Two immunoglobulin-like domains of the Z-disc portion of titin interact in a conformation-dependent way with telethonin

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Abstract The giant muscle protein titin/connectin plays a crucial role in myofibrillogenesis as a molecular ruler for sarcomeric protein sorting. We describe here that the N-terminal titin immunoglobulin domains Z1 and Z2 interact specifically with telethonin in yeast two-hybrid analysis and protein binding assays. Immunofluorescence with antibodies against the N-terminal region of titin and telethonin detects both proteins at the Z-disc of human myotubes. Longer titin fragments, comprising a serine-proline-rich phosphorylation site and the next domain, do not interact. The interaction of telethonin with titin is therefore conformation-dependent, reflecting a possible phosphorylation regulation during myofibrillogenesis.

Key words: Titin; Connectin; Telethonin; Z-disk; Myofibrillogenesis

1. Introduction

Myofibrillogenesis involves the ordered assembly of hundreds of protein subunits in a temporally and spatially defined manner. Titin [1], described also as connectin [2], acts as a molecular ruler for the assembly of the sarcomere by providing spatially defined binding sites for other sarcomeric proteins [3] over the distance of an entire half-sarcomere [4]. Titin is expressed as one of the earliest sarcomeric proteins and is localised to primordial sites of sarcomeric assembly on stress-fibre-like structures together with α-actinin [5]. Titin is composed largely of immunoglobulin-like domains and specific linker sequences involved in ligand binding and control functions [3]. In the Z-disc region, the molecular architecture of titin consists of two immunoglobulin-like (Ig-like) domains at the extreme N-terminus (Z1 and Z2; see Fig. 1), followed by a serine-proline-rich linker (zis1) containing several consensus phosphorylation sites for erk-like protein kinases [6]. This region is followed by a third module, Z3, putatively assigned as an Ig-like domain, and a novel 45-residue repeat which is differentially spliced [6]. These Z-repeats represent one of two types of sorting signals for α-actinin [7–9]. Recent investigations demonstrated that the titin N-terminus localises close to the barbed end of thin actin filaments in the Z-disc and might be involved in the control of thin filament capping [9]. Protein interactions with the N-terminal region of titin are therefore of great interest to understand the functions in primordial myofibrillogenesis, capping and crosslinking of actin filaments which ultimately lead to the assembly of the Z-disc.

We have previously shown in transfection assays with fragments of the titin Z-disc region that the two N-terminal Ig-like domains Z1 and Z2 exert a strong dominant negative effect on myofibrillogenesis in transfected myocytes, suggesting a protein interaction of that region which is crucial for sarcomere assembly [10]. To analyse this titin interaction, we have used the yeast two-hybrid system and identified telethonin as the specific ligand.

2. Materials and methods

2.1. Plasmids

The titin N-terminal Ig domains Z1–Z2, Z1, Z2, Z2–zis1, Z2–Z3, Z1–Z3, Z1–zr3 were isolated by PCR from primary cDNA of human heart muscle. Domain boundaries followed the nomenclature of the human cardiac titin sequence (EMBL X90568) [11]. The cDNA fragments were subcloned into a modified pLexA plasmid [12,13] with 4–10 aa N- and C-terminal fusions to lexA. The lexA fusion baits proved to be non-auto-activating when grown on His- and Trp-deficient selection media and to result in the translation of the expected fusion protein when assayed with a lexA monoclonal antibody (Clontech).

2.2. Yeast two-hybrid screening

The yeast strain L40 (MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ) was used [13]. L40 harbouring pLexA-Z1-Z2 were transformed with a human skeletal muscle library in pGAD10 (Clontech) using lithium acetate/PEG. Transformants positive for HIS3 and β-galactosidase reporter gene expression were selected after 4 days, and the library plasmids sequenced.

2.3. Protein expression and binding assays

Telethonin subfragments were amplified by PCR from a pGAD10 clone obtained in the two-hybrid screen and cloned into an N-terminally His_6-tagged pET3d vector. A C-terminal Glu-Glu-Phe tag was included for detection of the recombinant protein [14]. His_6 fusion protein expression followed standard procedures. Full-length telethonin was overexpressed solubly in fusion with maltose binding protein, purified and cleaved as described (New England Biolabs, Germany). Titin fragments were expressed as in [6,14,15]. Purity was assessed by SDS-PAGE [16]. Dot-blot assays were performed essentially as described [14]. Briefly, 9–88 pmol of recombinant titin fragments were spotted on nitrocellulose membranes, blocked with 5% bovine serum albumin (BSA) and incubated for 30 min at room temperature with recombinant full-length telethonin at 50 μg/ml in binding buffer (20 mM imidazole-hydrochloride pH 7, 100 mM NaCl, 1 mM ethylenediamine-tetraacetic acid, 1 mM dithiothreitol, 1% BSA). After several washes with binding buffer, the bound telethonin was detected using the anti-telethonin antibody (see below), and bound immunocomplexes visualised by standard alkaline phosphatase reactions.

2.4. Muscle cell cultures and myofibrils

Human skeletal muscle cells were isolated and cultured essentially as described previously [17,18]. In brief, satellite cells were enzymatically isolated from normal human skeletal muscle biopsies, passaged

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two to five times and frozen in liquid nitrogen. Cells were quickly thawed and plated on glass coverslips in DMEM supplemented with 20% foetal calf serum, 2% Ultroser G, and antibiotics (all from Life Technologies, Eggenstein, Germany) and the cells were grown until near confluence. Differentiation of the cells was induced by changing the high nutrition medium to a low nutrition medium (DMEM, 0.4% Ultroser G, and antibiotics). Fully differentiated cells were fixed for approximately 5 min in methanol and subsequently for 30 s in acetone both at −20°C. After air drying, cells were stored at −80°C. Myofibrils from rabbit psoas muscle were prepared as described [19].

2.5. Antibodies and immunochemistry

A rabbit polyclonal antibody, TthC-ra, was raised against telethonin Tth(104–167) following standard procedures. The antibody was affinity-purified on Tth(104–167) immobilised to NHS Hitrap columns (Pharmacia) as described [9]. Western blots were performed by standard procedures and confirmed the reactivity against telethonin. Indirect double immunofluorescence was performed as described [20] using the affinity-purified telethonin antibody at 1 μg/ml in undiluted hybridoma supernatant of anti-titin T12 [4]. Detection of bound antibody was performed with anti-rabbit FITC and anti-mouse Texas red conjugates (both Jackson Immunoresearch, USA). The reaction of anti-telethonin TthC-ra could be totally suppressed by preincubation of the antibody with surplus recombinant antigen.

3. Results

3.1. The two N-terminal titin Ig-like domains interact specifically with telethonin

The Z1–Z2 region of titin (see Fig. 1) was used as a bait construct for yeast two-hybrid analysis in fusion with the lexA DNA binding domain. A human skeletal muscle cDNA library in the pGAD-10 vector was cotransformed with the titin Z1–Z2 bait into L40 cells. After 3 days of incubation at 30°C, over 800 HIS3 and β-galactosidase positive clones were identified from a total of $\sim 8 \times 10^5$ clones. Library plasmids were isolated and rescued into *Escherichia coli*. DNA sequencing of six of these clones showed them to be telethonin, a recently identified muscle-specific protein [21]. These clones contain the complete coding sequence of telethonin starting with the putative translation-initiating methionine 1 [21]. Analysis of the library plasmids of a further 60 clones by specific PCR and restriction digest analysis showed that all clones analysed encode telethonin. Co-transformation of L40 reporter cells with other titin immunoglobulin constructs showed that this interaction is highly specific for Z1–Z2 (Fig. 1). To map the interaction site on titin, further N-terminal titin fragments were

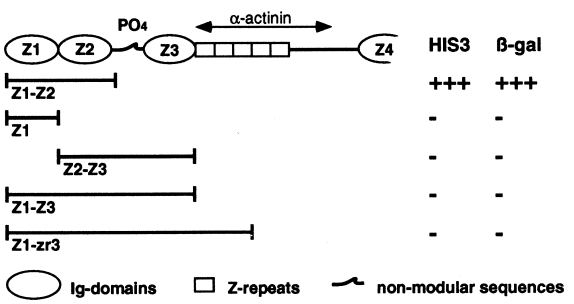

Fig. 1. The domain pattern of the titin N-terminus and bait constructs from this region. The region involved in α-actinin sorting is marked. Telethonin interaction is observed only with the Z1–Z2 bait. Longer baits from this region like Z1–Z3, and subfragments like Z2–Z3 or Z1 are negative. This implies that the two N-terminal domains are needed for telethonin-titin interaction, whereas the binding site on titin and/or telethonin is inaccessible in the long bait construct Z1–Z3.

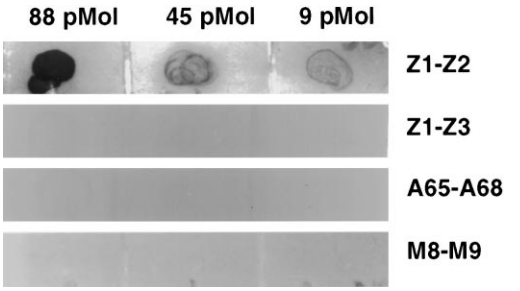

Fig. 2. Telethonin binding to tandem Ig fragments of human titin from the Z-disc and M-band. Dot-blot assays were performed as described in Section 2; 9–88 pmol of protein was spotted for both fragments. Recombinant telethonin binds to titin Z1–Z2 (top panel) but not to control constructs A65–A68 from the A-band and M8–M9 from the M-band. The Z1–Z3 construct, which is unreactive in the two-hybrid assay, shows no comparable interaction even at the highest protein amount spotted.

assayed for telethonin binding in the yeast two-hybrid system (Fig. 1). Truncation of Z1–Z2 abolishes telethonin interaction. This demonstrates that the two Ig-like domains Z1 and Z2 are both necessary and sufficient for telethonin binding. Interestingly, constructs longer than the original Z1–Z2 bait, including the serine-proline-rich linker in zis1 and Z3 (Fig. 1), do not interact in the two-hybrid assays. This suggests that in the entire titin N-terminal region, the telethonin binding site is in an inaccessible conformation in this assay.

To verify this interaction analysis further, we expressed telethonin in *E. coli* and assayed the recombinant protein for binding to titin fragments from Z-disc, A-band and M-disc in a dot-blot assay. The recombinant protein binds to Z1–Z2; control constructs of two tandem Ig domains from the M-band, M8–M9, or to titin domains A65–A68 from the A-band show no significant binding (Fig. 2). This confirms the Z-disc-specific interaction of titin with telethonin. In agreement with the two-hybrid binding assay, the longer titin Z-disc fragment Z1–Z3 shows no strong binding in the dot-blot assay (Fig. 2). Due to the strength of the interaction, a biosensor assay could not be used since the sensor surface could not be completely regenerated without losing binding capacity.

3.2. The titin N-terminus and telethonin colocalise at the sarcomeric Z-disc

To characterise the titin-telethonin interaction at the cellular level, cultured human skeletal muscle cells were investigated by indirect immunofluorescence with an affinity-purified antibody against the telethonin C-terminus (TthC-ra; residues 104–167) and the titin epitope T12 which maps near the Z-disc [4]. In addition, we assayed for the expression of the telethonin message in Northern blots of mRNA purified from these myocytes. The RNA analysis demonstrated that the telethonin mRNA is undetectable in undifferentiated cells and is strongly transcribed in differentiating cells (not shown). Immunofluorescence in day 6 myotubes detects highly regular myofibrils which stain in a narrow doublet for titin-T12 and a single band for telethonin TthC-ra (Fig. 3). At this stage, the telethonin label is localised central to the T12 doublet that can sometimes be resolved (Fig. 3A–C). This localisation is in excellent agreement with the ultrastructural layout of titin in the Z-disc [9], and the genetically and biochemically identified

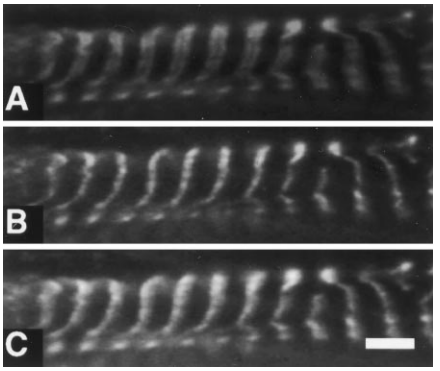

Fig. 3. Telethonin and titin colocalise at early stages of Z-disc formation during myofibrillogenesis. Double immunofluorescence visualised the anti-titin T12 antibody and anti-telethonin TthC-ra. In day 6 myotubes, localisation of the telethonin label to the central Z-disc is evident: the titin T12 antibody stains a narrow doublet band flanking the Z-disc (A) and telethonin TthC-ra stains a central narrow Z-disc band (B). Superposition of both channels results in a single broad band and demonstrates the localisation of telethonin central to the T12 label (C) and therefore at the Z-disc. Scale bar 3 μm.

telethonin binding site at Z1–Z2. Immunofluorescence in standard myofibril preparations [19] failed to detect telethonin, presumably because the protein is rapidly degraded or only transiently expressed.

4. Discussion

We demonstrate here that the novel muscle protein telethonin interacts with the Z-disc region of titin. More specifically, the two most N-terminal immunoglobulin domains Z1 and Z2 form a binding site for telethonin that requires the presence of both Ig-like domains. This prerequisite of at least two Ig-like domains on one ligand is similar to the binding sites of titin for myomesin [14], M-protein [22] or myosin binding protein C [15]. Our localisation data of telethonin in cultured myocytes show that telethonin localises to the nascent Z-disc. This localisation is in excellent agreement with the two-hybrid screening data and the biochemical binding analysis, but is in contrast to previous immunocytochemical data that suggested that telethonin protein was localised abundantly to the sarcomeric A-band [21]. The A-band localisation of telethonin in adult muscle still remains to be characterised. It is possible that the protein is redistributed in adult muscle, and that telethonin fulfils functions necessary for Z-disc as well as A-band function.

The telethonin gene encodes the twelfth most abundant muscle transcript; yet, only an estimated 0.05% of total muscle protein is telethonin [21]. This argues that telethonin protein underlies a rapid turnover with a small static pool of protein, or that its expression is strongly translationally controlled. The latter notion is supported by the observation that extension of the telethonin cDNA into the 5′ non-coding region failed ([21] and A. Mues, unpublished observation), suggesting a possible extension block by stem-loop structures in the 5′ untranslated region. Such translational control is frequently found in muscle proteins and is believed to represent a way of dynamic adaptation to turnover demands [23]. We observed furthermore that telethonin rapidly degrades in muscle extracts and is undetectable in aged muscle samples or myofibril preparations (not shown). This volatility may have contributed to the unduly late identification of the protein, and to the difficulties in immunocytochemical localisation ([21] and our observations).

The interaction of telethonin with the Z1–Z2 domains of titin is modulated by flanking sequences. In the two-hybrid protein interaction assays, constructs including not only Z1–Z2 but also the serine-proline-rich insertion zis1 and the putative Ig domain Z3 do not interact with telethonin (Fig. 1) whereas truncation of the C-terminal region activates the telethonin binding site in Z1–Z2. In agreement with our observations, the previous two-hybrid screens with a 63 kDa N-terminal fragment of avian titin [8] have detected no interactions with telethonin. This suggests that the N-terminal region of titin between Z1 and Z3 could adopt a 'closed' conformation which would block the telethonin binding site. In vivo, phosphorylation of the multiple serine-proline phosphorylation sites by developmentally controlled kinases [6] could be the modulating factor controlling titin-telethonin interactions and possibly further ligand interactions. In addition, telethonin contains putative phosphorylation sites in the C-terminal region [21], and dual phosphorylation of both titin and telethonin may be important in controlling the interaction of both proteins, serendipitously mimicked in the two-hybrid assays. We suggest therefore that telethonin does not represent a static component of the myofibril, but rather a protein involved in the dynamic control of very early events of myofibrillogenesis and myofibril turnover.

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