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Myofibrillogenesis in Skeletal Muscle Cells in Zebrafish

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Abstract

The “premyofibril” model of myofibrillogenesis, based on observations in cultured avian muscle cells, proposes that mature myofibrils are preceded by two intermediary structures: premyofibrils and nascent myofibrils. To determine if this model applies to zebrafish skeletal muscle development, we stained developing embryos with antibodies to sarcomeric alpha-actinin and myosin II. In the youngest muscle cells, sarcomeric alpha-actinin and non-muscle myosin II were each localized in linear arrays of small bands that resembled the premyofibrils in avian myocytes. The distribution of muscle-specific myosin II began as scattered short filaments followed in time by overlapping bundles of filaments and organized A-bands in the older somites. Alpha-actinin organization changed from small z-bodies to beaded Z-bands and ordered Z-bands in myofibrils that extended the length of the elongating somites. In older somites with mature myofibrils, premyofibrils were also present at the ends of the mature myofibrils, suggesting that as the cells and somites grew longer, premyofibrils were involved in the elongation of existing mature myofibrils. Fluorescence Recovery After Photobleaching showed that the exchange of proteins (actin, alpha-actinin, FATZ, myotilin and telethonin) between sarcoplasm and the Z-bands of mature myofibrils in zebrafish resembled that seen for the same proteins in cultured avian myotubes, suggesting that myofibril assembly and maintenance in zebrafish share common properties with avian muscle.

Keywords

Myofibrillogenesis; Myofibril; Alpha-actinin; Myosin; A-bands; Premyofibril; Nascent myofibril; Mature myofibril; Fluorescence Recovery After Photobleaching; FRAP

Introduction

Recent studies to identify precursors of myofibrils in cells assembling muscle have emphasized the use of fluorescence microscopy to define the initial arrangement of sarcomeric proteins prior to the formation of organized myofibrils [reviewed in Sanger et al., 2005]. The studies were carried out in live cells in culture [Sanger et al., 1986; Dabiri et al., 1997; Sanger and Sanger, 2001; Wang et al., 2005b], in fixed and permeabilized cells in cultures of single cells [Sanger et al., 1984; Rhee et al., 1994; Holtzer et al., 1997; Turnacioglu et al., 1997; Sanger et al., 2005], and in cultures of precardiac mesoderm tissue [Rudy et al., 2001; Du et al., 2003], as well as tissue fixed *in situ* [Ehler et al., 1999; Du et al., 2008]. Cultured muscle has the advantage that fluorescently labeled proteins can be

introduced via microinjection or transfection of GFP-tagged probes and visualized as they assemble into myofibrils in live cells. The disadvantage is that in culture, cells are not disposed as they are *in situ* and can be maintained for only short periods of time [Engler et al., 2008]. The ideal format for analyzing myofibril assembly would combine immunofluorescence and live cell observations *in situ* to determine how the sarcomeric proteins become arranged into the structural units of the myofibrils. Zebrafish provides such a system with its optical clarity and well-defined sequence of embryonic muscle formation [Felsenfeld et al., 1991; Kimmel et al., 1995; Stickney et al., 2000] in which the temporal progression of myofibril formation can be followed in a single fish in myotomes that form sequentially.

The first skeletal muscle formation in zebrafish occurs in the pairs of somites that form along the trunk of the animal. By 18 hours after fertilization (hpf), 18 somite pairs have formed sequentially and grown in size in an anterior to posterior direction. Over the next six hours, somites form at the rate of approximately two per hour to produce a total of 30 - 34 pairs [Waterman, 1969; Kimmel et al., 1995; Stickney et al., 2000]. Figure 1 illustrates the range of sizes of the somites in the caudal end of a one day-old embryo (24 hpf) stained with fluorescent phalloidin to label the forming myofibrils and somite boundaries. The first or oldest somite shown in the image (number 9 of 30) measures about 59 microns in length while the youngest somite in the image (number 30) is about 29 microns long, half the length. Organized myofibrils are aligned in the anterior or rostral somites where they extend between the two-somite boundaries, while at the same time in the posterior or caudal somites, sarcomeric proteins are present in the early stages of assembly. During the rapid growth in somite size, myofibril formation is followed closely by myofibril elongations and contractions.

Two recent studies that employed antibody staining of fixed zebrafish skeletal muscle cells [Costa et al. 2002, 2003] reported that there was no evidence for precursor structures prior to the appearance of mature myofibrils and speculated that in earlier studies of avian cultures [Dlugosz et al., 1984; Sanger et al., 1984, 1986; Rhee et al., 1994; Holtzer et al., 1997; LoRusso et al., 1997; Sanger et al., 2002], tissue culture had imposed an alternate pathway for myofibril assembly. Here we show that specific fluorescent probes for actin, sarcomeric alpha-actinin and muscle myosin II and non-muscle myosin IIA demonstrate that there are intermediate steps in the assembly of myofibrils in zebrafish skeletal muscle at 20 - 25 hours post-fertilization. We have also examined protein dynamics with FRAP experiments in embryonic zebrafish muscle expressing YFP-fusions of Z-band proteins. Both the arrangement of actin, alpha-actinin and the myosins during myofibril assembly and the dynamics of the Z-band proteins were similar to that found in forming myofibrils and Z-bands in cultured avian myotubes supporting a multi-step model for myofibrillogenesis in zebrafish and suggesting that myofibrillogenesis is a conserved process that follows a similar pathway in vertebrates.

Materials and methods

Preparation of embryos

Fertilized zebrafish eggs (*Danio rerio*, *Tübingen genetic background*) were obtained as described by Mullins et al. [1994] and Kimmel et al. [1995]. Embryos intended for live cell imaging were microinjected with plasmids encoding Yellow Fluorescent Protein ligated to one of four different Z-band proteins. Approximately 100 pg of plasmid diluted in 0.1M KCl was injected as a 2nl bolus into the yolk of 10-20 minutes post-fertilization embryos that were allowed to develop at 28.5 degrees Celsius. The embryos were later staged by observation of the numbers of somite pairs following the initiation of somitogenesis

beginning at 10 hours post-fertilization (hpf) [Kimmel et al., 1995] and manually removed from their enveloping chorion by micromanipulation.

Embryos used for photobleaching experiments were examined with confocal microscopy as described below. Embryos intended for antibody staining were transferred at 18–24 hpf into Eppendorf tubes containing 3.5% formaldehyde solution (pH7, 4°C) in a low salt solution containing 0.1 M KCl, 10 mM phosphate buffer and 1 mM Mg Cl₂ (standard salt solution) [Dabiri et al., 1999]. The embryos were fixed at room temperature for 15 minutes, and then permeabilized with 0.1% Triton X-100 for 15 minutes at room temperature. The unreacted aldehyde groups were blocked by a five to ten minute treatment with 50 mM NH₄Cl in the standard salt solution. The embryos were rinsed with the standard salt solution for five to ten minutes before antibody staining.

Immunostaining procedure

Intact embryos were processed in Eppendorf tubes. Primary antibodies (200 µl), diluted with standard salt solution, were added to the tubes to cover the embryos. After incubation at room temperature or 37 °C for one to two hours with primary antibodies, the embryos were rinsed several times in standard salt, and incubated with secondary antibodies one to two hours at room temperature. Fluorescently labeled phalloidin was applied for 30 minutes at room temperature. Unbound reagents were removed with several rinses of standard salt solutions. The embryos were then rinsed with distilled water to remove the salts. The stained embryos were mounted in Mowiol containing the anti-fade reagent, n-propyl gallate (Calbiochem, La Jolla, CA) [Dabiri et al., 1999]. The cover slip was placed gently on the embryo to align the embryos so that the zebrafish tails were flat on the bottom of the glass slide.

Antibodies

Monoclonal antibodies were purchased from the following sources: Monoclonal anti-sarcomeric α -actinin (IgG, A7811), Sigma (St. Louis, MO), and F59 (IgG antibody against muscle myosin II), Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Rabbit polyclonal nonmuscle myosin IIA (IgG, M8064) and IIB (IgG, M7939) was obtained from Sigma (St. Louis, MO). Alexa (488 and 594) phalloidins, probes specific for actin filaments, were purchased from Molecular Probes (Eugene, OR). DAPI was ordered from Sigma (St. Louis, MO). Fluorescently labeled secondary antibodies purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) were selected to be non-cross reacting.

Deconvolution Microscopy

Widefield fluorescent images of the skeletal muscle cells were acquired in Z-axis optical sections on a DeltaVision Restoration microscopy system (Applied Precision, Issaquah, WA) with an Olympus IX70 microscope and an Olympus 100× Plan Apo, 1.35 N.A. objective lens, or on a Leica AF 6000 LX system (Leica Microsystems, Exton, PA) with Leica HCX PL APO 100×/1.40 lens. The filters used for the labeled antibodies and phalloidin were fluorescein: 490/528 nm; rhodamine: 555/617nm; CY5: 640/685 nm; DAPI: 360/457 nm. Images were processed with constrained iterative deconvolution with SoftWoRx or Leica software. All images were assembled for publication with Adobe Photoshop (San Jose, CA). The myosin filaments and A-bands were measured in undeconvolved images with SoftWoRx image analysis software, and analyzed with Excel software.

Preparation of YFP plasmids for zebrafish injections

The cDNA of the human Z-band proteins, alpha-actinin, actin, FATZ, myotilin and telethonin, were cloned into the pEYFP-N1 (Clontech) plasmid using techniques previously reported [Ayoob et al. 2000; Wang et al. 2005b]. The DNA for the YFP- Z-band proteins was excised from the Clontech vector and transferred into a plasmid containing a 3.7 kb skeletal muscle-specific alpha-actin-promoter isolated from zebrafish genomic DNA [Higashijima et al., 1997]. Injection of plasmids into zebrafish embryos resulted in mosaic expression of the Z-band-YFP proteins only in skeletal muscle cells.

Fluorescence Recovery After Photobleaching

Myofibrils of skeletal muscle cells in microinjected zebrafish were imaged with a 63× objective and analyzed with the FRAP modules of a Zeiss LSM 510 or Leica SP5 confocal microscope. Regions of interest were photobleached with the laser at 100% intensity and post-bleach images were followed with 2 to 4% laser intensity for 20 minutes. FRAP data were analyzed as previously reported [Wang et al., 2005a, 2007, 2008]. Two to six different zebrafish were used for each Z-band protein FRAP measurement. The animals were anesthetized with tricaine just prior to and during the FRAP experiments so that the focus could be kept on the same Z-bands. The animals readily recovered their motility once the anesthetic was washed away. In several cases, an embryo that had been tested, was anesthetized several days later prior to photobleaching the Z-bands again, and the recovery rates of the five Z-band proteins were the same.

Results

Alpha-actinin z-bodies and Z-bands in growing somites

In one-day old zebrafish embryos, sarcomeric alpha-actinin is localized in linear arrays of small puncta in the somites at the caudal end of the embryo (Figure 2 A, small arrows). In older somites nearer the head of the same fish, the sarcomeric alpha-actinin was present in Z-bands spaced at mature sarcomere length (Figure 2 B). Some of the Z-bands had a beaded substructure (Figure 2 B, arrowheads). Alpha-actinin was also in z-bodies along the myosepta and at the ends of the myofibrils that extended to the somite boundaries (Figure 2 B, arrows). The distance between the z-body alpha-actinin puncta ranged from 0.3 - 1.2 microns with an average spacing of 0.7 (+/- 0.18) microns. In contrast the Z-band spacing in the older somites ranged from 1.9 - 2.7 microns, with an average length of 2.25 (+/- 0.11) microns. In zebrafish expressing YFP-alpha-actinin in the skeletal muscle cells, z-bodies were detected in the earliest muscle cells in the first few somites (Figure 3 A), and Z-bands marking the boundaries of the sarcomeres in the mature myofibrils were detected in older somites (Figure 3 B).

Non-muscle myosin IIA

Non-muscle myosin IIA was found in a punctate distribution in fibers of cells in the somites and in higher concentration, at the boundaries of the somites in one-day-old embryos (Figure 4). The staining along the zebrafish cells resembled the non-muscle myosin II staining of avian embryonic cardiac muscle cells in tissue culture [Rhee et al., 1994], and in embryonic cardiomyocytes *in situ* [Du et al., 2008]. Antibodies specific for the IIB isoform of non-muscle myosin did not stain the zebrafish skeletal muscle cells.

Muscle Myosin II in early and late forming somites

Muscle specific myosin was distributed in small bundles in the most recently formed somites of one-day old embryos (Figure 5A, 1-4). In Figure 5, the youngest somites (Figure 5 A, 1-2) had deposits of myosin II in only a few cells. In the next older somites, an

increasing amount of muscle myosin II was organized in unbanded bundles that extend along the long axis of the somite (Figure 5 A, 3-4). Anterior to the youngest somites, muscle myosin was arranged in bundles some of which were partially banded (Figure 5 B), and others that were aperiodic and often present at the ends of the banded fibrils (Figure 5 B, arrows). In the oldest somites, long stretches of A-bands were easily identified (Figure 5 C). However, even in the oldest 24 hpf somites, aperiodic muscle myosin II could be detected at the ends of the myofibrils.

In cells with unbanded bundles of myosin, myosin filaments, shorter than A-band length were also present (Figure 6 A). In some of the cells linear arrays of these short myosin filaments could be detected in the zebrafish muscle cells (Figure 6 A). Both the short filaments (Figure 6 A) and A-bands (Figure 6 C) lack midzone staining with the F-59 antibody, reflecting the absence in the middle of bipolar myosin filaments of myosin ATPase heads or cross-bridges [Huxley, 1963], the binding sites of the F-59 antibody [Miller and Stockdale, 1986]. The staining pattern of the filaments suggests they are precursors to the aligned 1.5-micron long thick filaments of A-bands in mature myofibrils.

Short filaments were first detected in embryonic quail hearts fixed *in situ* at the two-somite stage (HH stage 7+) when the first cardiomyocytes form, and also in the spreading edges of three day cultured cardiomyocytes isolated from 10-day embryonic hearts [Du et al., 2008]. The lengths of the zebrafish short myosin II filaments and A-Bands are plotted in Figure 7. The short filaments ranged from 0.4 to 1.1 microns (average lengths = 0.87 ± 0.17 microns) compared to A-bands that ranged from 1.3 to 1.7 microns (average lengths = 1.51 ± 0.11 microns). The expected frequency curves exhibit an excellent fit with the measured distributions of the short myosin filament and A-band lengths (Figure 7).

Distribution of YFP-actin in somites

Actin-YFP incorporated readily into the I-bands of assembling sarcomeres of the myofibrils (Figure 8). Fluorescence intensity was highest in the Z-bands measuring about twice that seen in the actin filaments that project into the middle of the sarcomere (Figure 8 A), consistent with an overlap of the barbed ends of the actin filaments in the Z-band. In sarcomeres 2.7 to 2.5 micron long where there was a gap, or H-zone, between the ends of antipolar actin filaments in the middle of mature sarcomeres (Figure 8 A and B), the I-bands measured 2.2 microns, consistent with a thin filaments length of about 1.1 micron. In sarcomeres 2.2 microns in length the H-zone was absent (Figure 8 B) and in shorter sarcomeres (Figure 8 C; sarcomere lengths of 1.9 microns), a fluorescent band of actin was positioned midway between the adjacent Z-bands of the mature myofibrils (Figure 8 C), representing an overlapping pattern of thin filaments in the middle of the sarcomere [Huxley and Hanson, 1954]. At the ends of the mature myofibrils, near the edges of the somite boundaries, the pattern of fluorescent actin was unbanded (Figure 8 C), and resembled the solid actin patterns that were detected in avian and rodent premyofibrils and in nascent myofibrils [Rhee et al., 1994; LoRusso et al., 1997; Golson et al., 2004; Wang et al., 2005b; Du et al., 2003, 2008] suggesting that the actin filaments overlap at the ends of the mature myofibrils. This is also the area of the somite boundaries, where we detect fibrils containing alpha-actinin z-bodies (Figure 2 B) and overlapping muscle myosin II filaments (Figure 5C). These somite boundaries represent areas where recently formed mature myofibrils can elongate in the growing somites [Kimmel et al., 1995].

Fluorescence recovery After Photobleaching (FRAP)

To determine how dynamic the sarcomeric proteins of zebrafish were, the technique of Fluorescence Recovery After Photobleaching (FRAP) was used to analyze five YFP-tagged Z-band proteins expressed from plasmids encoding a skeletal muscle actin promoter and

injected into one and two cell embryos (Figure 9). Incorporation of the five different fluorescent probes did not affect the assembly of the myofibrils or the contractility of the muscles in the zebrafish. A comparison of the recovery characteristics with similar FRAP experiments of the same proteins: alpha-actinin-YFP, myotilin-YFP, FATZ-YFP, actin-YFP and YFP-telethonin in quail cultured myotubes (Figure 10) indicates a similar pattern, although the recovery rates of the five Z-band proteins are slower in zebrafish skeletal muscles, than in quail muscle cells over the 20 minute observation period (Figure 10). The recovery rates remained unchanged when measurements were repeated in the same or different embryos during the first week of development of the zebrafish embryos, the time period of our FRAP measurements.

Discussion

Model to explain myofibril assembly

Studies of myofibrillogenesis in cardiomyocytes and skeletal muscle cells, primarily from embryonic chicks or quails [reviewed in Sanger et al., 2005; Du et al., 2008], led us to propose a multistep model to explain how sarcomeric proteins assemble, interact and organize into myofibrils [Rhee et al., 1994; Sanger et al., 2002, 2004, 2005]. The first step is the assembly along the myocyte plasma membrane of premyofibrils composed of minisarcomeres with alternating concentrations of non-muscle myosin II and small beaded arrays or z-bodies with muscle-specific alpha-actinin localized along filaments of muscle-specific actin and its associated proteins (tropomyosin, and troponins) [Wang et al., 2007]. The transition from premyofibrils to nascent myofibrils occurs when z-bodies align in register, forming beaded Z-bands, and titin and muscle-specific myosin II are incorporated into the fibrils. At this stage, muscle myosin II filaments are not aligned in A-bands but overlap producing a continuous staining pattern, whereas the non-muscle myosin II is in periodic bands. In the final stage when mature myofibrils form, the beaded Z-bands gradually become linear, non-muscle myosin II is no longer associated with the myofibrils, and muscle myosin II filaments are organized in aligned A-bands [Rhee et al., 1994; Du et al., 2008; Stout et al., 2008].

Similarity of myofibrillogenesis in zebrafish skeletal muscles to avian myofibrillogenesis

The present study of myofibril assembly in skeletal muscle in embryonic zebrafish demonstrates changes in the organization of muscle proteins that are similar to earlier findings in avian muscle. These results differ from an earlier study of myofibril assembly in zebrafish embryos [Costa et al., 2002; 2003], in which muscle proteins were seen only in fully organized sarcomeres in mature myofibrils with no indication of immature stages, suggesting that culturing avian muscle resulted in an alternate assembly sequence for formation of myofibrils. The formation of somites in a cephalic to caudal direction along the flank of the zebrafish results a temporal sequence of the stages of myofibrillogenesis in a single animal, in which the earliest stages are in the youngest somites at the distal end of the tail with assembly progressively more advanced toward the head in the oldest somites. This allows the order of events in myofibril assembly to be followed more easily than is possible in avian myocytes placed in tissue culture.

In the earliest stages of myofibril assembly, the linear arrays of small puncta of alpha-actinin (Figure 2A) mirrored the z-bodies of minisarcomeres in premyofibrils previously seen in cultured chick embryonic cardiac and skeletal muscle cells [Rhee et al., 1994; Dabiri et al., 1997; Golson et al., 2004; Siebrands et al., 2004], as well as in cultured adult rat cardiomyocytes [LoRusso et al., 1997]. In older somites, the pattern of alpha-actinin was punctate in the Z-bands (Figure 2B, arrowheads), before changing to uniformly stained bands. We have previously demonstrated in living avian myotubes and cardiomyocytes how

the distances between the z-bodies marking the boundaries of the minisarcomeres in the premyofibrils grow apart until the full mature sarcomeric spacings are obtained [Sanger et al., 1986; Dabiri et al., 1997; Wang et al., 2005b].

The progression of muscle-specific myosin II assembly began in the youngest somites of 24-hour embryos as small aggregates, followed by small, unbanded bundles (Figure 5A, 1-4) similar to those we termed nascent myofibrils in avian myocytes in culture [Rhee et al., 1994] and in hearts fixed *in situ* [Du et al., 2008]. In older somites, cells with mixed populations of banded and unbanded fibrils (Figure 5B) appeared before myofibrils with well-organized A-bands (Figure 5C). Some of the myosin (Figure 5C) and actin (Figure 8C) fibrils near the older somite boundaries were unbanded as in younger cells in an earlier stage of myofibril assembly. This and the concentration of z-bodies of alpha-actinin at the ends of fibers containing mature Z-bands (Figure 2B) may indicate that myofibril growth occurs near the boundaries of the older somites, concurrently with the elongation of the somites during embryogenesis (Figure 1). Thus this process of elongation of existing mature myofibrils via the intermediary steps of premyofibrils and nascent myofibrils [Dabiri et al., 1997] may be a common mechanism in both cultured and *in situ* muscle cells. We would predict that as demonstrated in cardiomyocytes [Danowski et al., 1992] that the Z-bands of the contracting mature myofibrils in zebrafish are attached to the sarcolemma via costameres, thus allowing addition of premyofibrils and nascent myofibrils to the ends of these elongating myofibrils.

The concentrations of non-muscle myosin IIA in the boundaries of the zebrafish somites (Figure 4) resemble a similar reported concentration of the non-muscle myosin II (*zipper*) in the formation of the intersegmental muscles in Stage 16 of *Drosophila* embryos [Bloor and Kiehart, 2001]. There was also diffuse lighter staining of the *Drosophila* non-muscle myosin II antibody distributed within the muscle cells. Cuticle deposited after Stage 16 prevented Bloor and Kiehart [2001] from being able to detect any structures in the muscle cells after Stage 16. However, flies lacking non-muscle myosin II had unbanded fibers of actin, as seen in premyofibrils or in cells arrested at the premyofibril stages by the use of reversible inhibitors [Du et al., 2003; Golson et al., 2004], and did not form mature myofibrils.

Non-muscle myosin IIA is the predominant non-muscle myosin II in embryonic skeletal muscles, while non-muscle myosin IIB is the predominant type in embryonic hearts [Rhee et al., 1994; Takeda et al., 2000]. In our studies on zebrafish skeletal muscles, only the non-muscle myosin IIA stained the somites. In future experiments we will determine whether or not the IIB non-muscle myosin antibodies stain embryonic zebrafish hearts. In contrast to the reports of the antibody stainings of non-muscle myosin II A and B in the Z-bands of cardiac and skeletal [Takeda et al., 2000] muscle cells, we have never observed non-muscle myosin II A or B in the Z-bands of cardiac or skeletal muscle cells [Rhee et al., 1994, Du et al., 2003, 2008, unpublished results]. We recently transfected quail cultured myotubes with GFP plasmids encoding non-muscle myosin II A or B or C. All of the transfected live muscle cells exhibited premyofibrils incorporating each one of these probes in identical patterns, but none of the three non-muscle myosin IIs (A, B, or C) were observed in any of the Z-bands in the myotubes (manuscript in preparation).

Short muscle myosin II filaments

Short myosin II filaments were discovered in the elongating ends of the skeletal muscle cells in the zebrafish embryos (Figure 6 A, C). Evidence was presented for the first time by Du et al. [2008] that myosin thick filaments can be detected at a much shorter length than detected in mature myofibrils in avian hearts. These short filaments were first discovered in embryonic quail hearts fixed *in situ* at the two-somite stage (HH stage 7+) when the first cardiomyocytes form. The small filaments of muscle myosin II were detected scattered

around the nuclei of these first cardiomyocytes. These first myosin filaments had an average length of 0.76 microns as compared to 1.6 micron A-bands in mature myofibrils [Du et al., 2008]. This novel discovery led to a search for these short myosin filaments in older cultured embryonic cardiomyocytes. The same types of short myosin rodlets were present in the spreading edges of three day cultured cardiomyocytes isolated from 10-day embryonic hearts [Du et al., 2008]. We have also found these short myosin II filaments in the elongating tips of elongating quail myotubes in tissue culture (unpublished results). Titin is believed to play a role in the determination of the final length of vertebrate thick filaments [Wang and Wright, 1988; Whiting et al., 1989; Du et al., 2008]. Mutations that disrupt the normal distribution of titin [Costa et al., 2002] result in disruptions of the Z- and A-bands of the myofibrils in both cardiac and skeletal muscle cells in zebrafish [Xu et al., 2002; Seeley et al., 2007]. Future analysis of these mutant zebrafish should be examined to determine if the thick myosin filament lengths have not achieved their final 1.5-micron lengths.

The three-step model of myofibrillogenesis appears valid for muscle cells in avian and rodent tissue culture [Rhee et al., 1994; Dabiri et al., 1997; LoRusso et al., 1997; Golson et al., 2004; Siebrands et al., 2004], in precardiac explants [Du et al., 2003], *in situ* in embryonic chick hearts [Du et al., 2008] and now, in fixed and living zebrafish skeletal muscle cells.

Dynamics and remodeling

Myofibrils have been thought of as very stable structures [reviewed in Sanger et al., 2004, 2005]. However, FRAP experiments have demonstrated that in both cultured myotubes and now in zebrafish there is an exchange of Z-band proteins between the bound sarcomeric proteins in the sarcomeres and similar components in the cytoplasm [Sanger et al., 2005; Wang et al., 2005a, 2007, 2008; Etard et al., 2008; Sanger and Sanger, 2008]. These exchanges are much faster than the five to ten days for the synthetic half-lives of different sarcomeric proteins [Zak et al., 1977], and are not affected by the inhibition of protein synthesis tested over a three-hour period [Wang et al., 2005a]. The molecular exchanges detected in FRAP experiments in living zebrafish and in other muscle cells would provide a means for the entry of newly synthesized sarcomeric proteins into existing mature myofibrils, without the need to disassemble existing myofibrils, and then reassemble the muscle proteins into sarcomeres and myofibrils. The exchange of proteins detected by FRAP may also provide a cellular mechanism for the remodeling of the different parts of the sarcomeres during myofibrillogenesis, the continual testing of the fitness of older sarcomeric molecules, and the repair of micro defects in myofibrils resulting from exercises.

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Figure 1.

A one-day-old embryo was fixed and stained with fluorescently labeled phalloidin. The youngest 21 of the total 30 somites at this time point are shown in this image. Phalloidin stains the chevron boundaries (arrows) of the somites, the myofibrillar actin fibers that extend from the somite boundaries, and the boundaries of the small cells in the zebrafish tail. Note the doubling of the lengths between one of the youngest and shortest caudal somites (left side) versus an older and longer cephalic somite. Scale = 50 microns.

Figure 2.

(A, B) Zebrafish somites in the tail of a 25-somite stage embryo stained with sarcomeric alpha-actinin. **A.** Two full somites near the caudal end of the zebrafish. The three large arrows mark the boundaries or myosepta of the somites. The z-bodies of premyofibrils (thin arrows) are aligned linearly from one myoseptum to the other. This image is at the same magnification as the image in B. **B.** Two somites near the cephalic end of the tail of the same embryonic zebrafish. The Z-lines of the mature myofibrils have sarcomeric separations of about two microns. Premyofibrils (short arrows) are detected at the ends of some myofibrils and at the boundary between the two somites (large arrow). The arrowheads indicate Z-bands that are beaded. Scale = 10 microns.

Figure 3.

Living muscle cells in the same one-day-old embryonic fish expressing YFP-alpha-actinin.

A. Single muscle cell in a very young somite exhibiting the short alpha-actinin spacings (z-bodies) of premyofibrils (minisarcomeres). The distances between z-bodies in series are about 0.8 microns. **B.** A single muscle cell within an older somite demonstrating the presence of the Z-bands of mature myofibrils. The distances between the Z-bands are about 2.0 microns. Scale = 10 microns.

Figure 4.

Somites stained with non-muscle myosin IIA and fluorescent secondary specific antibodies. The large arrows point out the nonmuscle myosin II staining in the chevron-shaped boundaries of the somites. The small arrows indicate the linear punctate fibers that resemble the premyofibrils of avian embryonic muscles. Scale = 10 microns.

Figure 5.

Embryonic zebrafish embryo stained with a muscle specific myosin II antibody. **A.** New somites at end of tail. Note that muscle myosin has just been deposited in the newest somite labeled 1. The myosin becomes more aligned into solid fibers in somites 3 and 4. **B.** A higher magnification of somite number 5 of the same embryo. The arrows point out fibers with overlapping arrays of myosin filaments, i.e., nascent myofibrils. **C.** One of the oldest somites near the head of the fish. Note the aligned A-bands in the mature myofibrils. Scales = 10 microns.

Figure 6.

(**A**) Zebrafish embryo stained with anti-muscle myosin II antibodies. Arrows indicate thick filaments of different lengths in an early somite. Arrows indicate unstained middle of the myosin II filaments. Insert in (**C**) is an enlargement of the arrowed area in (**A**). (**B**) Myosin II filaments are aligned into uniform A-band lengths in an older somite. Note the unstained area of the middle of the A-bands in the mature myofibrils, and the absence of any loose myosin II filaments outside the A-bands. Scale = 5 microns for (**A**) and (**B**).

Figure 7.

Bar graphs showing the frequency distributions versus lengths measured in microns of short muscle myosin II filaments in the last two or youngest caudal somites (in green) versus the distribution of the lengths of A-bands in older rostral somites (in blue). Note that the earliest short filaments have a length distribution from 0.4 to 1.1 microns with an average length of 0.9 ($\pm$ 0.17) microns. In contrast, the average length of the A-bands is 1.5 ($\pm$ 0.11) microns. The smooth curves plot the expected frequencies versus length distributions from the measurements.

Figure 8.

Incorporation of YFP-actin in sarcomeres of different lengths in skeletal muscles of living zebrafish. (A) Two cells each of which exhibits a single myofibril. The lower cell and its myofibril are almost entirely within the focal plane of observation. A scan of the myofibril reveals that the middle line of the I-band, the Z-bands (arrowheads), has a fluorescent intensity double that of the thin filaments extending away from the Z-bands. The lengths of the sarcomeres in this myofibril are 2.7 microns, and the I-bands are 2.2 microns. The dark zone between the fluorescent I bands is the H-zone, the region where thin filaments are absent at this sarcomere length. (B) The upper sarcomere whose Z-bands are indicated by arrowheads measures 2.5 microns. The lower myofibril exhibits a sarcomere whose Z-bands (arrowheads) are separated by 2.2 microns. At this shorter sarcomeric length, the H-zone disappears (single arrow). (C) These cells exhibit shorter sarcomeres, about 1.9 microns in length. Narrow bands of actin (arrows) are present between the Z-bands (arrowheads). (A-C) All of these actin patterns support thin filament lengths of 1.1 microns that overlap in the Z-bands (A) and in the former H-zones of the shorten sarcomeres (C) of 1.9 microns. Scales = 5 microns.

Figure 9. FRAP of YFP- α -actinin in the Z-bands in two different muscle cells in a living zebrafish (three days old); Prebleach, Bleach, 10 minutes after photobleaching and then 2.5 hours after the bleach. The recovery is almost complete by the end of the ten-minute period. Scale = 5 microns.

Figure 10.

Comparison of the dynamics of five different Z-band proteins (myotilin, FATZ, alpha-actinin and actin) in the Z-bands of mature myofibrils in tissue cultured quail myotubes versus those in skeletal muscle cells in living zebrafishes. Note that as in the quail myotubes, the proteins recover at different rates over a 20-minute period. Note also that the rates for each individual Z-band protein is similar to that in quail cells as well.