INTRODUCTION

When a non-activated striated-muscle cell is stretched, passive force develops which restores muscle length following release, limits sarcomere-length inhomogeneity along the muscle cell and A-band asymmetry within the sarcomere. One of the main determinants of passive muscle force is the filamentous intrasarcomeric protein titin (also called connectin) a 3.0 to 3.7-MDa protein that spans the half sarcomere. Titin is anchored to the Z- and M-lines and is attached to the thick filaments of the A-band. The I-band section of the molecule is constructed of serially-linked immunoglobulin (Ig)-like domains (proximal and distal tandem Ig regions) interspersed with unique sequences including a proline (P)-, glutamate (E)-, valine (V)- and lysine (K)-rich PEVK domain. Upon stretch of the sarcomere, passive force is generated by the extension of the I-band segment of titin. The extension of titin's I-band section occurs as a series of consecutive events: the extension of the tandem Ig segment is followed by the extension of the PEVK domain and by the N2-B unique sequence in cardiac muscle. In different muscle types different length isoforms of titin are expressed. Cardiac muscle contains the shortest titin isoform with a 163-residue-long PEVK domain. By contrast, in soleus muscle the PEVK segment is 2174 residues long.

The PEVK domain of titin has been suggested to acquire a random structure due to the preponderance of highly charged residues. Indeed, early immunoelectron microscopic analysis has shown that the PEVK domain probably behaves as a quasi-unfolded, random protein chain. Recent structural experiments have suggested that the PEVK domain may contain left-handed poly-proline helices. Furthermore, a repetitive motif structure of PEVK has been demonstrated based on sequence analysis. Two main motifs were identified in the PEVK sequence: a) PPAK and b) polyE motifs. The PPAK motifs are ~28-residue-long sequences which begin most often with the amino acids PPAK. PolyE motifs contain a preponderance of glutamate. Based on NMR and CD spectroscopic data recently suggested that the PEVK domain has a malleable structure which is capable of transition between various conformational states: poly-proline helix, beta turn and unordered coil

Titin has been shown previously to bind actin. Several different segments or elements of the molecule have been demonstrated to independently possess actin-binding properties. Near the Z-line titin is tightly associated with a 100-nm-long stretch of the thin filament. A group of globular domains from the A-band super-repeat region has been shown to possess actin-binding properties of yet unclear physiological significance The PEVK domain of the cardiac titin has been shown to bind F-actin in a Ca^{2+}-S100-regulated manner Additional

recent works have also demonstrated an interaction between cardiac PEVK and F-actin In a recent work a C-terminal PEVK fragment of the skeletal-muscle titin was shown to bind actin but with a lower apparent affinity than cardiac PEVK. Notably, the cardiac titin sequence is inclusive in the skeletal isoform, therefore there might be regional differences in the actin-binding of skeletal PEVK. The exact mechanisms of PEVK-actin binding and its physiological significance are largely unknown.

AIMS

Our general aims were the systematic characterization of mechanical and actin-binding properties of titin's PEVK-domain. Our specific aims were the following:

- cloning, expression, purification and fluorescent labeling of recombinant PEVK fragments;
- investigation of the F-actin-binding properties of the PEVK domain (in vitro motility assay, solid-state surface binding assay, actin crosslinking assay, actin-PEVK cosedimentation assay, steady-state actin activated ATP-ase assay);
- design of molecular handles to facilitate the specific and high affinity holding of the N- and C-termini of PEVK fragments;
- mechanical characterization of PEVK fragments with single-molecule atomic force microscopy.

MATERIALS AND METHODS

Cloning, expression and purification of human skeletal PEVK

Human skeletal-muscle cDNA library was a generous gift of Dr. Siegfried Labeit. The entire skeletal PEVK domain (largest, *m. soleus* isoform) was expressed in three contiguous segments, with each segment corresponding to about one third (~700 residues) of the PEVK length: N-terminal (PEVKI), middle (PEVKII) and C-terminal (PEVKIII). The nucleotide sequence boundaries of the PEVK segments, based on GenBank accession No. X90569.1 are as follows: PEVKI 16852-19075, PEVKII 19076-21193, and PEVKIII 21194-23373. A 150-residue-long polyE-rich and a 200-residue-long PPAK-rich fragment were also cloned and expressed. The boundaries of these PEVK fragments, based on GenBank accession No. X90569.1 are as follows: PPAK 17413-18015 and polyE 20305-20757. We denote PEVKI, II and III as PEVK "segments", whereas the PPAK and polyE-rich sequences as PEVK "fragments". Each of the segments and fragments was cloned into pET-28a vector (Novagen) between NheI and XhoI sites introduced independently with PCR by using specific oligonucleotides. Proteins were expressed solubly in *E. coli* (BL21(DE3)pLysS). His_6-tagged proteins were purified on Ni^{2+}-NTA columns under native conditions following manufacturer's instructions (Qiagen) and further purified on a Sephadex G-25 column. Concentrations were determined with Bradford reagent (Sigma).

Preparation of proteins

Actin, myosin, heavy meromyosin (HMM) and native tropomyosin were purified according to established methods. F-actin was fluorescently labeled with molar excess of tetramethylrhodamine-isothiocyanate-phalloidin (TRITC-phalloidin, Molecular Probes).

Methods used for investigation *of actin binding properties of PEVK domain* were: in vitro motility assay, solid-state surface binding assay, actin cross-linking assay, actin-PEVK cosedimentation assay, labeling and imaging of myofibrils and steady-state actin-activated ATPase measurements

Calculations

Stretch – release curves were fitted with (Wormlike Chain, WLC) equation:

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

where A is persistence length, k_B is Boltzmann's constant, L is contour length, and T is absolute temperature, z is end-to-end length. Usual fit range was 0 – 50 pN

PEVK as a polyelectrolyte chain was characterized based on Odijk-Skolnick-Fixman (OSF) theory.

RESULTS

To explore the elastic properties and structure of skeletal-muscle PEVK domain, we mechanically manipulated recombinant PEVK fragments. We expressed contiguous N-terminal ("PEVKI"), middle ("PEVKII"), and C-terminal ("PEVKIII") PEVK segments, each ~700 amino acid residues long. The mechanics of the three fragments were studied individually with single- molecule AFM. We found that each PEVK segment displays non-linear elasticity and their mechanical response could be well fitted with the WLC model. The persistence length ranged between 0.1 and 1.7 nm at an ionic strength of 0.2 M. At 500 nm/s stretch rate no force hysteresis was observed, suggesting that the PEVK segment is an ideal elastic spring. Interestingly, the mean persistence length decreased with decreasing ionic strength, suggesting that the elasticity of the PEVK domain could possibly be modulated via electrostatic mechanisms. Measurement of the apparent persistence lengths revealed a hierarchical arrangement according to local flexibility: the N-terminal PEVKI is the most rigid and the C-terminal PEVKIII is the most flexible segment within the domain. Immunoelectron microscopy supported the hierarchical extensibility within the PEVK domain. Thus, the PEVK domain may function as a molecular telescope.

Our further plan is the investigation of PPAK and polyE motif interaction with F-actin. We apply stopped-flow apparatus to characterize these interactions on millisecond scale. Our preliminary results show that the interaction between PPAK and polyE fragments with actin filament is a two-step process. The detailed analysis of fast and slow steps of the PEVK-fragment – actin interaction may results in better understanding of titin – thin filament interaction in sarcomere.

We found a differential actin binding along PEVK in solid-state binding, cross-linking and *in vitro* motility assays. We also observed that PEVKII binds in the I-band of the sarcomere, suggesting that that PEVK-actin interaction may take place *in situ* with a potential physiological significance. To explore which sequence motifs convey the actin-binding property, we expressed a fragment rich in PPAK and another rich in polyE motif. The polyE fragment had a stronger apparent actin binding, suggesting that a local preponderance of polyE motifs conveys an enhanced local actin-binding property to PEVK. The actin-binding of PEVK may serve as a viscous bumper mechanism that limits the velocity of unloaded muscle shortening towards short sarcomere lengths. By varying PEVK's motif structure the magnitude of the viscous drag might be tuned.

As a sequel of this work we plan to investigate the mechanical properties of full-length PEVK domain with atomic force microscope and laser tweezers. We plan to express the full-length PEVK in baculovirus system. The required DNA construct is successfully designed and prepared.

PUBLICATIONS:

1. Nagy, A., Cacciafesta, P., Grama, L., Kengyel, A., Málnási-Csizmadia, A. and Kellermayer, M S. Z. 2004 Differential actin binding along the PEVK domain of skeletal muscle titin. J Cell Sci. 117(Pt 24):5781-9. IF 7.25.

2. Nagy, A., Grama, L., Huber, T., Bianco, P, Trombitás, K, Granzier, H. L. and Kellermayer, M. S. Z. 2005. Hierarchical extensibility in the PEVK domain of skeletal-muscle titin. Biophys J. 89(1):329-36. IF 4.463.

3. Grama, L., Nagy, A., Scholl, C., Huber, T. and Kellermayer M.S.Z. 2005. Local variability in the mechanics of tandem Ig segments. Croat Chem Acta. 78: 405-411. IF 0.729.

ABSTRACTS

1. Grama, L., Nagy, A., Málnási-Csizmadia, A., Kellermayer, M.S.Z. Mechanical stability of an eight-domain segment in titin's differentially-spliced tandem-Ig region. Biophys J 84, 563a, 2003.

2. Nagy, A., Grama, L., Málnási-Csizmadia, A., Kellermayer, M.S.Z. Expression and molecular mechanics of skeletal-muscle titin's PEVK segment. Biophys J. 84, 563a, 2003.

3. Nagy, A., Cacciafesta, P., Grama, L., Kengyel, A., Málnási-Csizmadia, A., and Kellermayer, M.S.Z. Differential actin binding along the PEVK domain of skeletal-muscle titin Biophys J. 86, 567A, 2004.

4. Nagy, A., Cacciafesta, P., Grama, L., Kengyel, A., Málnási-Csizmadia, A., and Kellermayer, M.S.Z. Elastic and actin-binding properties of full-length skeletal muscle titin's PEVK domain. J. Muscle Res. Cell Motil. 25, 263, 2004.

5. Nagy, A., Cacciafesta, P., Grama, L., Kengyel, A., Málnási-Csizmadia, A., and Kellermayer, M.S.Z. Elastic and actin-binding properties of full-length skeletal muscle titin's PEVK domain. Biophys. J. 88, 2005.

6. Nagy, A., Grama, L., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Expression and molecular mechanics of giant muscle protein titin. XXXII. Membrane Transport Conference, Sümeg, Hungary, 2002.

7. Nagy, A., Grama, L., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Expression and single-molecule mechanics of skeletal muscle titin's PEVK segment. Biophysical Society 47th Annual Meeting, San Antonio, Texas, 2003.

8. Nagy, A., Grama, L., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Molecular mechanics of recombinant titin fragments. XXXIII. Membrane Transport Conference, Sümeg, Hungary, 2003.

9. Nagy, A., Grama, L., Cacciafesta P., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Differential actin binding along the PEVK domain of skeletal-muscle titin. Biophysical Society 48th Annual Meeting, Baltimore, 2004.

10. Nagy, A., Grama, L., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Hierarchical extensibility in the PEVK domain of skeletal-muscle titin. EMBO-HHMI Conference, Budapest, 2005.

11. Nagy, A., Bainco P., Grama, L., Málnási-Csizmadia, A. and Kellermayer, M S. Z. Elastic and actin-binding properties of skeletal muscle titin's PEVK domain. Biophysical Society 49th Annual Meeting, Los Angeles, 2005.

12. Nagy, A., Grama, L., Huber, T., Bianco P., Trombitas, K., Granyier H. L. and Kellermayer, M S. Z. Hierarchical extensibility in the PEVK domain of skeletal-muscle titin. Alpbach Workshop, "Coiled-coils, Collagen and Co-Proteins." September 17-22, 2005, Alpbach, Austria.

OTHER PUBLICATIONS AND ABSTRACTS

1. Nagy, A., Malnasi-Csizmadia, A., Somogyi, B., Lőrinczy, D. 2004. *Thermal stability of chemically denatured green fluorescent protein (GFP).* Thermochimica Acta, 410, 161-163. IF 1.161.

2. Szenthe, B., Gaspari, Z., Nagy, A., Perczel, A., Graf, L.. 2004. *Same fold with different mobility: backbone dynamics of small protease inhibitors from the desert locust, Schistocerca gregaria*. Biochemistry. 43(12):3376-84. IF: 4.278.

3. Kellermayer, M. S. Z., Grama, L., Karsai, A., Nagy, A., Kahn, A., Datki, Z. L., Penke, B. 2005. *Reversible mechanical unzipping of amyloid beta-fibrils.* J Biol Chem. 280(9):8464-70. IF 6.482.

4. Horvath, I., Harmat, V., Perczel. A., Palfi, V., Nyitray, L., Nagy, A., Hlavanda, E., Naray-Szabo, G., Ovadi, J. 2005. *The structure of the complex of calmodulin with KAR-2: a novel mode of binding explains the unique pharmacology of the drug.* J Biol Chem. 280(9):8266-74. IF 6.482.

5. Karsai, Á., Nagy, A., Kengyel, A., Mártonfalvi, Zs., Grama, L., Penke, B. and Kellermayer, M.S.Z. 2005. *Effect of lysine-28 side chain acetylation on the nanomechanical behavior of Alzheimer amyloid ß25-35 fibrils.* J. Chemical Information and Modeling. **45** (6), 1641-1646, 2005 IF 3.078.

6. Karsai, Á., Mártonfalvi, Zs., Nagy, A., Grama, L., Penke, B. and Kellermayer, M.S.Z. *Mechanical manipulation of Alzheimer's amyloid ß1-42 fibrils.* J. Struct Biol. In the press. IF 3.092.

7. Kellermayer, M.S.Z., Grama, L., Karsai, Á., Nagy, A., Kahn, A., Datki, Z. and Penke, B. Reversible unzipping of amyloid ß-fibrils. Biophys. J. 88, 198A-199A, 2005.

8. Kellermayer, M. S., Grama, L., Karsai, Á., Nagy, A., Kahn, A., Datki, Z., Penke, B. (2005) Structural dynamics of amyloid explored by manipulating individual fibrils FEBS Journal 272 (s1) F2-016P.