Titin aggregates associated with intermediate filaments align along stress fiber-like structures during human skeletal muscle cell differentiation

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SUMMARY

Differentiating human skeletal muscle cell cultures were used to study the association of titin with other sarcomeric and cytoskeletal proteins during myofibrillogenesis. Several developmental stages of these cultures were double stained with antibodies to titin in combination with antibodies to α-actin, α-actinin, myosin heavy chain (MHC), nebulin, desmin, and β-tubulin. The first indications of titin expression were found in postmitotic mononuclear myoblasts where it is located in a random, punctate fashion. At the light microscope level no evidence was found for an association of these titin spots with any of the other proteins studied, with the exception of MHC, which colocalized with titin in a small minority of the titin expressing cells. Subsequently the titin spots were found to be linked to longitudinally oriented stress fiber-like structures (SFLS), containing α-actinin and sarcomeric α-actin, but not MHC, nebulin or desmin. Upon further maturation titin antibodies seemed to stain SFLS in a rather heterogeneous fashion together with MHC, α-actin and α-actinin. Thereafter a more periodic localization of titin, MHC, α-actin and α-actinin on SFLS became obvious. From these structures myofibrils developed as a result of further differentiation. Initially only short stretches with a striated titin, MHC, F-actin and α-actinin organization were found. Nebulin was integrated in these young myofibrils at a later developmental stage. Desmin was not found to be incorporated in these myofibrils until complete alignment of the sarcomeres in mature myotubes had occurred. At the ultrastructural level titin antibodies recognized aggregates that were associated with intermediate filaments (IF) in postmitotic mononuclear myoblasts. At a later maturation stage, prior to the development of cross-striated myofibrils, the IF-associated titin aggregates were found in close association with subsarcolemmally located SFLS. We conclude that IF and SFLS play an important role in the very early stages of in vitro human myofibrillogenesis. On the basis of our results we assume that titin aggregates are targeted to SFLS through IF. The association of titin with SFLS might be crucial for the unwinding of titin necessary for the assembly of sarcomeres and the first association of titin with other sarcomeric proteins.

Key words: myofibrillogenesis, intermediate filament proteins, titin, myosin, nebulin, α-actinin, immunolocalization, immunoelectron microscopy

INTRODUCTION

In the sarcomeric structures of skeletal and cardiac muscle several proteins are ordered in unique, periodic arrays resulting in the typical cross-striated appearance of these tissues (Fischman, 1986). During embryogenesis or regeneration in skeletal muscle, multinuclear myofibers develop by fusion of myoblasts or satellite cells. Several authors (Fischman, 1986; Fürst et al., 1989; Fulton and Isaacs, 1991) have reported that during these processes the expression of myofibrillar proteins and their mutual associations, occur in an ordered sequence. Most authors agree that titin is one of the first sarcomeric proteins detected in the process of myofibrillogenesis (Tokuyasu and Maher, 1987a; Fürst et al., 1989; Schaart et al., 1989), and that it probably plays an important role in the supramolecular organization of other myofibrillar proteins (Fulton and Isaacs, 1991; Trinick, 1992). However, the results of several studies describing the intracellular localization of titin as compared to other sarcomeric or cytoskeletal proteins are contradictory. Recently it was suggested that the order
of events that occur during myofibrillogenesis is species-specific (van der Loop et al., 1992).

In a previous report (van der Ven et al., 1992), we showed that human skeletal muscle cells, cultured under conditions optimal for differentiation, reach a sufficiently high degree of maturation, to allow the study of virtually all stages of myofibrillogenesis. In order to obtain a better insight into the precise localization of titin during the early stages of skeletal muscle development, these studies were extended to the immunoelectron microscope level. The goal of this study was to compare the temporal and spatial organization of titin to that of other sarcomeric and cytoskeletal proteins within the same muscle cell, both at the light and electron microscope level.

MATERIALS AND METHODS

Muscle cell cultures

Human satellite cells were isolated and cultured mainly as described before (van der Ven et al., 1992; Benders et al., 1991). In summary, satellite cells were enzymatically isolated from normal adult human skeletal muscle. The cells were plated in 35 mm culture dishes (Costar, Cambridge, MA, USA) in a high-nutrition culture medium supplemented with 20% fetal calf serum (FCS, HyClone Laboratories Inc, Logan, UT, USA) and 2% chick embryo extract (Flow Laboratories, Irvine, UK), and grown in 4% Ultroser G (Gibco BRL, Paisley, UK) containing culture medium. Cells were trypsinized and stored in liquid nitrogen in culture dishes and grown to near confluency. Differentiation of the cells was induced by changing the culture medium to a low-nutrition medium with 0.4% Ultroser G, and 2% Ultroser G containing culture medium.

Antibodies and reagents

The following monoclonal and polyclonal antibodies were used in this study:

1. 9D10, a mouse monoclonal antibody specific for titin (Wang et al., 1985; Handel et al., 1989).
2. E7, a mouse monoclonal antibody to β-tubulin (Chu and Klymkowski, 1987).
3. An affinity-purified polyclonal antibody against titin, isolated from Physarum polycephalum (Gassner, 1986), which was a kind gift from Dr D. Gassner, Bonn, FRG.
4. The mouse monoclonal antibody NB2, specific for nebulin (Fürst et al., 1988), purchased from Sigma Chemical Company, St. Louis, MO, USA.
5. The mouse monoclonal antibody αs-1, specific for striated muscle α-actin (Skalli et al., 1988), which was a kind gift from Dr G. Gabbiani, Geneva, Switzerland.
6. The mouse monoclonal antibody MF20, recognizing all forms of myofibrillar MHC (Bader et al., 1982), which was a kind gift from Dr D. Fischman, New York, NY, USA.
7. A polyclonal rabbit antiserum (pDes) raised against chicken gizzard desmin (Ramaekers et al., 1985), available from Euro-Diagnostics BV, Apeldoorn, The Netherlands.
8. A polyclonal rabbit antiserum raised against α-actinin, reactive with muscle and non-muscle isoforms of α-actinin (van der Ven et al., 1992), which was a kind gift from Dr B. Jockusch, Bielefeld, FRG.

Tetramethylrhodamine isothiocyanate-labeled phalloidin (Molecular Probes Inc., Eugene, OR, USA) was used for staining of filamentous actin (F-actin).

Indirect immunofluorescence assays

Muscle cells, cultured in 35 mm tissue culture dishes, were rinsed three times with phosphate buffered saline (PBS) and fixed in methanol/acetone (1:1) at −20°C for 1 minute. The dishes were stored at −25°C. Before incubation with the first primary antibody (30 minutes, room temperature), the dishes were thawed and rinsed briefly with PBS/0.05% Tween-20 (PBST). After extensive washing with PBST for 30 minutes, the cells were incubated with the second primary antibody. The dishes were washed again in PBST and incubated with a mixture of the appropriate secondary antibodies, i.e., fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit Ig (dilution 1:50 in PBST; Dakopatts, Glostrup, Denmark), FITC-conjugated goat anti-mouse IgG, Texas Red (TXR)-conjugated goat anti-mouse Ig, or TXR-conjugated goat anti-mouse IgM, all from Southern Biotechnology Associates, Birmingham, AL, USA, and used in a 1:75 dilution in PBST. After washing in PBST, the dishes were mounted in Aquamount (BDH Chemicals, Poole, UK). The cells were viewed with a Zeiss Axioskop microscope with epifluorescence illumination (Carl Zeiss, Oberkochen, FRG) and photographed with an automatic camera using a Kodak TMY film. Immunostained cultured muscle cells were also studied with a Bio-Rad MRC 600 confocal scanning laser microscope (CSLM, Bio-Rad Laboratories), equipped with a crypton/argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT, USA) with two separate wavelengths for the excitation of FITC (488 nm) and TXR (568 nm) mounted on a Zeiss Axioskop microscope (Carl Zeiss, Oberkochen, FRG). To quantify fluorescence intensities, fluorescein-labeled muscle cells were excited with a single wavelength light beam of 488 nm, using a neutral density filter no 1.5, showing 3% transmission, and a fixed pinhole position. Image processing was performed to upscale the signal and obtain optimal contrast. Photographs were taken from the monitor with a Nikon FT3 camera using a Kodak Ektachrome film or a Kodak Tri-X-Pan film.

Electron microscopy

Muscle cells, cultured as described above in 35 mm culture dishes, were rinsed twice with PBS and prefixed for 1 hour at 20°C in 1% paraformaldehyde in 100 mM phosphate buffer, pH 7.4 (PB). The monolayers were rinsed twice with PBS and twice with 20 mM glycine in PBS (PBGS). The cells were permeabilized by incubation with 0.1% saponin in PBGS (PBGS/sap) for 20 minutes at room temperature. The permeabilization buffer was removed and the cells were incubated with the titin monoclonal antibody appropriately diluted 1:10 in PBGS/sap for 30 minutes. The dishes were extensively washed with PBGS/sap and the cells were incubated with FITC-conjugated rabbit anti-mouse Ig, in order to identify those cultures showing most cells with a punctate titin localization. The dishes were washed again with PBGS/sap and incubated with Protein A/10 nm gold particles diluted 1:400 in PBGS/sap (Slot and Geuze, 1981). After washing four times with PBGS/sap, cells were post-fixed with 2% glutaraldehyde in PB for 15 minutes, and routinely embedded as monolayers in Epon 812, except that propylene oxide was omitted. Sections (100 nm) were cut parallel to the substratum, stained with uranyl acetate and lead citrate, and studied in a Philips EM 301.
RESULTS

Human skeletal muscle cell cultures
During proliferative stages, the muscle cells demonstrated a spindle-shaped to polygonal morphology. Upon the change to the low-nutrition culture medium, the cells elongated, fused and matured as described and illustrated before (van der Ven et al., 1992).

Premyofibrillar stages
Proliferating satellite cells did not express titin or any other sarcomere-specific constituent. The cells were only stained with the antibodies against desmin, β-tubulin (both filamentous), α-actinin (stress fiber-like structures, SFLS), and with TRITC-phalloidin (SFLS). About two days after the change of the medium, the first titin expression was observed in a punctate fashion in postmitotic mononuclear myoblasts.

Fig. 1. Indirect immunofluorescent labeling of human postmitotic mononuclear myoblasts, with the monoclonal (A,E,G) or polyclonal (C,I) anti-titin antibodies, anti-β-tubulin (B), anti-myofibrillar myosin heavy chain (D,J), anti-α-actinin (F), and anti-nebulin (H). (I and J) Micrographs obtained after confocal laser scanning microscopy. Bar, 50 µm (C-H), 20 µm (A,B) or 10 µm (I,J).
Initially these spots were located in close proximity to the nucleus as described before (van der Ven et al., 1992), or dispersed throughout the sarcoplasm (Fig. 1A,C,E,G,I). In addition these cells showed filamentous tubulin (Fig. 1B) and desmin (Fig. 5B,C) staining patterns, and SFLS decorated by α-actinin (Fig. 1F) and TRITC-phalloidin (Fig. 5A), but no

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**Fig. 2.** Immunofluorescence micrographs of immature myotubes double stained with monoclonal anti-titin (A,C,E,G,I) on the one hand, and TRITC-phalloidin (B,H), anti-desmin (D), anti β-tubulin (F) or anti-myofibrillar myosin heavy chain (J) on the other hand. Bar, 20 µm (A-D) or 50 µm (E-J).
nebulin reactivity was observed (Fig. 1H). Only a few cells showed an obvious colocalization of the titin spots with myofibrillar MHC spots (compare Fig. 1C,D with I,J). Except for titin and occasionally MHC, the other proteins tested did not show random punctate distribution patterns. Part of the titin spots seemed to be associated with the SFLS (Fig. 5A). In further maturated postmitotic mononuclear myoblasts and young myotubes virtually all titin spots were found to be associated with SFLS, thus forming nascent myofibrils, also containing sarcomeric α-actin. Initially, at this stage titin is localized at random sites on SFLS (Figs 2A,B, 5D,E,F), but upon further maturation the titin reactivity was homogeneously spread over the complete SFLS (Fig. 2G,I). At this stage, these SFLS contained, besides α-actinin and sarcomeric α-actin, myofibrillar MHC in a titin-like, non-interrupted distribution (Fig. 2J). The localization of desmin IF and microtubules now appeared parallel to the nascent myofibrils (Fig. 2C-F). No reactivity was seen when the cells were incubated with the nebulin antibody (Figs 1H and 3F, upper cell).

**Development of the striated myofibril**

As maturation continued, nascent myofibrils developed short stretches showing the typical doublet staining pattern for titin as observed with the antibodies used in adult striated muscle. The titin staining pattern on other parts of the same structure in the cell remained non-striated (Fig. 3A). The former stretches also showed a cross-striation when stained with TRITC-phalloidin (Fig. 3B) or incubated with anti-MHC or anti-α-actinin (Fig. 3C,D). Nebulin was absent from these young sarcomeres, but was obviously introduced somewhat later in development (Figs 3E,F, 5G).

![Fig. 3. Immunofluorescence micrographs of double-stained myotubes developing the first cross-striated myofibrils. Monoclonal anti-titin staining patterns (A,C,E,G) are compared to the staining patterns of TRITC-phalloidin (B), anti-α-actinin (D), anti-nebulin (F) and anti-desmin (H). Bar, 50 µm.](image-url)
At this stage large amounts of desmin and β-tubulin were observed in all myotubes. Again, no staining of the nascent myofibrils was found with the antibodies reacting with these latter two components (Fig. 3G,H).
The mature myotube
The number of striated myofibrils rapidly increased during
the later stages of maturation (Fig. 3C-H), until almost all
of the sarcoplasm was occupied with striated myofibrils in

Fig. 5. Double immunofluorescence micrographs of several stages of human skeletal muscle cell differentiation, illustrating the staining
patterns of postmitotic mononuclear myoblasts (A,B), and myotubes without (C-F) or with (G,H) striated myofibrils. Monoclonal (A-C,
E-G) or polyclonal (D,H) anti-titin reactivity patterns (A,D, FITC; B,C,E-H, Texas Red) are compared to the reactivity patterns of
TRITC-phalloidin (A), desmin (B,C, FITC), sarcomeric α-actin (D, Texas Red), α-actinin (E,F, FITC), nebulin (G, FITC) and
myofibrillar myosin heavy chain (H, FITC). Bar, 50 µm (A-C), or 14 µm (D-H).
mature myotubes (Fig. 4A-I). These myofibrils were invariably stained in a cross-striated pattern with the antibodies to titin, MHC (Fig. 5H), α-actinin (Fig. 4B), nebulin (Fig. 4D) or when stained with TRITC-phalloidin (Fig. 4F). Only when the complete myotube showed a mature cross-striated pattern, with perfectly aligned myofibrils, desmin was

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**Fig. 6.** Immunoelectron microscope localization of titin at different stages of maturation of cultured human muscle cells. In (A) titin aggregates associated with IF (arrows) in a postmitotic mononuclear myoblast are shown, while (B) shows the association of titin aggregates with aligned IF (arrows). (C) depicts the periodic localization of IF-associated titin aggregates (arrows) with SFLS. A detail of this micrograph, clearly showing the association between titin and IF (small arrows), is shown in (D). The integration of titin into striated myofibrils on both sides of Z discs (arrows) is shown in (E). Bar, 0.2 µm (B,D), 0.45 µm (A), 0.55 µm (E) or 0.8 µm (C).
found to be localized in a striated fashion (Fig. 4H). Staining with the antibody to β-tubulin did not result in a cross-striated staining pattern (Fig. 4J).

**Titin aggregates at the ultrastructural level**

Skeletal muscle cell cultures at different stages of maturation were examined ultrastructurally for the presence of titin aggregates. Globular titin aggregates, with a diameter of 0.10 to 0.15 μm and decorated with up to 15 gold particles, were identified in postmitotic mononuclear myoblasts. These aggregates were found to be associated with dispersed (Fig. 6A) or aligned (Fig. 6B) 10 nm IF. Titin aggregates were not found near dispersed or bundled microfilaments or microtubules. When maturation proceeded the IF-bound titin aggregates associated with longitudinally oriented SFLS, in a periodic fashion, 1.2-1.5 μm apart from each other (Fig. 6C,D). The SFLS did not yet show a clear periodicity at this stage. Subsequently, the immunogold labeling for titin was found concentrated on both sides of identifiable Z disc regions of clearly striated fibrils (Fig. 6E).

**DISCUSSION**

In a previous report (van der Ven et al., 1992) we have described that human skeletal muscle cells in culture can reach a sufficiently high degree of maturation to study virtually all stages of myofibrillogenesis. Here we extended our studies by investigations on the association of titin with sarcomeric and cytoskeletal proteins. For this purpose immunofluorescent double labeling procedures were applied with specific antibodies to titin in conjunction with antibodies to other sarcomeric or cytoskeletal proteins, i.e. myofibrillar MHC, α-actinin, sarcomeric α-actin, nebulin, desmin and β-tubulin. Moreover, by applying immunoelectron microscopy we were able to clarify the nature of titin spots and the fate of titin molecules in particular during early stages of myofibrillogenesis at the ultrastructural level.

**Titin spots are initially associated with intermediate filaments**

Expression of titin is initiated very early in avian (Tokuyasu and Maher, 1987a; Colley et al., 1990) as well as mammalian myofibrillogenesis (Fürst et al., 1989; Schaart et al., 1989; van der Ven et al., 1992). Immediately after or even during synthesis, the titin molecules become resistant to Triton X-100 extraction and are therefore believed to associate with the cytoskeleton (Colley et al., 1990). In our immunofluorescent double labeling experiments we found no evidence for the anchorage of titin molecules to any of the cytoskeletal filament systems at the very early stages of muscle cell differentiation. The results of our ultrastructural studies however, indicate otherwise. Aggregated titin molecules seen in the differentiating human myoblasts by means of immunoelectron microscopy are the most probable counterparts of the titin spots found in the immunofluorescence experiments. These aggregates were associated with 10 nm most probably desmin filaments. These immunogold-labeled clusters, associated with IF, resemble epinemin, an IF-associated protein in 3T3 cells, at least as far as ultrastructural appearance is concerned (Lawson, 1983). Therefore the observed titin-IF association is most likely specific and not due to trapping of titin molecules in the cytoskeletal network as earlier suggested by Isaacs et al. (1989). Our results are in contrast to those of Tokuyasu and Maher (1987b), who could not identify any morphological features corresponding to our immunofluorescent titin spots in very early stages of avian cardiac myofibrillogenesis.

**Is titin associated with MHC in early stages of myofibrillogenesis?**

Expression patterns of titin and myosin were reported to be tightly linked in time as well as in space (Hill et al., 1986; Komiyama et al., 1990; Isaacs et al., 1992). Other authors presented evidence for an earlier expression of titin (Colley et al., 1990; Fürst et al., 1986) or an earlier organization of titin in aggregates (Tokuyasu and Maher, 1987a) when compared to MHC. Our double labeling experiments show that in most skeletal muscle cells titin and MHC are not simultaneously expressed during the earliest stages of human myofibrillogenesis. Although titin can associate with MHC in early stages of human myofibrillogenesis, the relatively low number of cells showing the punctate titin/MHC co-localization indicates that this interaction is either very short-term or non-obligatory. The fact that titin spots were found to be clearly associated with SFLS not containing myofibrillar MHC supports the assumption that the observed early titin/MHC association is non-obligatory. This observation favours the suggestion that titin is the first sarcomeric protein that associates with SFLS, thus forming a scaffold for the integration or remodeling of other sarcomeric proteins, including MHC, within the nascent myofibrils. An interesting model for myofibrillogenesis, in which myofibrils are assembled in close association with SFLS, was provided by Dlugosz et al. (1984). Considering the observed association between myofibrillogenesis and microfilament bundles in vivo (Kelly, 1969), Fischman (1986) concluded that SFLS and the suggested association are not an artifact of in vitro growth conditions.

**Titin molecules unwind after association with SFLS**

Aggregated titin molecules, initially located in close proximity to the nucleus (van der Ven et al., 1992), are targeted to SFLS that are localized closely to the sarcolemma of differentiating muscle cells. IF may have a function in this intracellular transport of titin molecules (see below). The association of the titin aggregates to SFLS in a clearly periodic fashion is probably mediated by specific molecules present within the SFLS. The binding of titin with actin in solid-phase binding assays (Soteriou et al., 1993), supports the assumption of Handel et al. (1989) that these specific molecules are actin. However, the presence of other titin-binding proteins cannot be excluded. Subsequent to this association, the aggregated titin molecules will have to unwind before sarcomeres can be assembled. The observations that antibodies recognizing titin epitopes close to the M line reveal a periodic staining pattern in later developmental stages as compared to antibodies against titin epi-
topes closer to the Z line (Fürst et al., 1989), are also suggestive of a gradual unwinding of titin molecules during myofibrillogenesis. (Auto)phosphorylation of titin has been described in vivo (Somerville and Wang, 1987) as well as in vitro (Takano-Ohmuro et al., 1992). Therefore, it is tempting to speculate that the unwinding process is connected to the state of phosphorylation of the titin molecules. A connection between the phosphorylation of certain sites on the titin molecules and titin’s structural role, has already been suggested by Somerville and Wang (1988).

**Nebulin is incorporated in the sarcomere at a relatively late developmental stage**

The transition of nascent myofibrils, with a non-interrupted homogeneous titin and MHC reactivity pattern, into striated myofibrils was shown to initiate in a distinct region, as structures were found that contained only a few sarcomeres when stained for titin. As soon as titin cross-striations were observed, α-actin, α-actinin and MHC also showed a cross-striated staining pattern. In contrast, nebulin was observed later and only in a striated fashion. Often myofibrils were present in differentiating myotubes that showed the mature, doublet titin staining pattern but not yet nebulin reactivity. The fact that nebulin is expressed at a later developmental stage and that it is one of the last myofibrillar proteins to be integrated within the sarcomere during mammalian myofibrillogenesis, has been described before (Fürst et al., 1989). However, Komiyama et al. (1992) suggested that in their cultures of embryonic chicken skeletal muscle cells, nebulin is assembled into nascent myofibrils before titin and myosin. We cannot confirm the latter results for human skeletal muscle cell cultures. This may be a further indication for a species-specific order of the expression and assembly of proteins during myofibrillogenesis.

**A role for desmin in the assembly of the myofibril?**

IF expression and localization change dramatically during muscle cell maturation. While proliferating myoblasts and young myotubes contain large quantities of filamentous desmin and vimentin, differentiated myotubes do not seem to express vimentin anymore (Bennett et al., 1979; Gard and Lazariades, 1980; van der Ven et al., 1992). Desmin expression remains during all further stages of muscle development. However, the distribution of the desmin filaments changes from a longitudinal filamentous orientation to a cross-striated pattern, due to its localization in the Z disc area. Vater et al. (1992) recently described that in regenerating rat muscle, desmin already links Z-discs of adjacent myofibrils before their alignment, which made these authors conclude that desmin plays an important role in the lateral alignment of myofibrils. Our results support the observation that desmin does not show a cross-striated localization pattern in myofibrils, until adjacent myofibrils are perfectly aligned (Gard and Lazariades, 1980; Hill et al., 1986). Whether the alignment of myofibrils is initiated by desmin filaments was questioned by Hill et al. (1986) and Schultheiss et al. (1991). These authors showed that in cells in which IF assembly was disturbed, myofibrils were normally formed and aligned. They concluded that keeping myofibrils in register after assembly and alignment might be the most probable function for desmin. In this respect IF and the skelemin network, a myofibril-linking filament system at the level of the M-line (Price, 1987) may collaborate in warranting a normal process of myofibrillogenesis in these cells. The fact that this process occurs normally in the absence of IF, does not necessarily mean that IF are non-functional in myofibrillogenesis. Skelemins and desmin may compensate for each others role in this respect.

So far little attention has been paid to a possible role for desmin in the earliest stages of myofibrillogenesis. Our experiments at the immunoelectron microscope level show that titin aggregates are bound to IF before the aggregates associate with SFLS, and that titin remains attached to IF during the titin-SFLS association process. This observation points to a function for IF in the intracellular translocation of titin molecules. Further support is provided by the observation that desmin filaments are redistributed from a dispersed localization in postmitotic mononuclear myoblasts to a longitudinally oriented localization in elongated cells and young myotubes (Bennett et al., 1979; Gard and Lazariades, 1980; van der Ven et al., 1992). The longitudinally oriented IF are laid down alongside developing myofibrils (Vater et al., 1992) at a point in time comparable to the developmental stage that titin spots associate with SFLS in our human skeletal muscle cell cultures.

In summary, we conclude that IF most likely play an important role in the translocation of titin molecules to SFLS during muscle cell differentiation. The association of titin aggregates with SFLS, being the initial event in the formation of sarcomeres, is of cardinal importance for further steps in human myofibrillogenesis in vitro. Unwinding of the titin molecules probably produces a scaffold for the integration of Z line and A band proteins. Nebulin is obviously incorporated into the myofibrils after this process has been completed. Especially, our new findings at the immunoelectron microscope level contribute substantially to the understanding of early stages of human myofibrillogenesis in vitro.

The authors thank the reviewers for their helpful comments and suggestions, Mr H. van der Lee for excellent technical assistance and Mr M. Coelen for photography.

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