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Striational autoantibodies in myasthenia gravis patients recognize I-band titin epitopes

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Abstract

Myasthenia gravis (MG) patients develop autoantibodies primarily against the acetylcholine receptor in the motor endplate, but also against intracellular striated muscle proteins, notably titin, the giant elastic protein of the myofibrillar cytoskeleton. Titin antibodies have previously been shown to be directed against a single epitope on the molecule, located at the A-band/I-band junction and referred to as the main immunogenic region (MIR) of titin. By using immunofluorescence microscopy on stretched single myofibrils, we now report that ∼40% of the sera from 18 MG/thymoma patients and 8 late-onset MG patients with thymus atrophy contain antibodies that bind to a more central I-band titin region. This region consists of homologous immunoglobulin domains and is known to be differentially spliced dependent on muscle type. All patients with I-band titin antibodies also had antibodies against the MIR. Although a statistically significant correlation between the occurrence of I-band titin antibodies and MG severity was not apparent, the results could hint at an initial immunoreactivity to titin's MIR, followed by reactivity along the titin molecule in the course of the disease. © 1998 Elsevier Science B.V.

Keywords: Connectin; Myofibrils; Striated muscle; Immunofluorescence microscopy; Immunoelectron microscopy; Epitope spreading

1. Introduction

Myasthenia gravis (MG) is an autoimmune disease characterized by a developing muscle weakness. The primary cause of this weakness, which occurs in striated muscles only, is the binding of autoantibodies to the nicotinic acetylcholine receptor (AChR) in the motor endplates, which leads to a destruction of the receptor's functional arrangement. Whereas the presence of AChR antibodies appears to be essential for the disease, some MG patients also develop antibodies against non-AChR striated muscle antigens. These antigens include the ryanodine receptor, actin, myosin, alpha-actinin, and titin (Williams and Lennon, 1986; Aarli et al., 1990; Ohta et al., 1990; Mygland et al., 1992). Titin antibodies occur in 80–90% of MG patients who also have a thymoma (MGT patients; cf. Gautel et al., 1993) — an epithelial tumor of the thymus — and in nearly 50% of late onset MG patients with thymus atrophy (MGA patients, cf. Skeie et al., 1995).

Titin, also known as connectin, is a giant filamentous protein (Maruyama et al., 1977; Wang et al., 1979; Labeit and Kolmerer, 1995) which spans half-sarcomeres in striated muscles from the Z-disc to the M-line (Fürst et al., 1988). Along the A-band, titin is bound to the thick filament and therefore functionally stiff (Fig. 1A). In contrast, titin's I-band section is extensible (Fürst et al., 1988; Itoh et al., 1988; Trombitas et al., 1991) and principally

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responsible for the elasticity of relaxed myofibrils (Labeit et al., 1997). Also, titin filaments keep the A-bands centered within the sarcomere during active force generation (Horowits et al., 1989). Thus, titin plays an important role in the mechanical functioning of striated muscle.

Titin antibodies from MGT patients' sera are known to stain the edge of the A-band, the so-called main immunogenic region (MIR) of titin. The MIR has been mapped by immunoscreening human cardiac titin cDNA expression libraries with MG sera (Gautel et al., 1993). Therefore, some sera have recently been used as markers for the MIR position in an immunofluorescence study on the elastic properties of titin (Linke et al., 1996a). Surprisingly, in addition to the known MIR epitope, some sera labelled a second, distinct, epitope within the I-band. To follow up, we screened nearly 30 selected sera obtained from MGT and MGA patients by using immunofluorescence microscopy on single myofibrils. I-band titin antibodies were detected in a substantial number of sera from both classes of patients. The titin antibodies presumably originate in thymoma cells, which are shown here to express titin mRNA sequences. Finally, the occurrence of I-band titin antibodies is compared with the clinical parameters of patients with regard to the severity of MGT.

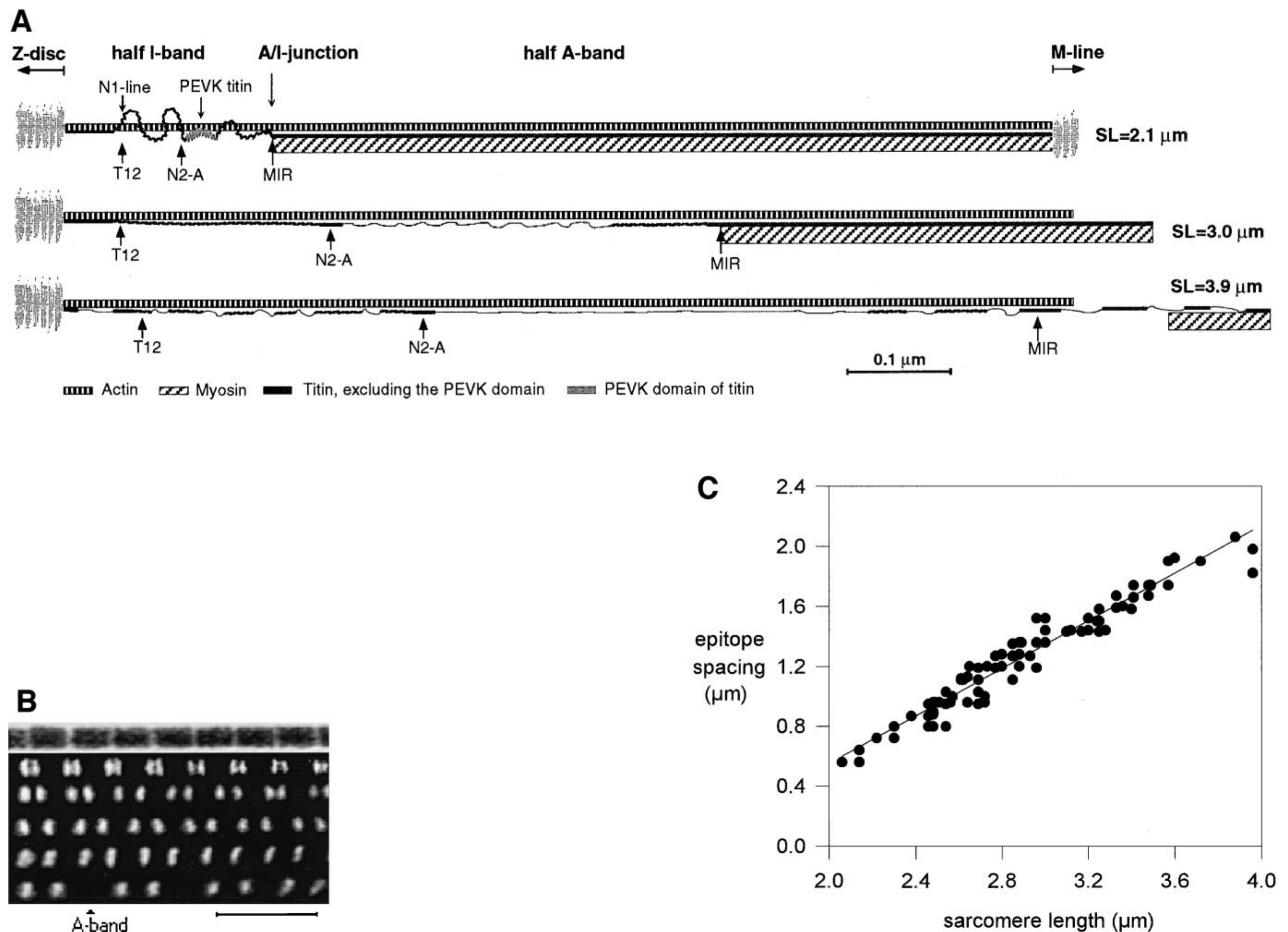

Fig. 1. Staining of the MIR epitope of titin by group I sera. (A) Layout of titin in the half-sarcomere and during stretch (adapted from Linke et al., 1996a). The epitope positions of titin antibodies used in this study, including the MIR, are indicated below each panel. Top: Half-sarcomere at slack length (model, psoas muscle). The titin filament, which spans from the Z-disc to the M-line, is elastic only in the I-band. Two major I-band segments, tandem-Ig domains and a sequence rich in Pro, Glu, Val, and Lys residues — termed the PEVK domain — confer extensibility to titin (Labeit and Kolmerer, 1995; Linke et al., 1996a). Middle: Sarcomere stretched to the maximum length presumably reached under physiological conditions. The tandem-Ig domain strand is straightened, whereas the PEVK domain is unfolded. Bottom: Highly stretched half-sarcomere. The PEVK domain is maximally unravelled and some individual Ig domains are unfolded. The MIR epitope has translated away from its original position at the A/I junction into the I-band section, indicating recruitment of previously stiff A-band titin sequences to the elastic titin region. (B) Representative fluorescence images of rat psoas myofibrils, stretched to SLs of 2.0, 2.4, 2.7, 3.1, and ~4.0 μm (from top to bottom), and stained with a group I MGT serum. Note that at extreme stretch (lowest image), the previously constant epitope–epitope distance across the A-band was increased dramatically in some sarcomeres, indicating mobilization of the previously stiff A-band titin portion (arrowhead). The top panel shows a phase-contrast image at 2.0 μm SL. Scale bar, 5 μm. (C) Summary of results of fluorescence measurements on psoas myofibrils ($n = 4$), using two group I MGT sera (I-1, I-2). Only the A/I-junction (MIR) was labelled. The MIR epitope spacing measured across the Z-disc is plotted against SL. The curve was fitted with a first order regression.

2. Materials and methods

2.1. Selection of antisera

Some 26 sera from myasthenia gravis patients were selected for analysis in this study. 18 sera were obtained from MGT patients and 8 from late-onset MGA patients (onset above age 40). Most of these sera were known to contain antibodies against the MIR of titin (cf. Aarli et al., 1990; Gautel et al., 1993; Skeie et al., 1995). As a control, we also investigated sera from 20 healthy blood donors. Previously, several tens of blood donor sera had been examined for MIR-titin antibodies in ELISA tests, and were all found to be negative (Gautel et al., 1993).

2.2. Myofibril preparation

Single myofibrils were prepared essentially according to the method of Knight and Trinick (1982). Briefly, thin strips from cardiac or skeletal muscles of male Wistar rats were dissected and either stored in ATP-free rigor solution mixed with 50% glycerol (v/v) at −20°C or processed for immediate use. To obtain single myofibrils, the strips were minced, skinned in rigor solution containing 0.25–0.50% Triton X-100 for 1–2 h, and homogenized in rigor solution in a blender at 4°C. From the suspension, a single myofibril (sometimes a doublet) was selected to be picked up under an inverted microscope by two glass microneedles, which could be controlled by hydraulic micromanipulators (Narishige, Japan). Relaxing solutions had an ionic strength of 200 mM, pH 7.1 and contained at least 20 μg/ml protease inhibitor leupeptin (cf. Linke et al., 1996a, 1997). All experiments were done at room temperature.

2.3. Antibodies

Polyclonal titin antibodies (N2-A) against the so-called N2-titin segment were obtained as in Linke et al. (1996a). To label titin at the N-terminus of its elastic I-band section, we used the monoclonal T12 antibody (Fürst et al., 1988). As secondary antibodies, we used TRITC-conjugated anti-rabbit or anti-mouse IgG (whole molecule, SIGMA, No. T-5269 and T-5393) and for the MG sera, TRITC-conjugated anti-human IgG (whole molecule, SIGMA, No. T-5903). The sera/primary antibodies were used in dilutions of 1:80, the secondary antibodies in dilutions of 1:100 (in relaxing solution); exposure time to myofibrils was 20–45 min.

2.4. Immunofluorescence microscopy

Fluorescence measurements were done under an inverted microscope (Zeiss Axiovert 135, 100 × oil immersion objective). A CCD video camera (FK440, Völker, Maintal, Germany) and a frame grabber board (Vision-EZ, Data Translation) including image capture software (GlobalLab Aquire, Data Translation) were used to record the myofibril image. Experimental protocols (antibody labelling, myofibril stretch) were as in Linke et al. (1997). Images of antibody-labelled myofibrils were recorded in the epifluorescence mode of the microscope and analyzed with image processing software (Global Lab Image, Data Translation; precision of detection, approximately ±80 nm; cf. Linke et al., 1997). As a control, we labelled myofibrils with secondary antibody only, but found no fluorescence.

2.5. Immunoelectron microscopy

Procedures were as in Aarli et al. (1990). Briefly, an adult rat was infused in vivo with 4% paraformaldehyde. Vibratome sections of thigh muscle (25 μm) were cut and stained in suspension with MG serum affinity-purified with whole titin, obtained by separating rat skeletal muscle proteins by SDS-PAGE. After transferral to nitrocellulose (NC), the titin-containing NC strip was cut from the sheet and incubated for 1 h with strongly titin-reactive MG serum (dilution, 1:100). After washout, IgG antibodies were eluted from the strip at pH 2.8 and used for further experiments. Controls were prepared in an identical manner by using NC strips from a region without titin; staining was done with normal human serum. The stained vibratome sections were incubated with diluted affinity-purified rabbit anti-human IgG antibodies (Vector Laboratories, Burlingame, CA), embedded in epon and thin-sectioned on an ultra-microtome.

2.6. RT-PCR analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described (Sorimachi et al., 1997) to test human thymoma tissue for the presence of I-band titin isoforms at the mRNA level. For amplification of cDNA, we used a set of four 30mer oligonucleotides covering altogether ~3 600 bp of the differentially spliced I-band section of the titin cDNA sequence (EMBL data library accession number x90569), and ~900 bp of the MIR region (accession number x90568). The primer pairs used were as follows (sequences all from 5′ to 3′): (1) ctgggccacc tagccaaatt cacctgtgag/ggaggttctt tgagtaagtg ggttgccatg; (2) gtgttgttgc taatgaagtc ggcaagtgtg/tgacatctat agacacagct tcctcggtga; (3) gtacatgtgt gaagctcaaa atgaggctgg c/ccctttaatg ctgtccattt tcttcagctt ttt; (4) gctggtgttt cagatccatc tgaaattctt/gcctcttgtg tgtccacagc ctggatt. Amplified sequences were separated on agarose gels and detected on Southern blots.

2.7. Disability scores of MG patients - statistical analysis

The diagnosis of MG was based on conventional clinical criteria, the results of edrophonium (Tensilon) test and

Table 1
Summary of results of MGT sera staining

Serum	Muscle	Number of epitopes (position in half-sarcomere)	Peak	End	Dead
Group I					
I-1	l.d., ps, l.v.	1 (A/I)	4	4	0
I-2	s, l.d., ps	1 (A/I)	4	3	1
I-3	s, ps, l.d.	1 (A/I)	4	4	1
I-4	ps	1 (A/I)	3	2	0
I-5	ps	1 (A/I)	4	3	0
I-6	ps	1 (A/I)	3	1	0
I-7	ps	1 (A/I)	2	1	0
I-8	ps	1 (A/I)	4	3	0
I-9	ps	1 (A/I)	4	2	0
I-10	ps	1 (A/I)	3	2	0
I-11	ps	1 (A/I)	2	0	0
Group II					
II-1	ps	2 (A/I, I)	3	2	0
	r.v.	1 (A/I), Z			
II-2	l.d.	2 (A/I, I)	2	2	0
	r.v.	1 (A/I)			
II-3	ps	2 (A/I, I)	2	1	0
	r.v.	1 (A/I), Z			
II-4	s, ps	2 (A/I, I)	no data available;		
	r.v.	1 (A/I), Z	severe MG		
II-5	l.d., ps	2 (A/I, I)	4	4	1
	l.v.	1 (A/I)			
II-6	s, l.d., ps	2 (A/I, I)	4	4	1
	r.v.	1 (A/I)			
II-7	l.d., ps	2 (A/I, I)	4	4	1
	r.v.	2 (A/I, I)			
Controls from healthy blood donors:					
1–5	ps	no labelling			
6–8	ps	weak unspecific staining			
9	ps	A/I			
10–13	l.d.	no labelling			
14–19	l.d.	weak unspecific staining			
20	l.d.	unspecific staining, Z, M			

Abbreviations: s, m. soleus; l.d., m. longissimus dorsi; ps, m. psoas; r.v., right ventricle; l.v., left ventricle; peak, disability score of MG patient at peak of illness; end, disability score of MG patient at latest follow-up; dead, MG-related death; A/I, A/I-junction; I, I-band; Z, Z-disc; M, M-line.

Table 2
Summary of results of MGA sera staining

Serum	Muscle	Number of epitopes (position in half-sarcomere)	Peak	End	Dead
Group I					
I-1	ps	1 (A/I)	2	1	0
I-2	ps	1 (A/I)	4	2	0
I-3	ps	1 (A/I), Z	3	1	0
Group II					
II-1	ps	2 (A/I, I)	4	4	1
	r.v.	2 (A/I, I)			
II-2	ps	2 (A/I, I)	no data available		
	r.v.	2 (A/I, I), M			
II-3	ps	2 (A/I, I), M	2	1	0
	r.v.	1 (A/I)			
Unclassified:					
1	l.d., ps	weak unspecif. stain.	3	1	0
2	s, l.d., ps	weak unspecif. stain.	3	1	0

Abbreviations: as in Table 1.

neurophysiological examination, and the presence of AChR antibodies. Clinical parameters were available from most patients. The patients were classified according to MG severity at peak of illness and at latest follow-up. They were grouped into five categories (0–4) modified from Oosterhuis (Mygland et al., 1995), with category 0 referring to complete remission without medication and category 4 to severe generalized symptoms. MG-related death was used as a separate criterion classified as death following deterioration of MG with respiratory distress and final respiratory failure or cardiac arrest.

An unpaired Student's t-test was used to evaluate statistical differences in the disability scores of patients between different groups of sera. A P value smaller than 0.05 was considered to be statistically significant.

3. Results

3.1. Classification of MG sera

Immunofluorescence microscopy on single, isolated myofibrils was employed to investigate the sera of MGT and MGA patients for the presence of antibodies against sarcomeric proteins. The results for the two classes of patients are summarized in Tables 1 and 2, respectively. Most sera labelled myofibrils from both skeletal and cardiac muscle strongly, whereas a few stained only weakly or not at all. Weak unspecific staining — which was also observed with many control sera from healthy blood donors (Table 1) — was taken as an indication of the presence of no or very low levels of antibodies against sarcomeric proteins. Sera with more-or-less strong staining were classified into two groups. The first group exhibited distinct antibody labelling at the ends of the A-band. The second group was found to stain both the A-band edge and another, distinct, epitope within the I-band.

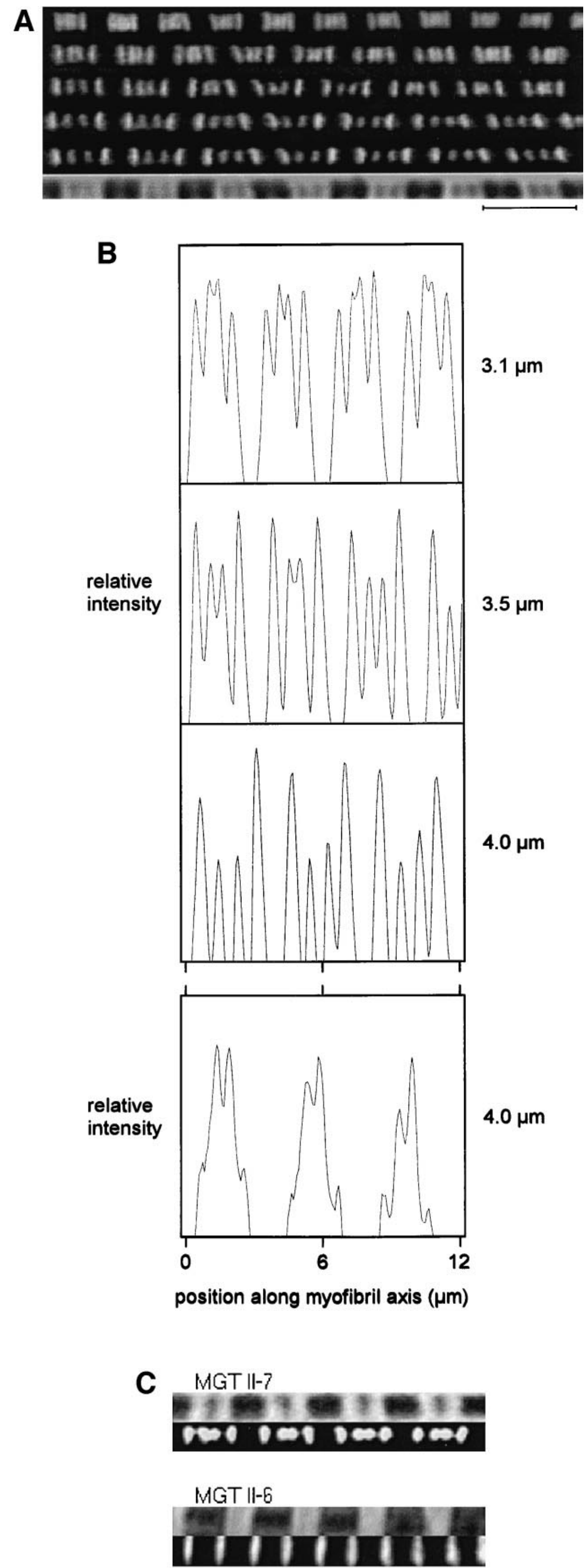

Fig. 2. Group II sera staining patterns. (A) Typical fluorescence images of psoas myofibrils stretched to 2.7, 3.1, 3.5, 3.8, and ~4.0 μm SL (from top to bottom), and exposed to a group II MGT serum and a fluorophore-marked secondary antibody. Two distinct epitopes per half-sarcomere are stained, one at the A/I-junction, the other in the I-band. For comparison, a phase-contrast image of the myofibril at SL ~4.0 μm is shown at the bottom. Note that the serum labelled so avidly that two dark stripes in the I-band could be seen in phase-contrast mode. (B) Upper three panels: intensity profile plots along part of the myofibril shown in (A), at three different SLs. The peak magnitudes differ by less than 50% from one another. Bottom panel: profile plot along part of a different, stretched, psoas myofibril stained with another group II MGT serum. This serum stained weakly at the A/I junction (small peaks) but strongly in the I-band (large peaks). (C) Fluorescence and phase-contrast images of highly stretched cardiac myofibrils labelled with two different group II MGT sera, one staining two epitopes per half-sarcomere (top panels), the other only one epitope (bottom panels). Scale bars in (A) and (C), 5 μm.

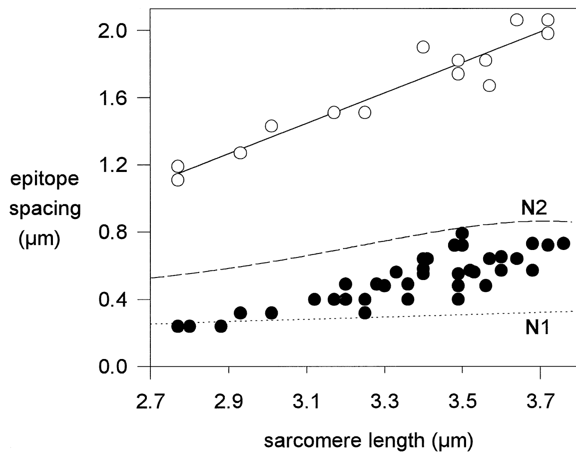

Fig. 3. Epitope spacing versus SL, as found with group II MGT sera. Summary of results of fluorescence measurements on stretched psoas myofibrils ($n = 6$). Data from six group II MGT sera (cf. Table 1) are included. The data points and fitted curve in the upper part of the graph indicate the MIR extension behavior measured with the same sera (see also Fig. 1C). For comparison, the dashed line shows the extension curve of an N2-titin antibody, the dotted line that of the T12 titin antibody in psoas myofibrils (cf. Linke et al., 1996a); these curves were obtained by third-order regression of pooled data points. Data points for the I-band epitopes (group II MGT sera) are sandwiched in between the dashed and dotted curves. Symbols: open circles, MIR epitopes; closed circles, I-band titin epitopes.

3.2. MG sera stain multiple titin epitopes

3.2.1. MIR epitope

When myofibrils were exposed to group-I sera, we observed avid labelling at the ends of the A-band in all four muscle types investigated (soleus, longissimus dorsi, psoas and cardiac tissue). This group comprised 11 MGT patients (Table 1) and 3 MGA patients (Table 2). With all but one serum (Z-disc labelling), distinct staining was absent in sarcomeric regions other than the A-band edge. Only in a very few cases was increased background staining of I-bands or A-bands apparent. The labelled epitope at the A/I-junction presumably corresponds to the previously described MIR of titin (Gautel et al., 1993; Bennett and Gautel, 1996). Since the MIR epitope is located within the functionally stiff titin region (Fürst et al., 1988), we expected that during myofibril extension, the position of this epitope relative to the M-line should remain unchanged. As shown in Fig. 1B, the expected behavior was indeed observed (data also implied in Fig. 1C). Conversely, when measured across the Z-disc, the distance between neighboring MIR epitopes increased steadily with myofibril stretch and was linearly related to changes in SL (Fig. 1B, C). To confirm that the stained sites represent titin epitopes, we utilized the property of A-band titin to become extensible at high degrees of myofibril stress (see the scheme in Fig. 1A; and cf. Wang et al., 1991; Linke et al., 1996b). With extreme stretch to beyond 3.7–4.2 and 3.3–3.4 μm SL in skeletal and cardiac myofibrils, respectively, we found that the previously stationary epitopes at the A-band edge translated into the I-band, indicating recruitment of A-band titin to the extensible titin portion (Fig. 1B, lowest image). This result confirmed that the sites labelled by group I sera are indeed titin epitopes.

3.2.2. I-band titin epitopes

In addition to the MIR epitope, 7 MGT sera and 3 MGA sera also stained a distinct I-band site (group II sera; Fig. 2A, Tables 1 and 2). Accordingly, the fluorescence intensity profiles measured along the myofibril axis showed two distinct peaks per half-sarcomere (Fig. 2B). The vari-

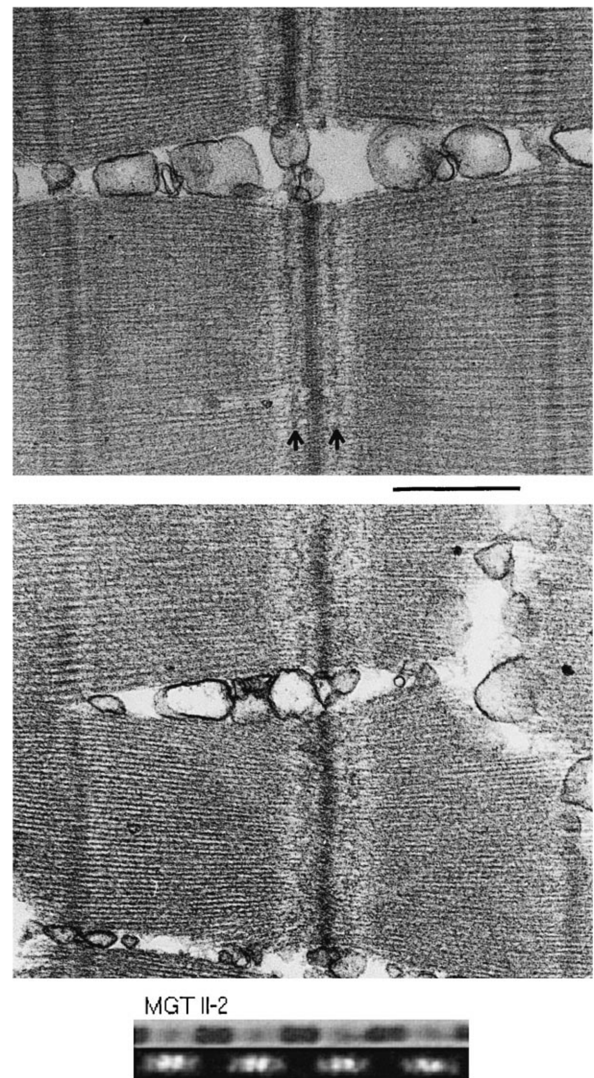

Fig. 4. Top: Representative electron micrograph (total $n > 10$) of part of a rat thigh muscle fiber at slack SL, labelled with a group II MGT serum. A distinct stripe appears in the I-band section of each half-sarcomere (arrows). Note that staining at the A/I-junction (MIR) was barely visible. Bottom: Control fibers labelled with normal human blood donor serum exhibited no distinct I-band staining, although the sarcomere's N1-line was stained weakly. Inset: phase-contrast and fluorescence image of rat psoas myofibril incubated with a group II MGT serum: staining at the A/I-junction was weak, while that in the I-band was strong, just as seen in the electron micrograph of the main figure. Scale bar, 0.5 μm.

ability in peak height could either be relatively small (less than 50%), as with MGT sera II-4 through II-7 (e.g. Fig. 2B, upper three panels), or very large, as with MGT sera II-1 through II-3 and group II MGA sera; in the latter case, staining at the A/I-junction was usually weak, whereas that in the I-band was very strong (Fig. 2B, bottom panel). Some of these sera also showed Z-disc/M-line labelling or increased background staining in the I-bands, as frequently seen with control sera from healthy blood donors (Tables 1 and 2). Importantly, however, with none of the control sera was labelling of a distinct I-band epitope observable.

Although group II MGT sera generally labelled I-band epitopes in all three types of skeletal myofibrils, only MGT serum II-7 stained the I-band also in cardiac myofibrils (Fig. 2C; Table 1). Similarly, all group II MGA sera labelled the I-band in skeletal myofibrils, whereas I-band staining was absent with MGA serum II-3 on cardiac specimens (Table 2). As for the I-band labelling in cardiac myofibrils, it appeared at the short SLs as if the Z-discs were stained; however, with moderate stretch, two stripes symmetrically about the Z-disc became clearly visible, indicating recognition of epitopes outside the Z-disc (cf. Fig. 2C).

The position of the I-band epitopes during sarcomere extension was examined in more detail. Upon SL increase, we found a steady increase in epitope-Z-disc-epitope distance (Fig. 3). Because such I-band-epitope mobility was observed with all group II sera, we concluded that the epitopes are located not on the stiff actin, but rather, on the extensible titin filaments. This conclusion was confirmed in experiments, in which we selectively removed the actin filaments from the sarcomere, by using a calcium-independent gelsolin fragment (cf. Linke et al., 1997). Even after actin extraction we found strong, distinct, staining of central I-band sites with group II MGT sera, clearly indicating recognition of epitopes on the titin filament.

The observed I-band epitope extension behavior was compared with that of other, epitope-mapped, I-band titin antibodies (cf. Fig. 1A). We used the T12 antibody, which binds at the N1-line of the sarcomere, ~ 100 nm from the center of the Z-disc (Fürst et al., 1988), and the N2-A antibody, which labels a more central I-band titin site (Linke et al., 1996a). We found that the I-band antibodies from MGT sera always recognized epitopes located between the T12 and the N2-A binding sites (Fig. 3). Similar results were obtained with MGA sera. In the cardiac myofibrils positive for I-band epitopes, staining appeared to be close to the Z-disc, presumably near the sarcomere's N1-line. In conclusion, the I-band antibodies apparently label within a titin segment that spans from the N1-line to the N2-A epitope.

3.2.3. Electron microscopy

We also prepared electron micrographs of rat thigh muscle fibers labelled with a purified group II MGT serum. The serum typically stained a distinct epitope in the I-band (Fig. 4). With this particular serum, the I-band epitope was labelled strongly, whereas the A-band edge was barely stained — similar to what had been seen with some group II sera in the immunofluorescence measurements (Fig. 4, inset, and Fig. 2B, bottom panel). At the slack SL of ~ 2 μm studied by EM, the I-band epitope was positioned ~ 100 nm from the center of the Z-disc, i.e. near the N1-line. However, it can be anticipated that the epitope moves away from the Z-disc during sarcomere stretch, as observed in the above experiments (Fig. 2A, 3). This result was taken as confirmation that group-II sera label titin within its elastic I-band section. To sum up, an overview of the suggested MG sera labelling sites on titin is shown in Fig. 5.

3.3. Thymomas express I-band titin isoforms

In an attempt to address the issue of a possible origin of the titin antibodies, we studied human thymoma tissue for the expression of titin at the mRNA level, by using the RT-PCR method. We aimed to detect whether the I-band titin sequences recognized by the antibodies from group II sera are present in thymoma cells. Partially overlapping

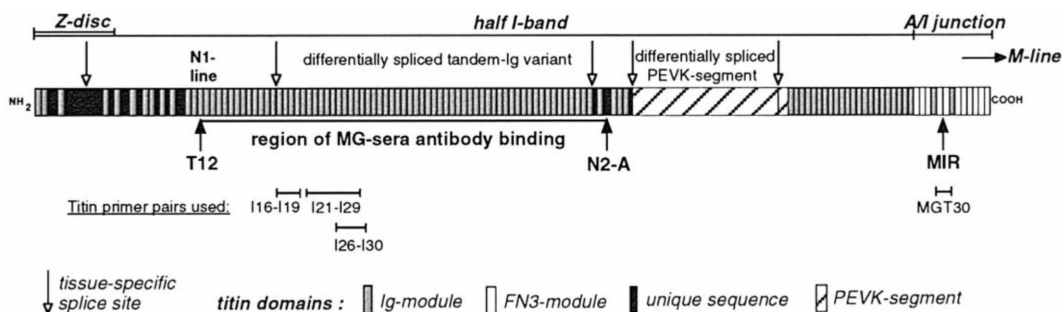

Fig. 5. Domain architecture of I-band titin (after Labeit and Kolmerer, 1995). The proposed sites of I-band titin labelling by group II sera between the N1-line (T12 epitope) and the N2-A position are shown. The MIR is located at the A/I-junction. Arrows below the figure indicate the positions of the antibodies used in this study. Also shown are the sites of titin primer pairs used for RT-PCR studies. Altogether four pairs were used, three from the differentially spliced N-terminal tandem-Ig region, and a primer pair covering the MIR (MGT 30). For titin domain designation, cf. Labeit and Kolmerer (1995).

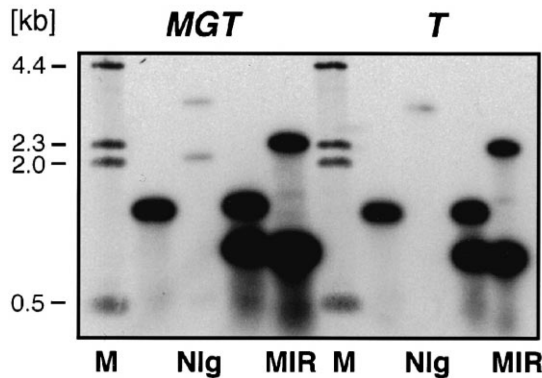

Fig. 6. Southern blot showing the results of RT-PCR analysis of human thymoma tissue with I-band titin primer pairs. Oligo pairs from four different regions of the titin sequence (for positioning, see Fig. 5) were used to amplify cDNA from the respective tissue. MGT, thymoma tissue from an MGT patient; T, thymoma tissue from a patient with a thymic tumor but no MG. All titin mRNA sequences studied are expressed in the thymomas. Multiple bands observed in a given lane could indicate isoform expression. M, molecular weight marker; NIg, N-terminal tandem-Ig sequences, covering the domains I16–I19 (left lane), I21–I29 (middle lane) and I26–I30 (right lane); MIR, MGT30 sequence (cf. Fig. 5).

I-band titin primer pairs were used, and their position is shown in Fig. 5. We also included a primer pair corresponding to the MIR of titin. We found that titin sequences both from the central I-band section (Ig domain region) and from the MIR position were present in thymoma cells of an MGT patient (Fig. 6, left five lanes) and of a patient with thymoma but no MG (Fig. 6, right five lanes). Thus, at least at the mRNA level, thymomas indeed express titin isoforms.

3.4. I-band titin antibodies and MG severity

The finding that MGT and MGA patients can have in their sera antibodies against multiple titin epitopes may be of particular interest with regard to the clinical parameters of those patients. We, therefore, compared the average disability scores of MGT patients with group I sera to those with group II sera. We found no statistically significant correlation between the occurrence of I-band titin epitopes and the severity of the disease (Fig. 7). Such indifference was evident for the disability scores both at peak of illness and at latest follow-up, and for the frequency of MG-related death. For the MGA patients, meaningful statistics could not be prepared, because the number of sera available was not high enough.

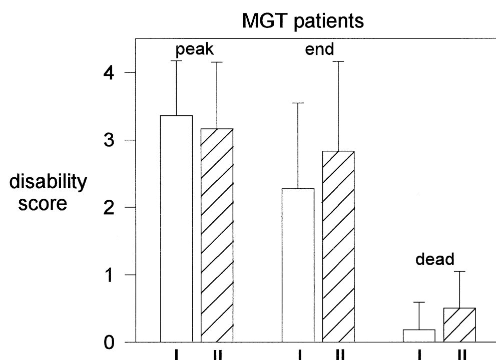

Fig. 7. Bar graph showing the average disability scores and frequency of MG-related death (mean ± S.D.) for MGT patients whose sera were either positive (patterned columns) or negative (open columns) for I-band titin antibodies. No statistically significant correlation between the occurrence of I-band titin epitopes and severity of the disease was apparent ($P > 0.2$ in Student's t-test). I: group I MGT sera; II: group II MGT sera; peak, end: disability score of patients at peak of illness and latest follow-up, respectively; dead: frequency of MG-related death.

4. Discussion

Already more than 35 years ago it was recognized that the sera of some MG patients contain antibodies against intracellular striated muscle proteins (Strauss et al., 1960). These antibodies were found to stain the A-bands and the I-bands of the sarcomeres in a cross-striational pattern (Vetters, 1965) and were, therefore, termed 'striational autoantibodies'. Since then, several intracellular muscle proteins — including titin — stained by such antibodies have been identified (e.g. Williams and Lennon, 1986; Aarli et al., 1990; Ohta et al., 1990; Mygland et al., 1992). In this study, we have characterized sarcomeric epitopes labelled by selected MG sera in terms of their mobility pattern during sarcomere extension, by applying a technique to mechanically manipulate isolated, single myofibrils (Linke et al., 1996a,b). Thereby, we confirmed the presence of antibodies against titin in the sera of MGT and MGA patients (Aarli et al., 1990; Williams et al., 1992; Skeie et al., 1995). Some of these sera (group I sera) labelled a single site on the titin filament at the A-band edge, the MIR of titin (Gautel et al., 1993). Others (group II sera) stained both the MIR and an I-band epitope. Whereas labelling of an epitope at the A/I-junction had been shown previously (Aarli et al., 1990; Bennett and Gautel, 1996), the observation that central I-band titin epitopes are recognized by a substantial number of MG sera is a novel finding.

Some interesting differences in the staining pattern were observed between cardiac and skeletal myofibrils. Since titin's MIR is highly conserved in different vertebrate species and constitutively expressed in striated muscle tissues (our own, unpublished results), it is not surprising that all MGT sera and most MGA sera labelled the MIR epitope in all myofibril types studied (Tables 1 and 2). In contrast, group II sera staining patterns varied to a much higher degree. I-band titin epitopes were recognized in all types of skeletal myofibrils exposed to group II sera from MGT or MGA patients, but rarely in cardiac myofibrils (Tables 1 and 2; Fig. 2C). This finding may be readily explained by the fact that the labelled I-band titin epitopes

are located between the N1-line and the N2 position of the sarcomere within a titin region that is expressed in different length isoforms in skeletal and cardiac muscle tissues (Fig. 5). The region is known to be made up of a family of homologous tandemly arranged immunoglobulin domains (Labeit and Kolmerer, 1995); only 15 of these domains are present in the sequenced titin isoform from human cardiac muscle, whereas up to 68 such modules are expressed in human skeletal muscles (Labeit and Kolmerer, 1995). Thus, the group II sera that did not stain I-band titin in cardiac myofibrils appear to contain antibodies to tandem-Ig domains which are spliced out in the cardiac titin sequence. The lack of expression of many I-band-titin Ig modules in cardiac muscle probably explains why the central I-band epitopes were not discovered in an initial screen for immunogenic epitopes on titin (Gautel et al., 1993), because in those experiments, a cardiac muscle cDNA expression library was used. Other possibilities are that the MGT patient's sera used in that study for cDNA screening did not contain I-band titin antibodies and/or that the cDNA expression system may have induced a protein with incorrect folding, resulting in failure to detect the I-band epitopes.

The occurrence of many striated muscle antibodies in MG patients' sera could in theory be explained by the phenomenon of epitope spreading (for review, see Vanderlugt and Miller, 1996), which has been shown for at least two antigens involved in autoimmune diseases, myelin basic protein and the AChR (Lehmann et al., 1992; Vincent et al., 1994). As for thymomas, it is known that they can express epitopes shared by the AChR, ryanodine receptor, and titin (Marx et al., 1992, 1996; Mygland et al., 1995). Such common sequence motif might sensitize T-cells within the thymoma, which shares morphological features with the normal thymus and, to some degree, retains the unique function of the thymus to attract pre-T-cells and promote their maturation (e.g. Marx et al., 1992). In light of this, our result that thymomas contain titin sequences both from the differentially spliced N-terminal I-band Ig region and from the constitutively expressed MIR position (Fig. 6) may ultimately help to uncover the stimulus for autoantibody production in MGT patients. Although an earlier report did not find complete titin molecules to be expressed in thymomas (Marx et al., 1992), our result now clearly shows expression of titin isoforms in such tissues. Thus, as far as titin is concerned, thymoma cells are potentially capable of self-antigen presentation. Whether full-length titin mRNA is expressed in a thymoma should be examined in the future.

Thymoma T-cells could migrate to the periphery and eventually initiate autoantibody production, if they encounter appropriate antigen-presenting cells. Indeed, AChR antigens can be presented to T-cells by epithelial cells from thymomas or non-neoplastic MG thymus in an immunogenic manner (Gilhus et al., 1995). This scenario might also hold true for titin: Williams et al. (1992) found that B-cell clones isolated from an MGT patient secreted antibodies reacting with two regions on titin, one near the Z-disc, the other close to the A/I-junction. These (monoclonal) antibodies had somatic mutations, suggesting an antigen-driven immune response. Thus, the presence of different active B-cell clones based on T-cell-mediated autoimmune responses may explain the phenomenon of epitope spreading in MG (cf. Elson et al., 1995). Evidence for a T-cell response to MIR-titin in MG patients has recently been presented (Skeie et al., 1997). Then, changes in the specificities of the T-cell epitope repertoire occurring in the course of the disease might explain the presence of antibodies that recognize titin epitopes apart from the MIR. Hence, a possibility worthy of further investigation is the occurrence of titin epitope spreading in MG.

Although it has been shown in this study and in previous reports that many MGT and MGA patients develop titin antibodies, there is no evidence for a pathogenic significance of these antibodies. On the other hand, in an interesting case study it has been reported that a patient, whose serum was also investigated by us (II-7), developed a severe form of MG after thymectomy for thymoma (Hassel et al., 1992); this patient was the only MGT patient, whose serum stained an I-band epitope also in cardiac myofibrils. This finding triggered our interest in studying whether certain titin epitope staining patterns could hint at the severity of the disease. Previously, the presence of MIR-titin antibodies in MG sera has been found to be a somewhat useful — though not highly specific — marker for the presence of a thymoma (Gautel et al., 1993). Also, the occurrence of MIR-titin antibodies may in fact indicate a more severe form of the disease in MGA patients (Skeie et al., 1995). However, it is emphasized that only for the AChR antibodies in MG patients, has a pathogenetic mechanism been clearly identified.

The search for a useful serological marker for MG severity appears to be particularly important considering that the level of AChR antibodies in MG patients' sera shows no correlation with the degree of muscle weakness in different patients, despite that there might sometimes be a weak correlation between the occurrence of AChR antibodies and the clinical condition of the patient (Oosterhuis et al., 1983). Also, in the present study, the concentration of antibodies against the AChR, ryanodine receptor and MIR titin was similar in the MGT sera (data not shown) and hence, did not correlate with the severity of the disease. When we studied the occurrence of I-band titin epitopes for a potential clinical significance, we found that MGT patients with group II sera were neither more disabled at the peak of their illness and latest follow-up, nor was their MG-related mortality different from that of patients with group I sera (Fig. 7). Although this result does not exclude a possible correlation in singular cases, it appears that there is no statistically significant correlation between the presence of I-band titin epitopes and severity of MGT in different patients. However, a possible scenario

is that in initial phases of the disease, the immune response may be restricted to titin's MIR only. Then, in the course of the disease, additional immunoreactivity to I-band titin epitopes could occur. If epitope spreading was confirmed, it could provide useful insights into the time course of MG.

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