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Early incorporation of obscurin into nascent sarcomeres: implication for myofibril assembly during cardiac myogenesis

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Abstract

Obscurin is a recently identified giant multidomain muscle protein whose functions remain poorly understood. The goal of this study was to investigate the process of assembly of obscurin into nascent sarcomeres during the transition from non-striated myofibril precursors to striated structure of differentiating myofibrils in cell cultures of neonatal rat cardiac myocytes. Double immunofluorescent labeling and high resolution confocal microscopy demonstrated intense incorporation of obscurin in the areas of transition from non-striated to striated regions on the tips of developing myofibrils and at the sites of lateral fusion of nascent sarcomere bundles. We found that obscurin rapidly and precisely accumulated in the middle of the A-band regions of the terminal newly assembled half-sarcomeres in the zones of transition from the continuous, non-striated pattern of sarcomeric α-actinin distribution to cross-striated structure of laterally expanding nascent Z-discs. The striated pattern of obscurin typically ended at these points. This occurred before the assembly of morphologically differentiated terminal Z-discs of the assembling sarcomeres on the tips of growing myofibrils. The presence of obscurin in the areas of the terminal Z-discs of each new sarcomere was detected at the same time or shortly after complete assembly of sarcomeric structure. Many non-striated fibers with very low concentration of obscurin were already immunopositive for sarcomeric actin and myosin. This suggests that obscurin may serve for organization and alignment of myofilaments into the striated pattern. The comparison of obscurin and titin localization in these areas showed that obscurin assembly into the A-bands occurred soon after or concomitantly with incorporation of titin. Electron microscopy of growing myofibrils demonstrated intense formation and integration of myosin filaments into the “open” half-assembled sarcomeres in the areas of the terminal Z–I structures and at the lateral surfaces of newly formed, terminally located nascent sarcomeres. This process progressed before the assembly of the second-formed, terminal Z-discs of new sarcomeres and before the development of ultrastructurally detectable mature M-lines that define the completion of myofibril assembly, which supports the data of immunocytochemical study. Abundant non-aligned sarcomeres in immature myofibrils located on the growing tips were spatially separated and underwent the transition to the registered, aligned pattern. The sarcoplasmic reticulum,

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This paper is dedicated to the memory of Professor Pavel P. Rumyantsev (1927–1988), a pioneer in studies of cardiac muscle differentiation, who is a lasting inspiration to all who worked with him.

the organelle known to interact with obscurin, assembled around each new sarcomere. These results suggest that obscurin is directly involved in the proper positioning and alignment of myofilaments within nascent sarcomeres and in the establishment of the registered pattern of newly assembled myofibrils and the sarcoplasmic reticulum at advanced stages of myofibrillogenesis.

Keywords

Cardiac myocytes; Myofibrillogenesis; Myosin; Obscurin; Sarcomere; Sarcoplasmic reticulum; Z-disks

Introduction

Despite all that has been learned during the last three decades concerning the control of contractile protein expression in cardiac myocytes, the mechanisms underlying the assembly and alignment of myofibrils into precisely regular longitudinal arrays remain incompletely defined. The current views regarding the structural aspects of this process considerably differ. Four major hypotheses seeking to explain the mechanism of myofibril formation have been recently reviewed by Sanger et al. (2005). These hypothetic models can be summarized as following: (1) template model that considers stress fiber-like structures as templates or scaffolds for assembly of myofibrils (Dlugosz et al. 1984); (2) the “stitching” model in which the actin filaments and Z-bands assemble independently from myosin filaments (Schultheiss et al. 1990; Holtzer et al. 1997; Ojima et al. 1999); (3) premyofibril model assuming the formation of premyofibrils as immediate structural precursors of myofibrils (Rhee et al. 1994; Du et al. 2003; Sanger 2005), and (4) direct assembly of myofibrils without any intermediate structures [for literature see Ehler et al. (1999, 2004)].

One of the major points of long-standing disagreements between these concepts concerns the mechanism of formation and integration of the thick (myosin) filaments into nascent myofibrils. Several authors noted the delay in myosin filament assembly, or integration that followed actin filament formation during differentiation of contractile structures (for discussion see Ehler et al. 1999, 2004; Sanger 2005). Surprisingly little is known about the cellular and molecular pathways regulating spatial and temporal dynamics of contractile filaments integration. The mechanisms of remodeling of non-striated precursors of myofibrils into the striated sarcomeric pattern also remain poorly understood. During the 1980–1990s, the limited optical resolution of immunofluorescence technique did not allow us to perform precise analysis of protein localization dynamics within the nascent sarcomeres. In recent years, the development of high resolution confocal microscopy, new generation of the equipment for digital imaging and the probes specific for different stages of myofibrillogenesis have opened new perspectives for a better understanding of this process.

The classical cross-bridge, actin–myosin sliding filament theory of sarcomere contraction proved to be more complex than it was initially suggested (for discussion see Huxley 2000). A significant progress in this field resulted from the studies of the third filament system of sarcomeres formed by titin and its associated proteins that extend from the Z-discs to M-lines of myofibrils (Tskhovrebova and Trinick 2003; Granzier and Labeit 2005). It was demonstrated that two giant muscle proteins, titin and nebulin/nebulette, are involved in patterning and defining the spatial dimensions of sarcomere compartments, ensuring the elasticity of myofibrils. These proteins are also involved in sensing stretch, signal reception and transduction under conditions of changing functional load and during adaptive responses (reviewed by Epstein and Davis 2003; Lange et al. 2006; LeWinter et al. 2007). These data led to the shift from the perception of myofibrils as purely mechanical, force-generating structures

to viewing them as highly adaptable and complexly regulated cross-points of several signaling pathways involved in cellular regulation of muscle function.

Obscurin is the third, recently identified, member of the family of giant muscle specific proteins. This is a giant multidomain protein (~800 kDa) localized in the areas of M-lines and Z-disks of myofibrils (Bang et al. 2001; Young et al. 2001; Russell et al. 2002; Bagnato et al. 2003; Kontogianni-Konstantopoulos et al. 2003; Borisov et al. 2003, 2004). The complex primary structure of the gene encoding obscurin suggests that it has multiple functions (Bang et al. 2001; Young et al. 2001; Russell et al. 2002; Fukuzawa et al. 2005). Obscurin interacts with titin (Bang et al. 2001, Young et al. 2001) and small ankyrin 1 (Bagnato et al. 2003; Kontrogianni-Konstantopoulos et al. 2003). Earlier we found that the expression of obscurin gene is rapidly up-regulated during progressive heart hypertrophy (Borisov et al. 2003). Downregulation of this protein in cardiac and skeletal muscle resulted in misalignment of myofibrils and multiple defects in the their assembly including the block of proper positioning of myosin filaments (Borisov et al. 2006; Kontrogianni-Konstantopoulos et al. 2006; Raeker et al. 2006). Thus, understanding of the functional role of obscurin in integration and registered alignment of sarcomeric filaments during myofibrillogenesis will provide new information regarding the mechanisms of assembly and structural support of the myocardial contractile apparatus.

In this study, we used immunolabeling for the sarcomeric isoform of α-actinin, a major protein of Z-discs, as a marker of nascent myofibrils and their precursors at different stages of their development. α-Actinin is one of the earliest proteins incorporating into differentiating myofibrils and defining their striated pattern at early stages of assembly (for literature see Tokuyasu and Maher 1987; Borisov 1991; Sanger et al. 2005). We examined the spatial patterns and the temporal dynamics of obscurin assembly into nascent terminal sarcomeres located on the tips of growing myofibrils in expanding myofibrillar clusters. Special attention was focused on the areas of remodeling, transformation and alignment of non-striated precursors of myofibrils into well registered cross-striated structures typical of differentiated cardiac muscle. To address these questions, we studied the dynamics and spatial interrelations of α-actinin and obscurin incorporation in the areas of transition from nonstriated to striated patterns located on the edges of developing myofibrils and compared these patterns to the localization of other sarcomeric components using confocal and electron microscopy.

Materials and methods

Primary cell cultures of cardiac myocytes

Primary cell cultures of rat cardiac myocytes were isolated from the hearts of neonatal rats by enzymatic dissociation of myocardial tissue as previously described (Borisov et al. 1985, 1989) and plated on glass coverslips in eight-well tissue culture plates (Corning, Inc., Corning, NY, USA). The cell cultures were cultivated in a CO_2 incubator (5% CO_2) in F-12 medium (GIBCO, NJ, USA) containing 10% of bovine fetal serum (GIBCO). The cells were collected for labeling every day beginning day 2 after isolation in culture.

Immunolabeling and confocal microscopy

Cell fixation, processing, and immunostaining were performed as previously described (Borisov et al. 2001). The development, characterization and specificity of the monoclonal antibody (MAb)EA-53 to the sarcomeric isoform of α-actinin was described by Fridlianskaia et al. (1989). This antibody is now available commercially from Sigma Chemical Company, St Louis, MO, USA). In our earlier publications, we demonstrated the applicability of this antibody for studies of skeletal and cardiac myofibrillogenesis (Fridlianskaia et al. 1989; Borisov et al. 1989). In recent years, this antibody was used in a large number of laboratories

for detection of sarcomeric α-actinin in cardiac and skeletal muscle in vivo and in cell culture (e.g. Bagnato et al. 2003; Sanger et al. 2005; Musa et al. 2006; Kontrogianni-Konstantopoulos et al. 2003, 2005).

A polyclonal antibody recognizing the carboxy-region of obscurin was prepared in a rabbit host using standard methods, and its specificity has been examined (Kontrogianni-Konstantopoulos et al. 2003). This antibody was kindly provided to us by Dr. A. Kontrogianni-Konstantopoulos and Dr. Robert Bloch (University of Maryland, Baltimore, MD, USA). An anti-titin monoclonal antibody, clone 9D10, binding to the PEVK domain of titin in the I bands near the I–A interfaces of sarcomeres (Trombitás et al. 1998) was obtained from the Developmental Studies Hybridoma Bank, University of Iowa (Iowa City, IA, USA). The monoclonal antibody MF20 specifically binding to all isoforms of sarcomeric myosin heavy chains (Bader et al. 1982) was also purchased from the Developmental Studies Hybridoma Bank. The secondary antibodies, FITC-conjugated anti-mouse and TRITC-conjugated anti-rabbit, were obtained from Sigma Chemical Company. Immunolabeled samples of cultured cardiac myocytes were mounted on slides and examined with a Carl Zeiss LSM 510 Meta confocal microscope using the 40× and 63× objectives.

Electron microscopy

Cells were rapidly fixed by immersion in ice-cold solution of 2.5% glutaraldehyde in 0.1 M isoosmotic cacodilate buffer, pH 7.4 for 4 h. After removal of the fixative, the samples were washed in three changes of 0.1 M phosphate buffer; each wash was for a period of 15 min. and were postfixed with 1% OsO_4 in 0.1 M phosphate buffer for 1.5 h at 4°C, washed again in the buffer solution three times (15 min each wash) and dehydrated through graded ethanol series to absolute ethanol and acetone. After infiltration with Epon/Araldite the samples were embedded in Epon/Araldite medium (Eponate 12-Araldite 502 kit, Ted Pella, Inc.). After polymerization of the medium in blocks, sections were prepared using a Reichert-Young Ultracut E ultramicrotome and mounted on grids. Following contrast staining with uranyl acetate and lead citrate, the sections were examined with a Philips CM 100 electron microscope at an accelerating voltage 60 kV.

Results

We found that in cell cultures of rat cardiac myocytes, sarcomeric α-actinin was localized in three types of structures (Fig. 1). The first type was represented by mature or nascent myofibrils expressing sarcomeric cross-striations. The second type is defined as non-striated fibers with a continuous intense labeling pattern of α-actinin localization. The third type consists of thin, narrow fibers with a variably spaced punctate or dotted pattern of α-actinin distribution with the periodicity that typically comprised one-fifth to two-thirds of the normal sarcomere length. The third type was localized mainly in the peripheral areas of the cytoplasm. We occasionally observed the areas of direct transition from the continuous to punctate or dotted pattern along the non-striated fibers (Fig. 1a, b). This fact indicates that the fibers expressing the dotted pattern appear to differentiate into continuously labeled structures.

Confocal microscopic analysis of obscurin and α-actinin localization following double immunofluorescent labeling of these proteins demonstrated that intense incorporation of obscurin occurred in nascent myofibrils that developed at the ends or in the middle of non-striated continuous fibers intensely immunopositive for α-actinin (Figs. 1a). These non-striated fibers represented the second type of α-actinin-positive structures mentioned above. At early stages of formation, myofibril clusters were small and consisted of two or three sarcomeres that contained obscurin in the middle of the A-bands (Fig. 1b, c). They were flanked, or “capped”, at both ends by non-striated regions immunopositive for α-actinin and negative or weakly immunopositive for obscurin, and were oriented at slightly different angles to one

another. These clusters were more abundant in the central, perinuclear areas of myocytes than at the cell periphery (Fig. 1a–c). The length of the non-striated regions that directly continued on the tips of myofibrils remained approximately constant and comprised 2–6 lengths of mature sarcomeres (Fig. 1b, c, 2a).

Further stages of myofibril assembly were associated with the formation of structural connections between spatially separated clusters. This process progressed as the result of growth and extension of longitudinally oriented non-striated bundles that linked individual developing clusters of myofibrils (Fig. 2b, 3). Independently of the sarcomere number in individual clusters, the myofibrillar cross-striated pattern always included obscurin in the areas of the A-bands. Transformation of non-striated bundles connecting the adjacent clusters into differentiating myofibrils advanced from their both ends toward each other gradually closing myofibril-free gaps (Fig. 3). At this stage, the longitudinal and transverse alignment of myofibrils quickly developed, and mature sarcomeres acquired precise parallel orientation in relation to one another (compare Figs. 1a, 3).

During this process, growing myofibrils laterally fused and formed large mature myofibril core that further expanded toward the peripheral areas of the cells as a result of longitudinal addition of new sarcomeres at the tips of its growing edges (Figs. 2b, 3). The intense immunopositivity of obscurin typically ended in terminal sarcomeres at the points of transition from striated to non-striated patterns (Fig. 1b, c, 2a, b). Interestingly, at this stage of myofibril assembly, the edges of obscurin-immunopositive regions at the lateral surfaces of sarcomeric A-bands frequently extended outside the contours of nascent Z-discs (compare Figs. 1b, c, 2a, b). Single separated obscurin-positive bodies were found on the lateral surfaces and between the nascent myofibril bundles (Figs. 1c, 2a, b). The formation of these discrete obscurin-positive structures preceded their lateral fusion. Thus, obscurin assembly into nascent contractile apparatus was directly associated both with the development of cross-striations in new nascent sarcomeres in elongating myofibrils and temporally and spatially coincided with the process of longitudinal and transverse alignment at the lateral surfaces of differentiating contractile structures at the level of M-lines and Z-discs.

To understand better the function of obscurin in this process, we examined the dynamics of its localization in the tips of growing myofibrils at the points of their direct transition into continuously labeled, non-striated fibrous structures using high resolution confocal microscopy. Structural analysis of these regions at high magnification revealed several important characteristics of obscurin integration during myofibril assembly. We found that incorporation of obscurin in the middle of A-bands did occur in the sites of transition from striated to non-striated pattern of α-actinin labeling (Figs. 4a–c, 5a–c). This process was topographically localized in the areas of transition from fork-like bifurcations of non-striated myofibrillar precursors to striated myofibrils (Figs. 4, 5). At least some bifurcations formed as the result of lateral contacts of fusing pairs of non-striated myofibrillar precursors before the development of striated pattern. It is important to note that obscurin was detected in nascent myofibrils immediately after morphological differentiation of the first-formed Z-bands of each terminal sarcomere as the first element of cross-striation (Figs. 4, 5). The assembly of the second-formed, terminal Z-discs of sarcomeres in such situations typically was not completed. It is essential to note that at the early stages of sarcomere assembly obscurin-immunopositive areas in the middle of A-bands were located closely to the persisting elements of non-striated structures that were intensely immunopositive for α-actinin and sometimes directly contacted these structures (Figs. 4, 5). In these regions, we did not observe distinct immunoreactivity for obscurin in the second-formed nascent Z-discs of terminal sarcomeres (Fig. 5b). This suggests that obscurin integrates early and rapidly in the middle of nascent sarcomeres, before assembly and differentiation of the complete Z- to Z-disc sarcomere structure.

In many cases at this stage of early cluster formation obscurin was expressed in the spaces between the pairs of laterally fusing non-striated structures at the interfaces of striated and non-striated regions of laterally fusing bifurcating non-striated myofibrillar precursors (Figs. 1c, 2a, b). High resolution optical analysis demonstrated that obscurin-containing dots can be found at the levels of mid-A-bands, close to the lateral surfaces of forming myofibrillar clusters (Figs. 1c, 2b). The dotted pattern was replaced by uninterrupted bands as the result of transverse expansion of obscurin at the level of mid-A-bands and Z-discs. This process leads to lateral binding of 2–4 neighboring nascent premyofibrillar structures together into one cluster at the level of A-bands and nascent Z-discs (Figs. 3, 4, and 5).

In these areas we found nearly synchronous incorporation of obscurin in 2–3 spatially separated bundles (Fig. 4a, b) with the subsequent expansion and lateral fusion of obscurin-positive areas into one continuous band expanding through a whole cluster (Fig. 4a–c). This supports our observations made using low- and intermediate-magnification microscopy and indicates that obscurin might be involved in the process of lateral addition of newly formed myofibrils and myofibrillar bundles during the expansion and growth in width of the developing contractile apparatus.

Thus, during the expansion of myofibril clusters, similarly to the early stages of cluster formation, the incorporation of obscurin in the tips of growing myofibrils occurred just after the formation of the first-formed Z-discs of each new sarcomere, before the formation of a distinct structural pattern of the second-formed terminal Z-discs (Figs. 4a–c, 5a–c). Obscurin integrated into the mid-A-band areas during or soon after lateral fusion of two or more neighboring non-striated bundles precisely at the sites of the transition to the striated pattern (Fig. 4a–c). Formation of α-actinin-negative spaces in the continuously labeled non-striated structures typically preceded the incorporation of obscurin in these regions (Fig. 4a). After formation of rounded obscurin-positive bodies in these spaces, the areas intensely immunopositive for obscurin expanded transversely and occupied the gaps located above individual nascent Z-bands and below parallel non-striated myofibril precursors. The dynamics of this process is illustrated in Fig. 4a–c.

An important structural characteristic of obscurin assembly into nascent myofibrils at all stages of myofibril cluster formation is its lateral incorporation in the gaps between fusing bundles of newly formed striated myofibrils as a result of its expansion outside their lateral surfaces (Figs. 2a, b, 4b). The next stage can be defined as the lateral fusion of laterally expanding obscurin dots. It is important to note that this process progressed before the formation of the second Z-disc of each forming sarcomere (Fig. 5). Therefore, we can conclude that the process of lateral stitching of bifurcated or trifurcated non-striated bundles and their longitudinal transformation into striated myofibrils underlies the progression of lateral expansion, differentiation and fusion of individual myofibrillar clusters.

A notable detail of this process is the preservation of the longitudinal orientation of the elements of non-striated fibers and their contact with the nascent Z-discs at the time of obscurin incorporation. In many cases, obscurin incorporated in square and rectangular “boxes” formed by nascent Z-discs from the side of nascent myofibril, two lateral sides formed by longitudinally oriented non-striated fibers on the sides, and by fusing two non-striated fibers on the top (Fig. 4). Fusing α-actinin-positive regions of non-striated fibers differentiated into the transversely oriented second Z-discs of newly formed sarcomeres. After this process was completed, the lateral surfaces of non-striated fibers progressively disassembled and individual obscurin-positive and α-actinin-positive bodies fused into transversely oriented bands at the level of mid-A-bands and Z-discs, respectively. The cross-striated pattern typical of mature myofibrils formed as a result of this fusion.

At the time of obscurin incorporation, most of the first Z-discs still had widened structure typical of immature muscle, and the second Z-discs were not clearly distinguishable. Their differentiation occurred after lateral fusion of nonstriated fibers and followed their transformation into striated pattern typical of nascent myofibrils in larger clusters.

It is essential to note that during sarcomere formation, the lateral expansion of obscurin approached and extended into the lateral elements of longitudinally oriented nonstriated structures (Fig. 4a–c) and was detected outside the lateral surface of sarcomeres following clearing the lateral spaces from the remaining fragments of non-striated fibers. High resolution microscopy also demonstrated that small obscurin-positive bodies were present in longitudinally oriented non-striated fibers close to the areas of terminal nascent sarcomeres (Fig. 4b, c, 5a, b). Incorporation of obscurin into nascent myofibrils can progress from both ends of non-striated premyofibrillar structures.

Thus, the dynamics of obscurin incorporation in such areas suggests that this protein is directly involved in a zipper-like fastening mechanism that longitudinally and transversely “stitches” nascent myofibrils to one another during myofibrillogenesis and participates in the establishment of the cross-striated morphology of the contractile apparatus typical of the differentiated muscle.

The comparison of the localization patterns of obscurin and α-actinin to the localization of sarcomeric actin and myosin, the major proteins of thin and thick filaments generating mechanical force, showed that the sarcomeric isoforms of actin and myosin were abundantly expressed in non-striated fibrillar structures that were only weakly immunopositive or immunonegative for obscurin (Fig. 6a–d). These illustrations demonstrate that despite low concentrations or the absence of obscurin in non-striated fibers, they do contain sarcomeric proteins necessary for formation of A- and I-bands, and Z-discs. This finding suggests that obscurin is not a critical element necessary for targeting and primary incorporation of sarcomeric filament proteins into the differentiating premyofibrillar structures. Thus, obscurin appears to be more important for establishment, organization and stabilization of myosin sarcomeric pattern.

Immunolocalization of titin, a known binding partner of obscurin, at the sites of active myofibrillogenesis demonstrated that the striated pattern of this protein in the precursors of myofibrils developed earlier than the striated pattern of obscurin (Fig. 7). Transverse striations of immunolabeled titin in nascent myofibrils were typically present in at least 3–4 nascent sarcomeres beyond the points of bifurcation of myofibrillar precursors, and evident residual striation was detectable even at greater distances from the points of their branching (Fig. 7a, b). Given the fact that obscurin incorporation in these areas only occurred very close to the points of bifurcation (Figs. 4, 5), the comparison of the localization patterns of these two proteins shows that the incorporation of titin precedes the incorporation of obscurin. Unlike obscurin, titin does not localize between the laterally fusing bundles, which suggest some difference in the functions of these two proteins. In the clusters of mature myofibrils, titin acquires uninterrupted laterally aligned pattern (Fig. 7c) that is similar to the uninterrupted pattern of laterally aligned obscurin (Fig 3). More detailed interrelations of temporal and spatial patterns of titin and obscurin incorporation are the subject of our current studies.

To further define the role of obscurin in myofibril assembly, we examined the ultrastructural characteristics of the differentiating contractile apparatus and the growing ends of myofubrils in cultures of cardiac muscle cells (Figs. 8, 9, 10). Using electron microscopy we found that myosin filaments were present in immature A-bands of newly formed myofibrils at early stages of their development (Fig. 8). Interestingly, at this time the elements of the sarcoplasmic

reticulum were already detectable in proximity to newly assembled sarcomeres (Figs. 8b, 10a, b).

The studies of more advanced stages of myofibril assembly showed that incorporation of myosin filaments typically occurred after the formation of half-sarcomere Z–I-interfaces on the tips of growing myofibrils (Fig. 9). Elongating myofilaments were usually associated with multiple large and small polyribosomes that were attached to their ends (Fig. 9a–d). These observations suggest that assembly of the A-bands, at least in this experimental model, progresses on pre-formed complexes of actin filaments attached to the newly formed Z-discs of the nascent sarcomeres. The presence of polyribosomes associated with myosin filaments on the lateral surfaces of nascent sarcomeres and free myosin filaments located close to the nascent myofibrils and oriented precisely along the long axis of forming myofibrils in muscle cells. (Fig. 9c, d) suggests that myofibrils can grow laterally by addition of contractile filaments from their sides. A significant difference in the width of two neighboring Z-discs frequently observed in the same sarcomere suggests that the process of lateral growth can also progress at the level of the Z-discs (Fig. 9c, d).

Misalignment of myosin filaments and nascent Z-discs in immature terminal sarcomeres located in the tips of myofibrils (Figs. 9a, 10a) suggests that the presence of obscurin detected by immunolabeling in these regions is required for the alignment of the sarcomeric components into the registered and aligned pattern. These data of ultrastructural studies strongly support our results obtained using confocal microscopy of immunolabeled cell samples. The presence of overlapping non-aligned or poorly registered nascent myofibrillar precursors containing clear elements of all sarcomere compartments, such as the Z-discs, and more or less differentiated actin and myosin filaments located at different levels and angles in relation to one another explains the structure of non-striated continuous fibers visualized by immunolabeling for all three major sarcomeric proteins (Figs. 8, 9, and 10). Myosin filaments in the terminal sarcomeres were also usually poorly aligned and were oriented at angles to one another.

An essential ultrastructural characteristic of nascent and newly assembled myofibrils in our experimental model is nearly complete absence of morphologically mature M-lines in these regions despite the fact that the electron-lucent H-zones were always well differentiated (Figs. 9, 10). The absence of structurally detectable M-lines in newly assembled cardiac myofibrils shows that the incorporation of obscurin into nascent sarcomeres precedes the advanced stages of assembly of A-bands and M-line complex.

Thus, the data of electron microscopy confirmed the fact the formation of the second Z-disc on each sarcomere occurred following integration of myosin filaments into the nascent structure. This observation is further supported by the fact that during differentiation of myofibrils, the periodicity of Z-disc to Z-disc spaces slightly increased from 1.3–1.6 μm in nascent terminal sarcomeres to 1.8–2.0 μm in more mature myofibrils (Figs. 2b, 4b, and 5c). This elongation can be explained by the elongation of myosin filaments and actin filaments in nascent sarcomeres during the process of myofibril maturation. The data presented above can be summarized in the schematic diagram that outlines the possible mechanism of the functional role of obscurin during the process of myofibrillogenesis (Fig. 11).

Discussion

The results of this study demonstrate that obscurin integrates into the middle area of A-bands on the tips of nascent myofibrils relatively early, soon after the assembly of the first differentiated Z-discs and before the completion of formation of the terminal Z-discs of each newly added sarcomere. Of special interest for the discussion is early incorporation of obscurin

in the areas of transition from a continuous to periodic pattern of α-actinin localization. The mechanism of formation of clear, "empty" spaces immunonegative for α-actinin within continuously labeled non-striated fibers that become the sites of obscurin accumulation remains unclear. Given the fact that electron microscopy shows the presence of superimposing free myofilaments and incompletely assembled, poorly registered clusters of sarcomeres in these areas, it appears that the periodicity of α-actinin develops as the result of the alignment of sarcomeric proteins into longitudinally and transversely registered pattern typical of nascent myofibrils. This process apparently includes significant spatial remodeling of contractile protein complexes and their progressive differentiation into pre-sarcomeric and sarcomeric structures. Thus, our data suggest that obscurin is involved both in the longitudinal addition of new sarcomeres on the tips of growing myofibrils and in their lateral alignment into the cross-striated register.

The continuous labeling pattern of non-striated myofibril precursors for the sarcomeric types of α-actinin, myosin, and actin (Fig. 6a–d) and considerably lower concentrations or the absence of obscurin in these structures (Fig. 5a–c) provide additional evidence that obscurin is primarily involved in the organization of myofilaments into registered and aligned sarcomeres at advanced stages of myofibrillogenesis rather than in the primary assembly of their non-striated, longitudinally oriented precursor structures. Double labeling for myosin/obscirin and actin/obscurin using the antibody to the carboxy-terminus of obscurin demonstrated that in newly assembled sarcomeres obscurin was localized in the middle of the A-bands and was not co-localized with actin-containing I-bands (Kontrogiann-Konstantopoulos et al. 2005, 2006). Differentiation of muscle cells and myofibril formation are accompanied by the progressive switches of contractile protein isoform expression (review Sanger et al. 2005). For this reason the incorporation patterns of different myosin and actin isoforms in relation to the structural dynamics of obscurin during myofibril assembly represent special interest. This issue is the focus of our current studies based on double and triple immunolabeling of these proteins and high resolution image analysis.

It is also important to mention that obscurin contains Rho-GEF domain that may activate small Rho GTPases and was shown to be directly involved in remodeling of actin cytoskeleton in several cell types (Burridge and Wennerberg 2004). It was recently shown that Rho-GTPases were up-regulated in developing chicken heart (Kaarbo et al. 2003) and their functional inhibition disrupts cardiac myogenesis in mice (Wei et al. 2002).

A progressive increase in the length of nascent sarcomere during differentiation has been reported by Sanger et al. in the avian embryonic cardiomyocytes (reviewed by Sanger et al. 2005). This observation became one of the key elements of the model of myofibrillogenesis suggested by these authors (Du et al. 2003; Sanger et al. 2005). The fact that the initial stage of obscurin incorporation is associated with the formation of a narrow, gradually expanding space above the first-formed terminal Z-disc of each sarcomere agrees well with these data. The presence of myosin filament–polyribosome complexes shows that elongation of myosin filaments can occur following the integration of shorter myosin filaments into the structure of nascent sarcomere before the completion of its Z-disc to Z-disc outline. During this process, obscurin together with other interacting proteins, such as myomesin and titin, might be involved in local fixation and stabilization of myosin filaments during their progressive differentiation. The location of free immature thick filaments near the sites of intense myofibrillogenesis shows that at least some of them can initially assemble outside the nascent sarcomeric structure. At the same time the transient preservation of persisting elements of α-actinin-positive longitudinally orientated non-striated fibers after development of transverse Z-discs suggests that non-striated structures may perform temporary template-like supporting functions.

It is essential to note that most of the information used for the development of the four current schemes of myofibrillogenesis was obtained in studies of early stages of muscle development and in experiments with embryonic muscle cells in situ and in culture. Interestingly enough, we observed the elements of all four schemes in our material. For example, it appears that there is a second, less frequent, structural pattern of obscurin integration that includes its incorporation between punctate, periodically arranged α-actinin-containing Z-bodies. This occurs at earlier stages of the contractile system formation at the time of assembly of first myofibrils (prepared for publication). Thus, the differences in current four schemes may be explained by the structural variations of this process in the cells of different stages of differentiation that belong to different striated muscle types in different species.

A paradoxical finding of this study is the fact that despite the presence of Z-, I-, A- and H-regions in newly formed myofibrils clearly detected by electron microscopy, the M-lines in the cells were absent or poorly developed. This suggests that obscurin incorporation into developing myofibrils occurs before formation of morphologically identifiable, differentiated M-lines. Assembly of mature M-bands in mammalian cardiac muscle occurs at terminal stages of differentiation. At prenatal stages of differentiation, the myofibrils of most of the mammalian ventricular myocytes, including rats and mice, are devoid of M-lines (for discussion see Rumyantsev 1976, 1977, 1991). Similar data were reported by Anversa et al. (1981) who found that in rat cardiac muscle, the formation of M-lines occurred during the postnatal period when the myofibrillar contractile apparatus was already very well developed. The results of immunolabeling experiments confirmed the data of electron microscopy. M-lines and their immunocytochemical marker, MM-creatine kinase, appeared in rat ventricular myocytes by week 4 of postnatal life (Carlsson et al. 1982).

The current views concerning the functions of M-lines assume that these structures serve as elastic web crosslinking myosin filaments in the center of sarcomere as “a safeguard” for sarcomere stability (Agarkova et al. 2003; Lange et al. 2005). Relatively little is known about the three-dimensional structure of protein interaction and detailed protein composition of M-lines (Agarkova et al. 2003). Emerging data suggest that at least some of their protein components work as molecular springs with adaptable elasticity (Schoenauer et al. 2005; Granzier and Labeit 2006) and are involved in signal transduction (Agarkova et al. 2005).

Yeast two-hybrid analysis demonstrated that obscurin is a binding partner of titin (Bang et al. 2001; Young et al. 2001). However, little is known as yet concerning the structural and functional interrelations of these two proteins in muscle cells. In this study, we found that titin incorporation at the sites of new sarcomere addition slightly preceded the incorporation of obscurin. Earlier expression of titin beyond the points of bifurcation and trifurcation of nascent myofibrils occurred before their lateral fusion, suggesting that these two proteins have distinct functions at different stages of myofibrillogenesis. The fact that the incorporation of titin may precede the accumulation of obscurin is consistent with the role of titin as a scaffolding protein for nascent myofibril assembly (Tokuyasu and Maher 1987; Trinick and Tskhovrebova 1999). These data also suggest that both obscurin and titin, possibly interacting to each other and other proteins, may be directly involved in the longitudinal and lateral determination of the sarcomere outline. It appears that these interactions involve the coordinated expression of the third muscle giant protein, nebulin/nebulette known to be involved in control of the assembly and length of actin filaments and Z-disc structure (Witt et al. 2006). Therefore, obscurin may play a role of a versatile linking adaptor involved in the process of integration and stabilization of sarcomeric filaments at the time of their incorporation into the nascent myofibrils. Of special interest is the fact that obscurin is present on the lateral surfaces of nascent myofibrils and between the forming narrow myofibril bundles. Yeast two-hybrid assay screening recently showed that obscurin interacts with small ankyrin 1, and immunolabeling revealed close localization of these two proteins in skeletal muscle fibers (Bagnato et al.

2003; Kontrogianni-Konstantopoulos et al. 2003; Armani et al. 2006). Small ankyrin 1 is a ligand protein structurally associated with the sarcoplasmic reticulum (Porter et al. 2005). The interaction of these proteins shows that obscurin may perform the function of a molecular link between myofibrils and the sarcoplasmic reticulum. This explanation is consistent with our recent finding that down-regulation of obscurin expression results in dissociation and structural disorganization of the sarcoplasmic reticulum and myofibrils in mouse skeletal myotubes differentiating in cell culture (Kontrogianni-Konstantopoulos et al. 2006) and in zebrafish skeletal muscle in vivo (Raeker et al. 2006).

In our experiments, electron microscopy showed that the formation of the sarcoplasmic reticulum occurred concomitantly with assembly of new myofibrils. This suggests that early incorporation of obscurin in these regions might be necessary for docking, clustering and lateral alignment of the Ca^{2+}-release apparatus at the spatially proper positions proximally to newly formed sarcomeres. Thus, the incorporation of obscurin may mediate the structural coupling of newly formed myofibrils with the calcium-transporting system. The data presented above provide an additional support to our hypothetic scheme illustrating the role of obscurin in myofibrillogenesis and maintenance of structural and functional integrity of the contractile apparatus and the sarcoplasmic reticulum outlined in Fig. 11.

This model suggests that obscurin is one of the key integrating proteins involved in stabilization of myofilaments and in the process of lateral alignment of myofibrils and structurally associated organelles. We did not include in the current scheme different possible modes of interactions between titin and obscurin within nascent and completely assembled myofibrils because very little is known about these interactions at the present time.

Finally, it is interesting to discuss briefly the data concerning the effects of down-regulation of giant muscle proteins, titin, nebulin and obscurin, on the structure of the contractile apparatus of striated muscle. The deletion of the M-band region of titin resulted in significant impairment of sarcomere assembly in cardiomyocytes derived from embryonic stem cells (Musa et al. 2006). Similar abnormalities of myofibrillogenesis have been reported in titin M-line deficient mice (Peng et al. 2005; Weinert et al. 2006) and in mouse skeletal myotubes that differentiated in culture following titin truncation (Miller et al. 2003). Reduction of titin expression using morpholino antisense oligonucleotides disrupted the assembly of Z-discs and A-bands of myofibrils in zebrafish (Seeley et al. 2007). Significant abnormalities of the Z-disk structure, shortened actin filaments, dysregulation of genes involved in calcium homeostasis and glycogen metabolism have been reported in nebulin-deficient mice (Witt et al. 2006). Several types of severe defects of myofibril assembly affecting different elements of the contractile system occurred in cardiac myocytes of rats (Borisov et al. 2006), mouse skeletal myotubes (Kontrogianni-Konstantopoulos et al. 2006) and in zebrafish cardiac and skeletal muscle (Raeker et al. 2006) following selective downregulation of obscurin expression. A recent report also suggests that mutations in the titin-binding domain of obscurin may cause hypertrophic cardiomyopathy (Arimura et al. 2007). Taken together, these studies demonstrate that the functional deficiency of each of three muscle giant proteins directly affects several sarcomere regions and disrupts myofibrillogenesis. This is consistent with the localization of these proteins at different sarcomeric compartments and their signaling properties in addition to the structural involvement in the process of myofibril assembly. Of special interest is the fact that functional impairment of obscurin expression in cardiac (Borisov et al. 2006) and skeletal muscle (Kontrogianni-Konstantopoulos et al. 2006) results in similar effects on the structure of the contractile apparatus as the down-regulation of titin function (Person et al. 2000; Musa et al. 2006; Seeley et al. 2007).

It is still diYcult to conclude whether the multiple effects of down-regulation of each of “three muscle giants” can be explained by the loss of a single protein expression per se, or these effects

are derived from the feedback disruptions of functional interactions of several binding partners and signaling pathways. The emerging role of the giant muscle protein networks as dynamic switchboards that regulate the function of the contractile apparatus and support control of signal transduction has been recently discussed by Lange et al. (2006).

The identification of the functional role of individual structural and signaling domains of obscurin during cardiomyogenic differentiation, in normal adult cardiac muscle and under conditions of pathology will be the focus of our future studies.

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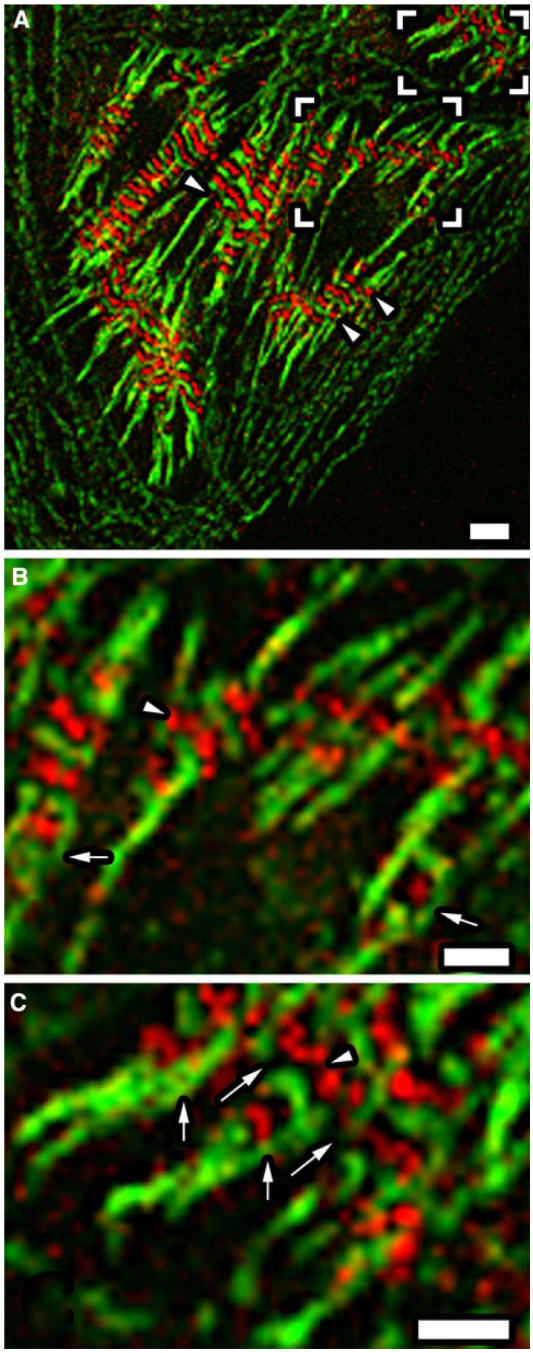

Fig. 1.

Obscurin incorporation during formation of individual myofibrillar clusters. **a** Assembling myofibrillar clusters in a cardiac muscle cell immunolabeled for sarcomeric α-actinin (*green fluorescence*) and obscurin (*red fluorescence*) shown at a low magnification. Immunolabeling for obscurin is observed as a striated pattern of myofibrils directly connected to non-striated continuous fibers intensely immuno-positive for the sarcomeric isoform of α-actinin. Note nearly complete absence of obscurin in the structures expressing the *dotted and dashed patterns* of α-actinin distribution. The *frame corners* indicate the areas shown in panel **b** (the lower set of *corners*) and **c** (the upper set of *corners*) at a high magnification. **b** The localization of sarcomeric α-actinin (*green*) and obscurin (*red*) in early myofibrils following formation of

the first 2–3 differentiated sarcomeres. Note the presence of non-striated fibers (*green*) bifurcating from a nascent myofibril. The *arrows* show transiently preserved longitudinally oriented regions of *α*-actinin-positive non-striated fibers on each lateral side of the terminal sarcomeres on the tips of growing myofibrils at the areas of transition from the non-striated to striated pattern. Obscurin in such areas is located within the *square or rectangular* "boxes" immunopositive for *α*-actinin. The *arrowhead* shows obscurin-containing rounded bodies that assemble in the middle of nascent A-bands. **c** Lateral fusion of nascent myofibrils into a bigger cluster. Note the presence of non-striated fibers originating from the striated regions and bifurcating on the tips. The *arrows* show that longitudinally oriented elements of non-striated pattern on the lateral surfaces of the nascent sarcomeres are still well preserved at the time of obscurin accumulation in the middle of the A-bands. *Long thin arrows* show the gaps in the structure of laterally fusing elements of the second-formed Z-discs. Note the presence of obscurin in the mid-A-bands in the same sarcomeres. The *arrowhead* indicates the beaded pattern of obscurin localization. Note the presence of closely apposed obscurin bodies. Day 4 in culture. *Bars* 4.3 μm (**A**), 2 μm (**b, c**)

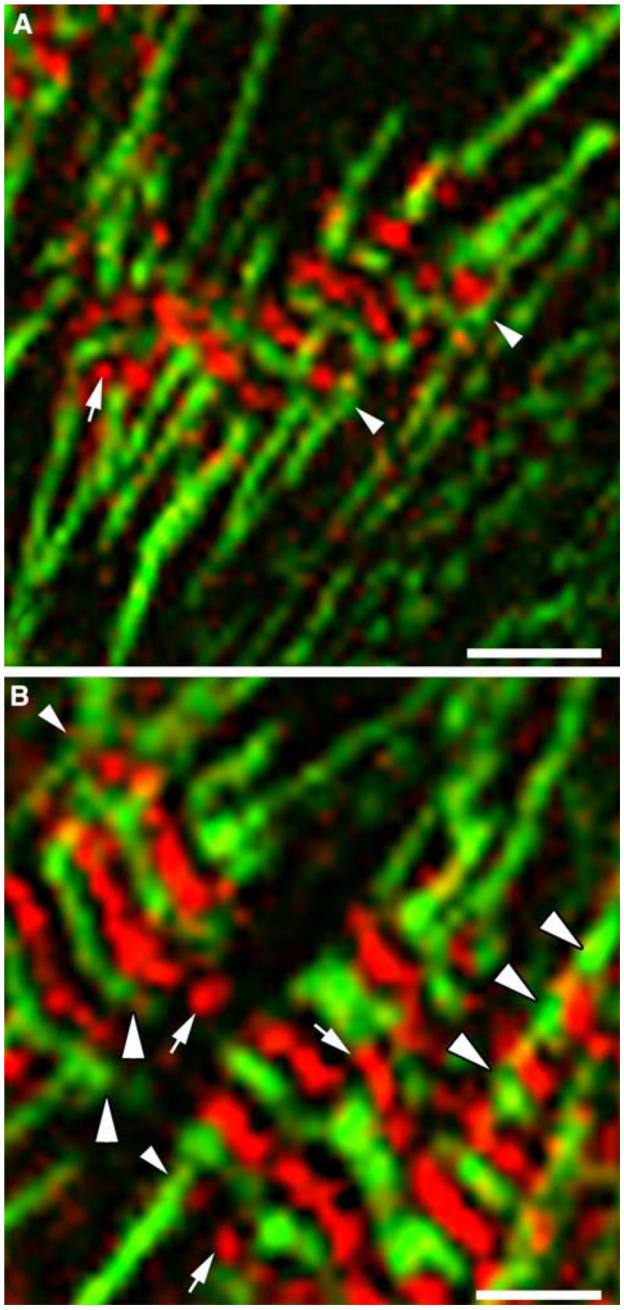

Fig. 2.

Progressive transverse and longitudinal expansion of fusing myofibrillar clusters. **a** The presence of longitudinally oriented *α*-actinin-containing material at the sides of the peripheral obscurin-positive sarcomeres in expanding myofibrillar clusters (*arrowheads*). The *arrow* shows the location of a rounded obscurin-positive body in the terminal sarcomere before the formation of the second transversely oriented Z-disc. Note that non-striated fibers are longitudinally oriented on the left and the right sides of the area marked with the arrow. **b** Presence of obscurin at the periphery of laterally aligning myofibrils (the *arrow* on the right) and close to the surface outside of the forming myofibril cluster (the *arrows* on the left). The areas of transition from myofibrils to non-striated fibrils are marked with the small *arrowheads*. Note the presence of obscurin in the A-bands, poor differentiation of the second-

formed Z-discs of terminal sarcomeres in these areas, and different width and length of the Z-discs in newly formed myofibrils. *Large arrowheads* mark the periodicity of nascent Z-disc material in newly formed sarcomeres containing obscurin (the left side of the photo) and in more mature sarcomeres (the lower right side of the photo). Note increasing spacing in more mature sarcomeres compared to the terminal sarcomere. *Bars* 4 μm (**a**), 2 μm (**b**)

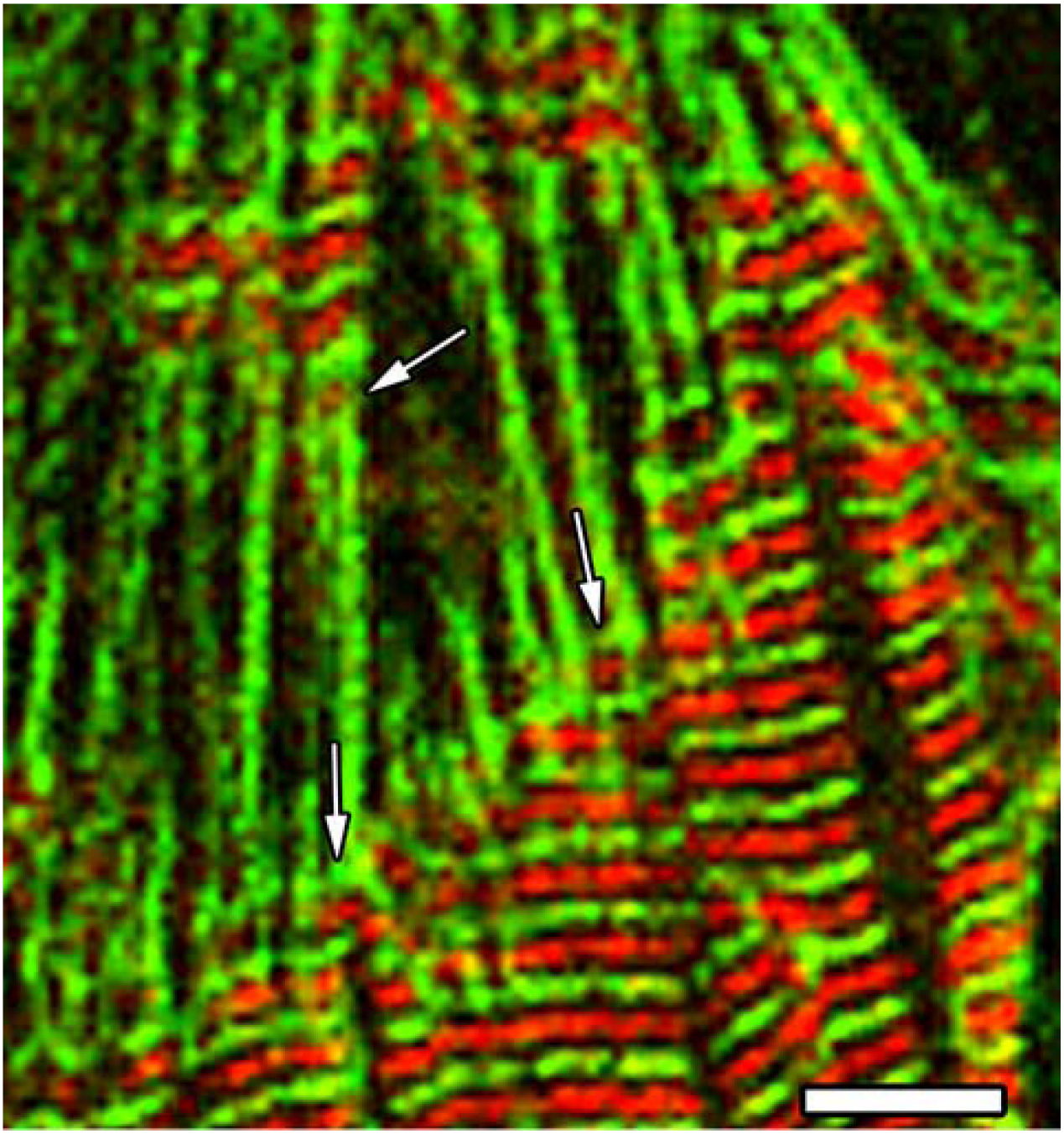

Fig. 3.

Obscurin incorporation (*red fluorescence*) into the contractile apparatus of a cardiac myocyte at the stage of formation of well-developed and laterally aligned myofibrillar clusters after 7 days in culture. myofibrils are labeled with the antibody to sarcomeric *α*-actinin (*green fluorescence*). The *arrows* show the sites of early incorporation of obscurin in the zones of lateral fusion of non-striated continuous fibers immunopositive for sarcomeric *α*-actinin. Note the presence of obscurin in the gaps between two fusing fibers and formation of the elements of nascent Z-discs in the zone of transition from the continuous pattern to sarcomeric cross-striations below and above obscurin-positive spots. Also note a progressive increase in

sarcomere length in more mature regions of the same myofibrils (the lower part of the photo). *Bar* 5 μm

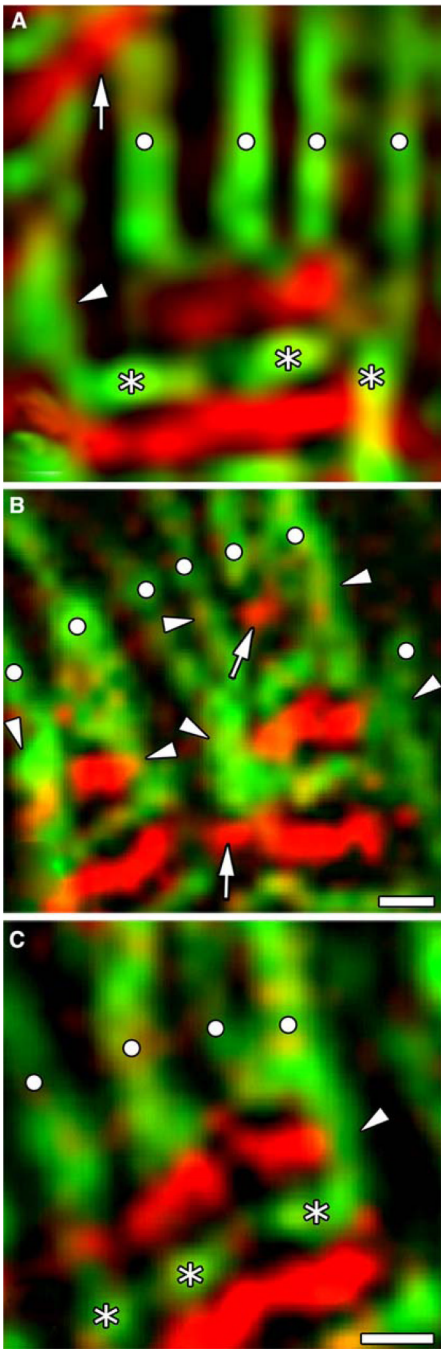

Fig. 4.

Patterns of obscurin (*red fluorescence*) and sarcomeric *α*-actinin (*green fluorescence*) localization in the areas of transition from non-striated fibrillar structures to cross-striated nascent myofibrils. **a** Non-striated fibrils continuously labeled for sarcomeric *α*-actinin are marked with *white circles*. The *asterisks* show the localization of the first-formed transversely oriented nascent Z-discs in each nascent myofibril. The *arrowhead* shows the preservation of a non-striated region of a longitudinally oriented continuously labeled fiber directly connected to a newly assembled Z-disc. Nascent Z-discs forming from individual non-striated fibers are still spatially separated from one another. The *arrow* indicates two closely opposed elongated, obliquely oriented obscurin-positive bodies located in the neighboring nascent myofibrils. Note

that the edges of the obscurin-positive bodies extrude outside of the contour of a non-striated structure labeled with the *white circle*. The presence of both obscurin and α-actinin in the lateral surface of the non-striated fiber visible on the right side of the photo below the *asterisk* as yellow fluorescence of superimposing green and red labels. A clear zone free of obscurin is seen below the termination points of non-striated fibers on the left side of the photo above the Z-disc. **b** The process of lateral fusion of two nascent myofibrillar bundles. The *symbols* are the same is in panel **a**. Obscurin (*red*) starts to concentrate in the prospective A-band region before or at the beginning of lateral fusion of non-striated bundles (*green*) just after initial formation of the first Z-discs of each new sarcomere (the *arrowheads* and the *arrow*, on the top of the photo). Note that after disassembly of the components of non-striated fibers on the lateral surfaces of nascent sarcomeres, the fusion of laterally growing obscurin bands forms an uninterrupted striated pattern in the middle of A-bands (the *arrow* at the bottom). **c** advanced stages of Z-disc formation. The *symbols* are the same as in **a**. Note that the second Z-discs in nascent myofibrils are assembled following incorporation of obscurin and formation of clear spaces for the A-band assembly. Note the morphological difference of the obscurin-positive bodies (*red*) spatially separated in nascent sarcomeres and laterally fused A-bands in more mature sarcomeres located below. *Bars* 0.9 μm

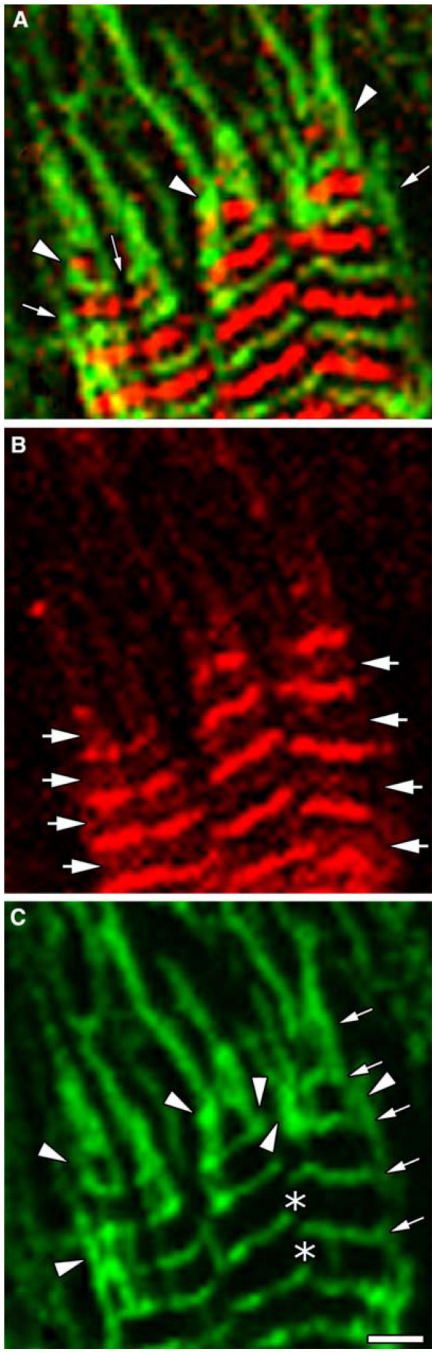

Fig. 5.

Localization of obscurin (*red fluorescence*) and *α*-actinin (*green fluorescence*) in the terminal sarcomeres of a growing myofibrillar cluster. **a** merged image showing the localization of both proteins. The *arrowheads* show the preservation of elements of non-striated fibrils on the lateral surfaces of newly formed early sarcomeres at the sites of obscurin incorporation. *Thin long arrow* shows the gap in the nascent Z-band at the site of lateral fusion of two early myofibrillar bundles. *Short arrows* show transient presence of longitudinally oriented non-striated structures contacting Z-discs of newly formed sarcomeres. **b** The localization of obscurin in the same field. The *arrows* show the presence of obscurin in the Z-discs. Note that obscurin incorporates first in the area of nascent A-bands of each terminal sarcomere. Also note the

absence of obscurin on the lateral surfaces of nascent sarcomeres in the areas immunopositive for sarcomeric α-actinin (see **c**). **c** Localization of α-actinin in the same field. The *arrowheads* indicate the presence of α-actinin on the lateral surfaces of the nascent sarcomeres on the tips of growing myofibrils. The *arrows* on the right show Z-disc periodicity along growing myofibrils. The *asterisks* show the gaps in nascent Z-discs that are located to newly formed myofibrils. The gap is nearly closed in the Z-bands located below the lateral fusion point. As one can see in photo **b**, obscurin bands in this area extrude to the periphery of sarcomeres and closely approach one another. Note a short distance between two nascent Z-discs in the terminal sarcomere at the initial stage of obscurin incorporation. *Bar* 1.8 μm

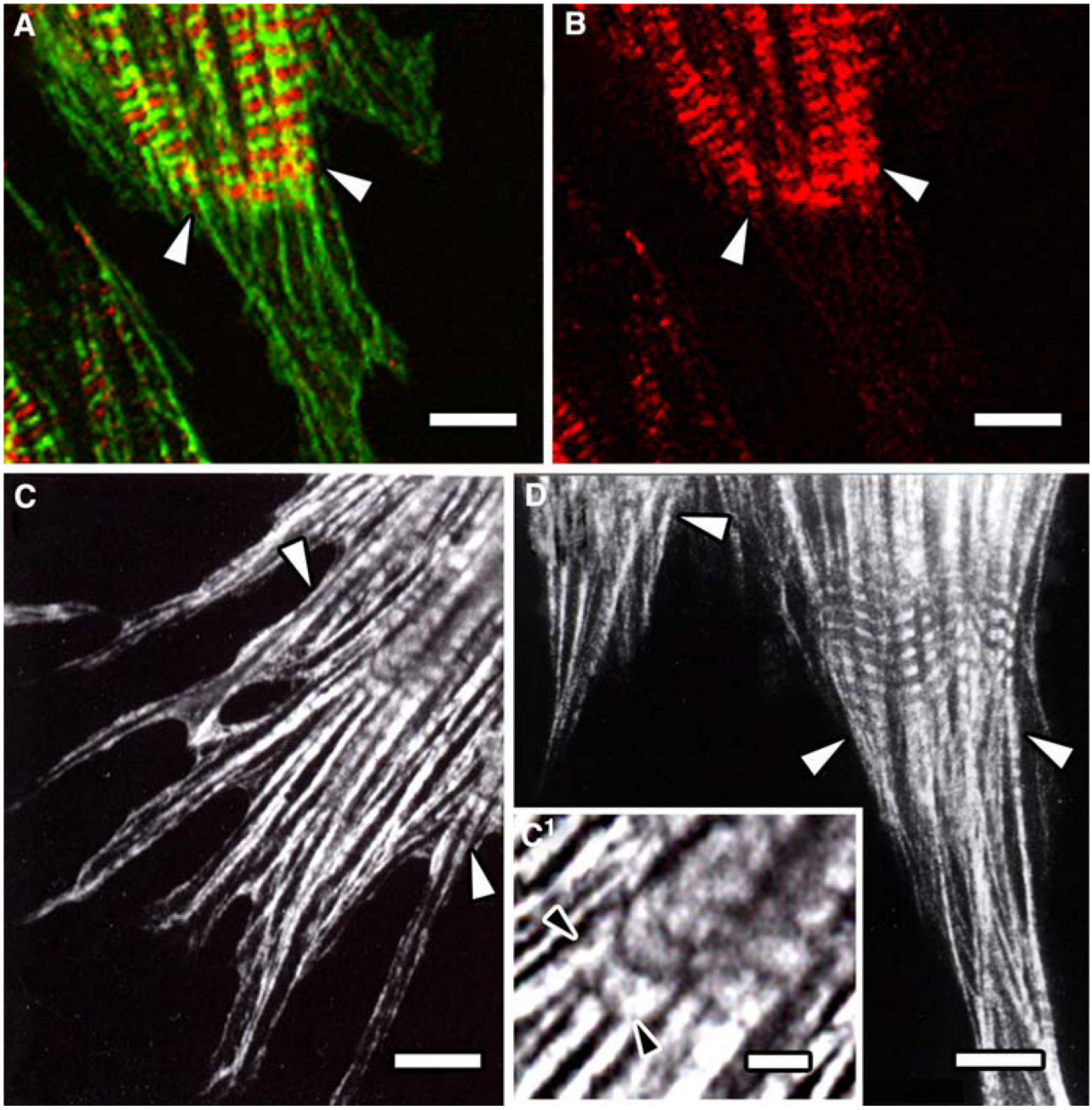

Fig. 6.

a Double immunofluorescent labeling of sarcomeric *α*-actinin (*green fluorescence*) and obscurin (*red fluorescence*) at the site of myofibrillogenesis in the peripheral cytoplasmic process of a cardiac muscle cell. **b** The localization of obscurin in the same field. Labeling of sarcomeric myosin heavy chains and sarcomeric actin in the areas of nascent myofibril formation at similar locations is shown in panels **c**, **c**^{1} and **d**, respectively. The *arrowheads* in **a**–**d** show the zone of transition of growing myofibril clusters into non-striated structural precursors of myofibrils. Note the presence of the sarcomeric isoforms of *α*-actinin, myosin, and actin in continuously labeled non-striated fibers and the abrupt termination of intense obscurin labeling in the zones of transition from the striated to non-striated pattern. **c**^{1} Shows the presence of sarcomeric myosin both in the newly formed sarcomeres and in continuously

labeled non-striated structures extending from assembling myofibrils. *Bars* 6 μm (**a, b**), 2 μm (**c**[1]), 10 μm (**c, d**)

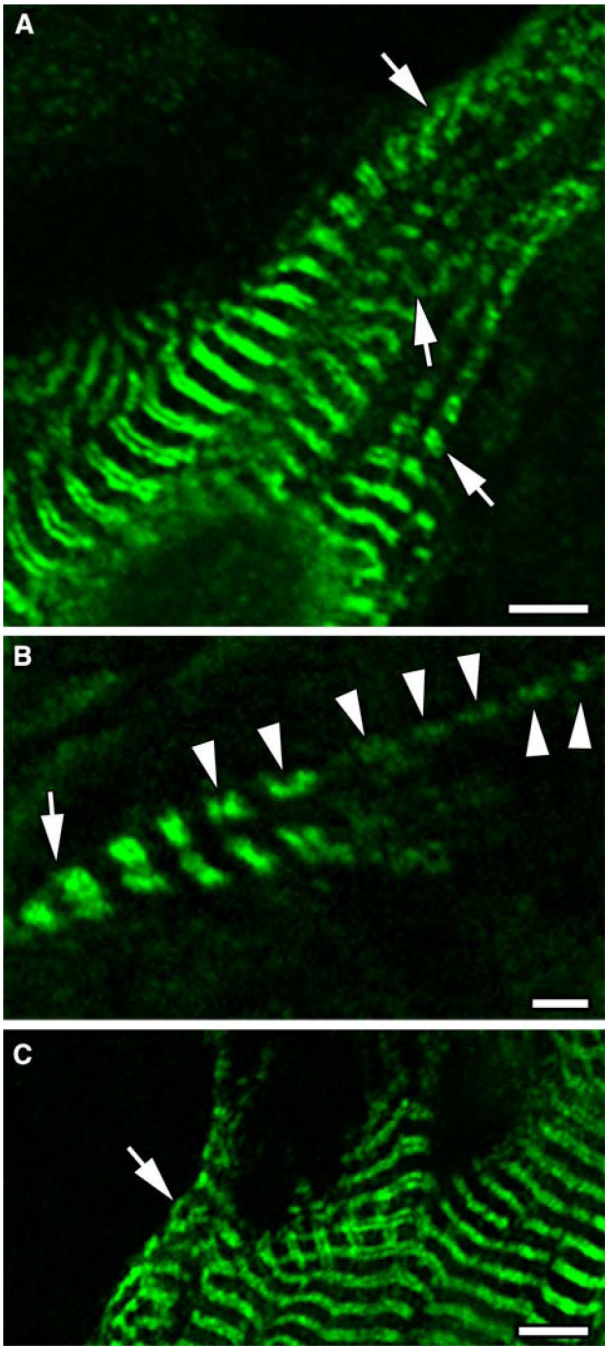

Fig. 7.

Localization of titin in the growing myofibrillar clusters. **a** A general view of a cardiac muscle cell after 7 days in culture in the area of transition from the differentiated and aligned myofibrils to nascent myofibrils *arrows* show the points of bifurcation and trifurcation of nascent myofibrillar bundles. **b** A bifurcating growing myofibril shown at a higher magnification. The *arrow* shows the point of bifurcation. The *arrowheads* indicate the presence of periodically arranged immunopositivity for titin in nascent sarcomeres beyond the point of bifurcation. **c** Uninterrupted cross-striated titin pattern illustrating complete lateral alignment of myofibrils in a muscle cell after 12 days in culture. Note that the gaps between individual myofibril bundles

are closed. The *arrow* shows a cytoplasmic ruZe with continuing myofibrollogenesis and spot-like periodic pattern of titin localization. *Bars* 4.2 μm (**a**), 1.7 μm (**b**), and 3.8 μm (**c**)

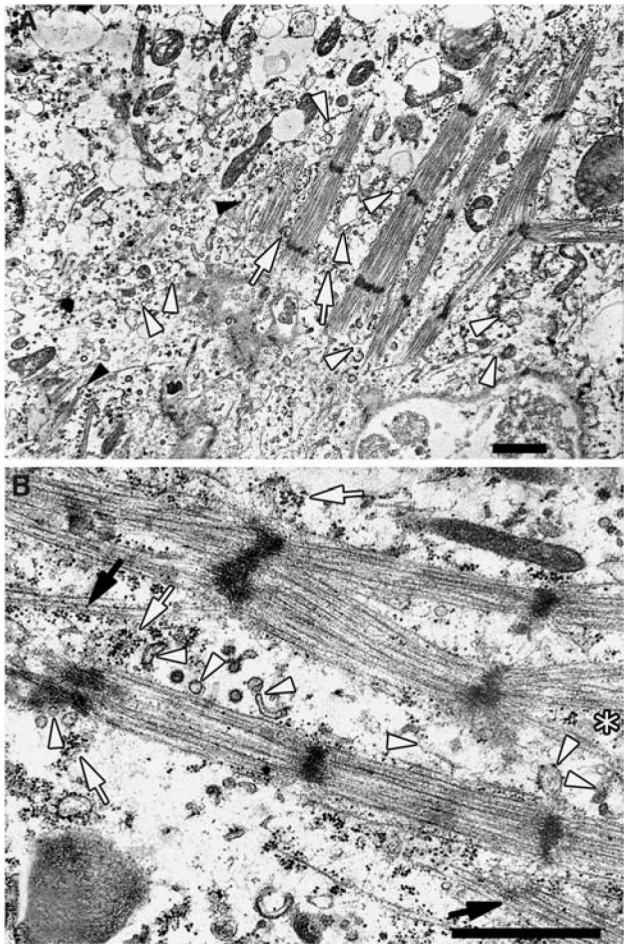

Fig. 8.

Early stage of formation of a myofibril cluster. **a** Nascent myofibrils are spatially separated from one another, but their Z-discs are aligned at approximately the same level. White *arrowheads* show the developing sarcoplasmic reticulum located closely to the lateral surfaces of myofibrils. *White arrows* show the presence of protein material connecting Z-lines at the neighboring myofibrils. *Black arrowheads* show free myosin filaments located close to the sites of assembly of nascent sarcomeres. **b** Branching myofibrils. The *arrowheads* indicate transversely and obliquely sectioned membranous tubular structures. Some of these structures represent the developing sarcoplasmic reticulum and are located closely to newly formed sarcomeres. *White arrows* show electron-dense protein deposits located near the lateral surfaces of Z-discs. *Black arrows* indicate the location of free myosin filaments. The *asterisk* shows myosin filaments located at angles to one another in a nascent terminal sarcomere. Four days in culture. *Bars* 1 μm

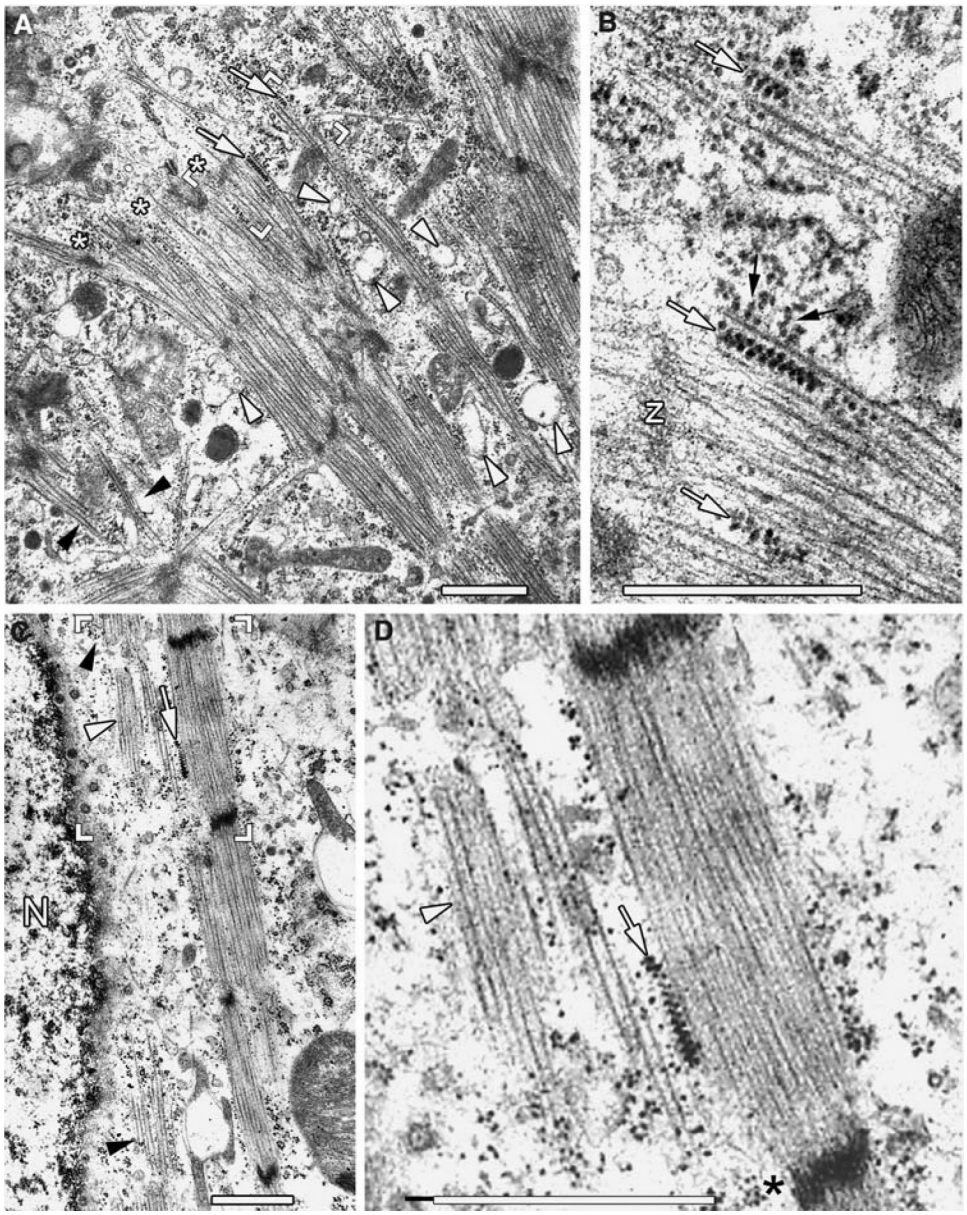

Fig. 9.

Electron microscopic images of myofibril assembly in rat cardiac myocytes. **a** Clusters of nascent myofibrils. Immature precursors of Z-discs are spatially separated and poorly aligned in relation to one another. Note that myosin filaments are loosely arranged and are oriented at different angles in the incompletely assembled terminal sarcomeres on the tips of growing myofibrils (*asterisks*). Also note the presence of transversely and obliquely sectioned cisterns of the forming sarcoplasmic reticulum (*white arrowheads*) located closely to the sites of myofibril assembly. *Black arrowheads* indicate free myosin filaments oriented at different angles and located closely to a nascent Z-disc. The *arrows* show the localization of polyribosomes on the tips of growing myofilaments of terminal sarcomeres. The frame *corners* show the area enlarged in panel **b**. The *arrows* in **b** show the same locations as in **a** at a higher magnification. **b** *White arrows* show the localization of polyribosomes attached to elongating sarcomeric filaments. *Black arrows* indicate free small spiral polysomes located closely to myosin filaments on the lateral surface of the forming sarcomere. A nascent Z-disc

at early stage of formation is marked with the letter *z*. Note the presence of free growing sarcomeric filaments in the upper part of the photograph. **c** Nascent myofibrils in the perinuclear area. *N* marks the nucleus. Electron lucent zones in the middle of newly formed A-bands show the absence of M-lines (also see **d**). *Arrowheads* show the groups of poorly aligned free myosin filaments. The *arrow* shows a polyribosome oriented along myosin filaments in the A-band and located on the lateral surface of the sarcomere. **d** A high magnification view of the sarcomere, free myosin filaments and the polyribosome marked with frame *corners* in photo **c**. The *arrow* and *white arrowhead* show the same locations as in photo **c**. Note the absence of Z-discs between the groups of longitudinally oriented myosin filaments of different length located on the left side of the photo and marked with the *arrowhead*. Also note that the Z-disc and the A-band located on the top of the photo are wider than the Z-disc and the A-band of the same sarcomere at the bottom located closer to growing tip of the myofibril. The presence of a polyribosome in the narrowing area of A-band at a close distance from the narrow region of the terminal Z-disc marked with the *asterisk* shows that the longitudinal and lateral growth of the second Z-disc occurs following the assembly of myosin filament and more or less complete formation of the A-bands in nascent myofibrils. Also note the absence of M-line in the middle of the A-band. *Bars* 1 μm

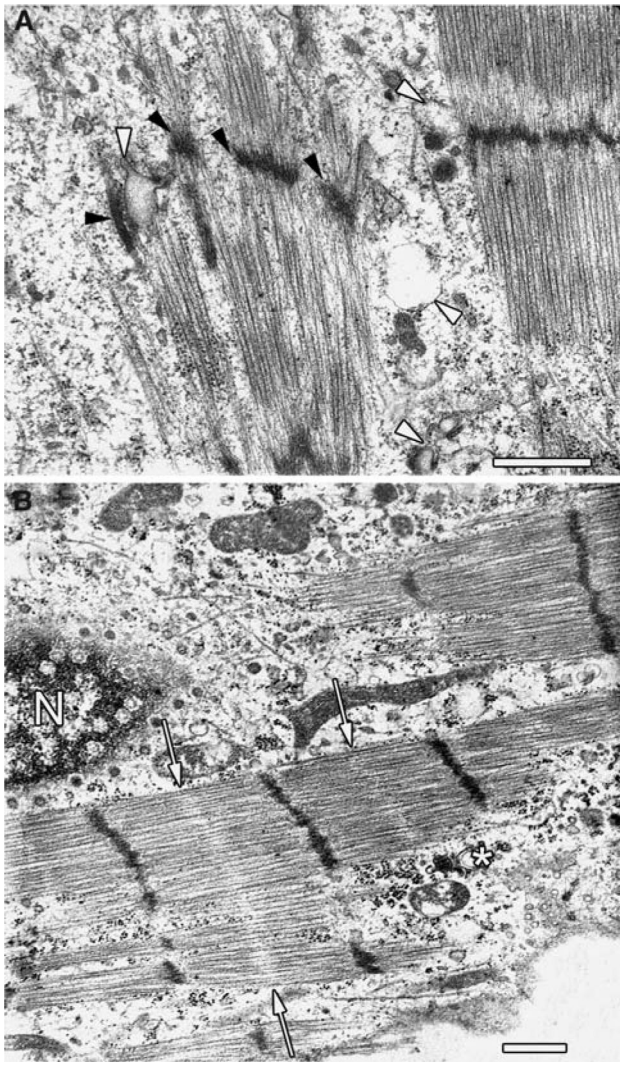

Fig. 10.

Non-aligned and aligned nascent myofibrils. **a** Non-aligned myosin filaments and spatially separated Z-discs (*black arrowheads*) in immature sarcomeres. Note that the nascent Z-discs are narrow, spatially separated, elongated, and obliquely oriented. The peripheral Z-discs are structurally associated with actin and nascent myosin filament forming the I–Z–I structures. A sarcomere at more advanced stage of differentiation is shown on the right. Note the presence of transversely oriented Z-disc and longitudinally aligned myosin filaments organized in a clearly visible A-band. *White arrowheads* show the elements of the developing sarcoplasmic reticulum. **b** Lateral alignment of smaller myofibrils into a larger myofibril. The *arrows* indicate the electron-lucent zones in the middle of A-bands, which shows the absence of M-lines. The *asterisk* shows the localization of the transversely sectioned membranous tubular structure morphologically resembling a forming element of the T-tubule system. *Bars* 1 μm

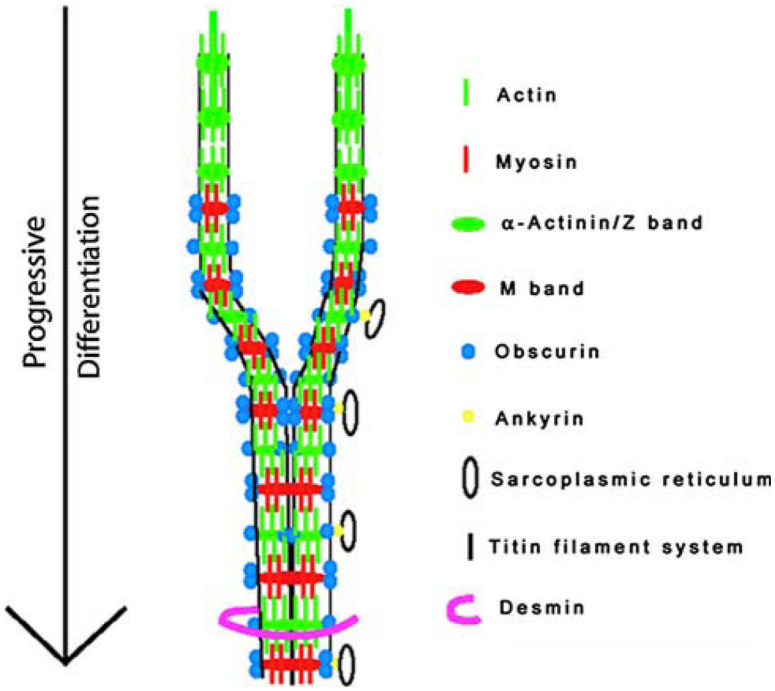

Fig. 11.

The hypothetic model illustrating the functional role of obscurin during myofibril assembly that illustrates the mechanism of maturation of myofibrils and the role of obscurin in this process Currently available data suggest that obscurin plays a role of a “stitching” linker protein involved in binding of sarcomeric filaments and the establishment of the longitudinal alignment of nascent myofibrils in relation to one another and to the sarcoplasmic reticulum