obscure the spectra below 205 nm and somewhat affect the accuracy of the isodicchoic point (Fig. 4B, inset). The third characteristic behavior of PPII is the nearly linear response to thermal titration, without the sharp, sigmodial transition commonly observed for α-helices, β-sheets, and other stable folds that display cooperative melting. As shown in Fig. 4C, thermal titration of polyproline from 2 to 90 °C, as monitored continuously at 201 nm, shows a linear response, with slight change in slopes at 28 and 80 °C. Such a titration curve suggests the gradual loss of PPII with raised temperature without cooperativity.

The CD of TP1 and its responses to thermal and urea titration bear striking resemblance to those of polyproline under identical conditions (Figs. 4, D–F). The strong negative band at 200 nm is slightly blue-shifted from the 205-nm band of polyproline. The negative shoulder at 220 nm may be derived from the same transition as the 229-nm (positive) band of polyproline (Fig. 4, D and E). Upon heating from 2 to 70 °C, a significant decrease of the magnitude of both bands of TP1 occurs (Fig. 4E, inset), showing the loss of stable folds. The presence of an isodicchoic point near 210 nm between 2 and 50 °C suggests that at least two conformations are in equilibrium between below 50 °C (Fig. 4D). The addition of urea up to 8 M causes progressive decrease in the 200-nm band and an increase in the shoulder at 220 nm, with an isodicchoic point at 210 nm (Fig. 4E), consistent with the increase of PPII at higher concentration of urea. Thermal titration of TP1 at 201 nm reveals a linear response from 20 to 70 °C, with a slight hint of a kink at 38 °C. Upon cooling, the CD returns to that observed before heating, with no sign of hysteresis (data not shown).

Based on the similarity of the CD spectra of TP1 to PPII and the unique and characteristic responses of the CD spectra of TP1 and PPII to urea and heat treatment, we suggest that TP1 contains a significant extent of ordered structures that resemble PPII helices. The absence of sharp, cooperative transition in thermal or urea titration (24) suggests that TP1 has an open structure within which the PPII helices are likely to be dispersed in an open polypeptide with very little long range interactions. Assuming that PPII is the only ordered structure that contributes to CD spectra, it can be estimated from residue ellipticity values at 210 nm that PPII content in TP1 at ~13% of the residues in TP1 is in the PPII conformation at 20 °C. The PPII content decreases gradually with rising temperature (~7% at 70 °C) and is slightly enhanced by high concentration of urea (~15% in 8 M urea). Further conformational analysis is required to evaluate or rule out the possible presence of other ordered structures in TP1.

Support of the open structure of TP1 came from the determination of its Stoke’s radius by gel filtration. The value of 85
electroblotting, and a majority of TP1 stayed behind in the gel after transfer (post-transfer gels for fractions 34–36). It also degraded rapidly during purification, giving rise to many minor bands below TP1 that were immunoactive to RT11. Nearly all smaller peptides in the final TP1 preparation are degradation products. TP1 resisted purification, giving rise to many minor bands below TP1 that were immunoactive to RT11. Nearly all smaller peptides in the final TP1 preparation are degradation products.

Reactivity Profile of TP1 with Proteins of Thin Filaments—A survey for potential interaction of TP1 with components of thin filament (actin, tropomyosin, troponin, and nebulin fragments (NA3, NA4, NA29, NC17, ND8, and ND66) titin motif I, and calmodulin) was done with both solid phase ELISA and plasmon resonance-based biosensor. As shown by ELISA (Fig. 6), TP1 on the plastic surface binds actin, NA4, and NC17 with moderate affinity ($K_d \sim 1–5 \mu M$). On the other hand, tropomyosin, troponin, three nebulin fragments (NA3, NA29, ND66), and calmodulin showed very little affinity toward TP1.

The reactivity of TP1 was investigated further with the surface plasmon resonance technique, which allows interaction of untagged proteins to a hydrophilic dextran surface-bound one to be observed at real time. Technically, due to the high affinity of the basic nebulin fragments toward the carboxylated surface layer, it was necessary, following protein coupling, to neutralize the excess anionic charges by a diamino compounds to lower background binding. As shown in Fig. 6B, there was no binding of actin to TP1 in buffers near physiological ionic strength. It is conceivable that the slow diffusion of F-actin into the sensor’s dextran gel layer may have hampered interaction. Two nebulin fragments, NA4 and NC17, bind to TP1 rapidly, and nebulin fragments NA3, NA29, tropomyosin, troponin, and titin motif I show no affinity under the same condition (Fig. 6B). To test potential for oligomerization of PEVK, binding of solution phase TP1 was also performed. No interaction was observed (data not shown).

The interaction of TP1 with human nebulin fragments confirms the ELISA data and suggests that PEVK may be involved in the interfilament interaction with thin filaments. To search for potential modulators for such interaction, we tested calmodulin/Ca$^{2+}$, which has been shown to dissociate nebulin from either actin or myosin (26). As shown in Fig. 7A, the binding of NA4 to TP1 was dissociated completely by 7 $\mu M$ calmodulin at pCa 3. Premixing NA4 and calmodulin also abolished binding. The addition of EGTA to the mixture to lower pCa to 8–9 immediately induced the binding of NA4 to TP1. As controls, we found that in the absence of calmodulin, NA4 binds to TP1 more effectively at pCa 8 than at pCa 3 (Fig. 7B). Since TP1 does not bind to calmodulin at either pCa 3 or 8, the data indicate that its dissociative effect of calmodulin at high calcium is mediated mainly through the binding of Ca$^{2+}$/calmodulin to NA4, perhaps by competing with PEVK for the same binding site on NA4. A direct effect of calcium on either nebulin or TP1 may also contribute to the lowered binding of NA4 to TP1 at pCa 3. In parallel experiments, we observed a similar dissociative effect of S100$\alpha$, a calcium sensor protein that is analogous to calmodulin. As shown in Fig. 7C, S100$\alpha$ diminished binding of NA4 to TP1 at pCa 8. The interaction is completely abolished by S100$\alpha$ at pCa 3.

In summary, NA4 binds to TP1 at pCa 8 with moderate, micromolar affinity. Their binding is weakened by either raising calcium to pCa 3 or by the presence of calmodulin (data not shown). This binding is completely abolished at pCa 3 in the presence of calmodulin. The S100$\alpha$ has a similar effect, indicating a general response of NA4/TP1 interaction to this class of calcium sensor proteins.

Elastic Stretch Response of PEVK in the Sarcomere—The reactivity of a panel of four monoclonal antibodies was tested against TP1. Only RT11 reacts specifically by ELISA (Fig. 8A). The same results were also observed by plasmon resonance assays (data not shown). This epitope is localized in rabbit psoas skeletal muscle by immunogold labeling at the electron microscopy resolution to a single band approximately at 0.35 $\mu M$ from the Z line in the I band of a resting sarcomere at 2.3 $\mu M$ (Fig. 8B). The antibody to Z line distance increases linearly from 0.35 to 0.54 $\mu M$ when the sarcomere was stretched from 2.3 to 3.5 $\mu M$. This stretch behavior, as illustrated in the linear

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**Fig. 3.** Expression and purification of cloned PEVK fragment, TP1. TP1 was expressed as a soluble protein in *E. coli*. The bacteria were lysed in 10 mM NaPi, 1 mM EDTA, 0.1 mM DTT, 0.1 mg/ml leupeptin, 1 mg/ml cassein, 1 mM diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0, and clarified for PC cellulose chromatography (A). TP1 was eluted between 0.4 and 0.5 M NaCl by a linear gradient (0–1 M NaCl) (B). Pooled fractions were then applied to a DEAE-Sepharose column (20 mM NaPi, 1 mM EDTA, 0.1 mM DTT, pH 7.0) (B). TP1 was eluted between 0.15 and 0.20 M NaCl. Yield was 15 mg per 4-liter culture. Note that TP1 migrated abnormally in SDS gels, with an apparent molecular mass of 86 kDa. Immunoblots of column fractions with monoclonal anti-titin RT11 were used to monitor purification and degradation. TP1 resisted electroblotting, and a majority of TP1 stayed behind in the gel after transfer (post-transfer gels for fractions 34–36). It also degraded rapidly during purification, giving rise to many minor bands below TP1 that were immunoactive to RT11. Nearly all smaller peptides in the final TP1 preparation are degradation products.
plots of Ab to Z line (or Ab to M line) distance versus sarcomere length (Fig. 8C), is typical of an elastic response, since the epitope moves away from both the Z line and the M line proportionally. In contrast, an A band titin epitope, RT15, was stationary and maintained its position relative to the M line (Fig. 8C). In a parallel experiment, the epitope location of another monoclonal antibody RT13 was also measured and found to be indistinguishable from the location of T11 epitope (8). Since the exact epitope sequences of RT11 and RT13 remain unknown, it is likely that both antibodies are directed to an adjacent region of the PEVK segment of rabbit psoas titin, despite the fact that only RT11 reacts with human fetal titin PEVK.

DISCUSSION

Modular Structure of PEVK Segment—The extension of elastic titin filaments in the I-band is thought to be responsible for the generation of passive tension of the sarcomere skeletal and cardiac muscle sarcomeres. Comparison of passive tension and sarcomere length curves of several skeletal muscles that express several different titin size isoforms led us to propose that the segmental extension of different lengths of I band titin accounts for the variation of sarcomere elasticity of these muscles (3, 8). It is now known that the I band titin consists of two structural motifs: tandem Ig domains and the PEVK region. Recent mechanical studies revealed that both elements contribute to passive tension. At low tension, the tandem Ig domains extend up to 3-4-fold without unfolding. At higher stretch, PEVK extends and develops higher tension. The two springs work in series to provide a broad range of forces (29, 30). PEVK is thought to be in a random coil or permanently unfolded conformation, and its mechanical behavior has been modeled as an entropic spring (10, 31, 32).

Our analysis of human fetal titin PEVK sequence provides
The first evidence of modular motifs consisting of a fundamental module that averages 28 residues. In addition, a higher order superrepeat consisting of seven PEVK modules is also evident. Examination of PEVK sequences of the 1800 residues in invertebrates. Indeed, the "random coil" or "completely unfolded" depiction of PPII. The enhancement of PPII content may be due to the effect of urea on water structure thus has no detrimental effect on PPII. The enhancement of PPII content may be due to the direct binding of urea to the peptides (23). We suggest that in aqueous solution PEVK contains multiple PPII helices in equilibrium. The chain is likely to be flexible and would make a significant entropic contribution to elasticity. On the other hand, the "random coil" or "completely unfolded" depiction of PEVK in the recent literature (1, 10, 33) is inadequate and perhaps misleading, since it ignores the possibility of ordered structure and stable folds. Further evaluation of these specific conformations and factors, which influence the folding of PPII and chain flexibility, is likely to facilitate understanding of the generation and modulation of titin elasticity.

**PPII Helices in PEVK**—Conformational studies of polyproline I and TP1 by CD indicate that PEVK in aqueous solution has open but somewhat folded conformations, most likely a chain of PPII-like helices with flexible joints. No conventional secondary structure such as β-helix or β-sheet is evident in TP1. Thermal titration causes gradual and reversible loss of PPII helices in both polyproline and TP1 without any evidence of sigmoid shaped cooperative melting commonly observed for compact protein domains. Urea titration causes both polyproline and TP1 to increase their helical content. This counterintuitive behavior of PPII results from the fact that this left-handed helix of trans-prolines does not form main chain hydrogen bonding as in α-helices or β-sheets. The chaotropic effect of urea on water structure thus has no detrimental effect on PPII. The enhancement of PPII content may be due to the direct binding of urea to the peptides (23). We suggest that in aqueous solution PEVK contains multiple PPII helices in equilibrium. The chain is likely to be flexible and would make a significant entropic contribution to elasticity. On the other hand, the "random coil" or "completely unfolded" depiction of PEVK in the recent literature (1, 10, 33) is inadequate and perhaps misleading, since it ignores the possibility of ordered structure and stable folds. Further evaluation of these specific conformations and factors, which influence the folding of PPII and chain flexibility, is likely to facilitate understanding of the generation and modulation of titin elasticity.

**PEVK and Interfilament Adhesion**—Studies with solid phase and surface plasmon resonance assays indicate that TP1 interacts with actin and cloned nebulin fragments. Interestingly, NA4/PEVK interaction is weakened by high calcium and is.
RT11 in the PEVK segment of rabbit psoas muscle. A, immunoreactivity of TP1. A panel of four anti-titin monoclonal antibodies (RT10, -11, -13, and -15) was tested for interaction with microtiter reactivity of TP1. A panel of four anti-titin monoclonal antibodies sarcomere. Furthermore, abolished by Ca/CaM or Ca2+/S100 in high calcium. These data suggest that the PEVK region of titin may be involved also in interfilament association with thin filaments in a Ca2+ and Ca2+/CaM- or Ca2+/S100-sensitive manner. This adhesion in turn may modulate titin extensibility. The lack of interaction of TP1 with some of the nebulin fragments, however, is puzzling. Fragments NA3, NA29, and ND66 either lack binding sites for PEVK or are not folded or dispersed properly for molecular contacts. The lack of binding to troponymosin and troponin suggests that the titin/thin filament adhesion is limited to actin and certain loci along the nebulin filament. Since NA4 and NC17 are normally located in the overlapping region of the A band in resting sarcomeres (18, 28), PEVK interaction would only occur when sarcomeres are stretched extensively for PEVK and NA4 to make contact. Whether the extensibility and the modular motif of PEVK play a role in the strength and sites of interaction with thin filaments are intriguing questions for future pursuits. It is tempting to speculate that the stretching of the PEVK segment might affect its avidity to interacting protein by changing accessibility and orientation. In this connection, the seven-module superrepeat structure is intriguing. The 7-fold repeat may match the seven-module nebulin superrepeat (12, 27) and the periodicity of the troponin actin filaments. A triple-stranded interaction between PEVK, nebulin, and actin might occur when titin is stretched to straighten the PEVK for a zipper-like adhesion with the thin filaments at the appropriate sarcomere length.

The dissociate effects of Ca2+/calmodulin and Ca2+/S100 on PEVK/nebulin interaction suggest that this interfilament connectivity may be modulated by calcium via calmodulin or S100 type sensor proteins. This modulation is in line with the observation that these molecules also dissociate nebulin from myosin and weaken their affinity to actin (26). Thus, calmodulin/S100 sensor proteins may unthread simultaneously adhesion of nebulin to both titin and myosin filaments in the sarcomere during muscle activation.

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