Forced Expression of Myocardin Is Not Sufficient for Induction of Smooth Muscle Differentiation in Multipotential Embryonic Cells

Tadashi Yoshida, Keiko Kawai-Kowase, Gary K. Owens

Objective—Myocardin, a coactivator of serum response factor, has been shown to be required for expression of multiple CArG-dependent smooth muscle cell (SMC) marker genes. The aim of the present study was to determine whether myocardin alone is sufficient to induce SMC lineage in multipotential stem cells as evidenced by activation of the entire SMC differentiation program.

Methods and Results—Overexpression of myocardin induced only a subset of SMC marker genes, including smooth muscle (SM) α-actin, SM–myosin heavy chain (MHC), SM22α, calponin, and desmin in A404 SMC precursor cells, whereas expression of smoothelin-B, aortic carboxypeptidase-like protein, and focal adhesion kinase-related nonkinase, whose promoters lack efficacious CArG elements, was not induced. Similar results were obtained in cultured SMCs, 10T1/2 cells, and embryonic stem cells. Moreover, myocardin inappropriately induced expression of skeletal and cardiac CArG-dependent genes in cultured SMCs. Stable overexpression of dominant-negative myocardin in A404 cells resulted in impaired induction of SM α-actin and SM–MHC by all trans-retinoic acid but had no effect on induction of smoothelin-B and selective aortic carboxypeptidase-like protein expression.

Conclusions—Taken together with previous studies, results demonstrate that myocardin is required for the induction of CArG-dependent SMC marker genes but is not sufficient to initiate the complete SMC differentiation program.

Key Words: smooth muscle cells • transcriptional coactivator • serum response factor • CArG element

Smooth muscle cells (SMCs) play pivotal roles in vascular development and in diseases such as atherosclerosis. Under normal circumstances, differentiated SMCs are highly specialized for their contractile function and express a unique repertoire of contractile proteins, contractile agonist receptors, and signaling molecules to perform this function. The expression levels of most of these SMC-specific/SMC-selective proteins reflect the differentiation state of SMCs. Therefore, these proteins are considered as useful markers for studying the control of SMC differentiation.

There has been considerable progress in elucidation of molecular mechanisms that regulate cell-selective expression of SMC differentiation markers in recent years. For example, expression of most SMC differentiation markers characterized to date, including smooth muscle (SM) α-actin, SM–myosin heavy chain (MHC), SM22α, calponin, and desmin, has been shown to be regulated at the transcriptional level and dependent on multiple CArG elements located within their promoter-enhancer regions. The CArG element has the general sequence motif, CC(A/T-rich)GG and binds the ubiquitously expressed transcription factor, serum response factor (SRF). CArG elements are also required for expression of many growth factor-inducible genes including c-fos and egr-1, and a major challenge has been to identify mechanisms by which CArG–SRF–dependent mechanisms can simultaneously contribute to the apparently disparate processes: activation of SMC-selective genes associated with cellular differentiation versus activation of growth-regulated genes. A major breakthrough for this field was the discovery of myocardin, a potent SRF coactivator that is expressed exclusively in SMCs and cardiomyocytes, which is extremely efficacious in activating multiple CArG-dependent SMC marker genes, including SM α-actin, SM-MHC, SM22α, and calponin. The promoter selectivity of myocardin appeared to be dependent on the cooperative interaction of multiple CArG elements. That is, growth-regulated genes such as c-fos that contain a single CArG element were not activated by myocardin. Short interfering RNA-induced suppression of myocardin in cultured SMCs was associated with marked decreases in transcription of SM α-actin, SM–MHC, and SM22α. Knockout of myocardin in mice resulted in embryonic lethality and was associated with failed SMC investment and differentiation, although the failure to form SM tissues may have been secondary to defects in...
formation of the extra-embryonic circulation. In addition, we recently provided evidence suggesting that myocardin served as a point of convergence in mediating effects of environmental cues on expression of SMC marker genes in that angiotensin II-induced expression of SM α-actin was associated with increased expression of myocardin, and short interfering RNA-induced suppression of myocardin attenuated angiotensin II-induced transcription of this gene. Taken together, the preceding results provide compelling evidence that myocardin plays a key role in regulation of SMC differentiation.

However, a major limitation in previous studies of myocardin, including our own, is that they did not examine the role of myocardin in regulation of expression of SMC-selective genes that appear to be CArG-independent yet critical for the differentiated function of SMC. For example, Layne et al13 showed that CarG elements were not required for expression of the SMC-selective aortic carboxypeptidase-like protein (ACLP) gene in SMCs using transgenic mice. Likewise, Smith et al16 demonstrated that a −163/+147-bp telokin promoter that contains only one CarG element was sufficient to drive SMC-specific expression in transgenic mice. Smoothelin is a cytoskeletal protein whose expression is highly specific for SMCs of contractile phenotype. There are 2 isoforms: a 59-kDa smoothelin-A expressed in the visceral SMCs and a 110-kDa smoothelin-B expressed in the vascular SMCs.18 In the smoothelin-B promoter, there are 2 CarG-like elements, but deletion of these elements had no effect on its promoter activity in PAC1 cells, indicating that expression of smoothelin-B is CArG-independent. Finally, the promoter of the SMC-selective gene, focal adhesion kinase-related nonkinase, also appears to lack CarG elements.19,20 As such, a number of SMC-selective genes that are thought to be critically important for SMC differentiation are not dependent on multiple CarG elements for their expression.

Therefore, the goal of the present study was to test whether myocardin is sufficient to induce the full SMC differentiation program in a variety of cultured cell systems including pluripotent embryonic stem (ES) cells, multipotent 10T1/2 embryonic fibroblasts that have potential to differentiate into SMC in response to transforming growth factor-β,21 a unique SMC progenitor line designated A404 cell,22 and adult rat aortic SMCs.

Methods

Cell Culture and Adenovirus Infection

A404 cells were cultured and differentiated as described previously.22 Rat aortic SMCs, 10T1/2 cells, and mouse ES cells were cultured as previously described.10,11 Mouse ES cells were kindly provided by Dr. Alfred Nordheim (Universität Tübingen, Germany). Approximately 24 hours before adenoviral infection, cells were seeded at 105 cells/cm2. Cells were infected with replication-deficient adenovirus encoding the flag-tagged myocardin (Ad/Myo) gene or an empty adenovirus (Ad/Emp) for 1 hour at a multiplicity of infection of 50. After 48-hour incubations, total RNA and total cell lysates were prepared.

Reverse-Transcription Polymerase Chain Reaction

To quantify the mRNA expression, semiquantitative or real-time reverse-transcription polymerase chain reaction (RT-PCR) analyses were performed. Sequences for primers and probes are shown (Table I, available online at http://atvb.ahajournals.org).

Western Immunoblotting

Cells were lysed in RIPA buffer (150 mmol/L NaCl, 50 mmol/L Tris HCl (pH 8.0), 0.1% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS). Cell lysates were centrifuged for 10 minutes at 10 000g to pellet insoluble material. Protein concentrations were determined by DC protein assay kit (Bio-Rad Laboratories, Inc, Hercules, Calif). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. The membrane was immunoblotted with anti-SM α-actin (1A4) antibody, anti-SM–MHC antibody (kindly provided by Dr. U. Grosse-Stewart), anti-ACLP antibody (kindly provided by Dr. Matthew D. Layne, Harvard Medical School, Boston, Mass), anti-telokin antibody (kindly provided by Dr. B. Paul Herring, Indiana University, Indianapolis), or anti-FAK carboxyl-terminal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). Bound antibodies were visualized using an ECL Western blotting system (Amersham Biosciences Corp, Piscataway, NJ).

Transient Transfection and Luciferase Assay

SM α-actin promoter-luciferase construct, pEA (−2.6/+2.8),10 ACLP promoter-luciferase construct, pACLP (−2502/+176), or pG3-basic plasmid (Promega Corp, Madison, Wis) was cotransfected with myocardin expression plasmid8 into 10T1/2 cells by FuGENE6 (Roche Diagnostics Corp, Indianapolis, Ind) and incubated for 48 hours. Luciferase activity was measured as described previously.10

A404 DN-Myo Cells

Stable cell lines expressing dominant-negative forms of myocardin were isolated after transfection of A404 cells with pcDNA–MyoCΔ581 (DN-Myo #321 and #322) or MyoCΔ585 (DN-Myo #356). Control A404 cells were isolated by transfection with an empty plasmid (control #431).

Results

Expression of Myocardin mRNA Was Increased During the Differentiation of A404 Cells Into a SMC Lineage

Previously, we showed that expression of myocardin mRNA was induced in A404 cells by all trans-retinoic acid (RA) as determined by semiquantitative RT-PCR. In the present study, we extended these results by real-time RT-PCR. A404 cells were treated with 1 μmol/L RA from day 0 to day 3 and with 0.5 μg/mL pyruvocin from day 4 to day 6 to induce differentiation into SMCs as described previously.22 As shown in Figure 1, SM α-actin and SM–MHC mRNA expression was significantly induced by 150-fold and 250-fold, respectively. Myocardin mRNA expression was also increased by 7.5-fold, whereas SRF mRNA expression was unaltered. Although the increase in myocardin expression was small relative to the increases in SM α-actin and SM–MHC expression, it seemed to precede the induction of SM α-actin and SM–MHC expression. These data are consistent with a model wherein myocardin may play a role in RA-induced expression of SM α-actin and SM–MHC genes.

Overexpression of Myocardin Failed to Activate a Subset of SMC Marker Genes in A404 cells

Our previous studies showed that RA treatment of A404 cells induced expression of all of SMC differentiation marker genes examined, including SM α-actin, SM–MHC, SM22α, SM22β, and SM130.
calponin, desmin, and smoothelin. To address whether myocardin also induces expression of these SMC marker genes, effects of adenovirus-mediated overexpression of myocardin on expression of SMC marker genes and transcription factors implicated in control of SMC differentiation were examined by semiquantitative RT-PCR. Myocardin substantially induced mRNA expression of CArG-dependent SMC marker genes, including SMα-actin, SM–MHC, SM22α, calponin, and desmin in A404 cells (Figure 2A). However, myocardin had no effect on a subset of SMC marker genes,

Figure 1. Expression of SMα-actin, SM–MHC, myocardin, and SRF mRNA in differentiating A404 cells as determined by real-time RT-PCR. A404 cells were treated with 1 μmol/L RA from day 0 to day 3, split on day 4, and treated with 0.5 μg/mL puromycin from day 4 to day 6 as described previously. U and D1 to D8 denote day 0 and day 1 to day 8, respectively. An arbitrary value of 1 was assigned to RNA samples from day 0. Values represent means±SEM.

Figure 2. Effect of adenovirus-mediated overexpression of myocardin on expression of multiple SMC marker genes in A404 cells. A404 cells were infected with Ad/Myo or Ad/Emp and incubated for 48 hours. A, Expression of multiple SMC marker genes was measured by semiquantitative RT-PCR. B, Expression of SMC markers was examined by Western blotting. Differentiated (D7), RA-treated (D2), and untreated (U) A404 cells were used as controls.
including smoothelin-B and ACLP. Lack of induction of smoothelin-B and ACLP expression by myocardin was also confirmed by quantitative real-time RT-PCR (data not shown). Taken together, these results indicate that myocardin is insufficient to initiate all of the SMC differentiation programs and that it also induces a subset of CArG-dependent skeletal and cardiac marker genes.

Myocardin Did Not Induce Several SMC Marker Genes in Rat Aortic SMCs, 10T1/2, and ES Cells

To address the capacity of myocardin to promote SMC differentiation/maturation, the effect of overexpression of myocardin was examined in rat aortic SMCs. As shown in Figure 4A, adenovirus-mediated overexpression of myocardin increased the expression of SM α-actin and SM-MHC mRNA, as reported previously. However, myocardin did not alter levels of smoothelin-B and ACLP transcripts. Myocardin also had no effect on focal adhesion kinase-related nonkinase protein expression levels (Figure 4B). However, myocardin increased telokin expression in SMCs. Moreover, myocardin inappropriately induced skeletal and cardiac α-actin and heart-specific atrial natriuretic factor expression in SMCs. Whereas the latter effects may be an artifact of expression of myocardin at supraphysiological concentrations, the fact that even high concentrations of myocardin failed to activate a subset of SMC marker genes suggest that it is not sufficient, even in SMC context, to induce the full repertoire of genes characteristic of differentiated SMC.

The effect of myocardin was also tested in 10T1/2, ES, and ES SRF cells. Consistent with the previous reports, expression of SM α-actin and SM-MHC mRNA was induced by myocardin in 10T1/2 and ES cells, whereas it was not induced in ES SRF cells (Figure I, available online at http://atvb.ahajournals.org). In these cells, neither ACLP nor smoothelin-B mRNA expression was induced. Moreover, cotransfection studies revealed that myocardin did not induce transcription of ACLP promoter-luciferase construct, whereas it markedly induced transcription of the SM α-actin gene (Figure 5). Because both constructs contain sufficient regions to drive expression in SMCs in vivo in transgenic mice, and because the ACLP gene has no CArG element in this promoter, these results suggest that the effect of myocardin in control of SMC differentiation is limited to activation of CArG-dependent SMC marker genes.

Dominant-Negative Myocardin Inhibited the Induction of SM α-Actin and SM–MHC Expression but Not the Induction of Smoothelin-B and ACLP Expression

To further test the role of myocardin in SMC differentiation, A404 DN-Myo cells that stably expressed dominant-negative forms of myocardin were developed (Figure II, available online at http://atvb.ahajournals.org) and tested to see whether RA induced expression of SMC marker genes in them. Dominant-negative forms of myocardin partially suppressed RA-induced expression of SM α-actin and SM–MHC (Figure 6A and 6B). However, RA induced smoothelin-B and
ACLP mRNA expression in A404-DNMyo cells to the same extent as in control cells (Figure 6C and 6D), suggesting that smoothelin-B and ACLP expression in A404 cells was not dependent on myocardin.

Discussion

Members of the MyoD family of basic helix-loop-helix (bHLH) transcription factors function to establish the myogenic lineage and activate genes that drive skeletal muscle differentiation. In contrast, the results of our present studies provide compelling evidence that myocardin alone is not sufficient to induce the full SMC differentiation program, but rather support a model whereby SMC differentiation is dependent on complex combinatorial interactions of multiple cis elements and their binding factors and modulation of their activity by environmental cues such as angiotensin II, transforming growth factor-β, and platelet-derived growth factor-BB. Indeed, myocardin failed to activate some of the SMC marker genes in undifferentiated A404 cells in which several transcription factors, including GATA family, MEF2B, and dHAND, are not expressed, whereas all of the SMC marker genes were expressed in RA-treated differentiated A404 cells with these transcription factors. Although myocardin plays a critical role in regulating multiple CArG-dependent SMC marker genes, coordinated activation of the entire repertoire of genes characteristic of fully differentiated SMC appears to be dependent on a variety of other transcription factors, including GATA family, MEF2 family, bHLH family, and homeodomain proteins. Although little information is available thus far concerning the regulation of CArG-independent gene expression, there are several E-boxes (binding sites for bHLH proteins), Sp-1 sites, a transforming growth factor-β control element, and GATA boxes in the smoothelin-B promoter. Layne et al showed that transcription of the ACLP gene was increased by ubiquitous factors, Sp-1 and Sp-3, in cultured SMCs. Identification of the cooperative mechanisms by which these transcription factors and their cofactors interact with SRF and myocardin to regulate SMC-specific expression represents a key challenge for the field.

Results of the present studies showed that endogenous expression of telokin, whose −163/+147-bp promoter that contains only one CArG element was sufficient to drive SMC-specific expression in transgenic mice, was also increased by myocardin in SMCs. This is consistent with our previous results using promoter-reporter assays in that retention of a single CArG B element within the context of the −2.6/+2.8-kb SM α-actin promoter was sufficient to confer partial myocardin responsiveness. Moreover, Wang et al showed that cysteine-rich protein 1 gene was also induced by
myocardin in 10T1/2 cells, although its 5-kb promoter-enhancer, which contained sufficient region in vivo to drive expression in arterial SMCs, contained only one CArG element. However, it is also true that single CArG-containing c-fos gene was not induced by myocardin and that retention of single CArG A or intronic CArG within 2.6/2.8 kb SMα-actin promoter had no responsiveness to myocardin. These findings suggest that specific DNA sequences both within and surrounding the CArG element may alter the responsiveness to myocardin. That is, although it is apparent that 2 CArG elements are enough to confer gene induction by myocardin, several kinds of single CArG elements alone or a single CArG element plus its flanking sequences also appear to be capable of inducing gene expression by myocardin. Further studies are required to explore regulatory mechanisms that are necessary for induction of single CArG-containing cell-selective differentiation marker genes in SMCs.

Of interest, expression of skeletal and cardiac CArG-dependent genes was also induced in SMCs by overexpression of myocardin. Chen et al. also showed that stable expression of myocardin induced endogenous atrial natriuretic factor expression, as well as SMα-actin and calponin, in L6 myofibroblasts. Moreover, Wang et al. showed that myocardin could activate an artificial promoter consisting of 4 c-fos CArG elements coupled to the basal promoter. Based on evidence accumulated thus far, it appears that myocardin can potently activate virtually any promoter-enhancer containing multiple CArG elements, although it must be appreciated that inappropriate induction of multiple CArG-containing non-SMC genes in the preceding studies may be a function of expression of myocardin at supraphysiological concentrations. Nevertheless, studies raise a critical question: what are the mechanisms by which myocardin selectively activates CArG-containing SMC genes but not skeletal and cardiac CArG-containing genes in SMCs? There are several potential mechanisms. For example, SMCs may contain regulatory factors/mechanisms that selectively repress expression of inappropriate CArG-containing genes through mechanisms that control accessibility of SRF binding to CArG elements. Consistent with this, we previously found that RA treatment of A404 cells was associated with selective enrichment of SRF binding to the CArG-containing regions of SMC promoters within intact chromatin, but not the CArG-containing skeletal α-actin promoter. It is also possible that subtle difference in DNA sequences within the CArG elements may play a role in determining not only SRF binding, but also subsequent recruitment of myocardin. Another possibility is that the phasing and spacing of CArG elements may be a critical determinant of myocardin gene selectivity. In this regard, it is interesting that the SRF promoter contains 2 highly conserved CArG elements, but results of our present study showed that expression of SRF gene was not altered by myocardin. Consistent with our results, Spencer et al. showed that mutation of these CArG elements had no effect on basal transcriptional activity, suggesting that they did not work as binding sites for SRF–myocardin complex. Because they are only 10 bp apart,
it may be too close to function as CArG-SRF-myocardin complexes. Taken together, results indicate that although myocardin plays a key role in controlling CArG-dependent gene expression, overall regulation of SMC differentiation is extremely complex and involves interactions of many factors and signaling pathways yet to be identified.

Although there is extensive evidence that myocardin plays a key role in regulation of SM CArG-dependent gene expression, overall regulation of SMC differentiation is extremely complex and involves interactions of many factors and signaling pathways yet to be identified.

As mentioned earlier, myocardin plays a key role in controlling CArG-dependent gene expression. For example, SM α-actin and SM22α also appear to be inconsistent with it being responsible for initial induction of SMC lineage during development. For example, SM α-actin and SM22α are expressed as early as E9.5 in the cells surrounding the dorsal aorta, whereas myocardin is not detectable in this tissue until E12.5. Because myocardin transcript is detected in the primitive heart at E9.5, initial induction of these early SMC markers may not require myocardin. In this regard, it is noteworthy that many CArG-dependent SMC marker genes that are activated by myocardin are induced at different time points in SMC development. These findings further support results of the present studies indicating that myocardin alone is insufficient to initiate the entire SMC differentiation program. Of interest, Wang et al showed that expression of the myocardin-related factors, MRTF-A/MAL/MKL1/BSAC and MRTF-B/MKL2, preceded the expression of myocardin, and they were expressed throughout the embryo at E10.5. In addition, they showed that MRTF-A/MAL/MKL1/BSAC potentiated SM22α transcription, even in the absence of myocardin. It is thus possible that these myocardin-related factors may also contribute to initial expression of early SMC markers.

In summary, the results of the present studies provide strong evidence showing that myocardin plays a critical role in regulation of expression of multiple CArG-SRF-dependent genes, but that myocardin alone is insufficient to induce the entire repertoire of genes characteristic of fully differentiated SMC in multiple cultured cell systems. Rather, results of this and previous studies suggest that myocardin is one key component of complex mechanisms involved in regulation of SMC differentiation.

Acknowledgments

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References

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## Supplemental Table

**Oligonucleotide sequences used in this experiment**

### Real-time RT-PCR

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<th>Oligonucleotide</th>
<th>Sense (S)</th>
<th>Antisense (AS)</th>
<th>Probe (P)</th>
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**Supplemental Figure I.** Effect of myocardin on SMC marker genes in 10T1/2 and ES cells. 

**A:** 10T1/2 cells were infected with Ad/Myo or Ad/Emp and incubated for 48 h. Expression of SMC marker genes was determined by semi-quantitative RT-PCR. **B:** ES and ES SRF-/- cells were infected with Ad/Myo or Ad/Emp and incubated for 48 h. Expression of SMC marker genes was measured by semi-quantitative RT-PCR.
Supplemental Figure II. Characterization of A404 DN-Myo cells. A: Expression of MyoCΔ381 and MyoCΔ585 was confirmed by Western blotting using anti-flag antibody. B: Immunofluorescence studies showing the expression of flag-tagged dominant-negative myocardin in A404 DN-Myo cells.