Myomaxin Is a Novel Transcriptional Target of MEF2A That Encodes a Xin-related α-Actinin-interacting Protein*

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The physiological targets regulated by MEF2 in striated muscle are not completely known. Several recent studies have identified novel downstream target genes and shed light on the global transcriptional network regulated by MEF2 in muscle. In our continuing effort to identify novel, downstream pathways controlled by MEF2, we have used mef2a knock-out mice to find those genes dependent on MEF2A transcriptional activity. 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 Z-disc/costameric region in striated muscle. This gene, called myomaxin, was identified as a gene markedly down-regulated in MEF2A knock-out hearts. Myomaxin is the mouse ortholog of a partial human cDNA of unknown function named cardiomyopathy-associated gene 3 (CMYA3). Myomaxin is expressed as a single, large transcript of ~11 kilobases in adult heart and skeletal muscle with an open reading frame of 3,283 amino acids. The protein encoded by the myomaxin gene is related to the actin-binding protein Xin and interacts with the sarcomeric Z-disc protein, α-actinin-2. Our findings demonstrate that Myomaxin functions directly downstream of MEF2A at the peripheral Z-disc complex in striated muscle potentially playing a role in regulating cytoarchitectural integrity.

The mechanisms by which MEF2 (myocyte enhancer factor-2) transcriptional activity is modulated, via specific signal transduction pathways and protein-protein interactions, to regulate gene expression has been extensively characterized (1–3). These findings have led to a better understanding of the involvement of MEF2 in a variety of developmental, physiological, and pathological pathways in muscle (4–6). Despite this extensive information regarding MEF2 function a gap remains in our understanding of the downstream cellular processes regulated by MEF2 in muscle.

The number of MEF2-regulated genes has been increasing, since recent reports have described novel, downstream target genes responsive to MEF2 activity, such as CHAMP (7), HRC (8), mef2c (9, 10), BOP (11), and Srpk3 (12). An elegant comprehensive genomic profiling analysis in combination with chromatin immunoprecipitation (ChIP2-on-Chip) has revealed a broad array of direct target genes for MEF2 during muscle differentiation (13). Likewise, DNA microarray approaches have identified MEF2-regulated genes that function in signal transduction pathways (14) and ion channel regulation (15) in skeletal muscle and heart, respectively, further highlighting the diversity of genes controlled by this transcription factor.

We recently reported that the myospryn gene is directly regulated by MEF2A (16). myospryn encodes an α-actinin- and dysbindin-interacting protein localized to the peripheral Z-disc region of striated muscle known as the costamere (16, 17). Myospryn is the mouse ortholog of CMYA5, a cardiomyopathy-associated gene of unknown function, whose transcripts are down-regulated in mef2a null mice (16, 18). Given the intriguing finding of a “cardiomyopathy-associated” gene dysregulated in mef2a null hearts, which have severe cardiac abnormalities (18), we set out to determine whether additional cardiomyopathy-associated (CMYA) genes existed and, if so, whether these are also sensitive to MEF2A dosage.

Using an *in silico* approach, we identified four such additional cardiomyopathy-associated genes named CMYAI, CMYA2, CMYA3, and CMYA4 in the NCBI data base. These additional CMYA genes are partial human sequences identified by genome scale expression profiling of cardiac muscle (Incyte Genomics) and deposited in the public data base. Interestingly, the full-length mouse orthologs have been reported for human CMYA1, -2, and -4, but those studies were published either prior to the identification of the CMYA genes or failed to point out the similarity to the CMYA genes. The mouse ortholog for CMYA1, -2, and -4, is Xin (19), Myomegalin (20), and Unc-45 (21), respectively. Xin is an actin-binding protein required for cardiac morphogenesis in the developing chick heart (19, 22). Myomegalin is a Golgi/centrosomal and sarcomere-localized protein that binds phosphodiesterase 4D3 (20). Unc-45 functions as a molecular chaperone for myosin and is modified by E3/E4-multisubunitlyation (21, 23). All of the mouse orthologs for the CMYA genes are expressed predominantly or exclusively in striated muscle.

A partial human cDNA for CMYA3 has been described and tentatively named Xirp2 (22). This particular sequence encodes...
a small portion of Xirp2 that binds F-actin through a novel 16-amino acid repeat motif named the “Xin repeat” because of its similarity to a repetitive motif present in the muscle protein Xin (19, 22). A recent paper has also reported the up-regulation of CMYA3 transcripts in hearts from mice exposed to angiotensin II, a known inducer of cardiac hypertrophy (24). In this paper, we describe the cloning and characterization of the full-length mouse ortholog of CMYA3/Xirp2, which we have named Myomaxin (myogenic MEF2-activated Xin-related protein). We show that myomaxin is dramatically down-regulated in mef2a knock-out hearts and that it is a direct MEF2 target gene. Furthermore, Myomaxin is localized at the periphery of the Z-disc in striated muscle, where it interacts with sarcomeric α-actinin in two separate subdomains within the actin-binding 16-amino acid repeat region. These findings indicate that Myomaxin may play a role in the regulation of muscle cytoarchitecture downstream of MEF2A.

**Materials and Methods**

*In Silico Analysis and Bioinformatics*—Given the similarity of *myospryn* to CMYA5 (16), we searched the NCBI data base for additional genes containing the name “cardiomyopathy-associated.” The mouse CMYA3 sequence was identified as one of four such genes whose sequences were deposited by Incyte Genomics. Continued searching of the database failed to identify additional CMYA genes, indicating that only five novel gene sequences were deposited by Incyte Genomics.

**Northern Blot Analysis and RT-PCR**—A mouse multiple-tissue poly(A)+ RNA blot (Clontech) was hybridized with a 32P-labeled Ncol-EcoRI fragment (nucleotides 8,219–9,051) of the Myomaxin cDNA. For RT-PCR, RNA was extracted from whole hearts using Trizol reagent (Invitrogen), according to the manufacturer’s instructions, and cDNA was prepared from total RNA using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega). Primers for amplification of the CMYA genes were as follows: CMA41 (Xin), 5′-CCAGGTA-GTCTCTAACAACTAGAT-3′ and 5′-TTGTAGCTTCAAGGA-TCCTCTAGG-3′; CMA2 (Myomegalin), 5′-GAGGAGCCG- CATTGACACAGATGTA-3′ and 5′-GTCGGTCTTCTTCT- GAGACAGCTTGCT-3′; CMA3 (Myomaxin), 5′-CCCGGCC- AAAGTTGAGAAGTTG-3′ and 5′-ACTGCCACCTCCTCC- TTTGGATTTC-3′; CMA4 (Unc45), 5′-CCTGCGGTGC- TCCAGAGGACC-3′ and 5′-CTTGAACCAAGGGCCTC- CCTTCA-3′. For RT-PCR analysis of myomaxin expression during C2C12 differentiation, C2C12s were seeded at a density of 1.5 × 10⁵ cells in 10-cm dishes and allowed to grow to confluence (~3 days) in normal growth medium (DMEM containing 10% FBS, 2 mM l-glutamine, and 1% penicillin/streptomycin). The medium was then replaced with differentiation medium (DMEM containing 2% horse serum, 2 mM l-glutamine, and 1% penicillin/streptomycin), and cells were maintained in differentiation medium for up to 7 days. Cells were harvested at days 0, 1, 3, 5, and 7 of differentiation in Trizol reagent (Invitrogen). RNA was extracted according to the manufacturer’s protocol, and cDNA was generated as described above. For analysis of myomaxin expression in primary neonatal rat cardiomyocytes, primary cardiomyocytes were isolated by collagenase digestion, as described previously (25). Cells were seeded in 60-mm dishes at a density of ~1 × 10⁶ cells and maintained for 24 h in DMEM containing 10% FBS, 2 mM l-glutamine, and 1% penicillin/streptomycin. After 24 h, the medium was replaced with DMEM containing one of the following treatments: 0.5× Nutridoma SP (Roche Applied Science), 0.5× Nutridoma SP plus 100 μM phenylephrine (Sigma), or 10% FBS. Cardiomyocytes were then incubated for 72 h, at which point cells were harvested, and RNA was extracted as described above. Primers used for amplification of myomaxin were the same as above. Primer sequences were as follows: atrial natriuretic factor (mouse and rat), 5′-ACCTGCTAGCCATGGAGAGGAGG-3′ and 5′-CTTGGA- TGGTTATCTTGGATCCCGG-3′; BNP (rat), 5′-ATCTCGAG- AAGTGCTGCCCGACTGATGA-3′ and 5′-GCCAGGGAGTT- TCATTCAAAACCACTCAG-3′; MCK (mouse), 5′-GATGTC- CATCCAGACTGGTTGGAACCC-3′ and 5′-TGAATCTG- GCGGCAGGGTGGTAG-3′; glyceraldehyde-3-phosphate dehydrogenase (mouse and rat), 5′-GCCATACAGCCCC- TTCATTG-3′ and 5′-ACTCCACGACATCTCAAG ACC-3′; α-Myosin heavy chain (mouse), 5′-CTCGTGAGAGGATTATT- CCTCG-3′ and 5′-GGAGAGGTGAGCAGGGCATCAAGG-3′.

**Transient Transfections, Chromatin Immunoprecipitations, and Western Blots**—COS-7 cells were maintained in DMEM containing 10% FBS, 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 myomaxin promoter in pGL3-Basic (Promega) and 0.2 μg of human MEF2A in pcDNA1 (Invitrogen). Cells were transfected using FuGENE 6 transfection reagent (Roche Applied Science) and were harvested after 36–48 h. Luciferase assays for the myomaxin promoter in C2C12 cells were performed in the same manner as for COS cells with the exception that 6-well plates were seeded at a density of 300,000 cells/well. Luciferase assays were performed using the Dual Luciferase reporter assay system (Promega), and protein concentrations were normalized by Bradford assay. For co-immunoprecipitations, COS cells in 100-mm dishes (at 25% confluence) were transfected with 20 μg of expression plasmids for full-length Myomaxin and 5 μg for truncated forms of Myomaxin and α-actinin, using FuGENE 6 reagent (Roche Applied Science). Myomaxin proteins were fused with an amino-terminial Myc epitope, and α-actinin was fused with an amino-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 Science)). Extracts were immunoprecipitated for 2 h at 4°C using protein A/G-agarose and 1 μg (or 1 μl) of monoclonal anti-Myc antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Subsequently, the pellet was washed with ELB buffer and subjected to SDS-PAGE, followed by transfer to Immobilon polyvinylidene difluoride membrane and immunoblotting using anti-FLAG antibodies (Sigma).

**Gel Shift and Chromatin Immunoprecipitation Assays**—For gel shift assays, in vitro translated MEF2A was produced in rabbit reticulocyte lysates (Promega) and incubated in the presence of radiolabeled, double-stranded oligonucleotides corresponding to each of the three MEF2 sites in the myomaxin promoter as described previously (16). For ChIP analysis,
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C2C12 myoblasts were seeded at a density of $1.5 \times 10^6$ in 10-cm dishes induced to differentiate. On day 7, cells were cross-linked with 37% formaldehyde, incubated for 10 min at room temperature, and subsequently harvested in cold PBS containing protease inhibitors (Roche Applied Science). ChIP assays were carried out according to the manufacturers’ suggestions (Upstate Biotechnology) with the following modifications. Cells harvested from one 10-cm dish were sonicated in 800 μl of SDS lysis buffer containing protease inhibitors. Each 800-μl sample was subsequently split into 200-μl samples and diluted 10-fold in ChIP dilution buffer containing protease inhibitors. Immunoprecipitations were performed using 4 μg of anti-MEF2A antibody (C-21; Santa Cruz Biotechnology). The rat, mouse, and human myomaxin promoter sequences were aligned using the MatInspector program (available on the World Wide Web at www.genomatix.de). Primers used for amplification of the −75 MEF2 site were as follows: 5′-GGTGG-CAACCTGGAAGGGAGTTTGAGTG-3′ and 5′-CCAGCTCCACCGAAGAAGTCTATGAG-3′.

Antibodies and Immunocytochemistry—Rabbit polyclonal anti-Myomaxin antibodies were raised against GST-Myomaxin fusion protein. Antibody BU2 (GST fusion containing nucleotides 1,699–1,999) was IgG-purified using MabTrap II (Amer sham Biosciences) and exhibited the highest specificity with heart and skeletal muscle cryosections. For Western analysis, tissues were extracted in ELB buffer and fractionated in a 6% SDS-PAGE with subsequent immunoblotting using anti-Myomaxin at a dilution of 1:500. For immunostaining, adult mice were perfused with 4% paraformaldehyde, hearts were dissected and cryoprotected by immersion in 30% sucrose at 4 °C for 24–48 h. Cryoprotected hearts were then placed in embedding Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek) and frozen at −80 °C. Hearts were cryosectioned at 15 μm and air-dried on Superfrost Plus Slides (Fisher). Primary antibodies for Myomaxin (BU2, IgG fraction, dilution 1:100), mouse monoclonal sarcomeric α-actinin (1:100), and secondary antibodies (1:200) for anti-Myomaxin (anti-rabbit Texas Red) and anti-actinin (anti-mouse fluorescein isothiocyanate) were used on heart cryosections. For blocking experiments, GST fusion proteins were constructed for Myomaxin (nucleotides 1,699–1,999) and Myospryn (nucleotides 9,600–9,900), were used on heart cryosections. For blocking experiments, GST fusion proteins were constructed for Myomaxin (nucleotides 1,699–1,999) and Myospryn (nucleotides 9,600–9,900), were used on heart cryosections. For Western analysis, tissues were extracted in ELB buffer and fractionated in a 6% SDS-PAGE with subsequent immunoblotting using anti-Myomaxin at a dilution of 1:500. For immunostaining, adult mice were perfused with 4% paraformaldehyde, hearts were dissected and cryoprotected by immersion in 30% sucrose at 4 °C for 24–48 h. Cryoprotected hearts were then placed in embedding Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek) and frozen at −80 °C. Hearts were cryosectioned at 15 μm and air-dried on Superfrost Plus Slides (Fisher). Primary antibodies for Myomaxin (BU2, IgG fraction, dilution 1:100), mouse monoclonal sarcomeric α-actinin (1:100), and secondary antibodies (1:200) for anti-Myomaxin (anti-rabbit Texas Red) and anti-actinin (anti-mouse fluorescein isothiocyanate) were used on heart cryosections. For blocking experiments, GST fusion proteins were constructed for Myomaxin (nucleotides 1,699–1,999) and Myospryn (nucleotides 9,600–9,900), were used on heart cryosections. For Western analysis, tissues were extracted in ELB buffer and fractionated in a 6% SDS-PAGE with subsequent immunoblotting using anti-Myomaxin at a dilution of 1:500. For immunostaining, adult mice were perfused with 4% paraformaldehyde, hearts were dissected and cryoprotected by immersion in 30% sucrose at 4 °C for 24–48 h. Cryoprotected hearts were then placed in embedding Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek) and frozen at −80 °C. Hearts were cryosectioned at 15 μm and air-dried on Superfrost Plus Slides (Fisher). Primary antibodies for Myomaxin (BU2, IgG fraction, dilution 1:100), mouse monoclonal sarcomeric α-actinin (1:100), and secondary antibodies (1:200) for anti-Myomaxin (anti-rabbit Texas Red) and anti-actinin (anti-mouse fluorescein isothiocyanate) were used on heart cryosections. For blocking experiments, GST fusion proteins were constructed for Myomaxin (nucleotides 1,699–1,999) and Myospryn (nucleotides 9,600–9,900), were used on heart cryosections. For Western analysis, tissues were extracted in ELB buffer and fractionated in a 6% SDS-PAGE with subsequent immunoblotting using anti-Myomaxin at a dilution of 1:500. For immunostaining, adult mice were perfused with 4% paraformaldehyde, hearts were dissected and cryoprotected by immersion in 30% sucrose at 4 °C for 24–48 h. Cryoprotected hearts were then placed in embedding Optimal Cutting Temperature (O.C.T.) compound (Tissue-Tek) and frozen at −80 °C. Hearts were cryosectioned at 15 μm and air-dried on Superfrost Plus Slides (Fisher). Primary antibodies for Myomaxin (BU2, IgG fraction, dilution 1:100), mouse monoclonal sarcomeric α-actinin (1:100), and secondary antibodies (1:200) for anti-Myomaxin (anti-rabbit Texas Red) and anti-actinin (anti-mouse fluorescein isothiocyanate) were used on heart cryosections. For blocking experiments, GST fusion proteins were constructed for Myomaxin (nucleotides 1,699–1,999) and Myospryn (nucleotides 9,600–9,900), expressed, and purified using glutathione-Sepharose columns (Amer sham Biosciences). After the immunostaining procedure, Vectashield (Vector Labs) was applied to heart cryosections and protected with coverslips.

RESULTS

In Silico Analysis and Identification of Myomaxin—Given the surprising finding that the MEF2-regulated myospryn gene is the mouse ortholog of human CMYA5 (cardiomyopathy-associated gene 5) we used an in silico approach to identify additional “cardiomyopathy-associated” genes and to test whether these genes are also dysregulated in mef2a knock-out hearts. By searching the NCBI nucleotide data base using the key words “cardiomyopathy-associated.” A total of four such additional genes were identified: CMYA1, -2, -3, and -4. By, expression levels of the CMYA genes in mef2a knock-out hearts. Total RNA was isolated from postnatal day 5 (P5) hearts of wild-type, heterozygote, and mef2a mutant mice, and transcripts were amplified by semi-quantitative RT-PCR. RT-PCR reactions were performed for 25 cycles and were fractionated on a 1% agarose gel. Similar amplification levels for each of the CMYA genes tested were observed between wild type and heterozygote cDNA. The CMYA genes are designated by their published names: Xin (CMYA1), Myomegalin (CMYA2), Myomaxin (CMYA3), and Unc-45 (CMYA4). α-Mysin heavy chain (α-MHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts, which are not dysregulated in mef2a null hearts, were used as controls. No transcripts were detected in the absence of reverse transcriptase. WT, wild type; KO, mef2a mutant.

**FIGURE 1. Bioinformatics identification of CMYA3 and analysis of CMYA transcripts in wild type and mef2a mutant hearts.** A, the NCBI public data base was searched for CMYA genes using the key words “cardiomyopathy-associated.” A total of four such additional genes were identified: CMYA1, -2, -3, and -4. B, expression levels of the CMYA genes in mef2a knock-out hearts. Total RNA was isolated from postnatal day 5 (P5) hearts of wild-type, heterozygote, and mef2a mutant mice, and transcripts were amplified by semi-quantitative RT-PCR. RT-PCR reactions were performed for 25 cycles and were fractionated on a 1% agarose gel. Similar amplification levels for each of the CMYA genes tested were observed between wild type and heterozygote cDNA. The CMYA genes are designated by their published names: Xin (CMYA1), Myomegalin (CMYA2), Myomaxin (CMYA3), and Unc-45 (CMYA4). α-Mysin heavy chain (α-MHC) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts, which are not dysregulated in mef2a null hearts, were used as controls. No transcripts were detected in the absence of reverse transcriptase. WT, wild type; KO, mef2a mutant.

Regulation of Myomaxin Transcription by MEF2A—To test whether myomaxin represents a direct transcriptional target for MEF2A, we identified the 5′-end of the gene using a combination...
of the expressed sequence tags database, 5′-rapid amplification of cDNA ends, primer extension, and RNase protection (data not shown). Based on this information, we isolated 5.0 kb of mouse genomic sequence upstream of the first exon and cloned this fragment into the pGL3BASIC-luciferase reporter. MEF2A responsiveness of this 5.0-kb region was tested in transfected COS cells. This 5.0-kb fragment alone exhibited background levels of activity in COS cells. However, when co-transfected with a MEF2A expression vector, the 5.0-kb fragment was induced nearly 5.0-fold (Fig. 2A), indicating that myomaxin is activated by MEF2A.

Given the ability of MEF2A to transactivate this upstream fragment, we examined this region for MEF2 binding sites and identified three putative MEF2 sequences exhibiting similarity to the consensus MEF2 site, (C/T)TA(A/T)TA(G/A). To determine whether MEF2A could bind these sequences, we performed gel mobility shift assays. The MEF2 sequences located at −75 (CTAAAATAG) and −2937 (TTATTATTTA) relative to the putative transcription start site bound to in vitro translated MEF2A (Fig. 2C), but binding to the −3096 site (TTATTATTA) was detectable only upon longer exposure.

FIGURE 2. Direct activation of myomaxin promoter by MEF2A and muscle specificity of the myomaxin proximal promoter. A, MEF2A activates the myomaxin promoter. A MEF2A expression vector was co-transfected into COS cells with the wild-type 5.0-kb myomaxin 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 pcDNA vector backbone. Values obtained for the mutant MEF2 sites are significant at p < 0.05. B, sequence of the mouse myomaxin proximal promoter. The sequence of 1.5 kb upstream of the putative transcription start site is depicted. The −75 MEF2 site and a putative E-box sequence are underlined. C, MEF2A binds two MEF2 sites present in myomaxin promoter. In vitro translated MEF2A was subjected to gel shift assays. Protein-DNA complexes were formed with each of the MEF2 sites within the myomaxin promoter with the exception that binding the most distal MEF2 site (−3096) was detected only upon longer exposure. MEF2A was unable to bind mutant MEF2 sites. D, the proximal myomaxin promoter is sufficient for MEF2A-dependent activation. A 1.5-kb myomaxin-luciferase reporter construct was co-transfected into COS cells with a MEF2A expression vector. MEF2A activated the wild-type (WT) but not the promoter with mutant −75 MEF2 site. E, the 1.5-kb upstream sequence is sufficient to confer muscle-specific gene transcription. The 1.5-kb myomaxin-luciferase vector was transfected into COS and C2C12 myoblasts, and luciferase units were measured. This fragment was sufficient to confer muscle-specific activity. The −75 MEF2 mutant exhibited basal activity in C2C12 myoblasts.
To determine whether the 1.5-kb fragment was sufficient to confer muscle specificity, we tested this reporter construct in the C2C12 muscle cell line and showed that this minimal region was highly active in C2 myoblasts as compared with COS cells (Fig. 2E). Moreover, the −75 site is essential for this muscle-specific responsiveness, since a mutation within this proximal MEF2 site nearly completely abolishes muscle-specific reporter activity (Fig. 2E). These results demonstrate that the −75 MEF2 binding site is necessary and sufficient for myomaxin transcriptional regulation.

Given the importance of the −75 site for MEF2-dependent transactivation of the myomaxin promoter, we searched for possible evolutionary conservation of the proximal MEF2 site. Indeed, the −75 site and neighboring sequences are conserved between rodents and humans (Fig. 3A). To determine whether the −75 MEF2 site is directly bound by MEF2A in vivo, we performed ChIP analysis on the myomaxin promoter. Nucleosomes were isolated from differentiated C2C12 myotubes and subjected to immunoprecipitation using anti-MEF2A antibodies (C-21; Santa Cruz Biotechnology). We designed primers that would amplify mouse genomic sequences surrounding the evolutionarily conserved −75 MEF2 site (Fig. 3A). As shown in Fig. 3B, we were able to demonstrate that the −75 MEF2 site in the mouse myomaxin promoter is bound by MEF2A in differentiated myotubes (left). Importantly, ChIP assays performed with IgG and two additional unrelated antibodies, anti-GAL4 (Santa Cruz Biotechnology) and anti-green fluorescent protein (Santa Cruz Biotechnology), failed to immunoprecipitate the −75 MEF2 site (Fig. 3B, right panel). Collectively, these results demonstrate that myomaxin is a direct transcriptional target for MEF2A.

**Cloning Full-length Myomaxin**—We isolated the full-length mouse Myomaxin cDNA from a Marathon skeletal muscle cDNA library (Clontech) using high fidelity PCR (Roche Applied Science) with gene-specific primers designed against predicted exons in the public data base. These PCR products were sequenced and assembled to generate the full-length cDNA (GenBank™ accession number EF122140). Conceptual translation of the Myomaxin cDNA revealed an open reading frame of 3,283 amino acids with a predicted size of 350 kDa (Fig. 4A). BLAST analysis using the open reading frame of Myomaxin confirms that it is the mouse ortholog of the partial human CMYA3/Xirp2 sequence and also exhibits modest sim-
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A schematic of the full-length mouse Myomaxin cDNA contains an open reading frame of 3,283 amino acids. SMART analysis of the entire protein sequence of Myomaxin revealed an actin-binding 16-amino acid repeat region (amino acids 309–1,599, indicated by ovals; 32 repeats total). Myomaxin and the partial human Xirp2 sequence exhibit 83% similarity within the actin-binding repeat region. Both Myomaxin and Xirp2 exhibit 34% identity to Xin within the actin-binding repeat region but no significant homology outside this region. Both Myomaxin and Xirp2 exhibit 34% identity to Xin within the actin-binding 16-amino acid repeat region (amino acids 309–1,599) (Fig. 4A).

Expression of myomaxin cDNA, revealed a transcript migrating above the 9.5 kb RNA marker and highly restricted to adult heart and skeletal muscle (Fig. 5A). Expression was not detected in any other adult mouse tissue. Moreover, myomaxin transcripts were not detected in either mouse aorta or stomach, representative tissues enriched for smooth muscle (Fig. 5A, bottom), indicating a striated muscle-specific expression of the myomaxin gene.

Myomaxin expression was also examined in the C2C12 myoblast cell line using RT-PCR. Myomaxin transcripts were detected in both proliferating C2C12 myoblasts and in differentiating myotubes (Fig. 5B). We observed a modest increase in myomaxin expression at the onset of myogenic differentiation, but surprisingly, the most mature myotubes (day 7) exhibited a robust up-regulation in myomaxin transcription (Fig. 5B). Examination of myomaxin transcripts in neonatal rat cardiomyocytes also showed that myomaxin is expressed in these cells and is up-regulated by hypertrophic stimuli, such as the β-adrenergic agonist, phenylephrine, and serum (Fig. 5C). Altogether, these results indicate that myomaxin is a striated muscle-specific gene induced by signals that promote muscle differentiation and growth.

Subcellular Localization of Myomaxin and Co-localization with Sarcomeric α-Actinin—To determine the subcellular localization of Myomaxin in striated muscle, polyclonal antibodies were raised against a GST-Myomaxin fusion protein (see “Materials and Methods”) and used for immunohistochemistry on cryosections from adult mouse hearts. As shown in Fig. 6A, Myomaxin antibodies revealed a robust, striated pattern (right), whereas preimmune IgG exhibited a diffuse, low level background signal (left). As an initial demonstration of specificity, Myomaxin antibodies were tested by immunoblot analysis. Anti-myomaxin antibodies detected specific signals in heart and skeletal muscle but not liver extracts (Fig. 6B). An essentially identical pattern was observed with two additional Myomaxin antibodies raised against different regions of the protein. This pattern is also observed in neonatal cardiomyo-
cyte and C2C12 myoblast extracts. The upper band represents full-length Myomaxin, since it comigrates with in vitro translated full-length Myomaxin cDNA (Fig. 6B, right). The lower molecular mass bands may represent degradation products or alternative isoforms of Myomaxin. To further demonstrate specificity of this signal, we performed blocking experiments on immunoblots and muscle cryosections. By Western analysis, preincubation of Myomaxin antibodies with recombinant protein completely blocked the striated pattern at 10- and 50-fold molar excess (Fig. 6C, bottom). The striated pattern observed with the Myomaxin antibodies suggested possible Z-disc localization. To rule out potential cross-reactivity of these antibodies to other proteins known to be localized at the Z-disc, we performed immunocytochemistry on COS cells transfected with representative Z-disc proteins. As shown in Fig. 7, the anti-Myomaxin antibodies specifically detected COS cells transfected with Myomaxin but not those cells expressing α-actinin, muscle LIM protein, or T-cap/telethonin, indicating that the striated signal in muscle cryosections corresponds to Myomaxin.

To more precisely determine that the striated signal actually represented Z-disc localization, we performed double label immunohistochemistry with anti-α-actinin-2 (Sigma). As shown in Fig. 8A (top), anti-sarcomeric α-actinin-2 (left; green) exhibits a periodic, Z-line staining pattern as expected. Anti-Myomaxin (middle; red) displays similar periodic striations in these myofibers. When these images are superimposed (right), the periodic striations are now yellow, indicative of co-localization of both proteins to the Z-disc. We also examined transverse sections of skeletal muscle using the anti-Myomaxin antibodies. These immunostaining results revealed specific reactivity along the periphery of myofibers indicating localization at the sarcolemmal region (Fig. 6A, bottom). Taken together, the immunostaining data from longitudinal and transverse sections indicate Myomaxin localization at the level of the Z-disc in the costameric region of striated muscle.

Myomaxin 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 Myomaxin and α-actinin-2. For these studies, COS cells were transfected with full-length MYC-Myomaxin (amino acids 1–3,283) 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. 8B
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Disruption of Z-disc/costamere function has been implicated in the pathology of various forms of cardiomyopathy. Myomaxin is a novel muscle protein with a new Z-disc localization, but its function and relationship to pre-existing Z-disc structures is not known. We used an in vivo approach to determine the possible role of myomaxin in Z-disc/costamere function.

Myomaxin was identified as a Xin-related MEF2A target gene in our recent publication. Xin is a Z-disc/costamere protein that is dysregulated in heart disease. MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.

**Discussion**

Myomaxin was identified as a Z-disc/costamere protein that is important for muscle development and function. Its expression is dysregulated in heart disease, and MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.

**Identification of Myomaxin**

Myomaxin was identified as a Xin-related MEF2A target gene in our recent publication. Xin is a Z-disc/costamere protein that is dysregulated in heart disease. MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.

**Expression Analysis**

Myomaxin expression is dysregulated in heart disease, and MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.

**Function Analysis**

Myomaxin was identified as a Z-disc/costamere protein that is important for muscle development and function. Its expression is dysregulated in heart disease, and MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.

**Conclusion**

Myomaxin is a novel muscle protein with a new Z-disc localization, but its function and relationship to pre-existing Z-disc structures is not known. We used an in vivo approach to determine the possible role of myomaxin in Z-disc/costamere function.

**Response to Questions**

Myomaxin expression is dysregulated in heart disease, and MEF2A is a transcription factor that regulates gene expression in striated muscle. Its activity is important for normal muscle development and function.
observed a robust expression of the gene. The reason for this increased expression at such a late stage in the myogenic differentiation process in vitro is unclear. In cardiac muscle cells, myomaxin is also activated by the β-agonist, phenylephrine, and serum, known stimulators of hypertrophic signaling pathways (27). Future studies will focus on whether the differentiation and growth signaling pathways that stimulate myomaxin gene expression in striated muscle are being controlled by a common MEF2-dependent mechanism.

Myomaxin Is a Peripheral Z-disc-restricted and α-Actinin-binding Protein—Myomaxin represents another MEF2A target gene whose gene product is restricted to the peripheral Z-disc/costameric region in striated muscle. These results are consistent with the cytoarchitectural defects observed in mef2a−/− hearts. Moreover, our results demonstrate that Myomaxin co-localizes and interacts directly with sarcomeric α-actinin. A previous study demonstrated that the partial human ortholog, Xirp2, interacts with F-actin (22). Our new findings raise the possibility that Myomaxin, by anchoring both actin and α-actinin, functions to position α-actinin in close proximity to its substrate, thereby facilitating its ability to cross-link actin.

Myomaxin interacts with α-actinin in two separate subdomains, XinA and PostXin, within the actin-binding repeat region. Both the XinA and PostXin fragments harbor repeat sequences and are situated within, but at each end of, the actin-binding region. Surprisingly, the XinC fragment also harbors repeat sequences and is located within the core actin-binding repeat region but does not interact with α-actinin. So what is the determining feature in Myomaxin that dictates interaction specificity? We initially considered the number of repeats as potentially influencing binding specificity, since it was previously shown that the number of repeats determines binding interaction with F-actin (22). However, the number of actin-binding repeats is probably not the determining factor, since the PostXin subdomain has four repeats, the fewest number of repeats in all fragments tested, and interacts effectively with α-actinin. In striking contrast, the XinC fragment has 10 repeats, a similar number of repeats compared with XinA. Another possibility is that the interrepeat sequence pattern is important for interaction specificity.
regions may dictate α-actinin binding specificity. This seems unlikely, because the interrepeat sequences in the XinA and PostXin fragments that bind to α-actinin are not any more similar to each other than to XinC. A final possibility is that secondary structure or a combination of secondary structure and repeat sequences may determine binding interaction with α-actinin. Interestingly, secondary structure analysis predicts the formation of numerous α-helices within the actin-binding repeat fragments, XinA and PostXin, whereas the XinC subdomain does not harbor any predicted α-helical structure.

Relationship to the Intercalated Disc Protein, Xin—Myomaxin exhibits modest similarity to Xin within the 16-amino acid repeat region but no obvious homology outside this region. It is within this region that Xin and the human ortholog of Myomaxin, Xirp2, interact with actin. Given that Myomaxin interacts with α-actinin within the actin-binding repeat region, it would also be of interest to determine whether Xin also interacts with α-actinin. So why is it that striated muscle has two related cytoplasmic proteins that serve to bind F-actin? This may be explained partly by the different subcellular localizations of Myomaxin and Xin. Immunolocalization studies have revealed that Xin resides within the adherens junction of the intercalated disc in cardiac muscle and the functionally equivalent myotendinous junction in skeletal muscle (28). In contrast, Myomaxin is precisely localized at the peripheral Z-disc/costameric region, a subcellular structure, coincidentally, completely devoid of Xin immunoreactivity (28). These results suggest that two related actin-binding proteins have evolved to perform unique functions, distinct from binding actin, which enables them to coordinate the assembly and organization of the actin cytoskeleton and associated protein complexes within their respective subcellular locations. Indeed, Xin has been demonstrated to exist in a complex with N-cadherin and β-catenin, two proteins found in the adherens junctions (28).

Potential Function of Myomaxin—The importance of the Z-disc and peripheral structures in the coordination of striated muscle contraction is well established (29–31). In recent years, many novel, muscle-specific proteins residing within these structures have been identified, thereby adding to the complexity of this subcellular region. The diversity of protein-protein interactions further adds an additional layer of specificity to this region.

The observation that myomaxin is down-regulated in 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. Also of note is the up-regulation of myomaxin under conditions that stimulate muscle hypertrophy, a condition that perturbs cytoskeletal remodeling events. This raises the possibility that perturbations in the expression and/or function of myomaxin may be a contributing factor in the pathogenesis of muscle disease. Future studies investigating the in vivo function of Myomaxin will provide insights into the role of this protein in striated muscle biology and contribute to an understanding of the myogenic pathways regulated by MEF2A.

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