Mechanisms Responsible for the Promoter-Specific Effects of Myocardin.

Jiliang Zhou and B. Paul Herring*

Department of Cellular and Integrative Physiology,
Indiana University School of Medicine,
635 Barnhill Drive
Indianapolis, IN 46202-5120

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*Send Proofs to:
B. Paul Herring, Ph.D.
Department of Cellular and Integrative Physiology
Indiana University School of Medicine
635 Barnhill Drive, Indianapolis, In 46202-5120

Telephone: 278-1785
FAX: 274-3318
Email: pherring@iupui.edu
SUMMARY

Understanding the mechanism of smooth muscle cell (SMC) differentiation will provide the foundation for elucidating SMC-related diseases such as atherosclerosis, restenosis and asthma. Recent studies have demonstrated that the interaction of SRF with the co-activator myocardin is a critical determinant of smooth muscle development. It has been proposed that the specific transcriptional activation of smooth muscle-restricted genes, as opposed to other SRF-dependent genes, by myocardin, results from the presence of multiple CArG boxes in smooth muscle genes that facilitate myocardin homodimer formation. This proposal was further tested in the current study. Our results show that the SMC-specific telokin promoter, which contains only a single CArG box, is strongly activated by myocardin. Furthermore, myocardin and a dimerization defective mutant myocardin, induce expression of endogenous telokin but not c-fos in 10T1/2 fibroblast cells. Knocking down myocardin by siRNA deceased telokin promoter activity and expression in A10 SMCs. A series of telokin and c-fos promoter chimeric and mutant reporter genes were generated to determine the mechanisms responsible for the promoter specific effects of myocardin. Data from these experiments demonstrated that the Ets binding site (EBS) in the c-fos promoter partially blocks the activation of this promoter by myocardin. However, the binding of Ets factors alone was not sufficient to explain the promoter specific effects of myocardin. Elements 3’ of the CArG box in the c-fos promoter act in concert with the EBS to block the ability of myocardin to activate the promoter. Conversely, elements 5’ and 3’ of the CArG box in the telokin promoter act in concert with the CArG box to facilitate myocardin stimulation of the promoter. Together these data suggest that the promoter specificity of myocardin is dependent on complex combinatorial interactions of multiple cis elements and their trans binding factors.

Index terms: gene regulation, telokin, c-fos, smooth muscle
Introduction

There is extensive evidence showing that altered control of the differentiated state of smooth muscle cells contributes to the development and/or progression of a variety of diseases, including atherosclerosis, hypertension and asthma. These diseases are all associated with decreased expression of proteins required for the differentiated function of smooth muscle cells. An understanding of the mechanisms that control smooth muscle cell differentiation is required before it will be possible to determine how these control processes are altered in pathological conditions.

Recent studies have demonstrated that the interaction of SRF with the co-activator myocardin is a critical determinant of vascular smooth muscle development (1). Myocardin is expressed in visceral and vascular SMCs, physically associates with SRF, and greatly potentiates SRF-dependent transcription of multiple SMC marker genes (2-5). The activity of myocardin is however, selective to cardiac and smooth muscle-specific genes as other SRF-dependent genes such as c-fos are not strongly activated by myocardin (6). A model has been developed to explain this promoter specificity of myocardin (6). According to this model, transcriptional activation of smooth muscle cell-specific genes by myocardin requires at least two CArG boxes to allow formation of myocardin homodimers. In contrast, ubiquitously expressed genes, such as c-fos, that contain a single CArG box, close to the transcription initiation site, are not activated by myocardin due to their inability to promote myocardin homodimer formation. However, this model does not explain why other growth factor regulated genes such as Egr1, which contains 5 CArG boxes is not activated by myocardin-related transcription factor A or by myocardin (current study) (7,8). In addition, fragments of the smooth muscle specific α-actin and myosin proximal promoters that contain two CArG boxes are not sufficient to direct expression of transgenes specifically to smooth muscle cells (9,10). In contrast, the smooth muscle-restricted telokin promoter that contains only a single CArG box has been shown to be sufficient to mediate smooth muscle-specific expression of a transgene in vivo (11). Telokin expression has also been shown to be
upregulated following myocardin infection of rat aortic smooth muscle cells (12). In order to further investigate the mechanisms underlying the promoter-specific effects of myocardin we have compared the ability of myocardin to activate two single CArG box-containing genes, the smooth muscle-specific telokin gene and the widely expressed c-fos gene.

Results demonstrate that myocardin and its dimerization deficient LZ (leucine zipper) mutant is capable of strongly trans-activating single CArG box containing, smooth muscle-specific, telokin promoter and to induce telokin expression in 10T1/2 cells although the myocardin LZ mutant is less effective than the wild type myocardin. In contrast, myocardin had no effect on c-fos promoter activity or c-fos gene expression in 10T1/2 cells. Knocking down endogenous myocardin in SMC cells by siRNA decreased telokin promoter activity and endogenous telokin expression. Analysis of a series of chimeric and mutant telokin and c-fos reporter genes demonstrated that in the c-fos promoter the Ets binding site (EBS), which binds ets factors, partially blocks the activation of this promoter by myocardin, however, an additional region between −300 and +39 is required to prevent myocardin activation of the c-fos promoter. Conversely, multiple cis-elements in telokin promoter are required for maximal myocardin activation. We propose that the gene specificity of myocardin is dependent on combinations of multiple positive and negative cis elements and their trans binding factors.
Materials and methods

Mammalian expression and reporter gene assays. The mouse myocardin pcDNA3.1-myc/his vector was kindly provided by Dr. Eric N. Olson (Southwestern Medical Center, Dallas, Texas). The myocardin LZ mutant was developed by QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA)(6). All promoter reporter genes were constructed by cloning fragments of promoters into the pGL2-B luciferase vector (Promega, Madison, WI). The mouse and rabbit telokin promoter-luciferase reporter gene used includes nucleotides –190 to +181 (T370) and -256 to +147 (T400), respectively, of the telokin gene as described previously (13). The SM22α-luciferase reporter gene includes nucleotides -475 to +61 of mouse SM22α (14,15). The SM α-actin promoter fragment extended from nucleotide –2,555 to +2,813 (9) and the SM-MHC promoter from –4,200 to +11,600 (16). The Egr1 and c-fos luciferase reporter genes spanned from –637 to +79 and –605 to +39, respectively. The minimal TK promoter used comprised nucleotides -113 to +20 of the thymidine kinase gene. All mutant reporter gene constructs were initially generated in pCR pBlunt vector (Invitrogen, Carlsbad, CA) by QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and then transferred to pGL2b vector. The resultant plasmids were sequenced to verify the integrity of the insert. Transfection was carried out as previously described (17). The level of promoter activity was evaluated by measurement of the firefly luciferase relative to the internal control Renilla luciferase using the Dual Luciferase Assay System essentially as described by the manufacturer (Promega, Madison, WI). A minimum of six independent transfections was performed and all assays were replicated at least twice. Results are reported as the mean ± SEM.

Reverse transcription coupled to PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). A pair of unique primers for telokin was designed as sense 5’-GACACCGCCTGAGTCCAACCTCCG-3’ and antisense 5’-GACCCTGTTGAAGATTTCCTGCCACTG-3’, yielding a 127-bp product. Other sequences of PCR primers are available upon request. 200ng of RNA was utilized as a
template for reverse transcription (RT) and PCR with respective specific primers using SuperScript One-step RT-PCR system (Invitrogen, Carlsbad, CA).

**siRNA.** A plasmid-based system for production of siRNA was generated by insertion oligonucleotides specific to myocardin AATGCAACTGCAGAAGCAGAA (3), downstream of an H1 promoter in the adenovirus shuttle vector pRNAT-H1.1/Shuttle (GenScript, Piscataway, NJ) that is compatible with the adeno-X system (Clontech, Palo Alto, CA). The shuttle vector contains the H1 promoter driving the siRNA cassette together with a CMV-driven coral GFP cDNA.

**Adenovirus construction and cell infection.** Adenovirus constructs were made using the Adeno-X vectors essentially followed the manufacturers instructions (BD Biosciences, Palo Alto, CA) as previously described (17). The recombinant adenovirus was packaged in HEK293 cells and amplified to obtain high titer stocks. For adenoviral infection A10 cells or 10T1/2 cells were seeded in 6-well plates at a density of $2 \times 10^5$ cells/well and grown overnight to near confluence. These cells were washed with PBS to remove serum and infected with adenovirus encoding LacZ, myocardin, myocardin LZ mutant, or myocardin siRNA in PBS for 4 h at 37°C. These conditions resulted in close to 100% infection of cells. 72 hours following infection cell protein extracts were prepared using RIPA buffer and protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

**Western blotting.** Western blotting analysis was carried out essentially as described previously (18). Fifteen micrograms of protein were fractionated on a 7.5% or 15% SDS-polyacrylamide gels. The protein sample was electrophoretically transferred to a PVDF membrane and verified by Ponceau S staining. The membrane was then probed with a series of antibodies. Antibodies used in this study were anti: $\beta$-actin (Sigma, 1:10,000), calponin (Sigma, clone hCP, 1:10,000), GFP (Clontech, 1:400), HA tag (BabCO, 1:1000), MLCK (Sigma, clone K36, 1:10,000), SM22$\alpha$ (a gift from Dr. Len Adam, 1:6000), SM $\alpha$-actin (Sigma, clone 3A1, 1:10,000), SRF (Santa Cruz, 1:10,000) and
telokin (1:6,000) (18).
Results

Myocardin *trans-activates the telokin promoter*. In contrast to many smooth muscle restricted promoters, the telokin promoter contains only a single CArG box. The effects of myocardin on reporter genes driven by the mouse minimal telokin promoter (-190+171, T370), the SM α-actin promoter, SM22α promoter or the SM myosin heavy chain (MHC) promoter were examined in 10T1/2 cells. Results from these luciferase assays revealed that the telokin promoter was strongly activated by myocardin, similar to other SMC-specific promoters in 10T1/2 cells. In contrast, myocardin only activated the Egr1 and c-fos promoters 4-fold, (Figure 1A). Similar activation by myocardin was observed in COS cells, however, myocardin activated the smooth muscle-specific promoters to a far lesser extent in A10 SMCs as compared to 10T1/2 cells or COS cells (Figure 1B, C).

**Over-expression of wild-type and LZ mutant myocardin induces endogenous telokin but not c-fos expression in 10T1/2 fibroblast cells.** Wild-type and LZ mutant myocardin expression plasmids were transfected into 10T1/2 cells and RT-PCR was carried out on RNA isolated from the transfected cells in order to determine whether forced expression of wild-type and LZ mutant myocardin can induce expression of endogenous telokin. This experiment demonstrated that both myocardin and myocardin LZ mutant were sufficient to drive telokin and SM22α mRNA expression without altering expression of c-fos in 10T1/2 cells. Consistent with these results, adenoviral mediated expression of wild type or LZ mutant myocardin was also sufficient to induce expression of several smooth muscle proteins including telokin, SM22α, SM α-actin and MLCK in 10T1/2 cells (Figure 2 B, C). A direct side-by-side comparison of the effects of the wild type and LZ mutant myocardin, demonstrated that much higher levels of expression of the mutant molecule are required to induce smooth muscle-specific gene expression as compared to the wild type myocardin (Figure 2C). Similarly promoter activation by the LZ mutant myocardin was only approximately 20% of that observed with the wild type myocardin (Figure 2D). Together these data show that although the dimerization
deficient myocardin can activate smooth muscle specific genes its activity is impaired as compared to the wild type myocardin.

**siRNA-mediated knock-down of myocardin decreases telokin promoter activity and endogenous telokin expression in A10 SMCs.** To determine the role of endogenous myocardin in regulating SM-specific gene expression endogenous myocardin level was decreased using siRNA. To demonstrate the efficacy of the myocardin siRNA constructs COS cells were co-transfected with myocardin expression plasmids and either control or myocardin siRNA plasmids. Analysis of myocardin levels in extracts from these cells demonstrated that the myocardin siRNA but not control siRNA decreased the exogenous myocardin expression (Figure 3A). To determine whether down-regulation of myocardin decreased endogenous SMC marker gene expression in SM A10 cells, myocardin siRNA was expressed using an adenovirus in A10 cells and endogenous SMC mRNA and proteins examined by RT-PCR and western blotting, respectively (Figure 3B and C). These data demonstrated that endogenous myocardin, telokin, but not GAPDH mRNA were significantly down-regulated as compared to siRNA control infected and non-infected cells (Figure 3B). At the protein expression level, 72hours following transduction with myocardin siRNA, telokin, SM22α and calponin were significantly decreased 20-40%, however, there were no significant changes in the levels of expression of SM α-actin, β-actin or the 130kDa MLCK (Figure 3C). To confirm that telokin promoter activity is myocardin dependent in SMCs, plasmid-based myocardin siRNA or a scrambled siRNA control pshuttle plasmid were transiently co-tranfected into A10 SMCs together with telokin promoter reporter genes and luciferase activity determined. As shown in figure 3D, the activity of the rabbit telokin promoter, but not the thymidine kinase promoter was significantly reduced to approximately 40% of control levels in A10 cells transfected with either 300ng or 600ng of myocardin siRNA plasmid.

**Maximal myocardin activity on the telokin promoter requires multiple cis-elements.** As both telokin and c-fos promoters contain single CArG boxes we
determined whether the specific sequences of the CArG box within the telokin promoter contributes to ability of myocardin to activate the promoter. Reporter genes were generated in which the telokin promoter CArG box was mutated to the c-fos gene CArG box sequence or the SM22\textsuperscript{a} gene CArG-near sequence or to a sequence no longer able to bind SRF. These mutant reporter genes were co-transfected together with myocardin and luciferase activity determined (Figure 4A). Mutant telokin promoter reporter genes containing either a c-fos or SM22\textsuperscript{a} CArG box were activated by myocardin similar to the wild-type telokin promoter. As expected a mutant telokin promoter that was unable to bind SRF showed no activation by myocardin, showing that the intact CArG is critical for the myocardin activation (Figure 4A). These data demonstrated that SRF binding to the CArG box is necessary for myocardin activation of the telokin promoter but the sequence of the CArG box does not explain the ability of myocardin to activate the telokin promoter as opposed to the c-fos promoter.

To define the minimal regions of the telokin promoter required for myocardin activation, the ability of myocardin to activate a series of deletion constructs was determined (Figure 4B). Results from this analysis suggest that the regions between –80 and –66 (an AT-rich region) and between +36 to +82 are important for myocardin activation. In contrast, deletion of residues –190 to –80 or +82 to +171 did not alter the ability of myocardin to activate the promoter, suggesting that these regions are not important for this effect. Deletion of the region from +36 to +82 or from –80 to -66 decreased the ability of myocardin to activate the promoter over 10- and 20-fold, respectively. These data demonstrated that the CArG box together with regions from +36 to +82 and –80 to –66 are necessary for myocardin activation of the telokin promoter. To determine if these regions are sufficient to confer myocardin activation, the telokin CArG box, –66 to –80 region (AT-rich region) and +36 to +82 region were fused to a minimal TK promoter, alone or in combination. Each of these regions alone was not sufficient to confer a large amount of myocardin activation (Figure 4C). Although the CArG element alone increased activation to 11-fold, when all three elements were present the ability of myocardin to activate the minimal TK promoter was increased to 50-fold. These data
suggest that multiple cis-elements of telokin promoter are necessary and largely sufficient to confer maximal activation by myocardin.

**The ets binding site (EBS) in c-fos promoter partially blocks myocardin activation.** It has been reported that the SRF binding affinity of the c-fos CArG box is higher than the SM22α CArG boxes and the variations among CArG boxes of c-fos and SM22α influence cell type specificity of expression (19). To determine if the specific sequence of the c-fos CArG box is important for the lack of response of this promoter to myocardin, the CArG box was mutated to the telokin CArG box sequence or to a sequence unable to bind SRF. Analysis of these mutant reporter genes demonstrated that c-fos promoters containing either the native or telokin CArG box sequence were poorly activated by myocardin (Figure 5B) and, as expected, a mutant c-fos promoter that was unable to bind SRF showed no activation by myocardin. These data together with those obtained from the mutant telokin promoters described in figure 4A, suggest that the precise sequence of the CArG boxes in the c-fos and telokin promoters does not account for the promoter-specific effects of myocardin.

It has recently been reported that growth signals can repress smooth muscle-specific genes by triggering the displacement of myocardin from SRF by ELK1, an ets family member that competes for the myocardin docking site on SRF through a structurally related SRF-binding motif (20,21). As Elk1 was first identified as a component of the c-fos SRE this raises the possibility that the binding of Elk1 to the ets binding site (EBS) adjacent to the CArG box in the c-fos promoter is sufficient to prevent myocardin activation of this gene. To examine this possibility, reporter genes were generated in which this site was either deleted or mutated and the effects on myocardin activation determined. Analysis of these mutant reporter genes revealed that deletion of the ets binding site within the c-fos promoter resulted in a partial activation of the promoter by myocardin to 40-fold as compared to the 4-fold activation of the wild-type promoter. This data would suggest that Elk1 binding to the c-fos promoter partially blocks myocardin activation (Figure 5B). Although the telokin promoter does not contain a consensus ets
binding site adjacent to the CArG box, sequence alignment between the telokin and c-fos promoters revealed a significant degree of sequence similarity in this AT-rich region (Figure 5A). This sequence similarity allowed us to determine if the sequences immediately 5’ of the CArG boxes are important for the promoter-specific effects of myocardin. When the EBS region in the c-fos promoter was mutated to the corresponding sequence in the telokin promoter the mutant c-fos promoter remained largely refractory to myocardin activation (activation was increased from 4-fold to 10-fold, figure 5B). In addition, when the corresponding region of the telokin promoter was mutated to match the EBS and surrounding nucleotides of the c-fos promoter this did not prevent myocardin from activating the promoter (Figure 5C). Similar results were obtained when the CArG box sequences were also switched in conjunction with the AT-rich/EBS sequences (Figure 5C). These data suggest that there are additional regulatory regions within the c-fos promoter that prevent myocardin activation of the promoter. To begin to further identify these regions a truncated promoter was generated (-324 to +39) in which the region 5’ of the ets binding site was deleted. This truncated construct had similar myocardin activation to the wild type c-fos promoter (Figure 6 B). In addition, changing the EBS and CArG box from the c-fos promoter to corresponding telokin promoter sequences, within the context of this truncated promoter, had no further effect on myocardin activation (Figure 5B). In a reciprocal experiment changing the AT-rich region and CArG box of the telokin promoter to the corresponding sequences in the c-fos promoter, within the context of a –80 to +82 minimal telokin promoter did not prevent myocardin from activating the telokin promoter (Figure 5C). Together these data suggest that sequences in the c-fos promoter between –300 and +39 and between –55 and +82 of the telokin promoter are responsible for the promoter specific effects of myocardin on these two genes.

To determine if the telokin +36 to +82 region is sufficient to confer myocardin responsiveness to the c-fos gene, this fragment was added to c-fos promoter and the ability of myocardin to activate the promoter determined. This chimeric promoter showed only a small increase in myocardin activation to 8-fold as compared to the 4-
fold activation of the wild-type c-fos promoter (Figure 5D). When the +36 to +82 region was added in combination with the telokin AT-CArG sequence, no further activation of the promoter was observed. These data imply that the positive elements within the telokin promoter are not able to override the negative elements located between −300 to +39 of the c-fos promoter.
Discussion

Our data demonstrate that myocardin increases telokin expression through a CArG-dependent mechanism that requires the cooperative activity of multiple cis-acting regulatory elements. Conversely the inability of myocardin to activate the growth factor responsive c-fos gene appears to result from both the lack of these key cooperative positive regulatory elements together with the presence of multiple negative elements that help prevent myocardins’ activation of the promoter.

It has been proposed that the ability of myocardin to specifically activate cardiac and smooth muscle-specific genes is dependent on cooperative interaction of pairs of CArG boxes. This would explain why growth-regulated genes such as c-fos, that contain a single CArG box, are not activated by myocardin (6). However, another early growth response gene, Egr-1, that has 5 CArG boxes located in the 5′ flanking promoter sequence, was not activated by myocardin (Figure 1A) or MRTF-A (7,8). In addition, the proximal SM α-actin or SM myosin heavy chain promoters, that each contain two CArG boxes are not sufficient to drive SMC-specific transgene expression (9,10) whereas the telokin promoter which contains only one CArG box is sufficient to drive SMC-specific expression in transgenic mice (22). In the current study, we have further shown that the telokin promoter is strongly activated by myocardin (Figure 1, 4A), that myocardin can activate endogenous telokin expression in 10T1/2 cells (Figure 2) and that knocking down endogenous myocardin in SMC cells decreases telokin expression (Figure 3). Furthermore, although a myocardin LZ mutant, which is not able to dimerize, activated the telokin promoter (Figure 2D) and induced endogenous telokin expression in 10T1/2 fibroblast cells (Figure 2A,C) it did so much less effectively compared to the wild type myocardin. These data suggest that a myocardin monomer is sufficient to induced telokin and other smooth muscle-specific gene expression when expressed at high levels. However, at more physiological levels of expression it is likely that the ability of myocardin to dimerize is important for its ability to activate smooth muscle genes, including those such as the telokin gene, that contain only a single CArG box in their
promoter regions. Taken together, these data would suggest that the ability of paired CArG box elements to promote myocardin dimerization is not sufficient to account for the smooth and cardiac muscle-specific effects of myocardin.

Although siRNA mediated knockdown of myocardin resulted in decreased telokin, SM22α and calponin expression in A10 cells no changes in the level of expression of SM α-actin or the 130kDa MLCK were observed (Figure 3). These latter findings are puzzling in light of our data (Figure 2) and that of others that have shown that myocardin induces expression of SM α-actin and the 130kDa MLCK in 10T1/2 cells. At least one explanation for this apparent discrepancy could be that in A10 cells much of the expression of SM α-actin and the 130kDa MLCK may be occurring through myocardin independent mechanisms. This is particularly likely for these two proteins as expression of neither protein is restricted to smooth muscle cells. For example, SM α-actin is expressed in skeletal muscle myoblasts that do not express myocardin and the 130kDa MLCK is expressed in most adult cell types.(23,24). Together these data suggest that the expression of SM α-actin and the 130kDa MLCK may occur by myocardin dependent pathways in some cell types and by myocardin independent pathways in other cells that do not express myocardin.

Consistent with previous reports (2-4), our results demonstrate that an intact CArG element is required but not sufficient for telokin promoter activation by myocardin (Figure 4). Although essential for myocardin activation the precise sequence of the CArG box has little effect on the ability of myocardin to activate the promoter. Within the telokin promoter at least two additional regions (-80 to -66 and +36 to +82) are required to act in concert with the CArG box to facilitate high levels of promoter activation by myocardin (Figure 4). Although this combination of elements is sufficient to confer a significant amount of myocardin activation to a minimal thymidine kinase promoter, these elements are not sufficient to confer increased myocardin responsiveness to the c-fos promoter (Figure 5D). These data would suggest that, in addition to lacking key positive acting cis-regulatory elements, the c-fos promoter also contains a negative
regulatory region located between nucleotides –300 and +39 that blocks the activity of these positive elements.

Based on a recent report demonstrating that growth signals can repress smooth muscle-specific genes by triggering the displacement of myocardin from SRF by ELK1, an ets family protein, it is logical to propose that the inability of myocardin to activate the c-fos promoter is likely to be due to binding of ets to the serum response element of the c-fos promoter (20,21). Similarly, there are multiple ets binding sites surrounding the CArG boxes in the Egr-1 promoter that may be responsible for the inability of myocardin to activate this promoter despite the presence of multiple CArG boxes(25). However, although our data demonstrated that the ets binding site in the c-fos promoter is partially involved in inhibiting the activation of this promoter by myocardin, when the ets-binding site and CArG box in the c-fos promoter were replaced with the corresponding sequences from the telokin promoter the mutant c-fos promoter remained refractile to myocardin stimulation (Figure 5). Conversely when the AT-rich region and CArG box from the telokin promoter were replaced with the ets binding site and CArG box from the c-fos promoter the mutant telokin promoter remained strongly activated by myocardin. Together these data would suggest that the regions 3’ of the CArG boxes of the c-fos (-300 to +39) and telokin (-55 to +82) promoters, rather than the EBS/AT-rich sequences or CArG boxes, are critical for determining the promoters’ responsiveness to myocardin. Presumably each region binds to specific trans-acting factors that interact with myocardin and/or SRF to modify their function. The –55 to +82 region in the telokin gene is highly conserved between species being 90% identical between mouse, rabbit and human genes also suggesting that this region contains important regulatory elements (26). The identity of the key regulatory factors is currently unknown, however, it is tempting to speculate that these factors may comprise part of the transcription initiation complex that forms on each gene. This proposal arises from the observation that the putative regulatory regions span the transcription initiation sites and that the telokin and c-fos promoters utilize different cis-elements to initiate transcription. The telokin promoter initiates transcription from multiple start sites spanning approximately
70-80bp in a TATA-independent manner whereas the c-fos gene is a classical TATA-dependent gene with a single transcription initiation site. Although the core components of the transcription initiation complex will be identical on both genes, additional accessory factors may be promoter specific. This raises the possibility that specific components of the transcription initiation complexes may be required for myocardin to strongly activate transcription.

In summary, our results suggest that the promoter-specific effects of myocardin, and likely also MRTF-A, result from a complex interaction of positive regulatory elements that include at least one CArG box in responsive genes together with negative regulatory elements in unresponsive genes that block the activity of myocardin family members.
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Figure Legends

Figure 1. Effects of myocardin on telokin promoter activity in different cell lines. 400ng of mouse myocardin, or an empty expression vector plasmid were co-transfected, together with 400ng of promoter-luciferase reporter genes containing either the mouse telokin promoter (T370, -190+171), the smooth muscle myosin heavy chain promoter (MHC, -4,200 to +11600), the smooth muscle α-actin promoter (SM α-actin, -2,555 to +2813), the SM22α promoter (-475 to +61), the c-fos promoter (-605 to +39) or the Egr1 gene promoter (-637 to +79) and 200ng of a thymidine kinase (TK) promoter driven renilla luciferase internal control plasmid into 10T1/2 fibroblast cells, COS cells or A10 SMCs, as indicated. The level of promoter activity was determined by measurement of the firefly luciferase activity relative to the control renilla luciferase. Fold-activation of promoter activity relative to vector control transfections is presented as mean ± SEM of 6 samples.

Figure 2. Effects of wild-type and LZ mutant myocardin over-expression on endogenous telokin expression in 10T1/2 cells. (A) Mouse wild-type or LZ mutant myocardin expression vector, or empty plasmid pcDNA3.1 was transiently transfected into 10T1/2 cells. 24h post-transfection total RNA was harvested from cells using TRIzol reagent and RT-PCR was performed to detect endogenous telokin, SM22α, and c-fos expression with SuperScript One-step RT-PCR system (Invitrogen, Carlsbad, CA). GAPDH house keeping gene served as an internal control showing the equal RNA input and RT-PCR reaction efficiency. (B) 10T1/2 cells were seeded in 6-well plates overnight
and then transduced with adenovirus encoding LacZ or myocardin as indicated, for 4 hours. 72 hours following infection protein extracts were prepared from infected cells and analyzed by Western Blotting to detect expression of endogenous proteins. (C) Wild type myocardin, myocardin LZ mutant or control LacZ adenovirus were used to transduce 10T1/2 cells and Western Blotting analysis was carried out as described above in ‘B’. (D) Myocardin (empty bars), myocardin LZ mutant (hatched bars) or an empty expression vector plasmid were co-transfected, together with promoter-luciferase reporter genes containing either the mouse telokin promoter, SM22α, MHC, SMα-actin promoter and a thymidine kinase (TK) promoter driven renilla luciferase internal control plasmid into 10T1/2 fibroblast cells. The level of promoter activity was determined by measurement of the firefly luciferase activity relative to the control renilla luciferase. An arbitrary value of 100 was assigned to the activation of the promoters by myocardin alone. Data presented are mean±SEM of 6 samples.

Figure 3. Effects of myocardin siRNA on telokin promoter activity and expression in A10 cells. (A). COS cells were co-transfected with plasmid encoding myocardin, myocardin siRNA or a scrambled siRNA control as indicated. 24 hours following transfection protein extracts were prepared from transient transfected cells and analyzed by Western Blotting. The level of myocardin siRNA or control siRNA transfection is indicated by GFP expression, which is encoded on the same plasmid construct. (B). A10 cells were transduced with adenovirus encoding scrambled siRNA control or myocardin siRNA, as indicated. 72 hours following infection total RNA was
prepared from non-infected or infected cells and analyzed by RT-PCR. The level of siRNA expression is indicated by the expression of GFP, which is encoded on the same virus. (C) A10 cells were transduced with adenoviruses as described in (B) and then total protein was harvested and analyzed by Western Blotting. Protein expression was quantitated by densitometric analysis of the blots and telokin, SM22\(^\alpha\) and calponin expression were found to be significantly decreased by myocardin siRNA treated cells (p<0.05, student nonpaired \(t\)-test). (D) Rabbit telokin promoter (T400) and minimal thymidine kinase promoter-luciferase constructs were transiently transfected into A10 cells, together with either 300 or 600ng of myocardin siRNA pshuttle or scramble siRNA control pshuttle plasmids or vector alone, as indicated. 24 hours later extracts were prepared for luciferase assays. Luciferase activity was normalized to empty vector transfections. Values presented are the mean±SEM and samples that were statistically different from controls are indicated by an asterisk (P<0.001).

**Figure 4. Multiple cis-elements in the telokin promoter are involved in the activation by myocardin.** (A) The mouse telokin promoter CArG box (CCTTTTATGG) was mutated such that it could not bind SRF (CCTTTTCTAG), or to c-fos promoter CArG box sequence (CCATATTAGG), or near CArG (CCAAATATGG) from the SM22\(^\alpha\) gene using the QuickChange Site-Directed mutagenesis kit (Stratagene, La Jolla, CA). Reporter genes constructed using these mutant promoters were co-transfected with mouse myocardin or empty expression vector into 10T1/2 cells. The fold change in promoter activity relative to vector control transfections is presented as mean±SEM of 6
samples. (B) Truncation mutants of telokin promoter reporter genes together with myocardin or empty vector as a control were transfected into 10T1/2 cells and luciferase activity measured 24hr later. The fold change in promoter activity relative to vector control transfections is presented as mean±SEM of 6 samples. (C) The –80 to –66 AT-rich region (AT), -66 to –56 CArG box (CArG) or +36 to +82 regions of the telokin promoter were fused individually or in combination to a minimal TK promoter as indicated. Promoter-luciferase reporter genes were transfected into 10T1/2 cells together with or without myocardin expression vector and then luciferase activity determined. The fold change in promoter activity relative to vector control transfections is presented as mean±SEM of 6 samples.

Figure 5. Mapping the elements in telokin and c-fos promoters responsible for myocardin selectivity. (A) Schematic diagram showing the sequences of the CArG box and adjacent 5’-sequence from c-fos and telokin promoters. The c-fos promoter (open bar) EBS binding site within the c-fos 5’ flanking region (dark gray) and CArG box (black) and the telokin promoter (light grey) AT-rich region (hatched) and CArG box (checkered) are indicated. The EBS core binding site and CArG box were underlined within the sequences. (B) A series of deletion or mutations of c-fos promoter reporter genes were generated and co-transfected together with myocardin or empty expression vector into 10T1/2 cells and luciferase activity determined. The wild type c-fos promoter is shown at the top of the graph. An ‘X’ through a domain donates a mutant element that is no longer able to bind to its’ cognate trans-factor. Elements that were mutated to
match the corresponding region of the telokin promoter are shaded and pattern coded
to match the telokin sequence shown in (A). The fold change in promoter activity
relative to vector control transfections is presented as mean±SEM of 6 samples. (C)
The wild-type (top of panel) or mutant telokin promoter reporter gene constructs were
transiently co-transfected together with myocardin or empty expression vector into
10T1/2 cells and luciferase activity measured. The promoter origin of each domain of
the mutant reporter genes is indicated using the same scheme as described in (A). (D)
Chimeric c-fos promoter reporter genes were generated as indicated and analyzed as
described in (A).
Figure 1

**10T1/2 cells**

- c-fos
- Egr1
- SMα-actin
- MHC
- SM22α
- T370
- T400

**Fold Activation**

**COS cells**

- c-fos
- SMα-actin
- MHC
- SM22α
- T370
- T400

**Fold Activation**

**A10 cells**

- TK
- c-fos
- SMα-actin
- MHC
- SM22α
- T370
- T400

**Fold Activation**
Figure 3

A

myocardin
GFP
β-actin
++
++
myocardin siRNA
control siRNA

B

non-infected
control
control siRNA
myocardin siRNA

myocardin
GFP
GAPDH
telokin

C

control
siRNA
myocardin
siRNA

GFP
β-actin
SM α-actin
telokin
SM22α
calponin
MLCK 130K

D

Relative Luciferase Activity

myocardin siRNA (ng) 300 600 300 600
control siRNA (ng) – – – –

T400 TK

*
Figure 4

A

T370

CArG mutant

Mutated to c-fos CArG

Mutated to SM22α near-CArG

Fold Activation

B

T370(-190+171)

T272(-190+82)

T172(-90+82)

T164(-80+82)

T148(-66+82)

T125(-90+36)

T226(-190+36)

Fold Activation

-190 -80 -66 -56 +36 +82 +171

AT-rich CArG

C

TK

2X(AT)-TK

(CArG)-TK

TK(+36+82)

(AT-CArG)-TK

(CArG)-TK(+36+82)

(AT-CArG)-TK(+36+82)

Fold Activation
Figure 5
Mechanisms responsible for the promoter specific effects of myocardin
Jiliang Zhou and B. Paul Herring

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