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Myofibril Assembly Visualized by Imaging N-RAP, Alpha-Actinin, and Actin in Living Cardiomyocytes

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Abstract

N-RAP is a striated muscle-specific scaffolding protein that organizes α -actinin and actin into symmetrical I-Z-I structures in developing myofibrils. Here we determined the order of events during myofibril assembly through time-lapse confocal microscopy of cultured embryonic chick cardiomyocytes coexpressing fluorescently tagged N-RAP and either α -actinin or actin. During de novo myofibril assembly, N-RAP assembled in fibrillar structures within the cell, with dots of α -actinin subsequently organizing along these structures. The initial fibrillar structures were reminiscent of actin fibrils, and coassembly of N-RAP and actin into newly formed fibrils supported this. The α -actinin dots subsequently broadened to Z-lines that were wider than the underlying N-RAP fibril, and N-RAP fluorescence intensity decreased. FRAP experiments showed that most of the α -actinin dynamically exchanged during all stages of myofibril assembly. In contrast, less than 20% of the N-RAP in premyofibrils was exchanged during 10–20 minutes after photobleaching, but this value increased to 70% during myofibril maturation. The results show that N-RAP assembles into an actin containing scaffold before α -actinin recruitment; that the N-RAP scaffold is much more stable than the assembling structural components; that N-RAP dynamics increase as assembly progresses; and that N-RAP leaves the structure after assembly is complete.

Keywords

myofibrillogenesis; heart; sarcomere

Introduction

A number of models have been proposed to describe the sequence of events by which actin, myosin, and titin-associated proteins assemble into linear arrays of sarcomeres [1,2]. There is broad agreement that the earliest myofibril precursors appear near the cell periphery as immature fibrils containing punctate α -actinin Z-bodies, α -actin and muscle tropomyosin [3–12]. Several studies also provide evidence that muscle myosin filaments assemble separately and suggest that they are subsequently interdigitated with the I-Z-I complexes (symmetrical actin filaments with their barbed ends anchored at a central Z-body or Z-line containing α -actinin) to form full-fledged sarcomeres [6,7,13,14]. Other aspects of sarcomere assembly remain controversial. Premyofibrils with Z-line spacings between 0.5 and 1 μ m have been

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observed in many experimental systems [3,4,15-17], but are less prevalent in embryos than in cultured cells [6,8]. Nevertheless, time-lapse microscopy of cardiomyocytes expressing α -actinin as a GFP fusion protein demonstrated that, over many hours, closely spaced dots of α -actinin (Z-bodies) increase their longitudinal spacing and fuse laterally to form Z-lines [4]. The requirement for nonmuscle myosin IIB, previously proposed to be necessary for premyofibril assembly [3,16,17], has also been disputed. Ablation of nonmuscle myosin IIB by gene targeting in vivo [18] or knockdown by RNA interference in cultured cardiomyocytes [19] suggest that this protein is dispensable for myofibril assembly.

In addition to the assembling structural components of sarcomeres, several other proteins are transiently associated with myofibril precursors during assembly [20]. These include scaffolding proteins such as N-RAP [8,19,21,22] and Krp1 [20], and chaperone proteins such as Hsc70 and Hsp90 [14]. N-RAP binds α -actinin and actin [22,23], and is associated with assembling myofibrils in both cardiac and skeletal muscle [8,19,21,22,24]. Several cell biological studies support a role for N-RAP in myofibril assembly [25-27], and a molecular mechanism has been proposed by which N-RAP scaffolds α -actinin and actin assembly into symmetrical I-Z-I structures [25,26].

Here we test details of the previously proposed model describing N-RAP scaffolding of premyofibril assembly. Using time-lapse confocal microscopy of cultured embryonic chick cardiomyocytes coexpressing fluorescently tagged N-RAP and either α -actinin or actin, we observed that N-RAP associates with newly forming actin filaments before incorporation of α -actinin. The results show that N-RAP assembles into an actin containing scaffold before α -actinin recruitment, and suggest a novel mechanism by which N-RAP might control I-Z-I assembly.

Materials and Methods

Expression Plasmids

The previously described full-length mouse cardiac N-RAP cDNA cloned into the pcDNA3.1/NT-GFP-TOPO plasmid vector [28] was PCR amplified (forward primer: 5'-CACACACAC**CGCG**TTTATGAATGTGCAGGCCTGCTCTAG-3'; reverse primer: 5'-CGCCACTGTG**GTTCGAC**ATCTGCAGAATTGCCCTT-3') and cloned using the MluI and SalI restriction sites (bold) into the pTRE2hyg2-Myc plasmid (Clontech Laboratories, Mountain View, CA). In order to remove mutations previously introduced by PCR cloning, we excised regions from various sequenced mouse N-RAP cDNA clones [28] and used them to replace regions in the pTRE2hyg2-Myc-N-RAP plasmid so that the final construct matched the exons embedded in the mouse genomic DNA sequence (NCBI document ID 20890983). The resulting pTRE2hyg2-Myc-N-RAP plasmid was used as a template for amplifying full length N-RAP for directional cloning using EcoRI and SalI restriction sites incorporated into the forward and reverse primers, respectively (forward primer: 5'-CACACACAC**GAATTCT**ATGAATGTGCAGGCCTGCTCTAG-3'; reverse primer: 5'-CGCCACTGTG**GTTCGAC**ATCTGCAGAATTGCCCTT-3'; restriction sites in bold). PCR was performed using the Clontech Advantage-2 PCR kit according to the manufacturer's protocol. The PCR product was gel purified, digested with SalI, ligated to the mCherry-C1 vector previously digested with SalI and EcoRI, digested with EcoRI, and ligated to form the final circular mCherry-N-RAP plasmid. The mCherry-C1 vector is mCherry [29] cloned into the pEYFP-C1 vector from Clontech to replace EYFP, and was generously provided by Drs. George Patterson & Jennifer Lippincott-Schwartz (NICHD, NIH). TOP10 Ultracompetent E. coli cells (Invitrogen, Carlsbad, CA) were transfected with the ligated plasmid and bacteria harboring the plasmid were colony-purified. Plasmid was gel purified and integrity of the cloning sites was verified by DNA sequencing.

Plasmids for expressing YFP- α -actinin (sarcomeric α -actinin cDNA cloned into the pEYFP-N1 vector from Clontech) and YFP-actin (skeletal muscle α -actin cloned into the pEYFP-N1 vector from Clontech) have been previously described [30] and were generously provided by Dr. Joseph Sanger (SUNY Upstate Medical University, Syracuse, NY).

Protein Isolation and Immunoblotting

HEK 293 cells were cultured in DMEM supplemented with serum and antibiotics for 24 hours and then transfected with the full length mCherry-N-RAP construct. The transfection mixture contained 2 μ g plasmid DNA and 6 μ l FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) in 100 μ l of DMEM without serum. Medium was changed after 20 hours and after 48 hours cells were washed with cold PBS and scraped into Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA). Proteins from transfected and untransfected cells were separated on a 7.5% SDS polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was cut so that proteins below ~75 kDa could be probed with a 1:50,000 dilution of mouse monoclonal antibody against α -tubulin (clone DM1A from Sigma, St. Louis, MO) and proteins above ~75 kDa could be probed with a 1:1000 dilution of rabbit polyclonal antibody against N-RAP [31]. Primary antibodies were detected with 1:2000 dilutions of horseradish peroxidase linked anti-mouse or anti-rabbit secondary antibodies and the ECL Western Blot System (Amersham, Piscataway, NJ).

Primary Culture and Transfection of Embryonic Chick Cardiomyocytes

Embryonated chicken eggs were obtained from CBT Farms (Chestertown, MD). Hearts from day 5-7 embryos were isolated, cut into 5 or 6 pieces, and incubated at 37°C for 2 $\times$ 11 minutes with 0.25% trypsin (Gibco) in DPBS. After washing in DMEM the tissue was treated with 2% collagenase (Sigma) in 10% FBS/DMEM for 15 to 20 minutes at 37°C with periodical shaking to dislodge the cells. Cells were centrifuged at 300-560 $\times$ g for 3 minutes and pre-plated for a minimum of 2 hours to minimize fibroblast contamination. The nonadherent cells were then collected by centrifugation, counted, and plated at a density of 1×10^5 cells per 35 mm round glass bottom culture dish (MatTek, Ashland, MA) coated with laminin at a concentration of 3-5 μ g/cm² (Gibco). Cells were grown in DMEM supplemented with 10% FBS, antibiotics and antimycotic as previously described [21].

Cardiomyocytes were transfected 36 hours after plating. Cardiomyocytes overlaid with 1 ml of fresh supplemented DMEM were transfected by the addition of 200 μ l of transfection mixture (4 μ g mCherry N-RAP plasmid, 2 μ g of either YFP- α -actinin or YFP-actin plasmid, and 24 μ l FuGENE 6 transfection reagent (Roche Diagnostics Corporation, Indianapolis, IN) in DMEM without antibiotics or serum). The cultures were incubated at 37°C in 5% CO₂ for 18- 20 hours after which the medium was removed and replaced with fresh medium.

Antibody Staining

Cardiomyocytes transfected with YFP-actin plasmid were fixed 36 hours after transfection for 20 minutes with 4% paraformaldehyde in PBS. The fixed cells were washed with PBS, permeabilized with 0.1% Nonidet-P-40 for 1 minute, washed again with PBS, and blocked with 5% horse serum in PBS for 30 minutes. Cells were stained with a 1:1000 dilution of monoclonal antibody specific for sarcomeric α -actinin (clone EA-53, Sigma-Aldrich, St. Louis, MO) for 45 minutes at 37°C, followed by detection with a 1: 1000 dilution of Alexa fluor 633-linked goat anti-mouse secondary antibody (Invitrogen).

Confocal Microscopy

Images were captured using a Zeiss LSM 510 META laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) fitted with an incubator to maintain temperature at 37 °C and deliver

5% CO₂. Cultures were imaged 24 hours after transfection using a 63×/numerical aperture 1.4 apochromatic oil immersion lens. YFP-tagged proteins were imaged using a 488 nm laser for excitation and a 505-530 nm bandpass emission filter. mCherry-NRAP was imaged using a 543 nm laser for excitation in combination with a 560 nm long pass emission filter. Alexa fluor 633 antibody label was detected using a 633 nm laser for excitation and a long pass 650 nm emission filter. Excitation lasers were activated sequentially to avoid channel cross-talk.

For time-lapse studies, 10 mM Oxyrase (Oxyrase, Inc., Mansfield, OH) was added to the medium to reduce photodamage [32]. Images were captured every 5 to 10 minutes for a period of 10 to 24 hours. This rate of image capture was sufficient to unambiguously track structures of interest. Cells were imaged in at least three different focal planes with an inter-plane spacing of 0.6 microns. Focus drift was minimized using the autofocus feature of the microscope. Captured images were concatenated using the Zeiss software for image analysis. Maximum intensity of mCherry and YFP fluorescence was typically found in the same single image plane, which was then used for analysis and presentation.

For fluorescence recovery after photobleaching (FRAP) experiments, regions of interest were imaged twice within a short time interval as detailed above, and then bleached using the 40 milliwatt, 488 nm argon laser set at 100% transmission. Total bleach time was optimized to yield an ~80% decrease in both mCherry and YFP fluorescence within the region, and an image was collected immediately after bleaching. Subsequent images were collected every 20 seconds for the first 2 minutes, and then at 1 minute intervals for a total period of 20 minutes.

Image Analysis

Each fluorophore was individually analyzed in standard time-lapse and FRAP experiments, and only cells that appeared healthy following imaging were included in the analysis. Fluorescence intensity values of defined regions were measured using Image J, a variant of the NIH Image software program developed at the U.S. National Institutes of Health and available on the internet at <http://rsb.info.nih.gov/nih-image/>. Calculations and curve fitting were performed using Kaleidagraph software (Synergy Software, Reading, PA). The fit parameters and their standard errors were used in a t-test to evaluate the significance of differences in the time course of accumulation of fluorophores in individual time-lapse experiments [33].

For each cell, mean fluorescence intensity values were measured for individual regions of interest (I), the entire cell or the whole region of the cell that was imaged (T), and an extracellular region (B). A normalized intensity I_N was calculated from the following:

$$I_N = (I - B) / (T - B)$$

For newly forming structures and FRAP experiments, a mean normalized scaled intensity I_{N-S} was calculated so that the minimum (I_{N-MIN}) and maximum (I_{N-MAX}) levels measured during the experiment were set equal to 0 and 1, respectively:

$$I_{N-S} = (I_N - I_{N-MIN}) / (I_{N-MAX} - I_{N-MIN})$$

For pre-existing structures, mean intensity values were scaled so that the maximum levels measured during the experiment were set equal to 1:

$$I_{N-S} = (I_N) / (I_{N-MAX})$$

I_{N-S} values were plotted versus time (t) and, as noted in the figure legends, were fit to the following equations, where a, b and k are fit parameters:

$$\text{Linear: } I_{N-S} = a \cdot t + b$$

Rising exponential: $I_{N-S} = a + b \cdot (1 - \exp(-k \cdot t))$

Exponential decay: $I_{N-S} = a \cdot \exp(-k \cdot t)$

In the case of rising exponentials, I_{N-S} values below 0.2 were omitted from the fits.

The recovery phase of FRAP data were fit to a single exponential:

$I_{N-S} = a \cdot (1 - \exp(-k \cdot t))$ where a is the percent recovery and k is the rate of recovery.

In the case of exponential fits, the half-times ($t_{1/2}$) of rise or fall were obtained by substituting the rate constants k in the exponential equations:

$$k = 0.693/t_{1/2}$$

Results

Timing of N-RAP and α -actinin Incorporation in Developing Myofibrils

In order to simultaneously image N-RAP and sarcomeric components expressed as YFP fusion proteins, the entire N-RAP coding sequence was cloned into a vector for expression as a fusion protein with N-terminal mCherry, an improved red fluorescent protein derived from DsRed [29]. The intact 220 kilodalton mCherry-N-RAP fusion protein was detected by immunoblot in HEK 293 cells transfected with the expression plasmid (figure 1A).

To determine the order in which N-RAP and α -actinin assemble into developing myofibrils, cardiomyocytes were doubly transfected with plasmids encoding mCherry-N-RAP and YFP- α -actinin and imaged by confocal microscopy over several hours. Overexpression of full length N-RAP can interfere with myofibril assembly in chick cardiomyocytes [28]. Therefore, cardiomyocytes were imaged within 24 hours after transfection of expression constructs, while fusion protein levels were low but increasing. We found that mCherry-N-RAP and YFP- α -actinin incorporated normally into myofibrillar structures. This was verified by morphometric measurement of mature myofibril content in cardiomyocytes, which showed that mCherry-N-RAP expression did not result in decreased myofibril content under these conditions (figure 1B). In addition, the myofibril content measured here is similar to levels previously reported in chick cardiomyocytes that were mock transfected after 24 hours in culture and observed 24 hours later [26]. The results suggest that myofibril assembly is not adversely affected by the low levels of recombinant N-RAP and α -actinin expressed during these experiments. To account for the changing specific activity of each fusion protein during the time-lapse experiments, measured fluorescence intensities in regions of interest were normalized to the mean fluorescence intensity measured simultaneously in the whole cell.

Figure 2 shows a cardiomyocyte expressing mCherry N-RAP and YFP- α -actinin imaged at 10 minute intervals over a 10 hour period. During this time the width of this cell increased dramatically via extension of lamellipodia (figure 2, arrows). The boundaries of these extensions were labeled by YFP- α -actinin, while mCherry-N-RAP was not incorporated in these structures. In contrast, mCherry-N-RAP was efficiently incorporated into myofibrillar structures labeled by YFP- α -actinin. Boxed regions A-D in the upper panels highlight examples of such structures and are shown enlarged in the lower panels (figure 2).

Box A in figure 2 shows a newly formed N-RAP fibril that was initially devoid of α -actinin. This N-RAP fibril translated to the right as the cell spread, and periodic dots of α -actinin subsequently organized on this structure. mCherry-N-RAP and YFP- α -actinin fluorescence accumulated in this region with half-times for the exponential fits of 31 ± 8 and 270 ± 190

minutes, respectively (figure 2, box A graph), but with an interval of several hundred minutes between assembly of the N-RAP fibril and subsequent α -actinin assembly.

The second highlighted region in figure 2 shows an area into which the cell spread and began accumulating linear arrays of α -actinin and N-RAP (box B). N-RAP accumulation appeared fibrillar with periodic substructure, whereas α -actinin was clearly punctate and exhibited submicron linear spacings. mCherry-N-RAP and YFP- α -actinin fluorescence accumulated in this region at nearly identical rates, with half-times for the exponential fits of 91 ± 8 and 71 ± 9 minutes, respectively (figure 2, box B graph).

The third highlighted region in figure 2 contained a fibrillar structure that had incorporated fluorescent N-RAP (box C). While N-RAP fluorescence remained constant in this structure over the course of the experiment, YFP- α -actinin became incorporated into this pre-existing structure, organizing as punctate dots with submicron spacings (figure 2, box C). The half-time for the exponential rise of α -actinin fluorescence in this region was 439 ± 121 minutes. The inset of the box C graph shows a line plot of N-RAP fluorescence intensity along the fibril at $t=160$ minutes. The line plot demonstrates a $1 \mu\text{m}$ periodicity in N-RAP fluorescence before significant accumulation and organization of α -actinin has occurred.

The fourth highlighted region in figure 2 contained an N-RAP fibril associated with striations of α -actinin spaced at $\sim 2 \mu\text{m}$ intervals, characteristic of fairly mature sarcomeres (figure 2, box D). During the course of the experiment α -actinin was added to these pre-existing striations, creating much broader striations. The associated N-RAP fluorescence remained constant over the first ~ 7 hours of observation, and then significantly decreased over the last few hours (figure 2, box D graph). The declining region of N-RAP intensity at times greater than 490 minutes was fit to a decreasing exponential with a half-time of 690 ± 130 minutes.

Figure 3A shows a cardiomyocyte expressing mCherry N-RAP and YFP- α -actinin imaged at 8 minute intervals over a ~ 6.5 hour period. The boxed regions in the top panels highlight an area in which both N-RAP and α -actinin accumulated, and these areas are shown below the main panels enlarged and with the color channels separated. Initial N-RAP accumulation appeared fibrillar, whereas α -actinin was clearly punctate. The α -actinin dots fused to form nascent Z-lines, after which the N-RAP fluorescence began to decrease. Figure 3B depicts the time course of fluorescence changes in this area, and the times corresponding to the images in figure 3A are labeled with numbered arrows. In this example low levels of N-RAP fluorescence accumulated slightly ahead of α -actinin, and the rising phase of N-RAP and α -actinin fluorescence were fit to single exponentials with half-times of 140 ± 83 minutes and 122 ± 19 minutes, respectively. The declining region of N-RAP intensity was fit to a single decreasing exponential with a half-time of 187 ± 27 minutes.

Dynamic Exchange of N-RAP and α -actinin in Developing Myofibrils

We conducted FRAP experiments to determine the dynamics of mCherry-N-RAP and YFP- α -actinin exchange in myofibrillar structures at various stages of assembly. The micrographs in figure 4 show typical structures subjected to photobleaching. Premyofibrils, nascent myofibrils, and mature myofibrils were operationally defined by the combination of N-RAP and α -actinin organization: Premyofibrils contained punctate α -actinin, and N-RAP was present; nascent myofibrils contained striated α -actinin, and at least some of the N-RAP present was longitudinally organized within the structure (i.e. N-RAP was not restricted to the Z-lines); and mature myofibrils contained striated α -actinin, and N-RAP was absent or restricted to the Z-lines. Periodicity of α -actinin intensity along these structures was $0.76 \pm 0.07 \mu\text{m}$ in premyofibrils, $1.76 \pm 0.04 \mu\text{m}$ in nascent myofibrils, and $1.70 \pm 0.07 \mu\text{m}$ in mature myofibrils, consistent with previous studies demonstrating shorter periodicities in premyofibrils [3,4]. The recovery curves shown in figure 4 show mean data from several experiments fit to single

exponentials, and the mean FRAP parameters are tabulated in table 1. The mobile fraction of N-RAP increased dramatically during myofibril assembly, from $19 \pm 4\%$ in premyofibrils to $72 \pm 14\%$ in mature myofibrils. In contrast, $61 \pm 6\%$ of the α -actinin fluorescence was recovered after bleaching in premyofibrils, and this fraction increased to $91 \pm 15\%$ in mature myofibrils. The half-times for recovery of N-RAP and α -actinin fluorescence showed little change during myofibril maturation, varying from 61 to 88 seconds for N-RAP and from 44 to 56 seconds for α -actinin.

Timing of N-RAP Incorporation into Actin Fibers

As documented by previous investigators, fluorescently labeled actin monomers introduced into striated muscle cells do not uniformly label actin filaments along their length [34-39]. In order to characterize incorporation of YFP-actin in living cardiomyocytes relative to different stages of myofibril assembly, sarcomeric α -actinin was detected by immunofluorescence in cardiomyocytes fixed 36 hours after transfection with the YFP-actin construct. Mature Z-lines marked by α -actinin staining were intensely labeled with YFP-actin (figure 5, arrowheads). Lower levels of YFP-actin fluorescence were present between the Z-lines. Less mature regions containing punctate α -actinin staining exhibited more continuous incorporation of YFP-actin (figure 5, arrows). These results agree with several previous studies utilizing microinjection of fluorescently labeled actin [34-39].

To determine the order in which N-RAP and actin assemble into developing myofibrils, cardiomyocytes were doubly transfected with plasmids encoding mCherry-N-RAP and YFP-actin and imaged by confocal microscopy over several hours. Figure 6A shows a cardiomyocyte expressing mCherry N-RAP and YFP-actin imaged at 6 minute intervals over a 2 hour period. Much of the YFP-actin appeared as closely spaced dots of fluorescence associated with N-RAP fibrils (figure 6, asterisk). The boxed region in figure 6 contains broad striations of YFP-actin associated with N-RAP fibrils; this region is shown below the main panels enlarged and with the color channels separated. YFP-actin fluorescence in this mature region increased over the course of the experiment, while mCherry-N-RAP fluorescence decreased with a half-time of 840 ± 269 minutes (figure 6B). Spreading lamellipodia in the same cell contained YFP-actin, but not N-RAP (figure 6A, arrows). Interestingly, this cell also contained a region of an N-RAP fibril that did not incorporate YFP-actin (figure 6, arrowhead).

Figure 7A shows a region of a cardiomyocyte expressing mCherry N-RAP and YFP-actin imaged at 6 minute intervals over a 140 minute period. During this period actin and N-RAP began to assemble in a new structure moving towards the right in figure 7 (boxed area). The time course of fluorescence accumulation in this region clearly shows YFP-actin assembling before mCherry-N-RAP (figure 7B). These proteins accumulated with half-times of 5 ± 1 and 14 ± 2 minutes, respectively.

The half-times of accumulation of mCherry-N-RAP and either YFP- α -actinin or YFP-actin observed in specific structures analyzed in figures 2, 3, 6 and 7 are summarized in table 2, along with the measured half-times of N-RAP removal. The tabulated values show a wide variation in α -actinin and N-RAP accumulation rates and N-RAP removal rates between the different regions analyzed.

Discussion

N-RAP Association with Actin Filaments and α -actinin during Myofibrillogenesis: The Order of Events

The results of our dual-label time-lapse imaging experiments indicate that during myofibrillogenesis, actin filaments first form and bind N-RAP, and then recruit α -actinin. In

double fluorophore time-lapse experiments with mCherry-N-RAP and YFP- α -actinin, we observed examples of delayed α -actinin accumulation relative to N-RAP assembly (figure 2, box A and box C), as well as examples of approximately simultaneous assembly of these proteins (figure 2, box B; figure 3), but in no instance did we observe α -actinin assembly preceding N-RAP accumulation in developing myofibrils. The time resolution of these experiments is determined by the ~ 10 minute interval between micrographs, which was chosen to optimize our ability to follow structures of interest while limiting cardiomyocyte exposure to laser light. From the available data, we conclude that N-RAP organizes into fibrils before assembly of α -actinin into Z-bodies. We also observed N-RAP and actin accumulation into newly forming fibrils, and the time course of incorporation was significantly faster for actin than for N-RAP (figure 7). The results are consistent with N-RAP incorporating into premyofibrillar actin filaments shortly after they are formed. The mCherry-N-RAP fluorescence intensity in these fibrils fluctuates with a periodicity of 0.5-1.0 μm , even before α -actinin accumulation (figure 2, box B and box C). The results suggest that N-RAP first incorporates into premyofibrillar actin filaments, and that the N-RAP/actin cofilaments have a short sarcomeric periodicity before α -actinin assembly into Z-bodies.

The interpretation of the present results assumes that each fluorescently tagged protein accurately reports the organization of the total pool of that protein present in the cell, including the endogenous unlabeled molecules. We find that plasmid-encoded recombinant N-RAP fused with either mCherry (this report) or GFP [26,28] incorporates into the same structures in cultured embryonic chick cardiomyocytes in which endogenous N-RAP is found [21]. Although previous studies emphasize colocalization of N-RAP and α -actinin in developing myofibrils, examples of endogenous N-RAP organized along developing myofibrils in regions without α -actinin can be found in the literature. (For clear examples of this in chick cardiomyocytes see Carroll and Horowitz, 2000, figure 3A-B.) Therefore, the key conclusion that N-RAP assembles in premyofibrils before α -actinin incorporation is supported by double immunofluorescence localization studies of the endogenous proteins.

During myofibril assembly YFP- α -actinin accumulated in punctate periodic Z-bodies, which then fused laterally to form Z-lines (figure 3A), consistent with a previous time-lapse imaging study reported by Sanger and colleagues [4]. We also observed broadening of nascent Z-lines by direct addition of α -actinin from the diffuse pool (figure 2, box D). The mCherry N-RAP fusion protein used in this study was incorporated into these assembling myofibrillar structures, consistent with the previously reported localization of endogenous N-RAP [8, 19, 21, 22, 24]. This incorporation was specific, as mCherry N-RAP was not observed at the leading edge of lamellipodia in which YFP- α -actinin and YFP-actin were concentrated (figures 2 and 6).

In addition to N-RAP, actin, and α -actinin accumulating in developing myofibrils, we observed mCherry N-RAP fluorescence decreasing in the later stages of assembly. These stages were characterized by mature sarcomere spacings revealed by the periodicity of YFP- α -actinin or YFP-actin fluorescence, as well as broadening Z-lines (figure 2, box D; figures 3 and 6). Therefore, double fluorophore time-lapse experiments utilizing mCherry-N-RAP and either YFP-actin or YFP- α -actinin both exhibit removal of N-RAP from nearly mature myofibrils, consistent with the absence of N-RAP in the myofibrils of fully mature sarcomeres [8,21,24, 31]. Interestingly, N-RAP periodicity was often difficult to detect in these later stages (e.g. see figure 6), suggesting less specific localization of N-RAP within the myofibril during the N-RAP removal phase of maturation.

Dynamic Exchange in Assembling Structures

FRAP experiments showed that the fraction of N-RAP that is rapidly exchangeable steadily increases during myofibril maturation (figure 4 and table 1). Less than 20% of mCherry-N-RAP exchanged over 10 minutes in premyofibrils, while more than 70% of mCherry-N-RAP

was exchanged in more mature sarcomeres. Like N-RAP, we found that the proportion of YFP- α -actinin that exchanged over 10 minutes increased as myofibrils matured. However, even in premyofibrils more than 60% of α -actinin was mobile, and this proportion increased to more than 90% in mature myofibrils. Therefore, most of the α -actinin was mobile regardless of the state of myofibril assembly, while N-RAP was largely immobile in the early stages of assembly. The results suggest that most of the N-RAP is tightly bound in the first steps of assembly, but becomes mobile as myofibrils mature. This increase in mobility correlates with the removal of N-RAP from mature sarcomeres observed in our longer time-lapse experiments.

The increasing mobility of α -actinin that we observed during myofibril assembly is inconsistent with a previous report of decreasing mobilities during myofibril maturation for a variety of Z-band structural proteins [30]. Several differences between the two studies may account for the discrepancy. Most notably, our experiments were performed in avian cardiomyocytes, while the previous study used avian skeletal myotubes. In addition, we found that a single exponential was sufficient to fit our fluorescence recovery data, while the previous investigators fit their data to two exponentials. The half-times of recovery that we observed for α -actinin fluorescence were similar to the fast phase reported by Sanger and colleagues [30], while the five to ten-fold slower phase reported by those authors was not observed in our experiments. Therefore, the results may be explained by a redistribution of α -actinin from faster to slower exchanging populations that occurs during myofibril maturation in skeletal muscle but not in cardiac cells. The molecular basis of this may depend on the presence of different forms of related proteins in the two muscle types, e.g. nebulin in skeletal muscle versus nebulin in cardiac muscle [40].

N-RAP Scaffolding of I-Z-I Assembly

Based on N-RAP's transient association with developing myofibrils [8,19,21,22,24] and cell biological studies indicating a functional role in assembly [25-27], we previously proposed a molecular mechanism to describe N-RAP scaffolding of α -actinin and actin assembly into symmetrical I-Z-I structures [25,26]. The previous studies relied on average measures of myofibril accumulation in populations of cardiomyocytes, whereas the current study addresses the specific order in which N-RAP and structural components assemble into individual developing myofibrils. Figure 8 illustrates a revised scheme by which N-RAP may perform its scaffolding function that is consistent with the order of events documented in our time-lapse experiments, as well as with the increase in N-RAP dynamics observed during myofibril assembly. In this model, actin filaments polymerize and associate with N-RAP, which forms antiparallel dimers that guide actin filaments into the antiparallel organization that is characteristic of the I-Z-I complex (figure 8, panels A-B). The FRAP results suggest that the actin-N-RAP complex is long-lived, allowing little exchange of N-RAP molecules. Like the previous model [25,26], this scheme posits that actin orientation is controlled by N-RAP super repeats, consistent with the linear directional organization of the homologous nebulin super repeats in sarcomeric actin filaments [41-43]. The antiparallel dimerization of N-RAP that we hypothesize could occur via its LIM domain, consistent with previously observed dimerization of LIM domains in other proteins [44,45]. By setting the antiparallel organization of actin filaments before α -actinin incorporation into Z-bodies, this step accounts for the periodic substructure we observed in N-RAP fluorescence along fibrils before recruitment of α -actinin. Subsequently, α -actinin is recruited to the antiparallel regions of actin filaments by interaction with the N-RAP LIM domain and adjacent single repeats (figure 8C), consistent with *in vitro* binding studies [22,46]. In the last step, Krip1 promotes broadening of myofibrils [20], and N-RAP leaves the structure (figure 8D). In this model, sequential binding of α -actinin and Krip1 may destabilize N-RAP binding to the developing myofibril, accounting for the increasing fraction of N-RAP that exchanges in FRAP experiments as myofibrils mature.

The model in figure 8 does not directly address the wide range of rates observed for the accumulation of N-RAP and α -actinin in premyofibril structures and for the removal of N-RAP during maturation (table 2), nor does it explain why in some cases there is a long delay between N-RAP assembly and α -actinin recruitment (figure 2, box A and box C) while in other instances these components assemble almost simultaneously (figure 2, box B; figure 3). These observations suggest that the rates of individual steps in assembly are subject to regulation, which is consistent with the varying amounts of assembly precursors observed in different experimental systems (discussed in Lu et. al, 2005).

The proposed role of N-RAP in controlling actin and α -actinin assembly into I-Z-I antiparallel structures is supported by the inhibitory effect of expressing individual regions of N-RAP or truncated N-RAP on myofibril assembly [25,26], as well as by the effects of N-RAP knockdown [27]. However, another protein with antiparallel organization in the Z-line region is titin, which also contains repeats that bind α -actinin and could in principle control the location of α -actinin cross-links between both titin and actin filaments [47]. The interplay between N-RAP and titin as well as other Z-line components during myofibril maturation remains to be explored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Immunoblot

B. Myofibril Content

Figure 1.

(A) Immunoblot analysis of mCherry-N-RAP expression in HEK 293 cells. Lanes were loaded with approximately equivalent amounts of protein isolated from untransfected cells (-) or cells transfected with mCherry-N-RAP expression plasmid (+). The upper and lower parts of the filter were probed with antibodies against N-RAP and α -tubulin, respectively. Note that the intact mCherry-N-RAP fusion protein is specifically detected in the transfected cells. (B) Morphometric measurement of myofibril content based on YFP- α -actinin organization into mature striations. Cells were transfected with YFP- α -actinin alone or in combination with the empty mCherry vector or the mCherry-N-RAP expression plasmid. Recombinant mCherry-N-RAP does not perturb myofibril content under the conditions of the present study. Each bar represents the mean $\pm$ sem of 5-10 cells. Mean values did not change significantly over an additional 10 hours of imaging.

Figure 2.

Time-lapse images of a single cardiomyocyte expressing YFP- α -actinin (green) and mCherry-N-RAP (red). Boxed regions A-D in the top panels are shown below enlarged and with the color channels separated. Plots to the right of the micrographs show the time course of mean YFP- α -actinin and mCherry-N-RAP intensities measured in the smaller boxed areas within the enlargements (boxes A, C and D) or in the total enlarged area (box B). Arrows: Spreading lamellipodia containing α -actinin, but not N-RAP. Box A: Dotted array of α -actinin organizing on a newly formed N-RAP fibril. The curves are least squares fits to a rising exponential ($t_{1/2} = 31$ minutes for N-RAP and 270 minutes for α -actinin). Box B: Dotted arrays of α -actinin organizing on newly formed N-RAP fibrils. The curves are least squares fits to a rising

exponential ($t_{1/2} = 91$ minutes for N-RAP and 71 minutes for α -actinin). Box C: Dots of α -actinin organizing on a pre-existing N-RAP fibril. The α -actinin data above 0.2 were fit to a rising exponential ($t_{1/2} = 439$ minutes). The N-RAP data were fit to a straight line. The inset in the graph shows a line plot of the mCherry-N-RAP intensity in the center panel, demonstrating periodic N-RAP organization before α -actinin organization. Box D: Maturation of Z-lines by addition of α -actinin, accompanied by decreasing mCherry-N-RAP fluorescence. The declining region of N-RAP intensity at times greater than 490 minutes was fit to a decreasing exponential ($t_{1/2} = 690$ minutes); the other curves are linear least squares fits. For newly forming structures, mean intensity values were scaled so that the minimum and maximum measured values are 0 and 1, respectively (box A, box B and α -actinin in box C). For pre-existing structures, mean intensity values were scaled so that the maximum values are 1 (box D and N-RAP in box C). The full time-lapse sequence of each enlarged region is contained in the accompanying video files (figure2-boxA.avi; figure2-boxB.avi; figure2-boxC.avi; figure2-boxD.avi).

A. N-RAP & α -actinin Time Lapse Images

B. N-RAP & α -actinin Fluorescence Intensity

Figure 3.

(A) Time-lapse images of a single cardiomyocyte expressing YFP- α -actinin (green) and mCherry-N-RAP (red). The boxed regions in the top panels are shown below enlarged and with the color channels separated. (B) Time course of mean YFP- α -actinin and mCherry-N-RAP intensities measured in the enlarged areas. Data were scaled so that the minimum and maximum mCherry-N-RAP and YFP- α -actinin levels coincide. Numbered arrows correspond to the images shown in part A (left to right). The curves are least squares fits to a rising exponential for the increasing N-RAP ($t_{1/2} = 140$ minutes) and α -actinin ($t_{1/2} = 122$ minutes) data with intensity greater than 0.2, and to an exponential decay for the decreasing region of

the N-RAP data ($t_{1/2} = 187$ minutes). The full time-lapse sequence of the boxed region is contained in the accompanying video file (figure3.avi).

Figure 4.

FRAP analysis of assembling myofibrils. Prebleach images (pre) were taken 15 seconds before photobleaching of the boxed area. The time in seconds after photobleaching is shown above the images. Premyofibrils, nascent myofibrils, and mature myofibrils were operationally defined by the combination of N-RAP and α -actinin organization; see text for details. Recovery curves, at right, are best fits of the mean data for each time point to a single exponential. Each point represents the mean and standard error of the mean of 4-9 bleached areas.

Figure 5.

A single cardiomyocyte expressing YFP-actin and stained with antibody against α -actinin. Arrowheads: Z-lines marked by α -actinin staining and intense labeling with YFP-actin. Lower levels of YFP-actin fluorescence are present between the Z-lines. Arrows: Less mature regions containing punctate α -actinin staining and more continuous incorporation of YFP-actin.

A. N-RAP & Actin Time Lapse Images

B. N-RAP & Actin Fluorescence Intensity

Figure 6.

(A) Time-lapse images of a single cardiomyocyte expressing YFP-actin (green) and mCherry-N-RAP (red). The boxed regions in the top panels are shown below enlarged and with the color channels separated. Asterisk: Closely spaced dots of YFP-actin associated with N-RAP fibrils. Arrow: Spreading lamellipodia containing actin, but not N-RAP. Arrowhead: Region of an N-RAP fibril that is unlabeled by YFP-actin. (B) Time course of mean YFP-actin and mCherry-N-RAP intensities measured in the boxed regions of the enlarged areas. Data were scaled so that the maximum mCherry-N-RAP and YFP-actin levels coincide. The curves are least squares fits to an exponential decay for the N-RAP data ($t_{1/2} = 840$ minutes) and to a straight

line for the actin data. The full time-lapse sequence of the enlarged region is contained in the accompanying video file (figure6.avi).

A. N-RAP & Actin Time Lapse Images

B. N-RAP & Actin Fluorescence Intensity

Figure 7.

(A) Time-lapse images of a single cardiomyocyte expressing YFP-actin (green) and mCherry-N-RAP (red). Arrows indicate colocalization of maximal concentrations of N-RAP and actin fluorescence, presumably at assembling Z-lines. (B) Time course of mean YFP-actin and mCherry-N-RAP intensities measured in the boxed regions. Data were scaled so that the minimum and maximum mCherry-N-RAP and YFP-actin levels coincide. The curves are least squares fits to a rising exponential for the N-RAP ($t_{1/2} = 14$ minutes) and actin ($t_{1/2} = 5$ minutes) data with intensity greater than 0.2. The full time-lapse sequence is contained in the accompanying video file (figure7.avi).

Figure 8.

Schematic model illustrating N-RAP scaffolding during assembly of the I-Z-I complex. (A) Actin filaments form. (B) Antiparallel N-RAP dimers cross-link actin filaments in an antiparallel orientation. The orientation of each actin filament is indicated by an arrowhead at the pointed end. (C) α -actinin binds to N-RAP and cross-links the actin filaments. (D) Krp1 promotes lateral fusion of the Z-bodies to form Z-lines, and N-RAP leaves the complex.

Table 1

FRAP Parameters

	% Recovery			Recovery Half-Time (sec)		
	Pre-	Nascent	Mature	Pre-	Nascent	Mature
N-RAP	19 ± 4	36 ± 6	72 ± 14	88 ± 28	61 ± 22	82 ± 13
α-actinin	61 ± 6	76 ± 7	91 ± 15	56 ± 9	44 ± 7	56 ± 8

Premyofibrils, nascent myofibrils, and mature myofibrils were operationally defined by the combination of N-RAP and α-actinin organization; see text for details. Fluorescence recovery data from individual bleached regions were fit to a single exponential function. Each point represents the mean and standard error of the mean of 4-9 bleached areas.

Table 2

Half-times of fluorescence changes in minutes

	Accumulation		Decrease
	α -actinin	N-RAP	N-RAP
figure 2, box A	270 $\pm$ 190	31 $\pm$ 8	
figure 2, box B	71 $\pm$ 9	91 $\pm$ 8	
figure 2, box C	439 $\pm$ 121	PE/constant	
figure 2, box D	PE/linear increase	PE/constant	690 $\pm$ 130
figure 3	122 $\pm$ 19	140 $\pm$ 83	187 $\pm$ 27
	actin	N-RAP	
figure 6	PE/linear increase	PE/decreasing	840 $\pm$ 269
figure 7	5.4 $\pm$ 0.9*	14.5 $\pm$ 1.8*	

PE/constant: pre-existing structure with a linear fit to the data exhibiting a slope that is not significantly different from 0; PE/linear increase: pre-existing structure with a linear fit to the data exhibiting a positive slope significantly greater than 0 ($p < 0.01$);

PE/decreasing: pre-existing structure with a decreasing exponential fit to the entire time period. *N-RAP and actin accumulation half-times from figure 7 are significantly different ($p < 0.001$). Paired N-RAP and α -actinin accumulation half-times (figure 2, box A and box B, and figure 3) are not significantly different.