Titin as a Giant Scaffold for Integrating Stress and Src Homology Domain 3-mediated Signaling Pathways

THE CLUSTERING OF NOVEL OVERLAP LIGAND MOTIFS IN THE ELASTIC PEVK SEGMENT

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The richness of proline sequences in titins qualifies these giant proteins as the largest source of intrinsically disordered structures in nature. An extensive search and analysis for Src homology domain 3 (SH3) ligand motifs revealed a myriad of broadly distributed SH3 ligand motifs, with the highest density in the PEVK segments of human titin. Besides the canonical class I and II motifs with opposite orientations, novel overlapping motifs consisting of one or more of each canonical motif are abundant. Experimentally, the binding affinity and critical residues of these putative titin-based SH3 ligands toward nebulin SH3 and other SH3-containing proteins in muscle and non-muscle cell extracts were validated with peptide array technology and by the sarcomere distribution of SH3-containing proteins. A 28-mer overlapping motif-containing PEVK module binds to nebulin SH3 in and around the canonical cleft, especially to the acidic residues in the loops, as revealed by NMR titration. Molecular dynamics and molecular docking studies indicated that the overlapping motif can bind in opposite orientations with comparable energy and contact areas and predicts correctly orientation-specific contacts in NMR data. We propose that the overlap ligand motifs are a new class of ligands with innate strength, and stereospecificity of receptor interactions. Proline-rich sequences of titins are candidates as major hubs of SH3-dependent signaling pathways. The interplay of elasticity and dense clustering of mixed receptor orientations in titin PEVK segment have important implications for the mechanical sensing, force sensitivity, and inter-adaptor interactions in signaling pathways.

Titin is a family of giant structural proteins (3–4 MDa) that constitute an elastic matrix in striated muscle sarcomeres (1), as well as smooth muscle (2) and some non-muscle cells (3). Titin and associated proteins in the elastic matrix may play major physiological roles, including the genesis of long range elasticity, the maintenance of sarcomere stability, the assembly of nascent sarcomeres in developing muscle cells, and the assembly of myosin in the microfilaments in non-muscle cells (4–7). In vertebrate skeletal and cardiac muscles, each titin molecule spans half a sarcomere from the M-line to the Z-line, adheres to myosin thick filaments along most of its length, and connects the thick filaments to the Z-line via an extensile I-band segment. In the I-band region, where titin extends during passive force development, titin is comprised of serially linked motifs of the following three types: (i) the folded 100-residue modular repeats of Ig or fibronectin type 3 (Fn3)4 domains, (ii) the N2-A or N2-B insert, and (iii) a unique elastic 28-residue PEVK region of which 70% is composed of proline, glutamate, valine, and lysine residues (4, 8). Titin PEVK segment, differing in length in titin isoforms, is constructed of modules that are differentially spliced from about 100 exons (4). Together with N2 inserts, they are the major spring elements in the physiological range of sarcomere extension (9, 10).

Rapid progress is being made in the roles of titin in several distinct signaling pathways (11). The kinase domain near the titin carboxyl terminus at the M-line region of the sarcomere is dually regulated by calcium/calmodulin and tyrosine phosphorylation. The agonist-sensitive phosphorylation of titin forecasted its involvement in signaling mechanisms (12, 13). The stress-sensitive titin kinase interacts with the zinc finger protein NBR1 through a force-induced conformation change and eventually controls muscle gene expression and protein turnover via p62, MuRF2, and serum-responsive transcription factor (14–16). At its amino terminus near the Z-line, repeats Z1-Z2 interact with telethonin (T-cap), which in turn interacts with a potassium channel subunit, myostatin, a muscle growth factor (17), and the muscle LIM protein, MLP (18). Z-line repeat Z4 and the 700-kDa titin isoform “novex-3 titin” interact with obscurin, an ~700-kDa protein that is involved in regulating Rho-like GTPases and A-band assembly (19, 20). In the I-band region, N2B domain and PEVK segment interact with crystallin that is known to be a chaperonin in assisting protein folding (21). The interaction of the polypeptide type II helices (PPII) in the PEVK segment with Src

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4 The abbreviations used are: Fn3, fibronectin type 3-like; SH3, Src homology domain 3; PPII, polypeptide type II helices; PVDF, polyvinylidene difluoride; HRP, horse radish peroxidase; SA, simulated annealing; r.m.s.d., root mean square deviation; MD, molecular dynamics; HSGC, heteronuclear single quantum correlation; nSH3, nebulin SH3 domain; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxymethyl]propane-1,3-diol; MOPS, 4-morpholinepropanesulfonic acid; PBS, phosphate-buffered saline.
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homology domain 3 (SH3) invokes SH3 signaling pathways and possibly in the assembly and targeting and orientation of the nebulin during sarcomere assembly (22).

Our recently detailed analysis of the conformational states of a representative PEVK module (human exon 172) and a 16-PEVK module (473-residue) protein fragment of human fetal titin (TP1) has clearly demonstrated the presence of PPII, unordered coil, and β-turn structure motifs in the largely unstructured PEVK segment (23–25). The facile interconversion of these conformational states under subtle environmental conditions such as temperature, ionic strength, and solvent polarity has revealed that the titin PEVK segment is conformationally malleable, without involving proline trans/cis isomerization. The elasticity of PEVK also varies with ionic strength, where the elasticity has been measured to either decrease (26, 27) or increase (28) with increasing ionic strength. This behavior implies that ionic interactions within the PEVK segment play an important role in the ensemble average of the polypeptide configuration and therefore the relative entropic and enthalpic contributions to molecular elasticity. We have proposed that the titin PEVK segment is an open and flexible polypeptide with its elasticity driven and modulated by the number and location of internal salt bridges (26). The elasticity of adult soleus titin PEVK appears to vary along its length, with the amino-terminal region being more rigid than its carboxy-terminal region (28).

The elastic PEVK segment is also a major binding region for protein interactions. The segment appears to be involved in interfilament adhesion between thin filaments in both skeletal and cardiac muscles. In skeletal muscle, PEVK binds actin and nebulin, with only the PEVK/nebulin interaction being inhibited by Ca²⁺/S100A (25, 29). This S100 sensitivity of the adhesion between PEVK and thin filaments was later observed in the cardiac muscle, resulting instead from its effect on PEVK/actin interaction (30). The interaction of titin PEVK with the thin filament proteins thus may be manifested as a calcium-sensitive and reversible resistance to stretching (i.e. viscous drag) of the muscle fiber (31).

Proline-rich peptide sequences in intrinsically disordered proteins or protein domains are hallmarks of protein-protein recognition sites in signaling pathways, by acting as ligands that bind modular adapter domains such as SH3, WW, and EVH1 (32). The SH3 domain, a family of small globular proteins of 60 amino acids found in more than 1500 eukaryotic proteins (33), has a hydrophobic cleft formed by two orthogonal β-sheets that bind to 7–10-residue polyproline II helices with a canonical ligand motif PXXP. The peripheral sequences around the core-binding motif of SH3 determine which of the two possible orientations each ligand takes in the binding cleft. Two classes of ligands, class I, with the consensus sequence R(K)XXPXXP, and class II, with the consensus sequence XPXPPXR(K) (34), bind to the surface cleft of SH3 domains in opposite orientations. Recently, several SH3 domains were found to recognize non-PXXP motifs: PXDDY (by Eps15) (35); WWQXF (by Pex13p) (36); VPMLR (by p53BP-2) (37); RKXXXYY (by SKAP55) (38); PXXRRXXKP (by Grb2 SH3 (C)) (39); PX(V/I)(D/N)RXKKP (by Hbp) (40); and RXXK (by Gads SH3 (C)) (41). Moreover, a few SH3 domains are known to bind ligands outside of the conventional hydrophobic cleft (42). Further insights of the determinants of ligand orientation have been provided by the “tryptophan switch” concept proposed by Fernandez-Ballester et al. (43). The authors classified the SH3-ligand complexes according to two distinct angles between the conserved Trp indole ring and Pro residues at the YPW triad in the binding site of the SH3, each correlating with one of the two ligand orientations (43).

The richness of natural proline sequences in the titin family qualifies titin as one of the largest intrinsically disordered proteins in nature and provides a unique opportunity to understand the biological function and structural basis of protein recognition based on natural selection. This search complements the powerful screening approaches based on combinatorial phage or bacterial display libraries. Our recent demonstration of nebulin SH3 domain interaction with the central PPII helix of a representative PEVK repeating module (22) raised the intriguing possibility that titin PEVK may contain multiple SH3-binding sites and even as tandem repeats in some titin isoforms. In this study, we address this question by carrying out an extensive search and analysis for SH3-binding motifs in the protein sequences of titin and titin-like proteins, including human titin, invertebrate connectin (titin), Drosophila titin PEVK and Caenorhabditis elegans titin PEVT/K segments. Besides classical class I and II motifs, novel motifs consisting of overlapping motifs with opposite orientation are abundant. Experimentally, we evaluated the binding affinity of these motifs toward nebulin SH3 domain and other SH3-containing proteins in muscle and non-muscle cells with the peptide array technology. The feasibility of binding the bipolar motifs to SH3 in opposite orientations was also demonstrated by molecular modeling. These findings have important implications for the signaling roles, mechanical sensing of and inter-receptor interactions of intrinsically disordered proline-rich domains in signaling proteins.

EXPERIMENTAL PROCEDURES

SH3-binding Motifs in PEVK Exons of Titin and Titin-like Protein Genes—The search for SH3-binding motifs was performed on the following sequences (from NCBI or GenBank™): complete DNA sequence for titin PEVK exons (AJ277892); human cardiac N2B titin PEVK (CAA62188, residues 4429–4614); human cardiac N2B titin PEVK (AF525413, residues 232–2887) and human soleus titin (CAD12456, residues 1–2154); D-titin PEVK1 (CAB93524, residues 5389–6535); D-titin PEVK2 (CAB93524, residues 9233–14474); I-connectin PEVK1 (AB055861, residues 4822–5144); I-connectin PEVK2 (AB055861, residues 13,675–14,985); and C. elegans titin PEVK (AY130758, residues 4600–6857) and human cardiac N2B titin A-band (CAA62188, residues 7386–26342).

Preparation of nSH3, Muscle, and HeLa Extracts—The soluble nSH3 (AA02622) was expressed using B21(DE3)pLysS host cells transfected with a PET3d plasmid (Novagen, Madison, WI) in LB medium and purified as described previously (44). For muscle extracts, an 8-pound New Zealand White rabbit was injected intramuscularly in the haunch with 3 ml of anesthetic (Ketaset/Xyla-Jet 9:1). After confirming euthanasia,
the rabbit was exsanguinated followed by dissection. Each of the four striated muscles were dissected: longissimus dorsi (6 g), psoas (5.7 g), soleus (2.3 g), and heart (5.6 g). Each of the four rabbit muscles was individually homogenized three times, 15 s each, with a Polytron homogenizer (Brinkmann Instruments) in 5–8 ml/g of muscle (v/w) in pyrophosphate relaxing buffer (0.1 M KCl, 2 mM MgCl2, 2 mM EGTA, 0.01 M Tris, pH 6.7, 2 mM Na2P2O7, pH 7.2, 0.5 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM diisopropyl fluorophosphate, 0.5% Triton X-100, v/v). Small aliquots of the homogenized material (whole muscle) were saved for further Western blotting analysis. The rest of the homogenates were centrifuged at 1500 × g for 15 min (Beckman, JA 25.50 rotor). The supernatants (S1) were then further centrifuged at 27,000 × g for 30 min using the same rotor and finally a high speed centrifugation at 65,000 × g for 15 min (Beckman TLA 100.4) to prepare soluble muscle extracts free of any traces of insoluble components. The pellets (P1) were resuspended by vortexing into 35 ml of low salt buffer (0.1 M KCl, 2 mM MgCl2, 2 mM EGTA, 0.01 M Tris, pH 6.7, 0.1% Triton X-100, v/v), followed by pelleting at 1500 × g for 15 min and washing nine times. These highly washed myofibrils were for Western blotting analysis. HeLa cell nuclear extract and HeLa cell cytoplasmic extract were generous gifts from Drs. Louis Schiltz and Vittorio Sartorelli of Laboratory of Muscle Biology, NIAMS, National Institutes of Health, which were prepared as described previously (45).

**Western Blotting Analysis**—The monoclonal anti-aminophy- sin, anti-BMX, anti-BRAMP2, anti-Btk, anti-CASK, anti-CDC42GAP, anti-cortactin, anti-Crk, anti-Dlg, anti-Eps8, anti-Fyn, anti-GRB2, anti-GRB14, anti-HS1, anti-Lck, anti-Lyn, anti-Nck, anti-Ntk, anti-p130cas, anti-P13-K, anti-Ras-GAP, and anti-Csk were obtained from BD Biosciences. Polyclonal anti-Abl SH3 domain and anti-JIP1⁄2 (SH3) were from Upstate Biotechnology, Inc., and Zymed Laboratories Inc., respectively. Monoclonal anti-α-I spectrin and anti-α-II spectrin were purchased from Signet Laboratories, Inc. Polyclonal rabbit anti-nebulin SH3 was prepared against the human nebulin (P20929) carboxy-terminal peptide 6652VQRTGRTGMLPANYVE 6667C in our laboratory and was affinity-purified by binding to peptide sulfolink-coupled agarose gel (Pierce), followed by elution using 1500 × g for 15 min and washing nine times. These highly washed myofibrils were for Western blotting analysis. HeLa cell nuclear extract and HeLa cell cytoplasmic extract were generous gifts from Drs. Louis Schiltz and Vittorio Sartorelli of Laboratory of Muscle Biology, NIAMS, National Institutes of Health, which were prepared as described previously (45).

**nSH3 Binding to Ligand Arrays**—The dry SPOTs membranes were first rinsed with methanol for 2 min and three times for 10 min in TBS-T (10 mM Tris, pH 7.2, 150 mM NaCl, 0.05% Tween 20, v/v) and then blocked overnight in the blocking buffer (1% alkali-soluble casein, Novagen) and then shaken at room temperature. The blocked membranes were then incubated with nSH3 (2–5 μM) in blocking solution for 4 h at 4 °C with gentle shaking. Unbound nSH3 was removed by washing with TBS-T (three times for 10 min), plus a final wash with H2O (5 min) at 4 °C, and the bound nSH3 was electrotransferred in a semi-dry blotter (Bio-Rad) at a constant power of 0.8 mA/cm2 for 20 min between 50 mM sodium phosphate, pH 7.0, soaked blotting papers onto activated PVDF membrane (pretreated with 1% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) for 30 min, followed by extensive washing with H2O (five times for 6 min)). Electrophoresis was conducted in buffer at pH 7.0 to allow the dissociated nSH3 (pI 4.3) to migrate toward the anode and be captured by the activated PVDF membrane. (The nonactivated PVDF membrane failed to capture the SH3 molecules.) After electrophoresis, the membrane was quenched in 0.1 M glycine, pH 7.0, for 30 min, followed by washing with H2O (5 min). The bound nSH3 was detected using anti-nSH3 rabbit IgG primary antibody (0.11 mg/ml) followed by a secondary goat anti-rabbit HRP-conjugated antibody and chemiluminescence measurement (ECL kit, Amersham Biosciences). The intensity of each positive spot was quantified by integrating the entire spot using ImageJ. Immediately after use, SPOTs membrane was regenerated according to the manufacturer’s instructions (Sigma). Briefly, the membrane was first washed three times with 20 ml of water for 10 min each time at room temperature, followed by three times incubation with 20 ml of buffer A (8 M urea, 1% SDS, 0.1% 2-mercaptoethanol), 30 min each time at
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A: Human PEVK

| Exon | Sequence (ligand) | Size | Isoform | Binding nSH3 extracts |
|------|------------------|------|---------|-----------------------|
| 112  | ETPFFAPFGPEPKRNK | 28   | s       | c                     |
| 113  | VTTEPKFVVEKPEVKK | 24   | s       | c                     |
| 116  | RKSPPVAKKPVVAKK | 21   | s       | -                     |
| 117  | VVPPAKKPVVAKK | 26   | s       | +                     |
| 118  | KPVVAKKPVVAKK | 26   | s       | -                     |
| 119  | PVKKPVVAKKPVVAKK | 26   | s       | +                     |
| 120  | VVPPKPVVAKKPVVAKK | 26   | s       | -                     |
| 122  | VVPPKPVVAKKPVVAKK | 26   | s       | -                     |
| 123  | PKPPHNREKSPGK | 27   | s       | +                     |
| 124  | PKPPHNREKSPGK | 27   | s       | -                     |
| 125  | PKPPHNREKSPGK | 27   | s       | -                     |
| 128  | PKPPHNREKSPGK | 27   | s       | -                     |
| 131  | PKPPHNREKSPGK | 27   | s       | -                     |
| 132  | PKPPHNREKSPGK | 27   | s       | -                     |
| 133  | PKPPHNREKSPGK | 27   | s       | -                     |
| 134  | PKPPHNREKSPGK | 27   | s       | -                     |
| 135  | PKPPHNREKSPGK | 27   | s       | -                     |
| 136  | PKPPHNREKSPGK | 27   | s       | -                     |
| 137  | PKPPHNREKSPGK | 27   | s       | -                     |
| 138  | PKPPHNREKSPGK | 27   | s       | -                     |
| 139  | PKPPHNREKSPGK | 27   | s       | -                     |
| 140  | PKPPHNREKSPGK | 27   | s       | -                     |
| 141  | PKPPHNREKSPGK | 27   | s       | -                     |
| 142  | PKPPHNREKSPGK | 27   | s       | -                     |
| 143  | PKPPHNREKSPGK | 27   | s       | -                     |
| 144  | PKPPHNREKSPGK | 27   | s       | -                     |
| 145  | PKPPHNREKSPGK | 27   | s       | -                     |
| 146  | PKPPHNREKSPGK | 27   | s       | -                     |
| 147  | PKPPHNREKSPGK | 27   | s       | -                     |
| 148b | PKPPHNREKSPGK | 27   | s       | -                     |
| 149  | PKPPHNREKSPGK | 27   | s       | -                     |
| 150  | PKPPHNREKSPGK | 27   | s       | -                     |
| 151  | PKPPHNREKSPGK | 27   | s       | -                     |
| 152  | PKPPHNREKSPGK | 27   | s       | -                     |
| 153  | PKPPHNREKSPGK | 27   | s       | -                     |
| 154  | PKPPHNREKSPGK | 27   | s       | -                     |
| 155  | PKPPHNREKSPGK | 27   | s       | -                     |
| 156  | PKPPHNREKSPGK | 27   | s       | -                     |
| 157  | PKPPHNREKSPGK | 27   | s       | -                     |
| 158  | PKPPHNREKSPGK | 27   | s       | -                     |
| 159  | PKPPHNREKSPGK | 27   | s       | -                     |
| 160  | PKPPHNREKSPGK | 27   | s       | -                     |
| 161  | PKPPHNREKSPGK | 27   | s       | -                     |
| 162  | PKPPHNREKSPGK | 27   | s       | -                     |
| 163  | PKPPHNREKSPGK | 27   | s       | -                     |
| 164  | PKPPHNREKSPGK | 27   | s       | -                     |
| 165  | PKPPHNREKSPGK | 27   | s       | -                     |
| 166  | PKPPHNREKSPGK | 27   | s       | -                     |
| 167  | PKPPHNREKSPGK | 27   | s       | -                     |
| 168  | PKPPHNREKSPGK | 27   | s       | -                     |
| 169  | PKPPHNREKSPGK | 27   | s       | -                     |
| 170  | PKPPHNREKSPGK | 27   | s       | -                     |
| 171  | PKPPHNREKSPGK | 27   | s       | -                     |
| 172  | PKPPHNREKSPGK | 27   | s       | -                     |
| 173  | PKPPHNREKSPGK | 27   | s       | -                     |
| 174  | PKPPHNREKSPGK | 27   | s       | -                     |
| 175  | PKPPHNREKSPGK | 27   | s       | -                     |
| 176  | PKPPHNREKSPGK | 27   | s       | -                     |
| 177  | PKPPHNREKSPGK | 27   | s       | -                     |
| 178  | PKPPHNREKSPGK | 27   | s       | -                     |
| 179  | PKPPHNREKSPGK | 27   | s       | -                     |
| 180  | PKPPHNREKSPGK | 27   | s       | -                     |
| 181  | PKPPHNREKSPGK | 27   | s       | -                     |
| 182  | PKPPHNREKSPGK | 27   | s       | -                     |
| 183  | PKPPHNREKSPGK | 27   | s       | -                     |
| 184  | PKPPHNREKSPGK | 27   | s       | -                     |
| 185  | PKPPHNREKSPGK | 27   | s       | -                     |
| 186  | PKPPHNREKSPGK | 27   | s       | -                     |
| 187  | PKPPHNREKSPGK | 27   | s       | -                     |
| 188  | PKPPHNREKSPGK | 27   | s       | -                     |

FIGURE 1. SH3 ligand motifs in human titin PEVK and A-band segments. Module (exon) number and amino acid sequences (and numbers) are highlighted with the SH3 ligand motifs in colors: class I motifs in green; class II motifs in red, and overlapping motifs in yellow; XPPXXXPV(A) in purple; and others in black boxes, total residue number; exon usage in titin isoforms (human soleus as S, human fetal titin as F, and human cardiac N2BA as C). Experimental binding data of nSH3 and signaling complexes in cell extracts (extracts) based on peptide arrays are shown as strong (+), weak (+), and absent (−). All others were not determined. A, human titin PEVK-based ligand motifs. A total of 116 PEVK modules, based on human titin PEVK exons (AJ277892, 11/23/2001), were classified into two groups as follows: 102 PEVK modules of 14–37 residues designated as group P and 14 PEVK modules of 25–135 residues designated as group E. Note that because all PEVK exons are phase 3 exons, simple translation of reported PEVK exons cDNA sequences would yield modules missing the first residue. Differential splicing connects the terminal guanosine in the preceding exon with the first two bases of the next exon and gives rise to the correct open reading frame and number of amino acid residues. For this reason, all PEVK modules here now start with Val, or occasionally Glu, Ala, or Gly, to give the correct length. Pro is thus the second residue of the VPE consensus sequence. We have previously designated PE as the start of each module based on fetal PEVK exons (25). Other investigators have phased the PEVK module in the middle of the exons (54). Two additional PEVK exons, 148b and 200b, were recently identified in our laboratory. B and C, human titin A-band based ligand motifs. Class I, II, and overlapping motifs are found near the edges of the Fn3 domains (B) or Ig domains or linkers between the β-barrels (C). Shapos serve as two SH3 binding motifs within one exon or domain.
40 °C, and three times washing with 20 ml of buffer B (50% ethanol, 10% acetic acid) for 30 min each time at 20 °C. Finally, the membrane was washed with methanol (20 ml, 10 min, two times) and TBS-T (20 ml, 10 min, three times). The regenerated membrane was routinely tested for complete removal of bound SH3 by anti-SH3 Western blotting before reuse.

Binding of SH3-containing Proteins of Muscle and Non-muscle Cells to Ligand Arrays—Muscle longissimus dorsi, soleus, heart, and psoas cell extracts, non-muscle HeLa cell nuclear extract, and HeLa cell cytoplasmic extracts were prepared according to the procedures described above. Cell extract mixture was generated by mixing 1.5 ml of soleus extract, 4 ml of psoas extract, 5 ml of longissimus dorsi extract, 2 ml of heart extract, 1 ml of HeLa nuclear extract, and 20 ml of HeLa cytoplasmic extract together with protease inhibitor mixtures (Sigma). The mixture was centrifuged at 40,000 × g for 20 min at 4 °C. The resulting supernatant (28 ml) was filtered through a 0.45-μm filter and concentrated to 8 ml by spinning in Millipore Ultrafree-15 centrifuge filter unit (2000 g, 4 °C, 10-kDa cutoff). After dialysis against 10 mM potassium phosphate, 150 mM NaCl, pH 7.0, the extract solution was centrifuged at 78,000 g for 30 min at 4 °C. The supernatant was mixed with 5% casein in 1/10 PBS to produce 1% casein as the final blocking solution in the cell extract mixture. The SPOTs membranes were first blocked with 1% casein overnight and then incubated with the cell extract mixture for 4 h at 4 °C with gentle shaking. Unbound proteins was removed by washing with TBS-T (10 times for 10 min), whereas bound SH3-containing proteins were detected directly on the SPOTs membrane using anti-SH3 antibody pool (anti-Abl SH3, anti-JIP.
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TABLE 1

SH3-binding motifs in proline-rich sequences of titin and orthologs

| SH3-binding motif | Human fetal skeletal titin (hits) | Human soleus titin (hits) | Human cardiac titin (hits) | D-titin (hits) | I-connectin (hits) | CE titin (hits) |
|------------------|----------------------------------|---------------------------|---------------------------|---------------|------------------|----------------|
| Residues        | PEVK                             | PEVK                      | PEVK                      | N2B           | Titin            | PEVK           |
| PXXP            | 786                              | 2153                      | 34,350                     | 186           | 962              | 26,926         |
| Class I         |                                  |                           |                           |               |                  |                |
| (R)KXXXPXP      | 11                               | 18                        | 32                         | 1             | 8                | 16             |
| Class II        |                                  |                           |                           |               |                  |                |
| XPPXXPVR(R)     | 14                               | 33                        | 76                         | 1             | 11               | 45             |
| XPPXXPVR(A)XP   | 5                                | 8                         | 9                          | 0             | 2                | 1              |
| PXXXKXXKP       | 0                                | 0                         | 2                          | 0             | 0                | 1              |
| PXXDY           | 0                                | 1                         | 5                          | 0             | 1                | 3              |

*NA indicates not available.

SH3, anti-nebulin SH3, and anti-α-II spectrin), followed by a secondary rabbit anti-mouse HRP-conjugated antibody and then a goat anti-rabbit HRP-conjugated antibody and chemiluminescence measurement (ECL kit, Amersham Biosciences). Films were quantitated with a Kodak 2000A system and Scion Image software.

Immunofluorescent Localization of SH3-containing Proteins in Split Rabbit Soleus Muscle Fibers—Single soleus muscle fibers were dissected from rabbit tissue essentially as described previously (47). Single muscle fibers were dissected in a relaxing buffer (150 mM potassium propionate, 5 mM potassium phosphate, 3 mM magnesium acetate, 5 mM potassium EGTA, 5 mM NaCl, 3 mM ATP, 20 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM dithiothreitol, 50 mM 2,3-butane-dione monoxime). They were then stretched to varying degrees (0, 50, and 100% resting length) and mounted onto one-hole gold EM grids and then blocked with 0.5% bovine serum albumin (globulin-free), 0.02% normal goat serum in a buffer (10 mM Tris maleate, 10 mM KCl, 0.5 M NaCl, 0.02 M MgCl₂, 0.02 M EGTA, pH 7.0) for 1 h. The grids were then incubated overnight with two dilutions (5 and 10 g/ml) of each of the following antibodies: anti-ABI SH3, anti-JIP SH3, anti-spectrin II SH3, anti-nebulin SH3, anti-cortactin, anti-GRB2, anti-p130Cas, anti-DLG, anti-titin RT11, anti-spectrin II SH3, anti-nebulin SH3, and anti-cortactin. Nebulin SH3 residues involved in the docking of PPII peptides were those identified with HSQC by Ma and Wang (22) and tabulated in Fig. 4. The nSH3 construct used by Ma and Wang (23), the third residue (Pro) was identified as the start of the exon and the peptide sequence (i.e. residue 1). In this work, residue 1 is the first residue (Lys) to be consistent with the structural data file.

Molecular Dynamics and Docking of nSH3 to Peptides in Two Orientations—The HADDOCK protocol (51) consists of the following four stages: topology and structure generation; starting orientation randomization and (semi) rigid body energy minimization; semiflexible simulated annealing (SA); and flexible refinement in a water shell. For the PR peptide, the SH3-binding motif KKAPVAPPK (residues 13–21) was assumed to be the binding site for SH3. Nebulin SH3 residues involved in the docking of PPII peptides were those identified with HSQC by Ma and Wang (22) and tabulated in Fig. 4. The nSH3 construct used by Ma and Wang (22) lacked two amino-terminal residues in the 1NEB Protein Data Bank structure (48), and the indices have been changed accordingly. During the course of semi-rigid docking (10K structures for each of the 50 starting structures), the side chains of residues 9–22 of PR and residues 11–18, 35–40, and 51–56 of nSH3 were allowed to move. Because the SH3-binding groove is rather shallow as compared with other protein/protein interactions, a large number (50,000) of semi-rigid docking structures were generated to increase the chance of obtaining an optimal configuration. The 1000 lowest energy structures were then used in the SA part of the protocol. The SA has four stages as follows: high temperature rigid body search; rigid body SA; semi-flexible SA with side chains at the interface; and semi-flexible SA with flexible side chains.
chains and backbone at the interface. To allow for motion of the portions of PR away from the interface to move and interact with the nSH3, residues 1–8 and 23–30 of PR were made flexible throughout the SA protocol. Allowing more of PR or parts of nSH3 to be flexible during the SA protocol leads to poor results with distortion of the SH3 fold or kinks in PR at the SH3-binding site. The 1000 structures were then hydrated in an 8-Å water shell and subjected to SA from 100 to 300 K and back to 100 K. The hydrated structures with the lowest interaction energies were compared with known structures of class I peptide bound to SH3 (1ABO) and class II peptide bound to SH3 (1SEM). For comparison of the structures, the conserved YPW triad in each SH3 was aligned, and the r.m.s.d. of the portion of the PR peptide in the binding groove was calculated. The structures with the lowest energy, the most similar conformation to the prototype structures, the greatest buried surface area, and a hydrogen bond to the conserved tryptophan in nSH3 were taken as the best fits.

RESULTS

Abundant SH3-binding Motifs in the Proline-rich Sequences of Human Titin and Titin Orthologs—Because human titin isoforms are produced by differential splicing of more than 300 exons (4), our initial survey of five types of SH3-binding motifs (class I KX

\[ \text{XXP} \text{XXP} \text{XXP} \] (34), class II X

\[ \text{PP} \text{XXP} \text{PP} \text{XXP} \text{XXP} \] (34), X

\[ \text{PPPPXXPV(A)XP} \] (53), P

\[ \text{XXDKX} \text{XXP} \] (40), and P

\[ \text{XXDY} \] (35)) focused on the 116 PEVK exons that constitute the repertoire of building blocks for the titin PEVK segments. Of the 363 human titin exons recently published by Bang et al. (4) (GenBank™ accession number AJ277892, 11/23/2001), 114 exons code for PEVK modules and two additional exons, 148b and 200b, were recently identified in our lab.5 These human PEVK modules can be grouped, based on

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\[ \text{FIGURE 2. Distribution of class I, class II, overlap, and non-PXXP SH3 ligand motifs and experimental verification. A, class I, class II, overlapping motifs with unipolar, bipolar, and mixed-polar ligand orientations, and non-PXXP ligands. SH3 ligand motifs of the class I and class II category are single motifs that bind to SH3 domain in the opposite orientation and polarity. SH3 ligand motifs that are a composite of more than one single classes (class I, II, and others) are designated as class O_{u,b,m} (for overlap), with the subscript n for the total length, the superscripts for the potential polarity relative to the SH3 domain (u for unipolar, b for bipolarity, and m for mixed polarity). Symbols of all types of motifs are color-coded, and their sequence distribution is indicated in the sarcomere maps in B. Their binding to nSH3 and SH3-containing proteins are indicated as strong (+ +) and weak (+ +) or absent (- -). B, sarcomere distribution of SH3 ligand motifs along titin and titin orthologs. Seven titin isoforms were compared: human fetal titin (partial, AF321609); human soleus titin (CAD12456); human cardiac titin N2B (I38344); human cardiac titin N2BA (AF525413); crayfish I-connectin (AB055861); Drosophila D-titin (CAB93524); and C. elegans titin (AY130758). The distribution of class I (green) and class II (red) SH3 ligand motifs in PEVK and A-bands are indicated with vertical bars above and below the line. Overlapping motifs are indicated by symbols as defined in A. PEVK segments in each titin are indicated in yellow. The relative position of the Z-line, M-line, and A-band are indicated relative to the N and C termini of titin. Note the dense clustering of motifs in human PEVK segments. aa, amino acids.} \]
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**A** Peptide synthesis on SPOTS membrane

- Semi-dry transfer
- Anti-nSH3
- Incubation with nSH3
- Antibody cocktails
- ECL

**B** nSH3

| Human PEVK exons (P) | Human cardiac N2A A band (A) |
|----------------------|-----------------------------|
| Stds a b c d e f g h i | 1 2 3 4 5 6 7 8 9 10         |

**C** Cell extracts

| Human PEVK exons (P) | Human cardiac N2A A band (A) |
|----------------------|-----------------------------|
| Stds a b c d e f g h i | 1 2 3 4 5 6 7 8 9 10         |

**D** Ce-titin & I-connectin (E)

| Human cardiac N2A A band (A) | Human cardiac N2A A band (A) |
|------------------------------|------------------------------|
| Stds a b c d e f g h i j k l | 1 2 3 4 5 6 7 8 9 10         |

**FIGURE 3. Screening of the binding of nebulin SH3 domain and SH3-containing signaling complexes in cell extracts by ligand arrays.** A schematic of the SPOTs experimental design. Arrays of ligand peptides were covalently synthesized onto cellulose membrane by the SPOTs technology (46). Several SH3-binding peptides with known affinities are included as positive controls. Incubation of these arrays with nebulin SH3 protein was followed by semi-dry electrophoretic transfer to dissociate and transfer the bound nSH3 to a glutaraldehyde-activated PVDF membrane. After transfer, the PVDF membrane was subject to peroxidase-based ECL detection using anti-nSH3 antibody. The SPOTs membrane was then regenerated and reused several times. For binding of SH3-containing proteins from cell extracts, the incubated SPOTs membranes were stained directly with two antibody mixtures, followed by ECL detection to increase sensitivity. B and C, binding of nSH3 (S) and SH3-containing complexes in cell extracts (C) to three sets of peptide arrays: array P for human titin PEVK ligands; array A for human A-band ligands from cardiac titin N2B; array E for ligands from D-titin (1b-10d), I-connectin (1e-4), and C. elegans titin (5j-2l). Their sequences and layouts are described in supplemental Table ST1. The 1st row of each array contains the control peptides, each with known dissociation constants as controls for quantitation. The relative intensity of each spot was classified as strong (+ +), weak (+), or absent (−).

sequence similarity, into two major groups as follows: group P exons contain 102 modules enriched in Pro, Glu, Val, and Lys, with an average of ~28 residues (Fig. 1A), (designated as PAKK by Greaser (54)), and group E exons include 14 glutamate-rich modules of more variable size (25–135 residues) (Fig. 1A), designated as poly(E) by Greaser (54). A search for the SH3-binding motifs in the PEVK modules revealed that there are 94 class I (Fig. 1A, green) or class II motifs (Fig. 1A, red). As indicated in the color-coded motifs within the aligned amino acid sequences of PEVK exons (designated as PEVK modules), there was no evidence of additional motifs when all PEVK exons are spliced together pairwise and translated to protein sequences. There are only five pure class I motifs scattered at different positions within the P group PEVK modules and none in the human titin PEVK and titin sequences. There are 5 and 8 copies in human fetal and soleus PEVK sequences, respectively. The 9-residue motif PXXXXX-PV(A)XP is much rarer and again is found mainly, if not exclusively, in glutamate-rich group E modules. Twenty seven of 60 class II motifs are found in the first 7 residues of two blocks of PEVK exons from 121 to 162 and from 209 to 214. The remaining motifs are mostly found in the central region of the modules 118, 131, 171–203, and 217–218 and at the end of modules 122, 148b, and 224.

Given that nearly 80% of the human PEVK exons contain one SH3 class I or class II SH3-binding motif, we next extended the analysis to the distribution and number of potential SH3-binding motifs in differentially spliced titin isoforms and related proteins from several species. The analyses of all six types of SH3 ligand motifs in both PEVK and the remainder regions of titins are summarized in Table 1. It is clear that the core PXXP motif is abundant. Because the 4-residue core motif is too short to be useful for identifying ligands, we have focused our analysis on the search for class I and II motifs, two well established SH3-binding motifs of 7 residues each. There are different copies of class I and II motifs in human fetal skeletal PEVK (25), human soleus PEVK (51), cardiac N2B PEVK (2), cardiac N2BA PEVK (19), I-connectin PEVK-1 and PEVK-2 (42), D-titin PEVK-1 and PEVK-2 (17), and C. elegans titin PEVT (0), respectively (note that the designation 1 and 2 refer to distinct PEVK segments and not SH3 ligand motifs).

The 10-residue motif XPXXX-XP(A) is much rarer and again is found mainly, if not exclusively, in human PEVK exons. There are seven copies in I-connectin PEVK-1 and PEVK-2 and three copies in D-titin PEVK-2. Interestingly, there are 18 copies in the remainder of I-connectin and only one or two in non-PEVK segments of human titins. There are five and three PXXDY motifs in human soleus and cardiac titin and four and one in I-connectin and D-titin, respectively.

Further analysis indicated that class I and II SH3-binding motifs are highly enriched in most PEVKs of titin orthologs, except C. elegans titin. Because some of the PEVK sequences are still incomplete, a density index (based on the copy number per 1000 residues) was used to normalize the values for compari-
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Overlapping Ligand Motifs with Uni-, Bi-, and Mixed Orientations—Closer examination of the two classes of motifs in the PEVK modules revealed a novel class of overlapping motifs (Fig. 1A and Fig. 2A); 10 pairs of the 35 copies in fetal PEVK and 7 pairs of the 48 copies in human soleus PEVK total class I and II motifs are overlapping pairs that give rise to a 9-residue motif, KXXPXXPXK. Such a motif, being much shorter than the sum of two motifs (14 residues), may potentially allow SH3 to bind in either of the two orientations. Because class I and class II motifs bind to SH3 domains in the opposite orientation, these overlapping motifs can be considered as bipolar SH3-binding motifs. Significantly, overlapping motifs with different degrees of overlap and mixed polarity are also found in I-connectin PEVK-1 (9-residue) and in human soleus PEVK and cardiac PEVK (10-residue).

We designate these ligand motifs as class \( O_{n}^{a,b,m} \) (for overlap), with the subscript \( n \) for the total length and the superscripts for the potential polarity relative to the SH3 domain (\( u \) for unipolar, \( b \) for bipolarity, and \( m \) for mixed polarity) (Fig. 2A). Most of the 24 class \( O_{n}^{a} \) in human titin are found in the middle of a cluster of exons 171–183 in fetal muscle. A search for a more generalized class \( O \) overlapping motifs with shorter overlap led to the detection of one 11-residue motif in exons 179, 188, and 197 (\( O_{11}^{a} \), KXXPXXPXPPXP), two 12-residue motifs in exon 130 (\( O_{12}^{b} \), KXXPXXPXPPXP) and exon 218 (\( O_{12}^{b} \), KXXPXXPXPPXP) in exon 217. Of these motifs, exons 138, 217, and 218-based ones are expressed in human soleus titin PEVK.

Binding of nSH3 to Ligand Motifs in Human PEVK, A-band, and Titin Orthologs—To evaluate experimentally whether these titin SH3-binding motifs do indeed bind to SH3 domains, we first screened the interaction of nebulin SH3 domain with an array of these motif peptides covalently synthesized onto cellulose membrane by the SPOTs technology (46). SPOTs technology, originally designed for mapping epitopes, is being applied increasingly as a high throughput method for screening protein/peptide interactions (56). As shown in Fig. 3 and supplemental Table ST1, these peptide arrays include class I, class II, and non-PXXP motifs contained in PEVK exons (array P), titin isoforms (array M), and A-band (array A). Several SH3-binding peptides with known affinities are included as positive controls. The high density of the peptides on the membrane thus allows for the detection of even moderate binding affinity as frequently reported for such proline-rich peptides to SH3 domains (57). Incubation of these arrays with nebulin SH3 protein was performed under conditions that favor binding of PEVK modules to nSH3, including low temperature, moderate ionic strength (22), and the avoidance of prior exposure of nSH3 to low ionic strength buffer. In order to ease the stripping of the costly SPOTs membrane for repeated probing, we have chosen to electropheretically dissociate and transfer the bound nSH3 to a glutaraldehyde-activated PVDF membrane, rather than the direct enzyme linked detection on the SPOTs membrane. The electrophoretic transfer was performed at pH 7.0, so the acidic nSH3 (pI 4.6) would migrate toward the PVDF membrane near the anode. Glutaraldehyde activation of PVDF was necessary, because nSH3 has very low affinity for PVDF alone. After transfer, the PVDF membrane was subject to peroxidase-based ECL detection using anti-nebulin SH3 antibody. The SPOTs membrane was then regenerated without having been subjected to oxidizing conditions of the peroxidase development (Fig. 3A). In our hands, this technique was very sensitive and could

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6 K. Ma and K. Wang, unpublished data.
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**Figure A:** 
- PR sequence: KVP-EPPKEVVPEKKAPVAPPKEVVPEKPVK
- nSH3 sequence: TAGKIFRAMYDEMAADAVSFKDCDAIINVQAEIDCMGTYVQTGRGTMHEMANVEAI
- Docking Input
- Class I: 3A
- Class II: 3A

**Figure B:** 
- nSH3 structure
- RT Loop
- nN Src Loop

**Figure C:** 
- Class I Docking
- Class II

**Figure D:** 
- SA + MD + Matching
- Class I: nN Src Loop

**Figure E:** 
- SA + MD + Matching
- Class II: nN Src Loop
detect the signal of a peptide with a dissociation constant of 100 μM, as indicated by the spot intensity versus solution binding constants of the eight standard peptides toward SH3 domains (Fig. 3B and supplemental Table ST1). For this study, we classified the positive spots into two categories: strong (++) for intensity higher than 120 (corresponding roughly to a dissociation constant of 80 μM) and weak (+) for spot intensity between 10 and 120, and (−) for those below 10.

Nebulin SH3 domain interacted with 36 peptides derived from human PEVK exons, totaling 29% of the total PEVK exons (Fig. 3B, array P). The distribution of both strong and weak nSH3-binding sites along human soleus, fetal (partial), and cardiac (N2BA) titin PEVKs, with 20, 8, and 6 sites, respectively, is shown in Fig. 1 and supplemental Table ST1. There are nine strong binding motifs, corresponding to seven class O overlapping motifs (Fig. 3B, array P, spots P5b, P4d, P5d, P6d, P2e, P3e, and P4e), one class II (Fig. 3B, array P, spot P4f), and one non-PXXP motif (Fig. 3B, array P, spot P1i, XPXXXXPAXP). The strong peptide at position 4d in array P is derived from exon 172 and is a duplicate of the positive control peptide (VPEKAVAPPK) in position P6a. This peptide, designated as PR2 in our solution conformational studies (23), thus serves as an internal control for correlating solid phase and solution phase binding data.

Besides these strong binding motifs, the weak binding motifs contain class O overlapping motif (P7d), class I (Fig. 3B, spots P9d, P2f, and P6f), class II (Fig. 3B, spots P6b, P5e, P7c, P5e, P8e, P9e, P1f, and P5g), and unconventional motifs (Fig. 3B, spots P8f, XXXXXPKX, P7g, VPKKKRPVP). Interestingly, peptides at P5g and P7g are based on exon 151 and exon 148 of the group E exons. It is noteworthy that in the I-band region, most class O overlapping motifs interact with nSH3 with higher affinity than other motifs. nSH3 also interacts with 45% of the predicted SH3 ligand motifs along the A-band (Fig. 3B, array A). Among these binding motifs, five show strong binding (four class II and one class I), and the remaining 16 are all weak binding motifs (12 class II, two class I, and two non-PXXP motifs). These nSH3-binding sites are evenly distributed, one site per 1250 residues along human titin A-band in the sarcomere (Fig. 2B).

In addition to human titin, nebulin SH3 also interacts with multiple SH3-binding motifs along the sequences of D-titin, I-connectin, and Ce titin. Twenty four of 30 predicted SH3-binding motifs in D-titin show positive binding to nSH3, and two-thirds of all confirmed nSH3-binding sites are located in its two PEVK regions, with the remainder located outside the PEVK region in the I-band (Fig. 3B, array E). I-connectin shows a similar SH3-binding site distribution pattern to that of human titin with class I and II nearly exclusively enriched in the PEVK-1 region (Fig. 2B). Although there is no class I and II motifs in I-connectin PEVK-2, six non-PXXP-binding motifs in PEVK-2 were found to interact with nSH3 (Fig. 3B, array E). In C. elegans titin, the 18 predicted SH3 ligand motifs are scattered along the sequence except in the PEVT region (Fig. 2B), with the identified nSH3-binding sites (Fig. 3B, array E) located either amino- or carboxyl-terminal to the C. elegans titin. It is noted that nearly all nSH3-binding sites in human titin PEVK were class II or overlapping motifs. In contrast, binding sites from other titin PEVKs involve both class I and class II motifs.

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FIGURE 4. Molecular dynamics simulation and docking of a conformation ensemble of an overlap ligand, O9, from human PEVK module 172 and nebulin SH3. PR, 30-mer PR peptide sequence. Residues assumed to interact with nSH3 are underlined in black. Green and red arrows indicate class I and II residues, respectively. PVAP core sequence has a yellow background. nSH3, nebulin SH3 peptide sequence. Conserved YPW triad residues are in purple boxes with a triangle above. Residues in the RT loop and n5rc loop are in boxes. β-Sheet regions have a bar over them with identifier as in Ref. 48. Residues that are identified at contact sites by HSQC, assigned as active in docking simulations (a subset of HSQC residues) and residues within 3 Å of the bound peptide for the two classes of binding (D and E), are underlined. A, ensemble of wild type PR structures aligned at residues 16–19 which have the lowest r.m.s.d. along the length of the peptide (−1 Å). Wild type PR peptide was simulated over 30 ns. B, nebulin SH3 structure following 1 ns MD in water box. C, ensemble of PR structures aligned with optimal docked structures. D and E, optimal class I (D) and class II (E) docked structure from an ensemble of 1000 docked structures (see “Experimental Procedures” for more details). Criteria include low interaction energy and close match of peptide backbone in binding pocket to that of prototype class I and II structures. Each of the complexed ligands in class I and class II orientations (colored as purple and green, respectively) is superimposed on the ensemble configurations in C, as indicated by the arrowheads.

NMR Titration and Molecular Dynamics of PEVK Ligand and Docking an O9 Overlap Ligand to nSH3 in Opposite Orientations—To investigate the conformational basis of the strength, stereospecificity, and polarity of ligand/nSH3 interactions, we carried out NMR titration experiments on nSH3 with a wild type PEVK module (28-mer) containing an O9 overlapping motif and applied molecular dynamics simulations to model its binding to nSH3 in opposite orientations. The NMR titration was performed essentially as described previously (22). The titration of isotope-tagged nSH3 with increasing concentrations of PR resulted in the perturbation of chemical shifts of selected residues very similar to those titrated with a shorter ligand peptide (12-mer, VPEKAVAPPK) of the same module (22). To identify which of these chemical shifts arose from the binding of ligand in either class I or the class II orientation or both (as a mixture of class I and II orientations), molecular dynamics modeling and ligand docking simulation were then carried out. Because the flexibility and the ensemble conformations of SH3 and the ligands are likely to play significant roles in determining the specificity and affinity of interaction, docking simulations with molecular dynamics are more insightful because they can be performed using fully hydrated, relaxed structures. The available structures of both the PR peptide and nSH3, derived from NMR data, were first hydrated and then subjected to MD simulation to optimize the conformations before docking.

Portions of the 30-mer PR peptide underwent large fluctuations, but the center of the SH3-binding region (PVAP, residues 16–19) had the smallest r.m.s.d. of any four sequential residues during the 30-ns simulation. This core peptide varied less than 1 Å and was used as a reference state to align the ensemble conformations of the peptides. Overall, the fluctuations of the carboxyl-terminal portion of the wild type peptide were less than that of the amino-terminal portion as visualized by the superimposed conformations around the fixed reference core peptide (Fig. 4A).

Molecular dynamics simulation of the starting structure of nSH3 (reported by Politou et al. (48) as 1NEB in Protein Data
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Bank) at 300 K in water resulted in significant improvement over the starting structure that was optimized originally in vacuo (Fig. 4B). In their starting in vacuo structure, 64.7% of the non-glycine and non-proline residues were in the most favored region, and none were in the disallowed regions of the Ramachandran plot (48). In our final structure, 90.2% of the non-proline residues were in the most favored region, and none were in the disallowed regions. In particular, the \( \phi - \psi \) angles of the conserved Trp (Trp-38 in nebulin SH3, code 1NEB), an important residue in the tryptophan switch that governs the class I and class II binding configurations (43), changes considerably from 69.05, -166.80, for the first in vacuo structure to -132.44, 132.32, after 1 ns of MD. During the first 32 ps of the MD simulation at 300 K, the \( \phi - \psi \) angles of Trp-38, moved up into the \( \beta \)-sheet region. Also, the \( C_\alpha - C_{\gamma} - C_{\gamma} \) angle of Trp-38 changes from 109.5° in the NMR structure to 117.3° following MD in this work.

The newly calculated structure of nSH3 was used to dock with the ensemble of peptide conformations to arrive at the complexes with the lowest interaction energy (van der Waals and electrostatic) in both class I and class II binding orientations by an information-driven docking approach (HADDOCK program) (51). This program uses binding interactions, such as the residues identified via NMR titration, sequence homology, or other biophysical/biophysical means to limit the conformational searching problem inherent in docking simulations. For the PR peptide, the SH3-binding motif KKAPVAPPK (residues 13–21) was assumed to be the binding site for SH3 (22). Our strategy was to use only a subset of the nSH3 contact regions, as determined by HSQC NMR experiments (22), as docking inputs and then evaluate whether contact regions of the docked complexes in both orientations predict the entire set of experimental contacts of the 30-mer PR peptide (summarized in Fig. 4, nSH3 sequence annotation).

To exclude those structures in the docking simulations that were dominated by electrostatic interactions and mostly showed no stereo-specific binding to the hydrophobic cleft on SH3, the 1000 docked structures were first screened by examining those with an interaction energy function \( E_{\text{vdw}} + 0.1 \times E_{\text{elec}} \) less than -99 kcal/mol. The docked structures were screened further by comparison of the peptide orientation with the Protein Data Bank 1ABO structure for class I and with the 1SEM structure for class II binding. The 1ABO and 1SEM crystal structures were assumed to represent the canonical binding configurations for the two types of ligand binding. The structures with the lowest energy, the most similar conformation to the prototype structures, the greatest buried surface area, as well as a hydrogen bond to the conserved tryptophan in nSH3 were taken as the best fits. For class I structures, the configuration with the lowest r.m.s.d. from the peptide in the 1ABO structure (3.47 Å) was also within the \( E_{\text{vdw}} + 0.1 \times E_{\text{elec}} \) cutoff (-100.9 kcal/mol) with a buried surface area of 1974.3 Å² (Fig. 4D). Three class II docked structures with an r.m.s.d. of less than 3.5 Å from the peptide in the 1SEM structure were identified within the \( E_{\text{vdw}} + 0.1 \times E_{\text{elec}} \) cutoff, -102.3, -100.9, and -99.9 kcal/mol, with interaction energies of -220.4, -260.3, and -286.6 kcal/mol, respectively. The structure with the lowest interaction energy also had the largest buried surface area at 2126.4 Å² and is shown in Fig. 4. The tryptophan indole ring is hydrogen-bonded to the peptide Pro-16 and Val-17 carbonyls in the class I structure and to the peptide Ala-18 carbonyl in the class II structure.

Our modeling results indicate that the 30-mer PEVK module that contains the \( \alpha_3 \) motif can indeed bind in a stereospecific fashion in both directions with low and comparable net interaction energy. Moreover, the ligand makes substantially more contact with nSH3 beyond the canonical PPII-binding sequences in the binding groove. It is interesting that a comparison of the optimal docked peptide configurations for class I and II orientations with the starting ensemble configurations (Fig. 4C, where the configuration ensemble of free peptide is superimposed on the docked class I (purple) and class II (green) configurations) shows that the complexed configurations resemble closely some of the free ligand configurations. These pre-existing configurations may well be sampled preferentially by nSH3, resulting in a minimal loss of entropy upon binding and a higher binding affinity.

Orientation-specific Molecular Contacts of an Overlap Ligand—Several other features of the docked complexes of nSH3 and PR peptide in two orientations are worth noting. First, there is substantial difference in the conformation of SH3 with the ligand bound in two opposite orientations. Overall, the negative surface potentials are more compact in the class II complexes, especially the ones in both RT loop and nSrc loop and those lining up the edge of the binding cleft (in red, Fig. 4, D and E). Second, the surface negative potentials indicated by the asterisks in Fig. 4D (near residues D11, D16, D35, and E58) are involved in the charge interactions with the ligand lysine pairs that flank the sides of the PXXP core motif. Close examination of the ensembles of these complexes during MD indicated that in class II complexes, the \( \epsilon \)-amino groups of both \( ^{21} \)KK \( ^{22} \) of the ligand flip-flop around these two negative charge centers between the RT and nSrc loops (Asp-16 and Asp-35). In addition, the \( ^{13} \)KK \( ^{14} \) from across the core PXXP flip-flop between the negative centers near residues Asp-11 and Asp-16 on the other side of the nSH3. Interestingly, class I ligands showed a similar charge interaction with the same pairs of negatively charge centers, but in the opposite orientation. These quadruple charge interactions undoubtedly reflect the symmetrical disposition of KK around the core motif and the presence of two symmetrically placed negative charge centers on both sides of the cleft of nSH3. Our initial mutagenesis studies of ligands confirmed the importance of these flanking double positive charges in binding affinity. Third, Tyr-10 at one end of the cleft appears to have flipped 35° between class I and class II complexes. The phenyl ring of Tyr-10 is rotated toward the protein core in class I binding and away from the protein core in class II binding. The phenyl rings in the free SH3 and in the class I binding are in the same plane, but in the class II binding the phenyl ring is rotated away from the PR peptide. These changes in the orientation of the Tyr-10 phenyl ring are due in part to hydrogen bonding of the phenyl OH to PR backbone carbonyls.

Overall, the PR peptide wraps around the nSH3 hemisphere in both orientations and forms contacts with nSH3 residues more broadly distributed than those in the hydrophobic cleft (Fig. 5, A and B). The validity of the modeled complexes is supported by the fact that the contact residues on nSH3 agree well with those determined by our NMR HSQC experiments,
including several that were purposely ignored in the original inputs for the docking and molecular dynamic calculations. The modeling also revealed orientation-specific contacts: Thr-1, Val-31, and Gln-32 for class I complex and Arg-7, Thr-42, Gly-47, Arg-48, and Thr-49 for class II complex. Closer examination of the complexes and additional modeling indicated that these residues are orientation-specific. The fact that these orientation- or polarity-specific residues are indeed perturbed by binding of an overlap ligand in NMR titration experiments strongly suggests that both complexes are present in an appreciable amount and that slow exchange on the NMR timescale is observed. However, the observation that no separate signal was observed for free nSH3 indicates that the complexed nSH3 and nSH3 are in rapid exchange.

Conformations of YPW Triad—Fig. 5C shows the conserved triad for nebulin SH3 with the PR peptide docked in class I orientation (pink) and class II orientation (cyan). Upon ligand binding, the indole of Trp-38 does indeed tilt toward the conserved Pro-53 from 117.3° (the Cα-H11002-Cβ-H11003 angle in nSH3) to 113.8° for both class I and class II complexes (with the ligand in opposite orientations). The lack of a pronounced difference in tilt angles between the two orientations (by as much as 15° as reported by Fernandez-Ballester et al. (43)) raises the question as to whether the tryptophan switch is operative in the current ligand/SH3 pair. It is noted, however, that the Pro-53 is puckered differently as two rotamers (58), from DOWN (Cα-H11002/H11003 28.1) in unbound nSH3 to DOWN (Cα-H11002/H11003 31.2) in class I binding and to UP (Cα-H11002/H11003 27.2) in class II binding. Thus, the prolines are puckered differently in these two ligand orientations. A difference in proline puckering was also observed between the ligand complexes of Abl SH3 (class I SH3, code 1ABO (59)) and of Sem SH3 (class II SH3, code 1SEM (60)).

In the class I ligand, PR residue Pro-19 projects into the pocket formed by the YPW triad, and both Ala-17 and Pro-16 are in a position to hydrogen-bond with the Trp indole ring. In class II ligand, PR residue Val-17 projects into the binding pocket formed by the YPW triad, and the Ala-18 carbonyl forms a hydrogen bond with the SH3 Trp indole ring. The difference in the sizes of the side chains projecting into the pocket near Pro-53 may have caused the change in puckering of the Pro-53 to accommodate the side chains. As expected, Trp-38 is hydrogen-bonded to a carbonyl in both orientations, as dictated in the original selection criteria.

Binding of SH3 Proteins and Signaling Complexes in Muscle and Non-muscle Cell Extracts to Ligand Arrays—The experiments described above established the binding characteristics of the abundant nSH3-binding motifs in proline-rich sequences of titin and orthologs. Whether these motifs bind to SH3-containing proteins was then addressed by investigating the interaction between these SPOT sequences and total proteins in cell extracts of skeletal muscle (longissimus dorsi, soleus, and psoas), heart muscle, and nuclear and cytoplasmic extracts of HeLa cells. To maximize the scope of screening, two antibody binding orientations. Class I is shown in purple (PR) and pink (SH3 residues) and class II is shown in green (PR) and cyan (SH3 residues). The four charged residues at the four corners of the YPW triad are also shown. Note the different puckering of Pro-53.
miches consisting of either four anti-SH3 antibodies or a mixture of 13 antibodies to the SH3-containing signaling proteins (with antigens outside the SH3 domains) were used in Western blots (supplemental Table ST2). Major SH3-containing proteins in these cellular extracts were first identified by Western blots with each of the antibodies against either SH3 domains or SH3-containing proteins. Additionally, a number of SH3-containing proteins were identified in both skeletal and solute muscle fibers. The SPOTs were incubated with a mixture of cellular extracts and then screened by a set of anti-SH3 antibody mixtures, which detected four major classes of SH3 domains in whole muscle heart and soleus extracts, respectively. It is significant that these anti-SH3 antibodies recognize distinct sets of bands, with the exception of perhaps a 50-kDa band by three anti-SH3 antibodies (anti-Ab1, anti-JIPm and anti-nebulin SH3; see supplemental Figs. SF1A and SF1B). Experimentally, the SPOTs were incubated with a mixture of cellular extracts and then screened by a set of anti-SH3 antibody mixtures, which detected four major classes of SH3 domains. A total of 85 positive spot signals were obtained from 219 spots of three SPOT membranes (supplemental Table ST1), suggesting the interaction between the SPOT peptides and SH3-containing proteins in the cell extract mixture.

It is striking that the two sets based on nSH3 screening and cell extract mixture screening do not completely overlap as follows: 39 of 109 nSH3 positive spots were negative in the cell extract screen and 15 cell extract spots were negative for nSH3 screen (Fig. 3C and supplemental Table ST1), perhaps because of either distinct binding specificity of distinct SH3 domains and/or low SH3-containing protein concentrations in the extracts. Among the 39 extra signals detected in the nSH3 screen, five were strong binding (four class I and one class II SH3-binding motifs) (Fig. 3B, supplemental Table ST1), and the other 34 spots showed weak binding. Among the 15 extra signals detected in the extracts screen, one strong binding motif (PERKPEPKHEEV) derived from PEVK exon 219 was obtained. This difference again suggested that the cell extract mixture might contain different types of SH3 domains.

**Immunolocalization of SH3-containing Proteins in Myofibrils**—To demonstrate that these SH3-containing proteins do indeed bind to the muscle sarcomere, immunofluorescent localization on mechanically split single fibers from rabbit soleus muscle was performed with this set of antibodies, where they labeled to various degrees. Anti-titin RT11 (PEVK-specific (25)) labeled the A junctions as a doublet per sarcomere (Fig. 4A), as expected from their known sarcomere location. The anti-Abl SH3, which detected nearly 20 bands on Western blots of rabbit soleus muscle, stained across the sarcomeres, intensely and in a granular form on the A-band and weakly at the Z-lines and occasionally on the I-band (Fig. 6C). Anti-nebulin SH3 and anti-α-actinin stained the Z-line (Fig. 6C). Anti-JIP3 SH3, anti-p130, and anti-cortactin all gave similar staining patterns as the anti-Abl SH3 (not shown). The Alexa-phalloidin labeled the actin in the Z-line and the I-band green (Fig. 6) and was used as an internal standard for all experiments. Even though the inherently low resolution of immunofluorescence did not allow us to resolve PEVK regions near the A junctions from the A-bands, the staining patterns of rabbit soleus muscle sarcomere with anti-SH3 domains were strikingly similar to the distribution of SH3-binding motifs along the length of the human titin molecule (Fig. 2). We believe that the staining patterns reflect, at least in part, the presence of bound SH3-containing proteins on the titin in situ.

**DISCUSSION**

**Titin as a Giant Scaffold for Signaling Proteins**—The motif analysis and experimental validation of naturally evolved ligands of the proline-rich sequences of titin isoforms and orthologs complement the engineering-based approach of designing and screening of combinatorial libraries to define “optimal” binding affinity and the structural basis of binding specificity of “proline-philic” domains such as SH3. Human titin PEVK segments, consisting of disordered PPII-coil, represent one of the richest sources of different types of SH3-binding motifs with the remaining I-band, consisting mostly of Ig domains, nearly devoid of such motifs. In contrast, the A-band region of vertebrate titin contains motifs at the edge of Fn3 domains dispersed throughout the A-band. Other novel motifs that do not conform to these well known ligand classes may well exist, and thus the current survey may represent a lower limit. The identification of the binding profiles of each of the human
SH3 Ligand Motifs of Proline-rich Titin

FIGURE 7. Stereo specificity of SH3 overlapping motif and potential inter-receptor interactions in clusters of overlapping motifs. A, SH3 bound to the overlapping motif of PR (residues 15–20 shown as fixed in one direction in the binding clefts) in both class I and class II orientations. Note that SH3 binds to different faces of the PPII helix in these two orientations. For a fixed ligand orientation, the SH3 flips and rotates and binds to the opposite face of the PPII helix (via a D2 symmetry operation). The schematic of the $O_9^{ab}$ motif (B) shows the relative orientation of the SH3 domain when bound to the core PXXP motif in two orientations. The schematic of a mixed overlapping motif $O_{11}^{ab}$ (C) shows that two class I-binding sites are shifted by two residues leading to a 120° azimuthal angle between the two possible class I configurations. The PXXP motifs for the first class I and the class II are shifted by six residues, so they will have the same orientation as for the $O_9^{ab}$ shown in A. The binding of SH3-containing receptors to a cluster of binding motifs is depicted along a string of three ligand motifs: The relative orientation of each of the two $O_9^{ab}$ and one class I motif is indicated above the receptors. Potential inter-receptor interactions occur when the receptor shapes complement each other at the interface. The potential flip-flopping (curved arrows) of receptors on the $O_9^{ab}$ motif provides a facile and efficient way of regulating the stereospecific interactions of adjacent SH3-containing proteins.

PEVK modules described here therefore allows the prediction of the SH3 binding capacity of the isoforms that are expressed by alternative splicing of PEVK and other exons in a developmental- and tissue-specific manner. In turn, the exon usage of PEVK modules in titin may be finely tuned to add signaling functionality beyond its role as an elastic spring in the sarcomere. The potential signaling role of SH3-containing proteins in A-band assembly, turnover, and contractile activities is also suggested by the presence of more than 20 binding sites along the A-band segment and the presence of SH3-containing proteins in the sarcomeres. For the smaller titins from invertebrates, e.g. fly, where the long A-band anchoring segment of vertebrate titin is absent, the binding motifs are again found exclusively in one or two of the PEVK segments, albeit at a much lower density than the vertebrate counterparts are. This sharp contrast indicates that despite the richness of prolines in these invertebrate titin PEVK segments, common ligand motifs are relatively rare, whereas human PEVK modules may have evolved to play more significant roles in SH3 or other proline-based signaling transduction pathways. The possible presence of additional ligand motifs to other families of SH3 domains and other proline-rich domains is well worth exploring in the future. Our extensive survey has already added a new dimension of complexity to the signaling capacity of titin (11).

**Tandem Repeats of Class I, II, and Novel Overlapping SH3-Ligand Motifs**—Besides the class I ((R/K)XXPXP) or class II (XPXXPXR/K)) motifs, human titin PEVK modules are rich in the $O_9^{ab}$ type of overlapping motifs, with class I and class II sharing the same central PXXP in opposite orientation at the binding cleft flanked by positively charged residues at the specificity site of the SH3. This type represents the shortest possible bipolar overlapping motif. One $O_{11}^{ab}$ was present as well, with two overlapping class I motifs, offset by four residues (modules 179, 188, and 197). In contrast, human A-band motifs are mostly single classes with just a few overlapping ones (supplemental Table ST1).

Our peptide array binding studies revealed several key features that affect ligand affinity to nSH3 by these natural ligands. For native PEVK modules that contain motifs (KXXPXP(P/A/I/K)) from exons 172, 173, 182, 191, 175, 184, 193, 200, 201, 202, 177, 186, 195, 181, 190, and 199, the interaction with nSH3 is strong. Natural evolution from Ala to Pro (as exons 175, 184, and 193) or Ile to Pro (as exons 177, 186, and 195) weakened their binding. The presence of Ser residue within PXXP motif (exons 201, 181, 190, and 199) may also have weakened the binding. A single residue mutation can in fact impart SH3 binding capacity to some of the negative PEVK modules, indicating that these silent ligand motifs are poised for action upon further evolutionary changes.

The nSH3 domain has been classified previously as a class II ligand SH3, based on NMR studies of human nebulin SH3 (6610–6669, AC: X83957) and modeling of selected peptide ligand sequences (61). As clearly revealed by the peptide array screenings, nebulin SH3 binds both class II ligands (mainly in D-titin and I-connectin). The origin of this dual ligand orientation specificity is still unclear. Fernandez-Ballester et al. (43) classified nebulin SH3 as an SH3-III domain, based on the tilting of the conserved Trp indole ring plane toward the conserved proline of the triad. Such SH3-III domains bind not only to class II ligand.

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motifs but also to a subset of class I ligands in the opposite orientation if the motifs mimic class II ligand by residues such as leucines at $P_{-1}$ and $P_2$ (43). In this regard, titin class I ligands contain Lys (21), Leu (3), Val, Thr, Glu, Pro, Ile (2), Arg, and His (1) at position $P_{-1}$ and Lys (15), Glu (6), Gln (4), Ala, Pro (3), Thr (2) and Leu, Arg, and Ser (1) at position $P_2$. Both positions $P_{-1}$ and $P_2$ are not occupied simultaneously by prolines (supplemental Table ST1). Thus, some of these class I ligands might be class II mimics. This may also explain why Pastore and co-workers (61) did not detect class I motif binding to nSH3, because these authors only investigated class I ligand sequences with Pro at positions $P_{-1}$ and $P_2$.

Our NMR and molecular modeling of a 30-mer ligand, however, suggest that the nSH3 may well be ambivalent in its ligand preference, given a choice of sufficiently long ligand to reach and embrace the sites outside of the main cleft. The acidic clusters near the RT and nSrc loops may play an important role for the module 172-based overlap ligand. It is reasonable to expect that other ligands and other SH3 domains may have distinct sets of outlier residues that allow a more embracing and pair-wise-specific contacts between the ligand and the SH3 domains. Such interactions are unlikely to reveal themselves by gazing at mutagenized ligand sequences, in the absence of high resolution structural data for the ligand-SH3 complexes. It is worth noting that such overlapping motifs are not unique to the titin family and have been alluded to occasionally in several SH3-binding proteins with proline-rich motifs (52).

**Structural Basis of Overlap Ligand/nSH3 Interactions**—The affinities and the detailed conformation of the nSH3-PEVK modules complexes varied with sequences and temperature. In particular, the 12-mer PR2, representing a typical class $O^b$ motif, binds to nSH3 with 77 $\mu$M affinity at 22 °C and at 31 $\mu$M at 2 °C. The increase of affinity at lower temperature may result from a higher stability of PPII helix in PR2 at lower temperature as well as a conformational change of nSH3 upon cooling, as detected by the change in intrinsic fluorescence of nSH3 (22). The crucial question whether the class $O$ motif binds SH3 in two orientations is demonstrated by NMR titration and docking and molecular dynamics modeling of PR to nSH3 (Fig. 4, C and D). Our preliminary fluorescence titration hinted at two binding constants of different affinity. $^5$ PR1 and PR3, representing the remainder of the peptides of the PEVK module (exon 172), do not by themselves bind to nSH3, and neither peptide contains standard SH3 motifs, despite the presence of a short PPII helix in each peptide (23, 24). Such flanking regions do indeed contribute to the affinity of the 30-mer PR to nSH3 as shown by the docking simulations (Fig. 4, D and E) where the peptide wraps around the SH3 domain allowing for oppositely charged residues to interact and thereby increasing the affinity of the peptide for nSH3.

**Overlap Ligand Motifs, Stereo Specificity, and Signaling Capacity**—The major presence of overlapping motifs in titin prompted us to examine their potential functional significance in the context of signaling proteins (Fig. 7). First, such a class of motifs could be unipolar (nI or nII), bipolar (nI + nII), or mixed polar (nI + mII), allowing SH3 the option to bind in either the same orientation (as in $O^b$), or different orientations with the same core motif (as in $O^b_1$, Fig. 7, A and B) or a shift in “reading frame” by only a few residues up or down the core PXXP recognition sequence (as in $O^b_2$, Fig. 7C). Second, depending on the degree of overlap and the structure of the ligand, the attached SH3 domains would be related spatially to each other by sliding laterally and/or rotating (for unipolar class $O$ motifs) or sliding and/or “flip-flopping” (for bipolar class $O$ motifs) (Fig. 7, B and C). As a result, the overlapping motifs would steer the attached SH3 to a different azimuthal angle at different core sites within the small span of the total motif. Third, such overlapping motifs may enhance the avidity of SH3 binding. Avidity of SH3 binding to these essentially bivalent or multivalent ligands is expected to be enhanced on a statistical basis relative to the affinity of each class I or class II alone, similar to the enhancement of avidity of decavalent IgM than the bivalent IgG. Fourth, these motifs may accelerate receptor binding by presenting more choices of ligand orientations and affinity. They may also decelerate dissociation by allowing the bound receptor to switch positions internally among local motifs, without dissociating completely and paying the kinetic penalty of additional translational diffusion-limited steps.

Our experimental demonstration that most of these ligand motifs do indeed bind to SH3 domains and SH3-containing proteins indicated that these modules or domains are fundamental building blocks for the construction of multiple ligand sites. For titin PEVK segments, the extensive alternative splicing would give rise to a broad range of signaling potential in the different titin isoforms. For the A-band region, in contrast, the ligand sites are encoded by the genomic sequence of the long titin exon, without splicing. The signaling potential of newly identified titin isoforms can thus be assessed, as a first approximation, by identifying the exon usage at the sequence level and ligand binding attributes from data presented in Fig. 1 and supplemental Table ST1. The preferential utilization of overlapping motifs in close proximity in human fetal muscle PEVK is notable and suggested that titin PEVK is more active and complex in its involvement in SH3-dependent pathways in the fetal stage.

**PEVK and Polyproline Regions as a Force-sensitive Controller and Integrator of Inter-SH3 Receptor Interactions**—The high density and clustering of motifs, as demonstrated by several stretches of tandem repeats of five to six motifs in human skeletal PEVK, may impart functionality and attributes to qualify PEVK as an attractive candidate for the role of an integrator or controller of SH3 signaling pathways. The numerous binding motifs along the long string of open and flexible PPII helices and coils (23) are ideally suited for PEVK to recruit and accommodate and promote interactions of a large number of SH3 proteins to form multireceptor signaling complexes. The signaling proteins interact with each other in ways that are dictated by the spacing, orientation, and strength of each binding motif, the class type of SH3 domains, and the relative orientation and flexibility of the SH3 domain and the linkage to its host protein, much in the similar manner that messenger RNAs recruit and coordinate binding of ribosomes, tRNAs, and a myriad of factors in the protein synthesis machinery. Another theoretical advantage, illustrated diagrammatically in Fig. 7D, is that the potential flip-flopping and sliding of the SH3 domains along the length the overlapping motifs provide a facile and efficient way.
of expediting binding and unbinding and fine-tuning of the stereospecific interactions of adjacent SH3 signaling proteins. The flaccid and elastic polypeptides of PEVK segments would thus impose a general spatial confinement of bound receptors while allowing structural flexibility or elasticity to optimize and modulate inter-receptor interactions.

We propose that the titin overlapping motifs with multiple orientations may well be the basis for its broad multireceptor avidity and its key role as a hub in the interaction network. We have also noted in a survey that overlapping motifs are present in other hubs of signaling pathways.\(^6\) We predict that such specially clustered overlapping motifs are the structural basis for integrating and coordinating SH3 signaling pathways in non-muscle cells as well.

The innate elastic nature of the PEVK region (5) raises the exciting possibility that PEVK could modulate the strength, the spacing of SH3/receptor interaction, or even the spacing and relative orientation of SH3 proteins by sensing and responding to cellular strain and stress. Elsewhere, we have proposed that the PEVK segment is a polyampholyte that wraps into a nanogel allowing structural flexibility or elasticity to optimize and modulate inter-receptor interactions. The force-induced partial unfolding/folding of these terminal strands from the \(\beta\)-barrels could be a prerequisite for the access and binding/unbinding of SH3-containing proteins. If this notion is correct, titin may well be a sophisticated signal integration molecule that acts as both as a stress sensor and an actuator, by utilizing its sensitivity to mechanical stress and strain to control the trafficking of multiple signaling molecules that are essential in myofibril assembly, cell motility, sarcomere turnover, and mechanical response (11).

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