INTRODUCTION

Actin filaments are polymerized to strikingly uniform lengths in a variety of elaborate supramolecular structures in differentiated cells. Some dramatic examples include the ~1 μm long thin filaments in striated muscle sarcomeres, the ~40 nm long actin filaments in the erythrocyte membrane skeleton, the ~2.5 μm long actin filament bundles in intestinal epithelial cell microvilli, and the ~10 μm long actin filament bundles in Drosophila cuticular bristles (for reviews, see Fowler, 1996; Mooseker, 1985; Robinson and Cooley, 1997). Capping of the fast growing (barbed) and slow growing (pointed) actin filament ends is believed to be important both for regulation of filament assembly into these specialized architectural arrays, and for stabilization of uniform filament lengths once the filaments have been assembled (for recent discussions, see Fowler, 1996, 1997; Littlefield and Fowler, 1998). In vitro, barbed and pointed end actin capping proteins can regulate filament elongation and depolymerization by nucleating new filament assembly as well as by inhibiting monomer association and dissociation from filament ends (Schafer and Cooper, 1995; Mullins et al., 1998; Weber et al., 1994). However, in vivo, the relative contributions of barbed and pointed end capping proteins to the initiation, regulation of rates and termination of filament growth are not well understood.

Striated muscle is an excellent system to address these questions because both the barbed (CapZ) and pointed (tropomodulin) end capping proteins for the actin filaments have been identified (Fowler, 1996), unlike other systems that demonstrate uniform actin filament lengths. Furthermore, the ~1 μm long thin filaments in muscle are organized into regular, periodic structures and are believed to depend on the regulated activity of capping proteins at both the fast growing (barbed) and slow growing (pointed) filament ends. In striated muscle, the pointed end capping protein, tropomodulin, has been shown to maintain the lengths of thin filaments in mature myofibrils. To determine whether tropomodulin might also be involved in thin filament assembly, we investigated the assembly of tropomodulin into myofibrils during differentiation of primary cultures of chick skeletal muscle cells. Our results show that tropomodulin is expressed early in differentiation and is associated with the earliest premyofibrils which contain overlapping and misaligned actin filaments. In addition, tropomodulin can be found in thin filament bundles at the distal tips of growing myotubes, where sarcomeric α-actinin is not always detected, suggesting that tropomodulin caps actin filament pointed ends even before the filaments are cross-linked into Z bodies by α-actinin. Tropomodulin staining exhibits an irregular punctate pattern along the length of premyofibrils that demonstrate a smooth phalloidin staining pattern for F-actin. Strikingly, the tropomodulin dots often appear to be located between the closely spaced, dot-like Z bodies that are stained for α-actinin. Thus, in the earliest premyofibrils, the pointed ends of the thin filaments are clustered and partially aligned with respect to the Z bodies (the location of the barbed filament ends). At later stages of differentiation, the tropomodulin dots become aligned into regular periodic striations concurrently with the appearance of striated phalloidin staining for F-actin and alignment of Z bodies into Z lines. Tropomodulin, together with the barbed end capping protein, CapZ, may function from the earliest stages of myofibrillogenesis to restrict the lengths of newly assembled thin filaments by capping their ends; thus, transitions from nonstriated to striated myofibrils in skeletal muscle are likely due principally to filament rearrangements rather than to filament polymerization or depolymerization. Rearrangements of actin filaments capped at their pointed and barbed ends may be a general mechanism by which cells restructure their actin cytoskeletal networks during cell growth and differentiation.

SUMMARY

Actin filament lengths in muscle and nonmuscle cells are believed to depend on the regulated activity of capping proteins at both the fast growing (barbed) and slow growing (pointed) filament ends. In striated muscle, the pointed end capping protein, tropomodulin, has been shown to maintain the lengths of thin filaments in mature myofibrils. To determine whether tropomodulin might also be involved in thin filament assembly, we investigated the assembly of tropomodulin into myofibrils during differentiation of primary cultures of chick skeletal muscle cells. Our results show that tropomodulin is expressed early in differentiation and is associated with the earliest premyofibrils which contain overlapping and misaligned actin filaments. In addition, tropomodulin can be found in thin filament bundles at the distal tips of growing myotubes, where sarcomeric α-actinin is not always detected, suggesting that tropomodulin caps actin filament pointed ends even before the filaments are cross-linked into Z bodies by α-actinin. Tropomodulin staining exhibits an irregular punctate pattern along the length of premyofibrils that demonstrate a smooth phalloidin staining pattern for F-actin. Strikingly, the tropomodulin dots often appear to be located between the closely spaced, dot-like Z bodies that are stained for α-actinin. Thus, in the earliest premyofibrils, the pointed ends of the thin filaments are clustered and partially aligned with respect to the Z bodies (the location of the barbed filament ends). At later stages of differentiation, the tropomodulin dots become aligned into regular periodic striations concurrently with the appearance of striated phalloidin staining for F-actin and alignment of Z bodies into Z lines. Tropomodulin, together with the barbed end capping protein, CapZ, may function from the earliest stages of myofibrillogenesis to restrict the lengths of newly assembled thin filaments by capping their ends; thus, transitions from nonstriated to striated myofibrils in skeletal muscle are likely due principally to filament rearrangements rather than to filament polymerization or depolymerization. Rearrangements of actin filaments capped at their pointed and barbed ends may be a general mechanism by which cells restructure their actin cytoskeletal networks during cell growth and differentiation.

Key words: Tropomodulin, Actin, Myofibril, Muscle
repeating, contractile units called sarcomeres whose periodic (striated) organization can be easily visualized by light microscopy. In particular, thin filaments are aligned in an anti-parallel, polarized fashion in each sarcomere with the barbed filament ends at the Z line and the pointed filament ends in the middle of the sarcomere (Huxley, 1960).

CapZ is believed to function early in myofibril assembly to nucleate actin filament assembly, and to target and align the barbed ends of the thin filaments at the Z line (i.e. to establish filament polarity). This idea was based initially on association of CapZ with nascent myofibrils in both skeletal and cardiac myocytes (Schafer et al., 1993, 1994). In a subsequent study, inhibition of CapZ's actin capping activity in skeletal myotubes by microinjection of anti-CapZ antibodies or by dominant negative transfection approaches resulted in disruption of nonstriated premyofibrils or delays in formation of actin striations, respectively (Schafer et al., 1995).

In contrast, several experimental approaches indicate that tropomodulin functions late in myofibril assembly to maintain actin filament lengths and thin filament stability in mature myofibrils in cardiac myocytes. Microinjection of an antibody which inhibits tropomodulin's actin capping activity into chick cardiac myocytes leads to continuous (nonstriated) actin filament staining along myofibrils, due to inappropriate elongation of actin from the pointed ends of the thin filaments (Gregorio et al., 1995). These cells are no longer capable of beating, demonstrating that maintenance of actin filament length by tropomodulin is required for contraction. Furthermore, recent experiments altering tropomodulin levels by infection with sense or antisense adenovirus vectors reveal that reduction of tropomodulin levels in neonatal rat cardiac myocytes isolated from hearts of day 6 chick myocytes (Gregorio and Fowler, 1995). Tropomodulin is one of the latest markers of myofibril assembly in this cell type, and is only found assembled into myofibrils after the appearance of actin filament striations; i.e. after acquisition of mature thin filament lengths. However, since primary cultures of cardiac myocytes isolated from hearts of day 6 chick embryos do not differentiate de novo in culture, the nature of myofibril assembly precursors and intermediates in this cell model are somewhat controversial (e.g. see Dlugosz et al., 1984; Lin et al., 1989; Rhee et al., 1994).

It thus remains an open question as to whether tropomodulin might play a role in the assembly of actin filaments into muscle sarcomeres, in addition to its role in maintaining filament length in the mature structures. To investigate this, we used primary cultures of chick skeletal myogenic cells, an experimental system in which myoblasts differentiate de novo and fuse to form multinucleated myotubes in a relatively synchronous manner. In these cells, contractile proteins are synthesized coordinately and assembled into mature myofibrils over a period of several days (Cappers, 1960; Cooper and Konigsberg, 1961; Stockdale and Holtzer, 1961). Here, we have investigated the spatial and temporal distribution of tropomodulin during myofibril assembly with respect to other thin filament proteins.

Our results demonstrate that tropomodulin is associated with the first nonstriated premyofibrils that appear during myofibril assembly, including actin filament bundles found at the extreme distal tips of young growing myotubes. Furthermore, tropomodulin staining along nonstriated premyofibrils appears as an irregular pattern of dots which are often located between closely spaced dots of α-actinin, a marker for nascent Z bodies where the barbed ends of the thin filaments are located. The presence of both tropomodulin and CapZ along early nonstriated premyofibrils indicates that both the pointed and barbed filament ends of the thin filaments are capped from the initial stages of myofibril assembly in skeletal muscle cells. We conclude that transitions from nonstriated precursors to mature striated myofibrils may be principally due to thin filament rearrangements and alignments rather than to processes involving extensive elongation or depolymerization of filaments. Restructuring the actin cytoskeleton by filament rearrangements may be a general mechanism for gradual remodelling of the cytoskeleton during cell growth and differentiation.

**MATERIALS AND METHODS**

**Cell culture**

Skeletal muscle cultures were prepared from day 12 chick embryonic pectoralis muscle basically as described ( Nawrotzki et al., 1995; Gilbert et al., 1996). For the biochemical studies, cells were plated at 2×10⁵ cells/ml on collagen-coated 35 mm tissue culture dishes. For immunofluorescence analysis, cells were plated at 5×10⁵ cells/ml on collagen- (Sigma, St Louis, MO) or Matrigel- (Beckton Dickinson) coated glass coverslips. The same results were obtained with either coating. Collagen was prepared as a 0.01% (w/v) solution in 0.1% acetic acid. Autoclaved coverslips were coated with collagen for 2 hours at 37°C. Excess collagen solution was removed by several washes in Dulbecco’s PBS (0.136 M NaCl, 2.6 mM KCl, 1.4 mM KH₂PO₄, 8 mM NaHPO₄, pH 7.4). Alternatively, coverslips were coated with a drop of Matrigel for 2 hours at room temperature, excess liquid was aspirated and the coverslips were dried before use.

The cells were fed 24 hours after plating and then every other day thereafter with Dulbecco’s modified Eagle’s medium (Gibco BRL, Grand Island, NY), supplemented with 10% (v/v) ‘selected’ fetal bovine serum (HyClone, Logan, Utah), 4% (v/v) chick embryo extract and 1% (v/v) antibiotics-antimycotics (Gibco BRL). On day three, the medium was supplemented with 1 μg/ml cytokine β-D-arabinofuranoside (Sigma) to limit proliferation of contaminating fibroblasts. Embryonic chick cardiac myocyte cultures were prepared from day 6 embryonic chick hearts (Gregorio and Fowler, 1995).

**Antibodies**

Monoclonal antibodies to tropomodulin were generated using recombinant chicken tropomodulin (E-Tmod) (Babcock and Fowler, 1994) as an antigen (Chris W. Grant, Custom Monoclonals, W. Sacramento, CA) and were purified over a Protein G-Sepharose column (Pharmacia, Uppsala, Sweden). Monoclonal antibody 95 (mAb 95) was used for immunofluorescence staining; the specificity of this antibody is demonstrated in Fig. 1 using western blot analysis and immunoprecipitation of [³⁵S]methionine labelled proteins from day 5 chick skeletal myotubes. Monoclonal antibody 23 (mAb23) was used...
for the western blotting analysis of tropomodulin isoforms on 2-D gels since it recognizes both the E and SK-tropomodulin isoforms present in chicken striated muscle (Almenar-Queralt et al., 1997). Polyclonal antibodies to recombinant chicken E-Tmod (Babcock and Fowler, 1994) were generated in rabbits and affinity purified by standard procedures (Fowler and Adam, 1992) and characterized for specificity as for mAb 95; it recognized a single ~40 kDa polypeptide (data not shown). The monoclonal anti-sarcomeric α-actinin antibody (clone 9A2B8; Schultheiss et al., 1990) was a gift from Drs S. and H. Holtzer (University of Pennsylvania, Philadelphia, PA). Bodipy-labelled phallacidin, fluorescein conjugated-goat anti-rabbit IgG, rhodamine (TRITC)-conjugated donkey anti-mouse IgG and Cy2-conjugated goat anti-rabbit antibodies were purchased from Molecular Probes Inc. (Eugene, OR), Boehringer Mannheim Biochemicals (Indianapolis, IN), Accurate Chemical and Scientific Corporation (Westbury, NY), and Pierce (Rockford, IL), respectively. mAb 95 was conjugated with Alexa using an Alexa-594 labelling kit from Molecular Probes, Inc.

**Immunofluorescence microscopy**

Skeletal muscle cells were fixed and stained as described (Gregorio and Fowler, 1995). To permeabilize the cells and minimize nonspecific binding of antibodies, the coverslips were pre-incubated in PBS containing 3% bovine serum albumin (BSA) (ICN, Aurora, OH) and 0.2% Triton X-100 for 30 minutes. For double staining of tropomodulin with F-actin, cells were incubated with mAb 95 at 2 μg/ml, followed by rhodamine-conjugated donkey anti-mouse IgG (1:200) and bodipy-phallacidin (1:200). For double staining of tropomodulin with F-actin, cells were incubated with mAb 95 at 2 μg/ml, followed by rhodamine-conjugated donkey anti-mouse IgG and Cy2-conjugated goat anti-rabbit antibodies (1:400), followed by rhodamine-conjugated donkey anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (1:200) and bodipy-phallacidin (1:200). For double staining of tropomodulin with F-actin, cells were incubated with mAb 95 at 2 μg/ml, followed by rhodamine-conjugated donkey anti-mouse IgG and Cy2-conjugated goat anti-rabbit antibodies (1:400), followed by rhodamine-conjugated donkey anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (1:200). Alternatively, cells were first incubated with Alexa-conjugated mAb95 followed by incubation in normal mouse serum (1:50), then incubated in the monoclonal anti-α-actinin antibody followed by Cy2-conjugated goat anti-mouse antibodies (1:400). Stained coverslips were observed using a x63 (1.4 NA) Plan-Apochromat or a ×100 (1.3 NA) Plan Neofluar objective lens on a Zeiss Axioskop microscope equipped with filters that exclude crossover between fluorochromes used. Digital images were acquired with a Princeton Instruments 1317K CCD camera with a KAF 1400 chip (pixel size, 6.8 μm²) or a Princeton Instruments 1300Y CCD camera with a Sony Interline chip (pixel size, 6.7 μm²), using IP lab software. Images were processed for presentation using Adobe Photoshop. For some images, we applied a high pass filter to reduce the background. Separate images of double labelled specimens were merged and aligned using Adobe Photoshop by superimposition of asymmetric identical features (e.g. the edge of the cell, intersecting myofibrils); images captured the same day had a constant offset within a pixel. In some experiments (indicated in the Fig. legends), images were acquired on a Bio-Rad MRC 600 Confocal Microscope. Similar results were obtained by acquiring images with a CCD camera, confocal microscope or by recording images on film.

**Electron microscopy**

Myotubes grown on collagen-coated plastic tissue culture dishes were fixed and processed for electron microscopy at different days in culture as previously described (Tilney and Tilney, 1994). Ultrathin sections were cut parallel to the plate, mounted on grids, and stained again with uranyl acetate and lead citrate (Reynolds, 1963). Sections were examined at 70 kV on a Hitachi HU600 electron microscope and photomicrographs taken at several different magnifications. Distances between Z bodies along premyofibrils or Z lines along myofibrils from early and late days in culture, respectively, were analyzed from prints of micrographs at ×20,000 to ×25,000 magnification. Straight lines were drawn between the centers of adjacent Z bodies (or Z lines), parallel to the long axis of the myofibrils, and measured directly on the prints; ≥100 measurements were made from prints of the central regions (shafts) of the myotubes.

**Metabolic labelling, immunoprecipitation and electrophoresis procedures**

Myotubes were metabolically labelled for 3 hours with 200 μCi/ml of Trans 35 S-label (ICN Pharmaceuticals, Inc., Irvine, CA) and proteins were solubilized and prepared for immunoprecipitation according to the method of Gregorio and Fowler (1995). 35 S-labelled tropomodulin was immunoprecipitated from the extracts by addition of 2 μg of mAb95 that had been preadsorbed to Protein A Trisacryl beads (Pierce) coated with rabbit anti-mouse IgG+IgM (H+L) (Pierce), electrophoresed on 12% acrylamide mini-gels (Laemmli, 1970; Fowler, 1990), and gels were dried down and exposed to film by standard procedures. For western blotting, gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) (Towbin et al., 1979), and then incubated for 2 hours at room temperature with monoclonal anti-tropomodulin mAb95 (10 μg/ml) (Fig. 1) followed by rabbit anti-mouse IgG+IgM (H+L) (1:1000) (Pierce, Rockford, IL) and 125I-Protein A (Fowler, 1990; Fowler et al., 1993).

The tropomodulin isoform composition in skeletal and cardiac myogenic cultures was characterized by 2-dimensional (2-D) gel electrophoresis followed by western blotting with mAb23 (see above). Briefly, chick skeletal and cardiac myogenic cells were washed with ice-cold PBS plus protease inhibitors (Gregorio and Fowler, 1995) and then extracted with 9.2 M urea plus 1% (v/v) β-mercaptoethanol for 30 minutes at room temperature on a rocking platform. The total extract was collected and then centrifuged at 15,000 g for 10 minutes. The resulting supernatants were electrophoresed on two-dimensional gels (O’Farrell, 1975; as modified by Granger et al., 1982). The tube gels (worms) for the first dimension contained 2% Ampholines, pH range 5-7, 1% Ampholines, pH range 3-10 (Pharmacia). For the second dimension, the worms were run on 7.5% to 15% linear gradient acrylamide gels, pH 8.6 (Laemmli, 1970; Fowler, 1990). Nitrocellulose blots were probed with mAb23 as described above.

**RESULTS**

**Thick and thin filaments reorganize during myofibril assembly in skeletal muscle cultures**

To provide a framework for subsequent interpretation of our biochemical and immunofluorescence analysis of tropomodulin assembly into myofibrils we, like others, used thin section electron microscopy to analyze myofibril assembly in our cultures of chick skeletal muscle myotubes (e.g. Antin et al., 1986; Shimada et al., 1967; for a recent review, see Franzini-Armstrong and Fischman, 1996). Examination of myotubes from different days in culture shows that thin and thick filaments are present in bundles from the earliest days in culture, but that a dramatic change in filament organization appears to take place during differentiation (Fig. 2). First, in young myotubes in early cultures (~days 2-3), premyofibrils which consist of loose bundles of thin (actin) filaments interspersed between thick (myosin) filaments are observed all along the length of the myotubes (Fig. 2A). The thin filaments insert into darkly stained dense bodies (Z bodies) which are irregularly distributed along the premyofibrils (Fig. 2A, long arrows); this, together with the misalignments of thin and thick filaments, accounts for the nonstrialed appearance of premyofibrils by immunofluorescence staining (see below, Fig. 3). Adjacent Z bodies are spaced at distances ranging from 0.6
Cells from a day 5 culture were also metabolically labeled with rabbit anti-mouse antibodies and [125I]-Protein A (Western, lane 2). SDS-extracts from day 5 embryonic chick skeletal myogenic cultures were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie Blue (Coomassie, lane 1) or transferred to a nitrocellulose membrane and labelled with mAb95, followed by monoclonal anti-tropomodulin antibody 95 (mAb95) (IP , lane 3). The positions of myosin and actin are indicated on the left side of Lane 1, and the arrowhead in lane 3 indicates the position of tropomodulin (Tmod) (~40 kDa).

Fig. 1. Monoclonal anti-tropomodulin antibody 95 (mAb95) specifically recognizes tropomodulin in chick skeletal muscle cells, SDS-extracts from day 5 embryonic chick skeletal myogenic cultures were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie Blue (Coomassie, lane 1) or transferred to a nitrocellulose membrane and labelled with mAb95, followed by Coomassie Blue (Coomassie, lane 1) or transferred to a nitrocellulose membrane and labelled with mAb95, followed by rabbit anti-mouse antibodies and [125I]-Protein A (Western, lane 2). Cells from a day 5 culture were also metabolically labeled with [35S]methionine and tropomodulin was immunoprecipitated with mAb95 (IP; lane 3). The positions of myosin and actin are indicated on the left side of Lane 1, and the arrowhead in lane 3 indicates the position of tropomodulin (Tmod) (~40 kDa).

to 2.8 μm apart along the long axis of the premyofibrils; thus, many of them are considerably closer together than are Z lines of mature myofibrils (2.2±0.15 μm). Occasionally, in early cultures, what appear to be nascent sarcomeres are observed in which small groups of 1.6 μm long thick filaments are partially aligned and positioned between adjacent Z bodies along the long axis of the premyofibril (Fig. 2A, short arrows). Thin filaments emerging from adjacent Z bodies appear to overlap with one another; however, their lengths cannot be measured because the filaments in the loosely packed premyofibrils are not perfectly straight and often leave the plane of section (Fischman, 1967; Allen and Pepe, 1965, 1973; Auber, 1969; Fischman, 1967; Kelly, 1969).

By days ~3-4 of culture, most cells have fused to form myotubes and structures resembling sarcomeres appear more frequently along the central portion (the shaft) of the myotubes (Fig. 2B). However, it is common to find myofibrils at apparently earlier stages of assembly adjacent to the more striated myofibrils (Fig. 2B, asterisk), as well as at the tips of myotubes (data not shown and see below). Z bodies are clustered in groups, often forming irregular Z lines (Fig. 2B, long arrows) and the thick filaments become fairly well aligned between adjacent clusters of Z bodies (Fig. 2B, short arrows). At this stage, the M line is apparent in the center of the thick filament array in some sarcomeres (Fig. 2B, arrowheads). The lengths of the I bands are very variable in these intermediate cultures; some of the thick filament ends are almost juxtaposed to the Z bodies, while in other cases they are more distant (Fig. 2B, bracket).

Finally, in later cultures (~day 5), many myotubes start to contract spontaneously (twitch), myofibrils grow in length and width, and the thin and thick filaments become aligned into mature sarcomeres with well defined Z lines and regular I bands along the shaft of the long myotubes (Fig. 2C). High magnification micrographs reveal that the thin filaments from opposite Z lines overlap in the middle of the sarcomere (Fig. 2D, arrowheads), thus an H zone (gap in the middle of the sarcomere) is not present. While thin filament pointed ends cannot be discerned with certainty in these thin sections, the striated fluorescent staining patterns for tropomodulin and phallolidin indicate that thin filaments are likely to be between about 1 and 1.1 μm long in the mature sarcomeres (Fig. 3E,F), in agreement with previous estimates (Allen and Pepe, 1965; Fischman, 1967; Ohtsuki, 1979). It is not known if thin filament overlaps at the pointed ends in the sarcomeres are due to contraction during fixation or are the normal state of the mature striated myofibrils in cultured chick myotubes.

These ultrastructural features of myofibril assembly are in agreement with previous studies of primary cultures of embryonic chick skeletal myotubes (Antin et al., 1986; Shimada et al., 1967; Shimada and Obinata, 1977), and closely resemble the ultrastructural features of myofibril assembly in developing vertebrate skeletal muscle in vivo (Allen and Pepe, 1965; Allen, 1973; Auber, 1969; Fischman, 1967; Kelly, 1969).

**Tropomodulin is associated with premyofibrils in a punctate pattern and becomes striated concurrently with actin during myofibril assembly**

We used immunofluorescence staining to visualize when and where tropomodulin was assembled into myofibrils with respect to thin filament alignments during skeletal muscle differentiation. Phallacidin staining for F-actin was used to reveal the location of thin filaments and whether they were organized into early nonstriated (premyofibril) or striated myofibrils (Epstein and Fischman, 1991; Franzini-Armstrong and Fischman, 1996; Gregorio and Fowler, 1996; Holtzer et al., 1997). In young myotubes in early days of culture (e.g. day 2), tropomodulin staining is already associated with nonstriated premyofibrils running parallel to the long axis of the cells (Fig. 3A, arrows). At this early stage, the bodipy-phallacidin staining of individual myofibrils is now somewhat uneven in appearance along their...
length (Fig. 3D, arrows) (also see McKenna et al., 1986; Sasaki and Kuroda, 1994). It is not clear what the relationship of uneven phallacidin staining is to the periodic bands of tropomodulin staining.

In yet older myotubes later in culture (e.g. day 7), when the myotubes have spontaneously begun to twitch, the bands of tropomodulin staining along myofibrils in the shaft of the myotubes now appear narrower and crisper and farther apart (~2.3 μm), taking on the typical striated appearance for tropomodulin staining in mature myofibrils (Fig. 3E, arrows) (Fowler et al., 1993). The inability to resolve tropomodulin staining as two distinct stripes in each half sarcomere is due to the overlapping of thin filament ends in the slightly contracted sarcomeres typical of these myotubes (Fig. 2D) (also see

Fig. 2. Ultrastructural analysis of the organization of thick and thin filaments during myofibrillogenesis in primary cultures of embryonic chick skeletal myogenic cells. Electron micrographs of longitudinal sections of myofibrils in myotube shafts (i.e. the central portion of myotubes) from Early (A), Intermediate (Int., B) and Late (C,D) cultures (days, 2, 4, and 6, respectively). Long arrows, Z bodies or Z lines (A-C). Short arrows, thick filaments (A-C). Arrowheads, M lines (A-C); thin filaments (D). Brackets, I bands (B,C). Asterisk, a myofibril at an apparently earlier stage of assembly (B). Bars, 0.5 μm (A-C); 0.2 μm (D).
Fig. 3. The distribution patterns of tropomodulin change concurrently with actin filament reorganization during myofibril assembly. Fluorescence micrographs of mAb95 staining for tropomodulin (A,C,E) and phallacidin staining for F-actin (B,D,F) in chick skeletal myotube shafts from Early (A,B), Intermediate (Int., C,D) and Late (E,F) days of culture (days 2, 4, and 7, respectively). Arrows, premyofibrils (A,B,D). Brackets, tropomodulin bands (C). Arrows, mature tropomodulin striations (E); mature phallacidin striations (F) at the thin filament pointed ends. Arrowheads, phallacidin staining of thin filament barbed ends at the Z line (F). C and D are confocal micrographs. Bar, 10 μm.

Gregorio and Fowler, 1995). The tropomodulin striations also grow in length perpendicularly to the long axis of the myofibrils (Fig. 3E), reflecting the growth in myofibril width and increasing lateral organization of adjacent myofibrils that is also evident in thin section electron micrographs (Fig. 2C).

Interestingly, bodipy-phallacidin also stains mature myofibrils in a pattern of narrow bright stripes (Fig. 3F, arrows) which colocalize with the tropomodulin stripes in merged images (not shown). In addition, a fainter stripe of phallacidin staining is present between the bright striations (Fig. 3F, arrowheads), corresponding to the location of the Z line, as demonstrated by colocalization with α-actinin (not shown). Preferential staining of thin filament pointed and barbed ends by phalloidin (or phallacidin) has been observed previously in mature myofibrils in cultured chick skeletal muscle cells (Antin et al., 1986; Sasaki and Kuroda, 1994) as well as in isolated myofibrils (Ao and Lehrer, 1995; Zhukarev et al., 1997 and references therein). This has been proposed to be due to the presence of nebulin along thin filaments (Ao and Lehrer, 1995).

These immunofluorescence staining results demonstrate that tropomodulin is associated with early nonstriated premyofibrils as well as with mature myofibrils, and implies that the thin filament pointed ends are capped by tropomodulin at all stages of myofibril assembly during skeletal muscle differentiation. Further, the irregular dot-like pattern of tropomodulin staining at early stages of differentiation suggests that tropomodulin-capped pointed ends are clustered in regions along premyofibrils. The changes in tropomodulin staining patterns observed in myotubes on later days of culture (Fig. 3B,C) suggest a temporal sequence of myofibril assembly in which the tropomodulin dots in premyofibrils coalesce and align to form broader bands, followed by a transition to narrow stripes in mature myofibrils. Such a rearrangement of pointed end clusters would be similar to rearrangements of tropomodulin bands (C). Arrows, mature tropomodulin striations (E); mature phallacidin striations (F) at the thin filament pointed ends. Arrowheads, phallacidin staining of thin filament barbed ends at the Z line (F). C and D are confocal micrographs. Bar, 10 μm.

Clusters of thin filament pointed ends (tropomodulin) appear to alternate with Z bodies (α-actinin) along premyofibrils

To investigate if the distribution of tropomodulin dots along premyofibrils (clusters of thin filament pointed ends) might be complementary to the distribution of Z bodies, (the presumed location of the barbed filament ends) we used antibodies to α-actinin as a marker for Z bodies. α-Actinin cross-links actin filaments at their barbed ends and is a classic marker for nascent Z bodies (Holtzer et al., 1997; Mckenna et al., 1986; Sanger et al., 1986). For this experiment, myotubes from day 2 in culture were double stained with a rabbit polyclonal antibody to tropomodulin (Fig. 4A) and a monoclonal antibody to α-actinin (Fig. 4B). As described above for the tropomodulin monoclonal antibody (Fig. 3A), tropomodulin staining is detected in a discontinous, punctate pattern along the myofibrils. α-Actinin is also present in a distinct punctate pattern irregularly distributed along premyofibrils (Fig. 4B), as expected from the irregular distribution of Z bodies along premyofibrils at early stages in culture (Fig. 2A). Comparison of red (α-actinin) and green (tropomodulin) staining in the merged image (Fig. 4C) reveals that in general there is relatively little overlap (yellow) between the tropomodulin and the α-actinin staining in early premyofibrils, except in regions where several myofibrils are superimposed or closely juxtaposed to one another (Fig. 4C, asterisks). In areas where single myofibrils can be clearly discerned, short stretches of alternating red and green dots are frequently visible (Fig. 4C, brackets). In some cases, gaps in between the individual tropomodulin or α-actinin dots are evident (Fig. 4C, left bracket), whereas in other cases the red and green dots are immediately adjacent to one another (Fig. 4C, bottom right bracket). Interestingly, the distance between the α-actinin (or...
tropomodulin) dots varies from ~1.0 μm up to ~1.7 μm, in the regions where separate red and green dots can be clearly discerned (Fig. 4A-C, brackets). In comparison, the distances between α-actinin (or tropomodulin) striations in mature striated myofibrils are considerably longer and more regular, at ~2.3 μm (Fig. 4D-F, brackets).

We also attempted to directly compare the location of barbed and pointed ends by double staining myotubes for tropomodulin and CapZ. Unfortunately, we were unable to reach a conclusion due to high background staining with the available polyclonal and monoclonal antibodies to CapZ (data not shown; also see Schafer et al., 1993).

Assuming that barbed ends are associated with Z bodies, the alternating pattern of tropomodulin and α-actinin stained dots indicates that actin filament pointed and barbed ends are not randomly distributed along the length of the premyofibrils. This in turn suggests that thin filaments whose barbed ends are inserted into Z bodies are restricted to a relatively narrow range of lengths. By contrast, if the filaments were polymerized to a wide range of random and variable lengths, then the location of the clusters of pointed ends would be unlikely to have had any particular spatial relationship to the locations of Z bodies along the premyofibrils.

**Tropomodulin is associated with nonstriated premyofibrils at the tips of growing myotubes, even in the absence of α-actinin**

We also compared the distribution patterns of tropomodulin and α-actinin at myotube tips, which are believed to be sites of new sarcomere addition during myofibril elongation (Holtzer et al., 1997; Peng et al., 1981; Sanger et al., 1986). In young myotubes obtained from early days in culture (days 2-3), punctate staining for tropomodulin is detected along actin filament bundles that extend all the way to the tips of the myotubes and stain continuously with phallacidin (Fig. 5A,B). While not visible in Fig. 5, tropomodulin may also be associated with cortical actin filaments underlying the plasma membrane, based on our recent observation that tropomodulin is also localized on the sarcolemma in adult skeletal muscle (A. Almenar-Queralt and V. M. Fowler, unpublished observations).

Double staining for tropomodulin and α-actinin demonstrates that tropomodulin staining invariably extends all the way to the distal ends of the premyofibrils at the tips of the young myotubes (Fig. 6A,C, short arrows). However, α-actinin staining extends to the tips of the cells only in some (Fig. 6B) but not other (Fig. 6D) young myotubes; in this case, the α-actinin staining terminates well before the end of the myotube (Fig. 6D; compare long arrow marking the end of α-actinin staining with short arrow marking the tip of cell). Myotube tips which are tropomodulin-positive but α-actinin-negative represent about 30% of the population of myotube tips from these early days of culture. Double staining for α-actinin and actin also demonstrates that α-actinin staining does not always extend to the distal tips of phallacidin stained actin filament bundles in young myotubes (not shown).

In contrast to the absence of α-actinin from the distal ends of premyofibrils in some myotubes from early days of culture, α-actinin and tropomodulin are both detected all the way to the distal ends of the myofibrils in most myotubes (>90%) obtained from late days of culture (e.g. days 5-7) (Fig. 6E, tropomodulin; F, α-actinin). Note that the closely spaced dots of α-actinin and tropomodulin in the distal region of a myofibril at the tip of the myotube (Fig. 6E,F, short arrows) are

**Fig. 4.** The pointed and the barbed ends of actin filaments are not randomly distributed along nonstriated premyofibrils. Fluorescence micrographs of chick skeletal myotube shafts on early (A-C) and late (D-F) days of culture, fixed and double-stained for tropomodulin (A,D) and α-actinin (B,E). The merged images of tropomodulin (green) and α-actinin (red) are shown in C and F. Brackets, positions of α-actinin dots (A-C); positions of α-actinin stripes at the Z line (D-F). Asterisks, overlaps of 2 or more adjacent premyofibrils (C). Staining for tropomodulin was with a polyclonal rabbit antibody. D-F are confocal micrographs. Bar, 5 μm.
continuous with mature striated myofibrils containing periodic α-actinin and tropomodulin striations (Fig. 6E,F, arrowheads). Interestingly, the transition between striated and punctate staining patterns is fairly abrupt, as reported previously (Holtzer et al., 1997; Peng et al., 1981). In higher magnification merged images of tips in myotubes from early and late days in culture (not shown), tropomodulin dots are detected in between α-actinin dots, as described above for nonstriated myofibrils along the shafts of young myotubes (Fig. 4A-C).

These observations indicate that actin filament pointed ends are capped by tropomodulin and partially aligned in the earliest appearing actin filament bundles that have assembled at the tips of the young growing myotubes. Interestingly, the presence of α-actinin at myotube tips in most myotubes late in culture, but the absence of α-actinin at myotube tips in some young myotubes early in culture, suggests that thin filament organization at the tips of myotubes containing mature striated myofibrils may not be the same as in immature myotubes containing nonstriated premyofibrils. One possibility is that the termini of mature striated myofibrils are adhesion plaques required for stable sites of contact with the substratum, rather than lamellipodial-like extensions responsible for myotube elongation in young myotubes. Exploration of this idea will require further study.

Embryonic chick skeletal and cardiac muscle cells express the same isoform of tropomodulin (E-Tmod)

Our previous study with embryonic chick cardiac myocytes demonstrated that tropomodulin is not detected along nonstriated actin filament bundles in these cells and is assembled late during myofibril assembly, after myofibrils have become striated (Gregorio and Fowler, 1995). To investigate whether the expression of a different tropomodulin isoform during early skeletal myofibrillogenesis could account for these differences, we examined the isoform composition of tropomodulin by 2-D gel electrophoresis followed by western blotting with a monoclonal antibody that recognizes the two tropomodulin isoforms that have been identified in chicken striated muscles (E-Tmod and Sk-Tmod) (Almenar-Queralt et al., 1997). The results from this experiment demonstrate that a single isoform of tropomodulin is present in early (day 2) and late (day 7) skeletal cultures, as well as in cardiac myocytes (Fig. 7a-c). Co-electrophoresis of either day 2 skeletal muscle cells and cardiac myocytes (Fig. 7d), or day 7 skeletal muscle cells and cardiac myocytes (Fig. 7e) demonstrates that the tropomodulin polypeptides expressed in both cell types comigrate identically on 2-D gels, regardless of the day in culture for the skeletal cells. These results suggest that a single tropomodulin isoform is present in embryonic chick skeletal muscle cells and in cardiac myocytes. This isoform is E-Tmod based on co-migration with E-Tmod from adult chicken heart (data not shown) (Almenar-Queralt et al., 1997). Therefore, isoform differences are unlikely to account for the different distribution patterns of tropomodulin during myofibrillogenesis in these cell types.
**Discussion**

The polymerization and perfect alignment of thin and thick filaments into myofibrils in striated muscle is a dramatic example of supramolecular assembly in eukaryotic cells; the mechanisms by which this occurs are still incompletely understood. In this study, we sought to contribute to what is known about the roles of actin filament capping proteins in thin filament assembly and length regulation in skeletal muscle by focusing on tropomodulin, the capping protein for the pointed ends of the thin filaments (Fowler, 1996). We found that tropomodulin is associated with nonstriated premyofibrils early in skeletal muscle differentiation, indicating that thin filament pointed ends are capped well before the precise alignments of thin filaments into organized, mature sarcomeres. The discontinuous, punctate appearance of tropomodulin staining along premyofibrils indicates that thin filament pointed ends are clustered into discrete regions and implies a greater degree of thin filament organization than had been heretofore suspected. Our observation that some tropomodulin-stained dots alternate periodically with α-actinin-stained dots (Z bodies) further indicates that clusters of pointed ends are positioned between the nascent Z bodies; i.e. that thin filaments originating from adjacent Z bodies are oppositely polarized with their barbed ends at the Z bodies and their pointed ends in the middle of the nascent sarcomeres. The regular distribution of pointed and barbed ends along nonstriated premyofibrils implies that newly assembled thin filaments are polymerized to a relatively restricted range of lengths from the earliest stages of myofibrillogenesis in skeletal muscle. Thus, transitions from nonstriated premyofibrils to mature striated myofibrils in skeletal muscle may be due in large part to filament rearrangements and alignments, rather than to extensive filament polymerization or depolymerization processes.

**Thin and thick filaments rearrange to form mature striated myofibrils**

The concept that filament rearrangements are a major operative process in vertebrate skeletal muscle myofibrillogenesis has been widely accepted for assembly of thick filaments into mature myofibrils (Epstein and Fischman, 1991; Fischman, 1970; Franzini-Armstrong and Fischman, 1996). Myosin appears to polymerize directly into long thick filaments and shorter thick filament precursors are not observed in differentiating skeletal muscle myotubes in vertebrates (Allen and Pepe, 1965; Auber, 1969; Fischman, 1967). Our observations in this study suggest that similar mechanisms may also be important for thin filament assembly; namely, that thin filaments may be preassembled and capped at both ends first, and then subsequently be integrated into mature myofibrils by a gradual process of filament rearrangements and alignments.

While our data do not allow us to determine directly the lengths of thin filaments in nascent or immature sarcomeres, it is likely that thin filament lengths are restricted to a relatively narrow range based on the central location of tropomodulin dots (i.e. clusters of thin filament pointed ends) between closely-spaced sets of α-actinin dots (i.e. clusters of barbed ends) (Fig. 4). This conclusion assumes that thin filament barbed ends are associated with Z bodies along early premyofibrils; this will clearly require direct testing with improved antibodies to CapZ. In Fig. 8, we have depicted two possibilities for thin filament assembly into mature myofibrils, a Variable Length model (A1) in which the nascent thin filaments are somewhat shorter or longer than their mature length of ~1 μm, and a Fixed Length model (A2) in which thin filaments have been polymerized initially to their final ~1 μm lengths in early nonstriated premyofibrils. A previous study demonstrating that heavy meromyosin-decorated thin filaments in nonstriated premyofibrils have mixed polarities (Shimada and Obinata, 1977) is consistent with either model. We propose that misalignments of thin filament ends at early stages (Fig. 8A), rather than large variations in thin filament lengths, can account for the irregular sizes, variable spacings and occasional overlaps of tropomodulin and α-actinin stained dots in early premyofibrils (Fig. 4). Next, we propose that the preassembled thin filaments are organized into striated myofibrils by a process of filament sliding to align their barbed and pointed ends at the Z line and at the central bare zone of the thick filaments, respectively (Fig. 8B,C). During this process, sarcomere lengthening occurs simply as a consequence of rearrangements and alignments of the preassembled thin filaments (Fig. 8A-C).

Sanger and colleagues have also proposed a model of myofibril assembly in which nascent myofibrils are composed of ‘mini-sarcomeres’ that increase in length during later stages of myofibril assembly (Sanger et al., 1986; Rhee et al., 1994). This model was based principally on elegant time-lapse observations of the movements of α-actinin dots in living skeletal or cardiac cells using rhodamine-labelled α-actinin or GFP-α-actinin, which directly demonstrate separations and realignments of adjacent dots to form the regular, striated Z
lines of mature sarcomeres (Dabiri et al., 1997; McKenna et al., 1986; Sanger et al., 1986). Increases in sarcomere lengths have also been observed previously by thin section electron microscopy in differentiating skeletal muscle in vivo in chickens (Allen, 1973), and in cultured Xenopus muscle cells (Peng et al., 1981). However, the Sanger group’s idea that sarcomere lengthening results from filament elongation differs from our model that sarcomere lengthening results principally from thin filament rearrangements. In contrast, in invertebrates, sarcomere lengthening during maturation does result from elongation of both thin and thick filaments (i.e. polymerization) rather than from filament rearrangements (Reedy and Beall, 1993, and references therein).

**What is responsible for early thin filament alignments and subsequent rearrangements during myofibrillogenesis?**

Initial alignments of pointed and barbed ends of thin filaments along nonstriated actin filament bundles are likely to require an actin-binding protein(s) which can recognize thin filament polarity and ends. Clues to what might be involved come from examination of components at the distal ends of nonstriated actin filament bundles near myotube tips; sites of new sarcomere assembly (Holtzer et al., 1997). Surprisingly, discontinuous dot-like patterns of tropomodulin (i.e. clusters of pointed ends) that are observed in distal portions of nonstriated actin filament bundles at myotube tips are not always accompanied by α-actinin dots in many young, growing myotubes (Fig. 6). While we cannot exclude the possibility that nonsarcomeric α-actinin isoforms could be present and be undetected by our antibody, electron dense Z bodies are also not observed in the distal regions of premyofibrils at the extreme tips of Xenopus myotubes by thin section electron microscopy (Peng et al., 1981). This suggests that α-actinin is not required for clustering and aligning filament ends during early stages of myofibril assembly. In nonmuscle cells, bipolar myosin filaments have been suggested to play a role in cross-linking and gathering actin filaments into oppositely polarized bundles in the leading lamellipodium of migrating cells (Verkhovsky et al., 1995). A similar mechanism could also operate in skeletal muscle, based on a report that nonmuscle (but not sarcomeric) myosin is enriched near the tips of young myotubes in primary cultures of rat skeletal myogenic cells (Fallon and Nachmias, 1980; for a discussion of this question, see Franzini-Armstrong and Fischman, 1996).

Another candidate which could orchestrate initial thin filament alignments as well as subsequent realignments of Z bodies into striated myofibrils is titin, a giant sarcomeric protein which extends from the Z line to the M line. Titin is proposed to function as a template to organize thin and thick filaments into mature sarcomeres based on its molecular size, layout in the sarcomere, ability to interact directly with multiple sarcomeric components and presence of different isoforms (i.e. different templates) in different striated muscle types (for recent reviews, see Gregorio et al., 1999; Trinick, 1994). Association of the N-terminal portion of titin with α-actinin and other Z band and I band components, together with association of the C-terminal portion of titin with thick filaments, could establish the central position of the thin filaments with respect to the flanking location of thin filaments.
in the sarcomere. Interactions of myosin heads with oppositely polarized thin filaments could then contribute directly to aligning thin filaments into their final positions. Thus, the final stages of thin filament alignment would likely depend on the precision of myosin thick filament alignment in the A band, which is predicted to be determined by the late-assembling myosin-binding proteins: C protein and M protein (Lin et al., 1994; van der Ven and Furst, 1997). Interestingly, thin filament lengths as well as alignments have also been proposed to be directly dependent on correct assembly of a myosin thick filament-titin scaffold (Littlefield and Fowler, 1998).

Thin filament assembly is different in cardiac as compared to skeletal muscle

The observations reported here are strikingly different from what we had earlier observed in cultured embryonic chick cardiac myocytes, where tropomodulin is not associated with nonstriated myofibrils (Gregorio and Fowler, 1995). Indeed, tropomodulin is one of the latest markers of myofibril assembly and is found assembled into myofibrils only after the appearance of actin filament striations. The differences in the assembly properties of tropomodulin in cultured skeletal and cardiac muscle cells cannot be explained by differences in their tropomodulin isoform, which is E-Tmod in both cell types. Furthermore, preliminary characterization of de novo myofibril assembly in explants from precardiac regions of avian embryos indicates that tropomodulin is assembled late, after the appearance of actin striations (D. Rudy, P. B. Antin and C. C. Gregorio, unpublished observations). This indicates that late assembly of tropomodulin in cultured cardiac myocytes is not an artifact of isolated cells cultured in vitro. Therefore, differences in the temporal and spatial pattern of incorporation of tropomodulin in cardiac as compared to skeletal myofibrils may reflect intrinsic differences in thin filament and myofibril assembly mechanisms (Gregorio, 1997).

What accounts for the ability of tropomodulin to cap thin filament pointed ends in nonstriated myofibrils in skeletal but not cardiac muscle? We had proposed earlier that newly polymerized actin filaments in nonstriated myofibrils in cardiac myocytes are of indeterminate and variable lengths (Gregorio and Fowler, 1996; Gregorio, 1997). Differences in nascent thin filament lengths and tropomodulin assembly in skeletal and cardiac muscle could be at least partly a consequence of the interaction (either directly or indirectly) of tropomodulin with the skeletal muscle-specific protein, nebulin (Gregorio, 1997; Littlefield and Fowler, 1998). Nebulin is a giant protein that extends along the entire length of thin filaments and is hypothesized to be a template for thin filament assembly (for reviews see: Trinick, 1994; Keller, 1995; Labeit and Kolmerer, 1995). A full length nebulin analogue has not been identified in cardiac muscle (Itoh et al., 1998; Moncman and Wang, 1995). Significantly, the C terminus of nebulin (Holtzer et al., 1997), tropomodulin (this study) and CapZ (Schafer et al., 1993) are all detected in discontinuous punctate patterns along nonstriated actin filament bundles located at the tips of skeletal myotubules. Thus, it is attractive to speculate that the thin filaments in skeletal muscle could assemble to their mature lengths by copolymerization alongside a nebulin template, and be capped concurrently by tropomodulin and CapZ. Changes in staining patterns of nebulin epitopes that have been observed during myofibrillogenesis (Komiyama et al., 1992; Moncman and Wang, 1996) could be explained by thin filament rearrangements and increasing alignments during myofibril assembly. In cardiac muscle, restriction of thin filament lengths and thus capping by tropomodulin may not occur until after the thin filaments have been aligned into polarized sarcomeres by a titin-myosin scaffold (Littlefield and Fowler, 1998).

Actin filament rearrangements in nonmuscle cells

Actin filament rearrangements are emerging as an important mechanism for restructuring the actin cytoskeleton in a variety of nonmuscle cell types. For example, in cytokinesis in animal cells, the contractile ring appears to be formed by recruitment of preexisting actin filaments into the cleavage furrow from polar regions of the cell cortex (Cao and Wang, 1990). Actin filaments in stress fibers also undergo continuous movements and reorganization in living cells (Wang, 1987). It has also been proposed that in budding yeast, cell cycle-dependent movements of actin patches and cables may take place largely by rearrangements of preexisting actin filaments rather than by actin polymerization and depolymerization processes (Karpova et al., 1998). All of these processes are distinct from the rapid and extensive bursts of actin polymerization that take place during activation of platelets or chemotaxis of amoeboid cells (Can et al., 1991; Eddy et al., 1997; Hartwig, 1992), or from the transient assembly of actin filaments in membrane-associated foci in the lamellipodia of cultured cells (Schafer et al., 1998). The relative contributions of regulated actin polymerization or filament rearrangements to assembling actin filaments with defined lengths (e.g. microvilli, erythrocyte actin filaments, Drosophila bristles) remain to be elucidated.

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