Diaphanous 1 and 2 Regulate Smooth Muscle Cell Differentiation by Activating the Myocardin-Related Transcription Factors

Dean P. Staus, Alicia L. Blaker, Joan M. Taylor, Christopher P. Mack

Objective—We have previously shown that smooth muscle cell (SMC) differentiation marker gene expression is regulated by the small GTPase, RhoA. The objective of the present study was to determine the contributions of the RhoA effectors, diaphanous 1 and 2 (mDia1 and mDia2), to this regulatory mechanism.

Methods and Results—mDia1 and mDia2 are expressed highly in aortic SMCs and in a number of SMC-containing organs including bladder, lung, and esophagus. Activation of mDia1/2 signaling by RhoA strongly stimulated SMC-specific promoter activity in multiple cell-types including primary aortic SMCs, and stimulated endogenous SM α-actin expression in 10T1/2 cells. Expression of a dominant negative Dia1 variant that inhibits both mDia1 and mDia2 significantly decreased SMC-specific transcription in SMCs. The effects of mDia1 and mDia2 required the presence of SRF and the activity of the myocardin transcription factors and were dependent on changes in actin polymerization. Importantly, stimulation of mDia1/2 signaling synergistically enhanced the activities of the myocardin-related transcription factors, MRTF-A and MRTF-B, and this effect was attributable to increased nuclear localization of these factors.

Conclusions—These results indicate that RhoA-dependent signaling through mDia1/2 and the MRTFs is important for SMC-specific gene expression in SMCs. (Arterioscler Thromb Vasc Biol. 2007;27:478-486.)

Key Words: diaphanous • RhoA • SRF • myocardin • smooth muscle

A large number of environmental cues including growth factors, contractile agonists, matrix components, and cell–cell interactions have been shown to regulate smooth muscle cell (SMC) differentiation (see Owens et al1 for review). However, the signaling mechanisms by which these factors regulate SMC-specific transcription are poorly understood. Expression of most SMC differentiation markers, including SM α-actin, SM myosin heavy chain (SM MHC), SM22, calponin, and telokin, requires serum response factor (SRF) binding to one or more conserved CArG (CC(A/T)GG) cis elements present within their promoters.2–7 However, because SRF is a ubiquitous transcription factor that also regulates the early response growth genes, c-fos and egr-1 (see Treisman et al8 for review), it is clear that additional factors are required to differentially regulate these gene programs.

See page 448

The identification of the myocardin family of SRF cofactors by Wang et al was an important advance in our understanding of the transcription mechanisms that regulate SMC-specific transcription (see9 and10 for reviews). The founding member of this family, myocardin, strongly enhances SMC differentiation marker gene expression through direct interactions with SRF. Myocardin is expressed specifically in heart and SMCs and its genetic deletion leads to embryonic lethality attributable to failure of SMC differentiation/investment of the developing aorta. The Myocardin-Related Transcription Factors, MRTF-A/ MKL-1 and MRTF-B/MKL-2, are also expressed in some SMC sub-types including the aorta, and like myocardin, can strongly activate SMC differentiation marker gene expression.11–15 Because the MRTFs are thought to be expressed more widely, their involvement in regulating cell-type-specific gene expression is less clear.

We have previously shown that the small GTPase, RhoA, regulates SM differentiation, and we hypothesize that RhoA acts as an integrator of many of the environmental cues that regulate this process.16 For example, SMC-specific transcription in primary SMC cultures was stimulated by expression of constitutively active of RhoA (L63), and completely abolished by the RhoA inhibitor, C3 transferase. Neither of these interventions had dramatic effects on c-fos expression, suggesting that this pathway may be important for differentially regulating SRF-dependent growth and differentiation. A number of laboratories including our own have demonstrated that the downstream effects of RhoA on the actin cytoskeleton were critical for activation of SRF-dependent transcription.11,16–19 The RhoA effector, Rho-kinase (ROK), has been the most thoroughly studied in this regard because it has major effects on actin...
polymerization, stress fiber formation, and contractility (see Hall for review). Interestingly, the ROK inhibitor, Y-27632, only partially inhibited SMC differentiation marker gene expression, strongly suggesting that other RhoA effectors are important.16,21

Two other RhoA effectors that have been shown to stimulate actin polymerization are the diaphanous formins 1 and 2 (mDia1 and mDia2).22–24 These proteins belong to the subfamily of diaphanous-related formins (DRFs) that also includes Dia3 and FHOD1. Previous studies have demonstrated that the DRFs act as potent actin and microtubule polymerizing factors that regulate a number of processes including cell migration and division (see Higgs and Wallar et al for reviews). The DRFs are identified by 3 highly conserved formin homology (FH) domains, a conserved GTPase binding domain (GBD) that interacts with Rho family GTPases, and a Diaphanous Auto-regulatory Domain (DAD). The molecular mechanisms that control DRF/mDia activity have been fairly well described. In the inactive state, the DRFs are inhibited by an interaction between the DAD and the N-terminal Diaphanous Inhibitory Domain (DID) (Figure 1a). High affinity binding of activated RhoA (or another GTPase) to the GBD disrupts the DAD-DID interaction to expose the catalytically active FH1/FH2 region. The precise mechanisms by which mDia1/2 stimulate actin polymerization are not completely understood. However, mDia1/2 seem to promote polymerization from actin filament barbed ends in cooperation with the actin binding protein, profilin.27–29

The goal of these studies was to determine the contributions of mDia1 and mDia2 to the regulation of SMC-specific transcription. Our results demonstrate that both of these RhoA effectors are highly expressed in SMCs, that they strongly activate SMC-specific transcription, and that their effects are mediated by the myocardin family of SRF cofactors.

Methods

Plasmids and Reagents
mDia1 and mDia2 cDNAs were generous gifts from Shah Narumiya (Kyoto University, Japan). All mDia cDNAs were subcloned into flag pcDNA3.1 and/or pEGFP-C3 (Clontech) and include: full-
length mDia1, full-length mDia2, ∆GBDmDia1 (AA 238 to 1255), ∆GBDmDia2 (AA 257 to 1171), mDia2DAD (AA1030–1171), mDia1FH1FH2Δ1 (AA567-1182 minus the 20AA from 750 to 770), EGFP-∆GBDmDia1, and EGFP-∆GBDmDia2. Myocardin, MRTF-A, and MRTF-B cDNAs were generous gifts of Da-Zhi Wang (University of North Carolina, Chapel Hill). All myocardin cDNAs were subcloned into flag pcDNA3.1 and pEGFP-C3. The dominant negative MRTF-A was generated by polymerase chain reaction (PCR) and consists of AA 1 to 630.

Cell Culture, Transfections, and Reporter Assays
SMCs from rat thoracic aorta were isolated and cultured as previously described.11 10T1/2 and A7r5 SMC were obtained from ATCC. SRF+/− embryonic stem cells were a generous gift from Alfred Nordheim (Tubingen University, Germany) and have been previously described.30 For transfections, cells were cultured in 24- or 48-well plates, maintained in 10% serum, and transfected 24 hours after plating at 70% to 80% confluence using the transfection reagent, TransIT-LT1 (Mirus), as per protocol. The SM22, SM α-actin, and c-fos promoter luciferase reporter constructs have been previously described.11 The SM α-actin promoter constructs containing CArG mutations were a generous gift of Gary Owens (University of Virginia, Charlottesville) and have been previously described.31 When transfecting expression constructs (mDia1/2, MRTFs, etc.) the total amount of expression vector in each well was equalized by addition of empty vector. For experiments involving pharmacological inhibitors, cells were serum starved for 6 hours and then treated with Y-27632 (10 μmol/L) (Calbiochem) or Latrunculin B (0.5 μmol/L) (Calbiochem) for 24 hours before luciferase measurements.

RT-PCR and Western Blots
Samples were obtained from adult C57/B6 mice and confluent dishes of primary rat aortic SMC, A7r5 SMC, and 10T1/2 cells. For Western Blots, tissue and cells were lysed in RIPA buffer plus inhibitors. Protein concentrations were determined using the BCA assay (Pierce). Protein lysates (40 μg) from each tissue and cell line were run on an 10% SDS polyacrylamide gel, transferred to nitrocellulose, and probed with anti-mDia1, anti-mDia2 (generous gifts of Henry Higgs, Dartmouth Medical School, Hanover, NH), anti-SM α-actin (Sigma), anti-tubulin (Sigma), or anti-flag (Sigma) antibodies. In some experiments cells were serum starved for 24 hours and then treated with transforming growth factor (TGF)-β (1 ng/ml) for 24 hours before lysate preparation.

Immunohistochemistry
For immunohistochemical visualization of mDia1/2, the MRTFs, or SM α-actin, cells fixed in 3.7% paraformaldehyde were permeabilized in 0.5% Triton X-100 for 3 minutes, blocked in 20% goat serum/3% BSA for 2 hours, then exposed to antibody for 2 hours at a dilution of 1:500. Texas Red or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were used for visualization at 1:1000. In some studies Texas Red-conjugated phalloidin (1:400; Molecular Probes) was used to detect actin stress fibers, and DAPI (1:10 000) was used to detect nuclei.

Results
mDia 1/2 Signaling Regulates SMC-Specific Transcription
To test whether the RhoA effectors, mDia1 and mDia2, contribute to the regulation of SMC-specific transcription, we cotransfected murine mDia1 and mDia2 along with SM22 or SM α-actin promoter/luciferase constructs into multipotential 10T1/2 mouse cells. We and others have shown that these cells upregulate the expression of many SMC-specific genes when treated with TGF-β or sphingosine 1-phosphate (SIP), making them very useful for studying the regulation of SMC-specific transcription.11,32 Figure 1b demonstrates that expression of full length mDia1 or mDia2 induced a modest (2- to 3-fold) increase in SM22 and SM α-actin promoter activity. However, coexpression of constitutively active L63RhoA with mDia1 or mDia2 strongly and synergistically activated SM22 and SM α-actin promoter activity to approximately 25-fold, suggesting that basal RhoA activity is a limiting factor for mDia activation. Previous studies have shown that N-terminal truncations that remove the GBD/DID inhibitory domain of mDia1 and mDia2 (∆GBD) resulted in constitutive activation of these proteins. Remarkably, ∆GBDmDia1 and ∆GBDmDia2 activated SM22 and SM α-actin by 30- to 40-fold, indicating that these signaling molecules are powerful activators of SMC-specific transcription. The increased efficacy of mDia1 versus mDia2 was most likely attributable to increased expression of the mDia1 constructs (data not shown). ∆GBDmDia1 or ∆GBDmDia2 had comparatively little effect on c-fos promoter activity. Both ∆GBDmDia1 or ∆GBDmDia2 also activated the endogenous expression of SM α-actin as shown by the Western blot or immunohistochemistry (Figure 1d). Given that transfection efficiency in the 10T1/2 cell line was typically around 25%, this increase was as strong or stronger than that seen on treatment of cells with TGF-β. Interestingly, TGF-β significantly upregulated the expression of mDia2, but not mDia1 (data not shown).

mDia1 and mDia2 Are Highly Expressed in SMCs
To further investigate the role of mDia1 and mDia2, we analyzed their expression in tissues and cell lines. The Western blot shown in Figure 2a demonstrates that mDia1 and mDia2 are very highly expressed in primary rat aortic SMCs, A7r5 rat SMCs, and 10T1/2 cells as well as in numerous mouse tissues that contain a high SMC component, such as aorta, bladder, and lung. Given that mDia1 and mDia2 could strongly activate SMC-specific transcription and that these proteins were highly expressed in SMCs, it is likely that they play important roles in regulating SMC differentiation.

Endogenous mDia1/2 Signaling Regulates SMC-Specific Transcription in SMCs
We next tested whether mDia signaling was important for regulating SMC-specific promoter activity in SMCs. Expression of ∆GBDmDia1 or ∆GBDmDia2 in SMCs significantly upregulated SM22 and SM α-actin promoter activity (Figure 2b). However, these effects were much less dramatic than those observed in 10T1/2 cells, most likely because of the relatively high levels of SMC-specific transcriptional activity already exhibited by primary SMCs. To determine whether endogenous mDia1/2 signaling was required for SMC-specific promoter activity we used a dominant negative form of mDia1 (FH1FH2Δ1) originally described by Copeland et al, which has been shown to inhibit the function of both of these proteins.33 This strategy also allowed us to avoid known redundancy between mDia1 and mDia2 signaling. FH1FH2Δ1mDia1 completely inhibited the effects of ∆GBDmDia1 or ∆GBDmDia2 on SM22 promoter activity supporting its effects as a dominant negative (data not shown). Importantly, FH1FH2Δ1mDia1 strongly inhibited SMC-specific promoter activity in a dose-dependent
manner in primary rat aortic SMCs, the A7r5 rat SMC line, and in 10T1/2 cells (Figure 2c). Results from previous studies have shown that expression of DAD peptides can activate endogenous mDia signaling by interfering with the intramolecular repression that is mediated by the DID-DAD interaction. Expression of an mDia2 DAD peptide (AA 1030-1171) in all 3 cell types resulted in a dose-dependent increase in SM22 promoter activity (Figure 2d). Taken together, these results suggest that endogenous mDia signaling is very important for regulating SMC-specific promoter activity in SMCs and other SMC-like cell lines.

The Effects of mDia1/2 Require SRF Binding to CArG Elements
It is clear that RhoA-dependent regulation of SMC-specific transcription involves activation of SRF. To test whether the effects of mDia1 and mDia2 were also dependent on SRF, we
performed similar experiments in an SRF knockout embryonic stem cell line. ΔGBDmDia2 did not significantly increase SM α-actin promoter activity in SRF−/− ES cells (Figure 3). Reexpression of SRF strongly activated the SM α-actin promoter activity whereas single mutations to any one CARG reduced ΔGBDmDia2-induced activity by about 50% (see supplemental Figure I, available online at http://atvb.ahajournals.org). Interestingly, the intronic CARG, by itself, could mediate a significant portion of the effects of ΔGBDmDia2, and any combination of mutations that included the intronic CARG resulted in complete loss of promoter activity.

The Myocardin Family of Transcription Factors Are Important for the Effects of mDia1/2

To test whether the myocardin transcription factors were critical for the effects of mDia1 and mDia2, we used a dominant negative form of MRTF-A lacking the C-terminal transactivation domain that inhibits all 3 myocardin family members. Expression of this dominant negative dose-dependently inhibited the effects of ΔGBDmDia1 and ΔGBDmDia2 on SM22 promoter activity (data not shown). In addition, ΔGBDmDia1 and ΔGBDmDia2 enhanced the abilities of the myocardin factors to transactivate the SM22 promoter activity (Figure 4). Interestingly, diaphanous signaling had a relatively greater effect on the activities of the MRTFs than on myocardin. For example, even though all 3 myocardin transcription factors were expressed at similar levels, myocardin, MRTF-A, and MRTF-B activated SM22 promoter activity by 110-, 60-, and 30-fold, respectively, a result in relatively good agreement with previous studies. However, in the presence of ΔGBDmDia2, all 3 myocardin factors stimulated SM-specific promoter activity to a similar extent. Taken together, these data strongly suggest that mDia1 and mDia2 signal through the myocardin transcription factors to activate SMC-specific transcription.

The Effects of Dia1/2 Are Mediated by Actin Polymerization

Because we had previously shown that RhoA-mediated stimulation of actin polymerization was important for SMC-specific transcription,16 we wanted to test whether the effects of ΔGBDmDia1 and ΔGBDmDia2 were also mediated by changes in actin dynamics. Thus, we examined F-actin in over 200 ΔGBDmDia2-expressing cells using Texas Red-conjugated phalloidin. The representative micrographs shown in Figure 5a demonstrate that ΔGBDmDia2 significantly enhanced actin polymerization in 10T1/2 (top panels). This effect was not as easily observed in SMCs because of high basal levels of actin polymerization in primary SMC cultures even under serum-starved conditions. However, expression of dominant negative FH1FH2Δ1mDia1 in SMCs significantly inhibited actin polymerization, indicating that mDia signaling was important for maintaining actin polymerization in this cell type (Figure 5a, bottom panels). As shown in Figure 5b, treatment of SMCs and 10T1/2 cells with the actin depolymerizing drug latrunculin B (LB) or coexpression of G-actin nearly completely inhibited the effects of ΔGBDmDia2 on SM22 reporter activity, suggesting that changes in G-actin pools were important. Treatment of cells with the ROK inhibitor, Y-27632, did not affect ΔGBDmDia2-induced promoter activity. Identical results were obtained with ΔGBDmDia1 (data not shown).

Figure 3. The effects of ΔGBDmDia2 required SRF. SRF−/− embryonic stem cells were transfected with ΔGBDmDia2 in the presence or absence of flag-SRF. SM α-actin promoter activity was measured after 24 hours.

Figure 4. mDia signaling stimulated myocardin factor activity. 10T1/2 cells were cotransfected with submaximal concentrations of myocardin, MRTF-A, or MRTF-B in the presence or absence of ΔGBDmDia2. Inset: myocardin, MRTF-A, and MRTF-B expression levels as measured by Western blot.
Dia1/2 Signaling Induces MRTF Nuclear Localization

The data presented so far indicate that mDia1 and mDia2 strongly upregulate SMC-specific transcription by stimulating actin polymerization and myocardin family transcription factor activity. Recent studies by Miralles et al indicated that the effects of RhoA on SRF-dependent transcription were mediated by changes in MRTF-A localization. These authors demonstrated that MRTF-A was excluded from the nucleus in serum-starved cells, through an interaction that involved G-actin binding to conserved RPEL domains present in the N-terminus of MRTF-A. Previous results from our laboratory suggested that stimulation of MRTF-A localization by the strong RhoA activator, sphingosine 1-phosphate (S1P), was important for S1P-induced upregulation of SMC differentiation marker gene expression. We also demonstrated that MRTF-A and especially MRTF-B localized to the cytoplasm in a large percentage of serum starved SMCs and 10T1/2 cells (Hinson et al, unpublished data). To test whether the effects of GBDmDia1 and GBDmDia2 were mediated by changes in MRTF nuclear localization, we cotransfected flag tagged-versions of MRTF-A and MRTF-B along with GFP-GBDmDia2 into primary SMCs and 10T1/2 cells. Results shown in Figure 6 demonstrate that MRTF-B was localized almost exclusively to the cytoplasm in serum-starved SMCs and 10T1/2 (left panels), and coexpression of GBDmDia2 in either cell type resulted in MRTF-B nuclear localization in nearly all cells (right panels). Expression of GBDmDia2 also increased the percentage of cells containing MRTF-A in the nucleus to nearly 100% (data not shown). When coupled with our previous results, these data strongly indicate that RhoA-dependent activation of mDia1 and mDia2 stimulates SMC differentiation marker gene expression by promoting nuclear localization of MRTF-A and MRTF-B.

Discussion

The molecular signaling mechanisms that regulate SMC differentiation are poorly understood. We have previously shown that RhoA regulates SMC-specific transcription by
stimulating actin polymerization, but the RhoA effectors involved have not been completely described. Results from the present study indicate that mDia1 and mDia2 are important regulators of SMC differentiation marker gene expression. First, both of these RhoA effectors were highly expressed in SMCs. Second, RhoA-mediated activation of mDia1/2 strongly stimulated SMC-specific transcription in SMCs and SMC-like cell lines. Third, a dominant negative version of mDia1 that inhibits endogenous mDia1 and mDia2 signaling strongly inhibited SMC-specific promoter activity in SMCs and SMC-like cell lines. Finally, the effects of mDia1/2 were dependent on SRF and were likely attributable to nuclear translocation of the SRF cofactors, MRTF-A and MRTF-B.

Our analysis of Dia expression is in relatively good agreement with a previous study of mDia1 expression by Northern blotting. Although neither mDia1 nor mDia2 is expressed specifically in SMC, relatively strong expression in isolated SMCs and many SMC-containing tissues suggests that these RhoA effectors play an important role in regulating SMC function. Given their abilities to stimulate SMC differentiation marker gene expression, it will be crucial to further characterize mDia1 and mDia2 expression (and perhaps activity) during all stages of development using methods that provide better cell-type-specific resolution. It is also important to emphasize that lack of SMC-specificity of mDia1 or mDia2 does not necessarily preclude their involvement in regulating SMC differentiation. Cell-type-specific signaling can also result from differences in proteins downstream of the more general signaling pathways, and we believe that the myocardin transcription factors may serve this function in SMCs (see below).

It is clear that actin polymerization mediated by the catalytically active FH1/FH2 domain is inhibited by an intramolecular interaction between the DAD autoregulatory domain and the DID inhibitory domain. Extensive structure-function analyses including two recent X-ray crystallography studies indicate that the GBD and DID domains overlap slightly and that GTP-RhoA binding to the GBD displaces DAD from the DID binding pocket to activate the mDia proteins. In the present studies, we used several different strategies to activate mDia signaling, including coexpression of constitutively active RhoA, deletion of the mDia GBD/DID domain, and expression of a competing DAD peptide. All three of these interventions significantly upregulated SMC-specific transcription in 10T1/2 cells and rat aortic SMCs. In 10T1/2 cells, expression of ΔGBDmDia1 or ΔGBDmDia2 transactivated the SM22 promoter by 45- and 30-fold, respectively, levels of transactivation usually only seen on overexpression of the myocardin transcription factors. Expression of ΔGBDmDia1, ΔGBDmDia2, or DAD peptide in SMCs also led to significant increases in SMC-specific transcription, but these effects were more modest. A similar difference in relative activities is observed on overexpression of the myocardin factors in SMCs and is most likely attributable to the relatively high levels of SMC-specific transcriptional activity already exhibited by primary SMCs. Our data would suggest that strong mDia expression (and activity) probably contributes to this high basal activity. Nevertheless, when taken together with the inhibitory effects of DN mDia1 in SMCs and the high level of mDia1 and mDia2 expression in SMCs, we feel these data provide strong support for the involvement of RhoA-dependent mDia signaling in SMC differentiation.

Our results also demonstrated that mDia1 and mDia2 had very little effect on the activity of the c-fos promoter, further supporting the idea that RhoA signaling differentially regulates SRF-dependent SMC differentiation versus SRF-dependent SMC growth. The selectivity and/or promiscuity of individual DRFs for various Rho family GTPases may also be interesting in regard to gene-specific regulation by SRF. For example, mDia1 and mDia2 have been shown to interact with RhoA, RhoB, and RhoC, but not with Rac1, whereas FHOD1 interacts with Rac1, but not RhoA. Interestingly, mDia2 has also been shown to bind Cdc42 and a newly described GTPase, Rif, and these interactions are thought to play a role in filopodial extension. Expression of constitutively-active Cdc42 did increase mDia2-dependent activation of the SMC-specific promoters, but these effects were not as great as those observed with L63RhoA (Staus and Mack, unpublished observation). Because the small GTPases are regulated by different signaling inputs, reside in different cellular compartments, and regulate the formation of slightly different actin structures, it is interesting to speculate that the DRFs may relay specific information to the nucleus that could lead to different levels of SRF-dependent gene expression or to differential expression of subsets of SRF-dependent genes.

The inhibitory effects of latrunculin B and overexpression of G-actin clearly implicate actin dynamics in mDia-induced regulation of SMC-specific transcription. RhoA also stimulates actin polymerization through ROK/LIMK/cofilin, and it is thought that these pathways interact functionally to regulate SRF activity. In the present study, Y-27632 did not affect transactivation by ΔGBDmDia1, suggesting that ROK activity was not required for this response. When coupled with the strong effects of constitutively-active mDia1 and mDia2 on SMC-specific promoter activity, these data suggest that RhoA signaling to mDia1 and mDia2 may be more important for regulating SMC differentiation than RhoA signaling to ROK. Another RhoA effector, PKN, has recently been shown to be important for the induction of SMC-specific promoter activity by TGF-β, but whether changes in actin dynamics were involved was not addressed. The present studies also demonstrated that mDia2 expression was upregulated on TGF-β treatment, suggesting additional cross-talk between these two pathways.

A growing body of evidence indicates that the effects of actin dynamics on SRF-dependent transcription are mediated by nuclear localization of the MRTFs. G-actin binding to the N-terminal RPEL domains found in MRTF-A and MRTF-B has been shown to be important for sequestering the MRTFs in the cytoplasm, and reduction of G-actin pools by RhoA-dependent signaling is important for relieving this inhibitory signal. Results from the present study provide further support for this model. Importantly, expression of constitutively-active mDia1 or mDia2 stimulated the nuclear localization of both MRTF-A and MRTF-B and synergist-
cally activated MRTF-A– and MRTF-B–dependent SMC-specific transcription. Interestingly, in the presence of constitutively active mDia1 or mDia2, the activities of MRTF-A and MRTF-B were equal to that of myocardin, suggesting that mDia1/2 activity may be a limiting factor for their activities. Somewhat surprisingly, constitutively-active mDia1 and mDia2 also increased myocardin-dependent trans-activation, even though myocardin was constitutively nuclear. This most likely reflects cooperative effects with endogenously expressed MRTFs, but it is possible that Dia signaling activates myocardin by a separate mechanism. Kuwahara et al have recently identified another protein called STARS (StRiated muscle Activator of Rho Signaling) that stimulates SRF-dependent transcription by regulating MRTF nuclear localization. Whether mDia1/2 activity is important for differentiation of other muscle cell-types is an interesting question for future studies.

It is clear that MRTF-A and MRTF-B, like myocardin, can upregulate a number of CArG-containing muscle-specific genes. Whereas the MRTFs are expressed in some SMC subsets including the aorta, these SRF cofactors are thought to be expressed more widely complicating their involvement in cell-type–specific gene expression. Several recent studies, however, indicate that the MRTFs are important for regulating SMC differentiation. Two separate groups have shown that genetic disruption of MRTF-B leads to a lethal embryonic defect in pharyngeal arch remodeling and that this phenotype is accompanied by a failure of SMC differentiation of the cardiac neural crest cells that populate the cardiac outflow tract. In addition, Pipes et al used a chimeric mouse model to demonstrate that myocardin–/– cells could populate the developing aorta suggesting that the MRTFs probably regulate SMC differentiation under certain circumstances.

Interestingly, several groups have reported somewhat contrasting results on the regulation of MRTF-A localization by signaling pathways, suggesting that this mechanism may be more complicated than originally described and that cell-type–specific differences or local environmental cues may be important parameters for regulating this process. Given the observation that MRTF expression does not necessarily correlate with MRTF transcriptional activity, it is possible that differential RhoA/mDia signaling could play an important role in the control of cell-type–specific gene expression by the MRTFs. Because SMCs never terminally differentiate, this mechanism could be particularly important for modulating SMC phenotype in response to environmental signals.

In summary, our results indicate that mDia1 and mDia2 are very important regulators of SMC-specific gene expression. These RhoA effectors strongly activate the MRTFs by increasing actin polymerization, which induces their nuclear localization. The precise roles that mDia1 and mDia2 play in the regulation of SMC differentiation during vascular development or in the regulation of SMC phenotype during the progression of cardiovascular disease will be an important area for future studies.

Sources of Funding
This work was supported by NHLBI Grants HL-070953 (to C.P.M.), HL-081844, and HL-071054 (to J.M.T.), and American Heart Association Grants 0130193N (to C.P.M.) and 0355776U (to J.M.T.).

Disclosures
None.

References
21. Wanhoff BR, Bowles DK, McDonald OG, Sinha S, Somlyo AP, Somlyo AV, Owens GK. L-type voltage-gated Ca2+ channels modulate...


Diaphanous 1 and 2 Regulate Smooth Muscle Cell Differentiation by Activating the Myocardin-Related Transcription Factors
Dean P. Staus, Alicia L. Blaker, Joan M. Taylor and Christopher P. Mack

Arterioscler Thromb Vasc Biol. 2007;27:478-486; originally published online December 14, 2006;
doi: 10.1161/01.ATV.0000255559.77687.c1
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/27/3/478

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2006/12/15/01.ATV.0000255559.77687.c1.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SM α-actin Promoter Activity (Relative to Wt Promoter)

- Wt
- a
- b
- i
- a+i
- b+i
- a+b
- a+b+i