The Hydrophilic Domain of Small Ankyrin-1 Interacts with the Two N-terminal Immunoglobulin Domains of Titin*

Aikaterini Kontrogianni-Konstantopoulos‡ and Robert J. Bloch
From the Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Little is known about the mechanisms that organize the internal membrane systems in eukaryotic cells. We are addressing this question in striated muscle, which contains two novel systems of internal membranes, the transverse tubules and the sarcoplasmic reticulum (SR). Small ankyrin-1 (sAnk1), a muscle-specific isoform of the erythroid ankyrin-1 gene that is concentrated in the network SR of striated muscle fibers, surrounding the Z-disks and M-lines (6). Ankyrins are a family of proteins that possess binding sites for diverse integral membrane proteins as well as cytoskeletal components (7–9). To date, molecular cloning has identified three distinct ankyrin genes in mammals (Ank1, Ank2, and Ank3) that are expressed as tissue-specific, alternatively spliced isoforms (10–12). Ank1 is expressed predominantly in erythroid cells, striated muscle, and brain (13–15); Ank2 in smooth muscle (16–19); and Ank3 in cells of epithelial origin and striated muscle as well as in lysosomes and Golgi membranes in a wide variety of cells (20–23). The large canonical ankyrins share a similar structure, consisting of an N-terminal ~89-kDa membrane-binding domain, a central ~62-kDa spectrin-binding domain, and a C-terminal ~55-kDa regulatory domain (10, 11).

In striated muscle, the products of the Ank1 gene include the large (~210 kDa) and small (~17–19 kDa) ankyrin isoforms (6, 15). sAnk1 lacks both the membrane- and spectrin-binding regions of the larger form and has a C-terminal domain that is significantly shortened (24, 25). The N-terminal portion of sAnk1 contains a unique 73-amino acid segment, whereas the C-terminal 82 residues are identical to the C-terminal sequence of the large ~210-kDa ankyrin-1. The first 29 residues of sAnk1 are highly hydrophobic and target the molecule to the SR membrane, whereas the remaining 126 amino acids extend into the myoplasm. Thus, the hydrophilic tail of sAnk1 is appropriately oriented in the cytoplasm of striated muscle fibers to serve as a binding site for sarcomeric proteins.

Here, we describe a direct and specific association between sAnk1 and titin, also known as connectin (25–29). Titin is a giant (~2.7–4 MDa) protein that extends from the Z-disk to the M-line within the sarcomere, which it helps to organize. It is highly modular: ~90% of its mass is composed of repeating Ig-C2 and fibronectin-3-like domains that provide binding sites for myofibrillar proteins (31, 32). The remaining ~10% consists of unique non-repetitive sequence motifs, including phosphorylation sites, binding sites for muscle-specific calpain proteases, and C-terminal Ser/Thr kinase domains (30, 33–35).

The C-terminal 2 MDa of titin are located within the A-band, where titin tightly associates with the myosin thick filaments and several A-band proteins such as C-protein, M-protein, and myomesin (36–38). The most C-terminal end of the molecule (~200 kDa), which is embedded in the M-line, contains a Ser/Thr kinase domain, which implicates titin in myofibrillar signal transduction pathways (37–40). In the I-band, titin (~800 kDa to 1.5 MDa) carries proline/glutamate/valine/lysine-rich sequences, which confer great extensibility to the titin filaments (35, 41–44), in addition to numerous Ig domains. At the

* This work was supported in part by National Institutes of Health Grant R01 HL64304 (to R. J. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of National Institutes of Health Fellowship T32 AR07293.

§ To whom correspondence should be addressed: Dept. of Physiology, University of Maryland School of Medicine, 685 W. Baltimore St., Baltimore, MD 21201. Tel.: 410-706-4410; Fax: 410-706-8341; E-mail: akons001@umaryland.edu.

1 The abbreviations used are: T-tubules, transverse tubules; SR, sarcoplasmic reticulum; sAnk1, small ankyrin-1; GST, glutathione S-transferase; MBP, maltose-binding protein.

2 N. Porter, W. Resneck, A. O'Neill, D. van Rossum, and R. J. Bloch, unpublished data.
Small Ankyrin Binding to Titin

**EXPERIMENTAL PROCEDURES**

**Generation of Titin, sAnk1, and T-cap Expression Constructs**—PCR amplification was used to obtain cDNA from the Z-disk (80–120 kDa) region of titin. Several copies of a 45-residue repeat, called the Z-repeat, bind α-actinin within the Z-disk (47–49). The two most N-terminal Ig domains of titin, which are constitutively expressed in all titin isoforms and reside in the periphery of the Z-disk, bind a recently identified, 19-kDa protein of striated muscle, referred to as T-cap or telethonin (47, 50).

Titin has two functions in striated muscle: as a “molecular blueprint” for sarcomeric protein assembly during myofibrillogenesis and as a “molecular spring” that maintains myofibrillar integrity during contraction, relaxation, and stretch (27, 30, 32). Our results show that, in addition to binding T-cap, the two N-terminal Ig domains of titin interact specifically with sAnk1, suggesting that titin also coordinates the assembly of the contractile apparatus with the network SR that surrounds the Z-disk.

**Generation of Titin, sAnk1, and T-cap Expression Constructs**—PCR amplification was used to obtain cDNAs from DNA fragments of the Z-disk (~80 kDa) and M-line (~200 kDa) (37) portions of titin. cDNA from human cardiac muscle (Origene Technologies Inc., Rockville, MD) was used as template, and the following sets of custom oligonucleotide primers were generated, based on the sequence of human cardiac titin (GenBank™/EBI accession number X90568). For amplification of ZIG1/2, the two most N-terminal Ig domains of titin, primers A (5′-ACGTGAATTCATGCAGACACCAAGGACACCCG-3′, sense) and B (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, antisense) were used. For ZIG2/3 (including ZIG1 only), the sense primer C was used in combination with the antisense primer C (5′-ACGTCCGAGCTCTTACAGGGAAGGCTC-5′, sense). For generation of ZIG2/3-B (containing ZIG2 only), primers D (5′-ACGTGAAATGGAGAAAGCTC-3′), B (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′), and E (5′-ACGTGCAGAGCCACCTCCGACCAACC-3′) was used along with the antisense primer B. For ZIG3, the Ig domain just C-terminal to ZIG2/3, primer F (5′-ACGTGAAATGGAGAAAGCTC-3′), and primers B (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense) were used. Amplification of ZIG2/3 (for Z-repeat), the region of titin that interacts with α-actinin, primers G (5′-ACGTGAAATGGAGAAAGCTC-3′), and primers H (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense) were used with primer H (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense). Amplification of Zr (for Z-repeat), the region of titin that interacts with α-actinin, primers I (5′-ACGTGAAATGGAGAAAGCTC-3′), and primers J (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense) were used. For generation of ZIG2/3-B (containing ZIG2 only), primers D (5′-ACGTGAAATGGAGAAAGCTC-3′), B (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense), and H (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense) were used in combination with primer F (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense). For amplification of MIg1/2, primer K (5′-ACGTGAAATGGAGAAAGCTC-3′), and primers B (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense) were used with primer H (5′-ACGTCTCGAGGAGTTACCCTTTCACC-3′, sense). All sense primers contained an EcoRI recognition sequence, whereas all antisense primers contained an XhoI site for insertion into the yeast vector (pMal-c2X). Glutathione S-transferase (GST) fusion proteins. The titin ZIG2/3 fragment was also introduced into the pMAL-c2X vector at XhoI sites after PCR amplification with primers 1 (5′-ACGTGAATTCATGGCTACCTCAGAGCTG-3′, sense) and 2 (5′-ACGTGAATTCGTCAAAAAGA-CTCTCCACCAGGGTG-3′, antisense). For generation of MIg1/2, for 12 h at 4 °C, beads were washed in the cold with 10 mM NaPO4 (pH 7.2), 120 mM NaCl, 10 mM NaN3, and 0.1% Tween 20 and heated for 5 min at 90 °C in 2× SDS Laemmli sample buffer. The soluble fraction was analyzed by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to sAnk1, diluted in buffer C. Immunoreactive bands were visualized with a chemiluminescence detection kit (Tropix Inc., Bedford, MA).

**Blot Overlay**—The blot overlay assay was performed as previously described with minor modifications (51). Briefly, 2× 0.5 μg of bacterially expressed, affinity-purified GST and GST-ZIG1/2 proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. Non-specific sites on the nitrocellulose membranes were blocked in buffer A (50 mM Tris (pH 7.2), 120 mM NaCl, 3% bovine serum albumin, 2 mM dithiothreitol, 0.5% Nonidet P-40, and 0.1% Tween 20) plus protease inhibitors for 3 h at 25 °C and then incubated with 3 μg/ml MBP-sAnk1 fusion protein in the same buffer for 16 h at 4 °C. Blots were washed five times (15 min each) with buffer A and once with buffer B (1× phosphate-buffered saline (pH 7.2), 10 mM NaN3, and 0.1% Tween 20). Subsequently, they were incubated in buffer C (1× phosphate-buffered saline (pH 7.2), 10 mM NaN3, 0.1% Tween 20, and 5% dry milk) and probed with HRP-labeled antibodies to sAnk1, diluted in buffer C. In a parallel set of experiments, GST-Ank1 and GST-T-cap bound to glutathione matrices were allowed to interact with 5 μg of bacterially expressed, affinity-purified GST-ZIG1/2 fusion protein (i.e., 5′-ACGTGAATTCATGGCTACCTCAGAGCTG-3′, sense) and 2 (5′-ACGTGAATTCGTCAAAAAGA-CTCTCCACCAGGGTG-3′, antisense). For generation of MIg1/2, for 12 h at 4 °C, beads were washed in the cold with 10 mM NaPO4 (pH 7.2), 120 mM NaCl, 10 mM NaN3, and 0.1% Tween 20 and heated for 5 min at 90 °C in 2× SDS Laemmli sample buffer. The soluble fraction was analyzed by 12% SDS-PAGE, transferred to nitrocellulose, and probed with antibodies to sAnk1, diluted in buffer C. Immunoreactive bands were visualized with a chemiluminescence detection kit (Tropix Inc., Bedford, MA).

**In Vitro “Competition” Assay—**Equal amounts of GST, GST-sAnk1, and GST-T-cap bound to glutathione matrices were allowed to interact with 5 μg of bacterially expressed, affinity-purified GST-ZIG1/2 fusion protein (i.e., 5′-ACGTGAATTCATGGCTACCTCAGAGCTG-3′, sense) and 2 (5′-ACGTGAATTCGTCAAAAAGA-CTCTCCACCAGGGTG-3′, antisense). For generation of MBP-T-cap fusion protein, the full-length pET9D-Tcap plasmid (a generous gift from Drs. S. Labeit (European Molecular Biology Laboratory, Heidelberg, Germany) and C. C. Gregorio (University of Arizona, Tucson, AZ)) was used as template to obtain a PCR fragment that contained amino acids 1–140 (47). Primers 7 (5′-ACGTGAATTCCATAGTGAAGACTCCGTG-3′, sense) and 8 (5′-ACGTGCACACTGATCTCTCCGCGCCGCG-3′, antisense), carrying EcoRI and SalI sites, respectively, were used for insertion into the pMAL-c2X vector. T-cap (1–140) was also introduced into the pEXKT4-T vector at EcoRI/XhoI sites (XhoI and SalI have compatible ends) to produce a GST fusion protein. The authenticity of the obtained constructs was verified by sequencing analysis. GST and MBP recombinant polyepitopes were expressed by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 3 h and purified by affinity chromatography on glutathione Sepharose 4B (GST) or an amylose resin (for MBP fusion proteins) (New England Biolabs, Inc.) columns following the manufacturer’s instructions.

**Yeast Two-hybrid and β-Galactosidase Assays—**The Matchmaker LexA two-hybrid system (Clontech) was used as recommended by the manufacturer. The pB42AD prey vector and the pLexA bait vector were used to express titin (i.e., ZIG1/2, ZIG2/3-B, ZIG2/3-A, Zr, ZIG1/2, ZIg4/5, MIg1/2, and MIg5/6) and sAnk1 (i.e., sAnk1-29–155, sAnk1-A, sAnk1-B, and sAnk1-C) hybrid peptides, respectively, as described above. Saccharomyces cerevisiae strain EGY48 was sequentially transformed with reporter pSpo-lacZ, bait, and prey plasmids. True transformants were selected by plating on an induction medium (i.e. synthetic dropout media lacking Ura, Tryp, and Leu), in the presence of 80 mg/liter 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Liquid β-galactosidase assays were performed as described in the Clontech Yeast Protocols Handbook using chlororphen red β-D-galactopyranoside as substrate. For each interaction tested, four independent colonies were assayed, and each experiment was repeated twice. Results represent average fold values.


**RESULTS**

**Binding of sAnk1 to the Two Most N-terminal Ig Domains of Titin Shown with the Yeast Two-hybrid Assay**—sAnk1 is an ~17–19-kDa integral membrane protein of the network SR that has a 126-aminio acid sequence extending into the sarcomplasm surrounding Z-disks and M-lines (6). We used the yeast two-hybrid assay to test the idea that the hydrophobic sequence of sAnk1 (sAnk1-(29–155)) interacts with the giant myofibrillar protein titin, which spans each half-sarcomere from the Z-disk to the M-line. We inserted cDNA encoding the hydrophobic cytoplasmic domain of sAnk1 (sAnk1-(29–155)) into the yeast two-hybrid bait vector (Fig. 1A) and assayed its ability to interact with the N-terminal ~80-kDa portion of titin that resides in the Z-line (47), expressed by a series of constructs inserted into the yeast two-hybrid pB42AD prey vector (Fig. 1B). Specifically, the PCR products of titin we assayed were ZIG1/2 (amino acids 1–200), ZIG3 (amino acids 201–557), the Z-repeats or Zr domain (amino acids 558–910), and ZIG4/5 (amino acids 911–1118) (see “Experimental Procedures”). Yeast two-hybrid analysis followed by qualitative liquid β-galactosidase assays (Fig. 1C) indicated that sAnk1-(29–155) specifically interacted with the two most N-terminal Ig domains of titin, ZIG1/2 (amino acids 1–200), which reside at the edge of the Z-disk (28, 47). No specific association between sAnk1-(29–155) and the remaining ~60-kDa portion of Z-disk titin could be detected (Fig. 1C).

In additional tests of the specificity of the interaction with ZIG1/2, we generated two additional “prey” constructs encoding tandem Ig domains that reside in the M-line region of titin (37–39). These included MIG1/2 (amino acids 25250–25422) and MIG5/6 (amino acids 26281–26478). When their ability to interact with sAnk1-(29–155) was tested in a yeast two-hybrid assay, no specific interaction was observed (Fig. 1C). Although we were unable to test the ability of other M-line domains of titin to interact with sAnk1 in this assay, our results suggest that sAnk1 interacts preferentially with Ig domains at the N-terminal region of titin, located at the Z-disk.

**Mapping the Binding Sites on sAnk1 and Titin ZIG1/2**—We used the yeast two-hybrid assay to identify more precisely the sequences required for the binding of sAnk1 to titin (Fig. 2). Three subfragments of the hydrophobic portion of sAnk1 were inserted into the pLexA bait vector: sAnk1-A (amino acids 29–89), sAnk1-B (amino acids 90–155), and sAnk1-C (amino acids 61–130) (Fig. 2A). Two truncated titin ZIG1/2 prey constructs, ZIG1 (amino acids 1–99) and ZIG2 (amino acids 100–200), were also generated to assay the ability of each of the N-terminal Ig domains of titin to bind sAnk1 independently (Fig. 2B). Yeast two-hybrid analysis followed by liquid β-galactosidase assays showed that the ZIG1/2, ZIG3, Zr, and ZIG4/5 or parts of the M-line region of titin (e.g. MIG1/2 and MIG5/6) were inserted into the pB42AD prey vector. C, yeast two-hybrid analysis followed by liquid β-galactosidase (β-gal) assay indicated that sAnk1 specifically interacted with the two most N-terminal Ig domains of titin, ZIG1/2. Other regions of titin from the N-terminal region, associated with Z-disks, or the C-terminal region, proximal to M-lines, failed to interact with sAnk1.
Small Ankyrin Binding to Titin

subfragments (i.e. amino acids 61–89) contain the binding site for titin ZIg1/2 (Fig. 2A). Furthermore, both ZIg1 and ZIg2 were required for titin to bind to sAnk1 because sAnk1 failed to interact with the individual Ig domains (Fig. 2B).

Binding of sAnk1 in Muscle Homogenates to GST-ZIg1/2 in a Pull-down Assay—To confirm the specificity of the interaction between sAnk1 and titin ZIg1/2, we performed a GST pull-down assay using homogenates of skeletal muscle from adult rats. We expressed ZIg1/2, ZIg3, Zr, ZIg4/5, MIg1/2, and MIg5/6 as GST fusion proteins (Fig. 3). The calculated molecular masses of the GST fusion proteins are ~47, ~65, ~64, ~48, ~44, and ~47 kDa, respectively (Fig. 3A). GST-ZIg3 and GST-Zr showed some degradation, probably due to endogenous bacterial proteases, whereas GST-ZIg4/5 migrated with an apparent molecular mass of ~60 kDa instead of the calculated ~48 kDa. Equivalent amounts of these proteins and control GST (25 kDa) were bound to glutathione matrices and incubated with homogenates of quadriceps muscle. The matrix-

FIG. 3. Binding of native sAnk1 from skeletal muscle homogenates to titin ZIg1/2. A, GST fusion proteins containing portions of the Z-disk and M-line regions of titin (i.e. GST-ZIg1/2, ~47 kDa; GST-ZIg3, ~65 kDa; GST-Zr, ~64 kDa; GST-ZIg4/5, ~60 kDa; MIg1/2, ~44 kDa; and MIg5/6, ~47 kDa) and control GST protein (25 kDa) were analyzed by SDS-PAGE and visualized by staining with Coomassie Blue. B, equivalent amounts of these recombinant proteins bound to glutathione matrices were incubated with homogenates of adult rat quadriceps skeletal muscle. Binding of native sAnk1 to the titin fragments was examined by immunoblot analysis with anti-sAnk1 antibodies. Only GST-ZIg1/2 specifically retained native sAnk1.
Small Ankyrin Binding to Titin

We generated the following fusion proteins for these experiments: GST-sAnk1-(29–155) (~39 kDa), MBP-sAnk1-(29–155) (~56 kDa), GST-T-cap-(1–140) (~40 kDa), MBP-T-cap-(1–140) (~57 kDa), and MBP-ZIg1/2 (~64 kDa) (Fig. 5A). We included the N-terminal 1–140 residues (~16 kDa) of T-cap in the fusion protein, as they contain the binding site for titin ZIg1/2 (47, 50).

Equivalent amounts of GST-sAnk1, GST-T-cap, and GST protein bound to glutathione matrices were incubated with MBP-ZIg1/2. Both GST-sAnk1 and GST-T-cap specifically retained recombinant titin ZIg1/2, whereas control GST protein did not, as shown by Western blot analysis with anti-titin ZIg1/2 antibody (Fig. 5B). In a set of parallel assays, GST-sAnk1 and GST-T-cap attached to glutathione beads were initially allowed to interact with MBP-ZIg1/2. Following removal of unbound recombinant titin ZIg1/2, affinity-purified MBP-T-cap and MBP-sAnk1 were added to GST-sAnk1-MBP-ZIg1/2 and GST-T-cap-MBP-ZIg1/2 complexes, respectively, or to control matrix-bound GST protein. The presence of MBP-ZIg1/2, MBP-T-cap, and MBP-sAnk1 was examined in each sample by immunoblot analysis with the appropriate antibodies (i.e. anti-titin ZIg1/2, anti-T-cap, and anti-sAnk1) (Fig. 5, B–D). Both recombinant sAnk1 (Fig. 5C) and T-cap (Fig. 5D) bound efficiently and specifically to GST-T-cap-MBP-ZIg1/2 and GST-sAnk1-MBP-ZIg1/2 complexes, respectively. To rule out the possibility of a direct association between sAnk1 and T-cap, equivalent amounts of GST-sAnk1 and GST-T-cap adsorbed to glutathione matrices were also incubated with affinity-purified MBP-T-cap and MBP-sAnk1, respectively, under the same experimental conditions. No association was observed between the two proteins (data not shown). Our results indicate that the two N-terminal Ig domains of titin (ZIg1/2) simultaneously bind sAnk1 and T-cap in vitro, forming a three-way complex.

Subcellular Distribution of sAnk1, Titin ZIg1/2, and T-cap in Adult Skeletal Muscle Fibers—It has been well documented that sAnk1, titin ZIg1/2, and T-cap are present at the Z-lines of sarcomeres (6, 47, 54). To examine their topography with re-
spect to the Z-disk, we used antibodies against sAnk1, titin ZIg1/2, and T-cap to label longitudinal sections and cross-sections of muscle fibers by immunofluorescence, followed by confocal microscopy. As expected, each of the antibodies labeled the Z-lines in longitudinal sections of adult rat quadriceps muscle (Fig. 6, A–C); in addition, anti-sAnk1 antibody also labeled M-lines (Fig. 6A) (6). Cross-sections of muscle labeled with the same panel of antibodies showed sAnk1 in a reticular pattern, consistent with its distribution in the network SR (Fig. 6D) (6). By contrast, both titin ZIg1/2 and T-cap concentrated at the Z-disks (Fig. 6, E and F). No labeling was detected when primary antibodies were replaced with nonimmune rabbit or goat IgG (Fig. 6, G and H). Thus, titin ZIg1/2 and T-cap are present at the Z-disk, whereas sAnk1 is concentrated in the SR surrounding the Z-disk. Thus, if a complex between sAnk1 and titin forms in skeletal myofibers, with or without T-cap/telethonin, it would be limited to the periphery of the Z-disk.

**DISCUSSION**

A key question in the biology of striated muscle is how the internal membranes of the SR and the T-tubules become precisely aligned with the contractile apparatus. We have begun to address this question by identifying ligands of sAnk1, a structural protein of the network SR that, we hypothesize, helps to coordinate the alignment of the SR with nearby M-lines and Z-discs. Because titin serves as a molecular blueprint for the assembly of other myofilibrillar elements, we postulated that it might also provide a site for anchoring the SR at the level of the Z-disk. We found that the hydrophilic sequence of sAnk1, which extends from the SR membrane into the sarcoplasm (6), specifically and directly interacted with the two most N-terminal Ig domains of the giant myofibrillar protein titin. These two domains, ZIg1 and ZIg2, are present in all titin muscle isoforms identified to date and are localized at the periphery of the Z-disk lattice (47). These domains of titin are therefore appropriately positioned to anchor sAnk1 in the network SR to the Z-disk. sAnk1 carries a C-terminal hydrophilic sequence that differs significantly from the C-terminal portion of the large canonical form of ankyrin-1. It is considerably shortened (−14 versus −55 kDa) and contains a unique peptide sequence (amino acids 29–73); the remaining 82 residues (amino acids 74–155) are shared by both small and large splice forms of ankyrin-1, followed by a common translation stop codon (24, 25). The results of our yeast two-hybrid experiments suggest that a peptide 29 amino acids long (residues 61–89) contains the minimal sequence in sAnk1 with binding activity for titin ZIg1/2. Thus, the titin-binding site on sAnk1 may be comprised of residues unique to sAnk1 (i.e. amino acids 61–73) and amino acids shared by both small and large forms of Ank1 (i.e. amino acids 74–89). It is of course possible that the binding of sAnk1 to titin ZIg1/2 requires only some of these 29 residues. Future studies will delineate more precisely whether amino acids that are unique to sAnk1 or shared with larger forms of Ank1 are needed to form the titin-binding site on sAnk1.

Both the ZIg1 and ZIg2 domains of titin were required for binding to sAnk1. This is not surprising, as the binding of many titin ligands, such as myomesin, M-protein, myosin-binding protein C, obscurin, and T-cap/telethonin, requires the presence of pairs of titin Ig domains (37, 38, 41, 47, 50, 54–56). Indeed, the Ig domains of the defining members of the Ig superfamily, the immunoglobulins, act in tandem to form the binding sites of antibodies (57). The requirement of both ZIg1 and ZIg2 implies that the binding site of titin for sAnk1 includes residues from both Ig domains. Preliminary observations from our laboratory indicated that sAnk1 may homodimerize or multimerize in vitro. Thus, a sAnk1 dimer or multimer may be the active ligand for the ZIg1/2 region of titin.

Interestingly, ZIg1 and ZIg2 share a highly conserved peptide (i.e. SGXYSΦXATX, where X is a nonconserved amino acid and Φ is a nonpolar hydrophobic residue) that could serve as the binding site of a potential sAnk1 dimer. Although further experimentation will be required to address this issue, the results of our in vitro binding studies suggest that dimerization of sAnk1 may not be required for it to bind to titin ZIg1/2.

The two N-terminal Ig domains of titin were previously shown to contain the binding site of a Z-disk protein referred to as T-cap or telethonin (47, 50). Similar to sAnk1, the binding of T-cap to titin requires the presence of both ZIg1 and ZIg2. Titin ZIg1/2 can bind simultaneously to both sAnk1 and T-cap, indicating that these proteins can form a three-way complex.

The subcellular location of these proteins places limits on where this complex could form. T-cap and titin ZIg1/2 co-localize at the edge of the Z-disk lattice, where their binding is believed to anchor the N-terminal portion of titin to the Z-disk (47). By contrast, sAnk1 is limited to the SR at the periphery of the Z-disk, where its C-terminal hydrophilic sequence extends from the SR membrane (6).2 sAnk1 is therefore likely to interact with titin and possibly form a three-way complex with T-cap only at the periphery of the Z-disk (Fig. 7). As postulated, this interaction would link the network SR, where sAnk1 is concentrated, to the Z-disk.

T-cap was recently shown to bind to MinK, the β-subunit of the potassium channel of the transverse tubular membranes (58). This suggests that, just as sAnk1 links titin at the periphery of the Z-disk to the SR, T-cap may link the same population of titin molecules to the T-tubules. These interactions can occur in mammalian cardiac muscle and in avian and amphibian striated muscle, where both the SR and T-tubules are located around the Z-disk. In mammalian skeletal muscle, however, this function is likely to be limited to sAnk1, as only the SR is concentrated around the Z-disk. The T-tubules in skeletal muscle are present at the junction of the A- and I-bands and so are unlikely to be anchored via T-cap to titin at the Z-disk. Thus, through its ability to bind simultaneously to sAnk1 and T-cap, titin may simultaneously serve as a scaffold for assembling not only the contractile apparatus, but also the network SR and, in many (but not all) striated muscles, the T-tubules.

Titin is the second myofilibrillar protein that we have identified as a major cytoplasmic ligand for sAnk1: obscurin also binds to sAnk1 (53). Obscurin is a giant (~800 kDa) sarcomeric Rho guanine nucleotide exchange factor protein with homology to titin (55). Immunolocalization studies have shown that obscurin closely surrounds the myofilaments at the Z-discs and M-lines of each sarcomere (53). Thus, it appears that, whereas titin associates with sAnk1 at the level of the Z-disk, obscurin interacts with sAnk1 around both Z-discs and M-lines.

We have previously shown that amino acids 61–130 of the C-terminal hydrophilic sequence of sAnk1 contain the binding site for obscurin (53). In the present study, we have shown that residues 61–89 of sAnk1 are likely to contain the binding site for titin ZIg1/2. These findings suggest that the sAnk1 binding sites for titin and obscurin may overlap. Future studies will examine whether titin and obscurin compete with each other for binding to sAnk1 around the Z-disk in developing or mature striated muscle and whether one or both of these giant sarcomeric proteins play an important role in aligning the SR with the contractile apparatus.

Acknowledgments—We thank Drs. S. Labeit and C. C. Gregorio for providing the full-length pET9D-T-cap plasmid and Dr. C. C. Gregorio for sharing anti-titin-x112 with x113 antibody. We also thank W. G. Resneck and A. O’Neill for expert assistance.
