A NOVEL ROLE OF BRG1 IN THE REGULATION OF SRF/MRTFA-DEPENDENT SMOOTH MUSCLE-SPECIFIC GENE EXPRESSION.

Min Zhang, Hong Fang, Jiliang Zhou and B. Paul Herring

From the Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, IN 46202-5120

Running title: Brg1 regulates MRTFA activity

Address correspondence to: B. Paul Herring, Department of Cellular and Integrative Physiology Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202-5120, Tel. (317) 278-1785; Fax (317) 274-3318; E-mail: pherring@iupui.edu

Serum Response Factor (SRF) is a key regulator of smooth muscle differentiation, proliferation and migration. Myocardin Related Transcription Factor A (MRTFA) is a co-activator of SRF that can induce expression of SRF-dependent, smooth muscle-specific genes and actin/Rho-dependent genes, but not MAPK regulated growth response genes. How MRTFA and SRF discriminate between these sets of target genes is still unclear. We hypothesized that SWI/SNF ATP-dependent chromatin remodeling complexes, containing Brahma-related gene 1 (Brg1) or Brahma (Brm), may play a role in this process. Results from Western blotting and qRT-PCR analysis demonstrated that dominant negative Brg1 blocked the ability of MRTFA to induce expression of smooth muscle-specific genes, but not actin/Rho-dependent early response genes, in fibroblasts. In addition, dominant negative Brg1 attenuated expression of smooth muscle-specific genes in primary cultures of smooth muscle cells. MRTFA over-expression did not induce expression of smooth muscle-specific genes in SW13 cells, which lack endogenous Brg1 or Brm. Reintroduction of Brg1 or Brm into SW13 cells restored their responsiveness to MRTFA. Immunoprecipitation assays revealed that Brg1, SRF and MRTFA form a complex in vivo and Brg1 directly binds MRTFA, but not SRF, in vitro. Results from chromatin immunoprecipitation assays demonstrated that dominant negative Brg1 significantly attenuated the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes, but not early response genes. Together these data suggest that Brg1/Brm containing SWI/SNF complexes play a critical role in regulating expression of SRF/MRTFA-dependent smooth muscle-specific genes but not SRF/MRTFA-dependent early response genes.

INTRODUCTION

There are many clinical diseases, such as atherosclerosis, hypertension and asthma that involve abnormal differentiation of smooth muscle cells. An important pathological process that occurs in these diseases is the disruption of the balance between differentiation and proliferation of smooth muscle cells (1-4). Serum Response Factor (SRF) has been shown to play an essential role in regulating smooth muscle differentiation, proliferation and migration through its interaction with various accessory proteins (5). Smooth muscle-specific genes, such as SM α-actin, SM MHC, 130kDa MLCK, SM22α, and telokin, are activated by SRF-myocardin, SRF/MRTFA, SRF/GATA6/CRP2 or SRF/Nkx complexes (6-21). The immediate early growth factor responsive genes, such as c-fos and egr1 are regulated by SRF/Elk (ets) complexes (22-24). The later early response genes, such as SRF itself and vinculin, that are actin/Rho-dependent, are regulated by SRF/MRTFA complexes (16,25,26). Myocardin Related Transcription Factor A (MRTFA, or Mkl1, MAL, BSAC) is a unique co-activator of SRF in that it is involved in the regulation of multiple SRF-dependent gene families (reviewed in (27)). MRTFA has been reported to induce SRF-dependent, smooth muscle-specific genes such as telokin, SM22α and SM α-actin and actin/Rho-dependent...
early response genes, but not proliferation related MAPK-dependent immediate early response genes (13,15,16,26). It still remains a mystery how MRTFA can discriminate between different SRF-dependent genes. One possible mechanism could involve genespecific restriction of promoter access due to chromatin structure. In support of this model, it has been shown that there is very little SRF detectable at the CArG boxes of smooth muscle-specific genes in nonmuscle cells, whereas SRF binding can be readily detected at CA rG boxes of early response genes such as c-fos (28). In addition, over-expression of myocardin in nonmuscle cells was found to lead to increased SRF binding to the promoters of smooth muscle-specific genes. In the current study we provide evidence supporting a role for ATP-dependent chromatin remodeling in regulating SRF binding to CA rG boxes within promoters of smooth muscle-specific genes.

Since chromatin is highly condensed, the regulation of chromatin accessibility to transcription factors and RNA polymerase is an essential step in gene activation (29-31). Although studies have shown that myocardin can recruit enzymes capable of modifying chromatin structure through covalent modification of histone tails (32,33), no studies have examined how chromatin structure affects promoter access by MRTFA. In addition, the role of ATP-dependent chromatin remodeling enzymes in the regulation of smooth muscle differentiation is unknown. The SWI/SNF complex is the best characterized, mammalian, ATP-dependent chromatin remodeling complex (30). It is comprised of 7 to 11 components, which assemble into distinct complexes containing either Brg1 (Brahma-related gene 1) or Brm (Brahma) ATPase subunits. SWI/SNF remodeling complexes have been shown to play an essential role in the differentiation of neurons, T cells, erythrocytes, hepatocytes, adipocytes, skeletal and cardiac muscle cells (34-44). Although, the role of Brg1 or SWI/SNF in smooth muscle development is largely unknown, Brg1 has been shown to be upregulated in vascular smooth muscle cells in primary atherosclerosis and in stent stenosis (45). A recent study has also demonstrated that Brg1 binding to CRP2 is critical for induction of smooth muscle-specific genes by CRP2 (18). During skeletal muscle differentiation it has been shown that the recruitment of Brg1 to MyoD, that is associated with DNA bound Pbx1, induces chromatin remodeling of the myogenin gene. This facilitates tight binding of MyoD to E boxes resulting in co-factor recruitment and transcription activation (46). By analogy, we propose that weak SRF binding to the CArG boxes of smooth muscle-specific genes may facilitate recruitment of MRTFA and that interaction of MRTFA with SWI/SNF may then remodel chromatin permitting tight binding of SRF.

Results from our study demonstrate that Brg1 is required for the induction of smooth muscle-specific gene expression but not for early response gene expression by MRTFA. Endogenous Brg1, SRF and MRTFA were found to form a complex in smooth muscle cells and tissue and Brg1 directly bound to MRTFA, but not SRF, in vitro. Chromatin immunoprecipitation assays revealed that SWI/SNF is required for MRTFA to increase SRF binding to the promoters of smooth muscle specific genes. Furthermore, expression of a dominant negative Brg1 in differentiated smooth muscle cells attenuated expression of smooth muscle-specific genes. Together these data indicate that SWI/SNF plays a critical role in regulating expression of SRF/MRTFA-dependent smooth muscle-specific genes but not SRF-dependent early response genes. SWI/SNF thus plays an important role in regulating the balance between the differentiation and proliferation roles of SRF.

**EXPERIMENTAL PROCEDURES**

**Cell culture and adenoviral transduction.** An MRTFA cDNA image clone was purchased from Invitrogen (Clone ID: 682130) and moved to Adeno-X vector according to the manufacturer’s protocol (BD Biosciences). Adenovirus encoding nuclear localized YFP (Yellow Fluorescent Protein) was used as negative control. B22 cells, which are NIH3T3...
cells that express a tetracycline inducible dominant negative Brg1 (DN-Brg1, K798R mutant) (47), were obtained from Dr. Anthony N. Imbalzano (University of Massachusetts Medical School, Worcester, Massachusetts). By withdrawing tetracycline from the growth media of these cells, the expression of DN-Brg1 can be induced. B22 cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (DMEM) (Mediatech) containing 2 μg/ml tetracycline, 350 units/ml Hygromycin B, 75 μg/ml G418 and 10% fetal bovine serum (FBS). B22 cells were seeded into 6 well dish at the density of 2×10^5 cells per well in the medium either with or without tetracycline and grown for 24 hours prior to adenoviral transduction. Cells were incubated with adenovirus encoding MRTFA or YFP for 4 hrs at 37 °C and then replaced with complete medium. 30-48 hrs after transduction, cells were harvested for protein, RNA or chromatin immunoprecipitation analysis. SW13 and HeLa cells were obtained from ATCC and grown in high glucose DMEM containing 5 units/ml penicillin, 50 mg/ml streptomycin and 10% FBS. 24 hours before transduction, SW13 and HeLa cells were seeded at the density of 2.5×10^5 cells per well in 6 well dish. Cells were then transduced with adenovirus as described above for B22 cells. Primary mouse colon smooth muscle cells were prepared from colons dissected from 4 week-old mice. The epithelial layer was removed and remaining smooth muscle layer was minced and digested with 1 ml of tissue digestion buffer per organ (0.4 units/ml Blendzyme #3 (Roche) in DMEM) at 37°C for 1-2 hours with shaking. The digested tissue was then passed through a cell sieve and the cells collected by centrifugation. Pelleted cells were washed in DMEM containing 10% FCS and penicillin/streptomycin and plated into dishes. After 4-5 days cells reached confluence, they were trypsinized and replated at 7x10^5 per well in 12 well plates. 12 hours after plating, cells were transduced by DN-Brg1 or YFP control adenovirus. 72 hours after transduction, mRNA was harvested and the levels of SRF dependent genes were measured by quantitative real time RT-PCR.

**Plasmids used and cell transfection.** Human Brg1 and Brm cDNA and DN-Brg1 in pBabe retroviral expression plasmids were obtained from AddGene (48). MRTFA was cloned into pcDNA myc His (Invitrogen) for transfection and in vitro translation experiments. HA-SRF pShuttle was generated by cloning the human SRF cDNA into a modified pShuttle (Clontech) vector that includes an amino-terminal HA epitope tag. 12 hrs prior to transfection, cells were seeded at the density of 2.5×10^5 cells per well in 6 well plates. Plasmids were transfected into cells using Fugene 6 (Roche Applied Science): cells were washed once with phosphate-buffered saline (PBS) (pH 7.4) then 2 μl of complete medium was added to each well together with 2 μg plasmid DNA and 4 μl Fugene in 100 μl DMEM.

**RNA analysis.** RNA was extracted with Trizol reagent (Invitrogen). 1.2 μg RNA was used as template for reverse transcription (RT) using Superscript first strand cDNA synthesis kit (Invitrogen). cDNA was dissolved in 20 μl H2O. The cDNA levels of specific genes were measured by quantitative real time PCR using SYBR green PCR master mix (Invitrogen) and a 7500 Real Time PCR system (Applied Biosystems) with gene specific primers (Table 1 in Supplemental data). 2 μl of 1:10 diluted cDNA was used to each reaction in 25 μl total volume. All PCR reactions were performed in duplicate.

**Western blotting.** Protein was extracted with RIPA lysis buffer. Protein concentrations were determined by using a BCA Protein Assay Kit (Pierce). 30 μg of proteins were fractionated on 7.5 or 15% SDS-polyacrylamide gels and transferred to nitrocellulose or polyvinyl difluoride membranes. Membranes were then probed with a series of antibodies. Antibodies used for Western blotting were against: Brg1 (Upstate, 1:5000), Brm (Abcam, 1:1000) MRTFA (Santa Cruz, C-19, 1:500), SRF (Santa Cruz, G20X, 1:6000), β-actin (Sigma, 1:10,000), Egr-1 (Santa Cruz, 1:1000), Flag tag (Sigma, M2, 1:5000), HA tag (Covance, 1:3000), myc tag (Invitrogen, 1:1000), MLCK (Sigma, clone K36, 1:10,000), SM α-actin...
(Sigma, clone 3A1, 1:10,000), SM22α (a gift from Dr. Len Adam, 1:6,000), telokin (1:6,000) (49), vinculin (Santa Cruz, 1:5,000), NMMHRC IIA (a gift from Dr. Patricia Gallagher). Primary antibodies were detected using horseradish peroxidase conjugated secondary antibodies and visualized using chemiluminescence.

Co-immunoprecipitation (Co-IP). Co-IP assays were performed using a nuclear complex Co-IP kit, essentially as described by the manufacturer (Active Motif). 250μg of nuclear protein extracts were incubated with 3μg of anti-Brg1 antibody (Upstate), anti-SRF antibody (Santa Cruz, G20X), anti-MRTFA antibody (Santa Cruz, C-19; or ProteinTech), or appropriate IgG control in 500 μl of low salt IP buffer (Active Motif) overnight at 4°. 60 μl of EZview protein A beads (Sigma) were added to the mixture and incubated for an additional hour with rocking. Beads were then washed 6 times with the low salt IP buffer. The immunoprecipitated proteins were dissolved in 35μl of 2XSDS sample buffer and boiled for 5 minutes, prior to analysis by western blotting as described above.

In vitro transcription/translation. Synthesis of proteins was carried out in a coupled transcription/translation system (Promega, Madison, WI) in vitro, programmed with 1μg of pShuttle-Brg1 (flag tag), pcDNAmyc/his-MRTFA (myc tag) and pShuttle-SRF (HA tag) plasmids. The TNT products were mixed together as combinations of: SRF with Brg1, MRTFA with Brg1, SRF with MRTFA, in 250μl low salt IP buffer (Active Motif). Proteins were immunoprecipitated with 3μg anti-SRF (Santa Cruz, G20X) or 15μl anti-MRTFA antiserum over night. A matching rabbit IgG or a MRTFA preimmune serum served as negative controls. The immunoprecipitated proteins were then incubated with EZ-view beads (Sigma) for 2 hours. Following 6 washes with IP buffer the beads were dissolved in SDS-sample buffer and subjected to Western blotting as described above.

Quantitative chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed according to the protocol of Upstate with minor modifications. Cells were fixed in 3.7% formaldehyde for 15 minutes at room temperature and harvested using cold PBS with protease inhibitors. After collecting cells by centrifugation, cell pellets were lysed using 1%SDS lysis buffer (200μl / 1x10^6 cells). For each group, 1ml of lysate was sonicated for 7 x30 seconds at setting 2.25 on a Sonic Dismembranator (Fisher Scientific). 200μl aliquots of chromatin were immunoprecipitated using 6μg of anti-SRF antibody (Santa Cruz, G20X), anti-H3Ac (Upstate) or rabbit IgG as negative control. The precipitated genomic DNA was purified and the presence of specific promoters was measured by real time quantitative PCR, using gene specific primers (Table 2 in Supplemental data).

RESULTS

DN-Brg1 inhibits the ability of MRTFA to induce expression of smooth muscle specific genes, but not SRF-dependent early response genes. To determine the role of SWI/SNF mediated chromatin remodeling on the induction of genes by SRF/MRTFA, we utilized a previously characterized 3T3 cell line that inducibly expresses a dominant negative Brg1 (B22 cells, (47)). The dominant negative K798R mutant Brg1 blocks the function of SWI/SNF complexes containing Brg1 or Brm catalytic subunits. B22 cells were transduced with MRTFA or YFP adenovirus. 30 hours after transduction, cells were lysed and mRNA and protein expression analyzed by quantitative real-time RT-PCR and Western blotting, respectively (Figures 1, 2). In agreement with previous reports (13-15,26,50) MRTFA induced the expression of several smooth muscle-specific genes in fibroblast cells (Figure 1, compare solid bars to open bars in control cells). In contrast, MRTFA did not significantly induce expression of the early response genes, c-fos, egr1 or vinculin, although it did result in a 2-fold increase in expression of SRF mRNA (Figure 1). As shown by western blotting (Figure 2) and more quantitatively by qRT-PCR (Figure 1) dominant negative Brg-1 significantly abrogated the ability of MRTFA
to increase expression of telokin, SM22α, and calponin but not SM α-actin or any of the SRF-dependent early response genes examined. Conversely, DN-Brg1 augmented the ability of MRTFA to increase SRF mRNA expression and increased the basal expression of egr1 and c-fos mRNA(Figure 1).

**MRTFA cannot induce smooth muscle-specific genes in SW13 cells that lack Brg1 and Brm.** It is possible that DN-Brg1 inhibits the activity of more than just Brg1 and Brm containing SWI/SNF complexes, hence we also examined the role of Brg1 in a Brg1/Brm null cell system. The ability of MRTFA to induce gene expression in human adrenal carcinoma SW13 cells that lack endogenous Brg1/Brm was determined. Human cervical cancer HeLa cells that express Brg1 and Brm were used as control for these experiments. Results from Western blot analysis showed that over-expression of MRTFA in SW13 cells could not induce the expression of most smooth muscle-specific genes, including 130kDa smMLCK, telokin, or SM22α, and could only weakly induce SM α-actin expression (Figure 3A). In contrast, MRTFA readily induced expression of all these genes in HeLa cells. MRTFA induced expression of SRF and vinculin in SW13 cells as well as in HeLa cells. Although Egr1 was not induced by MRTFA in either of these two cell types, the basal expression of egr1 appeared higher in SW13 cells compared with HeLa cells. Surprisingly, MRTFA was able to induce expression of c-fos in SW13 cells but not in HeLa cells (Figure 3A) and this induction was attenuated by reintroduction of Brg1 into the SW13 cells (Figure 3B). These results are consistent with the results obtained from the DN-Brg1 3T3 cell lines. To verify that the inability of MRTFA to induce smooth muscle-specific genes in SW13 cells is due to the lack of Brg1/Brm we reintroduced Brg1 or Brm1 back into SW13 cells. Western blot analysis demonstrated that restoration of Brg1 or Brm expression could rescue the ability of MRTFA to induce the expression of smooth muscle-specific proteins in SW13 cells (Figure 3B).

**DN-Brg1 attenuates smooth muscle gene expression in primary smooth muscle cells.** The above results suggest that Brg1 or Brm is required for MRTFA to induce the expression of smooth muscle-specific genes in non-smooth muscle cells. To determine if Brg1 is required for smooth muscle cells to maintain the expression of smooth muscle-specific markers, DN-Brg1 was over expressed in primary colon smooth muscle cells. Results from quantitative real-time RT-PCR analysis demonstrated that dominant negative Brg-1 significantly decreased the expression of smooth muscle-specific genes (telokin, SM MHC, calponin, SM22α and SM α-actin) and increased the expression of the SRF-dependent early response genes, such as egr1 and c-fos. The expression of SRF was not significantly changed (Figure 4).

**Brg1 interacts with SRF and MRTFA in intact cells.** We next determined if Brg1 could interact with MRTFA and/or SRF in intact cells. Co-immunoprecipitation (Co-IP) assays were performed from COS cells transduced with HA-tagged MRTFA and SRF adenoviruses. MRTFA, SRF and endogenous Brg1 were immunoprecipitated from the transduced cell extracts. Western blotting of immunoprecipitated proteins showed that MRTFA immunoprecipitates contained Brg1, MRTFA and SRF (Figure 5A). SRF immunoprecipitates contained MRTFA and SRF, and a very weak Brg1 signal. Similarly Brg1 immunoprecipitates contained Brg1 and MRTFA and a small amount of SRF. These data indicate that Brg1, MRTFA and SRF can form a complex in intact cells. To confirm this finding, we determined if the endogenous proteins can also be detected in a complex in A10 smooth muscle cells (Figure 5B) or in bladder tissue (Figure 5C). Endogenous Brg1, SRF and MRTFA were immunoprecipitated from nuclear extracts of A10 cells or mouse bladder and western blot analysis revealed that Brg1 can be co-immunoprecipitated with SRF and MRTFA. These data indicate that the 3 proteins can form a complex in A10 smooth muscle cells and bladder tissue in vivo (Figure 5B,C).

**Brg1 interacts with MRTFA but not SRF in vitro.** To determine if Brg1 directly binds to MRTFA and/or SRF, in vitro binding assays
were performed. Brg1, MRTFA and SRF were synthesized in vitro using a coupled *in vitro* transcription and translation system. pShuttle-Brg1 (Flag tag), pDNA myc/his-MRTFA (myc tag) and pShuttle-SRF (HA tag) were utilized as templates for the transcription/translation reactions. In vitro synthesized proteins were incubated together and immunoprecipitation assays were used to identify the interacting proteins (Figure 6).

Results from this analysis indicate that Brg1 could be co-precipitated with MRTFA but not with SRF, demonstrating that Brg1 can directly bind to MRTFA.

**DN-Brg1 inhibits the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes.** To explore why Brg1 is required for MRTFA induced smooth muscle-specific gene expression, but not for expression of the early response genes, chromatin immunoprecipitation (ChIP) assays were performed. B22 cells were grown in the presence or absence of tetracycline and transduced with adenovirus encoding MRTFA or YFP. ChIP assays were performed to examine SRF binding to SRF-dependent promoters as described in ‘Experimental Procedures’. Consistent with previous studies using myocardin (28), we found that MRTFA significantly increased SRF binding to the promoters of smooth muscle-specific genes, telokin, SM22α and SM α-actin, (Figure 7A). DN-Brg1 dramatically attenuated the ability of MRTFA to increase SRF binding to telokin and SM22α promoters, but not SM α-actin promoter (Figure 7B). In contrast, neither MRTFA nor DN-Brg1 affected the SRF binding to the promoters of the early response genes, SRF and c-fos (Figure 7A, B). In addition to increasing SRF binding to the telokin promoter, MRTFA also increased levels of acetylated histone H3 (Figure 7A, right panel). This increase in acetylated histone H3 on the telokin promoter was blocked by expression of DN-Brg1. In contrast, the small increase in acetylated H3 on the SM α-actin promoter was not blocked by DN-Brg1 (Figure 7B, right panel). Under control conditions (no DN-Brg1 expression), in YFP transduced B22 cells, there appeared to be more SRF bound to the SM α-actin, SRF, and c-fos promoters as compared to the telokin and SM22α promoters (Figure 7C, left panel). Similarly there was more acetylated histone H3 associated with the SM α-actin promoter as compared to the telokin promoter under control conditions (Figure 7C, right panel).

**DISCUSSION**

In this study, we found that Brg1 or Brm are required for the MRTFA-mediated induction of smooth muscle-specific genes, but not early response genes. DN-Brg1 attenuated the induction of smooth muscle-specific genes and reintroducing Brg1/Brm into SW13 cells restored their responsiveness to MRTFA. Brg1 appears to be required for MRTFA to increase the binding of SRF to the promoters of smooth muscle-specific genes, within intact chromatin. Brg1, MRTFA and SRF were found to form a complex within intact smooth muscle cells and Brg1 directly bound MRTFA but not SRF *in vitro*. Since previous studies (16,25,50), and data presented in figure 6 have shown that MRTFA directly binds SRF *in vitro*, MRTFA can thus act as a bridge to connect Brg1 and SRF.

Results shown in figures 1 and 2 demonstrate that DN-Brg-1 blocked the MRTFA-mediated induction of telokin, SM22α and calponin but did not significantly block the induction of SM α-actin. However, from results shown in figure 2 it is apparent that SM α-actin, but not other smooth muscle marker genes, was readily detectable in B22 3T3 cells prior to over-expression of MRTFA (YFP control group). This indicates that the SM α-actin locus is transcriptionally active in control cells and thus likely to be in an open chromatin conformation in these cells. In support of this hypothesis, there was significantly more SRF and acetylated histone H3 associated with the SM α-actin promoter as compared to the telokin promoter in control cells (Figure 7C). Thus it might be predicted that Brg1 containing SWI/SNF complexes may not be required to further open the chromatin structure of the SM α-actin promoter in order for MRTFA to increase SRF binding to the promoter. This suggests a model in which
Brg1 is required for MRTFA to increase binding of SRF to the promoters of transcriptionally silent genes but not to genes that are already transcriptionally active within native chromatin (Figure 8).

As the MRTFA regulated early response genes are also expressed in control cells, our model would predict that activation of these genes would also be independent of Brg1. This is consistent with data presented in figure 1 showing that MRTFA mediated induction of SRF was not inhibited by DN-Brg1. In addition, under control conditions there was markedly more SRF bound to the SRF promoter as compared to the telokin or SM22α promoters (Figure 7C). In our experiments only in SW13 cells did we detect any activation of vinculin by MRTFA (Figure 3A), although this activation was not altered by expression of Brg1 or Brm (Figure 3B), suggesting that it is independent of these proteins. In agreement with previous studies (26) we also observed no affects of MRTFA on expression of MAPK dependent SRF-target genes such as c-fos or egr1 (Figure 1). However, the basal expression of c-fos and egr1 is higher in cells expressing DN-Brg1 (Figure 1) and in cells lacking Brg1/Brm (SW13 cells compared to HeLa cells in figure 3A). This observation is consistent with a previous study that showed that Brg1 represses c-fos expression (51). It is not known why Brg1 inhibits c-fos expression, though it might be predicted that it either results in increased binding of a repressor or it may alter the positioning of the nucleosome, which has been shown to be located between key regulatory elements in the c-fos promoter (52). As Brg1 is specifically required for MRTFA to induce expression of smooth muscle-specific genes but not early response genes, then regulation of MRTFA/Brg1 interactions could provide a mechanism for a cell to switch between activation of these two groups of genes.

Previous studies showed that Brg1 knockout mice die during the periimplantation stage (53), however, Brm knockout mice develop normally, except that adult mice have higher body weight (54). These data suggest that Brg1 has non-redundant functions that cannot be replaced by Brm. However, in our experiments we found that either Brg1 or Brm were required for MRTFA to induce expression of smooth muscle-specific genes. This is in contrast to a recent study that demonstrated that Brg1 but not Brm is required for CRP-mediated induction of smooth muscle-specific genes in cardiac myocytes (18). Future analysis of smooth muscle-specific knockouts of Brg1 will be required to determine the specific role of Brg1 and Brm in smooth muscle cells in vivo.

Although Brg1 is ubiquitously expressed, Brg1 has been shown to selectively rather than broadly regulate gene expression (30). This can be explained, at least in part, by the recruitment of Brg1 to individual promoters through its interaction with specific transcription factors (18,38,46,55-60). Our results further support this idea as we found that Brg1, SRF and MRTFA form a complex in smooth muscle tissue in vivo and Brg1 directly interacts with MRTFA but not SRF (Figure 6). This would allow Brg1 to be recruited to MRTFA-dependent SRF target genes but not to Elk-dependent SRF target genes. A recent study has also shown that CRP2 directly binds to Brg1 (18), and CRP2 has previously been shown to form a complex with SRF in some smooth muscle cell types (17). Thus, CRP2 may act similar to MRTFA to facilitate recruitment of Brg1 complexes to mediate stable SRF binding to the promoters of smooth muscle-specific genes.

It is likely that the ATP-dependent chromatin remodeling catalyzed by Brg1/Brm acts together with covalent histone modifications to permit stable SRF/MRTFA binding and transcription activation. Although SWI/SNF itself does not covalently modify histones its’ remodeling of chromatin through nucleosome reorganization can facilitate the recruitment of other proteins that covalently modify histones. Previous studies have shown that over-expression of Myocardin, an MRTFA homologue, can induce histone acetylation through myocardin’s interaction with the HAT, p300 (33). Histone acetylation has also been shown to promote the binding of
SWI/SNF complexes to chromatin, as the acetylated histone tail can bind to the bromodomain of Brg1 (61). Thus MRTFA/p300 induced histone acetylation, may further stabilize Brg1 binding to the promoters of smooth muscle-specific genes (Figure 8). In a reciprocal fashion Brg1-induced changes in chromatin structure will facilitate increased binding of SRF/MRTFA complexes to the promoter and thus result in increased recruitment of p300. Together these interactions can then lead to transcription activation.

In summary, we propose a model in which the recruitment of MRTFA/BrG1 complexes to weakly bound SRF at the promoters of smooth muscle-specific genes, leads to chromatin remodeling which facilitates tight binding of SRF complexes and subsequent transcription activation (Figure 8, upper panels). In contrast, in cells grown under high serum conditions, SRF is tightly bound to the promoters of SRF-dependent early response genes. Brg1 is thus not required to permit MRTFA or MAPK-Elk signals to activate these genes (Figure 8, lower panels).

ACKNOWLEDGEMENTS
We thank Dr. Anthony N. Imbalzano for providing B22 cells. We also thank the members of the Herring lab, for helpful discussions and April Hoggatt for expert technical assistance. This work has been supported by National Institutes of Health grants HL-58571, DK-61130 and DK-65644. Min Zhang is supported by a fellowship from the Fortune Fry Foundation. Jiliang Zhou was supported by an AHA postdoctoral fellowship.

REFERENCES
FIGURE LEGENDS.

Figure 1. The expression of DN-Brg1 in 3T3 fibroblasts interferes with the induction of endogenous SRF-dependent smooth muscle-specific genes by MRTFA. Inducible DN-Brg1 3T3 (B22) cells were plated in media either with or without tetracycline. 24 hours later, cells were transduced with MRTFA (solid bars) or YFP (open bars) adenovirus. 30-48 hours following transduction cells were lysed for mRNA analysis. mRNA was isolated from cells and the levels of mRNA expression were measured by quantitative real time RT-PCR. Transcript levels was firstly normalized to acidic ribosomal phosphoprotein PO (RPLPO) internal loading control and then normalized to their respective YFP control group. The $\Delta \Delta C_t$ method was used to calculate the relative quantity values (RQ) of gene expression levels. $C_t$ is the threshold cycle where the amplification of template begins. RQ=$2^{\Delta \Delta C_t}$ and $\Delta \Delta C_t = (C_{t \text{ experimental}} - C_{t \text{ RPLPO}})-(C_{t \text{ control}} -C_{t \text{ RPLPO}})$. Data presented are the mean±SEM of 8-9 samples obtained from 3 independent experiments. * Indicates statistical significance as determined by a student T test (P<0.05).

Figure 2. The expression of DN-Brg1 in 3T3 fibroblasts interferes with the induction of endogenous SRF-dependent smooth muscle-specific proteins by MRTFA. Inducible DN-Brg1 3T3 (B22) cells were plated in media either with or without tetracycline. 24 hours later, cells were transduced with MRTFA or YFP adenovirus. 30-48 hours following transduction cells were lysed using RIPA lysis buffer and protein expression analyzed by Western blotting. 30 µg of protein were loaded in each lane.

Figure 3. MRTFA cannot induce smooth muscle-specific gene expression in SW13 cells that lack Brg1/Brm1. A. SW13 and HeLa cells were transduced with MRTFA or YFP adenovirus, 48 hrs later, protein extracts were prepared and analyzed by western blotting. 30µg of protein were loaded onto each lane. * The upper band seen on the c-fos blots is a non-specific signal. β-actin was used as a loading control. B. WT-Brg1 or Brm-pBabe expression plasmids or empty vector control plasmids were transfected into SW13 cells in duplicate. After 24 hrs, cells were transduced with MRTFA adenovirus (low amount +, higher amount ++). 48 hours later cells were lysed by RIPA lysis buffer and analyzed by western blotting. NM-MHC2A serves as a loading control.

Figure 4. DN-Brg1 interferes with smooth muscle gene expression in primary smooth muscle cells. Primary colon smooth muscle cells were transduced by DN-Brg1 or YFP control adenovirus. 72 hours after transduction, mRNA was harvested and the levels of SRF dependent genes were measured by quantitative real time RT-PCR. Transcript level was calculated and presented as in figure 1. Data presented are the mean±SEM of 9 samples from 3 independent experiments. * Indicates statistical significance as determined by a student T test (P<0.05).

Figure 5. Brg1 forms a complex with SRF and MRTFA in vivo. A. Adenovirus encoding HA-tagged SRF and HA-tagged MRTFA were transduced into COS cells, 48 hours later, nuclear proteins were harvested using a hypotonic lysis/nuclease digestion protocol (Active Motif). Proteins were immunoprecipitated using MRTFA (MA), SRF (S) and Brg1 (B) antibodies and goat IgG (Ig(G)) or rabbit IgG (Ig(R)) as a negative control. (MRTFA is a goat antibody, SRF and Brg1 are rabbit antibodies.) Immunoprecipitated proteins were then identified by western blotting, using the antibodies indicated at the right of the blot. Inp- 10% of input. B. Co-immunoprecipitations were performed from nuclear extracts prepared from A10 cells. C. Mouse bladder tissues were diced into small pieces and homogenized in a pre-chilled homogenizer prior to harvesting nuclear protein as described above for cells. Precipitated proteins were analyzed by western blotting using antibodies directed against endogenous, Brg1, MRTFA and SRF as described in Experimental Procedures.
Figure 6. Brg1 binds MRTFA but not SRF in vitro. SRF, MRTFA and Brg1 proteins were synthesized by using in vitro transcription and translation, as described in experimental procedures. The proteins were then incubated together as shown at the top of the blot and proteins were immunoprecipitated using the antibodies indicated below the blot (IP) (preimm: preimmune serum from rabbit used to make MRTFA antibody). The presence of individual proteins in each immunoprecipitate was determined by western blotting using antibodies to each of their respective epitope tags (indicated at the right of the blot). 10% of the input of each IP was loaded at the left of the blot.

Figure 7. DN-Brg1 attenuates the ability of MRTFA to increase SRF binding to the promoters of smooth muscle-specific genes. B22 cells grown in the presence (control) or absence (+DN-Brg1) of tetracycline were transduced with MRTFA or YFP control adenovirus. After 30 hrs, cells were fixed and harvested for chromatin immunoprecipitation assays. Chromatin was precipitated using an antibody against SRF (left panels), acetylated histone H3 (right panels) or using IgG negative control. The precipitated genomic DNA was purified and the presence of the promoters of SRF-dependent genes measured by real time quantitative PCR, using gene specific primers. A. The increase in SRF or H3Ac binding in samples transduced with MRTFA is indicated relative to those transduced with YFP. These data were calculated and normalized to input levels as follows: Relative SRF/H3Ac binding, RQ=2^(-\Delta\Delta Ct), with \Delta\Delta Ct = (C_t MRTFA - C_t input) - (C_t YFP - C_t input) B. The relative inhibition of MRTFA induced SRF or H3Ac binding by DN-Brg1 is shown. This was calculated as follows: Relative SRF/H3Ac binding, RQ=2^(-\Delta\Delta Ct), with \Delta\Delta Ct = (C_t DN-Brg1 - C_t input) - (C_t control - C_t input). SRF data shown in panels ‘A’ and ‘B’ are the mean±SEM of 7 samples obtained from 3 independent experiments. H3Ac data shown in panels ‘A’ and ‘B’ are the mean±SEM of 4 samples obtained from 2 independent experiments. A one sample t-test was performed and the asterisks indicates the results that are statistically different from 1 (P<0.05). C. The relative binding of SRF or H3Ac in control samples (no DN-Brg1, YFP transduced) is shown for each of the promoters indicated. Results were calculated as follows: Relative SRF/H3Ac binding, RQ=2^(-\Delta Ct), with \Delta Ct = C_t SRF IP group - C_t IgG IP group. Results presented are the mean±SEM of 4-5 samples.

Figure 8. Proposed model describing the regulation of MRTFA/SRF activity by Brg1. Upper panels: The telokin promoter is used as an example to describe the activation of smooth muscle-specific promoters by MRTFA. In unstimulated non-muscle cells the promoter is in a condensed inactive chromatin conformation that only permits weak or transient SRF binding (Left panel). Upon increased nuclear MRTFA, MRTFA binds to Brg1 and recruits the MRTFA/Brg1/HAT (p300?) complex to the weakly bound SRF. The binding of Brg1 to acetylated histone tails may also play a role in this recruitment process. Once the MRTFA/Brg1/HAT complex is recruited to the promoter, Brg1 utilizes the energy derived from hydrolysis of ATP to remodel the chromatin structure, permitting tight binding of SRF (Middle panel). Tightly bound SRF/MRTFA can then recruit additional co-activators such as p300, resulting in additional chromatin remodeling, facilitating binding of the RNA polymerase complex and subsequent activation of transcription (Right panel). Lower panels: On the promoters of MRTFA regulated early response genes, such as the SRF gene itself, SRF is tightly bound to the promoter under resting conditions (Left panel). MRTFA, therefore, does not require Brg1-mediated chromatin remodeling to be able to bind to the promoter, recruit co-activators and thereby activate transcription (Right panel).
Figure 1

- **telokin**
  - Control: [Graph]
  - DN-Brg1: [Graph]
  - * indicates significant difference

- **SM22α**
  - Control: [Graph]
  - DN-Brg1: [Graph]
  - MRTFA: [Color]
  - YFP: [Color]

- **calponin**
  - Control: [Graph]
  - DN-Brg1: [Graph]
  - * indicates significant difference

- **SM α-actin**
  - Control: [Graph]
  - DN-Brg1: [Graph]

- **vinculin**
  - Control: [Graph]
  - DN-Brg1: [Graph]
  - * indicates significant difference

- **SRF**
  - Control: [Graph]
  - DN-Brg1: [Graph]
  - * indicates significant difference

- **egr-1**
  - Control: [Graph]
  - DN-Brg1: [Graph]

- **c-fos**
  - Control: [Graph]
  - DN-Brg1: [Graph]
Figure 7

A

SRF

Relative SRF Binding

MRTFA/YFP

telokin SM22α α-actin SRF c-fos

B

H3 Ac

Relative H3Ac Binding

MRTFA/YFP

telokin α-actin

C

Relative SRF binding

Control/IgG

telokin SM22α α-actin SRF c-fos

Relative H3Ac Binding

IP/Input

telokin α-actin
A novel role of Brg1 in the regulation of SRF/MRTFA-dependent smooth muscle-specific gene expression
Min Zhang, Hong Fang, Jiliang Zhou and B. Paul Herring

J. Biol. Chem. published online June 28, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M701925200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2009/03/31/M701925200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2007/06/28/jbc.M701925200.citation.full.html#ref-list-1