Myospryn Is a Direct Transcriptional Target for MEF2A That Encodes a Striated Muscle, α-Actinin-interacting, Costamere-localized Protein*

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The full repertoire of proteins that comprise the striated muscle Z-disc and peripheral structures, such as the costamere, have yet to be discovered. Recent studies suggest that this elaborate protein network, which acts as a structural and signaling center for striated muscle, harbors factors that function as mechanosensors to ensure coordinated contractile activity. Mutations in genes whose products reside in this region often result in skeletal and cardiac myopathies, demonstrating the importance of this macromolecular complex in muscle structure and function. Here, we describe the characterization of a direct, downstream target gene for the MEF2A transcription factor encoding a large, muscle-specific protein that localizes to the costamere in striated muscle. This gene, called myospryn, was identified by microarray analysis as a transcript down-regulated in MEF2A knock-out mice. MEF2A knock-out mice develop cardiac failure during the perinatal period with mutant hearts exhibiting several cardiac abnormalities including myofibrillar disarray. Myospryn is the muscle ortholog of a partial human cDNA of unknown function named cardiomyopathy-associated gene 5 (CMYA5). Myospryn is expressed as a single, large transcript of ~12 kilobases in adult heart and skeletal muscle with an open reading frame of 3739 amino acids. This protein, belonging to the tripartite motif superfamily of proteins, contains a B-box coiled-coil (BBC), two fibronectin type III (FN3) repeats, and SPRY domains and interacts with the sarcomeric Z-disc protein, α-actinin-2. Our findings demonstrate that myospryn functions directly downstream of MEF2A at the costamere in striated muscle potentially playing a role in myofibrillogenesis.

The cytoskeleton of striated muscle is comprised of a complex and highly organized assembly of proteins, which ensures that contraction is appropriately initiated and transmitted in a synchronized fashion while maintaining cellular integrity (1, 2). Alterations in the stoichiometry of cytoskeletal proteins or mutations in any of these components can destabilize this highly structured network often resulting in cardio and/or skeletal myopathies (3–9). Whereas much progress has been made in elucidating the pathways of muscle dysfunction and in identifying essential structural and signaling proteins, a gap remains in our understanding of the true complexity of molecular interactions between known and novel proteins within this cytoarchitectural framework. The discovery of novel muscle-specific cytoskeletal proteins and their specific protein-protein interactions will undoubtedly reveal additional important pathways in the formation and maintenance of the cytoskeleton in muscle cells.

The myocyte enhancer factor-2 (MEF2) family of transcription factors regulates the expression of numerous muscle-specific genes that include components of the elaborate cytoskeletal network in striated muscle cells (10). Consistent with its role in the regulation of muscle-specific gene expression, MEF2 loss-of-function mutations exhibit defects in striated muscle development and function with associated alterations in the expression of genes encoding proteins of the contractile apparatus (11–13). One of the more complex MEF2 knock-out phenotypes is that of MEF2A. MEF2A is one of the four MEF2 genes in vertebrates, MEF2A, -2B, -2C, and -2D expressed in muscle and non-muscle cell lineages and activates genes involved in cell proliferation and differentiation (14). A null allele for MEF2A in mice results in cardiac sudden death with cytoarchitectural defects, mitochondrial deficiency, and conduction disturbances (15). In humans, a dominant negative mutation of the mef2a gene has been linked to cardiovascular disease arising from defects in the coronary arteries (16). Subsequent reports have failed to demonstrate linkage of this mutation to coronary artery disease (17, 18) though a different polymorphism in MEF2A is now implicated in myocardial infarctions (19). Nevertheless, whereas a considerable amount is known regarding the mechanisms by which MEF2 transcription factors are modified to regulate gene expression (14) the downstream pathways that they regulate are not completely understood. Thus, the identification of novel downstream target genes for MEF2A will provide further insight into the cellular pathways regulated by this transcription factor in the cardiovascular system.

To investigate the molecular mechanisms of cardiac abnormalities in mef2a mutant mice and identify novel MEF2A target genes we compared gene expression levels between wild type and mef2a knock-out hearts using DNA microarray (15). This gene expression profiling approach identified numerous dysregulated expressed sequence tags (ESTs) down-regulated in mef2a mutant hearts. One of these mouse ESTs is down-regulated ~2-fold and is homologous to a partial human cDNA in the NCBI data base named cardiomyopathy-associated gene 5 (CMYA5). CMYA5 was discovered by expression profiling of a human

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The abbreviations used are: MEF2, myocyte enhancer factor-2; EST, expressed sequence tag; GST, glutathione S-transferase; ChIP, chromatin immunoprecipitation assay; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; BBC, B-box coiled-coil; CMYA5, cardiomyopathy-associated gene 5; TRIM, tripartite motif; FN, fibronectin; DM, double mutant; TM, triple mutant.
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cardiac muscle library but its function has not been reported. Because the expression of this EST is dependent on MEF2A transcriptional activity we focused our efforts on the cloning and characterization of the mouse ortholog of the CMYA5 gene. While this work was in progress, the mouse ortholog of CMYA5, named Myospryn, was identified as a dysbindin-interacting protein (20). Dysbindin is a ubiquitously expressed, cytoplasmic protein that interacts with α-dystrobrevin, which itself associates with the dystrophin glycoprotein complex (21). Myospryn encodes a large, novel member of the tripartite motif (TRIM) superfamily of proteins (22). We demonstrate that Myospryn is a direct target for MEF2A and is localized specifically to the costamere of striated muscle cells where it interacts with the sarcomeric Z-disc protein, α-actinin-2. The characterization of this MEF2A-dependent gene will contribute to our understanding of the extensive protein interactions within the costamere and Z-disc complex and its potential role in muscle function and disease.

MATERIALS AND METHODS

Microarray Analysis and Bioinformatics—Total RNA was prepared using TRIzol reagent (Invitrogen) from wild type and mef2a mutant hearts and used with Mouse GEM1 array (Incyte Genomics) as previously described (15). The down-regulated EST AA413670 was used to search the NCBI and Celera data bases to assemble the full-length mouse cDNA by PCR from a Marathon skeletal muscle cDNA library (Clontech) and is identical to Myospryn (GenBank™ accession number AJ575748).

Creation of Mef2a Transgenic Mice—The mouse MEF2A cDNA was amplified from a Marathon cardiac muscle cDNA library (Clontech) and cloned into the αMHC-hGH poly(A) vector. The promoter-cDNA cassette was released from the vector backbone by digestion with NotI, gel-purified using QiaQuick columns (Qiagen) and injected into the male pronuclei of fertilized mouse oocytes. Two transgenic positive founders were obtained and both transmitted the transgene to their offspring.

RT-PCR Analysis—Total RNA was isolated from cardiac muscle using the TRIzol method. First strand cDNA was prepared from total RNA with random hexamers using reverse transcriptase. cDNAs were subjected to PCR, fractionated on a 5% native polyacrylamide gel (Clontech) and is identical to Myospryn (GenBank™ accession number AJ575748).

Northern Blot Analysis and in situ Hybridization—A mouse multiple tissue poly(A) + RNA blot (Clontech) was hybridized with a 32P-labeled HindIII fragment (nucleotides 5,391–6,455) of the Myospryn cDNA. An antisense cDNA fragment corresponding to nucleotides 5,391–5,915 was used for radioactive in situ hybridization. Embryos at various developmental time points were fixed according to standard procedures and sagittal mouse sections were hybridized with antisense Myospryn cDNA labeled with 35S-UTP.

Antibodies and Immunocytochemistry—Rabbit polyclonal anti-Myospryn antibodies were raised against GST fusion proteins (Cocalico Biologicals) generated from three different regions of the Myospryn cDNA. Antibodies UT266 (GST fusion-containing nucleotides 4,764–5,391), UT264 (nucleotides 5,391–6,455), and UT262 (nucleotides 9,583–9,868) were IgG-purified using MabTrap II (Amershaw Biosciences). All three antibodies exhibited specificity for Myospryn in transfected cells. However, the UT266 antibody exhibited the highest specificity with heart crossections. For immunostaining of heart crossections, adult mice were perfused with 4% paraformaldehyde, hearts were dissected and cryoprotected by immersion in 30% sucrose/phosphate-buffered saline at 4 °C for 24–48 h. Cryoprotected hearts were then placed in embedding compound (OCT) and frozen at −80 °C. Hearts were cryosectioned at 15 μm and air-dried on Superfrost Plus Slides (Fisher). Primary antibodies for Myospryn (UT266, IgG fraction, dilution 1:100), mouse monoclonal sarcomeric α-actinin (Sigma, 1:100) and secondary antibodies (1:200) for anti-Myospryn (anti-rabbit Texas Red) and anti-actinin (anti-mouse fluorescein isothiocyanate) were used on heart cryosections. After immunostaining procedure, Vectashield (Vector Labs) was applied to heart cryosections and protected with cover slips.

Gel Shift and Chromatin Immunoprecipitation Assays—For gel shift assays, whole cell extracts were isolated from adult mouse hearts by homogenization and incubated in the presence of radiolabeled, double-stranded oligonucleotides corresponding to each of the four MEF2 sites in the myospryn promoter as described previously (15). For ChIP analysis, primary neonatal rat cardiomyocytes were isolated and seeded at a density of 5 × 10⁶ cells in 10-cm dishes. C2C12 myoblasts seeded at a density of 1 × 10⁶ were transfected with 15 μg of FLAG-MEF2A expression vector. Cardiomyocytes and C2C12 myoblasts were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, and penicillin/streptomycin. For reporter assays, COS cells in 6-well plates, each well containing ~500,000 cells, were transfected with 0.2 μg of the wild-type or mutant forms of the myospryn promoter in pGL3-Basic (Promega) and 0.8 μg of human MEF2A in pcDNA 1 (Invitrogen). Cells were transfected using FuGENE 6 transfection reagent (Roche Applied Sciences), and were harvested after 36–48 h. Luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega), and protein concentrations were normalized via Bradford assay. For co-immunoprecipitations, COS cells in 100-mm dishes (at 50% confluency) were transfected with 20 μg of expression plasmids for full-length Myospryn and 5 μg for truncated forms of Myospryn and α-actinin, using FuGENE 6 reagent. Myospryn proteins were fused with an N-terminal Myc epitope, and α-actinin was fused with an N-terminal FLAG epitope. Forty eight hours after transfection, cells were harvested in ELB buffer (50 mM HEPES, pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Igepal, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Complete, Roche Applied Sciences). Extracts were immunoprecipitated for 2 h at 4 °C using protein A/G-agarose and 1 μg of monoclonal anti-Myc antibody (Santa Cruz Biotechnology). Subsequently, the pellet was washed with ELB buffer and subjected to SDS-PAGE followed by transfer to Immobilon polyvinylidene difluoride membrane (Bio-Rad) and immunoblotting using anti-FLAG antibodies (Sigma).

Northern Blot Analysis and in situ Hybridization—A mouse multiple tissue poly(A) + RNA blot (Clontech) was hybridized with a 32P-labeled HindIII fragment (nucleotides 5,391–6,455) of the Myospryn cDNA. An antisense cDNA fragment corresponding to nucleotides 5,391–5,915 was used for radioactive in situ hybridization. Embryos at various developmental time points were fixed according to standard procedures and sagittal mouse sections were hybridized with antisense Myospryn cDNA labeled with 35S-UTP.

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sequences that were found to be identical in both the rat and mouse promoters. 5’ primer sequence: 5’-CCT GGT TTT ATT TTC CTC ATG ATA GG-3’. 3’ primer sequence: 5’-CTG TAG GCC TAC AAG AAG TTT G-3’.

RESULTS

Microarray Analysis and Identification of Myospryn—In our ongoing effort to gain further insight into the cellular pathways regulated by MEF2A in the postnatal heart we have continued to analyze microarray data previously reported for the characterization of the mef2a knock-out phenotype (15). mef2a mutant mice die in the perinatal period and display severe cyto-architectural and mitochondrial abnormalities in cardiac muscle cells. Aside from the known MEF2 target genes dysregulated in mef2a mutant hearts, numerous novel ESTs also exhibited alterations in gene expression levels in these abnormal hearts. One of these ESTs (accession number AA413760) is down-regulated 1.9-fold. While this work was in preparation, the full-length gene product of mouse EST AA413760, named Myospryn, was identified as a dysbindin-interacting protein (20). Dysbindin is a ubiquitously expressed cytosolic protein that interacts with α-dystrobrevin, which itself is a component of the dystrophin glycoprotein complex (21). The down-regulation of Myospryn was subsequently confirmed by semi-quantitative RT-PCR with cDNA from postnatal day 5 (P5) hearts of mef2a mutant mice. Transcripts for Myospryn were expressed at 0.41 ± 0.09 (n = 3) of wild-type levels in P5 hearts of mef2a mutant mice (data not shown).

MEF2A-dependent Activation of Endogenous Myospryn Gene—To further demonstrate that endogenous myospryn gene expression is sensitive to MEF2A dosage, we generated mice that overexpressed the full-length mouse MEF2A cDNA in the heart under the control of the α-myosin heavy chain (αMHC) promoter (Fig. 1A). An observed induction of the myospryn gene would strengthen the notion that it is a relevant target for MEF2A. This complementary approach will be useful in dissecting the MEF2A-dependent transcriptional network in the postnatal heart. 3

Two independent lines of αMHC-MEF2A transgenic mice, MHCA19 and MHCA20, were obtained, and expression of the transgene was confirmed by Northern analysis (data not shown). Western analysis using a MEF2A antibody (C-21, Santa Cruz Biotechnology) demonstrated an approximately 2-fold increase in MEF2A protein over basal levels in these transgenic hearts (Fig. 1B). We noted that this increase in MEF2A expression resulted in a modest enlargement of the heart by three months of age (Fig. 1C) and the induction of atrial natriuretic factor (ANF) a known MEF2 target and hypertrophic marker gene (data not shown). These results are consistent with the observed phenotype in transgenic mice overexpressing human MEF2A in the heart. 4 Additional molecular characterization of this phenotype will be described elsewhere.

To examine transcript levels for endogenous myospryn, RNA was isolated from 2-month-old transgenic hearts, prior to any visible cardiac enlargement and subjected to quantitative real time (qRT)-PCR (Applied Biosystems). Measurement of transcripts for myospryn using qRT-PCR revealed a 2.9 ± 1.0 (n = 3) enhancement in basal expression levels (Fig. 1D) demonstrating that forced expression of MEF2A alone induces this gene in the heart. Other well characterized MEF2 target genes and novel ESTs, previously demonstrated to be down-regulated in mef2a knock-out hearts, were induced in transgenic mice overexpressing MEF2A validating that these genes are downstream of MEF2A and are likely to be direct targets for this transcription factor in the heart. These results demonstrate that MEF2A is sufficient to activate the endogenous myospryn gene and supports the notion that it is a bona fide gene in vivo target for MEF2A in the heart.

Regulation of Myospryn Transcription by MEF2A—To test whether myospryn represents a direct transcriptional target for MEF2A we identified the 5’-end of the gene using a combination of the EST data base, 5’-RACE PCR, primer extension, and RNase protection (data not shown). Based on this information, we isolated 3.5 kb of mouse genomic sequence upstream of the first exon and cloned this fragment into the pGL3BASIC-luciferase reporter. MEF2A-responsiveness of this 5’ region was tested in transfected COS cells. As demonstrated in Fig. 2A, this 3.5-kb fragment alone exhibited background levels of activity in COS cells. However, when co-transfected with a MEF2A expression vector, the 3.5-kb fragment was induced 5.2-fold (Fig. 2A) indicating that the myospryn promoter responds to MEF2A activity.

3 J. Reynolds and F. Naya, manuscript in preparation.
4 J. D. Molkentin, personal communication.
Given the ability of MEF2A to transactivate this upstream fragment we examined this region for MEF2 binding sites and identified four putative MEF2 sequences exhibiting similarity to the consensus MEF2 site, YTA(A/T)₄TAR (Fig. 2B). To determine whether MEF2A could bind these sequences we performed gel mobility shift assays. The MEF2 sequences located at +33 (CTATTTAAAG), −1452 (TTATAATTAG), −1985 (TTATAAATTA), and −2834 (TTATTTTTA) relative to the putative transcription start site bound to in vitro translated MEF2A (Fig. 2C, left panel), and MEF2A-specific binding was confirmed by supershifting the protein-DNA complex with an anti-MEF2A antibody. To validate that endogenous MEF2A from cardiomyocytes could also bind these sequences protein extracts from adult mouse hearts were subjected to gel shift assays. MEF2 DNA binding activity was readily apparent for each of the four MEF2 binding sites and incubation of these extracts with MEF2A antibodies resulted in a slower migrating supershifted complex (Fig. 2C, right panel). These results confirm that MEF2A protein directly binds to all four MEF2 sites within the myospryn promoter.

To determine whether the myospryn promoter was being activated through one or more of the MEF2 sites, mutations that disrupt MEF2 binding were introduced within each of the core sequences in the context of the 3.5-kb proximal promoter. As shown in Fig. 2A, mutations within each of the individual MEF2 binding sites had varying effects on transactivation potential. Mutations of the +33 and the −2834 MEF2 sites had no significant effect and a 20% reduction, respectively, in transcriptional activity directed by MEF2A. In contrast, mutations within the −1452 and −1985 MEF2 sites affected transcriptional activity by 30%.

FIGURE 2. Direct activation of myospryn promoter by MEF2A. A, MEF2A expression vector or pcDNA3 was co-transfected into COS cells with the wild-type myospryn promoter or a series of mutant promoter constructs. The fold activation is relative to the luciferase activity (normalized to 1) of the wild-type or mutant reporter transfected with pcDNA3 vector backbone. DM, −1452/−1985; TM1, +33/−1452/−1985; TM2, −1452/−1985/−2834. Error bars represent S.D. for three experiments. Values obtained for the −1452, −1985, DM, and TM mutants are statistically significant at p < 0.05. B, sequence of the myospryn proximal promoter and additional upstream sequences. The four MEF2 sites and a putative SRF binding site (CArG-like) are underlined. C, MEF2A binds four MEF2 sites present in myospryn promoter. In vitro translated MEF2A or whole cell lysates from adult mouse hearts were subjected to gel shift assays. Protein-DNA complexes were formed with each of the MEF2 sites within the myospryn promoter. MEF2 DNA binding activity was confirmed by incubation with a MEF2A-specific antibody resulting in a slower migrating supershifted complex. D, sequence alignment of rat, mouse, and human myospryn promoter regions. The conserved −1985 MEF2 site is noted in the boxed region. Also shown are the −1452 and −2834 MEF2 sites aligned with their rat and human counterparts. No significant alignment was obtained with the +33 MEF2 site. Upper right panel, the conserved −1985 MEF2 site of the myospryn promoter is located in a region of acetylated chromatin. Lower right panel, FLAG-MEF2A binds the mouse −1985 site in C2C12 myoblasts. ChIP analysis was performed on cross-linked chromatin isolated from neonatal rat primary cardiomyocytes and C2C12 myoblasts (transfected with a FLAG-MEF2A expression vector), and immunoprecipitations were performed using the anti-acetyl-histone H3 antibody and anti-FLAG respectively. A no-antibody immunoprecipitation serves as the control. Immunoprecipitated DNA from each sample, as well as aliquots of non-immunoprecipitated DNA used as input controls for each sample, were subjected to PCR using primers flanking the −1985 MEF2 site.
and 45%, respectively. Given the greater inhibitory effects of the −1452 and −1985 mutations we generated a double mutation (DM) within the 3.5-kb promoter and determined its ability to be transactivated by MEF2A. This double mutation resulted in an additional inhibitory effect (60% reduction relative to wild type) in transcriptional activity. Because the double mutation did not completely eliminate transcriptional response of this promoter to MEF2A, we asked whether mutations in either the +33 or the −2834 MEF2 sites in the context of the DM construct would result in a more pronounced inhibitory effect. Mutations in the +33 and −2834 MEF2 sites were introduced into the DM construct resulting in two different triple mutations, TM1 (+33/−1452/−1985) and TM2 (−1452/−1985/−2834). Each of these triple mutant promoter constructs were tested for their ability to respond to MEF2A. Both TM1 and TM2 mutations did not result in a further reduction in MEF2A responsiveness. To rule out the possibility that this residual transcriptional activity was cell type-dependent, we tested these mutant constructs in both 10T1/2 cells and C2C12 myoblasts. Similar results were obtained when the mutant constructs were tested in these additional cell lines (data not shown). These results demonstrate that the myospryn promoter is associated with acetylated histones and, by extension, transcriptionally active in primary rat cardiomyocytes.

To determine whether any of the four MEF2 sites are within a transcriptionally active region in vivo we performed ChIP analysis on the myospryn promoter. Nucleosomes were isolated from primary neonatal rat cardiomyocytes and subjected to immunoprecipitation using anti-acetyl H3 histone antibodies (Upstate Biotechnology). We identified the rat myospryn promoter in silico and designed primers that would amplify rat genomic sequences surrounding the −1985 MEF2 site, because it is the only site in the promoter that is evolutionarily conserved in mice, rats, and humans (Fig. 2D, left panel). As shown in Fig. 2D (top, right panel), we were able to demonstrate that the −1985 MEF2 site in the rat myospryn promoter is associated with acetylated histones and, by extension, transcriptionally active in primary rat cardiomyocytes. To show that MEF2A directly binds to this site we transfected C2C12 myoblasts with a FLAG-MEF2A expression construct and subjected these cells to a ChIP assay. We successfully immunoprecipitated MEF2A bound to the −1985 site using an anti-FLAG antibody (Fig. 2D, lower, right panel) but not with an anti-MEF2A antibody. Similarly, we were unable to immunoprecipitate a MEF2A-chromatin complex in cardiomyocytes. The above results indicate that the anti-MEF2A antibody is unable to immunoprecipitate MEF2A bound to chromatin under these experimental conditions. Collectively, these results clearly demonstrate that myospryn is a direct transcriptional target for MEF2A.

Identification of Rodent and Human Myospryn Loci—Using a combination of public (NCBI and Ensembl) and private (Celera) data bases we identified the entire mouse, human, and rat genomic loci for myospryn. Human and mouse myospryn localize to chromosome 5q12-q13 and chromosome 13, respectively, whereas the rat gene localizes to chromosome 2. Moreover, we have identified a partial cDNA for the apparent zebrafish ortholog of myospryn (GenBank™ accession number XM_684008). The rodent and human myospryn genes span a region covering more than 100 kb, exhibit a similar exon-intron organization, and have a total of thirteen exons (Fig. 3, A and B). The myospryn genes have an unusually large second exon, over 9,400 base pairs in length, which is located more than 35 kb downstream of the first coding exon. The remaining eleven exons, distributed over 50 kb, encode the C-terminal 524 amino acids containing the TRIM region (20). Although the human ortholog has been named a “cardiomyopathy associated” gene a search in the Online Mendelian Inheritance in Man (OMIM) data base does not map the 5q12-q13 chromosomal locus of human myospryn to a susceptibility region for cardiovascular disease.

Developmental and Muscle-Specific Expression of Myospryn—Northern analysis of mouse tissues, using a probe derived from the large second exon of the mouse Myospryn cDNA, revealed a transcript migrating above the 9.5-kb RNA marker and highly restricted to adult heart and skeletal muscle (Fig. 4A, top). Because this Northern blot did not have a representative smooth muscle tissue we performed RT-PCR on RNA from adult mouse aorta and stomach. We failed to detect expression of myospryn in these tissues (Fig. 4A, bottom, lanes 2 and 3). We
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FIGURE 4. Expression analysis of myospryn mRNA from adult mouse tissues. A, mouse multiple tissue Northern blot (Clontech) was probed with a 1.0-kb HindIII fragment (corresponding to nucleotides 5,391–6,455) of Myospryn cDNA sequence. A single myospryn transcript, greater than the 9.4-kb RNA standard, was detected exclusively in heart and skeletal muscle. Lower panel, RT-PCR on selected mouse tissues; heart (lane 1), stomach (lane 2), and aorta (lane 3). Asterisk indicates amplification of myospryn cDNA. B, developmental expression of myospryn transcripts. Myospryn transcripts were detected by radioactive in situ hybridization of mouse embryo sagittal sections at various developmental time points. Sagittal sections at E10.5, 12.5, and 15.5 reveal striated muscle-specific expression at all time points. A frontal section of the heart at E17.5 revealed myospryn transcripts only in myocardial cells throughout the atria and ventricles.

also examined embryonic expression of myospryn transcripts by radioactive in situ hybridization on mouse embryo sections at various time points during development. At embryonic day 10.5 (E10.5) strong expression was observed in the atria and ventricles of the heart (Fig. 4B, left panel). The signal was localized predominantly to the trabecular zone but not the compact zone of the ventricles. Weaker expression was detected in the developing somites at this time point. At E12.5 the intense hybridization signal was maintained in the heart with stronger expression in the atria compared with the ventricles (not shown). At E15.5, myospryn continued to be expressed in the heart with lower levels in the atria compared with the ventricles. In contrast, skeletal muscle displayed high levels of myospryn transcripts (Fig. 4B, middle panel). A sagittal section of an isolated heart from an E17.5 mouse embryo showed abundant expression of myospryn in myocardial cells with a more robust signal in the trabecular region (Fig. 4B, right panel). Myospryn expression in the cardiac valves was completely absent at this time point.

Myospryn expression was also examined in the C2C12 myoblast cell line and the transformed cardiac cell line, NkL-Tag (23) using RT-PCR. We failed to detect myospryn expression in proliferating C2 myoblasts or in differentiated C2 myotubes (data not shown). Similarly, we failed to detect myospryn expression in the NkL-Tag cardiac cell line (data not shown).

Subcellular Localization of Myospryn—To determine the subcellular localization of Myospryn in cardiac muscle cells, antibodies were raised against GST fusion proteins from three different regions of Myospryn (see “Materials and Methods”). We initially tested the specificity of the antibodies by immunostaining in tissue culture cells. COS cells were transfected with expression constructs for Myospryn (amino acids 73–3739) and FLAG-actinin-2 (corresponding to nucleotides 5,391–6,455) of Myospryn cDNA sequence. A single myospryn transcript, greater than the 9.4-kb RNA standard, was detected exclusively in heart and skeletal muscle. Lower panel, RT-PCR on selected mouse tissues; heart (lane 1), stomach (lane 2), and aorta (lane 3). Asterisk indicates amplification of myospryn cDNA. B, developmental expression of myospryn transcripts. Myospryn transcripts were detected by radioactive in situ hybridization of mouse embryo sagittal sections at various developmental time points. Sagittal sections at E10.5, 12.5, and 15.5 reveal striated muscle-specific expression at all time points. A frontal section of the heart at E17.5 revealed myospryn transcripts only in myocardial cells throughout the atria and ventricles.

These results indicate that the anti-Myospryn antibodies are specific for Myospryn and that Myospryn is a cytoplasmic protein.

We used these antibodies to determine the subcellular localization of Myospryn in cardiac muscle cells. We performed double label immunocytochemistry on longitudinal cryosections from adult mouse hearts using the anti-Myospryn antibody, UT 266, and anti-α-actinin-2. As shown in Fig. 5A, anti-sarcomeric α-actinin-2 (left panel; green) exhibits a periodic, Z-line staining pattern as expected. Anti-Myospryn (middle panel; red) displays similar periodic striations in these myofibers. When these images are superimposed (right panel) the periodic striations are now yellow indicative of co-localization of both proteins to the Z-disc. Immunohistochemistry was also performed on transverse sections of adult skeletal muscle, which revealed staining along the periphery of each myofiber (Fig. 5C) indicating a sarcolemmal (i.e. plasma membrane) distribution of Myospryn consistent with the previously published report (20). Taken together, these staining patterns indicate that Myospryn is localized precisely at the periphery of the Z-disc in a region known as the costamere, an elaborate protein network linking the sarcolemma to the Z-disc (24).

Myospryn Interacts Directly with the Sarcomeric Z-disc Protein α-Actinin—Based on the above co-localization studies we sought to demonstrate a direct interaction between Myospryn and α-actinin-2. For these studies COS cells were transfected with an N-terminal Myc-myospryn (amino acids 73–3739) and FLAG-α-actinin-2. These extracts were immunoprecipitated with anti-Myc antibody and subsequently used in Western blot analysis with anti-FLAG antibodies. As shown in Fig. 6A, a band corresponding to α-actinin-2 could be immunoprecipitated from extracts containing Myospryn (amino acids 733–739) but not in those extracts transfected with pCDNA3 vector. We then sought to delineate the minimal region in myospryn that interacts with α-actinin. Initially, we generated an epitope-tagged fragment expressing the C-terminal 773 amino acids of Myospryn (amino acids

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Myospryn Is a Costameric MEF2A Target Gene

Several dozen muscle-specific genes are known to have essential MEF2 binding sites yet only one of these, the intermediate filament protein desmin, is targeted, but not restricted, to the periphery of the Z-disc/macromolecular complex. The expression of desmin is controlled transcriptionally by a single, functional MEF2 site in its promoter region. MEF2 is also activated in response to elevated MEF2A levels.

Microarray analysis was used to identify candidate MEF2A-dependent genes using RNA from hearts of wild type and mef2a−/− mice. One of these down-regulated genes is myospryn (20) whose transcripts are also activated in response to elevated MEF2A levels. Myospryn is the mouse ortholog of a human cardiomyopathy-associated gene, CMYA5, whose function is not known. Myospryn is expressed exclusively in cardiac and skeletal muscle lineages during mouse embryogenesis and in adult striated muscle. In addition, Myospryn localizes to the costamere at the periphery of the Z-disc complex and interacts with sarcomic α-actinin-2. Because of its large size Myospryn has the potential to interact with additional sarcomeric and/or cytoskeletal proteins to regulate the assembly and biomechanical function of the Z-disc/costameric macromolecular complex. Furthermore, the localization of this MEF2A target gene product to this region suggests that MEF2A may regulate additional genes required for the formation and function of this specialized structure in muscle cells.

Responsiveness of Myospryn Promoter to MEF2A—Our studies have established for the first time that myospryn is a direct transcriptional target for MEF2A in the heart. The myospryn promoter contains four MEF2 cis-elements within 3.5 kb of the transcription start site. Mutation analysis of each of the four MEF2 cis-elements, individually and in various combinations, has revealed that the −1452 and −1985 MEF2 binding sites are the functionally important sites for full activation of the myospryn promoter. Moreover, the −1985 MEF2 site is the only site that is evolutionarily conserved in rodents and humans and it is also the site that has the most dramatic effect on transcriptional activity. Surprisingly, a myospryn promoter construct with two different triple mutations, TM1 and TM2, as described in Fig. 2A fails to completely abolish MEF2A responsiveness. These results suggest that MEF2A collaborates with other transcription factors present in COS cells, perhaps in a non-DNA binding manner, to activate the mutant promoter. It is well known that MEF2 factors can activate transcription, in a non-DNA binding manner, to activate the mutant promoter. Regardless of the mechanism, our results clearly demonstrate that the myospryn gene is a direct target for the MEF2A transcription factor and harbors functional MEF2 binding sites in its promoter.

Myospryn Is a Costamere-restricted and α-Actinin-binding Protein—Several dozen muscle-specific genes are known to have essential MEF2 binding sites yet only one of these, the intermediate filament protein desmin, is targeted, but not restricted, to the periphery of the Z-disc/macromolecular complex. The expression of desmin is controlled transcriptionally by a single, functional MEF2 site in its promoter region (27). Myospryn now represents the first MEF2 target whose gene product is restricted exclusively to the costamere in striated muscle.

Recent studies have shed light on the importance of the region consisting of the costamere and Z-disc in sensing stretch in striated muscle cells. The Z-disc and peripheral structures serve as a scaffold for numerous α-actinin-associated proteins and harbor proteins that act as bio-

DISCUSSION

Microarray analysis was used to identify candidate MEF2A-dependent genes using RNA from hearts of wild type and mef2a−/− mice. One of these down-regulated genes is myospryn (20) whose transcripts are also activated in response to elevated MEF2A levels. Myospryn is the mouse ortholog of a human cardiomyopathy-associated gene, CMYA5, whose function is not known. Myospryn is expressed exclusively in cardiac and skeletal muscle lineages during mouse embryogenesis and in adult striated muscle. In addition, Myospryn localizes to the costamere at the periphery of the Z-disc complex and interacts with sarco-

2966–3739), which includes the TRIM region because a previous study showed that the BBC motif of the TRIM/RBCC protein, BERP, was sufficient to interact with α-actinin-4, a non-muscle actinin isofom (25). This C-terminal Myospryn construct interacted efficiently with α-actinin-2 when immunoprecipitated with anti-Myc antibody (Fig. 6A). To demonstrate that the interaction between Myospryn and α-actinin is specific and that other proteins from the TRIM/RBCC superfamily do not interact with α-actinin COS cells were co-transfected with α-actinin and MURF, a muscle-specific RBCC protein (26). We failed to detect interaction between MURF and α-actinin (data not shown).

To map the interaction interface on Myospryn more precisely, several 5′ and 3′ deletions were generated from the aforementioned C-terminal expression construct and tested by co-immunoprecipitation (co-IP). The results from these co-IP experiments are summarized in Fig. 6B. These results show that removal of the SPRY (Δ1), the SPRY and FN3 repeats (Δ2), or the entire TRIM region (Δ3) does not abolish interaction with α-actinin (Fig. 6B). The ability of the Δ3 construct (amino acids 2966–3214) to interact with α-actinin was unexpected since it lacks the entire TRIM region. Surprisingly, a construct expressing the TRIM region (amino acids 3215–3739) but lacking those amino acids present in the Δ3 construct also interacted with α-actinin (Fig. 6B). Furthermore, constructs expressing each of the individual motifs that constitute the TRIM region interacted with α-actinin-2 demonstrating that any one of these motifs on their own is sufficient for interaction with α-actinin.

Regions upstream of the TRIM region were also tested for their ability to interact with α-actinin. As summarized in Fig. 6B, those constructs spanning amino acids 73–2072 all failed to interact with α-actinin whereas those N-terminal constructs extending beyond amino acid 2072 and two additional constructs spanning amino acids 2072–2966
mechanical sensors to monitor muscle contraction. Particular attention has been given to two Z-disc-restricted proteins, the cysteine-rich muscle LIM protein (MLP) and the titin binding protein telethonin/T-Cap, and their potential role in monitoring muscle stretch (9). Mutations in either of these genes results in the inability of cardiac muscle cells to sense muscle length and respond appropriately to stretch. Additional Z-disc proteins have been identified but their role in sensing muscle stretch or strain has not been elucidated. The complexity of mechanotransduction in striated muscle suggests that the stretch sensor will likely involve elaborate molecular interactions between Z-disc, peripheral structures, such as the costamere, and signaling molecules. Because Myospryn is a large protein, much of which remains to be characterized, it is tempting to speculate that Myospryn functions as a docking platform for additional structural proteins and signaling molecules within this complex. As such, it will be of considerable interest to determine whether Myospryn is an integral component of the stretch sensor machinery in striated muscle cells.

Our results further demonstrate that Myospryn interacts directly with sarcomeric α-actinin. Myospryn interacts with α-actinin within a broad region spanning the entire C-terminal half of the protein. Subfragments within this 1700 amino acid region, including each of the three individual motifs, which comprise the TRIM region, are all capable of interacting independently with α-actinin. This is not entirely unexpected since the BBC and FN3 domains are known to mediate protein-protein interactions and the SPRY domain has been postulated to also act as a protein-protein interaction interface (28, 29). The surprising finding is that multiple regions of Myospryn are capable of interacting with α-actinin. It is not clear why α-actinin would require multiple interactions with Myospryn at several different regions within the protein. However, this may be explained by the modular nature of α-actinin and its ability to form numerous interactions with a diverse group of proteins (1). The importance of each interaction interface within Myospryn and how each affects α-actinin and/or Myospryn subcellular localization and more generally myofibrillar organization remains to be determined.

**BBC, FN3, and SPRY Domains of Myospryn**—The BBC, FN3, and SPRY domains are all situated in the last 524 amino acids of Myospryn. Surprisingly, these are the only identifiable motifs in the entire Myospryn open reading frame using the current protein domain prediction programs. TRIM proteins are involved in diverse cellular functions such as patterning, differentiation, and in some cases transcriptional regulation (30). Several subgroups exist within the TRIM superfamily based on different combinations of the various motifs (22, 30). The striated muscle restricted, RING finger protein, MURF belongs to a subclass of the TRIM/RBCC family (26). MURF is required for myogenic differentia-
tion and is localized to the microtubular network in muscle cells. The TRIM domains of Myospryn are less similar to MURF than to Mid 1 suggesting that Myospryn and MURF are distantly related and are likely performing different functions in striated muscle. In that regard, while TRIM/RBCC proteins are capable of homo- and hetero-oligomerization, we have been unable to demonstrate hetero-oligomerization between Myospryn and MURF (data not shown).

Potential Function of Myospryn—While this work was in progress, Myospryn was identified as a dysbindin-interacting protein (20). Dysbindin forms protein-protein interactions with α-dystrobrevin which itself associates directly with the dystrophin protein complex (21). The authors of this previous study concluded that Myospryn is localized to the sarcosomal region of skeletal muscle but is not a component in those fractions enriched for the myofibrillar apparatus. In agreement with those results, we readily detect Myospryn immunoreactivity at the periphery of skeletal muscle myofibers. Interestingly, we demonstrate in longitudinal sections of cardiac muscle cells that Myospryn exhibits a striated pattern reminiscent of Z-disc localization and co-localization with sarcomeric α-actinin. Taken together, these data indicate that Myospryn is a costameric protein. The direct interaction between Myospryn and sarcomeric α-actinin is not entirely surprising since α-actinin also resides within the costamere. Furthermore, costameric proteins have been shown to interact with proteins embedded within the Z-disc complex and sarcollama (24).

The observation that myospryn was identified by expression profiling for genes dysregulated in several models of muscle physiology and pathology (Ref. 20 and references therein) including mef2a knock-out hearts suggests that it functions in a regulatory pathway required for the structural assembly and integrity of the cytoskeleton in striated muscle. This raises the possibility that perturbations in the expression and/or function of Myospryn may be a contributing factor in the pathogenesis of muscle disease. Future studies investigating the in vivo function of Myospryn will provide insights into the role of this protein in cardiomyocyte and skeletal muscle biology and contribute to an understanding of the intracellular pathways regulated by MEF2A in muscle function and disease.

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