J. Mol. Biol. (1997) **270**, 688–695

JMB

Tissue-specific Expression and α-Actinin Binding Properties of the Z-Disc Titin: Implications for the Nature of Vertebrate Z-Discs

H. Sorimachi[1], A. Freiburg[2], B. Kolmerer[2], S. Ishiura[1], G. Stier[2] C. C. Gregorio[3], D. Labeit[4], W. A. Linke[5], K. Suzuki[1] and S. Labeit[2]*

[1]*Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, 113, Tokyo, Japan*

[2]*EMBL Heidelberg Meyerhofstr. 1 69012, Heidelberg, Germany*

[3]*Dept. of Cell Biology & Anatomy, University of Arizona, 1501 N. Campbell Ave, Tucson, AZ 85724, USA*

[4]*Department of Gynaecology Universitätsklinikum Mannheim, Theodor-Kutzer-Ufer, 68167, Mannheim Germany*

[5]*Physiology II, University of Heidelberg, Im Neuenheimer Feld 326, 69120, Heidelberg Germany*

**Corresponding author*

Titins are giant filamentous proteins which connect Z-discs and M-lines in the sarcomeres of vertebrate striated muscles. Comparison of the N-terminal region of titin (Z-disc region) from different skeletal and cardiac muscles reveals a 900-residue segment which is expressed in different length variants, dependent on tissue type. When searching for ligands of this differentially expressed domain by a yeast-two hybrid approach, we detected binding to α-actinin. The isolated α-actinin cDNAs were derived from the C-terminal region of the α-actinin isoform (α-actinin-2) encoded by the ACTN2 gene. Therefore, the two antiparallel subunits of an α-actinin-2 homodimer will attach to actin at their respective C termini, whereas they will bind to the Z-disc titin at their N termini. This may thus explain how α-actinins can cross-link antiparallel titin and thin filaments from opposing sarcomeres. The α-actinin-2 binding site of the Z-disc titin is located within a sequence of 45-residue repeats, referred to as Z-repeat region. Both the N-terminal and C-terminal Z-repeats have α-actinin binding properties and are expressed in all striated muscles. By contrast, the more central Z-repeats are expressed in slow and fast skeletal muscles, as well as embryonic and adult cardiac muscles, in different copy numbers. Such alternative splicing of the Z-disc titin appears to be important for the tissue and fibre type diversity of the Z-disc lattice.

Keywords: titin (connectin); alpha-actinin; Z-discs; striated and smooth muscles; alternative splicing

Introduction

Titin, also called connectin, is a giant filamentous protein of vertebrate striated muscles (for reviews, see Maruyama, 1994; Trinick, 1994; Labeit *et al.*, 1997). Single titin molecules, when isolated in the native state, are approximately 1.3 μm long (Suzuki *et al.*, 1994) and *in situ*, span an entire half-sarcomere from the Z-disc to the M-line (Fürst *et al.*, 1988). The complete titin polypeptide is formed by a single strand of 27,000 to 34,000 residues with a molecular weight of 3.0 to 3.7 MDa (Labeit & Kolmerer, 1995). The C terminus of the titin strand lies at the M-line, whereas titin's N terminus localizes to the Z-disc (Labeit *et al.*, 1992). Recently, antibodies specific to the N-terminal section of titin have been used to study the lay-out of the Z-disc region in more detail. Immunoelectron microscopic studies with these antibodies suggested that the titin filament extends all the way to the centre of the Z-disc, with approximately 600 to 800 N-terminal residues being located inside the Z-disc (Yajima *et al.*, 1996; Gautel *et al.*, 1996).

Our understanding of the lay-out and possible ligand interactions of the Z-disc titin is incomplete. It has been shown that during early muscle cell differentiation in 9-somite stage chick embryonic hearts (Tokuyasu & Maher, 1987), Z-disc titin epitopes become organised into cross-striational patterns at the time of first myofibril formation, and to co-localise with α-actinin spots. Similarly, a cross-striated organisation of Z-disc titin epitopes at an early developmental stage was also found in

H. Sorimachi and A. Freiburg contributed equally to the work.

Abbreviations used: RT–PCR, reverse transcriptase–polymerase chain reaction; 3-AT, 3-amino-1,2,4-triazole.

0022–2836/97/300688–08 $25.00/0/mb971145

cultured cardiac myocytes (Schultheiss *et al.*, 1992a). These findings raised the possibility that titin may co-assemble with α-actinin, and perhaps other Z-disc components, during early myogenesis. On the other hand, gel-overlay studies, using labeled α-actinin, have reported that nebulin, but not titin, has α-actinin binding properties (Nave *et al.*, 1990). In another study, it was found that during preparation of native titin, a contaminant co-purifying with titin had a similar molecular mass as α-actinin (Soteriou *et al.*, 1993). However, it was suggested from peptide mapping data that this ~100 kDa protein was not α-actinin. And finally, recent *in vitro* surveys of the interactions between expressed Z-disc titin fragments and purified muscle proteins revealed contradicting results: one study failed to detect titin-α-actinin interaction in dot blots (Gautel *et al.*, 1996), whereas another report did show binding of expressed fragments of zeugmatin (which is homologous to Z-disc titin) to α-actinin, by employing an immunoprecipitation technique (Turnacioglu *et al.*, 1996). Clearly, to resolve the above differences, other biochemical approaches appear to be needed.

In this study, we have characterised the primary structure of rabbit Z-disc titins from different muscle tissues, to investigate the incorporation of titin into the Z-disc. We have identified a maximally 900-residue-long N-terminal segment of titin, which in different muscle types is present in different length variants. The studied titin segment contains α-actinin binding sites, as found by employing a yeast-two hybrid method. Since the original submission of this manuscript, two reports from S. Kimura and colleagues have appeared, which have mapped α-actinin binding sites on titin by using also the yeast-two hybrid approach (Ohtsuka *et al.*, 1997a,b). Our data are in very good agreement with their mapping results. Thus, the yeast-two hybrid technique appears to be a more sensitive and more specific method for detecting interactions of titin with its ligands, compared with *in vitro* approaches using expressed fragments. By characterising in detail the molecular lay-out of the differentially expressed Z-disc titin region, we aim to improve our knowledge of the vertebrate Z-disc structure and enhance understanding of the dynamic response of Z-discs to mechanical stress (cf. Goldstein *et al.*, 1986, 1989).

Results

Tissue-specific expression of the N-terminal region of titin

Based upon the available human titin cDNA sequence (Labeit & Kolmerer, 1995), the 5 kb of 5′ end rabbit titin cDNAs were isolated by cross-species PCR and sequenced. Within this 5 kb segment, the sequences are highly conserved between rabbit and human (for an alignment of the rabbit and human Z-disc titin, see http://www.embl-heidelberg.de/ExternalInfo/Titin/). We then surveyed the titin transcripts expressed in a collection of rabbit striated and smooth muscle tissues with a set of rabbit titin primer pairs. A smooth muscle tissue was included, because it had recently been suggested that also this muscle type may express partial titin sequences (Turnacioglu *et al.*, 1996). Titin transcripts from the extreme 5′ end (bp 1 to 904) and from the segment bp 3730 to 5000 of the titin mRNA, respectively, were found to be expressed in all striated muscle tissues. Also, the amplified PCR fragments were of uniform length (data not shown). In the region connecting these two segments, bp 904 to bp 3730, many different size variants were detected (Figure 1a). A set of differently sized fragments from several species was gel-purified and sequenced. In the striated muscles, three major isoforms were detected in a region comprising bp 1300 to 2300 (Figure 1b). This segment is composed of a family of 45-residue repeats, referred to as Z-repeats (Gautel *et al.*, 1996). In adult rabbit cardiac muscle, seven copies of Z-repeats were found to be expressed (Figures 1b and 2), and were termed Zr1 to Zr7 (Figure 3). In fast twitch rabbit psoas muscle, the three repeats, Zr4 to Zr6, are excluded by exon skipping. In the red muscles of soleus and tongue, this latter isoform was co-expressed with a third variant expressing six Z-repeats and skipping only Zr4 (Figure 1b). The expression of different Z-repeat copy numbers is also regulated during development: in the adult human and rabbit heart, a variant comprising seven copies of Z-repeats is present, whereas in the fetal human heart, a mixture of isoforms is expressed. In the latter tissue type, the variant mainly found comprises four copies of Z-repeats (Figure 3).

From rabbit whole uterus (smooth muscle) RNA, a fragment was amplified that lacked the entire segment bp 904 to 3730 (Figure 1b). At present, it is unknown in which compartment of the uterus (e.g. myometrium, endometrium, etc.) this transcript variant is transcribed, and if it leads to the expression of a functional protein. To investigate whether the 2.8 kb segment of the Z-disc titin excluded in the uterus cDNA may be important for the assembly of Z-discs in striated muscle tissues, this 2.8 kb cDNA was used as a bait to carry out yeast-two hybrid screens (see below).

Interaction of titin Z-repeats with α-actinin

In search of a possible biological significance of the differential Z-disc titin expression, the segment comprising bp 904 to 3730 was subcloned into the shuttle vector, pAS2-1, which allowed replication in *Escherichia coli* and identification of potential ligands in yeast cells by the two-hybrid approach (Fields & Song, 1989). Altogether 200,000 clones from both a human skeletal and a human heart cDNA library were screened with the recombinant bait, referred to as pAS2-ZAD (ZAD, Z-disc assem-

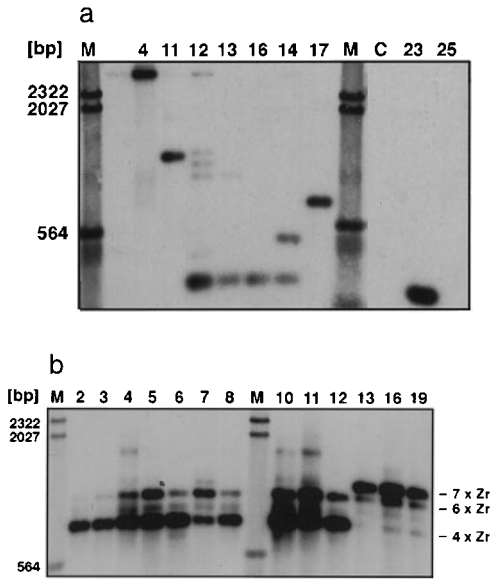

Figure 1. Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis of different rabbit muscles with Z-disc titin primer pairs. Primer pairs within the Z-disc titin segment bp 800 to 3800 (coordinates refering to X90568) were used to amplify cDNAs from a collection of striated and smooth muscle tissues. Amplified fragments were separated on agarose gels, and detected with specific probes on Southern blots. Tissues: 2, M. psoas; 3, M. longissimus dorsi; 4, M. soleus; 5, M. gastrocnemius; 6, M. plantaris longus; 7, diaphragm; 8, M. extensor digitorum longus; 10; M. rectus femoris; 11, tongue; 12, M. pectoralis major; 13, cardiac left ventricle; 14, proximal oesophagus; 16, cardiac right ventricle; 17, distal oesophagus (cardia); 19, left cardiac atrium; 23, uterus; 25, bladder; c, negative controls; M, λ *Hin*dIII size marker. a, RT–PCR analysis with a primer pair flanking the differentially expressed segment (bp 800 to 3800). Differently sized versions of the Z-disc titin domain are detected in 4, 11, 12, 14, 17 and 23. The smallest species amplified from uterus cDNA (23) completely excludes the expression of the 900-residue segment, bp 904 to 3730. The full-length version of this domain is expressed in the heart (13, 16), but this fragment is too large to be amplified under the PCR conditions used. b, RT–PCR analysis with a primer pair flanking the Z repeat region (bp 1500 to 2550). Different copy numbers of Z-repeats are expressed in the striated muscles, and the three major length variants observed correspond to seven, six, and four repeats, as indicated on the right. Skeletal muscle tissues containing predominantly fast fibres (psoas, longissimus dorsi, extensor digitorum longus) express the four-Z-repeat variant, cardiac tissues (13, left ventricle; 16, right ventricle; 19, left atrium) mainly the seven-Z-repeat version. Other muscle tissues co-express mostly mixtures of a four and a six Z-repeat isoform.

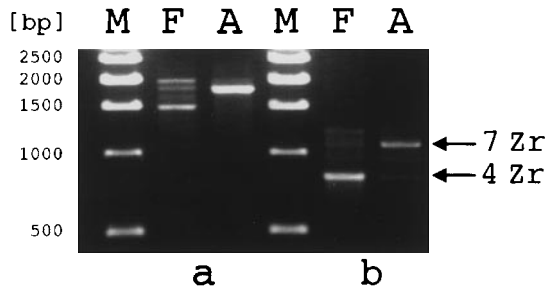

Figure 2. Developmental regulation of the cardiac Z-repeat copy number. Total cDNAs obtained from human fetal (F) and human adult (A) heart cDNA libraries were amplified with a primer pair flanking the Z-repeat region (a), and with a primer pair within the Z-repeat region (b). In adult muscle, a single species comprising seven copies of Z-repeats is detected. In cDNA from embryonic heart, mixtures of isoforms are detected. Within the Z-repeat region, a four-copy version is the most abundant species.

bly domain). From the human skeletal library, seven positive clones were identified and were all confirmed to interact with the pAS2-ZAD bait. From the human heart cDNA library, three of the eight clones selected in the primary screen were confirmed in the secondary screen. The inserts of all ten confirmed cDNA clones were derived from the ACTN2 gene (Beggs *et al.*, 1992) encoding a sarcomeric α-actinin isoform, in the following referred to as α-actinin-2. The ACTN2 gene is a member of the spectrin gene superfamily, which is characterised by an N-terminal actin-binding domain, a central rod domain composed of 4 to 24 repeated units, and a C-terminal domain with EF hand structures (Beggs *et al.*, 1992). All ten isolated ACTN2 cDNAs share a region of overlap in the C-terminal region of the α-actinin-2 (Figure 4). To assign the titin-binding site in the α-actinin clone with higher precision, 5′ and 3′ deletions were introduced into the ACTN2-14 construct (see Figure 4). Interaction of the shortened baits with the titin pAS2-ZAD construct was then monitored. The results indicate that the essential sequences for mediating Z-disc titin binding are located in the C-terminal region of the α-actinin-2 from about the second EF-hand up to the extreme C terminus (Figure 4).

To determine the sequences essential for α-actinin-2 binding within the Z-disc titin, a set of 5′ and 3′ deletions was introduced into the titin pAS2-ZAD bait (Figure 5). This allowed us to assign the region of α-actinin-2 binding within the titin bait to the Z-repeat region (Figure 5). A four Zr-motif-containing bait (Zr1,Zr2,Zr3,Zr7) showed strong interaction with ACTN2. Deletion of one motif, that of Zr7 in ZAD-Δ2 (bp 2119-2181), resulted in complete loss of binding on plates supplemented with 5 mM 3-amino-1,2,4-triazole (3-AT). In binding assays under less stringent conditions (without 3-AT) we also detected interaction of the motifs Zr1,Zr2,Zr3 to ACTN2 in the mutant ZAD-Δ3 (Figure 5), suggesting a weaker binding of the three Zr motifs which are contained in the ZAD-Δ3 clone. In conclusion, our data demonstrate a single strong α-actinin binding site in the titin Z-repeat Zr7. An additional potential α-actinin

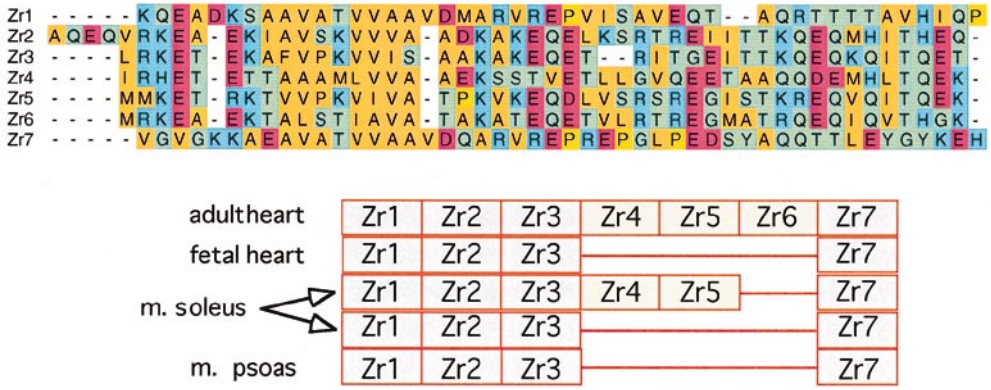

Figure 3. The family of Z-repeats and their differential expression. Top: The seven copies of the Z-repeats are shown which are expressed in the rabbit cardiac titin (for sequence alignment, the Wisconsin software package was used; see Devereux *et al.*, 1984). Hydrophobic residues, typical for the family of Z repeats, are coloured in orange. Bottom: Overview of the Z-repeat isoforms which are expressed in different types of striated muscles. The repeats Zr1,2,3 and Zr7 were found to be expressed in all striated muscles, whereas inclusion of Zr4,5,6 was dependent on tissue-type, and also reflected the developmental stage of the respective muscle.

binding motif appears to be present in the C-terminal Z-repeats.

Discussion

Z-disc structure and titin/α-actinin interactions

Vertebrate and invertebrate Z-discs are formed by the thin filaments from opposing half-sarcomeres, which overlap and are cross-linked to a stable network. Although the Z-disc architecture can vary considerably between muscle tissues and fibre types, all vertebrate Z-discs share common basic features (Squire, 1981): they are tetragonal and have a common structural unit, the Z-unit (Yamaguchi *et al.*, 1985). In the Z-unit, the opposing thin filaments from opposite sarcomeres are cross-linked by four pairs of Z-filaments (Yamaguchi *et al.*, 1985). These Z-filaments consist of α-actinin, a molecule which has been shown to form stable cross-connections of thin filaments *in vitro* (Stromer & Goll, 1972). However, under physiological conditions, α-actinin does not significantly cross-link thin filaments *in vitro* (Bennett *et al.*, 1984). Moreover, when Z-discs are degraded by calpain digestion, intact α-actinin and actin are released (Goll *et al.*, 1991). Therefore, it was suggested that, in addition to α-actinin, an unknown linker protein is involved in forming the Z-unit (for a review, see Vigoreaux, 1994). Also, it has remained unclear what structure specifies the invariable presence of four pairs of α-actinin filaments within a Z-unit (Vigoreaux, 1994). More recently, electron microscopic tomographic reconstructions of Z-discs from rat soleus muscle showed a substantial variation in the spacing between cross-connecting Z-filaments to axial filament connection points (Schroeter *et al.*, 1996). The authors concluded that the structure of the Z-disc may not be determined solely by the arrangement of α-actinin to actin-binding sites along the axial filament.

Our data now suggest that the Z-disc titin is the sought-after Z-disc linker protein. Titin may act to specify the attachment of the α-actinin-2 C terminus within the Z-disc lattice and thereby, determine the sites of thin filament cross-linking. Recent immunoelectron microscopic studies have posi-

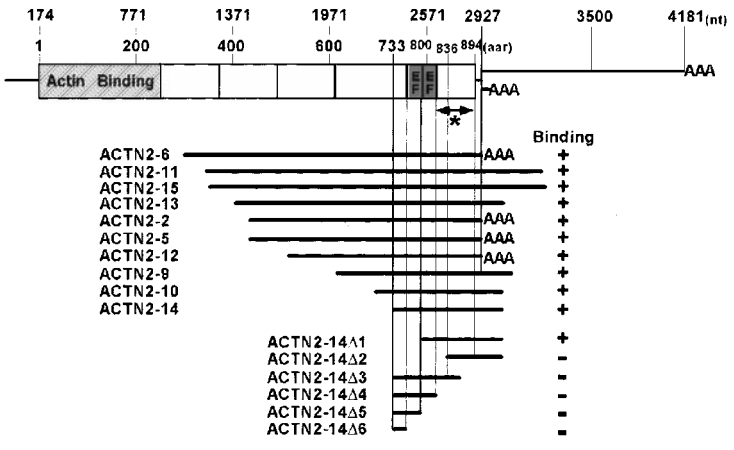

Figure 4. Interaction of ACTN2 cDNAs with the Z-disc titin bait. At the top the schematic structure of the ACTN2 cDNA is shown. AAA indicates a poly(A^+) tail. Thick lines below summarize the clones obtained from the two-hybrid screens (ACTN2-2 to ACTN2-15). Their shared overlaps assign the region of titin binding to the C-terminal region. A series of additional 5′ and 3′ deletions (ACTN2-14Δ1 to Δ6) allow to further narrow down the titin binding domain to the C-terminal 80 residues of the α-actinin-2 (double arrow with *).

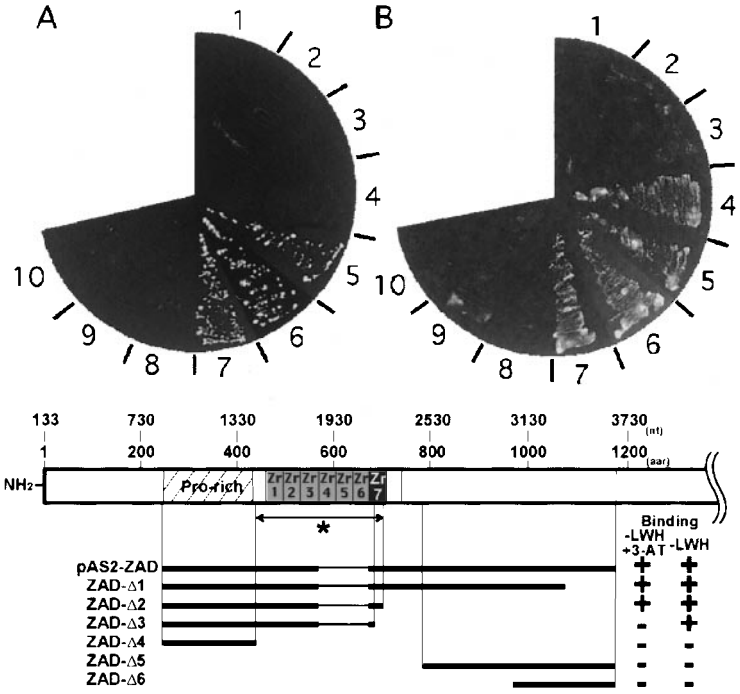

Figure 5. Binding assay of α-actinin-2 and the Z-disc titin by the two-hybrid method. The partial α-actinin-2 cDNA ACTN2-14 (see Figure 3) was co-transfected with partial titin cDNAs into the *S. cerevisae* strain CG-1945. Top: Interaction of target sequences was monitored on minus (Leu, Trp, and His) plates supplemented with 3-AT (A), or without 3-AT (B) after 4 days at 30°C. Co-transfected constructs were in: Sector 1, ZAD-Δ6; 2, ZAD-Δ5; 3, ZAD-Δ4; 4, ZAD-Δ3; 5, ZAD-Δ2; 6, ZAD-Δ1; 7, full length pAS2-ZAD; 8, μ-calpain large subunit as negative control (Sorimachi *et al.*, 1995); 9, pAS2-1-vector negative control. In section 10, co-transfection of pAS2-ZAD and ACTN2-14Δ was monitored. Bottom: Schematic summary of the modular structure of the Z-disc titin, the deletion constructs, and their respective binding properties to α-actinin-2. Truncation of the module Zr7 in the mutant ZAD-Δ3 results in loss of interaction on 3-AT-supplemented plates. Growth on minus-3-AT plates suggests residual weaker binding of the Zr1-Zr2- Zr3 motifs to α-actinin-2.

tioned the N-terminal titin residues 600 to 800 (bp 1900 to 2500) inside the Z-disc (Yajima *et al.*, 1996; Gautel *et al.*, 1996). The α-actinin-2 binding domain of titin, Zr7, locates to residues 647 to 695 (bp 2073 to 2218; Figure 5). Therefore, in good agreement with the electron microscopic studies, Zr7 appears to localise to the outermost sites of the Z-disc. We suggest that the Zr7 motifs of the two titin strands coming from opposite sarcomeres may be cross-linked by the respective C termini of an α-actinin-2 antiparallel homodimer, whereas the N termini of this homodimer, which have actin-binding properties (Hammonds, 1987; Levine *et al.*, 1990), could link the thin filaments from opposite sarcomeres (Figure 6). This model would explain why the expression of C-terminally truncated ACTN2 cDNAs disrupts the integrity of the Z-disc (Schultheiss *et al.*, 1992b).

Recently, it was proposed that the α-actinin binding site on the zeugmatin, which is presumably a titin fragment, is located within the charged peptide motif ''KIKK'' (Turnacioglu *et al.*, 1996), because this motif also shows α-actinin binding in other systems (e.g. in the case of the cytoplasmic domain of the ICAM-1 receptor; Carpen *et al.*, 1992). Our data on the presence of an α-actinin binding site in the titin are in agreement with this study (Turnacioglu *et al.*, 1996). However, the mapping of this binding site to a charged KIKK-motif (Turnacioglu *et al.*, 1996), or to the Z repeats (this study) appear to be in contradiction. We suggest that the titin-actin filament cross-links in the Z-discs are mediated by α-actinin-binding motifs, the Z-repeats, which are rather hydrophobic and which are distinct from the charged KIKK-motifs, which are presumably involved in making contacts between the submembraneous cytoskeleton and the membrane (Carpen *et al.*, 1992). It is also noteworthy that our yeast-two hybrid data and the independent studies of Ohtsuka *et al.* (1997a,b) predict the interaction of a single titin domain with a single α-actinin-2 domain. This suggests that the stoichiometry of titin within the Z-disc is four or a multiple of four, so that it would match the four pairs of Z-filaments per Z-unit.

Differential expression of Z-disc titin

Deletion of the Zr7 motif did not result in complete loss of interaction with ACTN2. Rather, the repeats Zr1-Zr3 apparently retained, to some degree, an affinity to α-actinin-2 (Figure 5). This suggests that several members of the Zr titin motif family have α-actinin-2 binding properties, and their binding to α-actinin-2 may be cooperative. This could perhaps explain why a recent study with single expressed Zr titin motifs failed to detect α-actinin binding properties (Gautel *et al.*, 1996). From our results, it is reasonable to postulate a biological function for the tissue-specific control of the copy number of expressed Zr repeats: such function could be a modulation of the strength of titin-α-actinin-2 interaction. In this

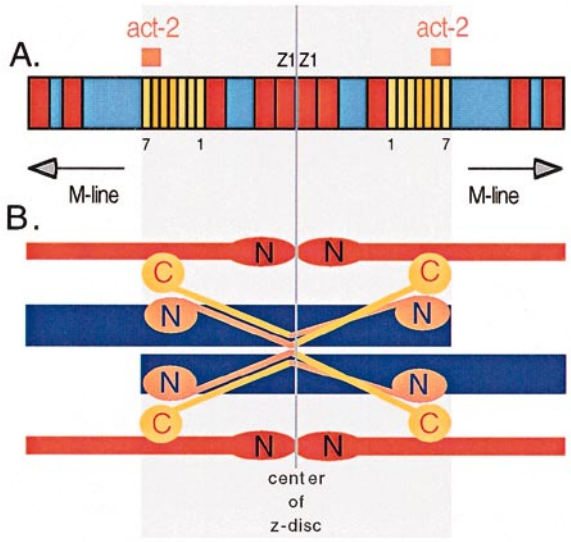

Figure 6. Model of the lay-out of the Z-disc titin and its assembly of Z-discs. Top: The N-terminal (~700) residues of titin are located within the Z-disc (Yajima *et al.*, 1996; Gautel *et al.*, 1996), and their modular structure is shown (Labeit & Kolmerer, 1995). Colour codes: Titin-Ig repeats, red; unique sequences, blue; cardiac Z-repeats 1 to 7, yellow/orange; skeletal muscle-type excluded Z-repeats, orange. The α-actinin-2 binding is assumed to locate at the edge of the Z-disc. The expression of the Z-disc titin in different length variants by alternative splicing between bp 904 (tissue-specific slice site) and Zr7 could acount for variable amounts of thin filament overlap and hence, Z-disc width. Bottom: Model for titin and Z-unit interactions (Z-units adapted from Yamaguchi *et al.*, 1985). Z-units are formed by overlapping thin filaments (blue) entering the Z-disc from opposite sarcomeres. The thin filaments are cross-linked by the N termini of an antiparallel α-actinin-2 homodimer (yellow/orange). Cross-links to antiparallel titin filaments (red) from opposing sarcomeres are made by the C-terminal region of a Z-filament composed of α-actinin-2.

context, it should be mentioned that also the expression of the ACTN2 C-terminal EF-hand domain is regulated by differential splicing (Parr *et al.*, 1992). Thus, multiple differential splicing events in both the titin and ACTN2 within their respective binding domains may support a sophisticated regulation of the titin-α-actinin-2 interaction. Finally, the skeletal Z-disc does not contain exclusively ACTN2-encoded sequences, but also ACTN3 sequences, and both actinin genes are expressed in similar levels (Beggs *et al.*, 1992). Therefore, in preliminary experiments, we have isolated ACTN3 C-terminal cDNAs and monitored their interaction with the titin Z-repeats by the yeast-two hybrid approach. Both the ACTN3 and the ACTN2 C termini were found to bind to Zr7 (data not shown). Considering the 90% of sequence similarity of the C termini from ACTN2 and ACTN3 α-actinins, this result may in fact not be surprising. At present, it is unclear why two highly similar α-actinins are involved in the formation of skeletal muscle Z-discs, whereas in cardiac muscle, exclusively ACTN2-encoded α-actinins are expressed. For the other members of the spectrin super family, spectrin, dystrophin and utrophin, the C-terminal sequences have much less similarity, which points to their distinct biological functions, e.g. their binding to membrane-protein assemblies (for a review, see Squire, 1997).

To further address a possible biological function of the differential splicing of Z-disc titin, we consider the important task of Z-discs in transmitting both active and passive tension between adjacent sarcomeres and to the ends of a muscle fibre. Then, the ability of Z-discs from different striated muscle types to withstand and transmit different levels of tension may be reflected in a variable strength of the Z repeat and the α-actinin-2 interaction. This study's results thus raise the possibility that the tissue-specific control of Z repeat copy number exhibits a fibre-type dependence with functional consequences. It should be interesting to study whether a modulation of the Z-disc titin and α-actinin-2 interaction could be involved in the dynamic response of Z-discs to mechanical stress through transitions in the Z-lattice structure, which has been reported for both skeletal and cardiac muscle (Goldstein *et al.*, 1986, 1989). As for uterus smooth muscle, the lack of the expression of the Zr7 α-actinin-2 binding site seems to be relevant for the muscle's inability to form Z-discs. Conversely, this could be a manifestation of the fact that smooth muscle tissue is not exposed to the high tensile forces striated muscle fibres have to bear. Similarly, the fetal cardiac Z-disc is not transmitting significant force between adjacent sarcomeres, wheres postnatally, the rise in blood pressure will exert forces onto the cardiac myofibrils. In sum, the expression of α-actinin-2 binding Zr titin repeats in different copy numbers in cardiac and skeletal muscles could indeed be relevant for a specific response of vertebrate Z-discs to mechanical stress.

The expression of the Z-disc titin in many different isoforms complicates the nomenclature. For future studies, it will be important to use a consistent terminology for the different titin domains. At present, it appears that the titin from the adult cardiac muscle is the full-length Z-disc version, whereas isoforms of the Z-disc titin are generated by skipping of some domains, both within and outside the Z-repeat region. Therefore, we suggest to ''count'' the Ig, Zr and other motifs from N-terminal to C-terminal according to the cardiac sequence. An isoform is then characterised by listing the skipped motifs (see Figure 3 for our suggestion).

Materials and Methods

Cloning and isoform studies of the rabbit Z-disc titin

For the cloning of the rabbit cDNAs, a set of 30mer oligonucleotides covering bp 1 to 5000 of the human titin cDNA sequence were used (EMBL data library AC No

X90568). Rabbit muscle RNA from M. soleus was prepared (MacDonald *et al.*, 1987) and reverse-transcribed by Mlu-LV essentially as described by manufacturer (BRL). Aliquots of the cDNA were PCR-amplified by the set of human titin 30mers (Saiki *et al.*, 1985). For the first two cycles, the annealing temperature was set to 50°C to allow mismatches of the human primers to the rabbit cDNA. For another 30 cycles, the annealing and the extension temperature was set to 68°C. Amplified rabbit titin cDNAs were gel-purified and extracted from native agarose gels with the Jetsorb kit (Genomed). Gel-purified DNA was sequenced with the PCR primers and subsequently with internal primers by a cycle sequencing method (for details of the protocol, see http://www.embl-heidelberg.de/ExternalInfo/Titin/).

For the isoform studies, rabbit RNAs were prepared from different muscle tissues (MacDonald *et al.*, 1987) and reverse transcribed by Mlu-LV-RT essentially as described by manufacturer (BRL). The collection of rabbit cDNAs was amplified with sets of primers derived from the rabbit cDNA sequence by PCR (Saiki *et al.*, 1985). The products were sized on native agarose gels, transferred to nylon membranes, cross-linked by UV (Church & Gilbert, 1984), and visualized by randomly labeled probes (Feinberg & Vogelstein, 1983). Total human adult and fetal cardiac cDNAs were obtained by the amplification of commercially available lambda phage libraries (from Stratagene and Clontech, respectively).

Yeast-two hybrid studies

A 2.6 kb rabbit titin cDNA fragment (corresponding to bp 904 to 3730 of the human entry, but lacking Zr4-6; EMBL data library X90568) was amplified from rabbit psoas muscle RNA by RT-PCR. The fragment was inserted into the pAS2-1 vector (Clontech) for obtaining a ''bait'' plasmid. The recombinant plasmid, referred to as pAS2-ZAD, was transformed into *Saccharomyces cerevisae* (strain CG-1945). For screening, the bait pAS2-ZAD was co-expressed in the CG-1945 cells with human skeletal and human heart cDNA clones in a pGDA10 vector backbone (Clontech HL4010AB and HL4013AB, respectively). Candidate clones from a total of 400,000 colonies screened were rescued on minus-Leu, Trp and His plates, supplemented with 5 mM 3-amino-1,2,4-triazole, sequenced and re-transformed with pAS2-ZAD to confirm positive binding (Sorimachi *et al.*, 1995).

Z-Δ1 to Z-Δ6 were constructed by digestion of pAS2-ZAD with *Age*I + *Bam*HI, *Bpu*1102I + *Bam*HI, *Pst*I, *Bst*PI + *Bam*HI, *Eco*RI + *Bpu*1102I, and *Nco*I + *Bst*PI, respectively, following self-ligation. ACTN2-14Δ was obtained from ACTN2-14 by *Bam*HI restriction and self-ligation. All constructs were expressed in CG-1945 and examined for growth on minus-(Leu, Trp, His) plates with or without 5 mM 3-amino-1,2,4-triazole (3-AT), and for β-galactosidase activity. β-Galactosidase activity of the cells was measured using chlorophenol red-β-D-galactopyranoside (CPRG) as described by manufacturer (Clontech).

Acknowledgements

We thank K. Maruyama and the Goho Science Foundation for getting this collaborative effort started, S. Kimura for communication of results prior to publication. This work was supported by the Human Frontier Science Programme, the Deutsche Forschungsgemeinschaft (La 668/2-3; Li 690/2-1), the European Union, and the ''Forschungsfond der Fakultät für klinische Medizin Mannheim''.

References

Beggs, A. H., Byers, T. J., Knoll, J. H. M., Boyce, F. M., Bruns, G. A. P. & Kunkel, L. M. (1992). Cloning and characterization of two human skeletal muscle α-actinin genes located on chromosomes 1 and 11. *J. Biol. Chem.* **267**, 9281–9288.

Bennett, J. P., Zaner, K. S. & Stossel, T. P. (1984). Isolation and some properties of macrophage α-actinin: evidence that it is not an actin gelling protein. *Biochemistry,* **23**, 5081–5087.

Carpen, O., Pallai, P., Staunton, D. E. & Springer, T. A. (1992). Association of intercellular adhesion molecule-1 (ICAM-1) with actin-containing cytoskeleton and alpha-actinin. *J. Cell Biol.* **118**, 1223–1234.

Church, G. M. & Gilbert, W. (1984). Genomic Sequencing. *Proc. Natl Acad. Sci. USA.* **81**, 1991–1995.

Devereux, J., Haeberli, P. & Smithies, O. (1984). A comprehensive set of sequence analysis programs for the VAX. *Nucl. Acids Res.* **12**, 387–395.

Feinberg, A. P. & Vogelstein, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **13**, 6–13.

Fields, S. & Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature,* **340**, 245–246.

Fürst, D. O., Osborn, M., Nave, R. & Weber, K. (1988). The organization of titin filaments in the half-sarcomere revealed by monoclonal antibodies in immunoelectron microscopy: a map of ten nonrepetitive epitopes starting at the Z line extends close to the M line. *J. Cell Biol.* **106**, 1563–1572.

Gautel, M., Goulding, D., Bullard, B., Weber, K. & Fürst, D. O. (1996). The central Z-disk region of titin is assembled from a novel repeat in variable copy numbers. *J. Cell Sci.* **109**, 2747–2754.

Goldstein, M. A., Michael, L. H., Schroeter, J. P. & Sass, R. L. (1986). The Z-band lattice in skeletal muscle before, during and after tetanic contraction. *J. Muscle Res. Cell Motil.* **7**, 527–536.

Goldstein, M. A., Michael, L. H., Schroeter, J. P. & Sass, R. L. (1989). Two structural states of Z-bands in cardiac muscle. *Am. J. Physiol. (Heart Circ. Physiol.),* **256**, H552–H559.

Goll, D. E., Dayton, W. R., Singh, I. & Robson, R. M. (1991). Studies of the α-actinin/actin interaction in the Z-disc by using calpain. *J. Biol. Chem.* **266**, 8501–8510.

Hammonds, R. G., Jr (1987). Protein sequence of DMD gene is related to actin-binding domain of α-actinin. *Cell,* **51**, 1.

Labeit, S. & Kolmerer, B. (1995). Titins, giant proteins in charge of muscle ultrastructure and elasticity. *Science,* **270**, 293–296.

Labeit, S., Gautel, M., Lakey, A. & Trinick, J. (1992). Towards a molecular understanding of titin. *EMBO J.* **11**, 1711–1716.

Labeit, S., Kolmerer, B. & Linke, W. A. (1997). The giant protein titin: Emerging roles in physiology and pathophysiology. *Circ. Res,* **80**, 290–294.

Levine, B. A., Moir, A. J. G., Patchell, V. B. & Perry, S. V. (1990). The interaction of actin with dystrophin. *FEBS Letters,* **263**, 159–162.

Maruyama, K. (1994). Connectin, an elastic protein of striated muscle. *Biophys. Chem.* **50**, 73–85.

MacDonald, R. J., Swift, G. H., Przybyla, A. E. & Chirgwin, J. M. (1987). Isolation of RNA using guanidinium salts. *Methods Enzymol,* **152**, 219–226.

Nave, R., Fürst, D. O. & Weber, K. (1990). Interaction of α-actinin and nebulin *in vitro*. *FEBS Letters,* **269**, 163–166.

Ohtsuka, H., Yajima, H., Maruyama, K. & Kimura, S. (1997a). Binding of the N-terminal 63 kDa portion of connectin/titin to α-actinin as revealed by the yeast two-hybrid system. *FEBS Letters,* **401**, 65–67.

Ohtsuka, H., Yajima, H., Maruyama, K. & Kimura, S. (1997b). The N-terminal Z repeat 5 of connectin/titin binds to the C-terminal region of α-actinin. *Biochem. Biophys. Res. Commun.*, in the press.

Parr, T., Waites, G. T., Patel, B., Millake, D. B. & Critchley, D. R. (1992). A chick skeletal-muscle α-actinin gene gives rise to two alternatively spliced isoforms which differ in the EF-hand Ca^{2+}-binding domain. *Eur. J. Biochem.* **210**, 801–809.

Saiki, R. K., Scharf, S. J., Faloona, F., Mullis, G. T. & Erlich, H. A. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* **230**, 1350–1354.

Schroeter, J. P., Bretaudiere, J.-P., Sass, R. L. & Goldstein, M. A. (1996). Three-dimensional structure of the Z band in a normal mammalian skeletal muscle. *J. Cell Biol.* **133**, 571–583.

Schultheiss, T., Lin, Z., Lu, M.-H., Murray, J., Fischman, D. A., Weber, K., Masaki, M., Imamura, M. & Holtzer, H. (1992a). Differential distribution of subsets of myofibrillar proteins in cardiac nonstriated and striated myofibrils. *J. Cell. Biol.* **110**, 1159–1172.

Schultheiss, T., Choi, J., Lin, Z. X., DiLullo, C., Fischman, D. A. & Holtzer, H. (1992b). A sarcomeric α-actinin truncated at the carboxyterminal end induces the breakdown of stress fibers in PtK2 cells and the formation of nemaline-like bodies and breakdown of myofibrils in myotubes. *Proc. Natl Acad. Sci. USA,* **89**, 9282–9286.

Sorimachi, H., Kinbara, K., Kimura, S., Takahashi, M., Ishiura, S., Sasagawa, N., Sorimachi, N., Shimada, H., Tagawa, K., Maruyama, K. & Suzuki, K. (1995). Muscle-specific calpain, p94, responsible for limb girdle muscular dystrophy type 2A, associates with connectin through IS2, a p94-specific sequence. *J. Biol. Chem.* **270**, 31158–31162.

Soteriou, A., Gamage, M. & Trinick, J. (1993). A survey of the interactions made by the giant protein titin. *J. Cell Sci.* **104**, 119–123.

Squire, J. M. (1981). *The Structural Basis of Muscular Contraction,* Plenum Press, New York.

Squire, J. M. (1997). Architecture and function in the muscle sarcomere. *Curr. Opin. Struct. Biol.* **7**, 247–257.

Stromer, M. H. & Goll, D. E. (1972). Studies on purified α-actinin. *J. Mol. Biol.* **67**, 489–494.

Suzuki, J., Kimura, S. & Maruyama, K. (1994). Electron microscopic filament lengths of connectin and its fragments. *J. Biochem.* **116**, 406–410.

Tokuyasu, K. T. & Maher, P. A. (1987). Immunocytochemical studies of cardiac myofibrillogenesis in early chick embryos. II. Generation of α-actinin dots within titin spots at the time of the first myofibril formation. *J. Cell. Biol.* **105**, 2795–2801.

Trinick, J. (1994). Titin and nebulin: protein rulers in muscle?. *Trends Biochem. Sci.* **19**, 405–409.

Turnacioglu, K. K., Mittal, B., Sanger, J. M. & Sanger, J. W. (1996). Partial characterization of zeugmatin indicates that it is part of the Z-band region of titin. *Cell Motil. Cytoskel.* **34**, 108–121.

Vigoreaux, J. O. (1994). The muscle Z band: lessons in stress management. *J. Muscle Res. Cell Motil.* **15**, 237–255.

Yajima, H., Ohtsuka, H., Kawamura, Y., Kume, H., Murayama, T., Abe, H., Kimura, S. & Maruyama, K. (1996). A 11.5 kb 5′-terminal cDNA sequence of chicken breast muscle connectin/titin reveals its Z line binding region. *Biochem. Biophys. Res. Commun.* **223**, 160–164.

Yamaguchi, M., Izumimoto, M., Robson, R. M. & Stromer, M. H. (1985). Fine Structure of wide and narrow vertebrate muscle Z-lines. A proposed model and computer simulation of Z-line architecture. *J. Mol. Biol.* **184**, 621–644.

Edited by J. Karn

(Received 2 January 1997; received in revised form 6 May 1997; accepted 9 May 1997)