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Identification of Skeletal Muscle Autoantigens by Expression Library Screening Using Sera From Autoimmune Rippling Muscle Disease (ARMD) Patients

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Abstract Novel forms of contractile regulation observed in skeletal muscle are evident in neuromuscular diseases like rippling muscle disease (RMD). Previous studies of an autoimmune form of RMD (ARMD) identified a very high molecular weight skeletal muscle protein antigen recognized by ARMD patient antisera. This study utilized ARMD and myasthenia gravis (MG) patient antisera, to screen a human skeletal muscle cDNA library that subsequently identified proteins that could play a role in ARMD. Based on nucleotide sequence analysis, three distinct ARMD antigens were identified: titin Isoform N2A, ATP synthase 6, and PPP1R3 (protein phosphatase 1 regulatory subunit 3). The region of titin identified by ARMD antisera is distinct from the main immunogenic region (MIR) recognized by classical MG antibodies. Sera from classical MG patient identifies an expressed sequence corresponding to the titin MIR. Although the mechanism of antibody penetration is not known, previous studies have shown that rippling muscle antibodies affect the contractile machinery of myofibers resulting in mechanical sensitivity. Titin's role as a modulator of muscle contractility makes it a potential target in understanding muscle mechanosensitive regulation. J. Cell. Biochem. 99: 79–87, 2006. © 2006 Wiley-Liss, Inc.

Key words: titin-like; autoantigen; rippling muscle disease; mechanosensitive

In rippling muscle disease (RMD) and myasthenia gravis (MG) the contractile behavior of skeletal muscle is altered. RMD, as first described by Torbergsen [1975] is a genetic disease involving an autosomal dominant mutation mapping to multiple chromosomal loci [Torbergsen, 1975; Ricker et al., 1989]. Recent evidence suggests a recessive form of RMD [Koul et al., 2001]. The hallmark characteristics of the disease are the percussion or stretch induced contractions displayed by the skeletal muscles [Torbergsen, 1975; Ricker et al., 1989]. The contractions appear electrically silent, that is, they display a lack of motor unit action potentials [Jusic, 1989; Ricker et al., 1989]. Interpretations of these data suggest that an alteration in calcium homeostasis is involved in the altered muscle contractility observed with RMD [Ansevin and Agamanolis, 1996].

Recent studies have mapped the chromosomal locations of specific genes associated with RMD. *RMD*1 is a rippling muscle gene that maps to 1q41 [Stephan et al., 1994; Stephan and Hoffman, 1999]. Another gene associated with RMD maps to a different location than that of *RMD*1 [Ricker et al., 1989]. This gene maps to the short arm of the human chromosome 3, a locus that contains *CAV*3. Mutations in certain regions of the *CAV*3 (caveolin-3) gene have been shown to result in rippling muscle [Betz et al., 2001; Vorgerd et al., 2001]. The *CAV*3 gene maps to 3p25 [Minetti et al., 1998; Sotgia et al.,

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1999]. Interestingly, mutations in other regions of the *CAV3* gene result in other distinctive muscle diseases [de Paula et al., 2001].

In 1996, Ansevin and Agamanolis described a MG patient who presented with peculiar wave-like contractions of skeletal muscles that were induced by both stretch and percussion [Ansevin, 1996; Ansevin and Agamanolis, 1996]. The patient, one of nine siblings, had no family history of rippling muscles (RM). The RM symptoms were alleviated by immunosuppressive therapy for MG, suggesting an autoimmune etiology for this form of RMD (autoimmune rippling muscle disease, ARMD). The mechanosensitive nature of these contractions and the fact that calcium is the direct initiator of skeletal muscle contraction suggests involvement of a mechanosensitive calcium ion channel in ARMD [Ansevin and Agamanolis, 1996; Muller-Felber et al., 1999].

MG is an extensively studied neuromuscular autoimmune disease. Autoantibodies to the nicotinic acetylcholine receptors are thought to be the causative agents in at least 85% of the cases [Vincent et al., 2001]. About 10%–15% of patients diagnosed with MG have a thymoma, a tumor of the thymus gland [Vincent et al., 2001]. In about 10% of the MG patients no anti-acetylcholine receptor antibodies are detected and are thus classified as seronegative (SNMG). They display anti-muscle specific kinase (MuSK) antibodies [Hoch et al., 2001]. These autoantibodies inhibit the action of MuSK presumably resulting in MG symptoms [Vincent et al., 2001]. Titin, myosin, and MuSK are among the types of antigens recognized by antibodies produced in MG patients.

Sera obtained from ARMD patients afforded this lab an opportunity to use antibodies as probes for proteins that play a role in a possible mechanosensitive contractile process. Studies using sera from RMD patients were used to identify and characterize immunoreactive skeletal muscle proteins. By immunoblot and immunoprecipitation analysis, proteins of large molecular mass were identified as potential ARMD antigens [Walker et al., 1999]. The antibodies from ARMD and MG/thymoma patients recognize a set of large proteins that remain trapped in the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) stacking gel. The sizes of these proteins are consistent with them being skeletal muscle proteins like titin or nebulin.

This study uses ARMD patient antisera as probes in the screening of a human skeletal muscle expression library. The skeletal muscle proteins, titin, ATP synthase subunit 6 and protein phosphatase 1 regulatory subunit 3 were identified as antigens by sequence analysis of immuno-selected cDNA clones. Regions of titin isoform N2-A (GenBank accession # NP596869) are identified as immunogenic sites possibly unique to ARMD.

METHODS

A Lambda Zap® human skeletal muscle cDNA library (Stratagene) was immunoscreened with antisera from patients with ARMD to identify proteins associated with rippling muscles. The λ-Zap phage library was plated out on *E. coli* XL-Blue MRF lawns at about 50,000 plaque-forming units per plate (150 mm NZY agar plates) and protein production was induced by 1.5% isopropyl-β-D-thiogalactopyranoside (IPTG) (SIGMA) plates were incubated at 37°C for 8 h. The plates were then blotted onto IPTG-saturated nitrocellulose membranes. The membranes were first blocked with 3% non-fat dry milk, 0.2% Tween 20 in tris buffered saline (TBS). Sera from ARMD, MG-T (MG with thymoma), or SNMG (seronegative MG) patients were used as primary antibodies. Primary antibodies were applied at a concentration between (1:500 to 1:1,000) and the membranes were incubated for 1 h. Unbound antibody was then removed by washing filters in TBS containing 0.2% Tween-20. The immunoreactive plaques were visualized by chromogenic detection with the use of a goat anti-human IgG secondary antibody, conjugated to horseradish peroxidase. The unbound secondary antibody was removed by washing with TBS containing 0.2% Tween-20. The filters were then incubated with the chromogenic substrate 4-chloro-1-napthol (SIGMA). The membranes were aligned with the plates and positive plaques were identified and cored. To establish the clonality of each positive phage, the positive plaques were cored by stabbing the agar containing the phage of interest with a pipette tip. The resulting agar plugs were injected into 1 ml of SM buffer with 40 μl of chloroform and vortexed gently. The $OD_{600} = 0.5$ XL-Blue MRF, cells were then infected with phage at a 1:1,000 dilution to produce well-spaced plaques, which were rescreened with the patient's serum used to

initially identify the plaques. Positive plaques were again selected, cored, and subjected to a tertiary round of screening before subjecting the positive phage to excision.

Excision of pBluescript Phagemids

The pBluescript phagemid encoding immunoreactive cDNA from each of these cloned phages was recovered by excision, with the use of the helper phage, ExAssist™ (Stratagene). Phagemids were propagated in *E. coli*. Cloned phagemids were analyzed by restriction endonuclease mapping and 1% agarose gel electrophoresis. Insert DNA sizes were determined based on electrophoretic mobility.

DNA Sequence Analysis and Database Searches

The Beckman–Coulter™ CEQ DCTS Quick Start kit was used for DNA sequence analysis using the isolated clones for PCR amplification. We used pBluescript specific primers M13 (5′-AATTAACCCTCTACTAAAGGG-3′) and reverse primer (5′-GGAAACAGCTATGACCA-TG-3′). Sequences were obtained with a Beckman/Coulter CEQ 2000 DNA Sequencer. The Basic Local Alignment Search Tool (BLAST); was used to compare the derived DNA sequences to existing databases (http://www.ncbi.nlm.nih.gov/BLAST).

Immunofluorescent Localization of Antigens

Human skeletal muscle was cryosectioned on a Leica CM 1800 cryostat microtome at -19°C. Sections of 10 μm thickness were stored frozen on glass microscope slides at -80°C. For analysis, cryosections were thawed and fixed for 30 min with 3.7% formaldehyde at room temperature. Fixed sections were washed with TBS three times for 10 min each followed by incubation in blocking buffer 5% powdered nonfat dry milk (PNDM) in TBS, to block nonspecific binding sites. The sections were made permeable by incubation in 0.25% Triton X-100 in blocking buffer. Sections were incubated 60 min in primary antibody (1:500) dilution in 1% PNDM/TBS) at room temperature with agitation. The sections were washed three times with TBS and then incubated 60 min at room temperature with FITC conjugated goat anti human Fab (1:1,000 dilution in 1% PNDM/TBS). The unbound secondary antibody was removed by washing sections three times with TBS. The tissues were examined by oil immersion microscopy using an Olympus Provis AX70 epifluorescence microscope. Micrographs where taken using Kodak 35 mm T-Max 400 film.

RESULTS

Identification of Immunoreactive Peptide Sequences

Autoantibodies from the sera from ARMD, SNMG, and MG-T patients were used to identify autoantigens in a Stratagene™ human skeletal muscle expression library. Ten immunoreactive cDNA were obtained from screens using these antibodies. The DNA sequences were determined and sequence identities were determined by BLAST database analysis. Five peptides in the databases searched show high degree of identity to; titin Isoform N2A, ATP synthase subunit 6, enolase, aldolase, and PPP1R3 (protein phosphatase 1 regulatory subunit 3) (Table I). The peptides all showed bit score above 70 and E-values below 1×10^{-10} indicating biological significance (data not shown).

ARMD Immunoreactive Sequences

Antisera from ARMG patients recognized expressed proteins that show identities to three muscle proteins (Table I). Plasmids pRMMG-1

TABLE I. Summary of GenBank Database Sequences Producing High Scoring Alignments With cDNA Sequences Identified by Patient's Sera

Possible autoantigen	Sequence Gen Bank Id. #	# of clones with identity	Immunoreactive clones		
PPP1R3	NM005398	1	pRMMG-1		
Titin N2-A	NP596869.1	6	pRMMG-5,-6,-8, -9,-11	pMG10-1	
ATP synthase	NP776050	1	pRMMG-4		
Enolase	NP001967.1	1		pMG10-2	
Aldolase	CAA30979.1	1			pSNMG-15

Clones are identified by the patient's sera used to identify the immunoreactive peptides produced by that cDNA clone. (RMMG, rippling muscle myasthenia gravis patient; MG, myasthenia gravis patient; SNMG, seronegative myasthenia gravis patient.)

```
pRMMG-6        LXELVDPPGCRNS--LAHNLTNESCKLTWFSPEDDGGSPITNYVIEKRESDRXXAWTPVT
Titin N2-A     IVDVLDVPGPVGTPFLAHNLTNESCKLTWFSPEDDGGSPITNYVIEKRESDRR-AWTPVT 17594

pRMMG-6        YTVTRQNATVQGLIQGKAYFFRIAAENSIGMGPFVVITSEALVIREPITVPERPEDLEVK
Titin N2-A     YTVTRQNATVQGLIQGKAYFFRIAAENSIGMGPFVE-TSEALVIREPITVPERPEDLEVK 17653

pRMMG-6        EVTKNTVTLTWNPPKYDGGSEIINYVLESRLIGTEKFHKVTNDNLLSRKYTVKGLKEGDT
Titin N2-A     EVTKNTVTLTWNPPKYDGGSEIINYVLESRLIGTEKFHKVTNDNLLSRKYTVKGLKEGDT 17713

pRMMG-6        YEYRVSAVXIVGXAXHHXAPNPL
Titin N2-A     YEYRVSAVNIVGQGKPSFCTKPI 17736
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Fig. 1. Plasmid pRMMG-6 amino acid sequence shows identity with skeletal muscle titin isoform N2-A (Gen Bank accession number NP596869). A translation of plasmid pRMMG-6 yields a amino acid sequence showing high identity to titin N2-A. The region of identity is in the +3 reading frame translation of sequenced cDNA. Amino acid residues in black highlights indicate identities, while gray highlighted residues indicate positives.

and pRMMG-4 are plasmids containing coding sequences showing identities with phosphoprotein phosphatase 1 subunit R3 and *Homo sapiens* ATP synthase 6, respectively (Table I, Gen Bank accession #s NM005398 and NP776050). The alignment between pRMMG-1 and PPP1R3 (data not shown) produces a high scoring pair with a bit score of 244 and an E-value 10^{-62}. This region of identity stretched over 109 amino acids. There were 99/109 identities (90%). An alignment between plasmid pRMMG-4 and *Homo sapiens* ATP synthase 6 produced a high scoring pair with a bit score of 70.1 and an E-value of 9×10^{-12}.

Sequences having homologies to the enzymes enolase (pMG10-2) and aldolase where identified with the use of MG and SNMG sera (Table I). No further characterization of these clones was done since, their relevance to muscle contractility is not obvious and thus not important to the focus of our current studies.

Five of the seven cDNAs obtained from the ARMG immunoscreen show specific identity to regions of titin. The highest scoring sequence alignment with a portion of the N2-A titin isoform, between amino acid numbers 17536 and 17736, and plasmid pRMMG-6 (Fig. 1). The alignment produces a bit score 342 in the +3 reading frame with an E-value of 3×10^{-93}. This region of identity stretches over 203 amino acids with 2-gap penalties. There were 176/203 identities (86%) (Fig. 1). There were also 182/203 positive amino acid matches in the high scoring pair.

Clone pRMMG-5 (data not shown) has sequence identify with the titin amino acid sequence 17562 and 17624. This sequence is contained within the pRMMG 6 sequence (Fig. 1). This sequence's highest scoring alignment produced a bit score of 132 with an E-value of 9×10^{-31}. This alignment covered a 63 amino acid region with 100% identity between the clone and the titin N2-A isoform.

Clone pRMMG-11 shows identity to the titin N2-A region, amino acids 13,329–13,412, (Fig. 2) giving a BLASTx bit score of 160 with an E-value of 2e−59. This region is close to the main immunogenic region (MIR) of titin recognized by autoantibodies from MG/thymoma patients [Gautel et al., 1993 #83], amino acids 14,432–14,495 (Gen Bank accession #AAB2 8119) (Fig. 2). It appears that all of the rippling muscle-associated immunogenic sequences fall outside this region. There is no sequence overlap between pRMMG-6 and pRMMG-11. They appear to be distinct regions of titin. Two additional clones, pRMMG-8 and pRMMG-9, have significant sequence alignments with titin

```
pRMMG-11       AKVKWFKDGAEIKKGKKYDIISXXGAVRILVINKCLLDDEAEYSCEVRTARTSGMLTVLE
Titin N2-A     AKVKWFKDGAEIKKGKKYDIISK-GAVRILVINKCLLDDEAEYSCEVRTARTSGMLTVLE 13387

pRMMG11        EEAVFTKNLANIEVSETDTIKLVCE
Titin N2-A     EEAVFTKNLANIEVSETDTIKLVCE 13412
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Fig. 2. Plasmid pRMMG-11 amino acid sequence shows identity with skeletal muscle titin isoform N2-A. Plasmid pRMMG-11 amino acid translation shows an identity with skeletal muscle titin isoform N2-A. The region of identity is in the +1 reading frame. Amino acid residues in black highlights indicate identities.

(Alignment 1)

```
pRMMG-9    SDIGQYTCDCGTDKTSGKLDIEDREIKLVRPLHSVEVMETETARFETEISEDDIHANWKL
Obscurin   ADRGFFGCETPDDKTQAKLTVEMRQVRLVRGLQAVEAREQGTATMEVQLSHADVDGSWTR 2111

pRMMG-9    KGEALLQTPDCEIKEEGNIYT-------------PLFCTTVAWTRRVGWISKLPMLNLVP
Obscurin   DGLRFQQGPTCHLAVRGPMHTLTLSGLRPEDSGLMVFKAEGVHTSARLVVTELPVSFSRP 2171

pRMMG-9    TSELSHEXLVFXGLXRMSPVTAGETATFDCELSYEDIPVEWV
Obscurin   LQDV--------------VTTEKEKVTLECELSRPNVDVRWL 2199
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(Alignment 2)

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pRMMG-11   VKWFKDGAEIKKGKKYDIISXXGAVRILVINKCLLDDEAEYSCEVRTARTSGMLTVLEEE
Obscurin   VHWLKDRKAIRKSQKYDVVCE-GTMAMLVIRGASLKDAGEYTCEVEASKSTASLHVEEKA 2915

pRMMG-11   AVFTKNLANIEVSETDTIKLVCEXFQ-----TWRRSDLVXRGXGDH
Obscurin   NCFTEELTNLQVEEKGTAVFTCKTEHPAATVTWRKGLLELRASGKH 2961
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Fig. 3. BLASTx alignments of amino acid sequences of immunoreactive cDNA clones that show a high scoring pair with obscurin. Plasmids pRMMG-9 and pRMMG-11 both show identity to skeletal muscle obscurin (GenBank Accession number CAC44768). Identities are indicated in black while positives are indicated by gray. Alignment 1 shows the results of pRMMG-9 in its +1 reading frame aligned with obscurin. Alignment 2 reports the results of a high scoring alignment between obscurin and plasmid pRMMG-11 in its +1 reading frame.

isoform N2-A (data not shown). These alignments are between amino acids 13,004 and 14,109. This is the region overlapping with the pRMMG- 11 region.

In addition to homology with titin, the pRMMG-11 clone also exhibits identity with obscurin (Fig. 3) (GenBank Accession number CAC44768). BLASTx produces an alignment with a bit score of 67 and an E-value of 2e−10 over amino acids 2,857 through 2,961 of obscurin shown in Figure 3. Moreover, the same pRMMG-9 open reading frame (ORF) that produced this high scoring N2-A alignment also shows a lower scoring alignment with skeletal muscle obscurin (Fig. 3) (Gen Bank accession number CAC44768). This alignment produces a bit score 63 with E-value 4e−9 and shows identity to 43 amino acids over a 162 amino acid region but also shows 69 positive matches over the same region between amino acids 2,052 and 2,199 of obscurin (Fig. 3).

Immunofluorescent Distribution of ARMD Autoantigens

The distribution of immunoreactive autoantigens using ARMD sera results in a striated pattern of immunofluorescence (Fig. 4C, arrows). The immunoreactivity displayed is intense and in patterns of cross-hatching from the lateral sides of the myofibers (Fig. 4C, arrows). Intense immunoreactivity is also observed along the margin of the sarcolemma showing straited and punctate patterns at the lateral margins of the cells (Fig. 4B, arrows). Immunofluorescence using normal is less intense compared to that observed for ARMG sera (Fig. 4A). With the normal sera there is a lack of striated or punctate staining. However, the striational banding pattern of this patient's sera corresponded to the striational banding pattern seen for dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) suggesting the presence of autoantibodies to the cellular location of the DHPR and RyR (data not shown).

MG Immunoreactive Sequences

Anti-sera obtained from an MG patient presenting with a thymoma identified a sequence (pMG 10-1) also showing close identity to a titin N2-A region (Fig. 5). The BLASTx alignment produces a bit score of 158 and an E-value of 4e−44. The alignment shows 89/131 identity (67%), while 95/131 (72%) is identical or positive (conservative difference). This region is immediately outside the MIR, often recognized by antisera from seropositive myasthenics.

The Relationship Between the Antigenic Sequences

The identified sequences can be assigned locations on the N2A titin linear sequence

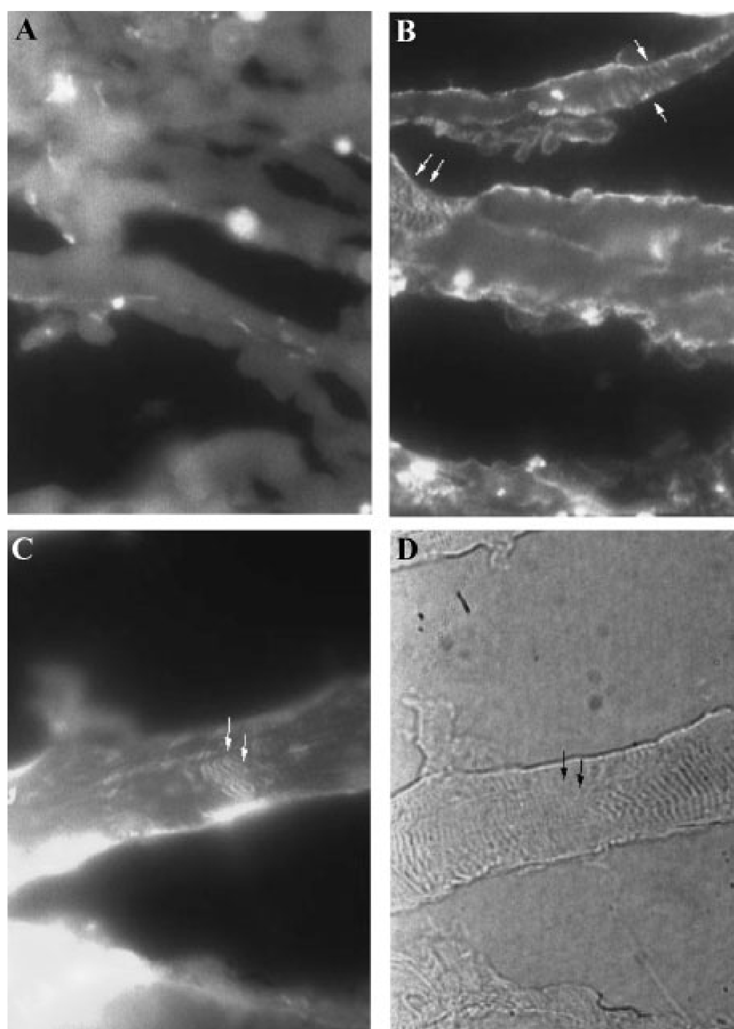

Fig. 4. Immunofluorescence labeling pattern of the rippling muscle disease (RMD) antisera. Skeletal muscle sections stained with ARMD patient anti-sera (**B–D**). or normal human sera (**A**). Arrows indicated striation-like fluorescence staining (A–C). The phase contrast image (D) also shows striations.

(Fig. 6). There are two distinct regions of the titin molecule act as immunogenic sites for MG and rippling muscle autoantibodies. Antibodies from the rippling muscle patients bind to both regions where as antibodies from classical myasthenic gravis patients bind only to the region close to the MIR (Fig. 6).

DISCUSSION

The presence of autoantibodies bind to regions of titin in ARMD patients suggests the possibility of a role for anti-titin antibodies in ARMD, although there is no clear understanding of the cellular effects of anti-titin antibodies. The immunogenic regions associated with ARMG, localize to two separate regions of titin (Fig. 6). Two of the selected cDNAs show high identity to a region within the myosin thick filament overlap area of titin (Fig. 6). The other ARMD immunoreactive clones, as well as a MG clone, show identity to a region near the MIR, between the MIR and the PEVK, regions of titin that are related to stiffness and elasticity [Gutierrez-Cruz et al., 2001] (Fig. 6) and outside of the thick filament overlap region.

Clearly our results demonstrate that ARMD patients have circulating autoantibodies to a protein that shares sequence with skeletal muscle titin isoform N2-A. This is in agreement with our previous work that showed ARMD autoantibodies recognized high molecular weight proteins (found in the SDS–PAGE stacking gels) [Walker et al., 1999]. The low

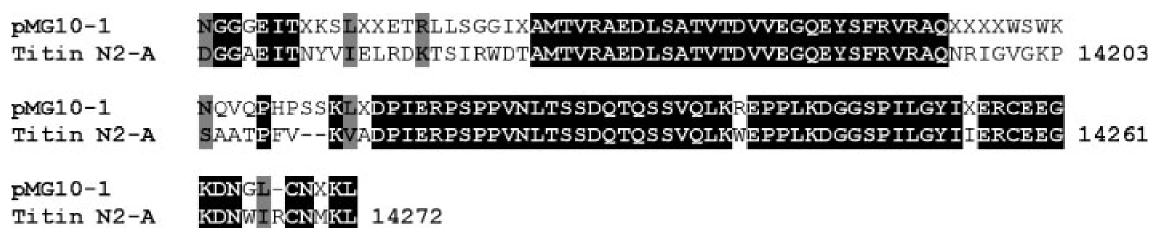

Fig. 5. Plasmid MG10-1 shows amino acid sequence identity with skeletal muscle titin isoform N2-A. The cDNA sequence of pMG10-1 translated in the -3 reading frame, shows identity with skeletal muscle titin isoform N2-A (Gen. Bank Accession number NP 596869.1). The identities are indicated in black highlights and the positives are shown in gray.

gel mobility of the immunoreactive bands in immunoblotting is consistent with the electrophoretic mobility expected for titin (with a molecular weight near 3,000 kDa) [Trinick, 1992]. The ARMD immunoblots on skeletal muscle using ARMD sera display a unique immunoblot pattern when compared to control sera immunoblots [Walker et al., 1999]. Analysis of this data suggests a possible role for autoantibodies in the apparent mechanosensitive muscle contractions in ARMD patients [Ansevin, 1996; Walker et al., 1998]. Interestingly, antigen sites occur in two clusters: one around the MIR found for other MG antibodies and the other is in the myosin overlap region past the A band/I band junction (Fig. 6). This last region seems unique to ARMD.

The MIR of skeletal muscle titin has been described as an antigenic region of titin [Gautel et al., 1993]. This MIR localizes to near the A/I junction of skeletal muscle although additional epitopes have been identified indicating antigenic spread [Gautel et al., 1993; Lübke et al., 1998]. Further, 95% of MG/T patients have autoantibodies to this region although a pathogenic potential for anti-titin antibodies has not been determined [Skeie, 2000]. Interestingly different muscle diseases result from different mutations in the same molecule, suggesting that autoimmune diseases may also be divergent due to antibodies binding different epitopes on the same protein.

Titin's role, in striated muscle as originally described, is to provide elasticity and support for the sarcomere [Wang and Greaser, 1985; Labeit and Kolmerer, 1995]. Recently, studies have shown that titin's role in the sarcomere is more dynamic than originally described. Titin along with T-cap and minK, is involved in the linkage of the T-tubules with the Z-disk in cardiomyocytes with a stretch sensitive role in K^+ channel activity [Furukawa et al., 2001]. It has also been shown that a negatively charged region of titin near the elastic PEVK region at the A abnd/I band interface may be involved in Ca^{2+} binding in μM quantities [Tatsumi et al., 2001]. Additionally, it has been suggested that physiological functions of

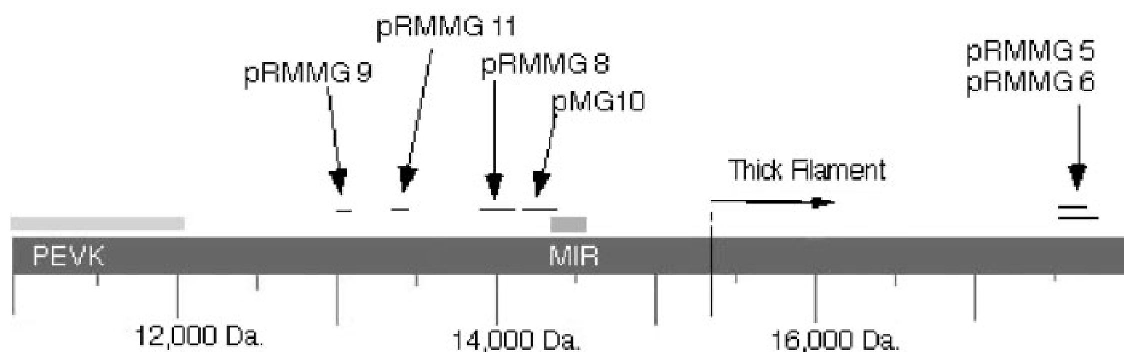

Fig. 6. Immunoreactivessquences locations relative to a section of titin isoform N2-A (amino acid 11,000 to amino acid 18,000). The sequence identities for each of the immunoreactive sequences are indicated by arrows to solid lines (showing relative distances in amino acid units). The horizontal arrow indicates the beginning of the myosin thick filament region.

titin are mediated by this Ca^{2+} binding [Kolmerer et al., 1999].

The increased understanding role of titin in muscle cell physiology has also led to its characterization in "titinopathies" of genetic disease in both cardiac and skeletal muscles. TTN, the gene coding for skeletal muscle titin, has been mapped to chromosomal locus 2q31 [Labeit and Kolmerer, 1995]. Henceforth, several striated muscle diseases have also been linked to this chromosomal locus. In skeletal muscle studies describing tibial muscular dystrophy [Udd et al., 1998] defects were linked to an 11 bp deletion/insertion mutation in TTN appearing to cause a defect in M-line titin [Hackman et al., 2002]. More dymamic effects due to titin mutations have been described in cardiomyocytes. Dilated cardiomyopathy has been linked to a truncation mutation resulting in tissue remodeling and hypertrophy in cardiac muscles [Gerull et al., 2002; Hein and Schaper, 2002]. Hypertrophic cardiomyopathy has been linked to the titin missense mutation, Arg740Leu, resulting in an increase in titin's binding affinity to alpha-actinin possibly leading to a functional alteration [Satoh et al., 1999]. Mutations of the sarcomeric protein troponin T have resulted in hypercontractility of cardiac muscle [Bonne et al., 1998].

The question arises as to the accessibility of antibodies to the cytoplasm. The plasma membrane is thought to an impenetrable barrier to large hydrophobic molecules such as antibodies. However, studies have demonstrated that under certain circumstances autoantibodies can cross the cell membrane in living cells, including fibroblasts, neurons, and epithelial cells [Alarcon-Segovia et al., 1996]. Additional studies have been conducted that used flow cytometry to demonstrate that antibodies enter viable lymphocytes [Ma et al., 1987]. The possibility exists that ARMD antibodies can cross the membrane and interact with titin. Such activity leads to the attenuation of titin interaction with ion channels or its own elastic/contractile properties explaining the mechanosensitive characteristics of skeletal muscle cells.

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