Organization of Protein and mRNA for Titin and Other Myofibril Components during Myofibrillogenesis in Cultured Chicken Skeletal Muscle

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ABSTRACT. Myofibril assembly requires the cell to join diverse components, correctly oriented to the rest of the cell. Titin, a huge elastic protein with a role in myogenesis, assembles during translation in vivo and may require spatially organized mRNA to allow assembly. By immunofluorescence, we examined titin and myosin protein organization early in skeletal muscle development in vitro; titin was the first organized, initially as spots, then as periodically spaced lines, and later as doublets. Titin mRNA organization during development was detected by fluorescent in situ hybridization. Only titin mRNA was seen in mononucleated myoblasts. Shortly after fusion, both titin protein and mRNA were diffuse. Titin mRNA remained diffuse when titin protein formed cables. Where titin protein formed linear arrays of spots, titin mRNA showed a colinear, continuous array. Titin mRNA remained in arrays colinear with young myofibrils until several slender myofibrils aligned laterally; then, titin mRNA formed periodic arrays. The titin probe encodes peptide sequence in the A band, where this region of titin mRNA is detected in the most organized cells. Nebulin undergoes a similar progression slightly later in development. This pattern, of narrowly spaced stripes, is too closely spaced to function in the soluble phase. Titin mRNA is the earliest mRNA to become so highly organized in muscle; it does so earlier and at a different location than do mRNAs for costamere proteins. These results, taken with earlier ones, suggest mRNA localization may be as key to somatic cell differentiation as it is to embryonic development.

Muscle development requires a cell to organize a large number of proteins into the complex structures of the myofibril, the sarcoplasmic reticulum and the costameres that anchor the contractile apparatus into the extracellular matrix. Myofibrillogenesis has been studied in embryos (6, 18, 19) and in tissue culture (1, 9). It involves the sequential organization of myofibril proteins into progressively more highly ordered arrays. Although both skeletal and cardiac cells contain striated myofibrils, there may be some differences in myofibrillogenesis in the two classes of muscle (14).

In the embryo, the earliest pattern for several myofibril proteins is a diffuse veil that gives rise to bundles and then periodic arrays of protein (reviewed in (5)). It appeared from these studies that titin was organized before other components, but direct comparisons between titin and other myofibril proteins were not made. We undertook to study titin organization relative to the timing of myosin organization by using directly conjugated monoclonal antibodies; this approach allowed identifying the component first organized into periodic arrays in cultured chicken skeletal muscle in vitro.

Titin (3 mD) is a huge protein (12, 21, 22). Transport of it after synthesis could be problematic, given the pore size of cytoplasm. Biochemical studies indicated that a significant fraction of titin appears to assemble during translation (9); this assembly was biochemically characterized as similar to the assembly state of mature titin (10). Some mRNAs for cytoskeletal proteins are highly localized, in both oocytes and somatic cells (reviewed in (4, 16)). In muscle cells, the mRNAs for several costamere proteins organize into narrow stripes with sarcomeric spacing late in development (13). Titin and other myofibril proteins organize into myofibrils much earlier. We asked whether titin or nebulin mRNA also becomes organized, and if so, when and where.

METHODS

Cell Cultures

For comparison of titin and myosin staining. Leg myoblasts of 12 day old chicken embryos were synchronized by a cycle of divalent cation depletion and repletion. The cultures were then prepared as previously (3). Each 35 mm culture dish was coated with collagen and plated with 2 mls of cells at a density of 1.5 × 10⁶ cells per ml, as determined by a hemacytometer.
For comparison of titin protein and mRNA staining. Cells were cultured and maintained as previously described (2) with the following modifications. Primary chicken thigh myoblasts were cultured onto 12 mm collagen coated glass coverslips at a concentration of 200,000 cells per coverslip. The cells were then left to attach for 1 hour in a 5% CO2/37°C incubator, then flooded with 1.5 milliliters of medium. Culture on glass coverslips was required to reduce background staining during in situ hybridization.

Antibody Purification. Mouse monoclonal antibody to titin, AMF-1 was a gift of D. Fischman (Cornell University, New York). The antibody was precipitated from 90 ml of medium with clean Staph-A for 30 minutes at room temperature and centrifuged. The antibody-Staph-A complex was washed by suspension in and centrifugation from low salt (20 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.5% Triton, 0.1% β-mercapto-ethanol), high salt (50 mM Tris, 500 mM NaCl, 5 mM EDTA, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS), and low salt buffer. The pellet was resuspended in 3.5 M MgCl2 for ten minutes to release antibody. The solution was centrifuged and the Staph-A pellet discarded.

Conjugation of Fluorophore to Antibody. Useful conjugation of fluorescent dye to the antibody was obtained with protein concentrations above 2 mg/ml. The absorbance at 280 nm of a 2 mg/ml solution of immunoglobulin is approximately 2.8.

Rhodamine conjugation. The protein solution was diazylated overnight at 4°C in a 750 mM sodium carbonate/bicarbonate buffer of pH 9. Approximately 1 mg of tetramethylrhodamine B isothiocyanate (TRITC, Sigma) in solution was slowly added at room temperature and stirred overnight at 4°C. Unconjugated rhodamine was separated from the conjugate using a 30 ml Sephadex G-50 column. The protein was eluted from the column in the void volume. The absorbance ratio at 515 nm/280 nm suitable for staining is 0.6 ± 0.4.

Fluorescein conjugation. A solution of 0.05 M sodium carbonate-bicarbonate at pH 8.5 with 2 mg of FITC on Celite (Sigma) was added to the protein solution and shaken for three minutes. The conjugate was then centrifuged in a microfuge for three minutes to remove the Celite containing excess FITC. This was most important for good separation when the conjugate was purified on a 30 ml Sephadex G-25 column. The column was prepared by pouring a Sephadex slurry into a column half filled with water and allowing the matrix to settle. The column was washed and eluted using 0.02 M sodium phosphate buffer at pH 6.5 (15). For staining, the necessary absorbance ratio at 495 nm/280 nm is 1.0 ± 0.5.

Immunofluorescence

Fixation and staining for comparison of titin and myosin protein. Cells were washed three times in PBS (phosphate buffered saline with 1 mM EGTA and 2 mM MgCl2) and fixed in 2 ml of 3.7% paraformaldehyde (Electron Microscopy Sciences) for 30 minutes at RT. After three rinses in PBS, cultures were fixed in absolute ethanol at −20°C for 10 minutes. The cultures that were to be stained for myosin were then incubated for 15 minutes in nonionic detergent NP-40.

After three rinses in cold (4°C) PBS, 2 ml of PBS with 0.1% BSA as a blocking agent was added at RT for 3 minutes. After removing blocking solution, 50 μl of each conjugated primary antibody was added to each dish and a piece of parafilm was placed on top of each culture to spread the solution. In experiments dealing with α-actinin, 0.5 ml of primary polyclonal rabbit antibody to this protein was used, along with 0.5 ml of appropriate unconjugated mouse mAb. The dishes were then incubated for 30 minutes at 37°C. After washing dishes 3 times in PBS, those dishes without directly labelled antibodies were again blocked with BSA. To each dish was added 0.5 ml of FITC-goat anti-rabbit IgG and 0.5 ml of RITC-sheep anti-mouse IgG. After incubation and rinsing, a drop of antibleach mounting medium (50% glycerol, 1 mg/ml paraphenylenediamine, 1 g/ml sucrose; pH 8) was added to each dish and a coverslip placed on top. The cells were then viewed using epifluorescent optics and a laser scanning confocal microscope.

Fixation for comparison of titin protein and mRNA. Cells were fixed with the proprietary fixative HistoChoice (Amresco) for 20 minutes at room temperature. A PBS/1 mM EGTA/2 mM MgCl2/1% Triton wash at room temperature followed fixation to make cells permeable to antibodies. HistoChoice was compared to 1:9 formaldehyde/methanol (vol/vol) fixation. HistoChoice fixed cells as well as or better than formaldehyde/methanol. Because HistoChoice is active at room temperature, cells were less likely to come off the coverslips.

Hybridization for comparison of titin protein and mRNA

Probes. A pBluescript SK- probe containing chicken titin sense cDNA was kindly provided by K. Tan and T. Huiatt (17). A 361 base pair probe, located between BamHI and HindIII restriction sites at the 3′ end of the insert, was generated by the polymerase chain reaction. The 5′ oligonucleotide began at nucleotide 918 (5′ CAGGCGTTGGCAGTAAGCTT 3′) and the 3′ oligonucleotide ended at nucleotide 1,279 of the cDNA insert (5′ AGATTCTCAGCTGCAACTCGG 3′).

A 429 base pair vimentin cDNA probe was generated from the pUC probe containing vimentin cDNA as previously described (2). The nebulin probe was generated from a plasmid kindly given by Kuan Wang.

Amplification for all probes used included a "hot start", heating all components (except for Taq Polymerase (BMB) and template) at 80°C for 5 minutes followed by cooling to 25°C under a wax layer provided by an AmpliWax Gem (Perkin-Elmer). After the polymerase and template were added, amplification was performed for 30 cycles with strand separation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute. The reaction mixture
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included a dNTP solution of 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.14 mM dTTP and 0.07 mM digoxigenin-11-dUTP. Incorporation experiments were previously established (2); however, agarose gels were run to determine if digoxigenin-11-dUTP incorporated into the probe.

Hybridization. Hybridization followed fixation after cells were washed as described in Table 1. of (2). Hybridization was performed as described in (2) with two exceptions. Step 3 of the published protocol was performed with 100 mM glycine /0.2 M Tris-HCl pH 7.4, with buffer chilled to 4° and sample left at RT during incubation. Step 4 was performed with PBS /5 mM MgCl₂ at RT for 10 min.

Immunofluorescent Staining of Titin Protein and mRNA. Titin protein was detected using a mouse monoclonal antibody, AMF1. The protocol for staining for both protein and message, described previously (2), was followed precisely. Cultures were then viewed under a Bio-Rad scanning laser confocal microscope.

RESULTS

Organization of titin and myosin protein during myofibrillogenesis. Titin and myosin are key proteins in the sarcomeres of striated muscle; titin provides elasticity and myosin, contractility. Titin interacts with isolated thick filaments and proteolytic titin fragments have been shown to interact with purified myosin (11). In situ crosslinking studies have shown that the two proteins interact structurally, even before periodic sarcomeres are visible (10). Those studies also demonstrated that during the synthesis of titin and myosin, they begin to interact (8).

To study the organization of these two proteins during development, monoclonal antibodies specific for each protein were directly conjugated to different fluorophores (15). Synchronized cultures of skeletal muscle cells derived from day 12 chicken embryo thigh muscle were then simultaneously stained with these antibodies. Using immunofluorescence, the organization of the two proteins relative to each other within a single cell was visualized. Titin was observed to organize into both cable and sarcomere structures slightly but clearly earlier than did myosin. Quantitation of these results indicates that localization of titin and myosin involves a transient intermediate structure in which titin is more organized. Titin and myosin tend to organize adjacent to the nucleus.

The first stage of titin and myosin organization observed in these cultured cells are dense linear arrays that run longitudinally in the cell. This linear, aperiodic structure, here called a cable, can be observed within hours after fusion. With time, the titin and myosin proteins begin to come into lateral register and form distinct sarcomeres, visible as regularly spaced patterns within a slender myofibril.

Double immunofluorescent labelling of titin and myosin in cultures day one and day two after fusion revealed the following. In all cultures, some cells contained titin organized into cables while myosin was still diffuse (not shown). Some cells contained titin organized into sarcomeres while myosin remained linear but aperiodic (Fig. 1). These stages of organization were infrequent, showing up in less than 10% of cells, but they were consistent over multiple observations.

In all cases where titin had sarcomeric periodicity in a cell, myosin was in either sarcomeres or cables at the same location. Often, in cells that had titin and myosin organized into a given cable, the titin in the cable would be slightly extended past the ends of the myosin. Similarly, there were cells which had both proteins in sarcomeres, in which the titin sarcomeres were more distinct and extended for some distance past the periodic myosin. Most significantly, two structures were never observed; myosin was never seen to be more organized than titin and titin was never formed into sarcomeres where myosin was still diffuse.

The nucleus appears to influence the organization of the proteins. The degree of protein organization was

Fig. 1. Developmental intermediate in myofibrillogenesis. Pictures show a muscle cell at 24 hours, labeled for both titin and myosin. Top shows titin immunofluorescence (labeled with AMF-1 and fluorescein). Bottom shows myosin immunofluorescence (labeled with MF-18 and rhodamine). Titin is largely periodic while myosin is still in a linear array.
greater in perinuclear regions than in the peripheral regions. In addition, both proteins were more organized in cells that had longer extensions.

These observations suggest a process in which titin molecules are assembled into a framework upon which the remainder of the myofibril can be constructed. This suggests further that not only is titin organized earlier than myosin, but that titin organization is prerequisite to myosin organization. Once this framework is under construction, other myofibril proteins interact with it (or simultaneously with myosin). The continued organization of titin, from an aperiodic cable into sarcomeric spacing, appears to require interaction with other myofibril proteins.

The abundance of intermediate stages (structures with titin more organized than myosin) gives an approximate idea of the rate of myofibril maturation (Fig. 2). Because the decrease in cells with diffuse titin and myosin from day one to day two is roughly equal to the fraction of cells displaying cables at day two, and because the increase in cells with sarcomeres is roughly equal to the fraction of cells in cables at day one, it appears that each stage transition takes 24 hours in culture, under these conditions. Since the intermediates (cables of titin with myosin diffuse; periodic titin with myosin in a cable) are found in about 4% of the cells in the cultures observed, these intermediate stages appear to last approximately one hour (Fig. 2A, B). These developmental stages are presented schematically in Figure 2, bottom.

**Organization of titin protein and messenger RNA during myofibrillogenesis.** The temporal priority of titin organization then lead us to ask whether the organization of titin mRNA yielded clues to the mechanisms by which this order was obtained. To address this, we examined cells labelled to detect titin protein and mRNA.

As before, titin was detected with the monoclonal antibody AMF-1, which stains titin protein at the A-I junction, where the thick and thin filaments of the sarcomere overlap (23). In individual myoblasts, titin mRNA is detected (Fig. 3e), but little protein (Fig. 3a). Upon fusion of myoblasts into multinucleated myotubes, protein levels increase and titin protein stains diffusely throughout the cell.

At 72 hours, the micromass cultures reveal a mixture

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**Fig. 2.** Proportion of cells in each developmental stage for titin and myosin. Graph A shows cells after 24 hours of growth; B, cells after 48 hours of growth. T and M denote protein (Titin, Myosin), and d, c, and s denote the levels of protein organization (diffuse, cables, sarcomeres.) Bottom displays a schematic for titin and myosin organization at a given point on the myofibril. The figure indicates the formation of transition intermediates by showing periods during which myosin organization lags behind that of titin.
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Fig. 3. Developmental stages of titin protein and mRNA. Cultures double stained with antibody AMF1 (protein, left hand panels) and antidigoxigenin antibodies to probe (mRNA, right). Panels a, e show myoblast containing mRNA but little protein. Panels b, f show young myotube containing linear arrays of protein and diffuse mRNA. Panels c, g show myotube containing periodic arrays of protein and linear mRNA. Panels d, h show myotube containing periodic and doublet arrays of protein and linear and periodic mRNA.

of organizational stages. Titin protein is observed in cable-like, nonperiodic structures and in myofibrils that have a punctate linear array of titin (examples in Figure 3b and c, respectively). At 72 hours, fewer than 50% of cells have punctate arrays. By day 5, >50% of cells have titin organized in either punctate arrays or as periodic doublets of protein, stained at the A-I junction on either side of the Z line of the sarcomere. By day 7, >80% of cells contain titin protein organized into doublets with the myofibrils beginning to come into register laterally (Fig. 3d). This lateral registration of slender myofibrils occurs before muscle cells begin to twitch spontaneously.

Where is titin messenger RNA when titin protein aligns into sarcomeres? Titin mRNA is visible in individual myoblasts as mRNA diffusely spread throughout the myoblast (Fig. 3e). Low or undetectable levels of protein are visible in myoblasts; somewhat more protein is visible in myotubes. During development, several organized patterns of titin mRNA are visible. In elongated, fused cells in which titin protein is in cables or punctate arrays, the titin mRNA is diffusely spread throughout the cytoplasm (Fig. 3f). At 72 hours, some titin mRNA is in cables (nonperiodic, linear arrays) aligned with the punctate arrays of titin protein (Fig. 3g). Titin mRNA is only found in punctate arrays in areas where titin protein is organized into doublets. However, some titin protein doublets are associated with linear arrays of titin mRNA.

With continued culture, titin protein is increasingly found in periodic arrays of doublets; in these regions of the cell, titin mRNA is diffuse or in cables that are colinear with the arrays of doublets. By day 7, most (>80%) myotubes have titin protein organized in sarcomeric doublets; over half have laterally aligned these doublets so that two or more slender myofibrils are in register. More than 50% of cells have titin mRNA in striations throughout the myotube. Striations of titin mRNA are only seen in association with laterally aligned myofibrils (Fig. 3h).

Thus, in the most highly organized patterns of protein and mRNA, titin mRNA and titin protein show sarcomeric periodicity and alignment within the sarcomere. The AMF1 antibody to titin protein stains titin only at the A-I junction although the titin molecule spans the half sarcomere. The probe to titin mRNA is detected at the A band; the probe is complementary to the region of titin mRNA that encodes sequences found in the A band portion of titin protein. The staining pattern is consistent with this portion of the titin mRNA being colocialized with the domain of its protein product. The confocal microscope allows collecting two fluorescent signals using different filters at two detectors, without touching the sample to change filters. In addition, at day 15 when myofibrils in the myotube are extensively in register, the titin mRNA and protein remain in register (Fig. 4).

Controls were performed for in situ hybridizations. Vimentin protein and mRNA, described before (2), were the positive control to set gain and pinhole of the confocal microscope (Fig. 5a, b). A sample prepared
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Fig. 4. Highly organized titin protein and mRNA. Cultures from day 15, double stained with antibody AMF1 (titin protein, top) and antidigoxigenin antibodies to probe (titin mRNA, bottom). Titin probe sequence is complementary to mRNA sequences encoding A band peptide sequences. Therefore, images indicate colocalization of probed region of mRNA with protein domain.

without probe was used as a negative control, following all steps of hybridization except adding probe DNA to the probe solution. When controls prepared in parallel with no probe present were examined at the same gain and pinhole as the titin and vimentin samples, there was no image or pattern (Fig. 5c, d). The mRNA patterns therefore were not due to nonspecific binding of the anti-digoxigenin-rhodamine antibody. Some samples were treated with RNase H, which digests only RNA that is in RNA-DNA hybrids. Such samples showed little or no staining (Fig. 5e, f). Finally, with the confocal microscope, the krypton/argon laser and selective filters prevent bleed-through from one channel to the other channel (Fig. 5g, h).

Nebulin is another giant myofibril protein; however, its assembly in vivo is less well characterized. Nebulin protein and mRNA underwent a similar progression to that for titin. The earliest stages showed linear arrays that became periodic, while the organization of the mRNA often lagged one stage behind that of the protein. In mature cells, both protein and mRNA were in sarcomeric periodicity (Fig. 6).

DISCUSSION

Terminally differentiated cells such as muscle and nerve have complex architectures that support the tissue-specific functions each cell performs. So complex a structure, composed of many components, is unlikely to form by the simultaneous assembly of all its components, and in fact these specific architectures develop over time.

For muscle, studies of embryos showed that titin, myosin and several other myofibril components assemble rapidly, with some support for temporal priority for titin. However, double immunofluorescent staining was difficult as the antibodies used were all mouse monoclonal antibodies. We circumvented this difficulty by directly conjugating antibodies to titin and myosin with
different fluorochromes. These antibodies permitted direct observation of titin and myosin during the first events of myofibrillogenesis.

These observations indicated that titin was in all cases the first of the pair to become organized, either as a longitudinal cable or in the periodicity of the sarcomere. However, this organization of titin did not appear to be autonomous of myosin, as in no case did we see titin advance more than one stage at a time. If myosin had not yet acquired the pattern of titin, the titin protein did not become more highly organized.

Titin has several characteristics that are consistent with a primary role in pattern formation for the myofibril. First, titin spans as a single molecule from the M-line to the Z-line. Thus it is long enough to provide half-sarcomere spacing by its own length. Second, titin is elastic; it is possible that titin elastically stretches during the alignment into cables and periodic structures, and in so doing constrains itself and associated molecules (including perhaps myosin) first into colinear bundles and later into periodic arrays.

Other characteristics of titin, however, place limits upon its utility as a template molecule. Its huge size (3 mD) and low solubility mean that it is unlikely to diffuse freely to encounter appropriate sites for assembly. For these reasons, the position of titin mRNA during myofibrillogenesis becomes particularly important. The mRNA is detectable in myoblasts, but the protein only becomes detectable after fusion. The titin protein has a half-life of 70 hours (9); thus, when the pattern of titin protein changes from diffuse to linear, or from linear to periodic, what is occurring is principally the rearrangement of protein after translation.

The titin mRNA at each stage except the last shows the spatial organization of the previous protein pattern. This suggests that titin protein that is being translated cotranslationally assembles into an assembly intermediate similar to the previous protein pattern, which then rearranges slightly to acquire the current pattern of the bulk, older protein. Only in the most mature myofibers, when lateral alignment of the slender myofibrils has occurred, does titin mRNA come into register with the cognate domain of the bulk of the titin protein. As only one titin probe was available, we cannot say that titin mRNA and protein are colinear; that would require probes to I band sequences.

Nebulin protein also displays sequential organization. Its mRNA is similarly delayed in organization, relative to the bulk nebulin protein.

Other aspects of the role of mRNA localization can also be inferred. Different mRNAs have different positions within the cell at any given time; therefore the specific mRNA contributes some information to its localization. Any one mRNA has different positions at different times; therefore the developing cytoskeleton also contributes some information to mRNA localization. Finally the patterns seen here are too localized to contribute to a function in the soluble phase, because the spacing is


