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Phosphorylation of KSP motifs in the C-terminal region of titin in differentiating myoblasts

Mathias Gautel, Kevin Leonard and Siegfried Labeit

European Molecular Biology Laboratory, Biological Structures Division, Meyerhofstrasse 1, 6900 Heidelberg, Germany

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Titin is a giant structural protein of striated muscle ($M_r \approx 3000$ kDa) and single molecules span sarcomeres from the M- to Z-lines. We have cloned and sequenced the C-terminal region of the titin molecule, which is an integral part of M-lines and forms intimate contacts with the 165 and 190 kDa M-line proteins. In contrast to the regular motif patterns of the A-band portion of titin, the 5.7 kb of titin sequences from the M-line show a complex structure of immunoglobulin-C2 repeats, separated by unique interdomain insertion sequences. As a striking feature, one interdomain insertion comprises four KSP repeats analogous to the multi-phosphorylation repeats of neurofilament subunits H and M. *In vitro* phosphorylation assays with expressed titin KSP sequences detect high levels of titin KSP phosphorylating kinases in developing but not in differentiated muscle. Since this kinase activity can be depleted from myocyte extracts by antibodies against cdc2 kinase and $p13^{suc1}$ beads, the titin KSP kinase is structurally related to cdc2 kinase. We suggest that titin C-terminal phosphorylation by SP-specific kinases is regulated during differentiation, and that this may control the assembly of M-line proteins into regular structures during myogenesis.

Key words: cdc2 kinase/sarcomere assembly/titin C-terminus/titin phosphorylation

Introduction

Titin, sometimes called connectin, is an abundant protein of vertebrate skeletal and heart muscle (for a review, see Trinick, 1991). Its molecular mass of over 3000 kDa makes it the largest known protein (Maruyama *et al.*, 1984; Kurzban and Wang, 1988). The primary structures derived from partial A-band titin cDNAs have shown that titin is a member of a family of modular muscle proteins (Labeit *et al.*, 1990) that are composed of multiple repeats, termed class I and class II, which share homology to fibronectin-type III and immunoglobulin-C2-like motifs, respectively (Benian *et al.*, 1989). Within the thick filament, the titin class I and class II motifs are grouped into highly ordered patterns of 11-domain super-repeats. Biochemical evidence from expressed sequences of the titin super-repeat suggests that there are multiple myosin and C-protein binding sites and thus implies that the A-band region of titin participates in the ordered integration of the thick filaments into the sarcomere (Labeit *et al.*, 1992). A feature whose function is unclear as yet is the presence of a serine/threonine protein kinase catalytic domain near the M-line (Labeit *et al.*, 1992), also found in the invertebrate giant muscle protein twitchin from *Caenorhabditis elegans* (Benian *et al.*, 1989). Titin itself is known to be phosphorylated *in vivo* and *in vitro* (Sommerville and Wang, 1987; Tanako-Ohmura *et al.*, 1992).

Electron microscope (EM) pictures of isolated native titin show a long filamentous molecule with a globular structure at one end. Immunohistochemical experiments demonstrate that the end of titin which forms the head is an integral component of the M-line (Fürst *et al.*, 1989a; Nave *et al.*, 1989). Purification and biochemical characterization of titin under native conditions, yielding the head-bearing species, demonstrate the co-purification of two other components of the M-line, the 165 and 190 kDa M-line proteins (Nave *et al.*, 1989). Especially noteworthy is the formation of titin dimers and higher multimers that seem to interact mainly via their respective M-line globular heads. These findings are strongly indicative of intimate protein–protein interactions of titin both with other titin molecules and with integral components of the M-line, especially the 165 and 190 kDa proteins.

The obvious polarity of the titin molecule is reflected functionally in the sequential array of Z-line and M-line regions of the molecule into ordered striated patterns in the nascent sarcomere. Analysis of the sarcomere formation in differentiating mouse somite myotomes with a set of titin monoclonal antibodies mapping near the Z- and M-lines, respectively, demonstrated that titin epitopes near the Z-line are organized into striated patterns earlier in myogenesis than those nearer or within the M-line (Fürst *et al.*, 1989b). This sequential formation of protein–protein interactions along the molecule requires controlled activation of binding sites. Obviously, the M-line region of titin should therefore possess an assembly switch to regulate titin/M-line protein interactions during development.

We have attempted to gain more information on the primary structure of this part of the molecule and to obtain data relevant to its function in sarcomere assembly during muscle development. Since the orientation of titin in the sarcomere has placed the N-terminus at the Z-disc and the C-terminal end of the molecule in the M-line (Labeit *et al.*, 1992), we have extended our titin sequences from those in the A-band recently described (Labeit *et al.*, 1992) to the titin C-terminus, thereby isolating the section of the molecule in the M-line. We find that the motif organization in the titin C-terminal region differs fundamentally from that of A-band titin. Structural and functional characterization of this region of the molecule is likely to help us elucidate the molecular basis of control of M-line assembly.

Results

Molecular cloning and sequencing of the titin C-terminus

To isolate M-line titin, we extended our human cardiac A-band titin contig mapping near the M-line (EMBL data

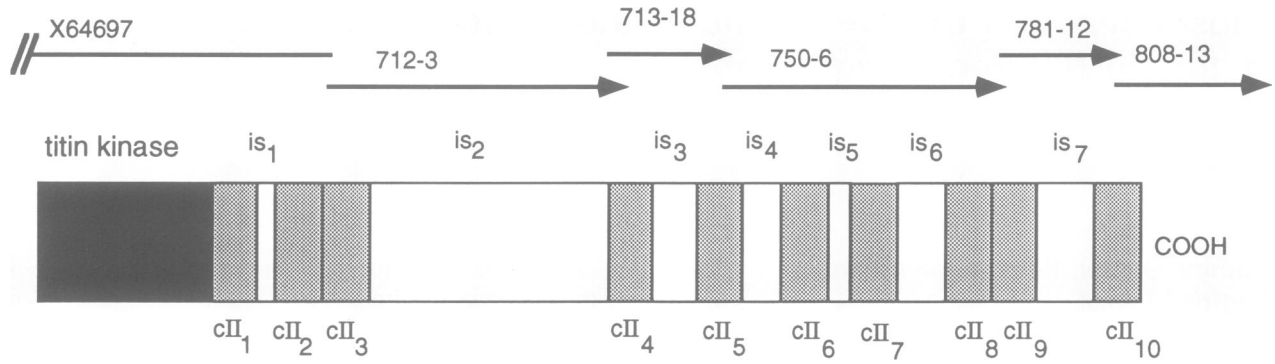

Fig. 1. Overview of the M-line titin cloning and sequencing project. The original 9.3 kb near M-line titin cDNA sequence X64697 (Labeit *et al.*, 1992) was extended up to its 3′ end by walking cycles yielding an additional 5.7 kb titin cDNA (arrows). The complete 15 kb C-terminal titin cDNA contig has been submitted to the EMBL data library (accession no. X69490, human cardiac M-line titin). Below, the domain organization within C-terminal titin is shown. Shaded boxes represent the C-terminal class II domains cII-1 to cII-9, and white boxes the interdomain insertions is1 to is7. The titin kinase domain is shown in black.

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CCGCCTAAAATTGAAGCTCTTCCATCTGATATCAGCATTGATGAAGGCAAAGTTCTAACAGTAGCCTGTGCTTTCACG
P  P  K  I  E  A  L  P  S  D  I  S  I  D  E  G  K  V  L  T  V  A  C  A  F  T

GGTGAGCCTACCCCAGAAGTAACATGGTCCTGTGGTGGAAGAAAAATCCACAGTCAAGAACAGGGGAGGTTCCACATT
G  E  P  T  P  E  V  T  W  S  C  G  G  R  K  I  H  S  Q  E  Q  G  R  F  H  I

GAAAACACAGATGACCTGACAACCCTGATCATCATGGACGTACAGAAACAAGATGGTGGACTTTATACCCTGAGTTTA
E  N  T  D  D  L  T  T  L  I  I  M  D  V  Q  K  Q  D  G  G  L  Y  T  L  S  L

GGGAATGAATTTGGATCTGACTCTGCCACTGTGAATATACATATTCGATCCATTTAA
G  N  E  F  G  S  D  S  A  T  V  N  I  H  I  R  S  I  *13,951

GAGGGCCTGTGCCTTATACTCTACACTCATTCTTAACTTTTCGCAAACGTTTCACACGGACTAATCTTTCTGAACTGT
AAATATTTAAAGAAAAAAAGTAGTTTTGTATCAACCTAAATGAGTCAAAGTTCAAAAATATTCATTTCAATCTTTTCA
TAATTGTTGACCTAAGAATATAATACATTTGCTAGTGACATGTACATACTGTATATAGCCGGATTAACGGTTATAAAG
TTTTGTACCATTTATTTTATGACATTTTACAATGTAAGTTTTGAAACTAACTGTTGGTAGGAGAAAGTTTCTTATGGA
ACGAATACCCTGCTCAACATTTAATCAATCTTTGTGCCTCAACATACTGTTGATGTCTAAGTATGCCTCAGTGGGTTG
AGAAAATCCCCATTGAAGATGTCCTGTCCACCTAAAAGAGAATGATGCTGTGCATATCACTTGATATGTGCACCAATA
CCTACTGAATCAGAAATGTAAGGCATTGGTGATGTTTGCATTTACCCTCCTGTAAGCAACACTTTAACGTCTTACATT
TTCTCTGATGATGTCACACAAAATTATCATGACAAATATTACCAGAGCAAAGTGTAACGGCCAACACTTTGTTCGCTC
ATTTTACGCTGTCTCTGACATAAGGAGTGCCTGAATAGCTTGGAAAAGTAACATCTCCTGGCCATCCCTTCATTTAAC
CAAGCTATTCAAGTATTCCTATGCCAGAGCAGTGCCAACTCTTGGAGGTCCCAGAGTGCAGCCAATGCCTTTTGTGGT
AGTTCTAAATTTTAATTGCACCTGAAAAACCTGGGCACCTAAGCAATGAGCCACAGCAAAAAGTAAAGAACAACAACA
AAATAAAGCTGTTGTTAAATTTTAAACAATATTACTAATTGCCCAAAATGTCAATTTGATGTAGTTCTTTTCATGCAA
GTATAAATTCAATTGTTAGTTATAATTGTTGGACCTCCTTGAGATAGTAACAACAAAATAAAGCAAGCTATCTGCACC
TCAAAAAAAAAAAAAAAAA • 14,970
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Fig. 2. The titin 3′ end. The last 1.3 kb of the 3′ end human cardiac titin message are shown and the translation of the last immunoglobulin-C2 domain up to the C-terminus is given. The 1 kb 3′ non-translated region includes two AATAAA polyadenylation signals (bold) raising the possibility of differential polyadenylation in different muscle tissues.

library accession no. X64697) from the 3′ end by cDNA extension methods. Five extensions yielded a total of 5.7 kb cDNA (Figure 1). Thereafter, no further extending fragments could be isolated from the human cardiac library. For the last extension, eight independent clones were sequenced, all of which were found to terminate at their 3′ ends within the same 30 nucleotides. Two of the eight clones possessed a 3′ poly(dA) tail confirming that the end of the titin cDNA had been reached. The assembled M-line titin contig was completely sequenced and merged with the 9.3 kb cDNA sequence of X64697, and the resulting 15 000 bp cDNA sequence has been submitted to the EMBL data library (human cardiac M-line titin, accession no. X69490). This contig encodes a single open reading frame terminating at position 13 951 with a TAA stop codon (Figure 2). The 3′ non-coding region extends to position 14 970 and contains two AATAAA polyadenylation signals (positions 14 813 and 14 946) raising the possibility of differential polyadenylation in different muscle types.

Domain structure of M-line titin

The peptide predicted by the M-line contig was analysed by computer consensus screening as well as by visual inspection for the presence of class I and class II motifs typical for A-band titin. A total of 10 class II motifs are present in the titin region 3′ of the kinase domain (Figure 3). NMR analysis of a single expressed M-line titin domain (cII-5) shows compact β-sheet folds similar to class II domains from A-band titin (data not shown) and myosin light chain kinase (Holden *et al.*, 1992) confirming that M-line class II repeats share structural similarity to the class II domain family. However, these M-line class II repeats appear to be a more diverse family than those within the A-band region of titin (Labeit *et al.*, 1990). In fact, conservation is restricted to the aromatic and hydrophobic side chains and their conserved spacing which are crucial to the folding of the β-barrel in intracellular immunoglobulin-C2 domains (Holden *et al.*, 1992).

In C-terminal titin, the class II repeats are separated by

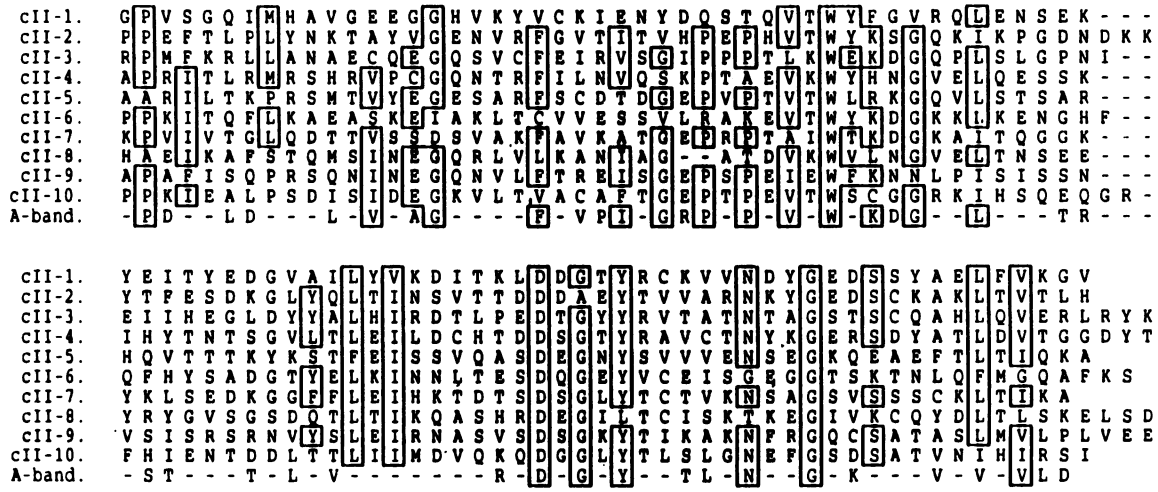

Fig. 3. Sequence alignment of class II domains in C-terminal titin. Residues conserved in at least six of the sequences are boxed. Comparison with the consensus sequence of A-band members of the same motif family (A-band) shows that the M-line family of type II domains shares less consensus than the A-band family. The nomenclature of the domains is as in Figure 1.

seven interdomain insertions which range from 30 to 490 residues in length (Figure 2). Data library searches with the seven interdomain insertion sequences is1−is7 do not detect significant homologies to other proteins in the data bank with the exception of is4. The sequence of is4 encodes four repeated KSP motifs in the N-terminal region (Figure 4) similar to KSP motifs found in neurofilament-H and -M subunits (Myers *et al.*, 1987; Lees *et al.*, 1988). The insertions 3, 5 and 7 are likely to be flexible linkers between the flanking class II domains, whereas especially the complex structure of is2, predicted to be mainly of α-helical fold, gives no obvious clues to its function.

In vitro phosphorylation assays with the titin KSP motif

To investigate whether the KSP sequences in is4 are potential phosphorylation sites in muscle, constructs containing the is4 sequence together with either the flanking N- or C-terminal class II domains cII-5 and cII-6, respectively, were expressed in *Escherichia coli* and purified as described by LeGrice and Grüninger-Leitch (1990). The purified fragment cII-5/is4 and is4/cII-6 was incubated with cell extracts from neonatal and adult mouse (BALB/c) heart and psoas myocytes in the presence of [γ-^{32}P]ATP. Both fragments were found to be phosphorylated efficiently by neonatal muscle tissue extracts, while extracts prepared from adult muscles showed significantly lower kinase activities (Figure 5). The synthetic peptide P4 (sequence in Figure 4) representing the four titin KSP repeats from is4 was similarly phosphorylated. In contrast, neither the expressed KSP-flanking domains cII-5 and cII-6 nor A-band titin class II domains were phosphorylated by the kinase activity in neonatal muscle (Figure 6a). We conclude from these experiments that *in vitro*, phosphorylation of titin in this region is restricted to is4 and is not modulated by flanking titin sequences. Phosphoamino acid analysis shows that serine residues are the target of phosphorylation in is4. No phosphotyrosine could be detected (Figure 6b).

To gain information on the accessibility of the KSP motifs within the is4 repeat to the KSP kinase activity and the stoichiometry of the phosphorylation, an ordered series of serine/alanine exchange mutants was created in the KSP

Fig. 4. The titin KSP repeat. Four copies of the tetrapeptide VKSP are present in the is4 sequence (underlined). The 28 residues shown are contained in the synthetic peptide P4 used in the *in vitro* phosphorylation studies.

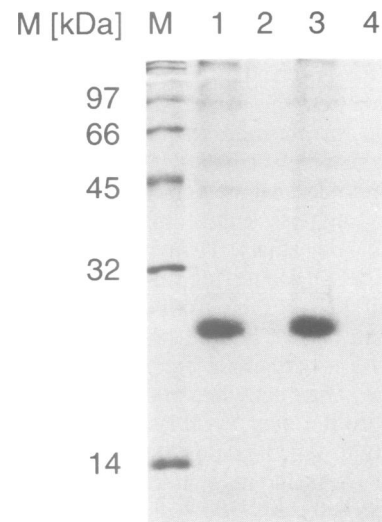

Fig. 5. Phosphorylation of titin KSP repeats in neonatal and adult muscle. Autoradiograph of an SDS−polyacrylamide gel of the *in vitro* phosphorylation of the titin construct cII-5/is4. Myocyte extracts were from neonatal heart and psoas (lanes 1 and 3, respectively) and corresponding tissues from adult animals (lanes 2 and 4). M = marker proteins.

repeat in the construct cII-5/is4, where cII-5/is4 was chosen for its better solubility. The mutants characterized in more detail represented four triple Ser/Ala and a total Ser/Ala exchange (Figure 7a).

All triple serine to alanine exchange mutants, S1 to S4, could be phosphorylated at levels above that observed for

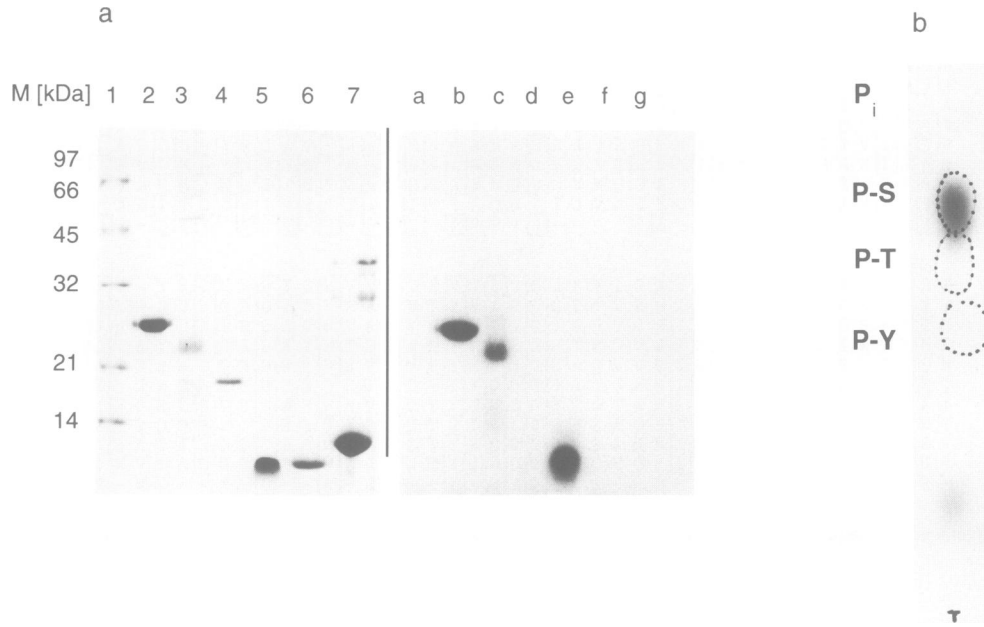

Fig. 6. (**a**) Localization of the C-terminal titin phosphorylation site in insertion is4. Expressed titin fragments around the insertion is4 were assayed for phosphorylation by neonatal myocyte extracts and reactions separated by SDS−PAGE. Left panel: Coomassie stained gel with: lane 2, cII-5/is4; lane 3, is4/cII-6; lane 4, the N-terminal flanking cII-5 alone; lane 5, is4 without flanking sequences. Lanes 6 and 7 show assays with two class II domains from A-band titin. Lanes a−g: autoradiograph of the corresponding lanes 1−7. 0.2 μl of cytosolic fractions from neonatal mouse myocytes were used in these assays. Lanes 1 and a: marker proteins (Bio-Rad). (**b**) Phosphoamino acid analysis of cII-5/is4. Autoradiogram of the TLC phosphoamino acid analysis of cII-5/is4; the positions of the three marker amino acids visualized by ninhydrin are indicated.

the complete exchange mutant S/A1−4 but below that of the wild-type. Residual phosphate incorporation observed for S/A1−4 is likely to be localized in an additional SP motif localized in the N-terminal region of is4.

We conclude from this experiment that all four KSP motifs are accessible for phosphorylation (Figure 7b).

The finding that high activities of a titin KSP motif-directed serine protein kinase can be detected in neonatal but not in adult tissues, suggests a developmental regulation of the muscle KSP kinase activity. To investigate the activity pattern of the titin KSP kinase during differentiation of myoblasts in more detail, cell extracts from a conditionally myogenic mouse cell line (Jat *et al.*, 1991) were assayed with expressed cII-5/is4 fragment before and after commitment to myogenesis. High activities of the titin KSP kinase were detected in proliferating myoblasts and in differentiating cells. Activity dropped gradually during differentiation of the respective clone into myotubes (Figure 8). Fractionation of cell extracts detected ~60% of the kinase activity in the myofibrillar, particulate fraction of the cells. To detect titin KSP phosphatases, the KSP motif was labelled with ^{32}P, purified as above and incubated with the same cell extracts. Low phosphatase levels showing no significant differences during differentiation were observed (data not shown).

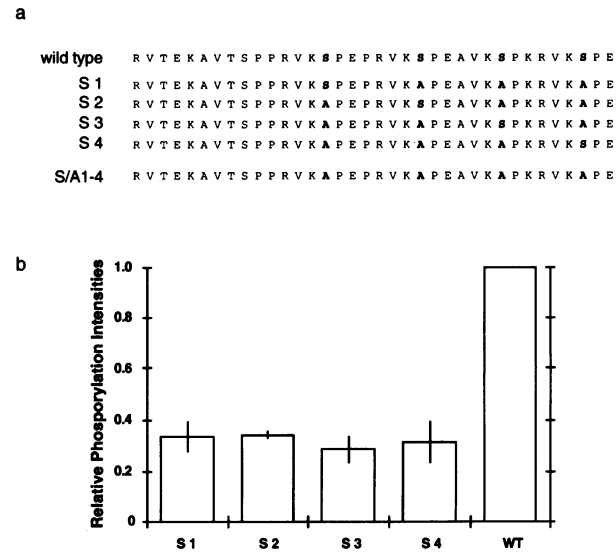

Fig. 7. Site-directed mutagenesis in the titin KSP repeat region. (**a**) The five mutants further characterized in *in vitro* phosphorylation assays are shown, the sites of serine to alanine exchange are printed in bold. (**b**) Relative phosphorylation intensities of the four triple serine to alanine mutants in is4 and the wild-type (WT) as described in Materials and methods. Means of three independent experiments are shown, with bars representing the standard deviation.

Characterization of the titin KSP kinase

To characterize the kinase activity involved in differentiation-regulated titin phosphorylation, screening experiments using purified kinases and specific inhibitors were performed. Purified cAMP-dependent protein kinase catalytic subunit from porcine brain (Sigma) did not phosphorylate the KSP motif. Inhibition assays with the kinase inhibitors and cofactor-antagonists as described in Materials and methods detected no inhibition of the kinase activity by these compounds. Therefore, the activity of titin KSP kinase in myocyte extracts is unrelated to cAMP-dependent kinase, protein kinase C or calmodulin-dependent kinase.

The similarity of the titin KSP motif to the multi-phosphorylation repeat of neurofilaments, a known substrate

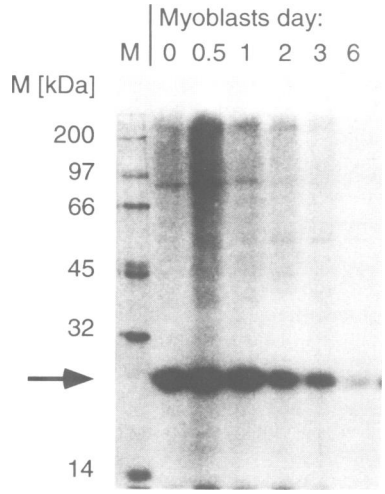

Fig. 8. Phosphorylation of titin KSP repeats in differentiating myoblasts. *In vitro* phosphorylation of the expressed titin construct cII-5/is4 by cytosolic fractions from the mouse myoblasts cell clone C30 at various differentiation stages as indicated. M: marker proteins.

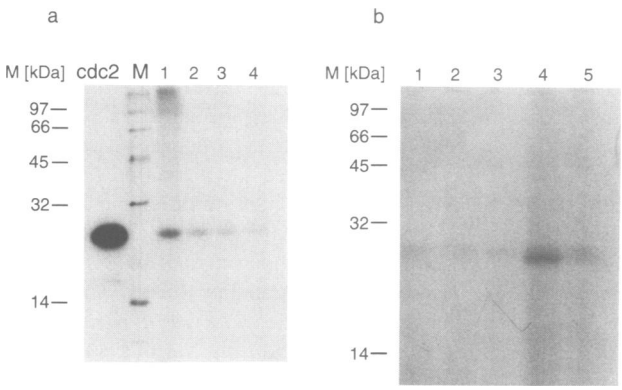

Fig. 9. (**a**) Depletion of kinase activity in myocyte extracts by immunoadsorption. *In vitro* phosphorylation of cII-5/is4 by neonatal mouse cardiac myocyte cytosol depleted by anti-cdc2 kinase antiserum against human cdc2 kinase C-terminus (G6) and *S.pombe* cdc2 kinase (G8). Lane 1, myocyte cytosol control, immunoadsorbed with pre-immune serum; lane 2, control, 5 mM EDTA; lane 3, immunodepleted by G8 serum; lane 4, immunodepleted by G6 sera; cdc2, control with cdc2 kinase; M, marker proteins.
(**b**) Phosphorylation of cII-5/is4 by bead-adsorbed kinase. Lane 1, phosphorylation assays containing pre-immune serum; lane 2, anti-MAP kinase serum-loaded beads, pre-incubated with myocyte extracts as described in Materials and methods; lane 3, phosphorylation assays containing BSA-conjugated beads; lane 4, p13 beads; and lane 5, anti-cdc2 kinase G6 serum-loaded beads, pre-incubated with myocyte extracts as above. M: marker proteins.

of cdc2 kinase, and the obvious differentiation dependence of the kinase activity led us to test the titin KSP motif as a substrate for cdc2 kinase. Using purified human cdc2 kinase/cyclin B complex from HeLa cells (a gift from G. Draetta, EMBL, Heidelberg), specific phosphate incorporation was observed indistinguishable from that obtained from myocyte extracts from neonatal mouse or a myoblast cell line. Depletion of cytosolic fractions of neonatal mouse myocytes using rabbit polyclonal antibodies against both the *Schizosaccharomyces pombe* (full-length, G8) and the human cdc2 kinase (C-terminus, G6) resulted in clearly decreased kinase activities using either the cII-5/is4 fragment (Figure 9a) or the P4 substrate. Depletion was more pronounced when immunoadsorption was carried out with the G6 antiserum to the human kinase.

When cytosolic cell fractions were incubated with $p13^{suc1}$ beads, a specific ligand of cdc2 kinases (Draetta *et al.*, 1989), washed and subsequently used in *in vitro* phosphorylation assays, specifically bound KSP-phosphorylating kinase activity could be detected (Figure 9b). In contrast, immunodepletion of myocyte extracts by a polyclonal serum against MAP kinase (provided by F.Rossi, EMBL, Heidelberg), resulted in no detectable reduction of kinase activity in the depleted fraction and showed no immunoadsorbed activity (Figure 9b). MAP kinase therefore does not contribute significantly to KSP phosphorylation in the system investigated.

We suggest that the major activity of titin KSP kinase in differentiating myocytes is identical to that of cdc2 kinase.

Discussion

The sequence of the titin C-terminal region presented here displays a remarkable degree of complexity compared with the A-band sequences of the same molecule (Labeit *et al.*, 1992). The regular molecular architecture of A-band titin is replaced by a complex array of 10 immunoglobulin-C2 repeats separated by seven insertions of unique sequence. The interdomain insertions range between 30 and 490 residues in length and are also heterogeneous in predicted secondary structures and charges, with theoretical pIs ranging from 4.5 to 11. Therefore, a clear change of motif organization in the molecule is observed at the transition from A-band to M-line sequences of titin. This supports the idea that local titin primary structure correlates with local sarcomere structure. At the level of single domains, motifs 5′ of the kinase domain are closely related in sequence and fall into the 11 subgroups of the A-band titin super-repeat (Higgins *et al.*, 1993). In contrast, the class II repeats C-terminal of the kinase domain share less sequence homology. Therefore, the titin kinase domain separates two titin regions with different sequence organization. It will be of great interest to determine the precise localization of the titin kinase domain in the sarcomere, and whether its position corresponds to the A-band/M-line transition.

Phosphorylation studies of the whole titin molecule have shown that titin is phosphorylated *in vivo* and *in vitro*. Injection of [^{32}P]inorganic phosphate into frogs leads to incorporation of the phosphate label into titin serine/threonine residues within a few days (Sommerville and Wang, 1987). Similarly, the 800 kDa projectin (a mini-titin) from *Drosophila melanogaster* is labelled when flies are grown in the presence of [^{32}P]inorganic phosphate (Maroto *et al.*, 1992). *In vitro*, β-connectin, a 2.2 MDa proteolytic A-band titin fragment, has been found to autophosphorylate (Tanako-Ohmura *et al.*, 1992). These *in vivo* and *in vitro* studies suggested that relatively few phosphate residues are incorporated into one titin molecule. Since the 50 kb of titin cDNA sequence data determined at present do not predict obvious phosphorylation sites with the exception of the KSP motifs in is4, and since all four serine residues in site-directed mutants are accessible to phosphorylation, we propose that the titin KSP motif is one major site of *in vivo* phosphorylation of titin.

KSP repeats have previously been found in higher redundancy in the multiphosphorylation repeat (mpr) of the C-terminal tail domain of neurofilaments (Myers *et al.*, 1987;

Lees *et al.*, 1988). In neuronal tissue, KSP phosphorylation of neurofilaments is under developmental control and, although the precise function and structural implications of their multiphosphorylation repeat are not fully understood, there is experimental evidence to support the idea that post-translational translocation or depolymerization of neurofilaments is controlled by phosphorylation (Nixon and Lewis, 1986; Shea *et al.*, 1990; Nixon and Sihag, 1991). Hisanago and co-workers (1991) demonstrated that phosphorylation of the NF 200 mpr by cdc2 kinase abolishes interaction with microtubules. The underlying general principle of phosphorylation-regulated inhibition of protein−protein interactions in the cytoskeleton is shared by other intermediate-type filament proteins such as lamin (Peter *et al.*, 1991) and vimentin (Inagaki *et al.*, 1987), which depolymerize after phosphorylation by cdc2 kinase. Similarly, the interactions of ankyrin−spectrin (Lu *et al.*, 1985), MAP−actin or MAP−tubulin are inhibited by phosphorylation (Jameson *et al.*, 1980; Nishida *et al.*, 1981; Selden and Pollard, 1983).

Do the KSP repeats serve analogous functions in neurofilaments and titin, molecules otherwise unrelated? The finding that the titin KSP kinase activity can be depleted from myocyte extracts by antibodies or specific ligands to cdc2 kinase suggests that the titin KSP motifs participate in signal transduction pathways controlled by cdc2 kinase, as has been suggested for the neurofilament KSP motifs (Hisanaga *et al.*, 1991). KSP motifs may therefore have emerged convergently in different functional contexts to sense changing levels of SP-directed protein kinase activities during differentiation.

To test in more detail whether the levels of titin KSP kinase activities are indeed related to myofibrillar assembly, we made use of a conditionally myogenic mouse cell line (Jat *et al.*, 1991). This cell line proliferates under permissive conditions, but undergoes myogenic differentiation upon temperature shift and γ-interferon withdrawal. The expression and assembly of contractile proteins into ordered sarcomeres and the formation of spontaneously twitching myotubes after induction suggest that this system can be regarded as a model for myogenic differentiation. High levels of titin KSP kinase activities are found before and after induction to myogenesis. A decline to almost undetectable levels is observed by days 5−7 (Figure 8), when the formation of ordered sarcomeres is completed as judged by EM. In contrast, the level of titin KSP phosphatases was found to be low, but approximately constant in all developmental stages (data not shown). Similarly, cell extracts prepared from neonatal mouse muscle contain activities of titin KSP kinase roughly two orders of magnitude higher than corresponding cell extracts obtained from adult animals. We propose that titin synthesized early in myogenesis is phosphorylated at its KSP serines but would later be converted to its dephosphorylated form during differentiation to mature sarcomeres.

What role does this process play in myogenic differentiation? Epitopes of monoclonal anti-titin antibodies near the Z-line form into a striated pattern earlier in myogenesis than those closer to the M-line (Fürst *et al.*, 1989b). This pattern is consistent with the splitting of 'singlet' to 'doublet' titin bands observed by other authors using mono- or polyclonal antibodies labelling only A/I junctions in cardiac (Tokayasu and Maher, 1987; Wang *et al.*, 1988; Handel *et al.*, 1991) or skeletal muscle (Handel *et al.*, 1989). Spatially separated attachment sites must therefore be sequentially activated proceeding from the Z- to M-line to coordinate titin integration into the nascent sarcomere. Controlled phosphate-turnover on the KSP repeats in the M-line region of titin could plausibly explain how M-line assembly is coordinated during myogenesis. The presence of such assembly switches would control how the ~30 000 residue protein participates in myofibrillar assembly in a coordinated way even though its synthesis must extend over a significant period of time, and also explain why nascent titin polypeptide chains leaving the ribosomes do not start assembly prematurely.

Recently, Vinkemeier *et al.* (1993) demonstrated the extension of the titin C-terminus into the centre of the sarcomeric M-line and the specific binding of the two titin-associated M-line proteins of 165 and 190 kDa. Consistent with this finding, co-purification of M-line proteins and titin has been observed previously (Nave *et al.*, 1989). We suggest that the differentiation-dependent phosphorylation of the four titin KSPs, flanked by two immunoglobulin-C2 binding motifs, introduces structural alterations relevant to myogenesis and rearrangement of the cytoskeletal lattice, possibly regulating the interaction of titin with the 165 and 190 kDa M-line proteins. Interestingly, the 165 and 190 kDa M-line proteins also consist of IgG-C2 domains (Noguchi *et al.*, 1992; Vinkemeier *et al.*, 1993). Clearly, biochemical studies characterizing the ligands binding to the flanking domains of the KSP motifs and the more detailed investigation of titin KSP kinase activity from myocytes undergoing structural rearrangement are required to elucidate the signal transduction pathways in which the titin KSP motifs participate. Sadoshima and Izumo (1993) recently reported the activation of MAP kinase, an SP-directed protein serine/threonine kinase (Gonzalez *et al.*, 1991), in mechanically stretched cardiac myocytes. At present, we cannot rule out the possibility that *in vivo*, titin may also be phosphorylated by kinases other than cdc2. The titin KSP repeat may therefore sense SP-directed kinases in an integrative fashion during various stages of muscle development.

The precise function of the other C-terminal titin domains and interdomain insertions is at present unclear. Since the total mass of the titin motifs C-terminal to the kinase domain is 210 kDa, the M-line section of titin could be of considerable length. Therefore, the immunoglobulin-C2 domains of C-terminal titin could provide binding sites for other components of the M-line than only the M-line proteins, as does A-band titin for a number of A-band proteins (Labeit *et al.*, 1992; Soteriou *et al.*, 1993). The significant potential length of M-line titin also raises the possibility that titin molecules coming from each half-sarcomere overlap and interact as antiparallel dimers. These hypotheses can be tested by functionally characterizing the interactions of over-expressed M-line titin and M-line protein fragments. In the long term, the functional and structural characterization of single domains from the different M-line components should provide us with detailed knowledge of the M-line architecture and the developmental control of its assembly.

Materials and methods

Molecular cloning and sequencing of titin

For C-terminal extensions, probes were prepared from the near M-line titin sequences (Labeit *et al.*, 1992) by randomly labelling with ^{32}P (Feinberg and Vogelstein, 1983) and used to screen a human heart cDNA library (Stratagene #936208). Twenty-four primary positives were picked and subjected to anchored PCR (Rasmussen *et al.*, 1989). For the specific primer, the sequence CAGGTGACAATGACAAG mapping 418 bp 5′ from the

current titin sequence 3′ end (EMBL data library accession no. X64697) was used, and sets of *Eco*RI flanking Bluescript sequences were used to amplify λZAP phage cDNA inserts from the other end. The longest cDNA fragment was subcloned into M13 (Yanisch-Perron *et al.*, 1985) and the sequence determined (Sanger *et al.*, 1977). Extending fragment DNA was again randomly labelled and used to re-screen the human heart cDNA library to isolate 24 further 3′ extending titin cDNAs. From the derived sequence, a new C-terminal extension primer was synthesized, and extension of cDNA inserts amplified by PCR as above. A total of five extension cycles yielding 5.7 kb were performed.

Sequence analysis and interpretation

Sequence editing, fragment assembly, prediction of ORFs, dotplot self-comparison and data library searches were performed with the UWGCG software package (Devereux *et al.*, 1984). The single predicted ORF in the human AB5 contig was used to translate the encoded peptide, and the C-terminal segments not consisting of class II modules were used to search the Swiss Prot data library (release 21) with Wordsearch. No significant homologies were found except for the KSP repeats. Alignment of M-line class II motifs was performed by eye, and conserved positions were boxed with Prettyplot (P.Rice, EMBL).

Mutagenesis

Serine to alanine mutations were introduced into the is4 KSP repeat by PCR amplification of the cII-5/is4 fragment with 3′ mismatch oligonucleotides introducing site-directed exchanges of the respective serine codon, TCT, to the alanine codon, GCT, in all four KSP positions. The amplified fragments were subcloned into M13mp18 and individual subclones were analysed by sequencing. Clones representing the desired Ser/Ala exchanges were subcloned into the expression system described below.

Expression of titin sequences in E.coli

Titin sequences in the KSP repeat region were isolated by PCR (Saiki *et al.*, 1985) using the original λZAP phage isolates as templates. Obtained fragments were subcloned into the pET8c vector (Studier *et al.*, 1990) and fused N-terminally with an oligonucleotide linker encoding a His_6 tag sequence. After induction of BL21 cells (Studier and Moffat, 1991) with 0.5 mM IPTG for 4 h, the harvested cell pellet was sonicated in 50 mM sodium phosphate pH 8.0, 500 mM NaCl, 0.2% Tween 20. After centrifugation at 25 000 *g*, soluble expressed products from the supernatant were purified by metal chelate affinity chromatography on Ni^{2+}-NTA agarose (Qiagen) essentially as described by LeGrice and Grüninger-Leitch (1990). Aliquots of purified products were assayed by circular dichroism, and obtained spectra showed predominantly β-sheet structures.

Preparation of cell extracts from muscle tissues and myotubes

Heart left ventricle and psoas tissue from adult and neonatal (day 0) BALB/c mice were dissected from freshly killed animals in ice. Tissues were cut into small fragments, washed briefly in PBS, shock-frozen in liquid nitrogen and pulverized in a pre-cooled mortar under nitrogen. Homogenized tissue (100–150 mg) was immediately transferred into ice-cold lysis buffer (25 mM HEPES pH 7.2, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 2 μg/ml leupeptin, 1 μM pepstatin, 0.5 mM PMSF, 0.02% Triton X-100). Cytosolic and myofibrillar particulate fractions were separated by centrifugation at 1500 *g*. Cell fractions were adjusted to 1 mg/ml total protein (modified Lowry assay, Sigma) by appropriate dilution in lysis buffer.

For cultured myoblasts, 10^6 cells from various myoblastic differentiation stages of the myoblast cell line, clone C30 (Jat *et al.*, 1991; a gift from Michelle Peckham, King's College, London) were harvested by pipetting off, washed in PBS and lysed in 100 μl lysis buffer by brief sonication with a Branson sonifier microtip. The particulate fractions containing nuclei and myofibrillar proteins were spun down as above and resuspended in 100 μl lysis buffer. Extracts were not adjusted to protein content but rather to cell number being 10^7 cells/ml buffer final.

Phosphorylation assay

Cytosolic fractions and myofibril-enriched particulate fractions were added to a phosphorylation assay using a final concentration of 0.1 μg/ml of the expressed substrate proteins or synthetic peptide, and cell lysate (2–4 μl) in 20 μl assay buffer (25 mM HEPES pH 7.2, 100 mM KCL, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, 0.2 mM ATP and 1 μCi [γ-^{32}P]ATP, 3000 Ci/mM). Assays were started by addition of a 10 × ATP mix and incubated at 30°C for 15 min. Reactions were stopped by addition of 10 μl of sample buffer (Laemmli, 1970) and heated to 95°C for 1 min. Analysis of phosphorylated proteins was performed on 14% polyacrylamide gels as described by Laemmli (1970). Gels were autoradiographed at −80°C with intensifying screens for 12–24 h.

For quantitative assays, bands from individual assays were cut out of polyacrylamide gels, homogenized and measured by liquid scintillation in Ready Safe (Beckman) in a Beckman LS 8100 liquid scintillation counter against the complete Ser to Ala mutant as background. Means of the incorporated counts were calculated from three independent experiments.

For the inhibitor screening assays, trifluoperazine (Sigma), an inhibitor of calcium/calmodulin-dependent kinases (Levin and Weiss, 1978), was added to phosphorylation assays at concentrations up to 50 μM. The protein kinase C-inhibitory peptide 19–36 (RFARKGALRQKNVHEVKN, Boehringer Mannheim) was added at 50 μM concentrations, as well as the inhibitory peptide of the calcium/calmodulin-dependent protein kinase II (LKKFNARRKLKGAILTTMLA, Sigma).

Phosphoamino acid analysis

Phosphoamino acid analysis of radiolabelled protein was carried out as described by Boyle *et al.* (1991). 10 μg of cII-5/is4 from phosphorylation assays as above was separated by metal chelate affinity chromatography and hydrolysed at 110°C in 5.7 M HCl for 2.5 h. The hydrolysate was lyophilized and dissolved in 20 μl of water, and 4 μl was applied onto Kodak TLC cellulose sheets. Phosphoamino acids were separated by electrophoresis in a pyridine/acetic acid/water system (4:40:756 by volume) pH 3.5, at 400 V for 50 min. Standard phosphoamino acids (Sigma) were visualized by ninhydrin and marked. Labelled phosphoamino acids were then visualized by exposure to Kodak X-AR 5 film, marked and identified by comparison with the standards.

Depletion of myocyte extracts by adsorption to anti-cdc2 kinase antisera and p13 beads

IgGs from the rabbit polyclonal sera G8 (raised against *S.pombe* cdc2 kinase full-length) and the serum G6 (raised against *Homo sapiens* cdc2 kinase C-terminal region, G6 and G8 serum provided by G.Draetta, EMBL, Heidelberg) were absorbed to protein A–Sepharose Cl-4B (Pharmacia). Beads were subsequently blocked with 25% fetal calf serum (FCS) and washed with lysis buffer. 5 μl of the packed beads in lysis buffer were then incubated with 20 μl of the cytosolic fraction from neonatal mouse heart. Supernatants were taken off after 30 min incubation at 4°C and the adsorption was repeated three times. Control samples were treated in the same way, with beads adsorbed with preimmune serum. 2 μl of the depleted extracts were used in phosphorylation assays as above. Similarly, rabbit anti-MAP kinase antibodies (provided by F.Rossi, EMBL, Heidelberg) were attached to protein A beads.

Bacterially expressed $p13^{suc1}$ was covalently coupled to CnBr-activated Sepharose at 1 mg/ml (provided by M.J.Marcote, EMBL). Beads were blocked in 25% FCS and washed as above, and 10 μl packed beads were incubated with 20 μl cytosolic myocyte fractions for 30 min at 4°C. Beads were washed vigorously and 5 μl packed beads were added to phosphorylation assays as described above. Control beads were coupled to bovine serum albumin.

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