Targeting and functional role of N-RAP, a nebulin-related LIM protein, during myofibril assembly in cultured chick cardiomyocytes

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SUMMARY

Targeting and functional effects of N-RAP domains were studied by expression as GFP-tagged fusion proteins in cultured embryonic chick cardiomyocytes. GFP-tagged N-RAP was targeted to myofibril precursors, myofibril ends and cell contacts, expression patterns that are similar to endogenous N-RAP. The GFP-tagged N-RAP LIM domain (GFP-N-RAP-LIM) was targeted to the membrane in cells with myofibril precursors and cell-cell contacts. The GFP-tagged super repeats (N-RAP-SR) and the GFP-tagged domain normally found in between the super repeats and the LIM domain (N-RAP-IB) were each observed at sites of myofibril assembly, incorporating into myofibril precursors in a manner similar to full length N-RAP. However, unlike full-length N-RAP, N-RAP-SR and N-RAP-IB were also found in mature myofibrils, associating with the sarcomeric actin filaments and the Z-lines, respectively. N-RAP-IB was also colocalized with α-actinin at cell contacts. Each of the N-RAP constructs could inhibit the formation of mature myofibrils in cultured cardiomyocytes, with the effects of N-RAP-SR and N-RAP-IB depending on the time of transfection. The results show that each region of N-RAP is crucial for myofibril assembly. Combining the targeting and functional effects of N-RAP domains with information in the literature, we propose a new model for initiation of myofibrillogenesis.

Key words: Chick, Cardiomyocyte, N-RAP, LIM protein, Myofibril assembly

INTRODUCTION

N-RAP is a 133 kDa actin-binding LIM protein recently discovered in skeletal and cardiac muscle tissues (Luo et al., 1997). At the C-terminal end of N-RAP there are nebulin-related super repeats that bind actin and vinculin, while at the N-terminal end, there is a LIM domain consensus sequence that binds talin (Luo et al., 1999; Luo et al., 1997). In between these two domains is a sequence unique to N-RAP that binds muscle LIM protein (MLP) (Ehler et al., 2001) and actin, although actin binding is tenfold weaker than in the super repeat region (Luo et al., 1999). Initially, in immunofluorescently stained frozen sections from mouse skeletal and cardiac muscle, N-RAP was found localized at sites of mechanical coupling between the myofibrils and the cell membrane, that is, at the myotendon junction in skeletal muscle and at the intercalated disks in heart muscle (Luo et al., 1997). Based on the localization of N-RAP and binding affinities, we proposed that N-RAP serves as a link between the terminal actin of the myofibril and the protein complexes at the cell membrane (Luo et al., 1999; Luo et al., 1997).

Immunofluorescent localization of N-RAP in cultured chick cardiomyocytes was consistent with this hypothesis, showing that N-RAP is found concentrated at the terminal ends of mature cells and at sites of cell to cell contact (Carroll and Horowits, 2000). In addition, N-RAP was found localized along myofibril precursors in immature cardiomyocytes (Carroll and Horowits, 2000). As cardiomyocytes spread in culture, immature fibrils containing punctate α-actinin Z-bodies, α-actin and muscle tropomyosin are formed (Dabiri et al., 1997; Dlugosz et al., 1984; Handel et al., 1991; Rhee et al., 1994; Schultheiss et al., 1990; Wang et al., 1988). This process appears to originate at the periphery of the cell, with the earliest myofibril precursors, the premyofibrils, containing nonmuscle myosin IIb (Rhee et al., 1994) and N-RAP (Carroll and Horowits, 2000). Time-lapse studies of living cardiomyocytes expressing α-actinin fused to GFP suggest that the closely spaced α-actinin beads in these structures aggregate laterally to form nascent myofibrils (Dabiri et al., 1997). The nascent myofibrils incorporate titin, and muscle myosin gradually replaces the nonmuscle isoform (Dabiri et al., 1997). The muscle myosin may exist as preformed bipolar thick filaments that are oriented and incorporated into the nascent myofibrils by their interactions with titin filaments (Holtzer et al., 1997; Schultheiss et al., 1990). Like nonmuscle myosin IIb, N-RAP is present in all of the myofibril precursors, but is not found in the mature sarcomeres (Carroll and Horowits, 2000).

In this study, we extend our investigation of N-RAP to the unique targeting and functional roles of the individual N-RAP domains during myofibril formation. By expressing N-RAP domains as GFP-fusion proteins in cultured cardiomyocytes, we found that each region of N-RAP exhibited targeting to

Table 1. PCR primer pairs

Amplified region (nucleotides)	Primer pair
N-RAP (1523-5047)	Forward, 5′-ATGAATGTGCAGGCCTGCTCT-3′; reverse, 5′-**TCA**CGGCCGTGTACTTGAATG-3′
N-RAP-LIM (1523-1693)	Forward, 5′-ATGAATGTGCAGGCCTGCTCT-3′; reverse, 5′-**CTA**GTGGGCGTGACAGTACGG-3′
N-RAP-IB (1694-3187)	Forward, 5′-AACCCTAAGAACAACACGTTCACTAG-3′; reverse, 5′-**CTA**CAGGGCTCTGCCCTTCATTTTC-3′
N-RAP-SR (3188-4948)	Forward, 5′-GGAGCCACAGACTCTAAGCTTCTGC-3′; reverse, 5′-**CTA**CACACAGGGAACACCACGCATG-3′

The nucleotides amplified refer to the corresponding regions in the full-length mouse N-RAP cDNA (Luo et al., 1997). The stop codon added to each reverse primer is indicated in bold.

specific areas within the cardiomyocyte, and could also disrupt myofibril assembly; these effects depended on the state of the cell, with systematic differences observed by varying the interval between culturing the cells and transfection with N-RAP constructs. We present a molecular model of myofibrillogenesis initiation that is consistent with the binding properties, targeting activities and phenotypic effects of N-RAP domains.

MATERIALS AND METHODS

Cloning of N-terminal GFP fusion proteins

RNA was isolated from adult mouse skeletal muscle and used as the template for cDNA synthesis, as previously described (Herrera et al., 2000). Specific regions of the N-RAP cDNA were PCR amplified using the primer pairs described in Table 1. PCR amplification of cDNA was performed using the Gibco BRL Elongase enzyme mix (Life Technologies, Gaithersburg, MD). The amplification protocol used for full-length N-RAP was 1 cycle at 94°C for 3 minutes; 45 cycles at 94°C for 30 seconds, then 59°C for 30 seconds and 68°C for 6.5 minutes; and 1 cycle at 72°C for 7 minutes. The amplification protocol used for the N-RAP LIM domain (N-RAP-LIM) was 1 cycle at 94°C for 5 minutes; 45 cycles at 94°C for 30 seconds, then 68°C for 5 minutes; and 1 cycle at 72°C for 7 minutes. The amplification protocol used for the N-RAP super repeat region (N-RAP-SR) and the region in between the LIM domain and the super repeats (N-RAP-IB) was 1 cycle at 94°C for 3 minutes; 45 cycles at 94°C for 30 seconds, then 59°C for 30 seconds, then 68°C for 5 minutes; and 1 cycle at 72°C for 7 minutes. PCR products were gel purified and cloned into the pcDNA3.1/NT-GFP-TOPO plasmid vector (Invitrogen, Carlsbad, CA). Plasmids were propagated in One Shot TOP10 E. coli cells (Invitrogen, Carlsbad CA) and purified using the Quantum Prep Plasmid Miniprep Kit (BioRad, Hercules, CA). Plasmids were partially sequenced to verify the integrity of the cloned inserts.

Culture and transfection of chick cardiomyocytes

Primary cultures of chick cardiomyocytes were prepared from 7-10 day chick embryos as previously described (Carroll and Horowits, 2000) and transfected after 1, 2 or 4 days in culture. The transfection mixture contained 1.5 μg of plasmid DNA and 6 μl of FuGene (Boehringer Mannheim, Indianapolis, IN), a non-liposomal transfection reagent, in normal growth media. Samples were processed for immunoblot analysis or fluorscence microscopy either 1 or 3 days after transfection, as previously described (Carroll and Horowits, 2000). Polyclonal antibody against N-RAP has been previously described (Luo et al., 1997). Monoclonal antibodies against sarcomeric α-actinin were obtained from Sigma (St Louis, MO), and monoclonal anti-GFP was obtained from Clontech Laboratories (Palo Alto, CA).

Data analysis

Digital micrographs were captured using a Hamamatsu CCD camera connected to a Zeiss Axiovert 135 microscope and interfaced with a Power Macintosh computer, as previously described (Carroll and Horowits, 2000). Quantitative image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

RESULTS

Characterization of GFP fusion proteins and GFP expression

Fig. 1A illustrates the different domains of N-RAP that were expressed as N-terminal GFP fusion proteins. From measurements of the fraction of cardiomyocytes exhibiting GFP fluorescence, we estimate that we achieved transfection efficiencies for GFP-N-RAP, GFP-N-RAP-SR, GFP-N-RAP-IB and GFP-N-RAP-LIM of 4%, 10%, 16% and 17%, respectively. To confirm that these constructs were properly expressed, the GFP fusion proteins obtained from lysed transfected cardiomyocytes were analyzed by immunoblot analysis. As shown in Fig. 1B, a monoclonal antibody against GFP specifically detected bands for GFP-N-RAP-SR (lane 1), GFP-N-RAP-IB (lane 2), GFP-N-RAP-LIM (lane 3) and GFP alone (lane 4) that migrated near the molecular weights predicted from their respective cDNA sequences. We were unable to detect the full-length GFP-N-RAP fusion protein by immunoblot (data not shown), possibly owing to the lower transfection efficiencies observed with this large construct. However, full-length double-stranded sequencing of the GFP-N-RAP plasmid insert verified that it contains the uninterrupted full-length open reading frame for N-RAP (data not shown) (Luo et al., 1997).

Transfection of cardiomyocytes with the plasmid vector alone, without any insert, resulted in a diffuse pattern of GFP expression throughout the cytoplasm and nucleus of the cardiomyocyte (Fig. 2A) that did not appear to affect myofibril formation, as detected by α-actinin staining (Fig. 2B). This indicates that neither the transfection procedure nor the introduction of GFP into the cardiomyocytes hinders myofibrillogenesis and that GFP alone was not specifically targeted to any areas within the cells.

The time course of myofibril assembly

Although embryonic chick cardiomyocyte cultures have been extensively used to study myofibrillogenesis, some investigators claim that this system is characterized by massive degradation of myofibrils that had been synthesized in ovo (Ojima et al., 2000). In order to assess the suitability of this system for studying myofibril assembly, we used a morphometric method to quantitate the time course of net myofibril accumulation. As illustrated in Fig. 3B, we measured the percentage of the area of each cardiomyocyte that

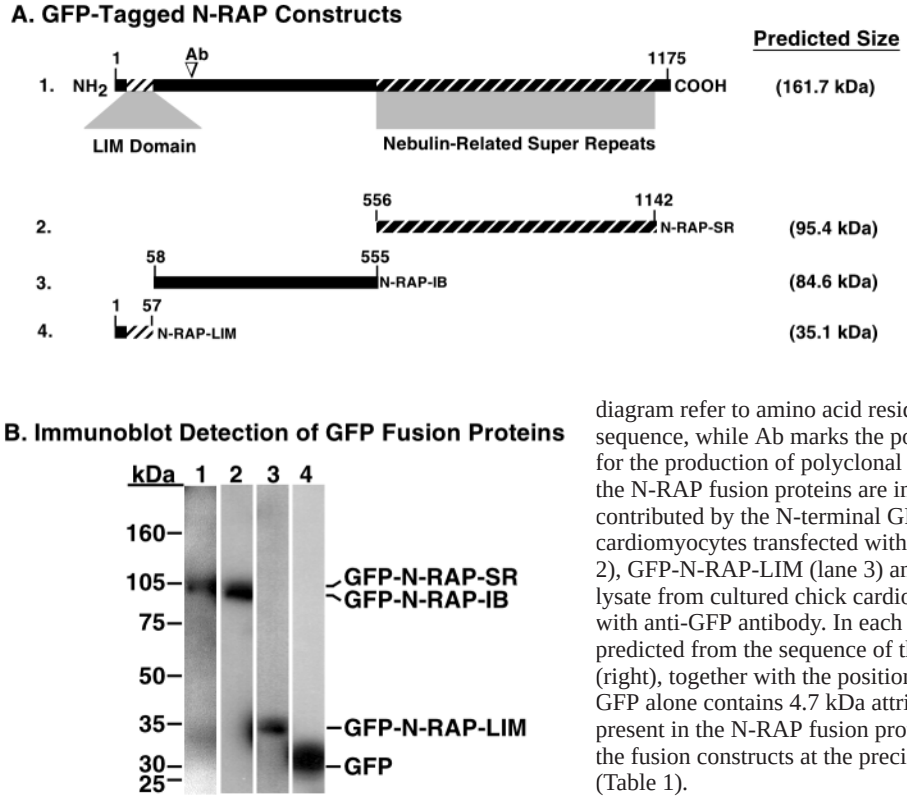

Fig. 1. (A) Regions of N-RAP expressed as GFP fusion proteins. GFP was fused to the N terminus of full-length N-RAP (1), the N-RAP super repeats (N-RAP-SR) (2), the region in between the super repeats and the LIM domain (N-RAP-IB) (3), and the N-RAP LIM domain (N-RAP-LIM) (4). Numbers above the diagram refer to amino acid residues from the full-length mouse N-RAP sequence, while Ab marks the position of a 30 residue peptide used as an antigen for the production of polyclonal antibodies. The predicted molecular weights of the N-RAP fusion proteins are indicated in parentheses, including the 28.6 kDa contributed by the N-terminal GFP. (B) Immunoblot analysis of chick cardiomyocytes transfected with GFP-N-RAP-SR (lane 1), GFP-N-RAP-IB (lane 2), GFP-N-RAP-LIM (lane 3) and GFP alone (lane 4). Equivalent volumes of lysate from cultured chick cardiomyocytes were loaded in each lane and probed with anti-GFP antibody. In each case, the detected band migrated near the position predicted from the sequence of the fusion plasmid. The detected bands are labeled (right), together with the position of the molecular weight markers (left). Note that GFP alone contains 4.7 kDa attributed to 46 C-terminal residues that are not present in the N-RAP fusion proteins, owing to the stop codons engineered into the fusion constructs at the precise end of the sequences derived from N-RAP (Table 1).

contained mature myofibrils as visualized by α-actinin staining. Fig. 2C shows the quantitative results for mock transfected cardiomyocytes, which were subjected to the transfection protocol with the omission of plasmid DNA (open symbols). The results for cardiomyocytes expressing GFP alone are also shown (Fig. 2C, closed symbols). Although the cells were isolated from beating embryonic hearts, 1-2 days after plating these cardiomyocytes contain few myofibrils (Fig. 2C). The myofibrillar markers α-actinin and titin are clearly present at this stage, but are diffusely distributed (Carroll and Horowits, 2000), demonstrating that the pre-existing myofibrils have been completely disassembled. The cardiomyocytes formed mature myofibrils between 2 and 3 days in culture, followed by degeneration of the mature myofibrils between 4 and 5 days (Fig. 2C). The time course of myofibril assembly and disassembly was unaffected by GFP expression (Fig. 2, closed versus open symbols), and was similar to that observed in untransfected cultures (data not shown), indicating that neither the transfection procedure nor GFP expression affected these processes. Our quantitative data demonstrating myofibril disassembly within 2 days of plating, followed by myofibril assembly between 2 and 4 days, and myofibril degradation after 4 days is consistent with previous reports on the myofibrillogenesis process in cultured chick cardiomyocytes (Handel et al., 1991; Rhee et al., 1994). Therefore, between 2 and 4 days in culture there is a net

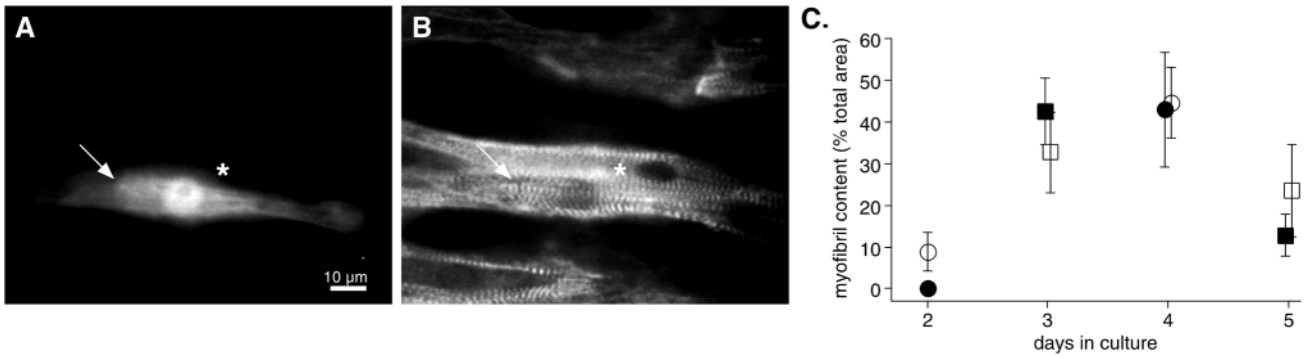

Fig. 2. Expression patterns of GFP (A) and of α-actinin in the same field (B). GFP is diffusely distributed in the single transfected cardiomyocyte (arrow, A). α-actinin (B) is clearly localized in striated myofibrils in both the GFP transfected cell (arrow) and the neighboring untransfected cell (asterisk). (C) Myofibril content versus days in culture. The open symbols show results for mock transfected cardiomyocytes, and the closed symbols show results for cardiomyocytes transfected with GFP alone. Cells were transfected after 1 day (circles) or 2 days (squares) in culture and viewed 1 day or 3 days post-transfection. Each point represents the mean±s.e.m. from 4 to 27 transfected cells. No significant difference was observed between the mock-transfected cardiomyocytes and cardiomyocytes expressing GFP.

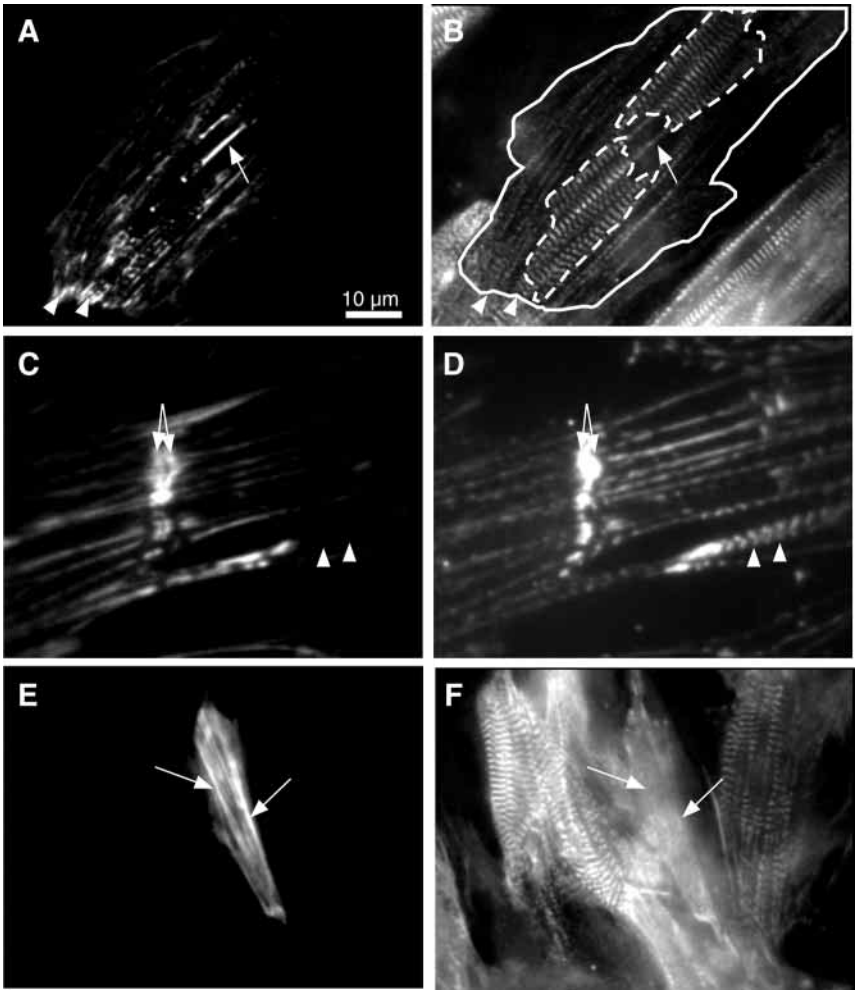

Fig. 3. Expression patterns of GFP-N-RAP (A,C,E) and of α-actinin in the same fields (B,D,F). Cardiomyocytes were transfected with GFP-N-RAP at 2 days and viewed 1 day post-transfection (A-B,E-F) or transfected at 4 days and viewed 3 days post-transfection (C-D). In cells containing mature sarcomeres linked by immature areas, GFP-N-RAP is localized only in the myofibril precursors (A,B, arrows), as well as at cell to cell contacts (A,B, arrowheads). In more mature cardiomyocytes that had well-established intercalated disks, GFP-N-RAP was highly concentrated at these sites, and often exhibited a doublet band of expression that appeared to bracket the α-actinin band (C,D, double arrows). Note that GFP-N-RAP was not expressed within mature myofibrils detected by α-actinin staining (C,D, arrowheads). Some cells exhibit specific localization of GFP-N-RAP along fibrillar structures that were not detected by α-actinin staining (E,F, arrows). Note the absence of myofibrillar α-actinin striations in this transfected cardiomyocyte compared with the surrounding untransfected cells (E,F). (B) The method for quantitating myofibril content in individual cardiomyocytes: the periphery of the transfected cardiomyocyte stained with α-actinin is outlined by the unbroken white line; areas containing mature myofibrils are outlined by broken lines. The area of each enclosed region was measured, and the percentage of the total area taken up by mature myofibrils was calculated.

assembly of myofibrils, and this period affords a suitable window in which to study the myofibrillogenesis process.

Expression of GFP-N-RAP

In contrast to GFP expression, transfection of the complete N-RAP protein fused to GFP yielded highly specific localization patterns that evolved throughout the culture period (Fig. 3). At early times in culture when most cardiomyocytes were immature, GFP-N-RAP was expressed along the myofibril precursors (data not shown). Later in culture, as cardiomyocytes matured and made contact with other cells, GFP-N-RAP was found in immature areas of myofibrils linking regions of mature sarcomeres (Fig. 3A,B, arrows), and along the cell to cell border (Fig. 3A,B, arrowheads). Eventually, towards the end of the culture period when intercalated disks were re-established, GFP-N-RAP was found highly concentrated at the intercalated disk sites (Fig. 3C,D, arrows). This pattern of GFP-N-RAP expression precisely mimicked the pattern of expression observed for endogenous N-RAP reported earlier at similar stages of cardiomyocyte myofibrillogenesis (Carroll and Horowits, 2000). The consistency of the patterns of expression observed for the endogenous N-RAP and GFP-N-RAP strongly suggests that fusion of GFP to N-RAP does not interfere with the intrinsic targeting and localization of N-RAP.

However, some cells exhibited specific targeting of N-RAP near the membrane and along fibrillar structures (Fig. 3E, arrows), even though no significant fibrillar structures were detected with α-actinin staining (Fig. 3F, arrows). However, it is not clear whether the apparent absence of α-actinin in these N-RAP-positive fibrillar structures is real or whether fibrillar α-actinin is obscured by the large amount of diffusely distributed α-actinin that is present. Cardiomyocytes shown in the top and bottom panels of Fig. 3 were transfected at the same day in culture and viewed the same days post-plating, so the differences in myofibril formation between the transfected cells in these panels cannot be attributed to the timing of transfection. Instead, the observed differences may be due to overexpression of GFP-N-RAP in the cardiomyocyte in the lower panels relative to the transfected cell in the upper panel, as inferred from the relative amount of GFP fluorescence observed.

Expression of GFP-N-RAP-LIM

Transfection of GFP-N-RAP-LIM into cardiomyocytes after four days in culture resulted in specific targeting of this construct to areas near the membrane (Fig. 4A). In cells where GFP-N-RAP-LIM was located in these areas near the membrane, the cardiomyocyte had typically developed myofibril precursors, indicated by punctate α-actinin staining (Fig. 4B), and the transfected cells were not easily distinguished from neighboring untransfected cells by α-actinin staining alone. By contrast, when cardiomyocytes were

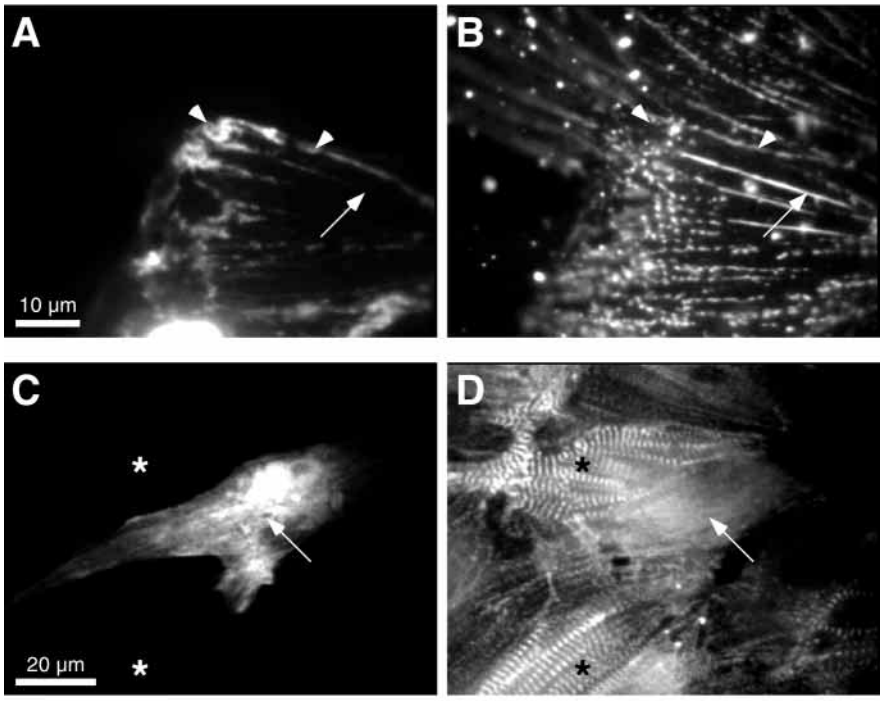

Fig. 4. Expression patterns of GFP-N-RAP-LIM (A,C) and of α-actinin in the same fields (B,D). Cardiomyocytes were transfected with GFP-N-RAP-LIM at 4 days and viewed 3 days post transfection (A,B) or transfected at 2 days and viewed 1 day post transfection (C,D). In cardiomyocytes transfected after 4 days in culture, GFP-N-RAP-LIM was targeted to the cell periphery and sites of cell to cell contact (arrowheads, A,B). Punctate or filamentous α-actinin staining revealed that myofibril precursors were present in these cardiomyocytes, but did not contain GFP-N-RAP-LIM (arrows, A,B). In cardiomyocytes transfected after 1 or 2 days in culture, both GFP-N-RAP-LIM and α-actinin were diffusely distributed (arrows, C,D), in contrast to neighboring untransfected cells exhibiting clear striations when stained for α-actinin (asterisks, C,D).

transfected with GFP-N-RAP-LIM at earlier times (1-2 days), this construct exhibited a diffuse pattern of expression (Fig. 4C), and the transfected cardiomyocyte contained significantly fewer α-actinin-positive myofibril structures compared with the neighboring untransfected cells, which contained large areas of well organized sarcomeres (Fig. 4D).

Expression of GFP-N-RAP-SR

GFP-N-RAP-SR expression in cells that were transfected after 2 days in culture was concentrated along myofibril precursors (Fig. 5A, arrows) detected by α-actinin staining (Fig. 5B, arrows), resembling the pattern of expression observed for GFP-N-RAP and endogenous N-RAP in similar immature areas. In areas containing well-organized sarcomeres, as indicated by the regular Z-band staining with α-actinin (Fig. 5B, arrowheads), GFP-N-RAP-SR exhibited a broad-banded pattern of expression (Fig. 5A). These broad bands spanned the area on either side of the Z-line into the I-band region (Fig. 5A inset, arrowheads). However, mature sarcomeres were rarely observed when GFP-N-RAP-SR was transfected into cardiomyocytes at the earliest time in culture (1 day). The transfected cardiomyocyte in the bottom panels of Fig. 5 is

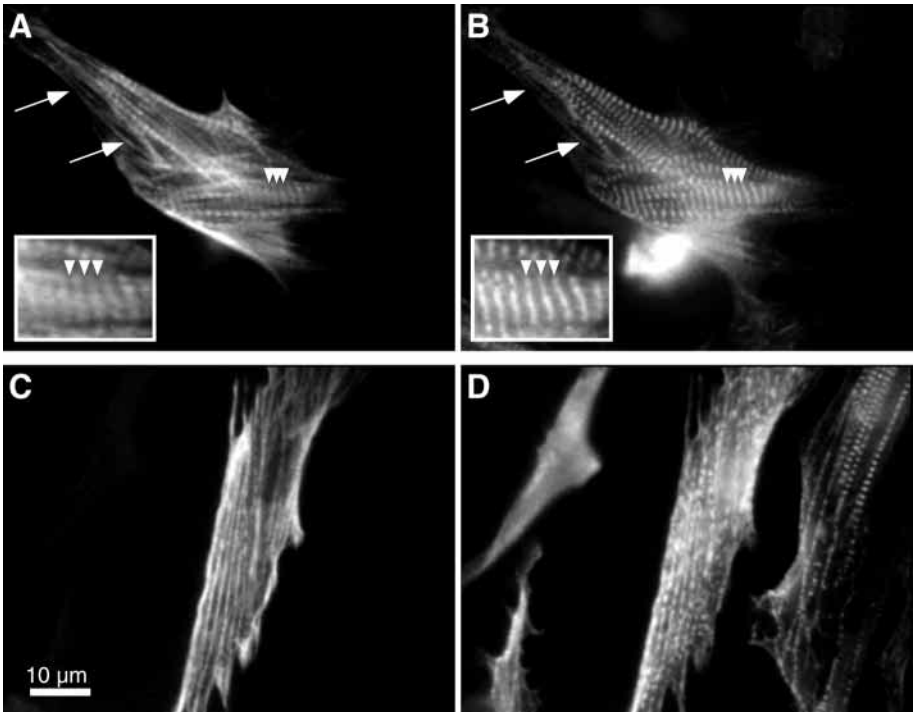

Fig. 5. Expression patterns of GFP-N-RAP-SR (A,C) and of α-actinin in the same fields (B,D). Cardiomyocytes were transfected with GFP-N-RAP-SR at 2 days and viewed 1 day post transfection (A,B) or transfected at 1 day and viewed 3 days post transfection (C,D). In cardiomyocytes transfected after 2 days in culture, GFP-N-RAP-SR is concentrated in myofibril precursors (arrows, A,B), as well as in mature sarcomeres (arrowheads, A,B). The inset (A,B) shows a magnified view of the α-actinin striations marked with arrowheads, showing that N-RAP-SR is concentrated within the myofilament-containing region of the sarcomere, but not in the Z-lines marked by α-actinin staining. The sarcomeres shown in the inset have an average length of 1.4 μm. Cardiomyocytes transfected with GFP-N-RAP-SR after 1 day in culture rarely form mature myofibrils (C,D).

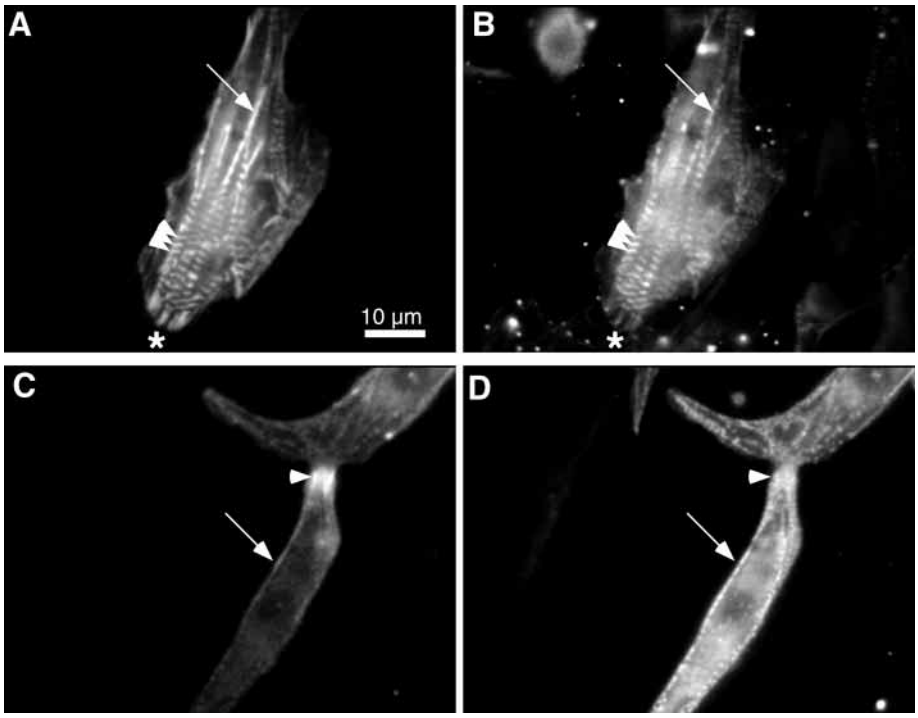

Fig. 6. Expression patterns of GFP-N-RAP-IB (A,C) and of α-actinin in the same fields (B,D). Cardiomyocytes were transfected with GFP-N-RAP-IB at 2 days and viewed 1 day post-transfection. GFP-N-RAP-IB was concentrated in myofibril precursors (arrows, A,B) as well as in Z-lines of mature sarcomeres (arrowheads, A,B). GFP-N-RAP-IB also colocalized with α-actinin at the cell periphery (asterisks, A,B; arrows, C,D) and at cell to cell contacts (arrowheads, C,D).

typical of this observation; it was packed with myofibril precursors containing both N-RAP-SR (Fig. 5C) and α-actinin (Fig. 5D), but did not contain mature myofibrils.

Expression of GFP-N-RAP-IB

Like GFP-N-RAP-SR, GFP-N-RAP-IB was observed along myofibril precursors (Fig. 6A,B, arrows) and within the sarcomere (Fig. 6A,B, arrowheads). However, sarcomeric GFP-N-RAP-IB was confined to the Z-lines. In addition to its sarcomeric localization, GFP-N-RAP-IB was often observed at the periphery of cells, particularly at sites of cell to cell contact (Fig. 6C), and appeared to co-localize with α-actinin staining (Fig. 6C,D).

GFP-N-RAP-IB could also interfere with normal myofibril formation. In some cells, GFP-N-RAP-IB appeared in periodic narrow striations resembling Z-lines (Fig. 7A), while α-actinin staining was intense but diffusely distributed (Fig. 7B). In other cases, GFP-N-RAP-IB appeared localized to structures resembling myofibril precursors and at the membrane along cell to cell borders (Fig. 7C), while no mature sarcomeres were detected by α-actinin staining (Fig. 7D). In this example, the α-actinin staining intensity was dramatically reduced in the transfected cell compared with the surrounding untransfected cells, which were packed with mature myofibrils (Fig. 7D).

The effect of N-RAP domains on myofibril assembly

We quantitated the functional effects of the GFP fused N-RAP domains on myofibrillogenesis by measuring the percentage of the area of each transfected cardiomyocyte that contained mature myofibrils (Fig. 3B). The results from cardiomyocytes expressing GFP-fused N-RAP and N-RAP domains were compared with cardiomyocytes transfected with GFP alone. Cardiomyocytes expressing GFP alone formed mature myofibrils between 2 and 3 days in culture, followed by

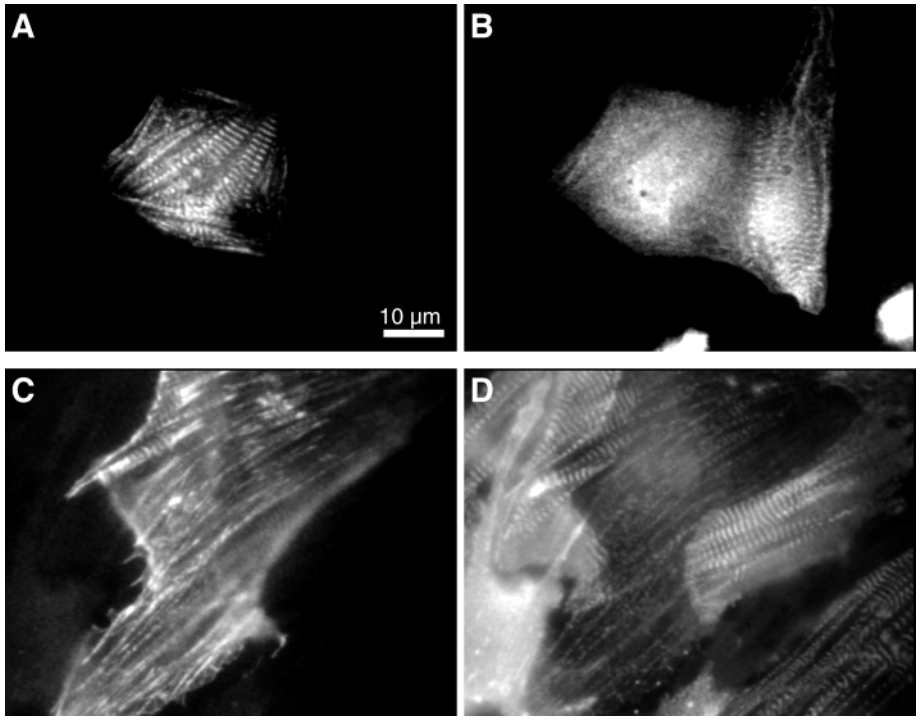

Fig. 7. Expression patterns of GFP-N-RAP-IB (A,C) and of α-actinin in the same fields (B,D). Cardiomyocytes were transfected with GFP-N-RAP-IB at 2 days and viewed 1 day post transfection. In some cardiomyocytes, GFP-N-RAP-IB is organized in striations resembling the Z-lines of mature sarcomeres (A), even though α-actinin is diffusely distributed (B). Note the presence of normal α-actinin striations in the neighboring untransfected cell (B). In addition, N-RAP-IB often inhibits myofibril formation (C,D). In this example, N-RAP-IB is localized at cell-cell contacts and cytoskeletal structures resembling myofibril precursors (C), but the transfected cell exhibits far fewer striations than neighboring cells (D).

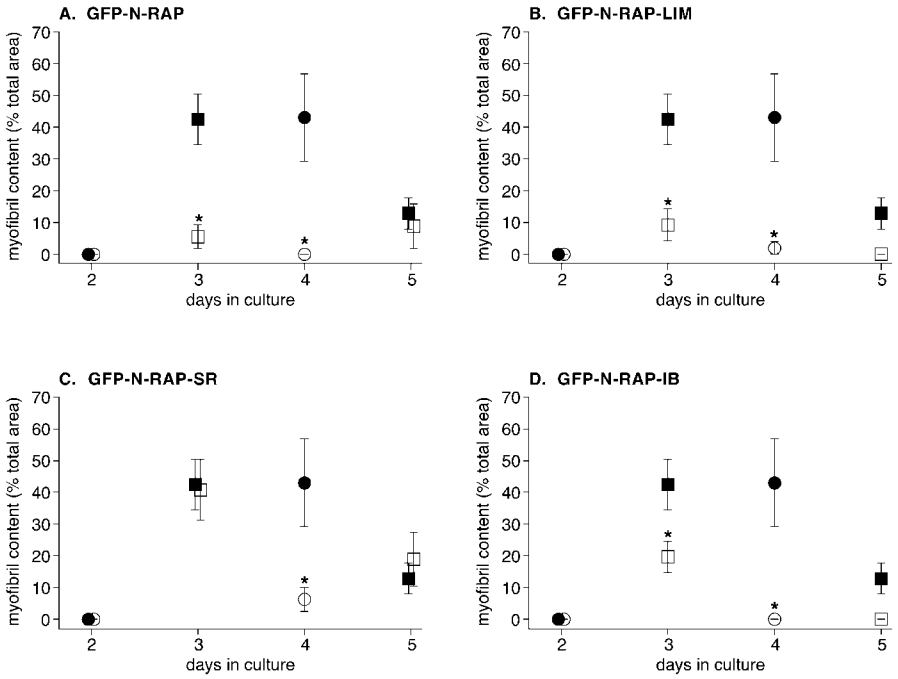

Fig. 8. Myofibril content versus days in culture. The open symbols show results for cardiomyocytes transfected with GFP-N-RAP (A), GFP-N-RAP-LIM (B), GFP-N-RAP-SR (C) and GFP-N-RAP-IB (D); in each case the results from cardiomyocytes transfected with GFP alone are shown for comparison (filled symbols). Cells were transfected after 1 day (circles) or 2 days (squares) in culture and viewed 1 day or 3 days post-transfection. Significant differences (P<0.05) between the N-RAP constructs and GFP alone were determined by unpaired Student's t-tests and are indicated by asterisks. Each point represents the mean±s.e.m. from 3 to 40 transfected cells.

degeneration of the mature myofibrils between 4 and 5 days (Fig. 8, solid symbols). This time course of myofibril assembly and disassembly was similar to that observed in mock transfected (Fig. 2C) or untransfected cultures (data not shown), indicating that neither the transfection procedure nor GFP expression affected these processes, and was also consistent with previous reports on the myofibrillogenesis process in cultured chick cardiomyocytes (Rhee et al., 1994).

Fig. 8 shows that each of the N-RAP domains, as well as full-length N-RAP, significantly inhibited myofibril formation when transfected into the cardiomyocytes after 1 day in culture (Fig. 8, open versus closed circles). By contrast, the effect of transfecting cardiomyocytes after 2 days in culture varied between the N-RAP constructs. GFP-N-RAP and GFP-N-RAP-LIM severely inhibited the formation of mature myofibrils within 1 day of transfection (Fig. 8A,B, open versus closed squares), while the effect of GFP-N-RAP-IB was significant but incomplete (Fig. 8D, open versus closed squares). By contrast, GFP-N-RAP-SR had no significant effect on the formation of mature myofibrils when transfected at day 2 (Fig. 8C, open versus closed squares).

The effect of N-RAP domains on endogenous protein levels

As each of the N-RAP constructs expressed as GFP fusion proteins could inhibit myofibril formation, we sought to determine if endogenous α-actinin and N-RAP levels were also affected. We measured the mean pixel intensity in micrographs of the α-actinin staining in transfected cells and normalized these values by the mean pixel intensity in untransfected cells present on the same slide. In general, the normalized values for the α-actinin levels did not significantly deviate from 1.0 for any of the constructs (Fig. 9, closed symbols). The results show that α-actinin levels are normal in the transfected cells, even under conditions where myofibril assembly has been inhibited by N-RAP constructs.

We also stained for endogenous N-RAP in cardiomyocytes transfected with GFP-N-RAP-SR and GFP-N-RAP-LIM. As our N-RAP antibody was raised against a peptide within the N-RAP-IB region (Fig. 1A), it could be used to specifically stain for endogenous N-RAP without cross-reacting with either GFP-N-RAP-SR or GFP-N-RAP-LIM, but could not be used to distinguish between endogenous N-RAP and either GFP-N-RAP or GFP-N-RAP-IB. Quantitating endogenous N-RAP levels by the same method used to determine α-actinin levels, we found that transfection with GFP-N-RAP-LIM led to a gradual decrease in endogenous N-RAP (Fig. 9C, open symbols). This effect occurred in cardiomyocytes transfected with GFP-N-RAP-LIM after either 1 day (open circles) or 2 days (open squares) in culture. By contrast, the effect of transfection with GFP-N-RAP-SR on endogenous N-RAP levels depended on when the cardiomyocytes were transfected; we found that when cardiomyocytes were transfected with GFP-N-RAP-SR after 2 days in culture there was no significant effect on the endogenous N-RAP staining compared with the neighboring untransfected cells (Fig. 9D, open squares). However, when cardiomyocytes were transfected with GFP-N-RAP-SR after 1 day in culture, the endogenous N-RAP levels were significantly reduced by day 4 compared with neighboring untransfected cardiomyocytes (Fig. 9D, open circles).

DISCUSSION

Targeting N-RAP domains

Table 2 summarizes the targeting behaviors of the individual N-RAP domains and of the full-length GFP-N-RAP construct, and compares this with the localization of endogenous N-RAP in untransfected cells. We found that GFP-N-RAP was specifically localized in myofibril precursors and at sites of cell to cell contact, the same structures where endogenous N-RAP

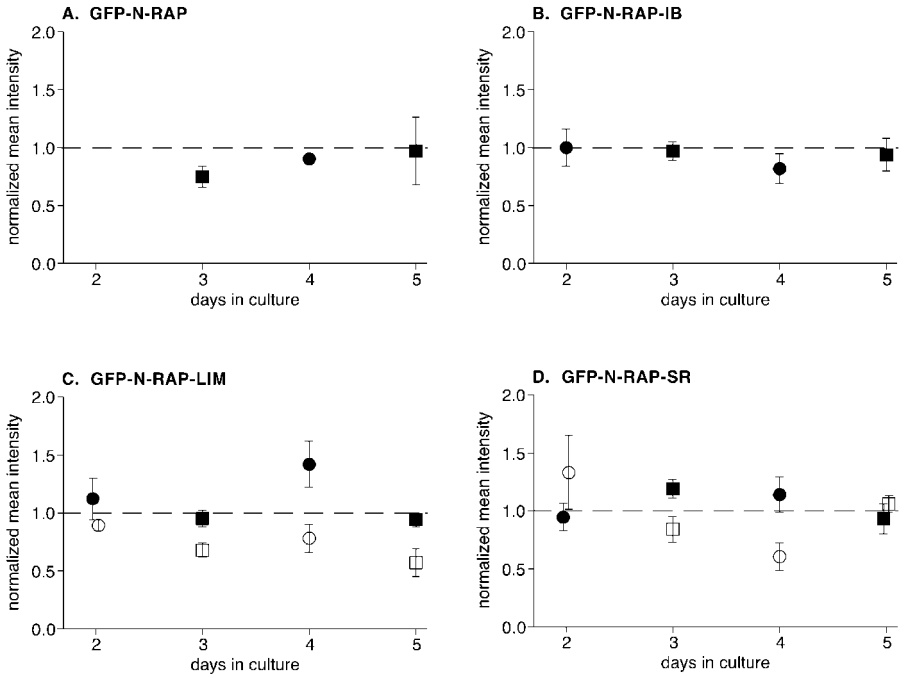

Fig. 9. α-actinin (filled symbols) and N-RAP (open symbols) staining intensity versus days in culture. Results are for cardiomyocytes transfected with GFP-N-RAP (A), GFP-N-RAP-IB (B), GFP-N-RAP-LIM (C) and GFP-N-RAP-SR (D). In each case the results were normalized to values obtained from untransfected cardiomyocytes on the same slides. Cells were transfected after 1 day (circles) or 2 days (squares) in culture and viewed 1 day or 3 days post-transfection. Each point represents the mean±s.e.m. from 2 to 14 transfected cells.

has been previously identified. Neither endogenous N-RAP nor GFP-N-RAP was observed within mature myofibrils.

By contrast, each of the partial N-RAP constructs exhibited localization patterns that differed from that of endogenous N-RAP, as well as from each other. GFP-N-RAP-LIM was specifically targeted to the cell periphery, but was not observed in any fibrillar structures. The results are consistent with the previously demonstrated binding of the N-RAP LIM domain to talin (Luo et al., 1999), an important molecular link between transmembrane integrins and actin filaments (Burridge and Chrzanowska-Wodnicka, 1996; Critchley, 2000).

In contrast to the N-RAP LIM domain, GFP-N-RAP-IB and GFP-N-RAP-SR were both targeted to myofibril precursors as well as to mature sarcomeres. However, their localization within the sarcomeres differed, with N-RAP-SR colocalizing with the myofilaments, and N-RAP-IB colocalizing with α-actinin at the Z-lines. As the N-RAP super repeats exhibited strong actin binding in vitro but did not bind myosin (Luo et al., 1999), the broad-banded myofibrillar localization of N-RAP-SR is probably due to its incorporation into the sarcomeric actin filaments. By contrast, the targeting data for N-RAP-IB suggest an interaction between N-RAP-IB and Z-line components. N-RAP-IB was also colocalized with α-actinin at the cell periphery and cell-cell contacts. N-RAP-IB directly binds muscle LIM protein (MLP) (Ehler et al., 2001), a Z-line protein (Arber et al., 1997; Flick and Konieczny, 2000) that directly binds α-actinin (Flick and Konieczny, 2000; Louis et al., 1997), and this interaction may account for the complex pattern of N-RAP-IB targeting that we observe.

Disruption of myofibrillogenesis

Each of the N-RAP constructs could inhibit the formation of mature myofibrils in cultured cardiomyocytes under certain conditions (Fig. 8), showing that each region of N-RAP is crucial for myofibril assembly. As overexpression of full-length GFP-N-RAP inhibits myofibril assembly as effectively as GFP-N-RAP-LIM, the stoichiometry between N-RAP and its binding partners must be a crucial factor that affects myofibrillogenesis.

In the case of N-RAP-SR and N-RAP-IB, the effect on myofibrillogenesis was crucially dependent on the time of transfection, with complete inhibition only observed in cardiomyocytes transfected within 1 day of plating. By contrast, GFP-N-RAP and GFP-N-RAP-LIM almost completely inhibited myofibril formation whether transfection occurred within 1 or 2 days of plating. The results suggest that, once formed, the association of the N-RAP super repeats and N-RAP-IB with their binding partners is less susceptible to competitive disruption than the association between the N-RAP LIM domain and its membrane associated binding partners. Consistent with this interpretation, their homology to nebulin suggests that the repetitive actin binding modules that compose the N-RAP super repeat region should bind actin cooperatively and strongly (Chen et al., 1993; Pfuhl et al., 1996). A repetitive motif has also been identified in the N-RAP-IB region (Luo et al., 1997).

Expression of the GFP-N-RAP domains did not result in a systematic change in α-actinin levels as estimated by quantitation of immunofluorescent images (Fig. 9). Our results show that α-actinin was present in approximately normal

Table 2. Targeting of N-RAP domains

N-RAP construct	Myofibril precursors	Myofibrillar Z-lines	Myofibrillar actin	Cell periphery
Endogenous N-RAP	✔	-	-	✔
GFP-N-RAP	✔	-	-	✔
GFP-N-RAP-LIM	-	-	-	✔
GFP-N-RAP-SR	✔	-	✔	-
GFP-N-RAP-IB	✔	✔	-	✔

Check marks signify targeting of the indicated N-RAP constructs to the indicated structures in cardiomyocytes.

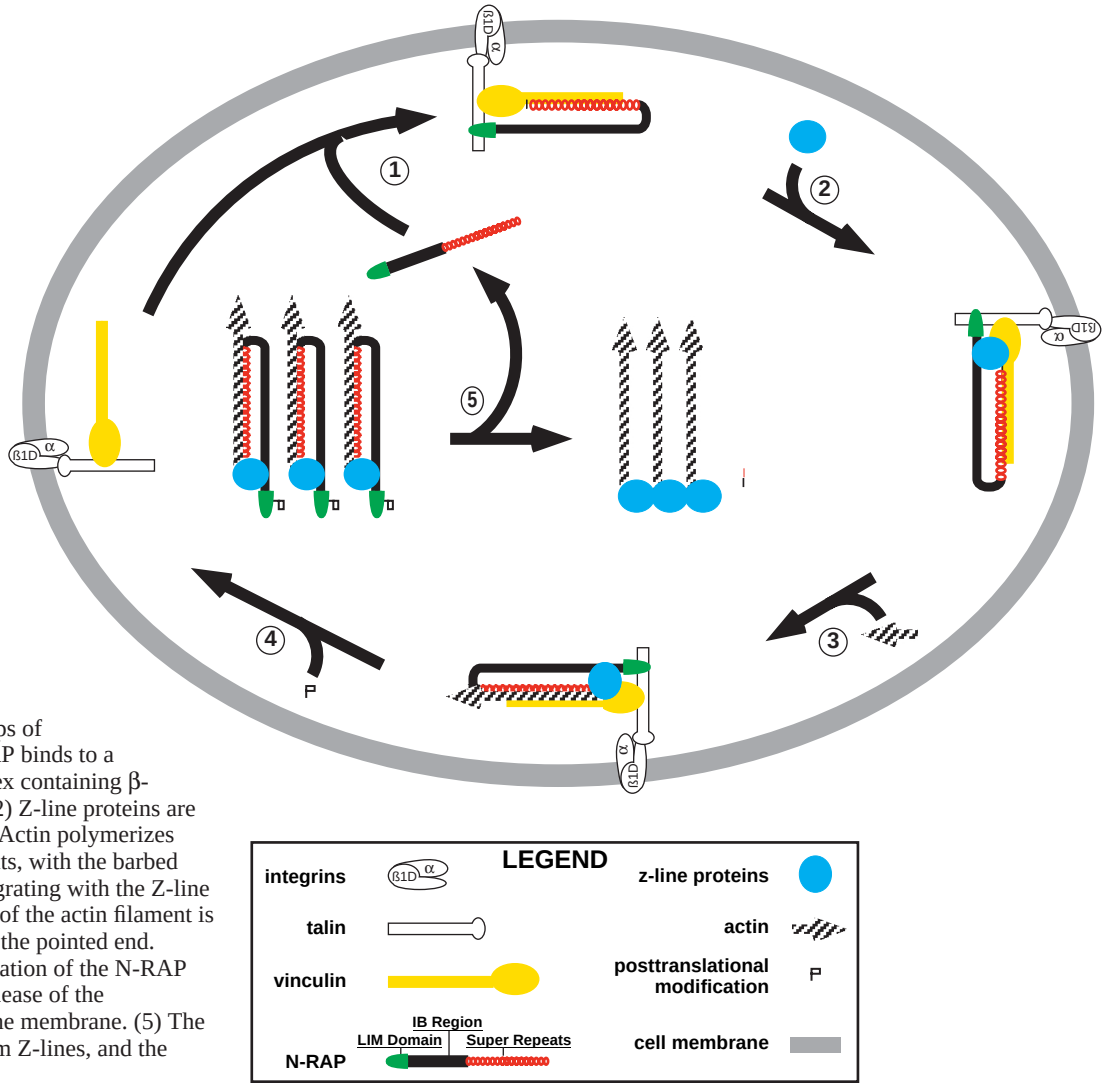

Fig. 10. The putative first steps of myofibrillogenesis. (1) N-RAP binds to a membrane-associated complex containing β-integrin, talin and vinculin. (2) Z-line proteins are recruited to the complex. (3) Actin polymerizes along the N-RAP super repeats, with the barbed end of the actin filament integrating with the Z-line components. The orientation of the actin filament is indicated by an arrowhead at the pointed end. (4) Post-translational modification of the N-RAP LIM domain accompanies release of the premyofibril complex from the membrane. (5) The Z-bodies fuse laterally to form Z-lines, and the modified N-RAP is removed.

amounts, but remained unassembled when myofibrillogenesis was disrupted by overexpression of N-RAP or N-RAP domains. By contrast, endogenous N-RAP levels appeared to decrease modestly in cardiomyocytes transfected with GFP-N-RAP-LIM or GFP-N-RAP-SR. The decrease in N-RAP levels appeared to be related to the inhibition of myofibril assembly, as cells expressing N-RAP-SR exhibited each of these effects only when transfected 1 day after plating.

A model for initiation of myofibrillogenesis

In cultured cardiomyocytes (Dabiri et al., 1997; Rhee et al., 1994), precardiac mesoderm explant cultures (Imanaka-Yoshida et al., 1998) and embryonic hearts (Ehler et al., 1999), the earliest myofibril precursors originate near the membrane, suggesting a role for a membrane-associated tethering molecule in the process. Although existing models of myofibrillogenesis address subsequent steps in some detail (Dabiri et al., 1997; Holtzer et al., 1997), the mechanism and role of the initial membrane association have remained unexplained. We hypothesize a crucial role for N-RAP in the first steps of myofibrillogenesis based on the following findings: (1) N-RAP binds tightly to the membrane associated proteins talin and vinculin, as well as to actin (Luo et al., 1999); (2) N-RAP is found in the earliest premyofibrils at the cell periphery (Carroll and Horowits, 2000); (3) N-RAP is expressed only in striated muscles (Luo et al., 1997); and (4) Each region of N-RAP can completely disrupt myofibril formation when expressed in cultured cardiomyocytes at particular times, consistent with a central role for N-RAP as an organizing center in the initial phase of myofibril assembly.

A schematic model illustrating one possible sequence of events leading to the initiation of myofibril assembly is shown in Fig. 10. In this scheme, N-RAP binds to a membrane-associated complex containing integrins, talin and vinculin (step 1). The N-RAP LIM domain is probably responsible for this initial interaction, as it is exclusively targeted to the cell periphery (Table 2) and binds directly to talin in vitro (Luo et al., 1999). The interaction between the N-RAP super repeats and the vinculin tail (Luo et al., 1999) may be important for appropriate positioning of the Z-line components and actin filaments that are recruited to the complex in subsequent steps.

Next, Z-line components are recruited to the membrane-associated complex by binding to N-RAP-IB and vinculin

(step 2); this is consistent with N-RAP-IB colocalizing with α-actinin at Z-lines and the cell periphery (Table 2), with a direct interaction between N-RAP and the Z-line component MLP (Ehler et al., 2001), and with α-actinin binding to the head region of vinculin (Kroemker et al., 1994).

Nebulin repeats can nucleate actin polymerization (Chen et al., 1993; Gonsior et al., 1998), and the strong actin-binding activity of the N-RAP super repeats (Luo et al., 1999), along with their homology to nebulin (Luo et al., 1997), suggests that they would have similar effects. Our model includes actin filaments polymerizing along the N-RAP super repeats and integrating with the Z-line components bound to N-RAP-IB (step 3). By analogy with nebulin, the super repeats are expected to assign directionality to the actin filaments; this is because the orientation of nebulin in skeletal muscle sarcomeres places its C terminus at the Z-line-associated barbed end of the actin filament and its N terminus in the center of the sarcomere at the pointed end of the actin filament (Herrera et al., 2000; Wang et al., 1996; Wright et al., 1993). We hypothesize that the interactions between N-RAP super repeats and the vinculin tail (Luo et al., 1999) serve to orient the complex in a way that allows for appropriate positioning of actin filaments polymerizing along the N-RAP super repeats relative to the Z-line components bound to N-RAP-IB and vinculin.

Finally, the N-RAP containing complex of Z-bodies with associated actin filaments is released from the membrane complex (step 4), and N-RAP is removed from the complex as the Z-bodies fuse laterally to form Z-lines (step 5). As we observed N-RAP-SR and N-RAP-IB retained in mature sarcomeres, it is likely that the N-RAP LIM domain targets N-RAP for removal from the nascent myofibrils. Release from the membrane complex may be accompanied by a post-translational modification of the N-RAP LIM domain, which serves as a signal for N-RAP removal.

The model proposed in Fig. 10 provides a framework for understanding the initial recruitment and assembly of myofibrillar components, including a mechanism for controlling the polarity of the actin filaments relative to the Z-line proteins. The initial linear arrangement of newly formed Z-bodies is not addressed, but might be controlled by a periodic arrangement of the membrane proteins, for example, by integrin clustering. Future tests of this model may illuminate the precise mechanism for the initiation of myofibrillogenesis, and link our understanding of the assembly of sarcomeric components to cellular control of the assembly process by signaling mechanisms.

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