Induction of Smooth Muscle α-Actin in Vascular Smooth Muscle Cells by Arginine Vasopressin Is Mediated by c-Jun Amino-terminal Kinases and p38 Mitogen-activated Protein Kinase*

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Exposure of vascular smooth muscle cells to arginine vasopressin (AVP) increases smooth muscle α-actin (SM-α-actin) expression through activation of the SM-α-actin promoter. The goal of this study was to determine the role of the mitogen-activated protein kinase (MAP kinase) family in regulation of SM-α-actin expression. AVP activated all three MAP kinase family members: ERKs, JNKs, and p38 MAP kinase. Inhibition of JNKs or p38 decreased AVP-stimulated SM-α-actin promoter activity, whereas inhibition of ERKs had no effect. A 150-base pair region of the promoter containing two CARG boxes was sufficient to mediate regulation by vasoconstrictors. Mutations in either CARG box decreased AVP-stimulated promoter activity. Electrophoretic mobility shift assays using oligonucleotides corresponding to either CARG box resulted in a complex of similar mobility whose intensity was increased by AVP. Antibodies against serum response factor (SRF) completely supershifted this complex, indicating that SRF binds to both CARG boxes. Overexpression of SRF increased basal promoter activity, but activity was still stimulated by AVP. AVP stimulation rapidly increased SRF phosphorylation. These data indicate that both JNKs and p38 participate in regulation of SM-α-actin expression. SRF, which binds to two critical CARG boxes in the promoter, represents a potential target of these kinases.

Vascular smooth muscle cells (VSMC) represent a highly plastic cell type that displays distinct changes in patterns of gene expression and proliferative rate during development and in association with vascular diseases (1, 2). During normal development VSMC undergo continuous differentiation from a proliferative phenotype, characteristic of embryonic and neonatal vessels, to a non-proliferating contractile phenotype associated with adult vessels. Smooth muscle α-actin (SM-α-actin) is probably the earliest marker of this process, and quantitative changes in SM-α-actin expression occur during development (3). In pathophysiologic settings such as atherosclerotic lesions (4) or following creation of intimal lesions by balloon catheterization, a subpopulation of VSMC undergo a phenotypic conversion to acquire many of the characteristics of neonatal SMC. This modulation of phenotype is associated with a decrease in expression of SM-α-actin and increases in non-muscle β-actin expression (5).

It is presumed that modulation of VSMC phenotype is mediated through activation of specific signal transduction pathways that act on transcription factors. In cultured VSMC, increases in SM-α-actin expression are observed in response to specific agonists. Vasoconstrictors such as arginine vasopressin (AVP) or angiotensin II (6–8) as well as transforming growth factor-β (9) increase expression, and these effects are mediated largely through increased transcription. We (10) and others (11) have previously demonstrated that a 150-base pair region of the rat SM-α-actin promoter is sufficient to mediate induction by vasoconstrictors in VSMC. This region contains two CC(A/T)G elements, known as CARG boxes: CARG-B (−121 to −112) and CARG-A (−71 to −62). Multiple CARG boxes have been identified in promoters of muscle-specific genes including skeletal and cardiac α-actin (12, 13) and myosin light chain (14). A CARG box also forms the core of the serum response element, controlling induction of c-fos. Serum response factor (SRF), a member of the MADS family of transcription factors (15), binds to the CARG box in the c-fos promoter (16) as well as to CARG boxes in promoters of muscle-specific genes (17–19). Induction of c-fos by mitogens involves formation of a ternary complex between DNA, SRF, and ternary complex factor (TCF), a member of the ets family of transcription factors (20). Phosphorylation of ternary complex factor (TCP) by members of the mitogen-activated protein kinase (MAP kinase) family is critical for formation of this complex (21). Although SRF binding to CARG boxes in promoters of muscle-specific genes appears to be required for increased expression (17–19), these genes are generally repressed by mitogenic stimuli. Conversely, induction of these genes during phenotypic modulation is often associated with withdrawal from the cell cycle and growth arrest. The mechanism whereby SRF acts on these two opposing patterns of gene expression remains to be determined but is likely to involve interactions with other transcriptional factors mediated through specific signaling pathways.

Earlier work from our laboratory showed that induction of SM-α-actin promoter activity by AVP is mediated through members of the Gα family of trimeric G-proteins (22). These studies implicated a role for MAP kinase in this regulation.

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¶ The abbreviations used are: VSMC, vascular smooth muscle cells; MEM, minimal essential media; SM-α-actin, smooth muscle α-actin; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; FCS, fetal calf serum; PDGF, platelet-derived growth factor; AVP, arginine vasopressin; SRF, serum response factor; EMSA, electrophoretic mobility shift assay; MAP, mitogen-activated protein, JNK, c-Jun amino-terminal kinase; PIPES, 1,4-piperaziniedethanesulfonic acid.

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Three major MAP kinase families have been described: extracellular-regulated kinases (ERKs), the c-Jun amino-terminal kinases (JNKs), and p38 MAP kinase family (23–25). Each of these is activated by specific upstream kinases: MKK1/2 for extracellular-regulated kinases (ERKs), MKK4/7 for JNKs, and MKK3/6 for p38 MAP kinase. Expression of dominant negative MKK4 inhibited the induction of the SM-α-actin promoter by vasoconstrictors (22). However, the role of other members of the MAP kinase family has not been examined in detail. The goal of the present study was to determine the role of the MAP kinase family in regulation of SM-α-actin expression and identify transcription factors that may mediate this effect.

MATERIALS AND METHODS

Reagents—The expression plasmid for SRF was a gift of Dr. Michael Gilman (Ariad Pharmaceuticals, Cambridge, MA). Full-length SRF was made as a polymerase chain reaction (PCR) product, cut with XhoI and BamHI, and inserted into pcDNA cut with XhoI and BamHI. The phosho-specific antibody against SRF was a gift of Dr. Michael Greenberg (Harvard Medical School, Boston, MA), and specifically recognizes SRF phosphorylated at serine 103 (26). Antibody against SRF used in electrophoretic mobility shift assays (EMSA) super-shift assays was purchased from Santa Cruz Pharmaceuticals (Santa Cruz, CA).

VSMC Isolation—Rat VSMC were isolated and cultured as described previously (27, 28). Briefly, thoracic aortas were dissected from Harlan Sprague-Dawley rats (250–300 g) and incubated in Eagle’s MEM containing 2 mg/ml collagenase for 1 h at 37°C. The adventitia was removed, and the aortas were minced and incubated in the MEM collagenase solution at 37°C for 2 h. The isolated cells were plated at a density of 1×10⁴ cells/ml culture media (Eagle’s MEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum) in 35-mm culture dishes. Cells were passed by trypsinization and used between passage numbers 3 to 9.

Reverse Transcription-PCR of JNK Isoforms and JNK Expression Constructs—mRNA was isolated from VSMC using the Promega polyAtract 1000 kit. Reverse transcription-PCR reactions were performed using primers specific for individual JNK isoforms: JNK1 5′ primer, GCAGGCTTATGATGCTATTCTTGAA; 3′ primer, TGGAGCTT- GAGGACCGCCTTATGATGCTATTCTTGAA; 3′ primer, TGGAGCTT- GAGGACCGCCTTATGATGCTATTCTTGAA; 3′ primer, TGGAGCTT- GAGGACCGCCTTATGATGCTATTCTTGAA. The resultant PCR products were confirmed to be the appropriate JNK isoform by direct sequencing.

In vitro arrays—JNKs in which the TPY sequence required for activation by upstream kinases was replaced by APF were prepared as described previously (29). Fusion proteins consisting of MKK7 fused to specific JNK isoforms were prepared as described previously (30) by fusing the murine MKK7 (31) to individual JNK cDNAs.

Assay of p38 MAP Kinase—VSMC grown to confluence and grown arrested for 3 days were treated with 0 or 100 ng/ml FCS. The cells were then exposed to AVP or vehicle (control) for the indicated times. Cells were harvested by trypsinization, and nuclear extracts were prepared by a modification of the method of Andrew and Fuller (34). Briefly, cells pellets containing 10⁶ cells were resuspended in a 5× volume of Buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride) at 4°C for 10 min, vortexed, and centrifuged at 25,000×g for 20 min. The pellet was resuspended in 100 μl of Buffer C (20 mM HEPES-KOH, pH 7.9, 250 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 M phenylmethylsulfonyl fluoride), homogenized with a Dounce homogenizer, and incubated at 4°C for 20 min. Samples were centrifuged for 20 min at 25,000×g, and the supernatant was recovered. Extracts were separated into aliquots and stored at −70°C.

Single-stranded oligonucleotide probes encoding the CArG elements plus 10 nucleotides of flanking sequence on both sides were custom-designed as follows: CArG-A sense, 5′-TGATCTTTGTTCCCTTTGTTTG- GAAAGCGAGT; CArG-Amut sense, 5′-TGATCTTTGTTCCCTTTGTTTG- GAAAGCGAGT; CArG-B sense, GTCTGAGGAGCTCCCTATAGGTTTG- GTTTAGA; CArG-Bmut sense, GTCTGAGGAGCTCCCTATAGGTTTG- GTTTAGA. The 5′ and 3′ oligonucleotides were annealed by heating for 5 min at 95°C and allowing the reaction mixture to slowly return to room temperature. Probes were radiolabeled using DNA polymerase I large (Klenow) fragment and [32P]dCTP for CArG-B and [32P]dGTP for CArG-A. Unincorporated 32P nucleotides were removed by polyacrylamide gel electrophoresis, and the labeled probe was eluted from the gel. A 30-min binding reaction was performed at 4°C (20 μl total volume; 5–10 μg of nuclear binding proteins, −75 pg of [32P]labeled probe) in Buffer D (50 mM HEPES-KOH, pH 7.9, 0.5 M NaCl, 1 mM dithiothreitol, 0.3 M phenylmethylsulfonyl fluoride, 0.3 M dithiothreitol, and 12% glycerol). For competition studies, the appropriate concentration of unlabeled DNA was added to the reaction 30 min before the addition of the radiolabeled probe. In the super-shift experiments, antibodies were preincubated at 4°C for 30 min with the nuclear binding proteins before addition of [32P]labeled DNA. Protein-DNA complexes were resolved on a 5% acrylamide gel (29:1 acrylamide: bisacrylamide, Life Technologies) and electrophoresed at 25 mAmp for 2 h in a 1× TGE (25 mA Tris, 1 mM EDTA, 190 mM glycine). The gels were subsequently dried and exposed to film for autoradiography.

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Expression of SM-α-actin is mediated by JNKs and p38 MAP kinase.

**RESULTS**

We previously demonstrated that expression of dominant negative MKK4, which specifically activates JNKs, partially inhibited the AVP-mediated increase in SM-α-actin promoter activity (22). Since multiple forms of JNK have been identified by molecular cloning, we sought to determine which isoforms were expressed in VSMC. By reverse transcription-PCR analysis and direct sequencing of the PCR product, all three gene products (JNK1, JNK2, and JNK3) were expressed in these cells (data not shown). Although the expression of JNK1 and JNK2 has been shown to be widespread, JNK3 expression has only been reported in a limited number of tissues including heart and brain (25). To test the role of individual isoforms in the regulation of SM-α-actin expression, VSMC were transiently co-transfected with the SM-α-actin promoter along with 5 μg of plasmids encoding MKK7 fusions with the indicated JNK isoform. Cells were then incubated for 3 days with media containing 0.2% FBS. Separate dishes were transfected without the MKK7 fusions and stimulated with AVP or 0.2% FBS for 3 days. Cells were harvested, and promoter activity normalized to β-galactosidase determined. Results represent the mean of three independent experiments performed in duplicate with the S.E. indicated.

Expression of constitutively active MKK7/JNK fusions stimulates SM-α-actin promoter activity. VSMC were transiently co-transfected with the SM-α-actin promoter along with 5 μg of plasmids encoding MKK7 fusions with the indicated JNK isoform. Cells were then incubated for 3 days with media containing 0.2% FBS. Separate dishes were transfected without the MKK7 fusions and stimulated with AVP or 0.2% FBS for 3 days. Cells were harvested, and promoter activity normalized to β-galactosidase determined. Results represent the mean of three independent experiments performed in duplicate with the S.E. indicated. *p < 0.05 versus basal.

Expression of dominant negative JNK isoforms inhibits induction of SM-α-actin promoter by vasoconstrictors. VSMC were transiently transfected with the SM-α-actin promoter (15 μg) along with plasmids encoding dominant negative forms (APF) of individual JNK isoforms (5 μg) or control plasmid (pDNA-3). Cells were then stimulated with AVP or angiotensin II (AII) for 3 d, and promoter activity normalized to β-galactosidase was determined. Results represent the mean of three independent experiments performed in duplicate, with the S.E. indicated.

Induction of SM-α-actin is mediated by JNKs and p38 MAP kinase.

**Fig. 1.** Expression of dominant negative JNK isoforms inhibits induction of SM-α-actin promoter by vasoconstrictors. VSMC were transiently transfected with the SM-α-actin promoter (15 μg) along with plasmids encoding dominant negative forms (APF) of individual JNK isoforms (5 μg) or control plasmid (pDNA-3). Cells were then stimulated with AVP or angiotensin II (AII) for 3 d, and promoter activity normalized to β-galactosidase was determined. Results represent the mean of three independent experiments performed in duplicate, with the S.E. indicated.

**Fig. 2.** Expression of constitutively active MKK7/JNK fusions stimulates SM-α-actin promoter activity. VSMC were transiently co-transfected with the SM-α-actin promoter along with 5 μg of plasmids encoding MKK7 fusions with the indicated JNK isoform. Cells were then incubated for 3 days with media containing 0.2% FBS. Separate dishes were transfected without the MKK7 fusions and stimulated with AVP or 0.2% FBS for 3 days. Cells were harvested, and promoter activity normalized to β-galactosidase determined. Results represent the mean of three independent experiments performed in duplicate with the S.E. indicated. *p < 0.05 versus basal.

**Fig. 3.** Stimulation of p38 MAP kinase in VSMC. VSMC were serum-restricted overnight and then stimulated with 10⁻¹ M AVP for 2, 5, 10, 15, or 30 min. A separate dish of cells was stimulated for 1 min with UV light and incubated for an additional 30 min. Cells were lysed and immunoprecipitated with anti-p38 antibodies. After washing, the immunoprecipitates were assayed for kinase activity using recombinant ATF-2. Samples were analyzed by SDS-gels followed by autoradiography and quantitated with a PhosphorImager. A representative experiment is shown. B, basal.

Dependent fashion, with half-maximal inhibition occurring between 2 and 5 μM (Fig. 4A). In contrast, PD98059, a specific MEK inhibitor (37), had no effect on either basal or AVP-stimulated SM-α-actin promoter activity (Fig. 4B). Conversely, expression of constitutively active MKK6, which specifically activates p38 MAP kinase (38), increased SM-α-actin promoter activity to the same level seen with AVP stimulation of cells not expressing constitutively active MKK6 (Fig. 5). This increase was blocked by treating the cells with SB203580. To identify potential targets of these kinase pathways, we sought to characterize critical elements of the SM-α-actin promoter and identify transcription factors that bind to these elements. Point mutations in each of the two CArG boxes were prepared by PCR and ligated into a promoterless CAT vector. VSMC were transfected with the respective constructs, and CAT activity was determined after incubation of cells for 3 days in the presence or absence of AVP, PDGF, or both agents. A point mutation in either CArG box resulted in a marked de-
crease in both basal and AVP-stimulated CAT activity (Fig. 6). Constructs encoding a mutation in CArG-B retained greater levels of promoter activity in response to AVP than mutations in CArG-A. A construct in which both CArG boxes were simultaneously mutated did not show any increased CAT activity in response to AVP. Mutations in the individual CArG boxes also blunted the increase in SM-α-actin promoter activity induced by transient expression of constitutively active α16(A16Q212L), (data not shown).

Complexes formed with the individual CArG boxes were analyzed by EMSA using nuclear extracts prepared from control and AVP-treated VSMC. Incubation of nuclear extracts with 32P-labeled CArG-B revealed a single major complex (Fig. 7A). Exposure of cells to AVP increased the intensity of this band by as early as 4–6 h, and this increase was maintained for at least 24 h(data not shown). The labeled complex was competed off by double-stranded cold oligonucleotides (Fig. 7, DS) but not by single-stranded sense (SS) or antisense oligonucleotides (not shown). Incubation of the same extracts with a

Previous studies indicate that one of the transcription factors binding to CArG boxes in this promoter is SRF (11, 39). Incubation of nuclear extracts with antibodies against SRF completely super-shifted the complex formed with both 32P-CArG-B and 32P-CArG-A in both extracts from control and AVP-stimulated cells (Fig. 7, A and B), indicating that SRF constitutively forms part of these complexes. To assess the functional role of SRF, VSMC were co-transfected with an expression plasmid encoding full-length SRF (a gift of Dr. Michael Gilman, Ariad Pharmaceuticals, Cambridge MA) along
with the SM-α-actin promoter construct. Overexpression of SRF increased basal promoter activity to levels greater than seen with AVP alone (Fig. 8). Exposure to AVP caused a further increase in promoter activity in cells overexpressing SRF. Overexpression of SRF also increased promoter activity with the CArg-BMUT activity in AVP-treated cells was about 70% that seen with the wild-type promoter in the absence of SRF overexpression. With the CArg-AMUT overexpression of SRF did not increase basal promoter activity but increased activity in AVP-stimulated cells to approximately 25% that of the levels detected using the wild-type promoter without SRF overexpression. These results indicate that increased SRF expression has a greater effect on CArg-B. To confirm this finding we employed a truncated version of the SM-α-actin promoter in which CArg-B had been deleted (~102). Overexpression of SRF had no significant effect on the promoter activity of this construct either in the absence of presence of AVP stimulation. Expression of SRF failed to increase promoter activity using the double mutant in which both CArg boxes have been altered (data not shown).

Expression levels of SRF protein were examined by immunoblotting. Exposure of VSMC to either AVP, angiotensin II, or PDGF for up to 72 h did not significantly alter SRF expression (Fig. 9A). Stimulation of fibroblasts by growth factors has been shown to increase phosphorylation of SRF at serine 103 (26). Using a phospho-specific SRF antibody (a gift of Dr. Michael Greenberg, Harvard Medical School), the effect of AVP on SRF phosphorylation was determined. AVP rapidly increased SRF phosphorylation by 5 min, and this effect was sustained for at least 30 min (Fig. 9B). By densitometry, the increase in phosphorylation at 15 min was 3-4-fold.

![Image](image_url)

**FIG. 7.** EMSA with oligonucleotides corresponding to CArg-B and CArg-A. Panel A, nuclear-binding proteins were prepared from basal cells (B) or cells stimulated with AVP (A) for either 4 or 6 h, as described under "Materials and Methods." Extracts were incubated with 32P-labeled oligonucleotides corresponding to CArg-B for 30 min, described under "Materials and Methods." Extracts were incubated which failed to form the complex. Panel B, extracts from control cells (B) or cells stimulated for 6 h with AVP (A) were incubated with 32P-CArg-Bmut, which failed to form the complex. Panel B, extracts from control cells (B) or cells stimulated for 6 h with AVP (A) were incubated with 32P-CArg-Bmut, which failed to form the complex. Panel B, extracts from control cells (B) or cells stimulated for 6 h with AVP (A) were incubated with 32P-CArg-Bmut, which failed to form the complex.

**DISCUSSION**

Data from our laboratory and those of others have demonstrated that vasoconstrictors increase expression of SM-α-actin in VSMC through transcriptional activation of the promoter. We have previously presented data suggesting that the JNK family of MAP kinases participate in induction of the promoter by AVP by co-transfection of a dominant negative JNK kinase, MKK4 (22). In this study we have confirmed a role for JNKs using two distinct strategies. Inhibitory forms of individual JNK isoforms (APF) blocked vasoconstrictor-mediated induction, and expression of MKK7/JNK fusions, which are constitutively active, increased promoter activity in the absence of vasoconstrictor stimulation. In both of these studies we did not detect any selectivity for individual JNK isoforms. Since the inhibitory constructs only partially inhibited induction of SM-α-actin, it is likely that additional signaling pathways are involved in regulation of the promoter. From these studies, the p38 MAP kinase pathway clearly plays an important role. Pharmacologic inhibition of p38 MAP kinase completely blocked induction of SM-α-actin promoter activity by AVP, and a constitutively active form of MKK6, a specific p38 MAP kinase kinase, induced promoter activity to a similar extent as stimulation with AVP. We would therefore propose that regulation of the SM-α-actin promoter is multifactorial, involving both JNKs and p38. The relative contributions of each pathway are difficult to assess. The magnitude and duration of kinase activation in AVP-stimulated cells is more transient than the sustained activation seen with expression of constitutively active isoforms. From studies using expression of Jun-Gal4 and UAS-luc, it appears that dominant negative forms of MKK4 or JNKs do not completely block the activation of wild-type JNKs (data not shown). It is therefore conceivable that the partial effects seen in Figs. 1 and 2 may be a result of incomplete inhibition of endogenous JNK activity by the dominant negative constructs. Finally, although the activation of JNKs by expression of MKK7/JNK constructs is sustained, the magnitude is less than the more transient peak of activity seen with wild-type JNKs activated by vasoconstrictors. The availability of specific pharmacological inhibitors of the JNK pathway will facilitate assessment of the role of this pathway in regulation of SM-α-actin expression by AVP.

We presume that the effects of these kinase pathways on regulation of SM-α-actin promoter activity is mediated through
phosphorylation of downstream transcription factors that bind to critical regulatory elements. We have previously shown that a 150-base pair region of the SM-α-actin promoter, which contains two CArG boxes, is sufficient to mediate stimulation of the promoter by vasoconstrictors (10). Our results employing point mutations demonstrate that both CArG boxes are required for AVP stimulation of the promoter and that mutations within the central A/T-rich hexamer are sufficient to disrupt regulated transcription. These mutations also blocked induction of the promoter by transient expression of constitutively active forms of α16 (data not shown), consistent with our previous results that vasoconstrictors and α16Q212L act through the same pathways to induce SM-α-actin expression (22).

SRF is a transcription factor that has been shown to bind to CArG boxes in a variety of promoters, including those of immediate early genes such as c-fos (16) as well as muscle-specific genes (11, 17, 40, 41). By EMSA analysis with nuclear extracts from VSMC, we have demonstrated that SRF forms a component of the complex detected with each of the two proximal CArG boxes of the SM-α-actin promoter. The relative intensities of the complexes formed suggest that SRF binds with greater affinity to CArG-B than to CArG-A, consistent with what has been observed by other investigators (11). This is confirmed by the finding that non-radioactive oligonucleotides corresponding to CArG-B displace complexes formed with [32P]-CArG-B or [32P]-CArG-A more potently than non-radioactive CArG-A. Formation of SRF-containing complexes appears to be critical for activation of the promoter, since mutations in the A/T-rich central region of the CArG boxes dramatically decreased both the amount of complex detected and promoter activity. This region has been shown to be critical for SRF binding to the CArG box contained within the c-fos promoter (42).

Although SRF appears to be constitutively bound to these elements, we observed increases in the intensity of the complexes formed within cells stimulated with AVP. Similar increases have been reported in extracts of cells stimulated with angiotensin II (11). Since AVP did not increase protein expression of SRF, we propose that post-translational modifications of SRF may result in increased affinity for individual CArG boxes. Studies examining growth factor induction of the c-fos promoter demonstrate that increased phosphorylation of SRF at serine 103 contributes to transcriptional activation by increasing the affinity of SRF for the CArG box forming the core of the serum response element (26). Using a phospho-specific antibody specific for serine 103, we have shown that AVP stimulation rapidly increases SRF phosphorylation at this site.

We have not determined how SRF phosphorylation mediates activation of the promoter. One possibility, analogous to what occurs in the c-fos promoter, is that phosphorylation increases the affinity of SRF for the CArG boxes in the SM-α-actin promoter. Increased steady state binding of SRF would then lead to increased transcription. Support for such a model is provided by the observation that overexpression of SRF, which increases the concentration of SRF and favors increased binding, was sufficient to mediate an increase in SM-α-actin promoter activity, even in the absence of vasoconstrictor stimulation (Fig. 8). However, AVP stimulation of cells overexpressing SRF resulted in further increases in promoter activity, suggesting additional mechanisms are operative. Phosphorylation of SRF may be required for formation of higher order complexes of transcription factors and co-activators. These factors remain to be identified, although several factors that may modulate SRF binding have been described.

YY1 has been shown to enhance the affinity of SRF binding to the c-fos promoter by causing structural changes in the DNA (43). However, other studies demonstrate antagonism of the effects of SRF by YY1 on expression of skeletal and cardiac α-actin genes (13, 44–46). Our studies to date have not detected complexes containing YY1 binding to either CArG box of the SM-α-actin promoter (data not shown). Homeobox proteins such as Phox1 have also been shown to enhance SRF binding (47). In this case this effect was not mediated through direct binding of Phox to DNA. A role for the homeobox protein Mhox has been proposed to mediate induction of the SM-α-actin promoter by angiotensin II (11). It is likely, as suggested by other workers, that binding of SRF to multiple CArG boxes coordinates the formation of a higher order complex necessary for increases transcription of the SM-α-actin gene (48). SRF phosphorylation may be critical for this process.

In summary, our studies indicate that increased transcription of the SM-α-actin gene by AVP is mediated through integration of both JNKs and p38 MAP kinase. These pathways act on factors binding to two CArG boxes that lie within the first 150 base pairs of the promoter region. SRF, which is rapidly phosphorylated following AVP stimulation, binds to both boxes. Increased SRF phosphorylation through either JNK or p38-de-
ependent pathways may be critical for forming larger complexes and engaging the transcriptional machinery.

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