Obscurin regulates the organization of myosin into A bands


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MYOFIBRILLOGENESIS IS A COMPLEX PROCESS that involves the expression and assembly of muscle proteins into sarcomeres, the basic contractile units of striated muscle (27). Myofibrillar assembly has been proposed to involve three morphologically and functionally distinct structures in developing myocytes, premyofibrils, nascent myofibrils, and mature myofibrils with organized sarcomeres (24, 26), but the molecules that drive the formation of these structures and their ability to mature in situ are still poorly understood.

Premeysin fibrils contain transitory arrays of I-Z-I complexes consisting of sarcomeric actin occupying primitive I bands attached to precursor Z bodies rich in α-actinin (9, 22, 24). The primitive I-Z-I complexes are connected to miniature A bands (12) that consist largely of filaments of nonmuscle myosin II (4, 24, 27). Precursor I-Z-I bodies of premyofibrils develop into maturing I-Z-I bands in nascent myofibrils with the cooperative binding and integration of at least five integral Z-band components, including nebulin, titin, and T-cap, along with the preexisting α-actinin and α-actin (22). Concurrently, muscle myosin II gradually replaces nonmuscle myosin in the developing A bands (4).

As premyofibrils become nascent myofibrils, M-line proteins, including myomesin and M protein, also accumulate in the cytoplasm and gradually organize into a periodic pattern of primordial M-line structures (6). In recent studies (31, 32), some investigators have postulated that the assembly of M-line proteins into primordial M lines occurs before the incorporation of the COOH-terminal portion of titin into the M line, suggesting that they are needed for this process and for myosin subsequently to assemble into A bands. As nascent myofibrils develop into mature myofibrils, they align parallel to each other to form sarcomeres with sharply delineated Z and M lines bisecting I and A bands, respectively.

A growing body of evidence indicates that the regular organization of sarcomeres is mediated by two structurally related muscle proteins: nebulin and titin (3, 29). Nebulin (~800 kDa) associates with actin filaments in I-Z-I complexes, driving their assembly and determining their length (30). Titin (3–4 MDa) associates with maturing I-Z-I complexes via its NH2 terminus and with myosin filaments through its COOH terminus, facilitating the coordinated integration of thin and thick filaments into sarcomeres (10).

In this study, we examined the role of obscurin, another giant, muscle-specific protein, in the assembly and organization of the A band. Obscurin (~800 kDa) is the third member of the titin family of proteins expressed in vertebrate striated muscle and, like titin and nebulin, is composed of adhesion modules and signaling domains (25, 33). Its NH2-terminal region contains 54 immunoglobulin (Ig) repeats and 2 fibronectin-III-like domains, followed by an IQ motif and a conserved SH3 domain next to Dbl homology and pleckstrin homology domains. The COOH terminus of the protein consists of 2 Ig domains followed by a nonmodular region of 420 amino acids that contains several copies of a consensus phosphorylation motif for ERK kinases. Unlike titin and nebulin, which are integral components of sarcomeres, obscurin intimately surrounds the myofibrils at the level of the Z disk and the M line (16). This unique distribution allows it to bind to a small isoform of ankyrin 1 in the sarcoplasmic reticulum (1, 16).

Obscurin closely resembles Unc-89, one of the giant muscle proteins found in Caenorhabditis elegans (2). Like obscurin, Unc-89 is a modular protein composed of Ig repeats and signal transduction domains and is localized at the M line. Mutations in the unc-89 gene result in nematodes with disorganized A bands devoid of M lines (2). This suggests that obscurin, too,
may help to organize the A bands and M lines in vertebrate striated muscle. We tested the role of obscurin in the assembly and organization of the sarcomere by overexpressing a portion of its COOH-terminal sequence through adenvirally mediated gene transfer in primary cultures of skeletal myotubes. We found that treated myotubes failed to assemble myosin into A bands but assembled other sarcomeric structures normally. Our results suggest that the COOH terminus of obscurin harbors a binding site that allows it to associate with myosin and regulates the formation of A bands.

**MATERIALS AND METHODS**

**Culture of Skeletal Myotubes**

Primary cultures of rat myotubes were prepared as previously reported (5). In brief, hindlimb muscles from postnatal day 1 (P1) rats were dissociated enzymatically and suspended at $10^6$ cells/ml in Dulbecco-Vogt modified Eagle’s medium (DMEM; GIBCO-BRL, Carlsbad, CA) containing 10% fetal bovine serum (FBS; GIBCO-BRL). Cell aliquots (0.5 ml) were applied to sterile glass coverslips and supplemented with 1 ml of the same medium the next day. Medium was replaced 48 h later with medium containing 2 $\times 10^{-5}$ M cytosine arabinoside (Sigma, St. Louis, MO) to kill dividing cells. Cultures were infected with adenovirus 5 days after plating.

**Generation of Recombinant Adenoviruses**

Infection of primary cultures. A COOH-terminal obscurin fragment containing nucleotides 1503–1863 (rat sequence Accession No. AY-167411; see Ref. 16) was generated by PCR with the following set of primers: 5′-ACTGAACTTACCGCTCTAGGCCC-3′ (sense) and 5′-ACGTGATCTGCTCTCTTCT-3′ (antisense). An additional fragment encoding the COOH-terminal tail of small ankyrin 1 (sAnk1) amino acids 29–155 (15) was also obtained after PCR amplification with the set of primers 5′-ACTGGATCTGCTCTCTTCTCTCTT-3′ (antisense) and 5′-ACTGTCCTGACGT-GCTGGGCTTTG-3′ (antisense). The sense primers carried a HindIII recognition sequence and the antisense primers contained a BamHI site for insertion into enhanced green dIII recognition sequence and the antisense primers contained a BamHI site for insertion into pAdlox. The authenticity of the constructs was verified by sequence analysis.

**Electron Microscopy**

Infected, permeabilized cultures were fixed with 2% paraformaldehyde for 15 min at 4°C, washed with PBS and mounted on slides. Infected cells, expressing the recombinant proteins in high amounts, were selected under confocal optics, and their locations were marked with a diamond stylus. Samples were then fixed overnight in 0.2 M cacodylate buffer, 2% glutaraldehyde, and 5 mg/ml tannic acid. After washing in 0.2 M cacodylate buffer, cultures were postfixed in 50 mM acetate buffer, 1% osmium tetroxide, stained en bloc with 1% uranyl acetate, dehydrated, and embedded in Araldite-Epon (EMbed-812; Electron Microscope Sciences, Fort Washington, PA). After hardening of the resin, the glass coverslips were separated from the sample with hydrofluoric acid, and sections were cut at a thickness of ~90 nm with an LKB MT5000 microtome. Subsequently, sections were picked up on 200 mesh copper grids, stained with uranyl acetate, followed by lead citrate, and analyzed under a Philips 201 electron microscope (Philips, Eindhoven-NL, The Netherlands) at $\times$20,000 magnification. Pictures were taken on Kodak 4489 film and digitally scanned at 720 dpi.

**Immunoprecipitation and Immunoblotting**

Infected myotube cultures were serially rinsed with DMEM containing 25 mM HEPES and ice-cold PBS and then scraped with a rubber policeman. Cells were resuspended in 10 mM NaPO$_4$, pH 6.8, 2 mM EDTA, 10 mM NaCl, and 1% Nonidet P-40 (NP-40), supplemented with protease inhibitors (Roche, Indianapolis, IN), and homogenized with a Dounce homogenizer. The lysate was subjected to centrifugation at 14,000 g for 15 min at 4°C. Protein in the supernatant was determined with the Bradford assay (Bio-Rad, Hercules, CA), and ~100 µg from each sample were fractionated by 10% SDS-PAGE. Blots were blocked in PBS, 10 mM Na$_2$CO$_3$, 1% Nonidet P-40, and 3% dry milk and probed with mouse antibodies to GFP (I4E 1 µg/ml; Medical and Biological Laboratories, Nagoya, Japan), myosin (MY-32, 1:500; Sigma), and rabbit antibodies to titin (x112/x113, labels at the Z line, 3 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA) or to myogenin (1:1000; Santa Cruz Biotechnology). Immune reactive bands were visualized with a chemiluminescence detection kit (Tropix, Bedford, MA). The intensities of bands of interest from three different experiments were quantified with the use of MetaMorph Imaging System software (Universal Imaging, Downingtown, PA).

**Immunofluorescence Staining and Confocal Microscopy**

Fixed, permeabilized cultures were blocked in PBS, 0.1% BSA, and 10 mM Na$_2$PO$_4$ (PBS/BSA) for 1–2 h at RT before immunofluorescent staining. Mouse antibodies to adult fast myosin II (MY-32, 1:500; Sigma), neonatal slow and fast Ila myosin (N2.261, 1:10; Developmental Studies Hybridoma Bank, Iowa City, IA), adult slow myosin (NOQ7.5-4D, 1:500; Sigma), titin (Clone T11, labels near the A-I junction, 1:500; Sigma), α-actinin (1:500; Sigma), myomesin (1:100), and rabbit antibodies to titin (x112/x113, labels at the Z line, 3 µg/ml) and phallolidin coupled to Alexa 488 (1:200; Molecular Probes, Eugene, OR) were added to cells for 12–16 h at 4°C. Samples were counterstained with goat anti-mouse Alexa 568 or goat anti-rabbit Alexa 568 (Molecular Probes), diluted 1:200, for 1 h at RT. Cells were washed with PBS/BSA, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and analyzed under a Zeiss 410 confocal laser scanning microscope (Carl Zeiss, Tarrytown, NY) equipped with a x63, NA 1.4 objective.
(15) with some modifications. Briefly, quadriceps muscle was homogenized in lysis buffer (10 mM NaPO₄, pH 7.2, 2 mM EDTA, 10 mM NaN₃, 120 mM NaCl, 1% NP-40) plus protease inhibitors in a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Protein lysates were incubated on ice for 1 h with occasional vortexing. Protein content was measured with the Bradford reagent and aliquots of 1 mg of total protein were pre cleared with either 50 μl of Dynabeads M-280 sheep anti-rabbit IgG (Dynal, Lake Success, NY) or 50 μl of Dynabeads M-450 goat anti-mouse IgG (Dynal) for 2 h at 4°C with gentle rocking. Aliquots (3 μg) of rabbit anti-obscurn (16), ChromaPure rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), mouse anti-myosin (MY-32, 1:500) or mouse IgG (Jackson ImmunoResearch Laboratories) were incubated with 30 μl of Dynabeads M-280 or Dynabeads M-450, respectively, for 6 h in PBS at 4°C with gentle mixing. Antibody-bound beads were incubated with 0.5 mg protein from the pre cleared homogenate for 12 h at 4°C. Samples were washed with 10 mM NaPO₄, pH 7.2, 10 mM NaN₃, 140 mM NaCl, 0.5% NP-40, and 0.5% Tween 20, solubilized
in 60 μl of 2× SDS-PAGE sample buffer, heated at 42°C for 30 min, analyzed by SDS-PAGE on 4–10% gradient gels, and processed for immunoblotting.

Materials

All restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Other chemicals were the highest grade available from Sigma.

RESULTS

Obscurin is a giant protein (Fig. 1A; see Ref. 33) that surrounds myofibrils at the level of the Z disk and M line (16). Previous work by our laboratory (16) and by others (1) has shown that it specifically and directly interacts with sAnk1, a muscle-specific isoform of ankyrin 1 that is selectively enriched in the network sarcoplasmic reticulum (SR). The binding of obscurin to sAnk1 is mediated by a sequence of obscurin located at its COOH terminus that contains nt 1503–1863 (16).

To study the role of obscurin in sarcomerogenesis and the architecture of the SR, we used adenovirally mediated gene transfer to overexpress the COOH-terminal fragment in primary cultures of skeletal myotubes (Fig. 1A). The obscurin fragment was expressed as a GFP fusion protein to facilitate the identification of transfected cells. In parallel experiments, primary cultures were infected with recombinant adenovirus carrying GFP alone. Infections were initiated on 5-day-old cultures, and their effects were assayed 2 days later. In this report, we focus on the morphological alterations observed in myofibril assembly after overexpression of the COOH terminus of obscurinnt1503–1863. The effects of this treatment on the organization of the SR will be discussed in a separate publication.

Examination of infected cells by fluorescence microscopy revealed ~90% infection efficiency (data not shown). To learn whether GFP-obscurinnt1503–1863 and GFP were efficiently ex-

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Fig. 3. Myosin failed to assemble into A bands in myotubes overexpressing GFP-obscurinnt1503–1863. A–C†: distribution and apparent levels of endogenous myosin were altered in myotubes that overexpressed GFP-obscurinnt1503–1863 (A–C). In ~15% of the infected cells, myosin expression was significantly diminished compared with control cells (D†), showing either diffuse distribution (A‡ and B‡, single arrows) or accumulations in nonstriated fibrils (B‡, two-headed arrow) and at the cell periphery (B‡, arrowhead). In 80% of myotubes, myosin was amply expressed but severely disorganized (C‡), either exhibiting punctate staining (C‡, arrow) or concentrating at structures resembling stress fibers (C‡, arrowheads). In cultures infected with control GFP virus (D) or GFP-sAnk1aa29–155 (E), myosin assumed its typical periodic organization at A bands (D′ and E′, respectively).
pressed as intact proteins in myotubes, we immunoblotted protein lysates prepared from infected cells. Both GFP-obscurinnt1503–1863 (~41 kDa) and GFP (~28 kDa) were abundantly expressed in myotubes infected with the appropriate virus (Fig. 1B).

At the time of viral infection (5 days), endogenous obscurin was efficiently expressed and organized periodically, primarily at M lines, as indicated by double immunofluorescence labeling with the Z-disk marker α-actinin (Fig. 2, A and A'). Titin and sarcomeric actin also assumed a striated distribution at the level of the Z disk and I band, respectively (Fig. 2, B and C). At that point, endogenous myosin was not yet organized into regular A bands, however. Instead, it accumulated in long, filamentous structures with periodic striations presumably corresponding to primordial A bands (Fig. 2D, yellow arrows).

Immunolocalization of sarcomeric proteins 2 days after infection indicated that the COOH-terminal fragment of obscurin dramatically altered the distribution of myosin (Fig. 3, A–C). A panel of antibodies against different myosin isoforms was used in these studies: MY-32, which recognizes adult fast myosin II; N2.261, which is specific for neonatal myosin; and NOQ7.5.4D, which recognizes adult slow myosin I (NOQ7.5.4.D). Specific labeling was observed only with the MY-32 antibody (Fig. 3). No staining was detected with either of the other two antibodies, suggesting that neonatal and adult slow myosin isoforms are expressed in low amounts in these cells, if at all (data not shown). Approximately 5% of infected cells expressing GFP-obscurinnt1503–1863 showed significantly reduced labeling for myosin, which appeared diffusely throughout the myoplasm (Fig. 3, A and A', arrow). In another

Fig. 4. Electron microscopic images demonstrating the absence of periodic A bands in myotubes infected with virus expressing GFP-obscurinnt1503–1863. After processing for electron microscopy, myotubes that overexpressed GFP-obscurinnt1503–1863 did not show myosin filaments assembled into regular A bands. The myosin filaments in these cells instead ran uniformly along the entire length of assembled sarcomeres, making the I bands less distinct (A, double arrow), or they accumulated in long, filamentous structures with no apparent periodicity (B, arrows). In control myotubes expressing GFP alone, sarcomeric myosin was organized into definitive A bands (C, double arrow) or accumulated in long, parallel, striated fibrils (D, arrows). Notably, Z disks and M lines were still discernible in myotubes infected with obscurinnt1503–1863 virus, although they were not as sharply defined as they were in control cells (A and C, arrows and arrowheads, respectively). Original magnification, ×20,000.
10% of infected myotubes, myosin was expressed in low amounts but exhibited punctate staining within the myoplasm (Fig. 3, B and B', single arrow) with occasional accumulations at nonstriated fibrils (Fig. 3, B and B', two-headed arrow) and near the cell periphery (Fig. 3B', arrowhead). The great majority (~80%) of the infected cells showed bright staining for myosin in cytoplasmic speckles (Fig. 3, C and C', arrow) or in long filaments with occasional periodicity (Fig. 3, C and C', arrowheads), but not in regular A bands. By contrast, ~95% of myotubes infected with control GFP virus showed regular organization of sarcomeric myosin in typical A bands (Fig. 3, D and D'). We have also generated a number of adenoviral GFP constructs carrying different parts of muscle cytoskeletal (e.g., spectrin), intermediate filament (e.g., cytokeratin), and membrane (e.g., s Ank1) proteins and used them to infect primary cultures of neonatal skeletal myotubes. Figure 3, E and E', show the normal distribution of sarcomeric myosin in A bands after overexpression of the cytoplasmic domain of s Ank1 aa29–155 (126 amino acids), which is similar in size to the COOH-terminal fragment of obscurin (120 amino acids) and contains the obscurin binding site. None of the other proteins (spectrin or cytokeratin) prevented myosin from assembling into regular A bands (data not shown). Moreover, we recently generated adenoviruses carrying the Dbl homology and pleckstrin homology domains of obscurin fused to GFP and successfully infected postnatal skeletal myotubes. Myosin also became organized into typical A bands in these cells (data not shown). These experiments further suggest that the inhibition
of myosin’s assembly into A bands after overexpression of GFP-obscurinnt1503–1863 was specifically caused by the COOH terminus of obscurin.

The failure of sarcomeric myosin to assemble into periodic A bands after overexpression of the COOH terminus of obscurinnt1503–1863 was further studied with the use of electron microscopy (Fig. 4). Consistent with the immunofluorescence data (Fig. 3), ultrastructural examination of treated myotubes revealed the total absence of regular A bands (Fig. 4, A and B). Myosin filaments either extended uniformly along the entire length of assembled sarcomeres, between neighboring Z disks (Fig. 4A, double arrow), rendering both A and I bands indistinct, or accumulated in long, filamentous structures with no obvious periodicity (Fig. 4B, arrows). By contrast, myotubes infected with control GFP virus contained sarcomeric myosin that was organized either into characteristic A bands (Fig. 4C, double arrow) or into long, striated fibrils (Fig. 4D, arrows). Notably, Z disks and M lines were still detectable in myotubes infected with obscurinnt1503–1863 Virus, although they were not as sharply delineated as they were in control cells (Fig. 4, A and C, arrows and arrowheads, respectively).

Several other myofibrillar proteins, including titin, myomesin, actin, and α-actinin (Fig. 5), were not affected by viral overexpression of the COOH-terminal region of obscurin. Titin was organized into regular striations at the A-I junction (Fig. 5, A and A') and the Z disk (Fig. 5, E and E'). Myomesin also assumed a periodic distribution at developing M lines (Fig. 5, B and B'), whereas actin and α-actinin were present in regular striations at I bands and Z disks, respectively (Fig. 5, C, C', D, and D'). Notably, endogenous obscurin was still organized in transverse striations at the level of the M line and the Z disk (Fig. 5, F and F', arrow and arrowhead, respectively), suggesting that the obscurinnt1503–1863 fragment neither mediates anchoring of native obscurin to the periphery of the contractile apparatus nor competes with the endogenous protein for binding to the M line and the Z disk. No labeling was detected when primary antibodies were replaced by nonimmune rabbit or mouse IgG (data not shown). Thus overexpression of GFP-obscurinnt1503–1863 specifically disrupted the assembly and organization of sarcomeric myosin into regular A bands without altering the subcellular distribution of several other sarcomeric markers.

To determine whether the disruption of myosin assembly into A bands reflected a general decrease in myosin content within myotubes, we collected homogenates of P1 myotubes infected with viruses expressing either GFP-obscurinnt1503–1863 or GFP and analyzed them by performing SDS-PAGE followed by immunoblotting for myosin and α-actinin, which served as controls (Fig. 6). Quantitation of the immunoreactive bands with MetaMorph Imaging System software showed a small but consistent decrease of ~20% compared with controls (P < 0.05) in the amount of myosin expressed in the cultures that overexpressed GFP-obscurinnt1503–1863 (Fig. 6A). In contrast, the levels of α-actinin were unchanged between the two infected cell populations (Fig. 6B), suggesting that the effect on myosin was specific. These observations are illustrated in Fig. 6C. The apparent reduction in the expression levels of myosin may be due to the failure of myosin to integrate into A bands, rendering it more susceptible to proteases present in the myoplasm.

To test the hypothesis that the COOH-terminal region of obscurin disrupts A bands by binding directly or indirectly to myosin, we used antibodies to obscurin or myosin to generate immunoprecipitates from homogenates of adult skeletal muscle (Fig. 7). Both immunoprecipitates contained a band at ~800 kDa that reacted with anti-obscurin (Fig. 7A, first and third lanes, top arrow) as well as a band at ~220 kDa that reacted with anti-myosin (Fig. 7B, first and third lanes, arrowhead). In control experiments in which nonimmune rabbit and mouse IgG were used, neither obscurin (Fig. 7A, second and fourth lanes) nor myosin (Fig. 7B, second and fourth lanes) was precipitated. The coimmunoprecipitation of obscurin and myosin is consistent with their presence in a complex within the myoplasm of adult skeletal myofibers.
DISCUSSION

Myofibrillogenesis is a highly complex process that depends on the coordinated assembly and integration of a number of cytoskeletal scaffolding and signaling proteins, including the giant proteins nebulin and titin (3, 10). Nebulin serves as a “molecular blueprint” for the assembly of thin actin filaments (29, 30), whereas titin acts as a “sarcomeric template” that interacts directly with both thin actin and thick myosin filaments, facilitating their coordinated integration into periodic I- and A bands, respectively (17, 29).

In the current study, we examined the role of obscurin, a recently identified giant sarcomeric protein, in the assembly and organization of the sarcomere, by overexpressing a portion of its COOH-terminal sequence through adenovirally mediated gene delivery in primary cultures of skeletal myotubes. We show that obscurin exists in a complex with sarcomeric myosin in homogenates of skeletal muscle and plays a critical role in the formation and maintenance of periodic A bands in developing muscle cells. Our study is the first to document a functional role for obscurin during myofibrillogenesis. Because obscurin is a bona fide M-line protein, our data also pinpoint the important role of obscurin as a scaffolding or regulatory molecule in the organization of thick myosin filaments into regular A bands.

Recent studies have postulated that M-line proteins, including myomesin and M-protein, assemble into primordial M-line structures before the incorporation of the COOH-terminal portion of titin into M lines (19, 28, 31, 32). Both myosin and M-protein bind directly to titin and are thought to promote the proper orientation and incorporation of its COOH terminus into developing M lines (20, 21). Both proteins also bind directly to sarcomeric myosin, suggesting that they may link titin to myosin filaments (18, 20). All of these events occur before A bands mature, suggesting an important role for M-line proteins in the assembly of A bands similar to the well-characterized role of α-actinin at the Z disk in the formation of I bands.

Obscurin, too, is likely to play an important role in organizing the A band, for several reasons. It is abundantly expressed early in embryogenesis and is readily detected in the somites of 9-day-old mouse embryos (unpublished observations). Obscurin becomes organized at M lines in neonatal cardiomyocytes (33) and skeletal myotubes (Fig. 2) before myosin aligns into definitive A bands. In C5C12 myotubes, it assembles at M lines concomitantly with the periodic organization of Z-disk epitopes of titin and slightly before the regular assembly of A band epitopes of titin (unpublished observations). Furthermore, like titin, myomesin, and M protein, obscurin also interacts with myosin, although at present we cannot be sure whether their interaction is direct or indirect. The multiple binding interactions among this group of proteins suggest that they are present in a complex at the level of the M line that plays a key role in the assembly of A bands.

Our present results show that the COOH-terminal region of obscurin harbors a sequence that, when overexpressed, prevents the formation of A bands but not of M lines. It is conceivable that during early myofibrillogenesis and before expression of sarcomeric myosin, M-line proteins, including endogenous obscurin and myomesin, form primordial M lines, perhaps together with nonmuscle myosin or another developmental myosin isoform, and that this process continues undisturbed in the presence of obscurinnt1503–1863. In this case, the COOH-terminal region of obscurin may interact with sarcomeric myosin, but not with nonsarcomeric or developmental myosins, to prevent its incorporation into A bands while permitting M-line proteins to assemble normally. Consistent with this notion, an earlier study postulated that there is a basic framework in the sarcomere established by structures that form the M line and the Z disk and that this framework is not affected when both thick and thin filaments are selectively removed (7).

The importance of obscurin in modulating the assembly of myosin into regular A bands is further underscored by the results of studies of its homolog, unc-89, in C. elegans (2). Deletion mutants of unc-89 show a normal number of thick filaments but severely disrupted A bands devoid of M lines, a property not shared by deletion mutants in the other ~50 genes that are essential to the assembly, organization, and function of muscle in C. elegans.

We must still determine whether obscurin interacts with myosin directly. In vitro binding studies have shown that obscurinnt1503–1863 and myosin interact weakly (unpublished observations). However, the COOH terminus of obscurin contains several copies of a consensus phosphorylation motif for ERK kinases (33), suggesting that its activity may be modulated by phosphorylation. The interaction between M-protein and myosin is also relatively weak in vitro and is regulated by protein kinase A phosphorylation of a single serine residue (21). Alternatively, obscurin and myosin may interact indirectly through an intermediate molecule. Likely candidates are myosin binding protein C and titin, both of which specifically and directly interact with myosin (13, 23) and obscurin (14, 33).

In summary, our results show that overexpression of obscurinnt1503–1863 in primary myotubes has a specific and profound effect on the organization of sarcomeric myosin. We propose that obscurin is essential for the organization and regular assembly of A bands in developing muscle cells.

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