PROTEINS: Structure, Function, and Genetics 43:145–149 (2001)

Identification of New Repeating Motifs in Titin

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ABSTRACT **Repeating motifs of 26–28 amino acids have been identified in the PEVK region of the giant elastic protein titin. These motifs, termed PPAK for the four amino acids that often constitute the beginning of the motif, occur 60 times in human soleus titin. PPAK motifs occur in groups of 2–12 that are separated by regions rich in glutamic acid (approximately 45%) and termed polyE segments. The fluctuation of the net charge between the PPAK and polyE regions suggests ionic interactions between these segments and their involvement in the elastic function of titin. Proteins 2001;43:145–149.**

Key words: protein domains; elasticity; PEVK; ionic interactions; secondary structure; muscle

INTRODUCTION

Titin, also called connectin, is a >3-million dalton protein found in heart and skeletal muscle.[1,2] The protein is involved in a number of functions: connecting the thick filaments to the Z-lines,[3] serving as a template for assembly of the thick filaments,[4] preventing the sarcomere from being overstretched,[5,6] acting as a serine/threonine kinase,[7,8] and playing a role in sarcomere assembly.[9–13] It is believed to be the major component responsible for passive tension.[14,15] Additional information on this unusual protein can be found in several recent reviews.[16–19]

Since the earliest titin cDNA sequence became available, it was clear that a number of repeating motifs were involved in its amino acid sequence.[20] Two different types of 100-amino acid motifs were found with similarities to immunoglobulin and the fibronectin III domains. Part of the A-band region of titin also consisted of an 11-motif super-repeat structure.[7] Additional super-repeats have been found in the tandem Ig domains in the I-band section.[21] A 45-amino acid repeat has been identified near the Z line end with varying numbers (≥7) expressed in different muscles.[22] Shorter serine–proline repeats considered as potential phosphorylation sites have also been found in regions of titin near the M-line[23] and Z-line.[24]

A unique feature of the titin sequence is the PEVK segment in the I-band region.[25] The PEVK is so named because approximately 75% of the amino acid residues are proline (P), glutamic acid (E), valine (V), and lysine (K). The length of the PEVK segment varies between 163 and 2174 residues, with the N2B isoform of the heart having the shorter length and the soleus muscle having the much longer segment.[25] Studies using antibodies to label muscle at various degrees of extension have demonstrated that PEVK lengthens with stretch.[26,27] Thus, the PEVK region is believed to serve an elastic function in muscle. Although several reports have mentioned sequence repetitions in the PEVK, the nature of such repeats has not been described. The current paper describes two new types of titin repeating sequence that constitute the bulk of the PEVK.

MATERIALS AND METHODS

A variety of gene and protein analysis software was used in the current study. These include BLAST,[28,29] MEME,[30] MotifSearch,[31] PeptideStructure,[32] PeptideSort, and Pileup. Most of these methods were used through SeqWeb Version 1.1 of the Genetics Computer Group Wisconsin Package Version 10.

RESULTS AND DISCUSSION

A search of the PEVK sequence for repeating structure was prompted by the observation that a titin monoclonal antibody (9D10)[33] labeled two 0.55-μm-wide zones per sarcomere that corresponded to the PEVK region of human soleus titin.[34] Since (1) there are only two sets of titin molecules per sarcomere,[3,35,36] (2) all the molecules are aligned in parallel in each set, and (3) most monoclonals label a zone no wider than 10 nm, the broad staining zone implied that multiple sequence regions were being recognized by the antibody. Numerous examples of the amino acid sequence PPAK were first found in the human soleus titin sequence (GenBank accession number X90569.1[25]) by visual inspection, and these occurred at 26–28 amino acid intervals.[37] A training set consisting of the best 23 sequences was used with the MEME program and a motif width of 28. The MEME output was then used in MotifSearch with the database. A total of 320 matches were recognized in the human soleus titin with this program, but there were numerous instances of overlap, presumably because of the amino acid redundancy and short within-motif sequence repeats. Motif borders were selected and overlap sequences eliminated with the help of the position *P*-values.[30] Motif positions 3–6 (typically KVP) were also useful in constructing a final alignment since this area of the motif showed the most limited variability.

Grant sponsor: College of Agricultural and Life Sciences, University of Wisconsin–Madison; Grant sponsor: National Institutes of Health; Grant number: HL62466.

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Received 17 July 2000; Accepted 8 December 2000

TABLE I. Selected PPAK Motifs in Human Soleus PEVK[†]

	1	10	20	28	
5949	PPPKVPELPE	KPAPEEVAPV	PIPKKVEP		5976
5977	PAPKVPEVPK	KPVPEEKKPV	PVPKKEPA		6004
6005	APPKVPEVPK	KPVPEEKIPV	PVAKKKEA		6032
6033	PPAKVPEVQK	GVVTEEKITI	.VTQREES		6059
6060	PPPAVPEIPK	KKVPEERKPV	PRKEEEVP		6087
6088	PPPKVPALPK	KPVPEEKVAV	PVPVAKKA		6115
6241	PPAKVPEAPK	KIVPEKKVPA	PVPKKEKV		6268
6269	PPPKVPEEPK	KPVPEKKVPP	KVIKMEEP		6296
6325	PKARVPEEPK	RAVPEEKVLK	LKPKREEE		6352
6353	PPAKVTEFRK	RVVKEEKVSI	EAPKREPQ		6380
6475	PPAKVL...K	KAVPEEKVPV	PIPKKLKP		6499
6531	PAAKVPMKPK	RVVAEEKVP.	.VPRKEVA		6556
6639	PPAKVPEVPK	KPEEKVPVL.	.IPKKEKP		6664
6665	PPAKVPEVPK	KPVPEEKVPV	PVPKKVEA		6692
6693	PPAKVPEVPK	KPVPEKKVPV	PAPKKVEA		6720
6721	PPAKVPEVPK	KLIPEEKKPT	PVPKKVEA		6748
6873	PPAKVPEVPK	KVEEK.RIIL	PKEEEVLP		6899
6930	APPRVPEVIK	KAVPEAPTP.	.VPKKVEA		6955
6956	PPAKVS...K	K.IPEEKVPV	PVQKK.EA		6978
6979	PPAKVPEVPK	KVPEK.KVL.	.VPKKEAV		7003
7130	PPAKVPEVPK	KIVVEEKVRV	PEEPRVPP		7157
7186	PPPKVPEAPK	EVVPEKKVPV	PPPKKPEV		7213
7214	PPTKVPEVPK	AAVPEKKVPE	AIPPKPES		7241
7264	PPVRVPEVPK	EVVPEKKVPA	APPKKPEV		7291
7292	TPVKVPEAPK	EVVPEKKVPV	PPPKKPEV		7319
7320	PPTKVPEVPK	VAVPEKKVPE	AIPPKPES		7347
7376	APPQVTVPPK	NPVPEKKAPA	VVAKKPEL		7403
7404	PPVKVPEVPK	EVVPEKKVPL	VVPKKPEA		7431
7432	PPAKVPEVPK	EVVPEKKVA.	.VPKKPEV		7457
7458	PPAKVPEVPK	KPVLEEKPAV	PVPERAES		7485
7522	PPPAVPEEPK	KIIPEKKVP.	.VIKKPEA		7547
Consensus	PPAKVPEVPK	KPVPEEKVPV	PVPKKPEA		
		K			

GenBank accession number X90569.1 includes the soleus PEVK (residues 5618–7791).[25] Vertical numbers indicate the amino acid starting and ending positions for each motif in the soleus sequence; horizontal numbers at the top refer to the motif position; dots in the sequences are gaps to maximize motif alignment.

A final total of 60 PPAK repeats were identified in the human soleus PEVK region. Repeat 55 (beginning at amino acid 7591) is truncated with only 17 amino acids, repeats 33 (6749) and 54 (7572) are 19 residues long, and the rest fall between 26 and 33 in size. The best 31 of these repeats are shown in Table I. Most of the motifs are 28 residues in length. The most invariant amino acid is the V at position 5 of the consensus sequence, and it occurs in 50 of the 60 repeats. The best fits with the consensus sequence generally occur with motifs in the middle region of the soleus PEVK. Six VP pairs are present in the consensus sequence and they usually occur at positions 5–6, 8–9, 13–14, 18–19, 20–21, and 22–23. Motifs with shorter lengths often can be aligned with the other sequences by using a VP deletion. Lysines are typically found at positions 4, 10, 11, 16, 17, 24, and 25 while glutamic acid residues occur mainly at positions 7, 15, and 27. This gives four clusters of positive charge and three clusters of negative charge per motif, each with a 6–8-amino acid spacing.

About one-half of the motifs have a lysine at position 15 instead of a glutamic acid; the position 15 lysines are more common in the C-terminal half of the PEVK. Substitutions for consensus residues are predominantly by amino acids of the same type (charged for charged, hydrophobic for hydrophobic). The PPAKs are clustered in a total of 10 groups with 2–12 motifs in each group (Fig. 1). Zones unusually rich in glutamic acid residues separate these

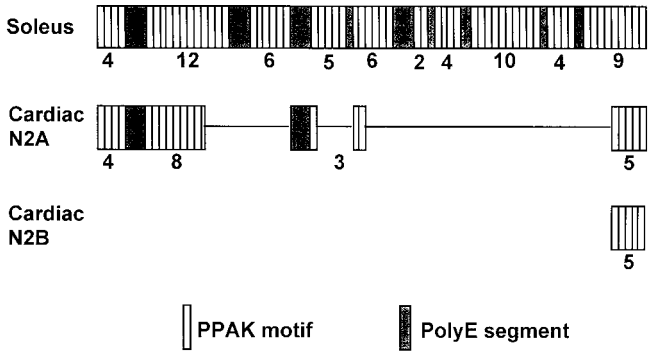

Fig. 1. Motif arrangement in different isoforms of the titin PEVK region. Thin horizontal lines depict regions deleted by alternative splicing.

TABLE II. Amino Acid Composition of Human Soleus PEVK, the PPAK Regions, and the polyE Regions

	PEVK total		PPAK total		PolyE total	
Residue	No.	mol %	No.	mol %	No.	mol %
A= Ala	131	6	115	7	16	4
C= Cys	0	0	0	0	0	0
D= Asp	11	1	4	0	7	2
E= Glu	480	22	275	16	205	45
F= Phe	25	1	13	1	12	3
G= Gly	16	1	11	1	5	1
H= His	18	1	11	1	7	2
I= Ile	82	4	65	4	17	4
K= Lys	344	16	334	19	10	2
L= Leu	51	2	38	2	13	3
M= Met	10	0	8	0	2	0
N= Asn	2	0	2	0	0	0
P= Pro	457	21	412	24	45	10
Q= Gln	28	1	21	1	7	2
R= Arg	62	3	51	3	11	2
S= Ser	37	2	27	2	10	2
T= Thr	54	2	46	3	8	2
V= Val	336	15	288	17	48	11
W= Trp	3	0	0	0	3	1
Y= Tyr	32	1	2	0	30	7

clusters. This leads to dramatic amino acid composition differences between the PPAK regions and the intervening sequences (Table II). These latter segments, with very high content of glutamic acid residues (45 mol %), will be referred to as polyglutamic acid or polyE regions. The polyE zones have much lower contents of lysine and arginine than the PPAK regions. They also contain 33 of the 35 tryptophan and tyrosine residues. Furthermore, there is a strikingly low frequency of aspartic acid residues in the whole PEVK region compared with the other charged amino acid residues. The polyE segments show less regularity of structure than the PPAK motifs. The most repetitive region begins at position 6769: (**EEE**V-LF**EEE**IVP**EEE**VLP**EEEE**VLP**EEEE**VLP**EEEE**VLP-**EEEE**IPP**EEEE**VPP**EEE**YVP**EEEE**FVP**EEE**VLP) and has 11 repeats containing 3 or 4 consecutive glutamic acid residues followed by $X_1X_2X_3$ with X_1 a hydrophobic side-chain amino acid (V, I, L, Y, F), X_2 a hydrophobic or proline, and X_3 usually a proline. A few such repeats are

also found in other regions, but more typical structures are 2–3 consecutive glutamic acids alternating with one or two hydrophobic and/or proline residues. The following example begins at residue 6398: (**EEE**AVSVQR**EEE**Y-**EE**Y**EE**YDYK**EF****EE**Y**E**PT**EE**YDQY**EE**Y). Often a single aromatic residue is sandwiched between two or three glutamic acids on both sides. There are also several regions where the glutamic acid residues alternate with other amino acids.

BLAST[28,29] and MotifSearch[31] searches were conducted to determine whether motifs similar to those found in the human soleus titin PEVK occurred in other proteins. The only similarity of sequence between the PEVK region and sequence patterns in the PROSITE database was with the proline-rich region found in keratinocyte proteins. The sequence KVPE occurs in several types of short motifs in these proteins (see http:/interpro.ebi.ac.uk entry IPR000694), but this protein group is typically high in cysteine and glutamine, amino acids that are virtually absent from the titin PEVK. A few PPAK-like domains can be identified in the partial chicken skeletal titin (connectin) sequence,[38] but the latter sequence is partial, extending only a short distance into the PEVK region. There appears to be some similarity of the PPAK sequence to the first 27 residues of a myosin alkali light chain[39] (1MPP-KKKEPKKAPEPKKE EPK PAPKPAEP28). A PolyE-like sequence is also found at the N-terminus of troponin T[40] (1MSDTEEQEYEEEQPEEEAAEEEEEAPEEPEPVAE-PEEERP KP41). In addition, neurofilament H[41] has weak sequence similarities to both the PPAK and polyE segments. The 9D10 titin monoclonal antibody cross-reacts with this latter protein on Western blots[37]; other workers have identified titin monoclonals that react with neurofilament H as well.[42–44] These observations suggest that the two proteins may assume some common folding arrangement. The greatest similarity between the titin PEVK and neurofilament H is the repetition of lysines (either single or pairs) spaced every 6–8 residues in both proteins.

Secondary structure predictions using PeptideStructure[32] for the PPAK repeat regions provided limited insight on the protein folding in the PEVK region. The high proline content precludes significant α-helix or typical β-sheet structure. However, predictions with the polyE segments consistently indicated considerable α-helical content. The significant number of prolines present would prevent the polyE segments from assuming a completely straight helical orientation. Regions rich in prolines often contain the polyproline II structure,[45] but the PPAK repeats lack the glutamine residues often found in such structures.[46] Further experimental work using circular dichroism (CD) or nuclear magnetic resonance (NMR), or both, on synthetic or expressed peptides will be necessary to better define the protein secondary structure in the PEVK region.

The transitions back and forth between the tandem PPAK-containing segments and the polyE regions result in a cycling of the isoelectric points between these regions. Figure 2 shows that the pI values fluctuate between near 10 for the PPAK segments to ~3–4 for the polyE regions.

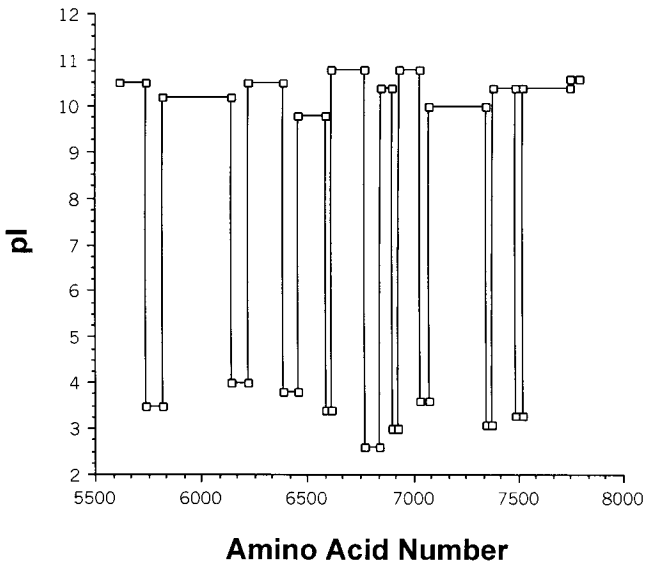

Fig. 2. Fluctuations in isoelectric point along the human soleus PEVK. Values for the different segments were obtained using PeptideSort.

The isoelectric points of the individual PPAK repeats are mostly in the 9–10 region, but a few have pI values of <7. The more negatively charged PPAKs tend to occur in the middle of the wider PPAK clusters, so there are more charge fluctuations than depicted in Figure 2. The isoelectric point of the PEVK region has previously been shown to be more negative than most of the rest of the titin molecule[47]; the calculated value for the human soleus PEVK is 5.06. The PPAK repeats constitute 75% of the amino acid number in the PEVK sequence while the polyE makes up 25%. Thus, the much smaller proportion polyE sequence dominates the charge for the PEVK region.

It is postulated that there may be charge–charge interaction between the positively charged PPAKs and the negatively charged polyE segments. Previous work has shown that there is an ionic strength effect on the stiffness of rat psoas myofibrils.[27] These workers suggested that this effect occurred in the PEVK region. Further work will be needed to validate or refute this hypothesis.

Elastic changes in other proteins upon stretch have been ascribed to several types of repeating amino acid patterns. The extensibility of the protein elastin is believed to involve a β spiral to extended chain transition of numerous repeats with the sequence VPGVG.[48–50] A similar β spiral produced from repetitive β turns is believed to occur in wheat gluten with GYYPTSPQQ and PGQGQQ repeat sequences.[51] Although glycines appear to be important in the flexibility of these structures, this amino acid is rare among the PPAK domains (Table I). A recent study of heptads with a sequence of YSPTSPS from RNA polymerase II[52] showed that polymers with this repeat contain both β turn and polyproline II helix structure. Proline, glycine, glutamine, serine, and threonine, are amino acids commonly found in β turn regions,[53] none of the latter four occurs in the PPAK consensus sequence (Table I). Thus,

it is unclear whether similar mechanisms of reversible folding–unfolding occur in titin.

Many of the PPAK repeats may arise from exon duplications. A family of 27 amino acid segments derived from single exons found in titin genomic DNA (many of which are not expressed in human soleus titin) was recently reported.[54] The start site for these exons does not align with the PPAK repeats; rather, it corresponds to the PPAK consensus valine at position 5. The 27-amino acid repeat[54] really should have 28 amino acids in the protein, but the highly conserved valine is missing because of incomplete codons at both ends of each exon. Although the PPAK repeats described above could be realigned to the exon start sites, beginning the motif with the position 1 proline appears to be more appropriate from a protein structure standpoint since it better separates the PEVK sequence into regions of different charge.

A number of different titin isoforms are expressed by alternative splicing.[25,54] Splicing removes all the polyE segments in the human cardiac N2B isoform (Fig. 1) plus all but 5 of the 60 PPAK repeats found in the soleus titin isoform. The cardiac N2A still has two polyE domains and 20 PPAK motifs. Other titin isoforms with varying PEVK length also occur in skeletal and cardiac muscle.[25,54] The lack of a polyE segment in the PEVK region of the N2B isoform suggests that interaction of the PPAK and polyE is not essential for the folding and unfolding of the PEVK region in this isoform. Further structural studies will be necessary to determine if the PPAK and polyE repeats interact under physiological conditions and to define their role in the elastic function of titin.

ACKNOWLEDGMENTS

The author thanks Dr. Jean-Yves Sgro for assistance with the GCG programs and Dr. John Trinick for comments and suggestions on an earlier version of this manuscript.

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