Regulation of 130-kDa Smooth Muscle Myosin Light Chain Kinase Expression by an Intrinsic CArG Element*

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Background: Mechanisms regulating transcription of MLCK are poorly defined.

Results: Deleting a CArG element from the mylk1 gene specifically decreased expression of the 130-kDa smMLCK isoform, resulting in decreased intestinal contractility and proliferation.

Conclusion: The 130-kDa smMLCK isoform has functions that cannot be compensated for by the 220-kDa MLCK.

Significance: Floxed mylk1 mice permit specific functions of the 130-kDa smMLCK to be determined.

The mylk1 gene encodes a 220-kDa nonmuscle myosin light chain kinase (MLCK), a 130-kDa smooth muscle MLCK (smMLCK), as well as the non-catalytic product telokin. Together, these proteins play critical roles in regulating smooth muscle contractility. Changes in their expression are associated with many pathological conditions; thus, it is important to understand the mechanisms regulating expression of mylk1 gene transcripts. Previously, we reported a highly conserved CArG box, which binds serum response factor, in intron 15 of mylk1. Because this CArG element is near the promoter that drives transcription of the 130-kDa smMLCK, we examined its role in regulating expression of this transcript. Results show that deletion of the intronic CArG region from a β-galactosidase reporter gene abolishes transgene expression in mice in vivo. Deletion of the CArG region from the endogenous mylk1 gene, specifically in smooth muscle cells, decreased expression of the 130-kDa smMLCK by 40% without affecting expression of the 220-kDa MLCK or telokin. This reduction in 130-kDa smMLCK expression resulted in decreased phosphorylation of myosin light chains, attenuated smooth muscle contractility, and a 24% decrease in small intestine length that was associated with a significant reduction of Ki67-positive smooth muscle cells. Overall, these data show that the CArG element in intron 15 of the mylk1 gene is necessary for maximal expression of the 130-kDa smMLCK and that the 130-kDa smMLCK isoform is specifically required to regulate smooth muscle contractility and small intestine smooth muscle cell proliferation.

The mylk1 gene is a large gene spanning ~250 kb, comprising 31 exons (1). mylk1 encodes at least three protein products: a 220-kDa MLCK,3 a 130-kDa MLCK, and a non-catalytic gene product, telokin. Each transcript from the mylk1 gene is derived from a unique independent promoter within the gene (1). The 220-kDa MLCK is also referred to as nonmuscle MLCK or endothelial MLCK, because it was first characterized in chick embryo fibroblasts and endothelial cells (2, 3). The 130-kDa MLCK is also called the smooth muscle MLCK (smMLCK), because it is most abundant in smooth muscle tissues; however, it is also widely expressed in other tissues at lower levels (1, 4, 5). Telokin is a non-catalytic product of the gene that is expressed at very high levels in intestinal, urinary, and reproductive tract smooth muscle; at low levels in vascular smooth muscle cells; and at undetectable levels in other tissues (6).

In the presence of Ca2+ and calmodulin, both the 220- and 130-kDa smMLCK can phosphorylate serine 19 of the 20-kDa myosin regulatory light chain of smooth muscle and nonmuscle myosin II. In smooth muscle cells, phosphorylation of the myosin regulatory light chain is an obligatory step for the initiation of contraction. In many other cell types, phosphorylation of regulatory light chain induced by MLCK is important for regulating actomyosin-based cytoskeletal functions, such as focal adhesion and stress fiber formation, secretion, cytokinesis, neurite growth cone advancement, endothelial and epithelial barrier formation, and cell migration (7–13). Alterations in MLCK expression have been linked to a variety of pathologies, including colitis (14), inflammatory bowel disease (15), asthma (16, 17), inflammatory lung disease (18), familial aortic dissection (19), and hypertension (20, 21). The specific functions of the various MLCK isoforms in these processes, however, are not clear. Global knock-out of the 220-kDa MLCK in mice results in numerous defects in epithelial and endothelial barrier function, suggesting that this isoform has a specific role in regulating these processes (22–26). Through specific targeting of a portion of the catalytic domain shared by the 220- and 130-kDa MLCKs, it has been possible to determine the combined roles of these kinases in specific tissues and cell types (27). As anticipated, ablation of both MLCK isoforms in smooth muscle cells resulted in impaired contractility and decreased myosin light chain phosphorylation (20, 27). Surprisingly, deletion of both 220- and 130-kDa smMLCK specifically from endothelial cells had very little effect on vascular permeability, bringing into question the importance of endothelial cell-expressed MLCK.

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3 The abbreviations used are: MLCK, myosin light chain kinase; smMLCK, smooth muscle MLCK; MLCL, myosin light chain; SUMO, small ubiquitin-like modifier; SRF, serum response factor; qRT-PCR, quantitative RT-PCR.

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in regulating endothelial barrier function (28). Because of the overlapping structure of the 220- and 130-kDa smMLCK, it is difficult to examine the function of the 130-kDa smMLCK without also affecting expression of the 220-kDa isoform. To address this issue, we examined regulatory elements that specifically regulate expression of the 130-kDa smMLCK with the hypothesis that deletion of these elements may attenuate expression of the 130-kDa smMLCK without affecting expression of the 220-kDa isoform. To address this issue, we examined regulatory elements that specifically regulate expression of the 130-kDa smMLCK with the hypothesis that deletion of these elements may attenuate expression of the 130-kDa smMLCK without affecting expression of the 220-kDa isoform.

The CArG element, CC(A/T)6GG, is the cis-regulatory element that binds SRF, an evolutionarily conserved MADS (MCM1, agamous, deficiens, and SRF) domain-containing transcription factor. SRF binding and crystal structure studies have shown that a functional CArG element can deviate by no more than 1 bp from the consensus sequence (30). Virtually all known CArG elements reside within 4 kb of the transcription start site of genes (30). Using computational algorithm prediction approaches with experimental validation, a genome-wide screen identified 60 target genes that are regulated by CArG elements. Among these, 26 of the validated SRF target genes encode for cytoskeletal/contractile or adhesion proteins (30, 31). When bound to a CArG element, SRF also provides a docking surface for interaction with numerous accessory co-factors to form ternary complexes, conferring tissue- or pathway-specific expression of target genes. For example, ternary complexes of SRF and Elk1 are important for growth factor regulation of immediate early genes such as c-fos (32). In smooth muscle cells, ternary complexes of SRF together with myocardin or myocardin-related transcription factors are very powerful activators of numerous smooth muscle-specific contractile and regulatory proteins, such as the 130-kDa smMLCK (33). SRF and myocardin enhanced the activity of the 130-kDa smMLCK promoter reporter genes and induced expression of the 130-kDa smMLCK in 10T1/2 fibroblast cells, whereas GATA-6 repressed promoter activity, possibly through disrupting SRF-myocardin complexes (29). Besides the CArG element in the promoter region, there is another highly conserved CArG element in the first intron of the 130-kDa smMLCK (intron 15 of the mylk1 gene). Chromatin immunoprecipitation assays confirmed that this intronic CArG element also binds to SRF in vivo in smooth muscle cells (29). However, the previous studies did not determine if this intronic CArG element affects the expression of the 130-kDa smMLCK in vivo. Here, we found that the intronic CArG element is important for regulating expression of transgenes driven by the 130-kDa smMLCK promoter in vivo and for driving expression of endogenous 130-kDa smMLCK in mice. Moreover, we show that targeting this element is an effective means to specifically decrease expression of the endogenous 130-kDa smMLCK without affecting expression of the 220-kDa MLCK or telokin.

EXPERIMENTAL PROCEDURES

Generation of Targeting Vector for Homologous Recombination—An mylk1 targeting vector was generated by inGenious Targeting Laboratory (Stony Brook, NY). A 7.56-kb fragment containing about 5.1 kb extending 5′ and 2.16 kb extending 3′ to the intronic CArG region, was subcloned from a C57BL/6 BAC clone (RP23: 55G1, Source BioScience) into pSP72 vector (Promega). To construct the targeting vector for homologous recombination, a loxP/FRT-flanked Neo cassette was inserted 54 bp 3′ of the intronic CArG element, and a single loxP site containing engineered AffIII and BamHI sites for Southern blot analysis was inserted 184 bp 5′ of the intronic element.

Generation of Transgenic Reporter Mice—The neomycin resistance cassette was removed from the targeting vector described above by FLP recombinase-mediated recombination in bacteria. The vector was then digested by FseI and PmlI to yield a 2.3-kb fragment that included the intronic CArG element and surrounding loxP sites. The 130-kDa smMLCK promoter, exon 1, intron 1, and a portion of exon 2 were cut from the pGL2B construct described previously (29) and ligated into the pWhere lacZ reporter vector (InvivoGen). The smMLCK(−389/+8427) pWhere vector was then cut by FseI and PmlI. The resulting 2-kb fragment that included the intronic CArG element was replaced with the corresponding 2.3-kb fragment isolated from the targeting vector to generate the 1CArG-smMLCK(−389/+8427) pWhere plasmid. The integrity of the plasmid was confirmed by restriction enzyme digestion and DNA sequencing. In order to delete the CArG element from the 1CArG-smMLCK(−389/+8427) pWhere plasmid, it was introduced into bacteria expressing Cre recombinase to generate plasmid Δ1CArG-smMLCK(−389/+8427) pWhere. Correct excision of the CArG element was confirmed by DNA sequencing. Δ1CArG-smMLCK(−389/+8427) pWhere and Δ1CArG-smMLCK(−389/+8427) pWhere plasmids were linearized and microinjected into pronuclei of fertilized oocytes by standard procedures by the Indiana University School of Medicine transgenic mouse facility. Neonatal founder mice were genotyped for the presence of the transgene and were analyzed at 1 month old by β-galactosidase staining as described previously (34). All animal experiments were conducted under the approval of the Indiana University School of Medicine Institutional Animal Care and Use Committee.

Generation of Knock-out Mice—Knock-out mice were generated by inGenious Targeting Laboratory. The targeting construct was linearized using NotI prior to electroporation into C57BL/6N embryonic stem cells. Positively selected ES cells were screened by PCR and then expanded for Southern blot confirmation of targeting. Correctly targeted ES cells were microinjected into BALB/c blastocysts. Resulting chimeras with a high percentage black coat color were mated to C57BL/6 FLP mice to remove the Neo cassette. The deletion of the Neo cassette was screened and confirmed by PCR and DNA sequencing. Germ line floxed mice were mated to smMHC-Cre mice (from Michael Kotlikoff (Cornell University, Ithaca, NY)) (also on a C57BL/6 background) to delete the intronic CArG region specifically in smooth muscle tissues. Genotyping primers were as follows: MLCK P1, GGC AAG CCA AAC CCT TAC
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Cell Proliferation—The intestines of littermate neonatal mice (day 9–10) were dissected, and the lowest portions of the ileum were incubated in 20% sucrose in PBS solution overnight at 4 °C. Tissue samples were frozen into Tissue-Tek O.C.T. compound (catalog no. 4583, Sakura), and 7-μm sections were cut. Sections were fixed with 3.7% formaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 10% FCS in 50 mM Tris, pH 7.6, 150 mM NaCl and then incubated with antibodies against Ki67 (catalog no. 15580, Abcam; 1:500) and smooth muscle α-actin (catalog no. A2547, Sigma; 1:500). Primary antibodies were visualized by incubation with rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:50) and FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch; 1:50) secondary antibodies.

Statistical Analysis—The χ² test was used to determine if the observed birth frequency of knock-out mice was lower than expected. For other statistical comparison, Student’s t tests were performed (Prism, GraphPad Software). A value of p < 0.05 was considered statistically significant.

RESULTS

Deletion of an Intronic CArG Element in the mylk1 Gene

Abolished Transgene Expression Driven by the 130-kDa smMLCK Promoter—Previously, we reported a highly conserved CArG element located in intron 15 of the mylk1 gene (first intron of the 130-kDa smMLCK) (29). To investigate the role of this intronic region in regulating 130-kDa smMLCK gene expression, we generated transgenic mice in which a lacZ reporter was driven by the 130-kDa smMLCK promoter, exon 1, intron 1, and a portion of exon 2 with (ICarG-smMLCK(−389/+8427) pWhere) or without (ΔICarG-smMLCK(−389/+8427) pWhere) this intronic CArG region, as described under “Experimental Procedures.” In two of the three independent founders harboring the wild type transgene, high levels of β-galactosidase staining were observed in visceral smooth muscle rich tissues, such as bladder, colon, small intestine, and ureters (Fig. 1). The third line had lower levels of expression but in a similar pattern (not shown). One of the two high expressing founders also exhibited staining in bronchi as well as in the lungs and small vessels of skeletal muscle and liver (Fig. 1). In contrast to the wild type transgenes, no β-galactosidase expression could be detected in any of the seven founder mice harboring the CArG-deleted transgene (Fig. 1). Weak background staining seen in bladder, kidney, and colon is similar to that seen in non-transgenic mice (Fig. 1, bottom right).

These data demonstrate that the intronic CArG region is critical for expression of a 130-kDa smMLCK-driven transgene.

Deletion of the Intronic CArG Region from the Endogenous mylk1 Gene Resulted in Decreased Expression of the 130-kDa smMLCK—To determine if deletion of the intronic CArG region would decrease expression of the endogenous 130-kDa smMLCK, we crossed mice harboring the floxed CArG region with mice expressing Cre recombinase under the control of the smooth muscle myosin heavy chain promoter (Fig. 2) (41). We have previously shown that this Cre transgene results in high levels of recombination specifically in smooth muscle tissues (35). PCR analysis of genomic DNA isolated from colon and aorta of knock-out and control mice further showed that there
is more efficient recombination of the floxed allele in colon smooth muscle as opposed to aortic smooth muscle (Fig. 2B). Although the recombined allele was readily detectable in both tissues, there also remained significant amounts of the non-recombined floxed allele. The latter probably represents a combination of less than 100% efficiency of recombination, together with contamination from other cell types in the sample. Of note, we did not detect the recombined allele in the majority (about 75%) of our control flox/flox mice. This is in contrast to a previous report, which showed that transient expression of Cre recombinase driven by the smMHC promoter in sperm resulted in recombination of the floxed allele derived from the male Cre-positive parent in almost all mice (43). Recombination of the floxed allele in sperm results in progeny that are CArGf+/H11002 (global heterozygous) rather than the expected CArGf/f. Both control (Cre−/− CArGf/f and Cre−/− CArGf−/) and smooth muscle-specific CArG knock-out (Cre−/+ CArGf/f and Cre−/+ CArGf−/) mice reached adulthood without any obvious growth and behavioral abnormalities. However, knock-out mice were born with a slightly lower than expected frequency of 19% as compared with 25% (χ² test p = 0.033, degree of freedom = 1; Fig. 2C). This suggests that deletion of the intronic CArG region from the native gene results in partial embryonic lethality or neonatal death. Quantitative real-time RT-PCR showed that there is a 40% decrease in 130-kDa smMLCK mRNA levels of CArGf/f Cre−/− knock-out mice compared with control CArGf+/H11002 Cre−/− mice, whereas there was no significant alteration in 220-kDa MLCK or telokin mRNA expression levels (Fig. 3A). Similar results were seen when CArGf+/H11002 Cre−/− knock-out mice were compared with CArGf+/H11002 Cre−/− control mice. Because we did not see any significant differences in 130-kDa smMLCK expression between
the two control strains (CArGf/f Cre−/− and CArGf−/− Cre−/−), and our standard genotyping does not distinguish between these strains, in all subsequent experiments, control mice were a mixture of CArGf/f Cre−/− and CArGf−/− Cre−/−. Similarly, all smooth muscle-specific knock-out mice were a mixture of CArGf/f Cre−/− and CArGf−/− Cre−/−.

Similar to colon, we observed ∼40 and 30% reductions in 130-kDa smMLCK mRNA levels in the bladder and aorta of knock-out mice, respectively (Fig. 3B). Moreover, using an antibody to the common carboxyl terminus of MLCK and telokin, we found that deletion of the intronic CArG region reduced 130-kDa smMLCK protein expression by ∼30 and 40% in colon and bladder, respectively (Fig. 3, C and D). Similar findings were observed using an MLCK antibody raised against the full-length bovine smMLCK (Fig. 3D). Additional experiments confirmed that the presence of the loxP sites in the control mice did not alter 130-kDa smMLCK expression compared with wild type mice (Fig. 3, E–G).

Deletion of the Intrinsic Region and Subsequent Decreases in 130-kDa smMLCK Expression Attenuated Smooth Muscle Contractility—In order to investigate whether the decreased expression of the 130-kDa smMLCK affects the contractility of both visceral and vascular smooth muscle, we analyzed the contractility of colon and aortic segments, ex vivo. Contraction elicited by high KCl-induced depolarization of colon from knock-out mice was dramatically decreased compared with control mice (Fig. 4, A and C). Similarly carbachol-induced contractions were also impaired in tissue from knock-out mice (Fig. 4, B and C). The L-type calcium channel inhibitor diltiazem blocked the contractile responses to high KCl in all mice (data not shown). ET1-mediated contraction of aortic segments was also decreased in knock-out mice compared with controls (Fig. 4D).

Decreased Contraction of Smooth Muscle Tissues in Knock-out Mice Was Associated with Decreased Myosin Light Chain Phosphorylation—The 130-kDa smMLCK induces contraction of smooth muscle by phosphorylating the regulatory myosin

FIGURE 2. Generation of the intronic CArG knock-out mice. A, schematic representation of the approach used to delete the intronic CArG region from the endogenous mylk1 gene. The native mylk1 gene is shown at the top with the targeting vector below it. The numbers above the native gene refer to nucleotide positions relative to the transcription start site of the 130-kDa smMLCK. The promoter and intronic CArG boxes are indicated (red and blue boxes, respectively). Yellow triangles, loxP sites; black triangles, FRT sites. Below the targeting vector are schematic representations of the correctly targeted allele (Flox+Neo), the targeted allele following FLP-mediated removal of the neomycin cassette (Flox), and the deleted allele generated following Cre-mediated recombination (Deleted allele). Positions of primers used for genotyping are indicated. B, ethidium bromide-stained agarose gel showing an example of PCR analysis of recombination of the intronic CArG element using primers P3 and P2 (A) and genomic DNA isolated from colon smooth muscle and aortic tissues. The bands corresponding to the floxed (F) and recombinant or deleted (Δ) alleles and an internal loading control (IC) are indicated. C, breeding scheme used to generate knock-out mice together with the genotypes of the progeny, their expected frequency, observed frequency, and total numbers of pups analyzed.
light chain. Thus, we sought to determine if the impaired contractile responses seen in the knock-out mice were associated with altered MLC phosphorylation. Under basal resting conditions, levels of MLC phosphorylation in both control and knock-out mice were very low and showed no significant difference (Fig. 4E). However, at the peak of contraction induced by high KCl, the level of phosphorylation of the MLC in knock-out mice was much less than that seen in control mice (18.3% as compared with 39.3%; Fig. 4E).

**DISCUSSION**

Results of this study clearly show that a CarG box-containing region in intron 15 of the mylk1 gene is required for expression of the 130-kDa smMLCK. Deletion of this intronic CarG box attenuated expression of the 130-kDa smMLCK without affecting expression of either the 220-kDa MLCK or telokin. Moreover, decreased expression of only the 130-kDa smMLCK isoform is specifically required to regulate not only smooth muscle contraction, but also intestinal smooth muscle cell proliferation. This demonstrates that the 130-kDa smMLCK isoform is specifically required to regulate not only smooth muscle contraction, but also intestinal smooth muscle cell proliferation.
Deletion of both the 220- and 130-kDa MLCK from smooth muscle tissues has been previously shown to impair contractility, MLC phosphorylation, and gastrointestinal motility in mice (27). The current studies suggest that it is primarily the 130-kDa smMLCK rather than the 220-kDa MLCK that is responsible for regulating contraction in gastrointestinal smooth muscle. This is consistent with the lack of reported effects on smooth muscle contractility in the 220-kDa MLCK knock-out mice (44). Results also suggest that the 130-kDa smMLCK has a specific role in regulating the proliferation of small intestinal smooth muscle cells during early neonatal growth (Fig. 6). Although MLCK and myosin light chain phosphorylation are known to be important in cell division, these are the first data that suggest a specific role for the 130-kDa smMLCK isoform in this process. This conclusion should, however, be viewed with caution because it is possible that the alterations in proliferation are secondary to impaired contractility. In vascular, airway, and bladder smooth muscle, mechanical strain can induce smooth muscle cell proliferation (45–47). A decreased mechanical stimulus in CArG knock-out mice may thus also impair intestinal smooth muscle cell proliferation, resulting in attenuated intestinal elongation.

Deletion of the intronic CArG region completely abrogated expression of a 130-kDa smMLCK-lacZ reporter transgene in the visceral smooth muscle tissues, whereas deletion of this element from the endogenous gene only decreased endogenous 130-kDa smMLCK expression by about 40%. The relatively small decrease in 130-kDa smMLCK expression may be partially due to incomplete recombination of the floxed alleles by Cre recombinase. In support of this, PCR analysis of genomic DNA isolated from colon and aorta demonstrates variable levels of floxed alleles remaining in these tissues (Fig. 2B). Alternatively, it is also possible that the reporter transgene is missing additional positive cis-acting regulatory elements that play a role in activating the endogenous 130-kDa smMLCK. In support of this possibility, a Notch-responsive element has been identified at \( \text{H11002}^{\text{3687}} \) that plays an important role in activating 130-kDa smMLCK expression in vascular smooth muscle cells (48). This element is not present in the reporter genes described in our study, which extend from \( \text{H11002}^{\text{389}} \).
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**Figure 5. Knock-out mice have shorter small intestines.** Representative pictures (A) and quantitative data (B) show the lengths of colon and small intestine of adult control (CArGf/f Cre<sup>-/-</sup>; Ctrl) and knock-out (CArGf/f Cre<sup>-/-</sup>; KO) mice. n = 5, *p < 0.05. C, body weights of adult control and knock-out mice. n = 6. D, qRT-PCR analysis of inflammatory cell markers in smooth muscle from the small intestine of control or knock-out mice. Transcript levels were quantitated as described in the legend to Fig. 3. n = 3–5. No significant differences in expression were observed in control and knock-out mice. Error bars, S.E.

**Figure 6. Knock-out mice have decreased intestinal smooth muscle cell proliferation.** A, Ki67 (red) and smooth muscle α-actin (green) staining of cross-sections of the lower portion of small intestines from neonatal control (CArGf/f Cre<sup>-/-</sup>; Ctrl) and knock-out (CArGf/f Cre<sup>-/-</sup>; KO) mice. White arrows and white arrowheads point to examples of Ki67 positive smooth muscle cells in the circular and longitudinal smooth muscle layers, respectively. B, quantitation of the number of positive Ki67 smooth muscle cells per field at ×40 magnification in the circular layer, longitudinal layer, and both smooth muscle layers of the small intestine. n = 6–7 mice. *p < 0.05. Error bars, S.E.
to +8427. This may also explain why the reporter genes exhibited very low level or undetectable lacZ expression in vascular smooth muscle tissues (Fig. 1). Although the endogenous 130-kDa smMLCK is expressed at lower levels in many nonmuscle tissues, we did not observe significant levels of lacZ transgene expression in many of these tissues. This may simply reflect the sensitivity of lacZ detection or the nonnative chromatin environment of reporter transgenes. Although the transgenic reporter mice were generated using a pWhere lacZ expression vector that is CpG-free and has H19 insulator elements flanking the transgene, we have previously shown that the telokin promoter also does not drive high levels of expression in many founder mice generated using this transgene vector (34). Because the promoter and regulatory elements analyzed are embedded within introns of the larger mylk1 gene, it is possible that transcription from the promoters that drive expression of the 220-kDa MLCK may modulate the chromatin structure of the gene to facilitate the activity of these internal elements. Although analysis of reporter genes suggests that the promoter and first intron of the 130-kDa smMLCK are not sufficient to fully recapitulate expression of the endogenous 130-kDa MLCK, deletion of the intronic CArG region from the endogenous gene decreases 130-kDa smMLCK expression by 40%. This demonstrates that this element is required for full activation of the gene. The region deleted following Cre recombinase-mediated recombination of the lacZ reporter or the endogenous mylk1 gene includes a conserved CArG box together with almost 300 bp of flanking sequence. The CArG element is located within a region of 63 bp that is highly conserved between species (29). Analysis using rVista identified conserved potential binding sites for the transcription factors SRF, Lun1, HoxA3, Oct, AP3, SRY, and DBP in this region. This raises the possibility that the decreased 130-kDa smMLCK seen following deletion of this region may be due to loss of not only SRF binding but also one or more of these other transcription factors. For example, Lun1, also named topoisomerase I binding, arginine-serine-rich, E3 ubiquitin protein ligase (TOPORS), is particularly interesting in this regard, because this protein also has SUMO ligase activity, and both SRF and myocardin are known to be regulated by sumoylation (49–51). This raises the possibility that Lun1 may further regulate SRF and myocardin activity to control expression of the 130-kDa smMLCK.

We have previously shown that a CArG element within the telokin promoter is also critical for expression of telokin transcripts through analysis of transgenic reporter mice and targeting the endogenous telokin promoter (34, 42). Together with the current findings, these data show that SRF plays a key role in regulating expression of multiple transcripts from the mylk1 gene. Although polymorphisms in these CArG elements have not yet been linked to diseases, an amplification of a CT repeat adjacent to the CArG element in the promoter of the 130-kDa smMLCK in SHR rats has been proposed to increase 130-kDa smMLCK expression and be the cause of the hypertension in these rats (21). A single nucleotide polymorphism (SNP) in intron 17 of the human MYLK1 gene (equivalent to intron 15 in mice) has also been shown to regulate 130-kDa smMLCK expression and to be linked to inflammatory lung disease (18). Because this SNP is not in the conserved region deleted in the current study, these data suggest that there may be multiple important regulatory elements within this intron of the mylk1 gene.

Although SRF is important for regulating expression of both 130-kDa smMLCK and telokin transcripts, it does so by binding to distinct CArG elements. These elements appear to be functionally separated from each other, because deletion of a single CArG element affects expression of one transcript but not the other. Deletion of the CArG element in the telokin promoter (in mylk intron 28) abolished telokin expression without affecting expression of transcripts encoding the 200- or 130-kDa smMLCKs (42). Similarly, deletion of the CArG element from intron 1 of the 130-kDa smMLCK gene (in mylk1 intron 15) decreased expression of the 130-kDa smMLCK without affecting expression of the 220-kDa MLCK or telokin (Fig. 3). These data suggest that either the CArG elements are simply too far from the other promoters to affect their activity (e.g. the telokin CArG element is about 73 kb from the 130-kDa smMLCK promoter) or that there are perhaps insulator elements within the mylk1 gene that restrict the activity of the elements to specific promoters. Additional studies are required to resolve these possibilities.

In summary, data from both transgenic reporter mice and a knock-out mouse model demonstrate that a CArG region within intron 15 of the mylk1 gene plays an important role in specifically regulating expression of 130-kDa smMLCK. Moreover, the ICArG flox mice provide a novel model system for further interrogating the specific functions of the 130-kDa smMLCK isoform in different cell types in vivo.

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