130-kDa smooth muscle myosin light chain kinase is transcribed from a CArG-dependent, internal promoter within the mouse mylk gene

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Submitted 14 July 2005; accepted in final form 10 January 2006

Yin, Feng, April M. Hoggatt, Jiliang Zhou, and B. Paul Herring. 130-kDa smooth muscle myosin light chain kinase is transcribed from a CArG-dependent, internal promoter within the mouse mylk gene. Am J Physiol Cell Physiol 290: C1599–C1609, 2006. First published January 11, 2006; doi:10.1152/ajpcell.00289.2005.—The 130-kDa smooth muscle myosin light chain kinase (smMLCK) is a Ca\(^{2+}\)/CaM-regulated enzyme that plays a pivotal role in the initiation of smooth muscle contraction and regulation of cellular migration and division. Despite the critical importance of smMLCK in these processes, little is known about the mechanisms regulating its expression. In this study, we have identified the proximal promoter of smMLCK within an intron of the mouse mylk gene. The mylk gene encodes at least two isoforms of MLCK (130 and 220 kDa) and telokin. Luciferase reporter gene assays demonstrated that a 282-bp fragment (−167 to +115) of the smMLCK promoter was sufficient for maximum activity in A10 smooth muscle cells and 10T1/2 fibroblasts. Deletion of the 16 bp between −167 and −151, which included a CArG box, resulted in a nearly complete loss of promoter activity. Gel mobility shift assays and chromatin immunoprecipitation assays demonstrated that serum response factor (SRF) binds to this CArG box both in vitro and in vivo. SRF knockdown by short hairpin RNA decreased endogenous smMLCK expression in A10 cells. Although the SRF coactivator myocardin induced smMLCK expression in 10T1/2 cells, myocardin activated the promoter only two- to fourfold in reporter gene assays. Addition of either intron 1 or 6 kb of the 5′ upstream sequence did not lead to any further activation of the promoter by myocardin. The proximal smMLCK promoter also contains a consensus GATA-binding site that bound GATA-6. GATA-6 binding to this site decreased endogenous smMLCK expression, inhibited promoter activity in smooth muscle cells, and blocked the ability of myocardin to induce smMLCK expression. Altogether, these data suggest that SRF and SRF-associated factors play a key role in regulating the expression of smMLCK.

myocardin; serum response factor; GATA; transcriptional regulation; gene expression; telokin

MYOSIN LIGHT CHAIN KINASE (MLCK) phosphorylates the 20-kDa regulatory light chain (RLC) of smooth muscle and nonmuscle myosin in the presence of Ca\(^{2+}\)/CaM, which facilitates myosin interaction with actin filaments (12). In smooth muscle, phosphorylation of the myosin RLC is an obligatory step involved in the initiation of contraction. In many cells, phosphorylation of RLC by MLCK is also important in regulating cellular migration and division (22).

The 130-kDa smooth muscle MLCK (smMLCK) is encoded by the MYLK gene, which is highly conserved among different species, including birds, mice, and humans (1, 3, 14). In the mouse, a single functional mylk gene is located on chromosome 16B4–16B5, spanning >200 kb and containing 31 exons that encode at least three different proteins from a common open reading frame. Exons 1–31 encode the 220-kDa MLCK, which has been referred to as the nonmuscle or endothelial cell MLCK; exons 15–31 encode smMLCK; and exons 29–31 encode telokin or kinase-related protein (KRP) (14, 31). Of these, smMLCK is the principal regulator of the myosin II molecular motor in the initiation of smooth muscle contraction. Although the smMLCK is expressed at the highest levels in smooth muscle tissues, it is ubiquitous in all adult tissues, including skeletal and cardiac muscle (16). RLC phosphorylation induced by smMLCK is also important in regulating actomyosin-based cytoskeletal functions in nonmuscle cells, including focal adhesion and stress fiber formation, secretion, ion exchange, cytokinesis, neurite growth cone advancement, cell spreading, and migration (22).

Given the pivotal role of smMLCK in regulating smooth muscle contractile activity and its myriad functions in nonmuscle cells, changes in the expression of smMLCK are likely to have profound effects on the physiological functions of cells. Several studies have described changes in smMLCK expression during development (6, 10, 16) and under various pathological conditions (13), but the mechanisms responsible for mediating these changes have not been elucidated. In the current study, we have identified the proximal promoter of the mouse smMLCK, which is located within an intron of the mylk gene. Functional analysis of the smMLCK promoter revealed that a single CArG box is required for basal promoter activity in smooth muscle and nonmuscle cell types. The smooth and cardiac muscle restricted serum response factor (SRF) coactivator myocardin robustly induced smMLCK expression in 10T1/2 cells, although it increased the activity of the proximal smMLCK promoter only twofold in reporter gene assays. In contrast to SRF and myocardin, GATA-6 repressed the activity of the smMLCK promoter and inhibited smMLCK protein expression in vascular smooth muscle cells. Altogether, these studies indicate that expression of the 130-kDa smMLCK is regulated by a CArG-dependent promoter located within an intron of the mouse mylk gene.

EXPERIMENTAL PROCEDURES

Plasmid constructs and promoter-reporter gene assays. Mouse GATA-6 wild-type cDNA and adenovirus (25) were both kindly provided by Dr. Jeffery D. Molkentin (Department of Pediatrics, University of Cincinnati and Children’s Hospital Medical Center, Cincinnati, OH). Mouse myocardin wild-type cDNA in pcDNA3.1-myc/His vector (32) was kindly provided by Dr. Eric N. Olson.
GeneRacer RNA oligo was ligated to the 5’ end of the decapped mRNA samples using T4 RNA ligase. RT-PCR were then performed to amplify the 5’-end using GeneRacer 5’ primer and the gene-specific primer (5’-CTGAAGAGTTGCGGCGAAAATCCATG-3’). The PCR products were extracted from agarose gels, cloned into the pCR2.1 vector (Invitrogen), and sequenced.

**Gel mobility shift assays.** Nuclear extracts were prepared from COS cells transfected with GATA-6 or SRF expression plasmids as described previously (8). Nuclear extracts were prepared from A10, 10T1/2, and mouse proximal colon smooth muscle cells (LC) as cells described previously (7). Gel mobility shift assays were performed in a final volume of 15 µl. Binding mixes contained 0.2 ng (1.5 × 10^4 counts per minute) of end-labeled, double-stranded DNA probe, 200 ng of poly(dI-dC), 4.5 µg of BSA, and various amounts of nuclear extract protein. Annealed oligonucleotides were labeled using [32P]dCTP and Klenow DNA polymerase (Promega). Unincorporated nucleotides were removed by agarose gel electrophoresis. All binding reactions were incubated for 20 min at room temperature, and then the DNA-protein complexes were resolved by performing electrophoresis through 4% polyacrylamide gels containing 6.75 mM Tris (pH 7.9), 3.3 mM sodium acetate (pH 7.9), 1 mM EDTA, and 2.5% glycerol. The gel was dried and autoradiographed with intensifying screens at −80°C overnight. Sequences of the sense and antisense strands of the oligonucleotide probes are as follows: SRF probe sense, 5’-CGTCCCTTTAAGGCTAATCTTCCATAGTATTAATAAAAAC-3’; smMLCK probe sense, 5’-AGCTGTTTATATATC-GTAAAGTTTCTAGCCTTATAAGGG-3’; smMLCK probe antisense, 5’-GTCCTCATTAATCTTGGAATACATG-TAAC-3’. The specificities of these probes were verified by Western blot analysis and by performing gel mobility shift assays.

**SRF shRNA.** Oligonucleotides specific to SRF (AGAGAAT-GAGTGGCACCAG) or a negative control (ACTACCGTTGGATATTTAA) were inserted downstream from an H1 promoter in the adenoviral shuttle vector pRNAT-H1.1/Shuttle (GenScript, Scotch Plains, NJ), which is compatible with the Adeno-X system (Clontech Laboratories, Palo Alto, CA). The shuttle vector contains the H1 promoter, which drives the small interfering RNA cassette, together with a cytomegalovirus-driven viral green fluorescent protein (GFP) cDNA. Adenoviral constructs were then created using the Adeno-X vectors essentially as the manufacturer instructed and as described previously (39). The recombinant adenovirus was packaged in human embryonic kidney HEK-293 cells and amplified to obtain high-titer stocks.

Adenoviral infection and Western blot analysis. For adenoviral infection, A10 cells or 10T1/2 cells were seeded onto six-well plates at a density of 2 × 10^5 cells/well and grown overnight to near confluence. The cells were washed with PBS to remove serum and infected with adenovirus in PBS at a multiplicity of infection of 100 for 4 h at 37°C. These conditions resulted in close to 100% infection of cells. Seventy-two hours after infection, cell protein extracts were prepared using RIPA buffer and protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed essentially as described previously (35). Thirty micrograms of protein were fractionated on 7.5% or 15% SDS-PAGE gels. The protein sample was electrophoretically transferred onto a polyvinylidene difluoride membrane and verified using Ponceau S staining. The membrane was then probed with a series of antibodies. Antibodies used in this study were anti-GATA-6 (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), anti-MLCK (clone K36, 1:10,000 dilution; Sigma Chemical, St. Louis, MO), anti-SRF (1:10,000 dilution; Santa Cruz Biotechnology), anti-GFP (1:1,000 dilution; Clontech Laboratories), and anti-hemagglutinin epitope tag (1:1,000 dilution; Covance Research
Cells were then washed in ice-cold PBS and harvested in 6 ml of PBS sequences were as follows: smMLCK promoter sense (304/H11002/H9262 l of each were used as the template for standard PCR. The primer from the immunoprecipitated and input samples were purified, and 2 SRF (Santa Cruz Biotechnology) and anti-rabbit IgG control. DNA specific antibodies. Antibodies used for this experiment were anti-precipitation of the supernatant was performed overnight at 4°C with slurry for 30 min at 4°C with rotation. After centrifugation, immuno-precleared by incubation with salmon sperm DNA-protein A agarose supernatants were diluted 10-fold with ChIP dilution buffer and indicated. The 5-ends of truncated fragments of the promoter analyzed in experiments shown in Fig. 2 are indicated.

Fig. 1. Cloning of the smooth muscle myosin light chain kinase (smMLCK) promoter. A: agarose gel showing the products generated by rapid amplification of 5’-cDNA ends (5’-RACE) using mRNA isolated from stomach or bladder as indicated. B: schematic representation of a portion of the mouse mylk gene (derived from alignment of the 220-kDa MLCK cDNA with the mouse genome; see Ref. 14). 5’-RACE analysis identified that transcription initiation site of the 130-kDa smMLCK, 5’-RACE analysis was performed using mouse bladder and stomach total RNA as the template. From each tissue, two major RACE products were obtained (Fig. 1A). Cloning and sequencing of these products revealed heterogeneity in the transcription initiation sites, the most 5 initiation site was designated +1 and was located 364 bp upstream of the translation start codon (Fig. 1, B and C). Other major initiation sites were identified at +47, +54, and +118. There are no consensus TATA boxes within 30–40 nt of the transcription initiation sites, suggesting that the smMLCK promoter, similar to the majority of mammalian promoters (4), is likely to be TATA independent and uses other cis-acting elements to recruit the transcription initiation complex to the promoter. For example, multiple GC-rich regions

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**RESULTS**

**Cloning of the smMLCK promoter.** To determine the transcription start site of the 130-kDa smMLCK, 5’-RACE analysis was performed using mouse bladder and stomach total RNA as the template. From each tissue, two major RACE products were obtained (Fig. 1A). Cloning and sequencing of these products revealed heterogeneity in the transcription initiation sites, the most 5 initiation site was designated +1 and was located 364 bp upstream of the translation start codon (Fig. 1, B and C). Other major initiation sites were identified at +47, +54, and +118. There are no consensus TATA boxes within 30–40 nt of the transcription initiation sites, suggesting that the smMLCK promoter, similar to the majority of mammalian promoters (4), is likely to be TATA independent and uses other cis-acting elements to recruit the transcription initiation complex to the promoter. For example, multiple GC-rich regions
may represent binding sites for Spl within the core promoter, and Spl was previously shown to promote transcription initiation from TATA-less promoters (4). In addition, although none of the transcription initiation sites conform to a perfect consensus initiator sequence (PyPyANPyPy), each of the initiation sites deviate from this consensus at only one or two positions. These data suggest that the GC-rich elements, possibly together with degenerate initiator elements, may be important for transcription initiation of the smMLCK. The mylk gene spans >200 kb in the mouse genome (14) and contains at least 31 exons encoding at least three transcripts (220-kDa MLCK, 130-kDa smMLCK, and telokin) that are expressed in a cell-specific manner from alternative promoters (18). The smMLCK transcript starts from within intron 14 of the mylk gene such that the first exon of the smMLCK includes exon 15 of the 220-kDa MLCK together with 59 bp of a unique 5'-untranslated sequence from intron 14 (Fig. 1B). The translation initiation site of the smMLCK is located in exon 15 of the mylk gene, 364 bp from the most 5' initiation site of the 130-kDa smMLCK transcript.

To analyze the transcriptional regulation of smMLCK, we isolated the genomic region containing intron 14 and part of exon 15 of the mylk gene, representing the putative proximal promoter (−389 to +115) of the smMLCK transcript, by performing PCR with mouse genomic DNA. We also cloned a longer 5'-flanking region (−6,476 to +115) containing all of intron 13 and exon 14 in addition to the proximal promoter region. The proximal promoter region from −389 to +115 exhibited similar levels of activity in A10 and 10T1/2 cells (Fig. 2A). The longer 5'-flanking region from −6,476 to +115 showed promoter activity similar to that of the proximal region (Fig. 2B).

A single CArG box contributes to basal activity of smMLCK promoter. To map the minimal region required for activity of the smMLCK promoter, we generated luciferase reporter gene constructs that contain a series of deletions of the 5'-flanking region (Fig. 1C) and then analyzed their activity in A10 and 10T1/2 cells. In A10 cells, truncations of the promoter down to −167 bp had no effect on promoter activity and further deletion of the 16-bp region between −167 and −151 drastically decreased promoter activity (Fig. 2B). In 10T1/2 cells, truncation of the promoter from −289 to −174 resulted in a small (20%) but statistically significant (P < 0.01) decrease in promoter activity, suggesting that this region may contain a weak positive element (Fig. 2C). However, similarly to the situation in A10 cells, truncation of the promoter from −167 to −151 decreased promoter activity to 20% of wild-type levels. Interestingly, a CArG box, the consensus binding site for SRF,
was found in this region. To further determine whether this CArG box is required for basal smMLCK promoter activity, four subsequent mutations or deletions were generated that spanned this 16-bp region (Fig. 2D). Each of the mutant reporter genes that disrupted the CArG box (mCarG-1, ΔCarG-2, and ΔCarG-3), but not deletion of adjacent residues (ΔTACT), significantly decreased promoter activity to levels ranging from 34.2% to 61.1% of those observed in the wild-type construct in both A10 smooth muscle cells and 10T1/2 fibroblasts. Altogether, these results indicate that the 283-bp fragment (−167 to +115) acts as the minimal proximal promoter for the smMLCK gene and that a single CArG box is critical for basal promoter activity. In support of this assertion, comparison of the minimal mouse smMLCK promoter (−167 to +115) with sequences of the rat mylk and human MYLK genes revealed a remarkable homology of 96.8% and 89.6% identity, respectively, with the CArG box, GATA site, Sp1 site, CAAT box, and Forkhead box-binding sites almost entirely conserved. There is a conservative A-G substitution in the GATA site in the human gene; however, the GATG sequence also has been shown to bind weakly with GATA proteins (23).

SRF and GATA-6 bind to adjacent sites within the smMLCK promoter. A GATA transcription factor-binding site (−136 to −139) was found adjacent to the CArG box (−166 to −157) (Fig. 1C). Because GATA-6 is the major GATA factor present in smooth muscle cells, gel mobility shift assays were performed to examine the ability of SRF and GATA-6 to bind to their respective consensus sequences in the smMLCK promoter. Gel mobility shift assays using a probe that encompasses −167 to −128 of the mouse smMLCK promoter (Fig. 3A) demonstrated that SRF and GATA-6 nuclear extracts both bound specifically to this fragment. These mobility-shifted complexes could be supershifted by antibodies to the respective proteins, but not by nonspecific anti-Sp1 antibody (Fig. 3B). To further confirm whether these consensus binding elements were necessary for SRF and GATA-6 binding, two probes were generated that contained nucleotide mutations (mGATA probe) or a 3-bp deletion (ΔCarG probe) within these consensus sequences (Fig. 3A). The results of these assays have demonstrated that deletion of the CarG consensus sequence abolished SRF binding. As a control, we have demonstrated that the ΔCarG probe was able to bind to GATA-6 with similar efficacy (Fig. 3C). In addition, mutation of the GATA consensus sequence (mGATA probe) (Fig. 3D) abolished GATA-6 binding without interfering with SRF binding. These results demonstrate that SRF and GATA-6 are able to bind to their adjacent consensus sequences in the core of the smMLCK promoter.

SRF regulates smMLCK protein expression in vivo. Gel mobility shift assays were also performed to examine the ability of endogenous SRF present in nuclear extracts from different cell lines to bind to the smMLCK promoter. One major mobility-shifted band was detected on the smMLCK

Fig. 3. Serum response factor (SRF) and GATA-6 bind to adjacent consensus sites within smMLCK promoter. A: sequence of probes used for gel mobility shift assays. Wild-type probe encompasses −167 to −128 of mouse smMLCK promoter. Putative CarG and GATA consensus sequences are underlined. B: 32P-labeled, double-stranded, wild-type smMLCK probe was incubated with nuclear extracts from cells transfected with SRF or GATA-6 as indicated. After incubation for 20 min at room temperature, anti-SRF, anti-GATA-6, or anti-Sp1 antibodies were added and incubated for an additional 1 h on ice. Samples were run on a 4% polyacrylamide gel, and mobility-shifted complexes were visualized using autoradiography. Positions of specific mobility-shifted complexes are indicated by arrows, and complexes that resulted from antibody supershifts are indicated by asterisks. C: to confirm that CarG box represents binding site for SRF within smMLCK promoter, 32P-labeled wild-type or ΔCarG smMLCK probes were incubated with SRF (top) or GATA-6 (bottom) nuclear extract and analyzed using gel mobility shift assay. Positions of SRF and GATA-6 mobility-shifted complexes are indicated. D: to confirm that consensus GATA sequence is binding site for GATA-6 within smMLCK promoter, 32P-labeled wild-type or mutant GATA (mGATA) smMLCK probes were incubated with GATA-6 (top) or SRF (bottom) nuclear extract and gel mobility shift assays were performed.
promoter probe using nuclear extracts from A10 rat vascular smooth muscle cells, LI mouse proximal colon smooth muscle cells, and 10T1/2 mouse embryonic fibroblasts. The identity of SRF in this complex was confirmed by the supershift of the band observed after addition of an antibody specific for SRF (Fig. 4A). In addition, the major mobility-shifted complex observed in the cell extracts was not visible when a probe containing a deletion in the CArG box was used (Fig. 4A).

ChIP assays also demonstrated that SRF bound to the endogenous smMLCK promoter in vivo in A10 cells (Fig. 4B). This binding was specific because no binding to an adjacent region from exon 1, which does not contain a CArG box, was detected. To determine the physiological role of SRF in regulating the expression of smMLCK in A10 cells, the effects of SRF knockdown by short hairpin RNA (shRNA) on endogenous smMLCK protein expression was determined. Seventy-two hours after transduction of A10 cells with control or SRF shRNA, cells were harvested and endogenous protein expression levels were assessed using Western blot analysis. This analysis revealed that knockdown of endogenous SRF resulted in parallel downregulation of smMLCK expression in A10 cells (Fig. 4, C and D). These results suggest that SRF is physiologically important in regulating smMLCK protein expression.
Effect of GATA-6 on endogenous smMLCK protein expression. Because we observed that GATA-6 interacts with the consensus GATA element in the smMLCK promoter (Fig. 3), the effect of GATA-6 on smMLCK promoter activity was examined using reporter gene assays. The results showed that GATA-6 significantly decreased the activity of smMLCK promoter (−389 to +115) in A10 and 10T1/2 cells (Fig. 5A). This repression of promoter activity was abolished when the GATA consensus site was mutated within the promoter (Fig. 5B). The effect of GATA-6 on smMLCK protein expression was also examined after adenovirus-mediated overexpression of GATA-6 in A10 cells. Seventy-two hours after infection with GATA-6 adenovirus, expression of the smMLCK was significantly downregulated, whereas the expression of the 220-kDa MLCK was markedly increased (Fig. 5, C and D).

Effect of myocardin on endogenous smMLCK protein expression. Myocardin is a smooth and cardiac muscle-restricted transcriptional coactivator of SRF (24, 32, 37). Previously, smMLCK was shown to be induced by myocardin expression in 10T1/2 cells (34, 38). The effects of myocardin on smMLCK promoter activity were therefore further examined. The results showed that myocardin upregulated smMLCK promoter (−389 to +115 and −6,476 to +115) activity approximately two- to fourfold in A10 and 10T1/2 cells (Fig. 6, A and B). This activation of the smMLCK promoter was SRF dependent because deletion of the CArG box abolished myocardin activation (Fig. 6C).

Despite the fact that myocardin induced expression of the smMLCK in 10T1/2 cells (34, 38) (Fig. 7B), the direct effects of myocardin on the smMLCK promoter were minimal compared with its 500- to 1,000-fold activation of smooth muscle-restricted promoters such as telokin, SM22-α, smooth muscle α-actin, and smooth muscle myosin heavy chain (smMHC) (5, 38). Because expression of smMLCK is not restricted to smooth muscle cells, we also examined the role of a more widely expressed myocardin family member, MRTFA (33), on the smMLCK promoter. The results of this analysis demonstrated that MRTFA also activated the smMLCK promoter only approximately fourfold in A10 cells (Fig. 6C) and approximately ninefold in 10T1/2 cells (data not shown). This activation could also be abolished completely by deletion of the CArG box in the smMLCK proximal promoter. One interpretation of these data is that the proximal promoter or 6.5 kb of the 5′-flanking sequence is not sufficient to mediate high levels of activation of the promoter by myocardin family members. Indeed, when the genome sequence containing the mouse smMLCK locus was aligned with corresponding regions of the human, rat, dog, and chimpanzee genomes, two short regions of 63 and 103 bp within the 7,174-bp intron 15 of the mylk gene (intron 1 of smMLCK) were observed to be highly conserved. Interestingly, in the most proximal 63-bp region located between +1,680 and +1,742, there is a consensus CArG box (Fig. 6D). ChIP assays confirmed that SRF binds specifically to this intronic CArG box in A10 cells in vivo (Fig. 6E), suggesting that this highly conserved intronic CArG box may be important in regulating smMLCK expres-

antibodies specific for indicated proteins. D: densitometric quantitation of data obtained from 2 independent experiments indicated smMLCK was significantly decreased and 220-kDa MLCK was increased in A10 cells overexpressing GATA-6. *P < 0.05.
sion. However, inclusion of the intronic CArG element in a luciferase reporter gene did not enhance the ability of myocardin to activate the smMLCK promoter (Fig. 6F).

Myocardin-induced activation of smMLCK expression is repressed by GATA-6. Previous studies showed that GATA-6 can either augment or repress myocardin’s activation of promoters in a promoter-specific manner (29, 35). The ability of GATA-6 to modify the effects of myocardin on the smMLCK promoter was thus examined. In luciferase reporter gene assays, the myocardin-induced activation of the promoter was found to be repressed completely by cotransfection of GATA-6 (Fig. 7A). In addition, the myocardin-dependent induction of the smMLCK in 10T1/2 cells could be attenuated by coinfecting cells with GATA-6-expressing adenovirus (Fig. 7, B and C).

DISCUSSION

In this study, we have identified and characterized an internal promoter within the mylk gene that is responsible for
transcription of the 130-kDa smMLCK. These studies have demonstrated that expression of smMLCK is regulated by SRF and SRF-associated proteins. The SRF-associated proteins myocardin and GATA-6 act as positive or negative regulators of smMLCK expression, respectively.

Three protein products of the mouse mylk gene were described previously, including telokin, 130-kDa smMLCK, and 220-kDa MLCK (2, 16, 17). The amino acid sequences of telokin and smMLCK are identical to the carboxy termini of the 220-kDa MLCK. The sequence identity between these proteins suggests that they are all encoded by the same mylk gene, either by alternative splicing or through alternative promoters. Previously, we demonstrated that the telokin transcript is derived from an internal promoter within the mylk gene rather than by alternative splicing (18). Similarly, in the current study, we have demonstrated that the smMLCK transcript is also produced from an internal promoter within the mylk gene. Both telokin and smMLCK mRNA contain short stretches of unique sequences in their 5′-untranslated regions that are not present in 220-kDa MLCK mRNA. These unique sequences are encoded within introns of the mylk gene. These results suggest that the mylk gene must contain at least three independent promoters. Although the presence of multiple promoters in a single gene is unusual, it is by no means unique and in fact is common within genes that encode other protein kinases related to MLCK, such as Unc-89, obscurin, and striated preferentially expressed gene (SPEG) (20, 21, 30). Interestingly, similarly to the promoters in the mylk gene, each of the promoters in these multipromoter genes also directs different patterns of cell-specific expression of the individual gene transcripts. For example, the SPEG gene encodes SPEG-α and SPEG-β, which are expressed in striated muscle; BSPEG, which is expressed in the brain and the vasculature; and APEG, which is expressed in vascular smooth muscle (20). Within the mylk gene, we previously showed that telokin, which is transcribed from an internal promoter within intron 28 of the mylk gene, is expressed exclusively in smooth muscle cells and that this expression pattern is a property of a 370-bp fragment of the telokin promoter (18, 19). The smMLCK and 220-kDa MLCK transcripts have distinct tissue distributions, with the 130-kDa MLCK being the predominant form expressed in most adult tissues, whereas the 220-kDa MLCK is the most abundant form in most cultured cells (2). The 130-kDa smMLCK is expressed at its highest levels in smooth muscle tissues and cells such as A10 vascular smooth muscle cells compared with other cell types, including REF52 fibroblasts or 10T1/2 fibroblasts (9, 11, 38). None of the smMLCK promoter fragments analyzed recapitulated this cell-specific pattern of expression of the endogenous gene (Fig. 2 and data not shown). This finding may suggest that additional, more distal elements are required to mediate cell-specific expression; alternatively, cell specificity may manifest fully only in vivo. In support of the latter possibility, similarly to the smMLCK gene, the smooth muscle α-actin gene contains a conserved intronic CArG element that is not required for myocardin activation of the promoter in reporter gene assays and is not required for promoter activity in smooth muscle cells in vitro (36), but is absolutely required for expression of the promoter in smooth muscle cells in vivo in transgenic mice (26).

We have found that individual transcription factors can exert distinct effects on individual promoters within the mylk gene.

Fig. 7. GATA-6 inhibits the myocardin-induced activation of smMLCK. A: A10 or 10T1/2 cells were transfected with smMLCK reporter gene (−389 to +115), together with either empty expression vector (open bars), myocardin (filled bars), or myocardin and GATA-6 vector (stippled bars). Promoter activity data relative to vector control transfections are means ± SE; n = 6 samples. B: 10T1/2 cells were infected with adenovirus encoding LacZ or myocardin in presence or absence of GATA-6 virus (multiplicity of infection of 100 for each virus) for 4 h at 37°C. Seventy-two hours after infection, protein extracts were prepared from infected cells and assessed using Western blot analysis. C: densitometric quantitation of data obtained from 2 independent experiments indicated that myocardin-induced expression of the smMLCK could be attenuated significantly by GATA-6. *P < 0.05.
Overexpression of GATA-6 in A10 vascular smooth muscle cells significantly downregulated the expression of smMLCK (Fig. 5C) and telokin (35), whereas the 220-kDa MLCK was markedly increased. Conversely, myocardin induced expression of smMLCK and telokin in 10T1/2 cells without altering expression of the 220-kDa MLCK (38) (Fig. 7). These data thus describe molecular mechanisms that begin to account for the differential activity of the promoters within the mylk gene.

Although smMLCK is ubiquitously expressed in mouse tissues, it is expressed at much higher levels in smooth muscle tissues than in any other tissue. This pattern of expression suggests that the smMLCK promoter may have properties analogous to those of promoters of smooth muscle-specific genes in addition to a basal housekeeping type of activity. Consistent with this proposal, we have demonstrated that, similarly to promoters of many smooth muscle-restricted genes, the smMLCK promoter is CArG dependent, and also that SRF binds to the CArG box within the promoter in vitro and in vivo. Accumulating evidence suggests that as a ubiquitously expressed transcription factor, SRF regulates various growth-responsive and muscle-specific genes through its interaction with different coregulators, such as myocardin, GATA family members, and Elk1. Myocardin is an extraordinarily powerful SRF cofactor that is expressed specifically in smooth and cardiac muscle cells (5, 24, 32, 37). It was previously shown that myocardin can induce the expression of many genes, including smMLCK, that are characteristic of smooth muscle cells in 10T1/2 fibroblasts (34). We have confirmed these results in the current study and also have shown that in contrast to smMLCK, the 220-kDa MLCK is not induced by myocardin in 10T1/2 cells (Fig. 7B). Interestingly, it has been reported that in a balloon-injured rat carotid artery model, expression of smMLCK and SM-2, one of the contractile smooth muscle myosin heavy chain isoforms, are both negatively regulated between 24 h and 7 days after injury (13). This decline in expression of smMLCK correlates well with the decline in expression of myocardin mRNA after vascular injury (15). This finding suggests that the loss of myocardin expression could account for a preferential decrease in the expression of the smMLCK and other smooth muscle-specific proteins compared with SRF-dependent but myocardin-independent genes, such as c-fos (15).

Despite the ability of myocardin to induce expression of the smMLCK in 10T1/2 cells at both the protein (Fig. 7B) and mRNA levels (34), reporter gene assays in the present study indicated that myocardin could increase smMLCK promoter activity only two- to fourfold (Fig. 6). This small stimulation of promoter activity is more similar to the levels of stimulation of myocardin-independent promoters such as c-fos than it is to the high levels of stimulation of smooth muscle-restricted promoters. This finding suggests that additional CArG boxes located more distally within the mylk gene may be required to mediate the myocardin activation of the smMLCK promoter. Alternatively, myocardin may induce expression of endogenous smMLCK through an indirect mechanism. Further studies, including analysis of the smMLCK promoter in vivo in transgenic mice, are required to resolve these questions.

We also found that the ability of myocardin to regulate smMLCK expression could be attenuated by GATA-6. GATA-6 is a zinc finger transcription factor that plays essential roles in development through its interaction with DNA-regulatory elements that contain a consensus WGATAR motif (28). The results of reporter gene assays and overexpression studies demonstrated that GATA-6 repressed the ability of myocardin to activate the smMLCK promoter or to induce smMLCK expression in 10T1/2 cells (Fig. 7). These effects of GATA-6 are similar to the reported effects of GATA-6 on the telokin promoter (35). The GATA-binding sites in both the smMLCK and telokin promoters are closely apposed to a critical CArG box, and GATA-6 binding to these sites is required for GATA-6 to exert its inhibitory effects. It also was shown previously that myocardin and GATA-6 compete for binding to SRF (35). Altogether, these data have led us to propose a model in which the recruitment of GATA-6 adjacent to the CArG box may be sufficient to allow GATA-6 to compete more efficiently with myocardin for binding to SRF and thereby inhibit the activity of a myocardin-dependent promoter. This would account for the ability of GATA-6 to inhibit these promoters while activating other promoters, such as the smMHC promoter, in which the GATA site is not adjacent to the CArG box. However, because myocardin activates the smMLCK promoter minimally in vitro, GATA-6 also could inhibit the smMLCK promoter via other mechanisms, such as by recruiting a corepressor or histone deacetylase. Additional studies examining the binding of myocardin and GATA-6 to the endogenous smMLCK promoter and identifying the factors that interact with these proteins on the smMLCK promoter are required to determine the mechanisms by which myocardin and GATA-6 regulate this promoter.

In summary, we have identified and characterized the smMLCK promoter within the mylk gene. Our results show that SRF, myocardin, and GATA-6 play critical roles in the transcriptional regulation of smMLCK. These novel findings provide important insights into the complex regulation of the mylk gene that occurs during development and differentiation.

ACKNOWLEDGMENTS

We thank Drs. Ron Prywes, Eric N. Olson, and Jeffery D. Molkentin for providing reagents. We are grateful to all members of the Herring laboratory for helpful discussions.

GRANTS

This work was supported by National Institutes of Health Grants HL-58571, DK-61130, and DK-65644 (to B. P. Herring). J. Zhou is supported by an American Heart Association postdoctoral fellowship.

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