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A functional knock-out of titin results in defective myofibril assembly

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SUMMARY

Titin, also called connectin, is a giant muscle protein that spans the distance from the sarcomeric Z-disc to the M-band. Titin is thought to direct the assembly of sarcomeres and to maintain sarcomeric integrity by interacting with numerous sarcomeric proteins and providing a mechanical linkage. Since severe defects of such an important molecule are likely to result in embryonic lethality, a cell culture model should offer the best practicable tool to probe the cellular functions of titin. The myofibroblast cell line BHK-21/C13 was described to assemble myofibrils in culture. We have now characterized the sub-line BHK-21-Bi, which bears a small deletion within the *titin* gene. RNA analysis revealed that in this mutant cell line only a small internal portion of the *titin* mRNA is deleted. However, western blots, immunofluorescence microscopy and immunoprecipitation experiments showed that only the N-terminal, approx. 100 kDa central Z-disc portion of the 3 MDa titin protein is expressed, due to the homozygous deletion in the gene. Most importantly, in BHK-21-Bi cells the formation of thick myosin filaments and the assembly of myofibrils are impaired, although sarcomeric proteins are expressed. Lack of thick filament formation and of ordered actin-myosin arrays was confirmed by electron microscopy. Myogenisation induced by transfection with MyoD yielded myofibrils only in myotubes formed from wild type and not from mutant cells, ruling out that a principal failure in myogenic commitment of the BHK-21-Bi cells might cause the observed effects. These experiments provide the first direct evidence for the crucial role of titin in both thick filament formation as a molecular ruler and in the coordination of myofibrillogenesis.

Key words: Titin/connectin, BHK-21C/13 cell, Null mutation, Myofibrillogenesis, Myofibroblast, Myosin assembly

INTRODUCTION

Titin (also called connectin) is a giant protein, present in all vertebrate cross-striated muscle cells, which spans the distance from the sarcomeric Z-disc to the M-band (Maruyama, 1976; Wang et al., 1979; Fürst et al., 1988). Not only is titin a major structural myofibrillar protein (comprising approximately 10% of the total myofibrillar protein; see Wang et al., 1979; Trinick et al., 1984), but it was also shown to interact with numerous sarcomere proteins (Fig. 1A), such as α-actinin (Sorimachi et al., 1997; Ohtsuka et al., 1997; Young et al., 1998), telethonin (Mues et al., 1998; Mayans et al., 1998; Gregorio et al., 1998), C-protein (also called MyBP-C; Fürst et al., 1992; Soteriou et al., 1993; Weber et al., 1993; Koretz et al., 1993; Freiburg and Gautel, 1996), myomesin (Nave et al., 1989) and M-protein (Nave et al., 1989). The fact that the domain organisation of titin mirrors several fundamental features of sarcomere structure served as a basis for the 'molecular ruler' concept of titin function (Whiting et al., 1989; Trinick, 1994). Thus, titin was suggested to direct the assembly of thick filaments and to integrate thick and thin filament assemblies into contractile myofibrils.

All current models of myofibrillogenesis imply important roles for titin at several stages of myofibrillogenesis. During early stages of this process, titin is colocalized with α-actinin in the Z-bodies of nascent myofibrils, and titin is thought to be responsible for the anchorage of α-actinin (Schultheiss et al., 1990; Rhee et al., 1994; Turnacioglu et al., 1996). These nascent myofibrils align, and fuse with adjacent nascent myofibrils to form solid Z-discs of mature myofibrils (Dabiri et al., 1997). Again, titin was suggested to play a key role in this lateral association (Dabiri et al., 1997). At the same time, the spacing between the α-actinin containing Z-bodies gradually increases from less than 1 μm in premyofibrils and nascent myofibrils to up to more than 2 μm in mature myofibrils. Titin epitopes that initially were colocalized in Z-bodies are pulled apart during this process (van der Loop et al., 1996), indicating that the stretching of the titin molecule, and thus the exposure of the binding sites for other myofibrillar proteins, is essential for the ultimate assembly of sarcomeres. Recently, it was shown that the exposure of titin's M-band epitopes coincides with the assembly of myomesin in M-bands (van der Ven and Fürst, 1997) and that mature A-bands only integrate into sarcomeres after the assembly of the cytoskeletal scaffold, consisting of Z-discs, titin and M-bands (Ehler et al., 1999; van der Ven et al., 1999). All these observations support the thesis that titin is a molecular ruler during myofibrillogenesis.

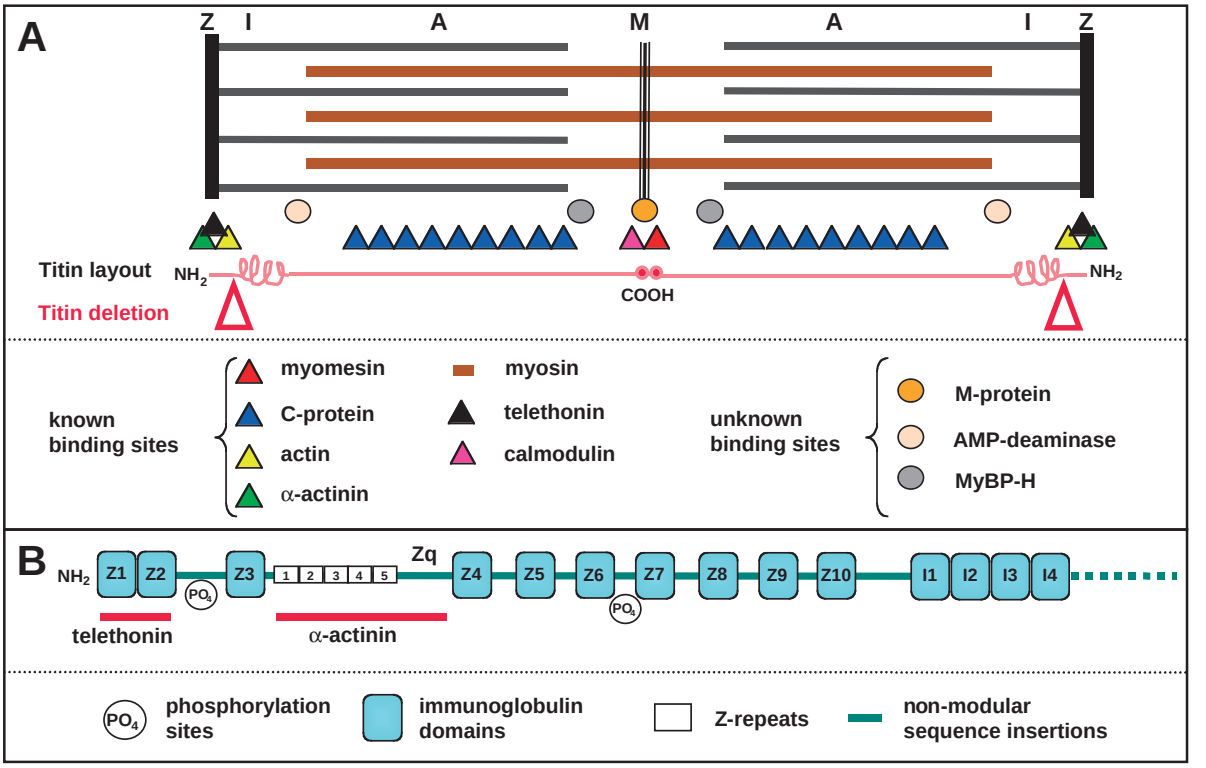

Fig. 1. Schematic representation of the structure of the sarcomere, the organisation of titin and its ligands, and the molecular layout of the Z-disc portion of titin. (A) A sketch of a sarcomere including the organisation of thick and thin filaments. The letters at the top refer to Z-discs (Z), I-bands (I), A-bands (A) and the M-band (M). The layout of titin and the location of titin-binding proteins are indicated below this scheme, with the protein symbols explained below. An arrowhead marks the position of the deletion in the *titin* gene in BHK-21-Bi cells. (B) Schematic depiction of the molecular layout of titin in the Z-disc region. The positions of titin's binding sites for telethonin and α-actinin are given.

If titin is indeed of such importance for muscle function, it will probably be difficult to achieve proof of this directly by using the knock-out technology in the mouse, since this can be expected to result in embryonic lethality. In line with this assumption, no titin-linked diseases have yet been confirmed. Therefore, a cell culture model might offer the best practicable in vivo tool to investigate the function of titin. Various aspects of the biology of muscle have been dealt with successfully using cultured muscle cells (Emerson and Sweeney, 1997). More recently, the myofibroblast cell line BHK-21/C13 was shown to express sarcomeric proteins (Schaart et al., 1991; Mayer and Leinwand, 1997) and even to assemble myofibrillar structures under suitable culture conditions (van der Ven and Fürst, 1998). At the same time, the subline BHK-21-Bi, in which a small part of the *titin* gene encoding the central part of the Z-disc region of titin is spontaneously deleted, was isolated (Jäckel et al., 1997). Here we have mapped the deletion with greater precision, demonstrated that only a truncated titin protein is expressed, and found a dramatic impairment of myofibrillogenesis.

MATERIALS AND METHODS

Hybridization probes and PCR primers

As hybridization probes, specific parts of human *titin* cDNA (Labeit and Kolmerer, 1995) were used. HH2, HH3b and HH5 are derived from the extreme carboxy-terminal, central and most amino-terminal parts of the Z-disc region of the titin cDNA, respectively, while HH3 is taken from a part of the cDNA encoding the C-zone of the A-band (for sequence descriptions, see http://www.embl.heidelberg.de). The approximate postions within the Z-disc portion of titin of these probes and those used for fine-mapping experiments are shown schematically in Figs 2 and 3. A 1.5 kb *Eco*RI fragment was used as probe for 18S RNA (Catalog number 77242, ATCC, USA).

Total RNA was prepared from cells grown as described below, using the RNeasy kit, following the instructions of the manufacturer (Qiagen, Hilden, Germany). RNA samples (20 μg) were heat-denatured for 5 minutes at 65°C and cooled on ice. After addition of 20× SSC to a final volume of 200 μl, the RNA samples were applied to a Minifold II Slot Blotter (Schleicher und Schüll, Dassel, Germany) using Nylonbind B membrane (Serva, Heidelberg, Germany). Probes were ^{32}P-labeled as described (Ausubel et al., 1995). Each pair of RNA samples (BHK-21/C13 and BHK-21-Bi) was probed with specific probes, or with an 18S RNA probe used as a control.

RT-PCR

Titin fragments were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) from total RNA isolated from BHK-21-Bi or BHK-21/C13 cells that were allowed to differentiate for 2 days using the SUPERSCRIPT One-Step RT-PCR System, according to the instructions of the manufacturer (LifeTechnologies, Eggenstein, Germany).

Transient transfection experiments

BHK-21/C13 cells (ATCC # CCL-10) and BHK-21-Bi cells (Jäckel et al., 1997) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Germany). For myogenisation, cells were seeded onto collagen-coated coverslips (50000 cells/30 mm plate) and transfected

with 5 μg of pSBC-MyoD (*Eco*RI fragment of MyoD cloned into the *Eco*RI site of pSBC1) using a calcium phosphate precipitation method (Chen and Okayama, 1987). 24 hours after transfection, cells were allowed to differentiate by the addition of DMEM supplemented with 2% FCS. 3-5 days later cells were fixed with ice-cold methanol for 6 minutes and subsequently used for immunohistochemistry as described (van der Ven et al., 1993).

Antibodies

The following antibodies were used in this study: *T20* and *T21*, both mouse monoclonal antibodies directed against z2, the second Ig-like domain in the Z-disc region of titin (Fürst et al., 1988; Gautel et al., 1996); *T-SPARMra*, a rabbit antiserum recognizing the linker located between z6 and z7 of Z-disc titin (Gautel et al., 1996); *T12*, a mouse monoclonal antibody directed against domains I2 to I4 just carboxy-terminal from the inextensible Z-disc region of titin (Fürst et al., 1988; Labeit and Kolmerer, 1995); *T30*, a mouse monoclonal antibody directed against a repetitive epitope of titin in the A-band (Fürst et al., 1989a); *T41*, a mouse monoclonal antibody directed against insert 4 between m5 and m6 domains near titin's carboxy terminus (Obermann et al., 1996); *MF20*, a mouse monoclonal antibody recognising all isoforms of sarcomeric myosin heavy chain (sMyHC; Bader et al., 1982); *MyBB78*, a mouse monoclonal antibody against domain 12 of myomesin (Vinkemeier et al., 1993; Obermann et al., 1996); *BM-75.2*, a mouse monoclonal antibody recognising all isoforms of α-actinin (Abd-el-Basset et al., 1991), purchased from Sigma (Deisenhofen, Germany); *EA-53*, a mouse monoclonal antibody specific for striated muscle α-actinin (Goncharova et al., 1992), purchased from Sigma (Deisenhofen, Germany); *NbSH3-ra*, a rabbit polyclonal antibody directed against the SH3 domain of nebulin that is localized in Z-discs (Young et al., 1998); *panC*, a polyclonal rabbit antibody recognizing all isoforms of C-protein (Gautel et al., 1998).

Immunoblotting

BHK-21/C13 cells and BHK-21-Bi cells were cultured as described above. Dishes containing proliferating cells or cells differentiated for 2 days were washed twice with phosphate-buffered saline (PBS), and the cells were collected in preheated SDS-sample buffer. Proteins were separated on 8% polyacrylamide gels as described (Fürst et al., 1988), and electroblotted to nitrocellulose using a BioRad semidry blotting apparatus and standard procedures. Membranes were blocked in 4% non-fat dry milk in PBS containing 0.05% Tween-20 (PBST) and subsequently incubated for 1 hour at room temperature with several titin antibodies directed against distinct parts of titin (see above and Fig. 3). All monoclonal antibodies were diluted 1:10 in PBST. Secondary antibodies (horseradish peroxidase-conjugated goat anti-mouse immunoglobulins from Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were applied diluted 1:17500 in PBST. Immune complexes were detected using enhanced chemoluminescence according to the instructions of the manufacturer (Amersham, Buckinghamshire, UK).

Immunoprecipitation

Proliferating or differentiated BHK-21/C13 and BHK-21-Bi cells, grown in 10 cm culture dishes, were collected by scraping in 1.5 ml of cold lysis buffer (1% Triton X-100, 0.2% SDS, 0.5% sodium desoxycholate, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM 2-mercaptoethanol, 15 mM Tris-HCl, pH 7.5). Cell extracts were homogenised by sonication and repeated pipetting and stored at –80°C until use. Magnetic beads with immobilised sheep anti-mouse IgG antibodies (Advanced Biotechnologies, Hamburg, Germany) were washed three times with 0.1% BSA in PBS (PBSB), and incubated overnight at 4°C in a rotator with 0.5 ml of T20 hybridoma supernatant diluted with 0.5 ml PBSB. The magnetic beads were washed twice with PBSB before addition of 250 μl lysis buffer and 750 μl cell extract. After an incubation for 1 hour at 4°C in a rotator, the beads were washed three times with lysis buffer and once with PBS. Subsequently, the beads were boiled in SDS-sample buffer. Samples were separated on an 8% polyacrylamide gel, transferred to nitrocellulose and incubated with antibodies as described above.

Electron microscopy

Cells grown on glass coverslips, as described above, were fixed with 2.5% glutaraldehyde in PBS, dehydrated in a graded series of ethanol and embedded in Epon. After removing the coverslip, ultrathin sections were cut on a Reichert Ultracut microtome, positively stained with aqueous uranyl acetate and lead citrate and viewed in a Philips CM 10 electron microscope at an accelerating voltage of 80 kV. Pictures were taken with a slow scan CCD camera (Gatan, Pleasanton, CA, USA).

RESULTS

Mapping of the titin deletion in BHK-21-Bi cells

BHK-21-Bi cells were shown before to bear a small internal deletion within the part of the *titin* gene that encodes its central Z-disc region (Jäckel et al., 1997). Here, we further characterized the deletion and analysed its effects on both *titin* transcript and protein expression. Total RNAs prepared from wild-type and mutant cells were hybridised to a panel of *titin* cDNA probes. Thus, only probe HH3b, which was derived from the center of titin's Z-disc region, was completely negative on BHK-21-Bi cells (Fig. 2A). HH2 (extreme amino terminus) and HH33 (A-band titin) were positive on both cell lines, and HH5 produced a considerably weaker signal in BHK-21-Bi cells, indicating that the deletion included the parts of the *titin* gene encoding domains Z4 to Z7, and extends into the region from Z8 to I2 (Figs 2A, 3). Fine-mapping achieved with labeled BHK-21-Bi derived cDNA, which was hybridized to a panel of Z-disc titin clones, allowed us to localize the deletion from Zq to Z10 (not shown; summarized in Fig. 3). These data were in part confirmed by RT-PCR experiments with different pairs of domain-specific primers (not shown).

Protein expression was probed by western blotting and immunoprecipitation experiments. While all the antibodies tested reacted with the 3 MDa titin polypeptide in total extracts of differentiated wild-type BHK-21/C13 cells (not shown; see van der Ven et al., 1998), only mAbs T20 and T21 recognised a 100 kDa band in samples of BHK-21-Bi-cells (Fig. 2B; for antibody epitope positions see Fig. 3, and Materials and Methods). In contrast, antibodies T-SPARMra, T12, T30 and T41 lacked any reactivity in BHK-21-Bi cells (Fig. 2B). These immunoreactivities were confirmed by immunofluorescence experiments on cultured cells (not shown). Immunoprecipitation of the expressed truncated titin polypeptide further corroborated the molecular mass found by western blotting (Fig. 2C). The results of these experiments (summarized in Fig. 3) mapped the deletion to a region of the *titin* gene that encodes the part of the protein that is located at the periphery of the Z-disc. Furthermore, they imply that the mutation most likely results in a frame-shift leading to a premature translational stop. Thus, BHK-21-Bi cells contain only an incomplete part of the amino-terminal Z-disc portion of titin; the remainder of the polypeptide is not translated.

Expression of sarcomere proteins in BHK-21-Bi cells

Initially, the expression of several sarcomeric proteins in BHK-

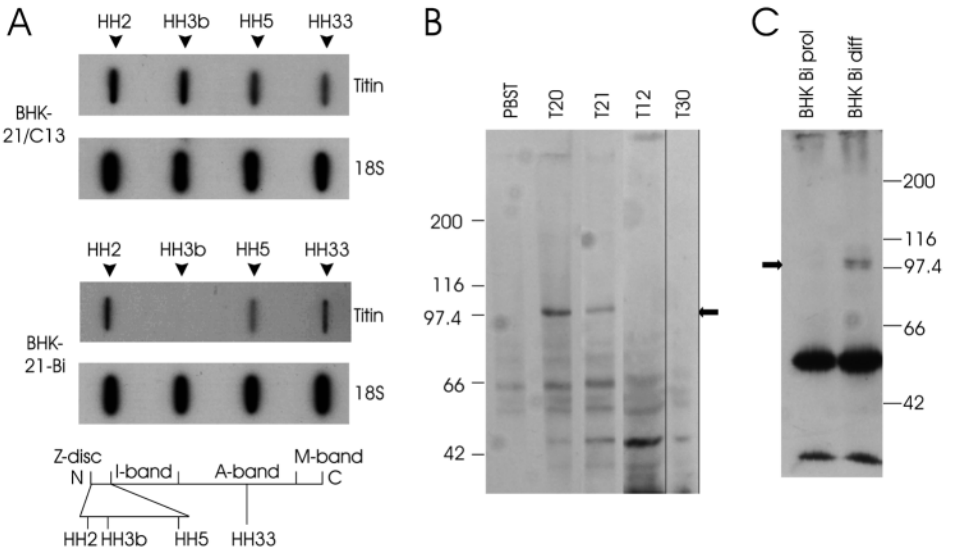

Fig. 2. Analysis of titin expression in BHK-21-Bi cells by RNA slot blots, western blots and immunoprecipitations. (A) Slot blots of total RNA isolated from BHK-21/C13 cells (upper panel) or BHK-21-Bi cells (lower panel) hybridized with four different titin probes representing different parts of Z-disc titin or A-band titin, as depicted below (see also Fig. 3). An 18S rRNA probe was used as a control. Note that only HH3b does not hybridise to BHK-21-Bi total RNA, and that the hybridisation signal for HH5 is considerably lower for BHK-21-Bi RNA when compared to BHK-21/C13 RNA. (B) Immunoblots of total protein extracts of differentiated BHK-21-Bi cells incubated with the titin antibodies noted at the top. Molecular masses of standard proteins are indicated at the left. (C) SDS-polyacrylamide gel showing immunoprecipitates from proliferating (prol) and differentiated (diff) BHK-21-Bi cells. The precipitations were performed with T20, while the blots were stained with T21. Note that both western blots and immunoprecipitations reveal expression of an amino-terminal 100 kDa titin fragment in BHK-21-Bi cells (arrows).

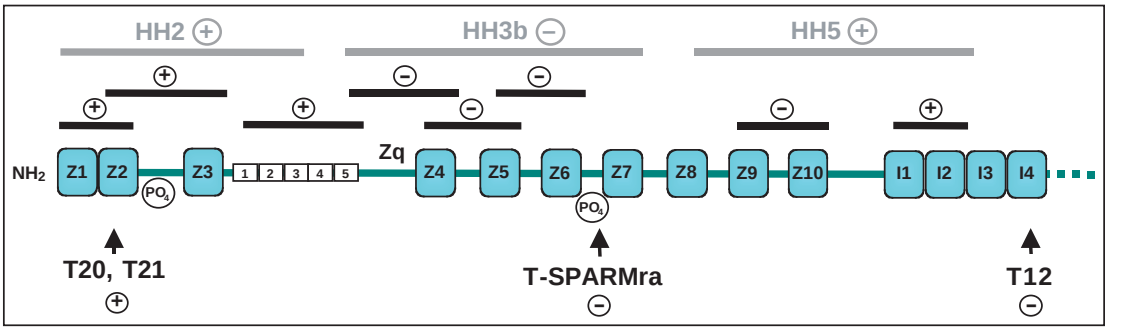

Fig. 3. Mapping of the titin deletion in BHK-21-Bi cells. Bars above the sketch give the positions and reactivity of overlapping probes from the Z-disc used for the slot-blot experiments, and the fragments that were amplified by RT-PCR to fine-map the deletion. Below the sketch, the epitopes and reactivity of sequence-assigned antibodies are shown. Note that only the extreme amino-terminal end of titin is detected at the RNA as well as the protein level. While the reactivity with titin-specific RNA probes is again positive in the I-band portion of the molecule, all further antibodies are negative. Thus, the titin deletion in BHK-21-Bi cells must comprise the region between Z-repeat 5 and I1 and result in a premature translation termination.

21/C13 and BHK-21-Bi cells was compared at the transcript level. Thus, the quantity of mRNAs encoding telethonin, sarcomeric α-actinin, slow C-protein and nebulin were found to be essentially identical in both cell types (Fig. 4). Likewise, the amount of titin transcript was comparable in both cell types (Fig. 4).

Expression of the respective proteins was assayed by immunofluorescence microscopy. As described for transcript expression, the levels of α-actinin and of the amino terminus of titin appeared to be similar (Fig. 5). Epitopes within the deleted region (T-SPARM-ra), and epitopes located carboxy-terminally to the deleted region (T12, T30, T41), were not revealed in BHK-21-Bi cells (Figs 3, 5F). Nebulin and C-protein were detected both in lower quantity and in fewer BHK-21-Bi cells (Fig. 5J,K). This is in line with earlier observations in the mouse embryo, which showed, for instance, early expression of nebulin mRNA (Moncman and Wang, 1999), while detection of the protein occurred relatively late (Fürst et al., 1989b; see also Discussion).

Most strikingly, the subcellular distribution of all the proteins mentioned was significantly altered in BHK-21-Bi

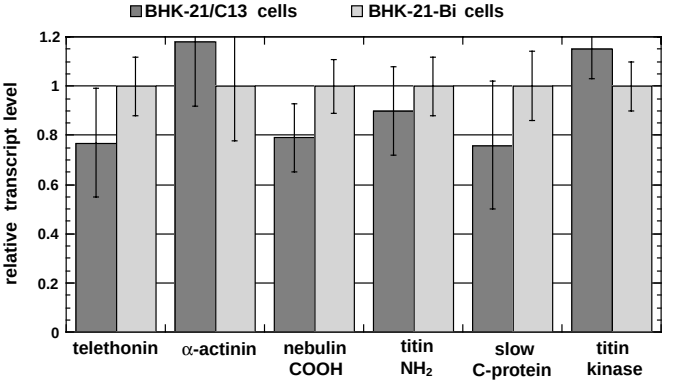

Fig. 4. Relative expression levels of sarcomeric protein mRNAs in BHK-21/C13 and BHK-21-Bi cells. Total mRNA was hybridised to probes of slow skeletal C-protein, telethonin, nebulin C terminus, titin C terminus and sarcomeric α-actinin 2A, and the bound probe quantified by densitometry of the exposed blots. The means of three experiments are shown ± s.d. Note that in BHK-21-Bi cells the transcript levels of all proteins are not markedly different from those in BHK-21/C13 cells.

cells: in the wild-type BHK-21/C13 cells myofibrillar banding patterns were found with all antibodies subsequent to 2 days of differentiation, but no sign of sarcomere formation was evident in BHK-21-Bi cells (Fig. 5; see also below). The truncated titin peptide was revealed partly in nuclei, but mainly in a punctate fashion along stress fibers (Fig. 5E), similar to the distribution pattern of sarcomeric α-actinin (Fig. 5L). Double stainings showed that both proteins colocalised (not shown). Interestingly, the spacings between the fluorescent dots in differentiated BHK-21-Bi cells (0.8-1.2 μm) were not

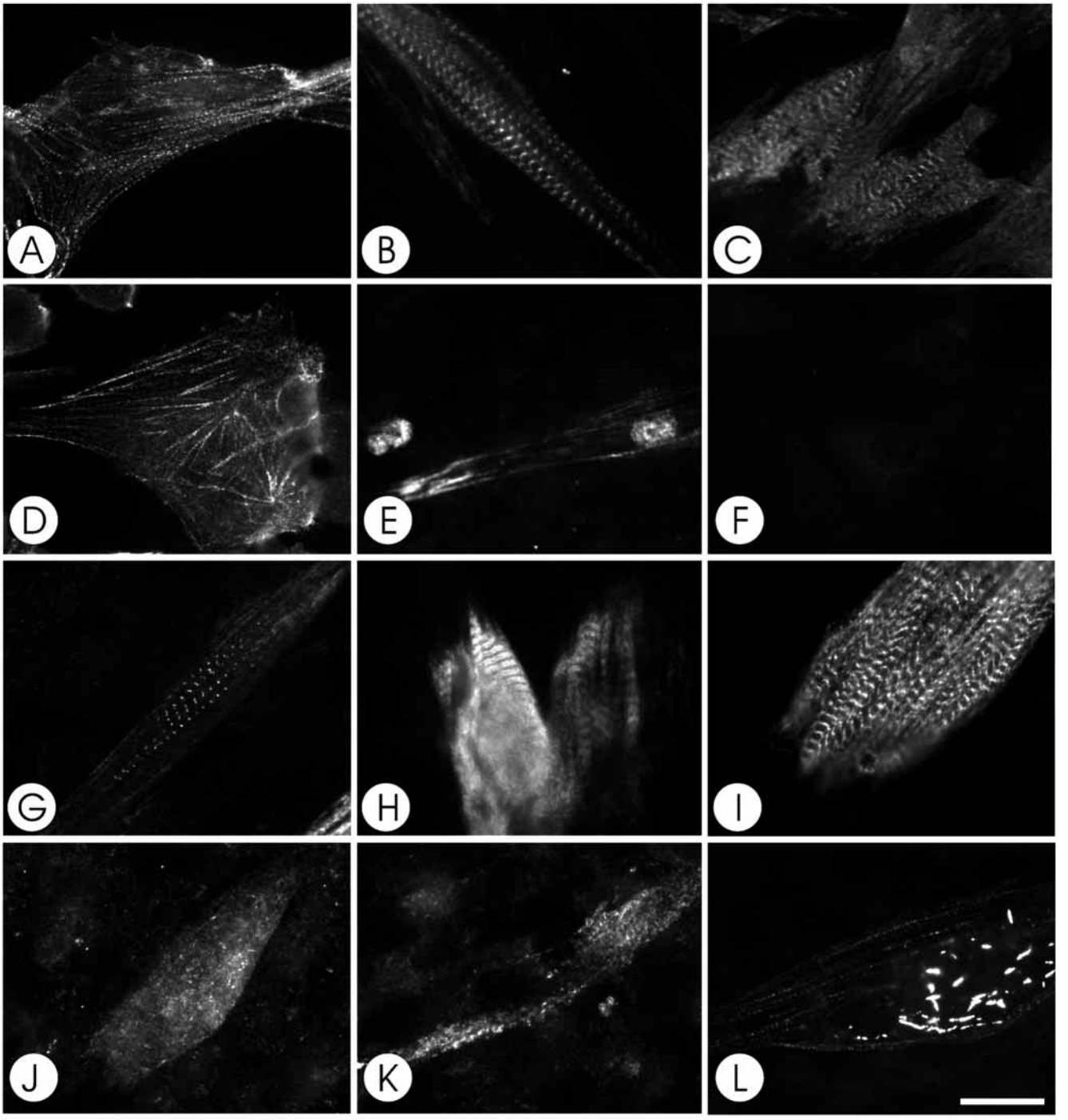

Fig. 5. Localisation of myofibrillar proteins in proliferating (A,D) or differentiated (B,C,E-L) BHK-21/C13-cells (A-C,G-I) and BHK-21-Bi cells (D-F,J-L) by immunofluorescence microscopy. The following antibodies were used: pan α-actinin (A,D), titin T20 (B,E), titin T41 (C,F), nebulin SH3 domain (G,J), C-protein (H,K) and sarcomeric α-actinin (I,L). All antibodies show a sarcomeric organisation in differentiated BHK-21/C13 cells exclusively, but not in the titin mutant BHK-21-Bi cells. Note that in proliferating cells, the spacing of the α-actinin dots is similar in both cell types (A,D; see text). Upon differentiation and sarcomere assembly, BHK-21/C13 cells show a spacing of 1.8-2.3 μm (C,G-I), while the spacing of titin (E) or sarcomeric α-actinin (L) in differentiated BHK-21-Bi cells does not differ from the α-actinin spacings (0.8-1.2 μm) in non-differentiated cells. Bar, 20 μm.

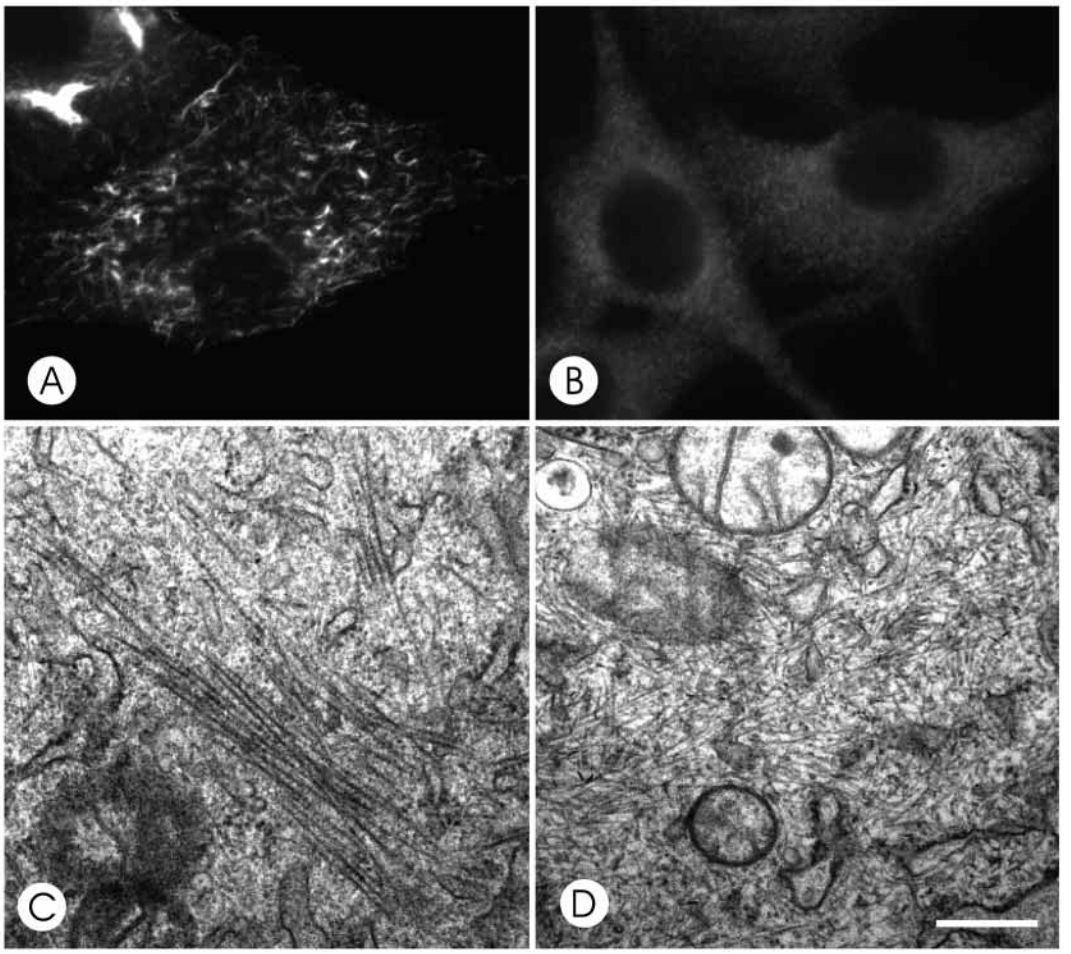

Fig. 6. Immunofluorescence and electron microscopy of BHK-21/C13 (A,C) and BHK-21-Bi cells (B,D) at early differentiation stages. Samples in A and B were stained with an antibody directed against sMyHC. Note assembly of stacks of thick filaments only in BHK-21/C13 cells (A) and not in BHK-21-Bi cells where sMyHC is diffusely distributed (B). Likewise, thick filaments can be identified by electron microscopy exclusively in wild type (C) and not in the mutant BHK-21-Bi cells (D). Bar, 20 μm (A,B); 0.4 μm (C,D).

different from those in non-differentiated cells or from those in undifferentiated BHK-21/C13 cells. In contrast, in differentiated BHK-21/C13 cells these spacings increased from 0.8-1.2 μm to 1.8-2.3 μm in cells where sarcomeres developed. Furthermore, in the BHK-21-Bi cells that express C-protein and nebulin, no myofibril formation was detected, ruling out that a downregulation or increased degradation of these proteins could have caused the observed defect.

Defective myofibrillogenesis in BHK-21-Bi-cells

The distribution patterns of sarcomere proteins were significantly altered in the titin mutant BHK-21-Bi cells (see above; Figs 5-7). The most dramatic difference in organisation was revealed for sarcomeric myosin heavy chain (sMyHC). In BHK-21/C13 cells, rod-like structures were revealed by immunofluorescence microscopy at the earliest stages of differentiation (Fig. 6A). Electron micrographs demonstrated stacks of thick filaments aligning in parallel (Fig. 6C). In contrast, in BHK-21-Bi cells, sMyHC was distributed in an almost diffuse fashion (Fig. 6B). Electron microscopy confirmed the absence of thick filaments in these cells (Fig. 6D). After longer periods of differentiation, BHK-21/C13 cells assembled sarcomeres with regular cross-striated patterns of sMyHC and the M-band protein myomesin (Fig. 7A,B). The distribution of the same proteins was strikingly different in BHK-21-Bi cells: both labels were patchy and did not form any discernable structure. In addition, the localisation of sMyHC and myomesin did not appear to be coherent (Fig. 7C,D).

Myogenisation of BHK-21-Bi cells

To exclude the possibility that the myogenic potential of the mutant BHK-21-Bi cells has been compromised, we transiently transfected the myogenic transcription factor MyoD both into BHK-21/C13 and BHK-21-Bi cells. As a result, fusion into multinuclear cells occurred within 2 days. In BHK-21/C13 myotubes, cross-striation was already evident with phase-contrast optics (Fig. 8A) and myofibrillar organisation was demonstrated by immunofluorescence microscopy using an antibody against the Z-disc portion of titin (Fig. 8B) and several other antibodies specific for other titin epitopes or other sarcomeric proteins, including components of the Z-disc, I-band, A-band and M-band (not shown). In contrast, the myotubes formed from BHK-21-Bi cells did not show any sign of myofibril formation upon staining with the same set of antibodies, but rather resembled the cells described above that have been subjected to ‘standard’ differentiation conditions (Fig. 8C-F).

DISCUSSION

The high degree of regularity of cross-striated myofibrils is one of the most impressive supramolecular assemblies in nature.

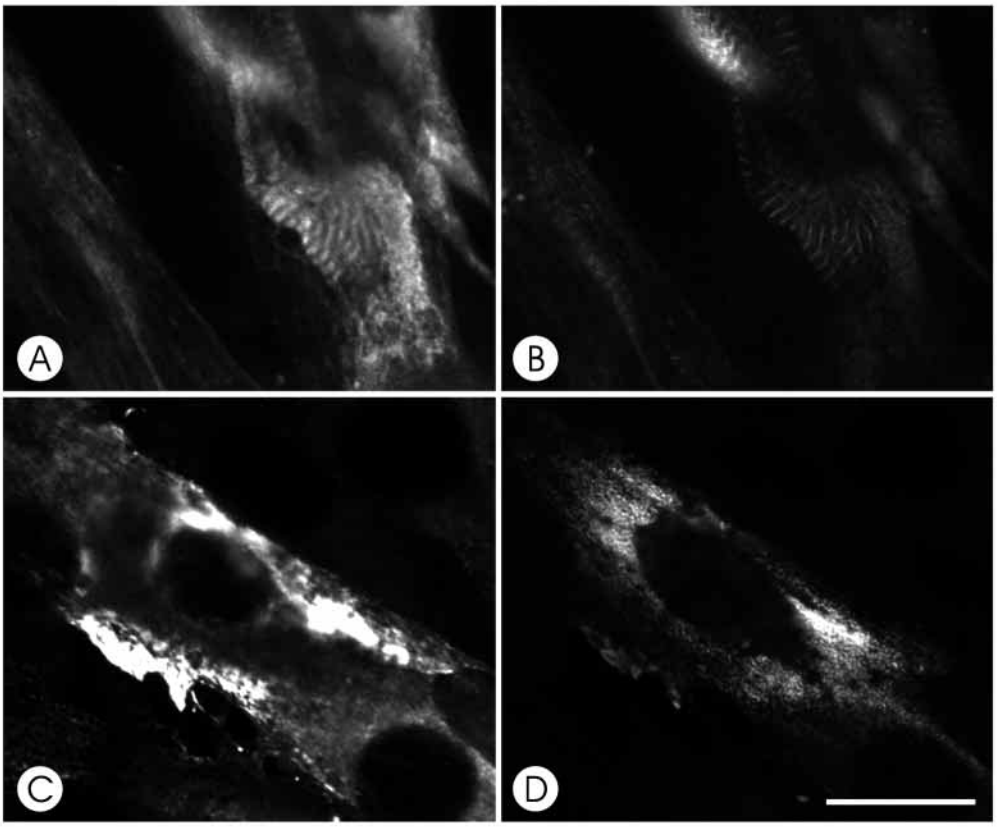

Fig. 7. Double immunofluorescence microscopy using antibodies directed against sMyHC (A,C) and myomesin (B,D) in BHK-21/C13 (A,B) and BHK-21-Bi (C,D) cells differentiated for 3 days. Note the presence of sarcomere structures only in BHK-21/C13 cells. The localisations of sMyHC (C) and myomesin (D) are largely independent in BHK-21-Bi cells. Bar, 20 μm.

Thousands of proteins have to 'self-assemble' in a co-ordinated fashion within a very short period of time – a few hours during early cardiac development (Tokuyasu and Maher, 1987; Ehler et al., 1999). Since this precision cannot be explained simply by the properties of actin and myosin, the process must require additional components. It has been suggested, therefore, that cytoskeletal proteins could have such a function (Small et al., 1992; Trinick, 1994; Stromer, 1998). Titin was identified as a molecule that can, as a single entity, span an entire half-sarcomere from Z-disc to M-band (Fürst et al., 1988). Furthermore, titin was suggested to provide elastic resting tension (Yoshioka et al., 1986), to keep adjacent thick filaments in register (Horowits et al., 1986) and to act as a ruler for thick filament assembly (Whiting et al., 1989; Trinick, 1994), which is supported by the identification of a plethora of titin-binding proteins (Fig. 1). More recently, the characterisation of phosphorylation sites and of the kinase domain have implied titin in signaling cascades that are possibly involved in the assembly of myofibrils during muscle differentiation (Gautel et al., 1993; Maruyama et al., 1994; Sebestyén et al., 1995; Mayans et al., 1998).

While some of the suggested physiological roles of titin could be probed directly (Trombitás and Pollack, 1993; Linke et al., 1996), its exact function in thick filament assembly and myofibril formation has remained elusive. Several models on the one hand predicted a role for titin in the fusion of Z-bodies of adjacent premyofibrils leading to the formation of Z-discs (Rhee et al., 1994; Dabiri et al., 1997). On the other hand, titin is probably involved in the assembly of a cytoskeletal scaffold consisting of Z-discs, titin and the M-line protein myomesin, which enables the integration of thick filaments into developing sarcomeres (Ehler et al., 1999; van der Ven et al., 1999). We found a model for approaching this question more directly in the serendipitously isolated myofibroblast cell line BHK-21-Bi. For some time it was known that BHK-21/C13 cells express a number of sarcomeric proteins (Schaart et al., 1991; Mayer and Leinwand, 1997). Recently it was demonstrated that BHK-21/C13 cells are capable of forming myofibrils (van der Ven and Fürst, 1998). In this report we describe that in these cells the assembly of myofibrils follows a general rule: initially α-actinin and titin are found to be colocalized in Z-bodies with an initial spacing of 0.8-1.2 μm, whereas mature Z-discs that develop upon culturing the cells in low-nutrition medium for at least 2 days are spaced 1.8-2.3 μm apart, indicating that normal-sized sarcomeres are assembled.

Most interestingly, in the subline BHK-21-Bi, which was shown to bear a defect in the *titin* gene (Jäckel et al., 1997), myofibrillogenesis is compromised. Our results demonstrate that in this mutant cell line only a truncated titin polypeptide comprising the extreme amino-terminal 100 kDa of Z-disc portion of titin is synthesised, which corresponds to a size of less than 3% of the intact protein. In contrast, the expression of other sarcomeric proteins is, at least at the RNA level, essentially unaffected (Fig. 4), making a defect in the general myogenic capacity in these cells unlikely. In order to exclude the possibility that the observed structural changes could be due to a secondary defect in the BHK-21-Bi cells, i.e. one in the myogenic commitment, we transiently transfected these cells with the myogenic transcription factor MyoD. Such transfections were shown to force cells of mesodermal or fibroblastic origin to transform into muscle cells, inducing

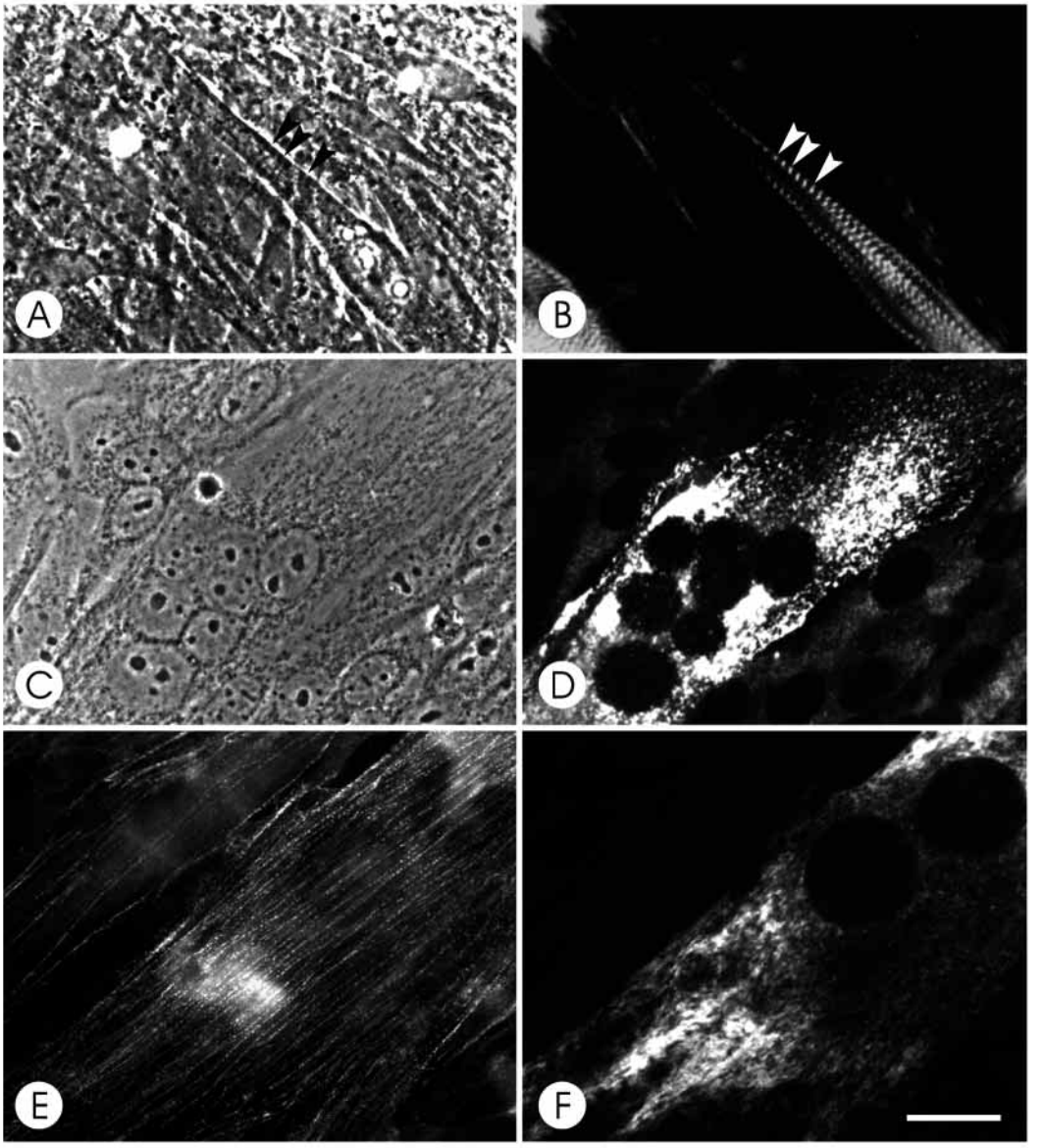

Fig. 8. Effect of myogenisation by MyoD transfection on BHK-21/C13 and BHK-21-Bi cells. (A,B) MyoD-transfected BHK-21/C13 cells in phase contrast (A) and immunostained for Z-disc titin (B). (C-F) Transfected BHK-21-Bi cells in phase contrast (C) and immunostained for sMyHC (D), or double stained with pan α-actinin- (E) and sMyHC-specific antibodies (F). As a result of myogenisation, BHK-21/C13 cells develop myotubes with cross-striated myofibrils, which are already evident with phase-contrast optics (arrowheads in A,B). In contrast, BHK-21-Bi cells develop multinucleated myotubes that do not show any sign of myofibril formation (C-F). Bar, 25 μm (A-D); 20 μm (E,F).

both the fusion of these cells into multinuclear myotubes and the expression of a wide range of sarcomeric proteins (Choi et al., 1990). The results of our transfection experiment show that the titin mutation in BHK-21-Bi cells does not affect the ability of these cells to form multinuclear myotubes. Instead, the striking consequence of a lack of functional titin is a diffuse-to-patchy myosin (sMyHC) distribution, indicating a defect in the assembly of ordered thick filaments (see Figs 6-8).

Since the first purification of myosin, the assembly of thick filaments and their length determination in vivo have been an enigma. No in vitro method allows myosin to assemble into filaments of the exactly tailored length of 1.6 μm (Trinick, 1994). This dilemma was one of the main arguments for the proposition that titin would fulfil this role, once its precise sarcomeric layout was revealed (Whiting et al., 1989; Trinick, 1994). This basic question can also be extended to several proteins with dual binding capacity like, for instance, C-protein and myomesin, which were previously shown to bind to both titin and myosin heavy chain (Obermann et al., 1998; Moos et al., 1975; Starr and Offer, 1978; Bähler et al., 1985; Nave et al., 1989; Fürst et al., 1992; Soteriou et al., 1993; Koretz et al., 1993; Okagaki et al., 1993; Freiburg and Gautel, 1996; Obermann et al., 1997) or the Z-disc cross-linker α-actinin (Sorimachi et al., 1997; Ohtsuka et al., 1997; Young et al., 1998). The exact molecular mechanism of sarcomeric targeting, however, is only poorly understood (see, for example, Gilbert et al., 1996; Auerbach et al., 1999). The lack of colocalisation of C-protein and myomesin with sMyHC in the titin-negative cells therefore implies that correct sarcomeric targeting largely depends either on titin itself or on the assembly of thick filaments directed by titin. Despite the presence of the C-protein and nebulin transcripts in BHK-21-Bi cells, these proteins are detectable in only relatively few cells. It is conceivable that the unassembled sarcomeric proteins are rapidly degraded, a situation which was also proposed for the unassembled mutant C-protein found in cases of familial hypertrophic cardiomyopathy (Rottbauer et al., 1997).

Considering the fact that transfection experiments of cDNA constructs encoding different parts of titin demonstrated that a short region of titin's Z-disc is sufficient for binding to dense bodies of stress-fibers of non-muscle cells and to Z-bodies and Z-discs in cardiomyocytes (Turnacioglu et al., 1996, 1997; Ayoob et al., 2000), our finding that the expressed truncated titin fragment binds to stress fiber-like structures in a periodic pattern identical to that of sarcomeric α-actinin is not surprising. The fact that no progression to real Z-discs occurred, is, however, remarkable. Despite the presence of the α-actinin and telethonin binding sites that were shown to be involved in the assembly of the Z-disc (see Fig. 1), the progression of myofibrillogenesis towards the cross-striated arrangement of Z-disc proteins does not occur. This process seems to require the Z-disc portion of titin that is deleted in BHK-21-Bi cells. This indicates that the Z-disc region of titin between Z4 and Z10, containing phosphorylation sites which can be phosphorylated by cyclin-dependent protein kinases and ERK2 (Sebestyén et al., 1995), might contain as-yet-unidentified binding sites for further Z-disc proteins. Alternatively, more carboxy-terminal parts of titin might be indispensable for the assembly of mature Z-discs that develop by the fusion of Z-bodies of neighboring nascent myofibrils (Rhee et al., 1994; Dabiri et al., 1997). This idea is supported by our earlier observations that, during the initial stages of myofibril assembly, the carboxy-terminal and amino-terminal parts of titin colocalize in Z-bodies (Mayans et al., 1998). Furthermore, the Z-disc protein telethonin is one of the first identified substrates of the titin kinase and constitutively active titin kinase disrupts myofibril formation (Mayans et al., 1998). While the amino-terminal region of titin is stably anchored to maturing Z-discs, the carboxy-terminal M-band region is gradually reorganized (van der Loop et al., 1996) and is found in a cross-striated pattern at a later developmental stage that coincides with the assembly of M-bands (van der Ven and Fürst, 1997; van der Ven et al., 1999). The formation of this cytoskeletal scaffold precedes the integration of A-bands into sarcomeres (Ehler et al., 1999; van der Ven et al., 1999). The lack of binding sites for M-band proteins and the deletion of the catalytic kinase domain in the truncated titin molecule expressed in BHK-21-Bi cells might prevent the formation of this cytoskeletal scaffold and thus, the assembly of mature myofibrils. The exact nature and further elements of these signaling pathways still need to be established.

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