Journal of Cell Science 108, 3029-3037 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

Characterization of a 5.4 kb cDNA fragment from the Z-line region of rabbit cardiac titin reveals phosphorylation sites for proline-directed kinases

Magdolna G. Sebestyén[1], Jon A. Wolff[1] and Marion L. Greaser[2,*]

[1]Departments of Pediatrics and Medical Genetics, Waisman Center, University of Wisconsin-Madison, Madison, WI 53705, USA
[2]Department of Meat and Animal Science, Muscle Biology Laboratory, University of Wisconsin-Madison, Madison, WI 53706, USA

*Author for correspondence

SUMMARY

Titin is an approximately 3 MDa protein that spans from the M- to the Z-line in the sarcomeres of vertebrate striated muscle. The protein is presumably encoded by unusually large mRNAs of 70-80 kb. Although titin has been studied by several laboratories, barely more than half of the cDNA sequence (≈45 kb) has been published, most of it obtained from the A-band and M-line region (corresponding to the C-terminal half of the molecule). A special cDNA library was constructed using size selected total RNA from adult rabbit cardiac muscle in order to obtain sequence data from titin's unknown N-terminal region. A monoclonal antibody (T12), which binds to an epitope close to the Z-line, was used to identify initial cDNA clones. Additional overlapping clones were isolated and sequenced yielding a 5.4 kb contig. The encoded polypeptide contains 16 Type-II domains and four unique intervening segments. Polyclonal sera, raised against an expressed protein fragment encoded by the 5′ end of the contig, strongly stained the Z-line of myofibrils of different species. However, the sequence of this fragment is 83% identical at the amino acid level with the previously reported C-terminal (i.e. M-line) end of chicken embryonic skeletal muscle titin. The expressed protein fragment could be phosphorylated in vitro by embryonic skeletal muscle extract and by the purified proline-directed kinase ERK1, presumably at the xSPxR recognition sites located in the first interdomain segment.

Key words: titin, cDNA sequence, Type-II domain, immunoglobulin superfamily, phosphorylation

INTRODUCTION

Titin, also known as connectin, is an exceptionally large single chain protein with a molecular mass of ≈2.5-3.0 MDa (Maruyama et al., 1984; Kurzban and Wang, 1988). It is expressed in large amounts in striated skeletal and cardiac muscle tissues. Immunohistochemical studies with a set of monoclonal antibodies revealed that titin molecules run parallel to the muscle fiber axis and span between the Z-line and the M-line in the sarcomere (Fürst et al., 1988; Whiting et al., 1989). Further immunological data imply that at physiological sarcomere length only the I-band region is elastic, while the A-band region seems to be rigidly bound to the thick filaments (Itoh et al., 1988; Fürst et al., 1989; Whiting et al., 1989; Pierobon-Bormioli et al., 1989). Titin has been identified as the responsible element for the resting tension of stretched myofibrils (Wang et al., 1991; Horowits, 1992; Granzier and Irving, 1995), and by connecting their ends to the Z-disc titin is thought to maintain the thick filaments in the center of the sarcomere (Horowits and Podolsky, 1987). Beyond the now well-established mechanical role of titin it is assumed to be crucial also in the morphogenesis of the myofibril (Handel et al., 1991; Isaacs et al., 1992; Behr et al., 1994; Rhee et al., 1994).

Several investigators have isolated and sequenced cDNA clones covering different regions of titin from different species and tissues (see Fig. 1 for a summary). The orientation of the titin molecule in the half sarcomere has been determined; the N-terminal end of the molecule is near the Z-disc and the C-terminal end is close to the M-line (Labeit et al., 1992). Like many other cytoskeletal proteins, titin has a characteristic domain structure consisting of repetitions of two types of motifs termed Type-I (homologous to the fibronectin Type-III domain) and Type-II (homologous to the immunoglobulin C2 domain) (Labeit et al., 1990). In the A-band region they are arranged in (-I-I-I-II-I-I-I-II-I-I-II-) super-repeats (Labeit et al., 1992). The Type-I and Type-II motifs 5′ to a kinase domain (close to the C-terminal end) show different arrangement (Labeit et al., 1992), and sequence 3′ to the kinase domain has only Type-II motifs separated by unique interdomain insertions (Gautel et al., 1993b). One of these interdomain segments codes for four KSP motifs which could be phosphorylated in vitro by the cytosolic fractions of differentiating myoblasts. The major activity of the titin KSP kinase was proposed to be identical to that of the proline-directed cdc2 kinase (Gautel et al., 1993b). Comparison of corresponding regions of A-band titin sequence data from human, rabbit, pig and mouse shows high (>90%) homology (Labeit et al., 1992; Fritz et al., 1993b).

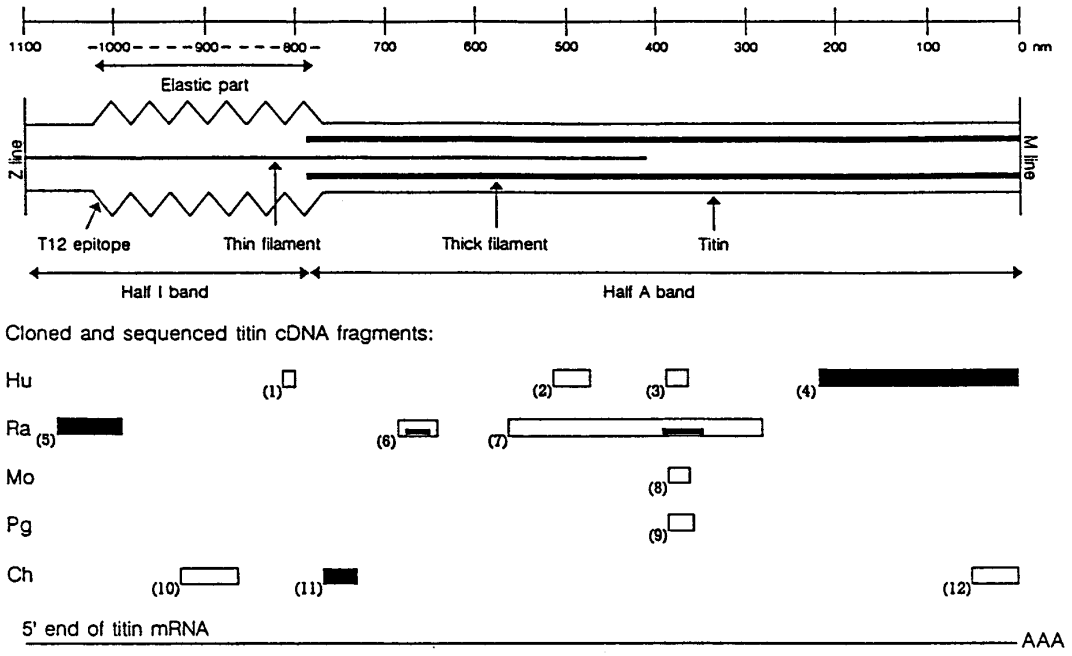

Fig. 1. Sequenced titin cDNA fragments from skeletal or cardiac muscle of different vertebrate species; relation to titin position in the half sarcomere. GenBank accession numbers and references: (1) no accession number (Gautel et al., 1993a), (2) X64698 (Labeit et al., 1992), (3) X64699 (Labeit et al., 1992), (4) X64697 and X69490 (Labeit et al., 1992; Gautel et al., 1993b), (5) U28657 (subject of the present study) (6) X17330 (Labeit et al., 1990; Fritz et al., 1993a), (7) X17329, X64696, X59596, M95596 and M98338 (Labeit et al., 1990, 1992; Fritz et al., 1991, 1993a), (8) X64700 (Labeit et al., 1992), (9) M97767 (Fritz et al., 1993b), (10) D16541 (Maruyama et al., 1993), (11) L19140 (Tan et al., 1993), (12) D16844 (Maruyama et al., 1994). Open boxes identify skeletal, filled boxes identify cardiac muscle titin sequences (where filled boxes are inside the open boxes, cDNA sequence was determined for both tissues). Hu, human; Ra, rabbit; Mo, mouse; Pg, pig; Ch, chicken. The approximate location of the T12 epitope is also marked.

Regions of titin near the A/I-band junction also contain alternating Type-I and Type-II motifs, but neither of the sequenced fragments covers a whole super-repeat (Gautel et al., 1993a; Tan et al., 1993). cDNA data from the I band region of titin (Maruyama et al., 1993) show the presence of Type-II motifs only, arranged either consecutively, or separated by unique interdomain sequences. This finding suggests that the domain structure of the I-band titin is essentially different from that found in the A-band or at the A-I junction and is consistent with the fact that the two parts play different roles in the sarcomere.

In the present paper we report data about the primary structure of a portion of titin near the Z-line and the identification of a second region for phosphorylation by proline-directed kinases.

MATERIALS AND METHODS

Construction and screening of the cDNA library

Total RNA was isolated from snap-frozen heart pieces of adult New Zealand White rabbits using the guanidinium isothiocyanate method (Sambrook et al., 1989). Acidic phenol:chloroform mixture (pH 4.7; Amresco) was used for the extraction in order to reduce DNA contamination. As the titin mRNA is unusually large (70-80 kb) and its fragmentation may occur during RNA isolation, poly-A selection of mRNAs may considerably reduce the representation of the 5′ end. To avoid this, the total RNAs were size-selected on a native 0.7% agarose gel in Tris-Acetate buffer (Sambrook et al., 1989), and transcripts larger than 5 kb were isolated from the gel using the RNaid Kit (Bio 101). Random primed cDNA synthesis was performed with the RiboClone cDNA Synthesis Kit (Promega). The double stranded cDNA fragments were inserted into *Eco*RI cut Lambda ZAP Express vector arms (Stratagene) using *Eco*RI adaptors (Promega). The library was immediately amplified after in vitro packaging (Gigapack II, Stratagene). Nitrocellulose membranes (Schleicher-Schuell, BA85, 0.45 μm) were used for both the immuno- and hybridization screening. Immuno-screening was conducted with the T12 anti-titin monoclonal antibody (Boehringer Mannheim) using horseradish peroxidase labelled goat anti-mouse IgG secondary antibody (Cappel) and the standard H_2O_2/DAB/$NiCl_2$ substrate solution (Sambrook et al., 1989). The *Eco*RI cut insert from clone T12/19 (nucleotides 2744-3474) and a PCR fragment amplified by the T and N primers (nucleotides 1557-2294; see Fig. 2) were [α-^{32}P]dCTP labelled by random primed synthesis (Prime-It Kit, Stratagene) and used for hybridization screening of the library. Phage clones giving positive signal by either screening method were plaque purified to 100% homogeneity; then the pBK-CMV phagemids were in vivo excised using the ExAssist/XLOLR system (Stratagene). The manufacturers' protocols were followed for all the above steps.

DNA sequencing and sequence analysis

Inserts of the pBK-CMV phagemids were sequenced by the dideoxy chain termination method, using the Sequenase Version 2.0 DNA sequencing kit (US Biochem. Corp.) and the manufacturer's protocol. The ends were initially read using the T3 and T7 promoter primers (Gibco-BRL) carried by the vector; the sequences were then completed using synthetic insert specific oligonucleotides. The following oligonucleotides were used as forward primers (numbers refer to nucleotide positions in the submitted cDNA sequence): FE 13-35, T 1557-1579, U 1870-1892, A 3382-3399, C 3963-3980, F 4316-4333, I 4585-4603, J 4922-4939. Oligonucleotides below served as reverse primers: Q 838-818, RE 1443-1425, N 2293-2268, M 2731-2713, K 3058-3034, H 3646-3628, B 3888-3870, E 5306-5288. Double stranded PCR fragments were sequenced without cloning using the method of Anderson et al. (1992).

Sequence data were assembled, translated and analyzed using the University of Wisconsin GCG software package (Devereux et al., 1984). GenBank Searches were conducted through the electronic mail system using the NCBI Blast Server (Altschul et al., 1990). Amino acid sequences of the Type-II domains were aligned manually based on highly conserved residues.

Polymerase chain reaction

PCR was performed using the GeneAmp PCR Core Reagents Kit (Perkin Elmer) and the Thermal Cycler (M. J. Research, Inc.). The standard method described by the Perkin Elmer protocol was followed. Initial denaturation was done at 94°C for 2 minutes, followed by 30-35 cycles of 94°C for 45 seconds, 50-54°C (depending on the melting temperature of the primers) for 2 minutes and 72°C for 3 minutes. The final extension step was 10 minutes at 72°C. The primers used for PCR included some of the synthetic oligonucleotides listed above. Primers FE and RE contained an *Nde*I and a *Bam*HI restriction site on their 5′ end, respectively, for directional cloning into the pET16b expression vector.

For RNA-PCR total RNAs isolated from skeletal or cardiac muscle by the guanidinium isothiocyanate method (Sambrook et al., 1989) were reverse transcribed using random primers and a method described by Noonan and Roninson (1988).

Cloning and expression of a 1.4 kb cDNA fragment

The 1.4 kb PCR product amplified with the FE and RE primers on the TN1 phagemid DNA template (nucleotides 13-1444) was cloned into the pCRII vector using the TA Cloning Kit (Stratagene). The inserts were subcloned into the pET16b (Novagen) expression vector using the restriction sites included in the primers. Fusion proteins were expressed following the manufacturer's protocol, and were analyzed on SDS-polyacrylamide gels (Neville, 1971).

Immunization

Inclusion bodies from the *E. coli* lysate were serially washed with 2 M, 4 M and 6 M urea in PBS, pH 7.4. Particles insoluble in the final 6 M urea were washed and resuspended in PBS, pH 7.4. This suspension showed a single dominant band at 56 kDa plus some minor contaminating proteins on SDS-PAGE. Six-week-old female Balb/c mice were immunized i.p. with 60 μg/mouse antigen in complete Freund's adjuvant (Sigma). Three and six weeks later the same amount of antigen was injected i.p. with incomplete adjuvant (Sigma). The animals were sacrificed 13 days after the last boost. Titer and reactivity of the sera were evaluated with western blotting (Sambrook et al., 1989) using the 56 kDa titin fragment as antigen.

Immunofluorescence

Bovine cardiac and rabbit psoas myofibrils were obtained using methods described previously (Wang and Greaser, 1985; Pan et al., 1986) and stored in 50% glycerol plus RB (75 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 5 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol). Myofibrils were washed free of glycerol by resuspension in 10 volumes of RB plus 1 mg/ml bovine serum albumin (BSA) and pelleted by centrifugation for 10 seconds in a microfuge. The washing was repeated with fresh solution and the myofibrils were resuspended in RB plus BSA to a concentration of approximately 1 mg/ml. The myofibril suspension was then gently smeared over No. 1.5 coverslips and incubated with mouse serum diluted 1:400 (in RB plus BSA) for 1 hour at room temperature. After washing in RB, the coverslips were incubated in Texas Red labelled secondary antibody (Cappel) diluted 1:20 for 1 hour. Unbound antibody was washed away, the myofibrils were fixed in 4% formaldehyde for 10 minutes, and the coverslips mounted on glass slides in 70% glycerol/RB containing *p*-phenylenediamine (1 mg/ml). Control samples without primary antibody were run in parallel. The slides were viewed with a Nikon Diaphot microscope equipped for phase contrast and epifluorescence illumination. Images were obtained using a 100× objective (NA 1.4) and a cooled CCD camera (Thomson 7883) controlled via a Macintosh IIfx with a Matrox board using IPLABS software. Exposure times were typically 0.2 seconds for phase contrast and 2 seconds for fluorescence.

Purification of expressed titin fragment from clone FE-RE

Inclusion bodies were suspended in 7 M urea, 10 mM sodium phosphate, pH 7.0, 1 mM EGTA, 1 mM DTT and dialyzed versus the same solution overnight at 4°C. The solubilized protein was then applied to a carboxymethyl cellulose (CM52) column equilibrated with the same buffer. The titin fragment was eluted using a gradient of the starting buffer and the starting buffer plus 0.3 M NaCl. Pooled fractions were then dialyzed versus solutions with decreasing urea concentrations (5 M, 4.5 M, 4.0 M for 2 hours each; then 3.5 M overnight, 3 M, 2 M for 2 hours each and finally overnight versus 0.15 M NaCl, 10 mM sodium phosphate, pH 7.0, 1 mM EGTA, 1 mM DTT, 1 mM NaN_3. The final solution was clarified in the microfuge (15 minutes) and concentrated in a Centriprep 10 (Amicon).

Phosphorylation of the solubilized FE-RE protein

ERK1-glutathione-S-transferase agarose conjugate was purchased from Upstate Biotechnology Inc. Chicken embryonic skeletal muscle extract was prepared by pulverizing the frozen tissue in a mortar under liquid nitrogen followed by homogenization in ice-cold lysis buffer (40 mM HEPES, pH 7.2, 1 mM EDTA, 1 mM DTT, 2 μg/ml leupeptin, 1.5 μM pepstatin, 0.5 mM PMSF, 0.05% Triton X-100). The cytosolic fraction was clarified by centrifugation (1,500 ***g***, 10 minutes at 4°C) and was used as a source of kinase activity. Reaction mixtures contained either 0.2 mg/ml ERK1 kinase in 25 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.1 mg/ml BSA, 100 μM ATP, or 0.4 mg/ml chicken embryonic skeletal muscle extract in 40 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 100 μM ATP. After preincubation at 30°C for 20 minutes, the substrates (500 ng of the FE-RE titin fragment, or 500 ng bovine histone H1 protein as a control) and 2.5 μCi [γ-^{32}P]ATP (Amersham, 6,000 Ci/mmole specific activity) were added in the same buffers. The mixtures were incubated at 30°C for an additional 20 minutes. The agarose beads were sedimented by a brief spin; then an equal volume of 2× sample buffer for SDS-PAGE was added to the supernatants and to the muscle cell extract samples to stop the reaction. Samples were heated to 100°C for 2 minutes and were loaded on a 10% SDS-polyacrylamide gel (Neville, 1971). The dried gel was autoradiographed at –80°C for 24 hours with an intensifying screen.

RESULTS

A rabbit heart partial (size-selected) cDNA library was constructed in λZAP-Express vector. It contained 2.5×10^5 independent clones with about 1 kb average insert size. Screening of this cDNA library with the anti-titin T12 monoclonal antibody yielded positive clones with 1×10^{-4} frequency. Three clones carrying different inserts were characterized: T12/2, T12/18 and T12/19 (Fig. 2). Sequence analysis of the inserts revealed that the three clones overlapped in a 300 bp region (presumably encoding the T12 epitope) and resulted in a 2.7 kb continuous open reading frame. *Eco*RI cut inserts from clones T12/18 and T12/19 were used for northern hybridization in order to confirm their titin origin. Both bound to >23 kb RNAs (data not shown), a typical binding pattern of titin cDNA fragments.

The insert of clone T12/19 was used for hybridization screening of the same library in order to extend the sequence. Two of the positive clones isolated in this second screening yielded new sequence data in the 5′ direction. Clone 19/1 added 82 bp to the 5′ end of clone T12/19 and also overlapped with the two other T12 clones (Fig. 2). The 3′ end of the insert in clone 19/5 overlapped with the known sequence in a 411 bp region and extended it to 4 kb. The cDNA library was screened once more with a 740 bp PCR fragment (amplified using the T and N primers; Fig. 2) to further extend the coding sequence

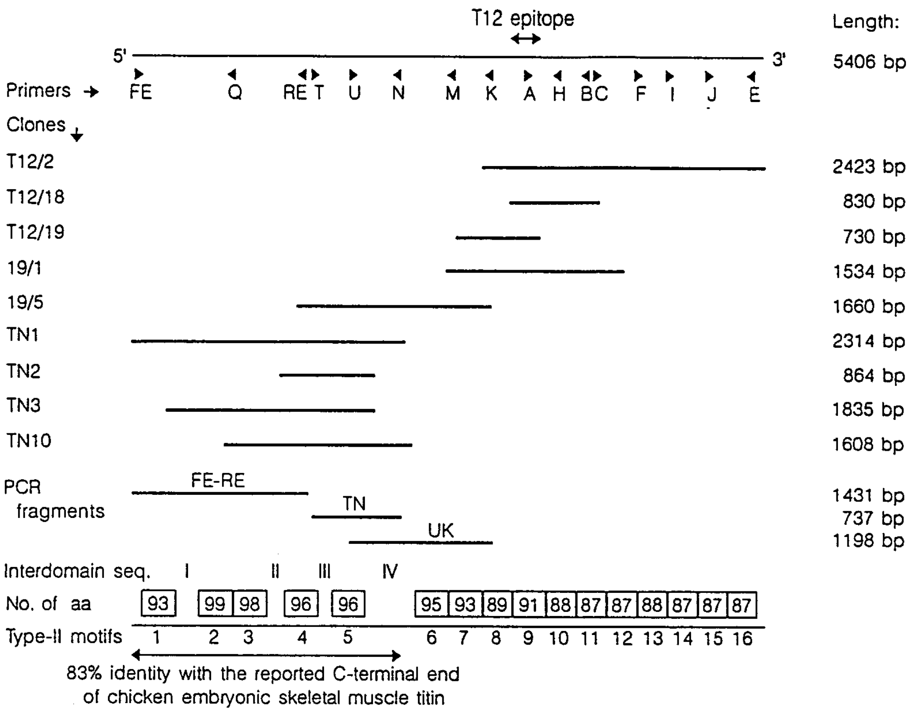

Fig. 2. Summary of the overlapping lambda ZAP Express cDNA clones for the 5.4 kb rabbit cardiac titin contig. The domain structure and the most significant homology of the predicted polypeptide chain is also presented. The positions of the synthetic oligonucleotides (used for sequencing and PCR) and the PCR fragments are also marked. Boxes represent the Type-II motifs; the number inside indicates how many amino acid residues are found in the motif.

```
1         SGFELRIKNYRI-LEGMGVTFHCKMS--GYPLPKIAWYKDGKRIKPGERYQMDFLQ-DGRASLRIPVVLPEDEGIYTAFASNIKGNAICSGK-LYVEP
2         PVFVLKPTSFKC-LEGQSARFDLKVV--GRPMPETFWFHNGQQVVNDYTHKVVIKE-DGTQSLIIVPATPSDSGEWTVVAQNRAGKSTISVI-LTVEAVEHQVK
3         PVFVEKLKNVNV-KEGARLEMKVRAT--GNPNPDIVWLKNSDIIVPHKYPKIIIEGTKGQAALKIEFHCRQDSAWYTATAINKAGRDTTRCK-VNVEVEFAE
4         PFFKKKLSSLRLKRFG-PAHFECRLTPIGDPTMVVEWLHDGKPLEAANRLRMINE--FGYCSLDYGVAYSRDSGVITCRATNKYGTDHTSAT-LIVKDEK
5         PDIVLYPEPVRV-LEGETARFRCRVT--GYPQPKLNWYLNGQLIRKSKRFRVRY---DGIHYLDIVDCKSYDTGEVKVTAENPEGVIEHKVK-LEIQQREDFR
6         PKIFERIQSQTV-GQGSDAHFRVRVV--GKPDPECEWYKNGVKIERSDRIYWYWPE-DNVCELVIRDVTAEDSASIMVKAINIAGETSSHAF-LLVQAKQ

Cons      P-Fx--x--x-x--EG--A-F-xRx---G-P-P-x-Wx-NG--I----Rx-x-x---DG---L-I--x---DSG-xTx-A-N--G-----x--L-V--------
                         *            * * *                                                *

          ____     ______   _____________    ___________    ________  _________     _________  _____________
β-sheet    A        A'           B                C              D         E              F              G

ChTe      PYFTKTILDMEV-VEGSAARFDCKIE--GYPDPEVMWYKDDQPVKESRHFQIDYDE-EGNCSLTISEVCGDDDAKYTCKAVNSLGEATCTAE-LLVETMGKEGE

7         LINFTQELQDVVAKEKDTMATFECETSEPF---VKVKWYKDGVEIHE-GDKYRMHSDRKVHF-LSVLTIDTSDAEDYSCVLVED-ENIKTTAK-LIVEGA
8         VVEFVKELEDIEV-PESFSGELECIVSPEN---IEGKWYHNNVELKS-NGKYTITSRRGRQN-LTVKDVTKEDQGEYSFVVD---G-KKTTCK-LKMRPR
9         PIAILQGLSDQKV-CEGDIVQLEVKVSLEN---VEGVWMKDGQEVQS-SDRVHIVI-DKHSHMLLIEDMTKEDAGNYSFTIPAL-G-LSTSGR-VSVYSV
10        --DVITPLKDVNV-LEGTKAVLECKVSVPD--VTSVKWFLNDEQIKP-DDRVHAIV-KGTKQRLVINRTHASDEGPYKLMV----GRVETSCN-LSVEKI
11        --KIIRGLRDLTC-TETQNVVFEVELSHSG---IDVLWNFKDKEIKP-SSKYKIEA-HGKIYKLTVLNMMKDDEGKYTFYA----GENMTSGK-LTVAGG
12        --AISKPLTDQTV-AESQEAVFECEVANPD---SEGEWLKEGKHLPL-SNAVRSES-DGHKRRLIIAATKLDDNWRIHLQ-----GATSKTSAKLKVETV
13        --KIKKTLKNLTV-TETQDAVFTVELTHTN--VKGVQWIKNGVVLKS-NDKYDISV-KGTIYTLRVKNCAIVDESVYGFKL----GRLGASAR-LHVETV
14        --KIIKKPKDVTA-LENTTVAFEVSVSHDT---VPVKWFHKNVEIKP-SDKHRMIS-ERKVHKLMLQNIAPSDAGEYTAVV----GQLECKAK-LFVETL
15        --HITKTMKSIEV-PETKTASFECEVSHFN---VPSMWLKNGVEIEM-SEKFKIVV-QGKLHQLIIMNTSSEDSAEYTFVC----GNDQVSAT-LTVTPI
16        --MITSMLKDINA-EEKDTITFEVTVNYEG---ISYKWLKNGVEIKS-TDRCQMRT-KKLTHSLNIRNVHFGDAADYTFVA----GKATSTAT-LYVEAR

Cons      ---I---L-Dx-V--E---xxFEx-VS------x-x-WxK-GVEIK---DKx-x----G----L-x--x---D-x-Y-Fxx----G-x--TSx-L-VE-x

D-11      PVTLIRDIENQTV-LTDEDAIFECEIKINYPE-IKLSWYKGTQKLDS-SDKYKIKI-EGDRHILKIKNCQLEDQGNYRIVC----GPHIASAR-LTVIE-
D-12      PA-VERHLHDTTF-KEGNTCTLSCQFSIPN---AKSQWYRNGRPIKI-GGRYSTQV-SDKVHKLIIKDVRTEDQGQYTCKL----DNLETTAD-LTIEAE
D-13      PIQFTKSIQNIVV-SEHQSATFECEVSFDD---AVVTWYKGPTELTE-SPKYSFRS-EGRCHYMTIHNVTAEDEGVYSVIA-----RLE----------
```

Fig. 3. Alignment of the 16 Type-II motifs of the 5.4 kb contig. Domains 1-6 are homologous with the immunoglobulin domain of chicken telokin (ChTe). The β-sheets of chicken telokin are also identified (Holden et al., 1992; Harpaz and Chothia, 1994). The glycine, proline and asparagine residues labelled with asterisks are proposed to play an important role in stabilizing the A′B, BC and FG turns. These residues are missing from most of domains 7-16, and from the short Type-II motifs of the only other I-band titin region reported to date (D-11, D-12 and D-13; Maruyama et al., 1993). These domains share a different set of consensus residues, and presumably have a different, potentially less stable tertiary structure. In the consensus sequences 'x' identifies hydrophobic positions. Residues in bold are the most conserved key positions used for the alignment.

in the 5′ direction. Four new lambda clones, TN1, TN2, TN3 and TN10 (Fig. 2), carried inserts of different sizes, and all the four inserts overlapped with clone 19/5. These clones extended the cDNA sequence to 5.4 kb; the contig (GenBank accession number U28657) had a single continuous open reading frame encoding 1802 amino acids.

Analysis of the domain structure of the predicted amino acid sequence revealed 16 Type-II motifs and 4 unique interdomain segments (Fig. 2). Alignment of the Type-II like domains (Fig. 3) suggested that the motifs can be placed into two groups based on their length and their conserved residues. GenBank search results indicated that the first 6 motifs had highest homology with the telokins of different species, with some Type-II motifs of twitchin, and with many different Type-II motifs from the A-band section of titin. Motifs # 7-16 showed the best homology to Type-II motifs of many different C-proteins, to the I-band titin region reported from chicken, to twitchin, and to the C-terminal (M-line) part of human cardiac titin. All homologies listed above showed only short stretches (50-100 residues) of 20-40% amino acid identity. However, the N-terminal 762 amino acids of the contig were 83% identical with the previously reported C-terminal end of the chicken embryonic skeletal muscle titin (Maruyama et al., 1994; see (12) in Fig. 1). At the nucleotide level identity is 77%. Although this homology is exceptionally high, there are several remarkable differences. The gap alignment (UW GCG) of the two sequences shows both substitutions and insertions. Altogether 7 gaps were found in the chicken sequence when it was aligned with the rabbit nucleotide sequences. The largest gap was 18 nucleotides in length, resulting in the insertion of 6 amino acid residues at position 159-164 in the first unique interdomain region of rabbit cardiac titin (Fig. 4). There are three triplet insertions randomly distributed along the 2.3 kb segment (Fig. 4, residues 17, 339 and 698), and there are three single nucleotide insertions causing a shifted reading frame in a short segment of the first Type-II domain (Fig. 4, residues 43-63). As there are three insertions, the frame is finally restored. The amino acid sequence coded by the rabbit version actually shows more homology with the consensus Type-II domain sequence than the chicken version (where even the most conserved tryptophan is missing), suggesting deletions in the chicken titin gene rather than insertions in the rabbit gene in the course of evolution. In spite of the gaps the two sequences show the same domain structure and can easily be aligned up to amino acid position 762 (Fig. 4). At that point the homology drops to an insignificant level. It is interesting to note, that from the very same point the last short C-terminal segment of the chicken sequence shows high homology (67%) to human porin, a channel-forming membrane protein (Maruyama et al., 1994). In our rabbit cDNA contig the interdomain sequence continues for another 40 residues and then is followed by 11 Type-II motifs.

When homology searches were performed using the individual interdomain sequences, II and III were found to be homologous only with the above-mentioned chicken titin sequence. Interdomain segment I also shared 40-50% sequence identity to short stretches of different histone proteins (regions similar to the xSPxR motifs; Fig. 5). Such sequences fit the consensus pattern for phosphorylation by proline-directed kinases and are found in histones and many other proteins (Langan et al., 1989). Interdomain segment IV shared 19-25% identity at a significantly low Poisson probability ($8.4\times e^{-10}$-$9.6\times e^{-9}$) with two *Plasmodium falciparum* sequences

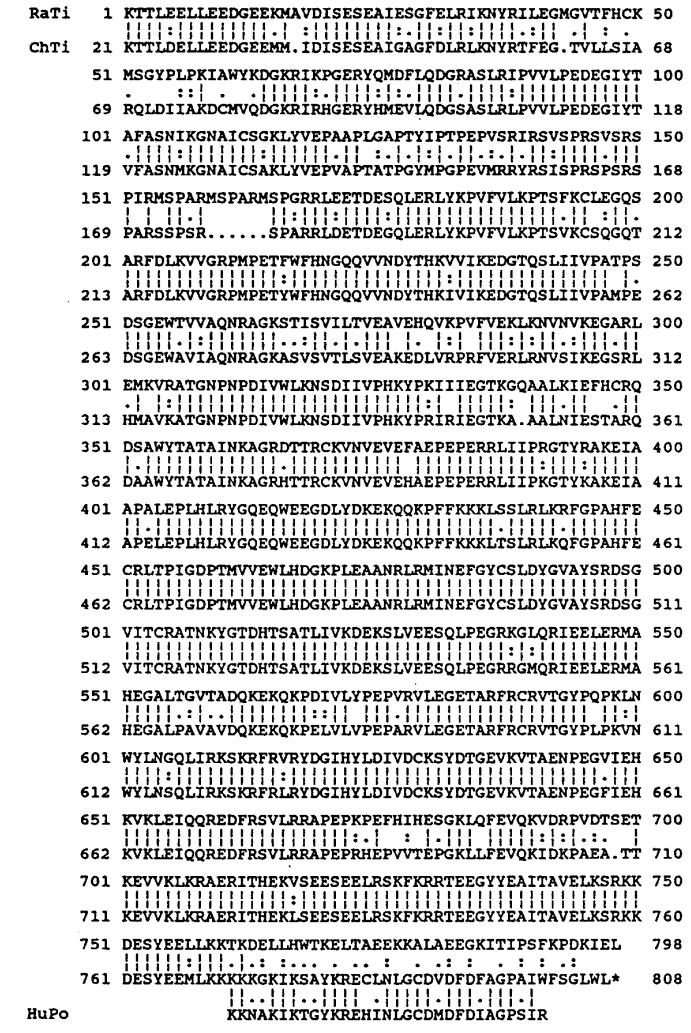

Fig. 4. Amino acid sequence alignment between the most N-terminal 804 residues of the novel rabbit cardiac I-band titin fragment and residues 21-807 (C-terminal end of the open reading frame) of the previously reported C-terminal sequence from embryonic chicken skeletal muscle titin (Maruyama et al., 1994). Identity is 82.6% and similarity is 89.4% until position 762, using 5 gaps. The rest of the two sequences do not share significant homology. The last short segment of chicken titin (residues 771-800) shows 67% identity with human porin (HuPo).

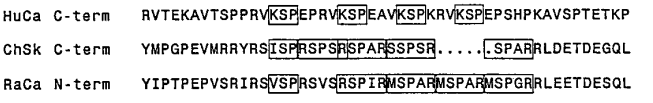

Fig. 5. Phosphorylation sites for proline-directed kinases in three titin cDNA sequences. The KSP motifs of the human cardiac muscle titin C-terminal end (HuCa C-term; Gautel et al., 1993b), the RSP motifs of the embryonic chicken skeletal muscle titin C-terminal end (ChSk C-term; Maruyama et al., 1994) and the rabbit cardiac muscle titin N-terminal (RaCa N-term) xSPxR motifs are aligned. The human KSP motifs and a protein fragment carrying the rabbit xSPxR motifs have been shown to be in vitro phosphorylated by cdc2 (Gautel et al., 1993b) and by ERK1 (Fig. 7) kinases, respectively.

(Accession numbers L04159 and M69183). These proteins are extremely rich in glutamic acid, lysine and arginine, drawing attention to the fact, that interdomain segment IV is also abundant (47%) in charged residues. Secondary structure prediction algorithms (UW GCG software; Chou and Fasman, 1978) suggested that this region of the rabbit titin would form α-helical structure. A recently published study (Musco et al., 1995) identified an α-helical motif near the N terminus of titin. The amino acid sequence of that interdomain segment (numbers 1888 to 2031; Musco et al., 1995) is 94% identical with interdomain segment IV of the rabbit titin contig presented above and represents the corresponding segment of human cardiac titin (Siegfreid Labeit, personal communication). Thus the predicted α-helical structure of this region is indirectly confirmed by the structural studies performed on the human fragment.

Besides homologies listed above the GenBank search showed high (89-92%) homology to three short human cDNA clones submitted as unpublished sequences by Genexpress (accession numbers Z28527, Z24824 and Z17410). Based on the degree of homology found between the A-band region of human and rabbit titin, these three uncharacterized human clones may represent the first sequence data from the human I-band part of titin, presumably from the region described in this paper.

Since the 5′ half of the contig showed surprising homology to a previously characterized titin clone identified as the C terminus, and as only a single cDNA clone (19/5) had been isolated which spanned that region (all TN clones) and the region encoding the T12 epitope (all T12 clones, and 19/1), further evidence was obtained to exclude the possibility of a cloning artefact. RT-PCR was performed on adult rabbit cardiac and skeletal total RNAs using the U and K primers (Fig. 2) in order to prove that the two halves of the 5.4 kb contig are really related. Rabbit genomic DNA and phagemid DNA from clone 19/5 were also used as controls. On both RNA templates and on the phagemid DNA the same fragment of the expected 1.2 kb size could be amplified (data not shown). A 2.1 kb fragment was amplified when using the genomic DNA template. These data confirm the proximity of the studied regions both in the mRNA and in the titin gene and indicates the presence of 0.9 kb intron sequence. The 1.2 kb PCR product amplified on cardiac muscle total RNA was sequenced with primers U, N, M and K (Fig. 2). The sequence was 100% identical with the corresponding region of clone 19/5.

PCR on rabbit genomic DNA template using the FE and RE primers (Fig. 2) resulted in the amplification of a 2.6 kb fragment although the cDNA sequence is only 1.4 kb. This indicates the presence of at least one intron in the region, meaning that the fragment homologous to the chicken C-terminal titin sequence is a multi-exon unit in the rabbit.

Polyclonal mouse serum was raised against an expressed 56 kDa protein fragment (encoded by the PCR-amplified 1.4 kb FE-RE fragment; Fig. 2) to map the sarcomere-position of the polypeptide encoded by the 5′ end of the contig. The fragment contains the first four Type-II motifs and the first two interdomain segments, one of them carrying the four xSPxR potential phosphorylation sites. Isolated myofibrils from rabbit skeletal and bovine cardiac muscle showed strong staining at the Z-line in immunofluorescence (Fig. 6), confirming that the cDNA

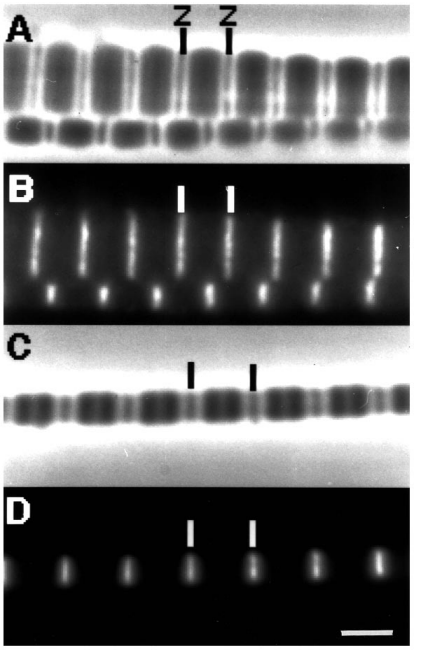

Fig. 6. Phase contrast (A and C) and fluorescent (B and D) images of myofibrils decorated with the polyclonal mouse serum raised against the N-terminal 56 kDa fragment of the contig. Antibodies show strong Z-line staining on both rabbit skeletal (B) and bovine cardiac (D) myofibrils. Bar, 2 μm.

characterized above truly encodes a segment of titin in this region of the sarcomere. Similar results were obtained using rabbit cardiac and embryonic chicken skeletal muscle myofibrils (data not shown).

The expressed FE-RE protein was solubilized and purified. Incubation with the proline-directed kinase ERK1 and [γ-^{32}P]ATP resulted in the phosphorylation of the fragment (Fig. 7A; Alessandrini et al., 1992; Gonzalez et al., 1993). Chicken embryonic skeletal muscle cell extracts have endogenous kinase activity and in the presence of [γ-^{32}P]ATP numerous proteins of the extract become phosphorylated. Addition of the FE-RE titin fragment to this complex system resulted in an additional labelled band. The electrophoretic mobility of the band is identical with the FE-RE titin fragment phosphorylated by the purified ERK1 kinase (Fig. 7B).

DISCUSSION

The 5.4 kb cDNA fragment presented above is the most 5′ titin sequence reported so far. Based on the staining pattern of the T12 monoclonal antibody and the polyclonal anti-FE-RE serum, the encoded titin segment is believed to be near to the N terminus. It contains exclusively Type-II motifs, a finding similar to that in the only other published I-band fragment (Maruyama et al., 1993). Comparison of the 16 domain sequences with the consensus immunoglobulin ‘I’ set (Harpaz and Chothia, 1994) reveals excellent homology at most of the critical positions. However, domains 7-16 are somewhat shorter and several critical residues are absent. The invariant glycine of the telokin family at position A′B1 is present in domains 1-6, but missing in all but two of domains 7-16. In addition the conserved B9 glycine, BC1 and BC3 prolines, and FG1 asparagine (designation of residues as in Harpaz and Chothia, 1994) are all present in domains 1-6 but are absent or misaligned in domains 7-16 (residues labelled with asterisks on Fig. 3). Most of the difference in domain length between

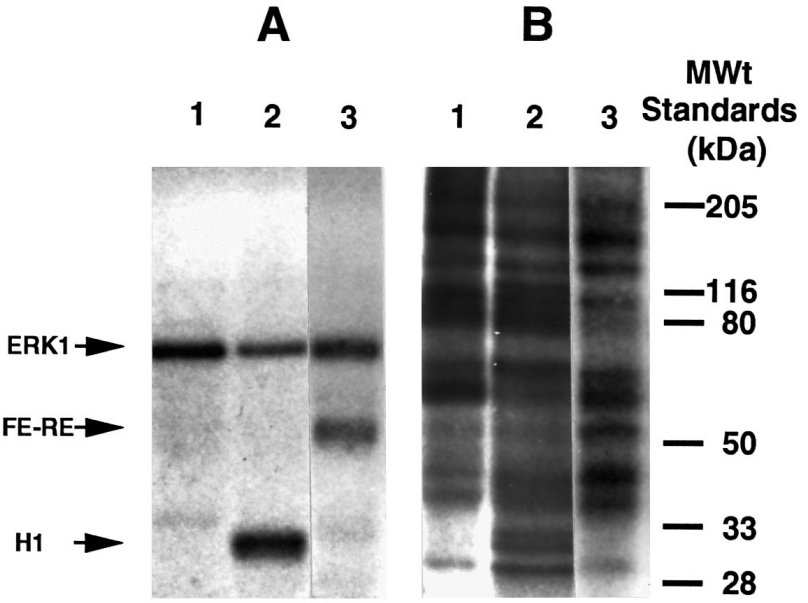

Fig. 7. Autoradiograms showing the phosphorylation of the expressed 56 kDa titin fragment with the purified proline-directed protein kinase ERK1 (A) and with chicken embryonic skeletal muscle extract (B). Lanes 1, no substrate controls (a small amount of the autophosphorylated ERK1 leaks from the beads, and there are many proteins phosphorylated or autophosphorylated in the muscle extract). Lanes 2, 500 ng bovine histone H1 substrate. Lanes 3, 500 ng purified 56 kDa titin fragment. Molecular mass markers are shown on the right.

the two sets appears to be in the loop between β-sheets F and G (Fig. 3).

The T12 monoclonal antibody interacted with the section of titin corresponding to domain 9 (based on the clones obtained by expression screening). Immunoelectron microscopic studies with skinned muscle fibers have suggested that this antibody's decoration position in the sarcomere (approximately 0.1 μm from the Z line) does not change with stretch (Fürst et al., 1988; Trombitás and Pollack, 1993). Thus the antibody binding region is near the transition point in the sequence for the two types of immunoglobulin domains. Although different subtypes of immunoglobulin-like domains are known to show extreme sequence divergence while still able to fold into stable β-barrels (Bork et al., 1994), it is tempting to speculate that the first six domains constitute the inextensible region of titin while the three dimensional structure of the remaining domains changes as the sarcomere is stretched. The reduced number of hydrogen bonds in the region at the C terminal end of β-sheet B, the C terminal end of β-sheet F and the N terminal end of β-sheet G (which are all tightly clustered at one end of the telokin β-barrel), could lead to a less stable domain that unravels more easily with stretch. The three short I-band Type-II motifs reported by Maruyama et al. (1993) have very similar features to domains 7-16 (Fig. 3). However, they are also preceded by longer, telokin-like motifs, which reside in the elastic part of the sarcomere. Finding potentially more and less stable domains within the extensible segment of the I-band would suggest that not all domains contribute equally to the elasticity of the region. Erickson (1994) proposed that, even in rest-length sarcomeres, some I-band domains may be unfolded to span the 250-400 nm distance. It is possible that some domains have lost so many residues of critical importance for normal folding (e.g. D-13 on Fig. 3), that they may not form the β-barrel at all. These may contribute to the non-elastic rest-length of the I-band, together with the unique interdomain segments. As the force increases on the ends of the folded β-barrel domains, the weakest will start to unfold and denature first (Erickson, 1994). As such 'weak' domains seem to be present at different sites of the I-band, the region stretches evenly, as determined by immunoelectron microscopic studies (Itoh et al., 1988; Trombitás et al., 1993). On the other hand, based on the findings of Soteriou et al. (1993), differences in domain-stability are subtle, resulting in an approximately simultaneous unfolding of all 'weak' and 'stable' domains, with a calculated average of 10 kcal/mol domain free energy change, meaning probably a range between 7-14 kcal/mol (Erickson, 1994). The thermodynamic stability of three telokin-like Type-II domains from the A-band region was measured to be less than the above-mentioned values (3.15-5.8 kcal/mol compared to 7-14 kcal/mol; Politou et al., 1994). These A-band domains presumably do not unfold when the sarcomere is exposed to force because of titin binding to the thick filament. The force required to pull apart protein-protein interfaces was calculated to be considerably higher than the folding energy of individual domains (Erickson, 1994). Based on these data and speculations, the inflexible nature of the N-terminal ≈100 nm of titin may be due to: (1) protein-protein bonds formed between neighboring titin molecules or between titin and other proteins; (2) the potentially higher stability of the domains found in that region; or (3) some combination of these factors.

Interdomain segments may play a crucial role in forming and/or regulating protein-protein interactions. The first unique interdomain sequence of the titin fragment reported here contains four x-S-P-x-R motifs. As the 56 kDa expressed protein carrying these sites could be in vitro phosphorylated by the purified proline-directed kinase ERK1, and also by chicken embryonic skeletal muscle cell extract, we speculate that there are active kinases in the developing muscle which can phosphorylate the corresponding segment of titin in vivo. The x-S-P-x-R motifs are similar to the KSP motifs found at the C-terminal end of human cardiac muscle titin (Gautel et al., 1993b), the RSP motifs reported at the C-terminal end of chicken skeletal muscle titin (Maruyama et al., 1994), and the recognition sites of different proline-directed kinases on neurofilaments, histone proteins, lamin, bradykinin etc. Gautel and coworkers (1993b) showed the in vitro phosphorylation of all four KSP motifs of the C-terminal end of human cardiac titin

by neonatal muscle tissue extract, and suggested that the major activity of the titin KSP kinase is identical to that of the proline-directed cdc2 kinase. The developmentally regulated phosphorylation of the KSP motifs was proposed to play a role in the controlled activation of binding sites during myogenesis (Gautel et al., 1993b). As the region carrying the x-S-P-x-R motifs in the rabbit sequence is N-terminal to the N1 line of the sarcomere, residing in the non-elastic part of the I-band, we hypothesize that phosphorylation of these sites may regulate the binding capacity of the neighboring domains to other proteins in or near the Z-disk. Thus there appear to be two potential phosphorylation regions in titin, one at each end of the molecule. Their in vivo significance in the regulation of titin attachment to other Z-line and M-line proteins is yet to be determined.

The high homology found between the N-terminal 762 amino acids of this I-band fragment and the reported C-terminal end of chicken embryonic skeletal muscle titin is puzzling. The average identity among pairs of Type-II motifs of the A-band region was found to be around 30% (Labeit et al., 1992). The highest homology (around 50%) was always found between Type-II motifs 11 domains (i.e. a super-repeat) apart. Different parts of titin have never been found to show higher homology. Homology for corresponding regions of titin sequences of different mammalian origins is about 90% (Labeit et al., 1992; Fritz et al., 1993b). Unfortunately there has been no previous data about corresponding fragments of avian and mammalian titin, except for the reported C-terminal end (Maruyama et al., 1994) which has no significant homology with the human cardiac C terminus (Gautel et al., 1993b). Many titin epitopes are well conserved from cold-blooded vertebrates to man as determined by using a set of monoclonal antibodies (Fürst et al., 1988, 1989). The T12 monoclonal antibody used in the present study cross reacted with every species in the study from fish to man. The T33 antibody, which decorates the sarcomere close to the M-line, reacted with fish, amphibian, avian and some mammalian titins, but failed to cross-react with human muscle. The T20 antibody binds to the Z-line and also failed to cross-react with human titin. These results allow for significant differences in the N-terminal and C-terminal ends of avian and human titin. It is noteworthy though, that the Pc1200 polyclonal antibody (which was used to identify the clone encoding the chicken C-terminal end as well as other clones encoding an I-band fragment of chicken titin) binds mainly to the Z-line, and only very weakly to the edge of the M-line (Maruyama et al., 1993, 1994). While the position of the embryonic chicken I-band region was confirmed by staining myofibrils with antibodies raised against the expressed titin fragment (Maruyama et al., 1993), there was no immunological evidence presented concerning the position of the C-terminal sequence within the sarcomere (Maruyama et al., 1994). The polyclonal serum raised against the N-terminal 56 kDa of the rabbit titin fragment presented above stains the Z-lines of embryonic chicken myofibrils with similar intensity as the rabbit and bovine Z-lines. This indicates that titin molecules in the embryonic chicken skeletal muscle contain a homologous region close to the Z-line. Further work will be needed to determine whether there are large sections of similar structure at both ends of embryonic chicken titin or if the localization of that clone to the C terminus has to be re-evaluated.

Results presented in the current study define the cDNA sequence for a 204 kDa titin fragment which has been localized to the Z-line region of the sarcomere. The in vitro phosphorylation of its expressed N-terminal segment by embryonic skeletal muscle extract and by the purified proline-directed kinase ERK1 raises the possibility that interactions made by this part of titin are in vivo regulated by proline-directed kinases.

This work was supported by the College of Agricultural and Life Sciences of the University of Wisconsin-Madison and by grants from USDA (91-37206-6743) and NIH (HL47053). We thank Dr Jeffery Fritz and James Ludtke for helpful comments and discussions. The assistance of Mangeng Cheng and Melanie Cobb in a preliminary ERK1 phosphorylation experiment is gratefully acknowledged.

REFERENCES

Alessandrini, A., Crews, C. M. and Erikson, R. L. (1992). Phorbol ester stimulates a protein-tyrosine/threonine kinase that phosphorylates and activates the ERK-1 gene product. *Proc. Nat. Acad. Sci. USA* **89**, 8200-8204.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403-410.

Anderson, R. D., Bao, C.-Y., Minnick, D. T., Veigl, M. and Sedwick, W. D. (1992). Optimization of double-stranded DNA sequencing for polymerase chain reaction products. *USB Editorial Comments* **19**, 39-58.

Behr, T., Fischer, P., Muller-Felber, W., Schmidt, A. M. and Pongratz, D. (1994). Myofibrillogenesis in primary tissue cultures of adult human skeletal muscle: expression of desmin, titin and nebulin. *Clin. Invest.* **72**, 150-155.

Bork, P., Holm, L. and Sander, C. (1994). The immunoglobulin fold. Structural classification, sequence patterns and common core. *J. Mol. Biol.* **242**, 309-320.

Chou, P. Y. and Fasman, G. D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. *Advan. Enzymol.* **47**, 45-147.

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

Erickson, H. P. (1994). Reversible unfolding of fibronectin type III and immunoglobulin domains provides the structural basis for stretch and elasticity of titin and fibronectin. *Proc. Nat. Acad. Sci. USA* **91**, 10114-10118.

Fritz, J. D., Greaser, M. L. and Wolff, J. A. (1991). A novel 3′ extension technique using random primers in RNA-PCR. *Nucl. Acids Res.* **19**, 3747.

Fritz, J. D., Wolff, J. A. and Greaser, M. L. (1993a). Partial titin cDNA sequence isolated from rabbit cardiac muscle RNA. *J. Muscle Res. Cell Motil.* **14**, 347-350.

Fritz, J. D., Wolff, J. A. and Greaser, M. L. (1993b). Characterization of a partial cDNA clone encoding porcine skeletal muscle titin: comparison with rabbit and mouse skeletal muscle titin sequences. *Comp. Biochem. Physiol.* **105B**, 357-360.

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

Fürst, D. O., Nave, R., Osborn, M. and Weber, K. (1989). Repetitive titin epitopes with a 42 nm spacing coincide in relative position with known A band striation also identified by major myosin-associated proteins. *J. Cell Sci.* **94**, 119-125.

Gautel, M., Lakey, A., Barlow, D. P., Holmes, Z., Scales, S., Leonard, K., Labeit, S., Mygland, A., Gilhus, N. E. and Aarli, J.A. (1993a). Titin antibodies in myasthenia gravis: identification of a major immunogenic region of titin. *Neurology* **43**, 1581-1585.

Gautel, M., Leonard, K. and Labeit, S. (1993b). Phosphorylation of KSP motifs in the C-terminal region of titin in differentiating myoblasts. *EMBO J.* **12**, 3827-3834.

Gonzalez, F. A., Raden, D. L. and Davis, R. J. (1993). Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J. Biol. Chem.* **266**, 22159-22163.

Granzier, H. and Irving, T. C. (1995). Passive tension in cardiac muscle: contribution of collagen, titin, microtubules and intermediate filaments. *Biophys. J.* **68**, 1029-1044.

Handel, S. E., Greaser, M. L., Schultz, E., Wang, S.-M., Bulinski, J. C., Lin, J. J.-C. and Lessard, J. L. (1991) Chicken cardiac myofibrillogenesis studied with antibodies specific for titin and the muscle and nonmuscle isoform of actin and tropomyosin. *Cell Tissue Res.* **263**, 419-430.

Harpaz, Y. and Chothia, C. (1994). Many of the immunoglobulin superfamily domains in cell adhesion molecules and surface receptors belong to a new structural set which is close to that containing variable domains. *J. Mol. Biol.* **238**, 528-539.

Holden, H. M., Ito, M., Hartshorne, D. J. and Rayment, I. (1992). X-ray structure determination of telokin, the C-terminal domain of myosin light chain kinase, at 2.8 Å resolution. *J. Mol. Biol.* **227**, 840-851.

Horowits, R. and Podolsky, R. J. (1987). The positional stability of thick filaments in activated skeletal muscle sarcomere length: evidence for the role of titin filaments. *J. Cell Biol.* **105**, 2217-2223.

Horowits, R. (1992). Passive force generation and titin isoforms in mammalian skeletal muscle. *Biophys. J.* **61**, 392-398.

Isaacs, W. B., Kim, I. S., Struve, A. and Fulton, A. B. (1992). Association of titin and myosin heavy chain in developing skeletal muscle. *Proc. Nat. Acad. Sci. USA* **89**, 7496-7500.

Itoh, Y., Suzuki, T., Kimura, S., Ohashi, K., Higuchi, H., Sawada, H., Shimizu, T., Shibata, M. and Maruyama, K. (1988). Extensible and less-extensible domains of connectin filaments in stretched vertebrate skeletal muscle sarcomeres as detected by immunofluorescence and immunoelectron microscopy using monoclonal antibodies. *J. Biochem.* **104**, 504-508.

Kurzban, G. P. and Wang, K. (1988). Giant polypeptides of skeletal muscle titin: sedimentation equilibrium in guanidine hydrochloride. *Biochem. Biophys. Res. Commun.* **150**, 1155-1161.

Labeit, S., Barlow, D. P., Gautel, M., Gibson, T., Holt, J., Hsieh, C.-L., Francke, U., Leonard, K., Wardale, J., Whiting, A. and Trinick, J. (1990). A regular pattern of two types of 100-residue motif in the sequence of titin. *Nature* **345**, 273-276.

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

Langan, T. A., Gautier, J., Lohka, M., Hollingsworth, R., Moreno, S., Nurse, P., Maller, J. and Sclafani, R. A. (1989).Mammalian growth-associated H1 histone kinase: a homology of cdc2+/cdc28 protein kinases controlling mitotic entry in yeast and frog cells. *Mol. Cell. Biol.* **9**, 3860-3868.

Maruyama, K., Kimura, S., Yoshidomi, H., Sawada, H. and Kikuchi, M. (1984). Molecular size and shape of β-connectin, an elastic protein of striated muscle. *J. Biochem.* **95**, 1424-1433.

Maruyama, K., Endo, T., Kume, H., Kawamura, Y., Kanzawa, N., Nakauchi, Y., Kimura, S., Kawashima, S. and Maruyama, K. (1993). A novel domain sequence of connectin localized at the I band of skeletal muscle sarcomeres: homology to neurofilament subunits. *Biochem. Biophys. Res. Commun.* **194**, 1288-1291.

Maruyama, K., Endo, T., Kume, H., Kawamura, Y., Kanzawa, N., Kimura, S., Kawashima, S. and Maruyama, K. (1994). A partial connectin cDNA encoding a novel type of RSP motifs isolated from chicken embryonic skeletal muscle. *J. Biochem.* **115**, 147-149.

Musco, G., Tziatzios, C., Schuck, P. and Pastore, A. (1995). Dissecting titin into its structural motifs: identification of an α-helix motif near the titin N-terminus. *Biochemistry* **34**, 553-561.

Neville, D. M. (1971). Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* **246**, 6328-6334.

Noonan, K. E. and Roninson, I. B. (1988). mRNA phenotyping by enzymatic amplification of randomly primed cDNA. *Nucl. Acids Res.* **16**, 10366.

Pan, K.-M., Roelke, D. L. and Greaser, M. L. (1986). Isolation and characterization of a new 40-kilodalton protein from bovine cardiac muscle. *J. Biol. Chem.* **26**, 9922-9928.

Pierobon-Bormioli, S., Betto, R. and Salviati, G. (1989). The organization of titin (connectin) and nebulin in the sarcomeres: an immunocytolocalization study. *J. Muscle Res. Cell Motil.* **10**, 446-456.

Politou, A. S., Gautel, M., Labeit, S. and Pastore, A. (1994). Immunoglobulin-type domains of titin: same fold, different stability? *Biochemistry* **33**, 4730-4737.

Rhee, D., Sanger, J. M. and Sanger, J. W. (1994). The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskel.* **28**, 1-24.

Sambrook, J., Fritsch, E. F., Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Soteriou, A., Clarke, A., Martin, S. and Trinick, J. (1993). Titin folding energy and elasticity. *Proc. R. Soc. Lond. B* **254**, 83-86.

Tan, K. O., Sater, G. R., Myers, A. M., Robson, R. M. and Huiatt, T. W. (1993). Molecular characterization of avian muscle titin. *J. Biol. Chem.* **268**, 22900-22907.

Trombitás, K., Pollack, G. H., Wright, J. and Wang, K. (1993). Elastic properties of titin filaments demonstrated using a 'freeze-break' technique. *Cell Motil. Cytoskel.* **24**, 274-283.

Trombitás, K. and Pollack, G. H. (1993). Elastic properties of the titin filament in the Z-line region of vertebrate striated muscle. *J. Muscle Res. Cell Motil.* **14**, 416-422.

Wang, K., McCarter, R., Wright, J., Beverly, J. and Ramirez-Mitchell, R. (1991). Regulation of skeletal muscle stiffness and elasticity by titin isoforms: a test of the segmental extension model of resting tension. *Proc. Nat. Acad. Sci. USA* **88**, 7101-7105.

Wang, S.-M. and Greaser, M. L. (1985). Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J. Muscle Res. Cell Motil.* **6**, 293-312.

Whiting, A., Wardale, J. and Trinick, J. (1989). Does titin regulate the length of muscle thick filaments? *J. Mol. Biol.* **205**, 263-268.

(Received 31 March 1995 - Accepted 14 June 1995)