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Characterization of the *in vitro* expressed autoimmune rippling muscle disease immunogenic domain of human titin encoded by TTN exons 248–249

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ABSTRACT

Autoimmune rippling muscle disease (ARMD) is an autoimmune neuromuscular disease associated with myasthenia gravis (MG). Past studies in our laboratory recognized a very high molecular weight skeletal muscle protein antigen identified by ARMD patient antisera as the titin isoform. These past studies used antisera from ARMD and MG patients as probes to screen a human skeletal muscle cDNA library and several pBluescript clones revealed supporting expression of immunoreactive peptides. This study characterizes the products of subcloning the titin immunoreactive domain into pGEX-3X and the subsequent fusion protein. Sequence analysis of the fusion gene indicates the cloned titin domain (GenBank ID: EU428784) is in frame and is derived from a sequence of N2-A spanning the exons 248–250 an area that encodes the fibronectin III domain. PCR and EcoR1 restriction mapping studies have demonstrated that the inserted cDNA is of a size that is predicted by bioinformatics analysis of the subclone. Expression of the fusion protein result in the isolation of a polypeptide of 52 kDa consistent with the predicted inferred amino acid sequence. Immunoblot experiments of the fusion protein, using rippling muscle/myasthenia gravis antisera, demonstrate that only the titin domain is immunoreactive.

1. Introduction

In striated muscle, titin provides elasticity and support functions for the sarcomere [1,2]. More dynamic roles for titin have been proposed involving minK and T-cap stretch activated processes [3]. It has also been shown that a negatively charged region of titin, near the elastic PEVK region, is modulated by Ca^{2+} binding [4] at the A/I junction region. Additionally it has been suggested that physiological functions of titin are mediated by this Ca^{2+} binding [5]. Studies of tibial muscular dystrophy [6] defects have been linked to deletion/insertion mutation in titin (TTN). These deletions appear to cause defects in M-line titin, [7]. There is a growing number of genetically based "titinopathies" being found associated with both cardiac and skeletal muscle diseases. Titinopathies may involve developmental pathways during myogenesis and these may be linked to titin's role in chromosome structure [8,9].

An autoimmune form of rippling muscle disease [1] was discovered in myasthenia gravis (MG) patients, possibly involving specific antigenic sites on titin. Myasthenia gravis is caused by anti-acetylcholine receptor antibody interference with neuromuscular transmission resulting in inhibition of muscle activation and consequently sensations of fatigue. Autoimmune rippling muscle disease (ARMD) is characterized by an immune system induced response in skeletal muscle involving stretch activation of muscle contraction, similar to that initially described for a genetic rippling muscle syndrome [11]. The first clue to the possible involvement of titin in this autoimmune variant of MG came from immunoblotting studies indicating that a very high molecular weight protein was a major autoantigen [12]. In addition, anti-sera from ARMD patients appear to display a distinct immunoblot pattern in skeletal muscle when compared to that found with sera from more characteristic myasthenia gravis patients [12].

Subsequent screening studies, probing skeletal muscle derived expression cDNA libraries, specifically identify titin as one of the significant auto-antigens [13]. Several clones were isolated from a human skeletal muscle expression library, sequenced and subjected to BLASTx database searches and subsequently shown to be titin specific sequences covering two regions of titin. The two regions shown to be antigenic can be grouped into two general clusters, one group outside the I/A-band boundary, in the I-band region and the other group past the I/A-band boundary in the thick filaments region. The antibodies show some striated patterns of antigen distribution within the muscle fiber in immunofluorescent studies.

This study characterizes a subcloned version of the putative ARMD specific antigenic domain of titin. The original analysis was carried out using pBluescript constructs [13] and this vector

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is not conducive to expression of and direct analysis of the immunogenic domain. In order to achieve better sequence data and allow for purification and analysis of the expressed titin antigenic domain we subcloned into a pGEX-3X fusion vector. This study reports the characterization of both the expressed immunogenic domain and characterizes in more detail the cDNA and the specific antibody titin interactions involved in autoimmune rippling muscle disease.

2. Materials and methods

2.1. Bacterial strains and plasmids

Chemically competent *Escherichia coli* cells (Top10, Invitrogen) were used for all transformations. The genotype is F-*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

The rippling muscle titin antigenic peptide clone #6 derived from pBuescript Phagemids [13] was subcloned into pTOPO-CR (pT-RMMG6) and maintained as TOPO-CR plasmid containing bacterial stocks. The expression vector pGEX-3X (GE Healthcare Sciences) was used to construct gst-affinity fusion genes.

2.2. Cloning of pG3RMMG6 and bacterial transformation

Plasmids were purified from 100 ml overnight bacterial cultures containing either pGEX-3X or pT-RMMG6. Plasmid purification was carried out using the Qiagen® Plasmid Midi Purification Kit following the manufacturer's instruction. The final purified DNA was resuspended in 600 μL of nuclease free water heated to 70 °C. Absorbance at 260 and 280 nm by spectrophotometry (SmartSpec, BioRad) was used to quantify and assess the purity of DNA.

After purification of both the pGEX-3X and pT-RMMG6 DNA EcoR1 restriction enzyme digestion was performed to cleave pGEX-3X and pT-RMMG6, to release the RMMG6 cDNA. The reaction mixture included approximately 750 ng of plasmid DNA, 50 μL of H buffer (Sigma®) and 30 μL of EcoR1 (SIGMA®) in a total volume of 500 μl. The digestion reactions were carried out at 37 °C. After 1 h 120 μL of stop buffer (AMRESCO) was added to inhibit the restriction enzyme and in preparation for agarose gel electrophoresis.

The restriction digest products were isolated by preparative gel electrophoresis using 2% agarose. Gel wells were loaded with 340 μL (666 ng) of pT-RMMG6 or 130 μL (366 ng) of pGEX-3X and the gels were run at 120 V for about 3.5 h. The linearized pGEX-3X plasmid and the RMMG6 cDNA bands were isolated and excised from the gel and the DNA extracted using the QIAquick Gel Extraction Kit (Qiagen®) kit, following the manufacturer's protocol. The final DNA samples were quantified by absorbance at 260 nm.

Construction of pGST-RMMG6 was accomplished by ligation of RMMG6 cDNA to the linearized pGEX-3X. Ligation buffer was made by adding 1 μL of ATP (SIGMA®) and 1 μL of 10× T4 ligase buffer (SIGMA®) in a small PCR tube. Afterward in a separate small PCR tube the following was added: 0.7 μL of nuclease free water, 1 μL of previously made ligation buffer, 7.1 μL (60 ng) of RMMG6 DNA insert, 0.7 μL (10 ng) pGEX-3X vector and 0.5 μL of well mixed ligase (SIGMA®). The ligation reaction was vortexed and then placed the ligation tube in the PTC-200™ Peltier thermal cycler DNA Engine Gradient cycler (MJ Research, Inc). The ligation reaction was carried out in two phases; first samples were incubated at 22.5 °C for 30 min.

The ligation reactions were followed immediately by transformation of *E. coli* One Shot TOP 10 (Invitrogen) cells. The ligation reaction mixture (2 μL) was added directly to the *E. coli* One Shot TOP 10 cell and then placed on ice for 45 min. Next the transformation mixture was transferred to a 42 °C water bath for 1 min to heat shock the cells and then transferred back to ice for 5 min. Room temperature SOC medium (250 μL) was added to the cryovial and incubated at 37 °C with agitation for 1 h and 15 min. Finally, 50 and 200 μL of the ligation reaction/transformation mixture were plated on LB Amp plates and incubated overnight at 37 °C.

The next day several colonies from each plate were transferred to LB Amp plates. The plate was incubated at 37 °C overnight. A sterile toothpick was used to transfer eight colonies in 5 ml of LB Amp broth. The broth was incubated overnight at 37 °C. The next day an AMRESCO Cyclo-Prep plasmid purification was performed according to the manufacturer's protocol. The DNA was eluted with 50 μl nuclease free water heated to 70 °C.

An EcoR1 restriction enzyme digest was performed on the purified plasmid DNA. In an eppendorf tube 20 μL of DNA was combined with 1 μL of nuclease free water, 2.5 μL of H buffer (SIGMA®) and 1.5 μL of EcoR1 (SIGMA®). The eppendorf tube was vortexed and incubated at 37 °C for 30 min and then vortexed again and incubated at 37 °C for another 30 min. After incubation, 6 μL of stop buffer was added.

When the restriction enzyme digest products were analyzed by agarose gel electrophoresis (2% agarose, 120 V for 3.5 h). Clones that did not contain the RMMG6 DNA insert were discarded. Clones that contained the RMMG6 DNA insert were grown overnight in LB Amp broth.

2.3. DNA sequencing

Sequencing reactions were performed using the Beckman–Coulter CEQ™ Quick Start Kit. Plasmid purified samples were sequenced according to the manufacturer's suggested procedure. Roughly 1.5 μg of pure plasmid DNA (dilutions were made and approximately 2 μL) was added to 2 μL of (1.6 pmol) primer, 8 μL of nuclease free water and 8 μL of DTCS Quick Start Master Mix and then gently mixed. The samples were run according to the manufacturer's directions. The products were analyzed with the Beckman–Coulter CEQ™ 2000XL automated sequencer. The technique used for sequence analysis was the LFR-1 + 30. Sequence data was subjected to BLAST alignment analysis using four Peaks and Geneius software.

The following primers were used for PCR and Sequencing
pGEX primers

Reverse (3′ end primer): 5′-CCGGGAGCTGCATGTGTCAGAGG-3″
Forward (5′ end primer): 5′-GGGCTGGCAAGCCACGTTTGGTG-3″

2.4. Protein expression of pGST-RMMG6

An overnight culture of pGST-RMMG6 was grown for 20 h at approximately 22 °C in 300 ml of LB Amp broth. Then 300 μl of Isopropyl-ß-D-thio-galactoside (IPTG) was added to the culture (to 0.1 mM) to induce the *lac* operon to express our protein. The culture remained at 22 °C for 4 h after the IPTG addition. After the IPTG induction the culture was poured into 50 ml conical Falcon tubes and centrifuged at 4 °C for 30 min at 3800 rpm.

2.5. Glutathione affinity chromatography purification

The bacterial cell pellet was washed by re-suspending with 4 °C TBS and then centrifuged again at 4 °C for 30 min at 3800 rpm. The supernatant was decanted and the cells were re-suspended in 5 ml of 4 °C TBS then 100 μl of Sigma P-7626 FW 174.2 phenylmethylsulfonyl fluoride α-toluenesulfonyl fluoride was added and point sonicated at 50% power for 10 s on and 10 s off for four cycles. Next, 0.65 ml of 10% Triton X-100 was added and sonicated for