Expression of α-actin in smooth muscle cells (SMCs) is regulated, in part, by an intronic serum response factor (SRF)-binding CArG element. We have identified a conserved nuclear factor of activated T cells (NFAT) binding site that overlaps this CArG box and tested the hypothesis that this site plays a previously unrecognized role in regulating α-actin expression. A reporter construct prepared using a 56-bp region of the mouse α-actin first intron containing SRF, NFAT, and AP-1 sites (SNAP) acted as an enhancer element in the context of a minimal thymidine kinase promoter. Basal reporter activity following expression in SMCs was robust and sensitive to the calcineurin-NFAT pathway inhibitors cyclosporin A and FK506. Mutating either the NFAT or SRF binding site essentially abolished reporter activity, suggesting that both NFAT and SRF binding are required. Basal activity in non-smooth muscle HEK293 cells was SRF-dependent but NFAT-independent and ~8-fold lower than that in SMCs. Activation of NFAT in HEK293 cells induced an ~4-fold increase in activity that was dependent on the integrity of both NFAT and SRF binding sites. NFATc3-SRF complex formation, demonstrated by co-immunoprecipitation, was facilitated by the presence of SNAP oligonucleotide. Inhibition of the calcineurin-NFAT pathway decreased α-actin expression in cultured SMCs, suggesting that the molecular interaction of NFAT and SRF at SNAP may be physiologically relevant. These data provide the first evidence that NFAT and SRF may interact to cooperatively regulate SMC-specific gene expression and support a role for NFAT in the phenotypic maintenance of smooth muscle.

Smooth muscle cells (SMCs), unlike terminally differentiated cardiac and skeletal myocytes, are phenotypically dynamic and maintain their differentiated phenotype through the regulated expression of a repertoire of smooth muscle-specific genes (1). This phenotypic flexibility is consistent with the physiological demands placed on vascular tissue and may underlie changes in smooth muscle structure and function that accompany pathological processes, such as those that occur in hypertension and atherosclerosis (2–4).

SRF transcriptional activity may be modulated by additional cofactors, some of which form complexes with SRF-CArG DNA, whereas others act to enhance SRF binding to CArG boxes without forming a detectable ternary complex (6, 14, 15). Although SRF activity is critically important in the regulation of SMC differentiation and SMC marker gene expression, CArG boxes alone are not sufficient to direct SMC-specific gene expression; additional cis-elements and trans-acting factors are required (reviewed in Ref. 6). Mechanisms that exist in parallel with the SRF-CArG pathway, as well as SRF-intersecting mechanisms, contribute to SMC phenotypic modulation and maintenance.

One of the more recent entries in the smooth muscle phenotypic maintenance derby is the calcium (Ca\textsuperscript{2+})-dependent transcription factor nuclear factor of activated T cells (NFAT), which regulates the expression of genes in a diverse array of immune and non-immune cells and is closely linked to developmental processes that involve modulation of cellular phenotypes (16–24). NFAT also contributes to pathological processes, as exemplified by its role in the etiology of pathological, but not exercise-induced, cardiac hypertrophy (25–27).

NFAT activation is regulated primarily through control of its subcellular localization (16, 28, 29). In response to Ca\textsuperscript{2+}-elevating stimuli, NFAT is dephosphorylated at multiple N-terminal phosphoserines by the Ca\textsuperscript{2+}-dependent phosphatase, calcineurin, which promotes NFAT nuclear translocation and DNA binding competence (30, 31). NFAT binds DNA with very low affinity in the absence of a cofactor, and in general, forma-
tation of an NFAT/cofactor complex is required for significant NFAT-mediated transcriptional activity. The NFAT family consists of four members (NFAT1/c2, NFAT2/c1, NFAT3/c4, and NFAT4/c3) that share the property of Ca\(^{2+}\)-dependent nuclear translocation and a fifth member, NFAT5, which is Ca\(^{2+}\)-independent and shares limited homology with the other family members.

NFAT activity has been shown to contribute to the maintenance of the differentiated smooth muscle phenotype through regulation of α1 integrin and caldesmon expression (32) and has been implicated in regulating the expression of the SM MHC gene (33). More recently, NFAT has been proposed to functionally disrupt SMC-specific expression also resulted in disruption of

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**Construction of Plasmids**—A 56-bp region of the α-actin first intron (corresponding to nucleotides 1035–1090 in GenBank\(^{22}\) accession number U83312) containing a previously identified SRF-binding CArG box, an overlapping NFAT binding motif, and an AP-1 binding site, designated SNAP (SRF/NFAT-AP1), was used as a starting point in the design of reporter constructs (see Fig. 1). A double-stranded oligonucleotide covering this region was prepared by combining equimolar amounts of single-stranded sense and antisense oligonucleotides (IDX Technologies), heat denaturing (95 °C, 5 min), and allowing them to slowly anneal at room temperature. SNAP oligonucleotides, synthesized in our laboratory, were cloned into an Nhel site present in the multi-cloning region of a luciferase reporter plasmid containing a minimal thymidine kinase (TK) promoter (pTAL; BD Biosciences). After transforming competent *Echerichia coli* (Invitrogen) and preparing DNA minirep from isolated colonies (Qiagen), individual clones were screened by restriction analysis to determine the orientation of each insert. Reporter constructs containing mutations in the NFAT (AGGGTT), SRF (AAATATAGG), or AP-1 (TCAACA) binding sites were similarly prepared.

Full-length NFATc3 was prepared from a plasmid (pSH205A) containing an alternatively spliced murine NFATc3 cDNA (37) kindly provided by Dr. Gerald Crabtree. The N terminus of native NFATc3 was cloned from mouse smooth muscle total RNA by reverse transcription-PCR and ligated into an XbaI site defining the 5′ end of the NFATc3 cDNA. A Tet-operated EGFP-NFATc3 fusion protein expression plasmid (pTetOP-EGFP-NFATc3-HGHpA) was prepared by cloning full-length NFATc3 into a pTetOP-EGFP-HGHpA plasmid constructed from pTetOP-HGHpA (containing a Tet operator and human growth hormone poly(A) tail) and pEGFP-C3 (Clontech). Transient Transfection and Luciferase Assay—HEK293 cells were transfected with TK-luciferase reporter constructs containing one, two, or three tandem copies of the SNAP element in the all-forward or all-reverse orientation using the FuGENE 6 transfection reagent (3:1 ratio of transfection reagent (μl)/total plasmid DNA (μg)) according to the manufacturer’s protocol (Roche Applied Science). Luciferase activity in cell lysates was determined using the Luciferase Assay System (Promega) and expressed relative to empty pTAL vector controls. Luciferase activity was normalized to protein concentration.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The rat A7r5 aortic smooth muscle cell line and an HEK293 human embryonic kidney-derived cell line (BD Biosciences) were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum and penicillin-streptomycin (10 units/ml; Invitrogen). G418 (2 mg/ml) was included in cultures of this reverse tetracycline transactivator (rtTA)-expressing HEK293 derivative (used in experiments involving co-transfection of a Tet operator-EGFP-NFATc3 construct) to maintain stable rtTA expression. The cultures were maintained in a humidified incubator at 37 °C and 5% CO\(_2\).
agarose beads (Pierce) were added (25 nucleotide. The beads were pelleted by centrifugation (2000 g). H10003
suspensions were placed on a gyrating shaker platform at 4 °C for 1–3 h. The proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed for SRF or NFATc3 by Western blotting. The absence of nonspecific binding to NFATc3-agarose beads was confirmed by stripping and reprobing for glyceraldehyde-3-phosphate dehydrogenase (primary antibody from Chemicon Interna-
tional; 1:1000 dilution for 1 h at room temperature; data not shown).

Western Blot Analysis—Protein samples were separated by SDS-PAGE (7.5% acrylamide gels in standard Laemmli buffer) and transferred to nitrocellulose membranes (Bio-Rad). After blocking for 1 h at room temperature in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20, blots were exposed to primary antibodies (diluted in blocking solution) overnight at 4 °C or for 1 h at room temperature. The primary antibodies used were mouse mono-
clonal NFATc3 (1:200; Santa Cruz Biotechnology) and rabbit polyclonal SRF (Santa Cruz Biotechnology). After multiple washes in Tris-buffered saline, horseradish peroxidase-conjugated secondary antibody (from Santa Cruz Biotechnology (1:10,000) or Pierce (1:2000) in blocking solution) was applied for 1 h at room temperature. Bands were visualized using standard or high sensitivity chemiluminescence detection systems (Pierce Supersignal West Dura or Femto, respectively).

Immunofluorescence—HEK293 and A7r5 cells were grown in an 8-well Lab-Tek chamber slide (Nalge Nunc). Cells were fixed with 4% formaldehyde in PBS for 15 min, permeabilized in 0.1% Triton in PBS for 10 min, and then blocked in 3% donkey serum in PBS for 1 h. Primary antibody (rabbit polyclonal anti-NFATc3, 1:100; Santa Cruz Biotechnology) was prepared in 0.1% gelatin in PBS and applied overnight at 4 °C. Secondary antibody (anti-rabbit Cy5, 1:500; Jackson Immunoresearch Laboratories) was prepared in 0.1% gelatin in PBS and applied for 1 h at room temperature. Nuclei were stained using SYTOX green (1:5000 in PBS).

Cells were examined at >40 magnification using a Bio-Rad 1000 laser scanning confocal microscope. NFAT and nuclear staining were detected by sequentially monitoring the Cy5 and SYTOX fluorescence using excitation wavelengths of 650 and 488 nm and emission wave-
lengths of 670 and 520 nm, respectively. Specificity of immune staining was confirmed by the absence of fluorescence in cells incubated with secondary antibody alone. For scoring of NFAT-positive nuclei, multiple fields for each replicate of each experimental condition were imaged and counted by two independent observers under double-blind conditions with the aid of Metamorph software (Universal Imaging Corp). The software was programmed so that individual pixels within a given image would appear white if co-localization of the green nuclear acid stain and the Cy5-NFAT stain occurred. Thus, for quantification pur-
pose, a cell was considered positive if co-localization (white) was dis-
tributed throughout the nucleus, whereas a cell was considered nega-
tive if no co-localization (green only) or only isolated co-localization was observed.

In-cell Western Analysis—SM α-actin expression in SMCs was quanti-
tified using an infrared-based in-cell Western assay system as described by the manufacturer (Li-Cor Biosciences). Briefly, confluent cultures of A7r5 cells were trypsinized and seeded into 96-well plates (70% conflu-
ence). After 48 h in 1% fetal bovine serum, cells were treated as described in the text, washed once with PBS, fixed with 4% formalde-
yde in PBS for 15 min at room temperature, and blocked (Aqua Block with 0.1% Tween 20; Rockland) for 1 h. TOTO-3 (1:1000; Molecular Probes) was used to counterstain DNA. Infrared fluorescence was monitored using an Odyssey Imager (700 nm for TOTO-3 and 800 nm for IRDye 800). The intensity of both channels was set at 7.5, with a focus setting of 3

mm. Integrated intensity of SM α-actin or β-actin (housekeeping protein) stain was normalized to the integrated intensity of DNA stain. Because of the ease and accuracy with which multiple individual rep-
lies can be independently analyzed, this approach is especially use-
ful where quantitation is the goal.

RESULTS

A CArG Box-containing Region of the Mouse α-Actin First Intron Acts as an NFAT- and SRF-dependent Enhancer Ele-
ment—We have identified a putative NFAT binding site in the α-actin first intron that overlaps a functionally important SRF-
binding CArG box (10). The sequence of this SRF-NFAT binding site-containing region is remarkably conserved among rod-
ent, human, and chicken α-actin and includes a completely conserved AP-1 binding site at a position ~24 bp downstream of the NFAT site (Fig. 1). To test these putative binding sites for potential enhancer activity, an oligonucleotide encompassing this mouse α-actin first intronic region containing SRF, NFAT, and AP-1 binding sites (SNAP) was cloned into a luciferase reporter plasmid upstream of a minimal TK promoter. Constructs containing one, two, or three tandem copies of SNAP in either forward or reverse orientation were tested in HEK293 cells and SMCs for luciferase expression. The minimal TK promoter-luciferase construct containing three tandem copies of the insert in the reverse orientation showed the highest reporter activity of those constructs tested (data not shown) and was used in subsequent experiments. The wild-type reporter construct (SNAP-Luc) and variants containing mutated SRF (SNmutNAP-Luc), NFAT (SNmutAP-Luc), or AP-1 (SNAPmut-
Luc) binding sites are depicted schematically in Fig. 2A.

Fig. 2, B and C, shows luciferase activity, normalized to the minimal TK promoter-containing (empty) pT.AL vector, in SNAP-Luc-transfected SMCs and HEK293 cells, respectively. In both cell lines, the SNAP element promoted a significant increase in reporter activity under basal (unstimulated) conditions, demon-
strating that this small region of the mouse α-actin first intron is capable of acting as a functional enhancer element. Inhibition of the calcineurin/NFAT pathway with FK506 (1 μM) and CsA (1 μM) significantly decreased reporter activity in SNAP-Luc-transfected SMCs (Fig. 2B), suggesting NFAT involvement.

To determine the relative contribution of SRF, NFAT, and/or AP-1 sites to the enhanced reporter activity, HEK293 cells and SMCs were transfected with SNAP-Luc constructs containing mutations at each of these sites, designed such that mutations in one binding element were independent of each of the other binding elements. Mutating either the NFAT site (SNmutAP-Luc) or the SRF binding site (SNmutNAP-Luc) almost completely abolished basal enhancer activity in SMCs, suggesting that both NFAT and SRF binding sites were required for this activity (Fig. 2B). Mutating the AP-1 binding site (SNAPmut-Luc) partially decreased basal activity in SMCs (Fig. 2B), suggesting that full activity may require formation of an SRF-NFAT-AP-1 complex. In contrast, only SRF binding appears to be required for basal activity in HEK293 cells because a mutation in the
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CARG box alone (SNmutAP-Luc) abrogated enhancer activity, whereas a construct containing a mutated NFAT site (SNmutAP-Luc) or a mutated AP-1 site (SNAPmut-Luc) retained full basal activity (Fig. 2C).

NFAT/AP-1 Pathway Stimulation Increases SNAP Enhancer Activity in HEK293 Cells—Concurrent treatment with the Ca²⁺ ionophore, ionomycin (Io), and phorbol 12-myristate 13-acetate acetate (PMA) is a stimulus paradigm that is commonly used to simultaneously activate NFAT and NFAT cofactor pathways (41–43). Dephosphorylation by the Ca²⁺-dependent phosphatase, calcineurin, serves to promote both NFAT nuclear accumulation and DNA binding (30). Stimulation of SNAP-Luc-transfected HEK293 cells with Io (1 μM) and PMA (100 nM) induced an ~4-fold increase in luciferase activity over unstimulated conditions (Fig. 3). This inducible luciferase activity was significantly inhibited by pre-treatment with the calcineurin inhibitors CsA and FK506, suggesting that dephosphorylation-dependent translocation of NFAT into the nucleus contributes to the observed increase in activity. The Io/PMA-induced increase in reporter activity, like the basal enhancer activity in SMCs, was dependent on the integrity of both SRF and NFAT binding sites, as indicated by the virtual absence of inducible activity in HEK293 cells transfected with reporter constructs containing mutations in the NFAT (SNmutAP-Luc) or SRF (SNmutAP-Luc) sites. Similar to results obtained under basal conditions in SMCs, a mutation in the AP-1 site (SNAPmut-Luc) partially but significantly reduced the response to Io/PMA stimulation in HEK293 cells. Interestingly, the high basal activity of the SNAP-Luc construct in SMCs (~8-fold higher than that in similarly transfected HEK293 cells) cannot be further enhanced by treatment with Io/PMA (data not shown; see “Discussion”).

NFATc3 and SRF Simultaneously Bind to the SNAP Enhancer Element—Functional effects of individual site mutations on SNAP enhancer activity strongly suggest that NFAT and SRF bind simultaneously at overlapping SRF and NFAT sites within the α-actin intronic regulatory element under study. To determine whether this was the case, we performed ABCD assays to pull down SNAP-associated proteins from cellular extracts, followed by Western analysis. Fig. 4A shows a representative ABCD assay employing whole-cell lysates from non-stimulated and Io/PMA-stimulated HEK293 cells. Consistent with the functional data, only SRF was bound to the oligonucleotide probe under non-stimulated conditions (Fig. 4A, lane 1). Under stimulated conditions, both SRF and NFATc3 were present in the DNA-protein complex (Fig. 4A, lane 2). Thus, the overlapping CARG box/NFAT site in the α-actin first intron can bind both NFATc3 and SRF in response to an NFAT-activating stimulus.

In competition assays employing either HEK293 or SMC whole-cell lysates, SRF binding to biotinylated SNAP was abrogated in the presence of an excess of unlabeled wild-type SNAP oligonucleotides. Unlabeled probes containing a scrambled sequence or a mutation in the SRF binding site (SNmutAP), however, failed to compete for SRF binding, demonstrating that the observed binding is sequence-specific (Fig. 4, B and C). To rule out the possibility that mutations in the adjacent NFAT or AP-1 sites might disrupt SRF binding, even though they do not disrupt the CARG box site itself, we performed competition binding assays using unlabeled SNAP containing mutated NFAT (SNmutAP) or AP-1 (SNAPmut) binding sites. In each case, an excess of unlabeled oligonucleotides was able to prevent SRF binding to the labeled probe, indicating that an intact CARG box alone is sufficient to bind SRF.

FIG. 2. A CARG box-containing region of the mouse α-actin first intron acts as an NFAT- and SRF-dependent enhancer under basal conditions. A, reporter constructs containing three tandem copies (depicted in forward orientation) of the SM α-actin first intronic enhancer element (SNAP) used in transfection experiments: wild-type (SNAP-Luc), NFAT-mutated (SNmutAP-Luc), SRF-mutated (SNmutNAP), or AP-1-mutated (SNAPmut). B, A7r5 SMCs were transfected with the SNAP-Luc construct or its mutants, as described under “Experimental Procedures.” Activity is expressed relative to the empty pTAL vector, which contains a minimal thymidine kinase promoter-luciferase expression cassette (100 nM) induced an ~4-fold increase in luciferase activity over the empty vector. *, p < 0.01 versus all; n = 6–9. C, HEK293 cells were transfected with the SNAP-Luc wild-type and mutant constructs, as described. Luciferase activity was normalized to reporter activity of cells transfected with empty vector. *, p < 0.01 versus all; n = 6.

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absence (lanes 3) relative to unstimulated SNAP-Luc without FK506/CsA. *, under non-stimulated conditions. Also shown is Io/PMA-stimulated SNAP-Luc activity in the presence of 1 pTAL/no insert) served as a control. Luciferase activity of each construct under stimulated conditions is expressed relative to reporter activity of wild-type SNAP-luciferase construct (SNAP-Luc) or constructs containing mutations in NFAT (SNmutAP-Luc), SRF (SmutNAP-Luc), or AP-1 (SNAPmutp-Luc) sites and incubated for 24 h with or without 1 μM Io and 100 nM PMA. HEK293 cells transfected with a minimal TK-Luc vector (pTAL/no insert) served as a control. Luciferase activity of each construct under stimulated conditions is expressed relative to reporter activity under non-stimulated conditions. Also shown is Io/PMA-stimulated SNAP-Luc activity in the presence of 1 μM FK506 and 1 μM CsA expressed relative to unstimulated SNAP-Luc without FK506/CsA. *, p < 0.01 versus no insert + Io/PMA, FK506/CsA + Io/PMA, SNmutAP + Io/PMA, SNmut + Io/PMA, and SNAPmut + Io/PMA; #, p < 0.05 versus no insert + Io/PMA. n = 4–10.

These experiments do not rule out the formal possibility that NFATc3 and SRF are binding individually to separate populations of probe molecules rather than binding simultaneously to the same probe. To address this, we immunoprecipitated NFATc3 in the presence or absence of unlabeled SNAP oligonucleotide and probed for associated SRF by Western analysis (Fig. 5). Under standard detection conditions (Fig. 5A), a strong SRF band is evident in the presence of the SNAP probe (lane 1), whereas no SRF band is evident in the absence of SNAP (scrambled oligonucleotide, lane 2; no oligonucleotide, lane 3). By increasing the gain (i.e. using a higher sensitivity chemiluminescence detection system), we are able to show that SRF is present in NFATc3 immunoprecipitates in the absence of SNAP, albeit to a lesser extent (Fig. 5B). These results indicate that SNAP acts as a sequence-specific scaffold to facilitate NFATc3 and SRF interactions, but they also suggest that NFATc3 and SRF may interact in solution.

NFATc3 Exhibits Constitutive Nuclear Localization in A7r5 Cells but Not HEK293 Cells—In general, NFAT is localized to the cytosol of unstimulated cells. However, in the A7r5 cells used in this study, we found that in an overwhelming majority of cells (95.33 ± 2.53%, n = 3 images (81 cells)), endogenous NFATc3 is constitutively localized to the nucleus under basal conditions (Fig. 6A, right panel). Inhibition of calcineurin activity by treatment with CsA and FK506 (15 min) induced a substantial redistribution of nuclear NFATc3 to the cytoplasm (NFATc3-positive nuclei = 27.40 ± 7.40%, n = 3 images (43 cells); p < 0.001 versus basal). Overexpressed EGFP-NFATc3 also exhibits nuclear localization in SMCs in the absence of an NFAT-activating stimulus (Fig. 6B, right panel). This distribution is strikingly different from the primarily cytosolic and perinuclear localization of both endogenous (Fig. 6A, left panel) and exogenously expressed (Fig. 6B, left panel) NFATc3 in HEK293 cells. These results are consistent with both the elevated basal activity of SNAP-Luc in SMCs and the requirement for an NFAT-activating stimulus for maximal reporter activity in HEK293 cells.

Calcineurin/NFAT Pathway Inhibition Reduces α-Actin Expression in Smooth Muscle Cells—The fact that NFAT and SRF can simultaneously bind to a CArG/NFAT site corresponding to a region of the α-actin intron known to be important for regulating SMC-specific α-actin expression (8, 10) suggests that NFAT cooperates with SRF in the regulation of α-actin expression. To confirm that the calcineurin/NFAT pathway is involved in regulating the expression of SM α-actin, we treated confluent, serum-deprived A7r5 SMCs with the calcineurin inhibitors FK506 and CsA, and we measured changes in SM α-actin protein expression by in-cell Western analysis (see *Ex-
Fig. 5. SNAP facilitates co-immunoprecipitation of SRF and NFAT. NFATc3 was immunoprecipitated from whole-cell lysates (500 μg of protein) of 10 (1 μM/PMA (100 nM)-treated (1 h) HEK293 cells in the presence or absence of SNAP followed by Western blotting for SRF using standard sensitivity (A) or high sensitivity (B) chemiluminescence detection systems (see “Experimental Procedures”). In C, Western blotting of NFATc3 immunoprecipitates for NFATc3 demonstrates uniform loading and immunoprecipitation efficacy. Lane 1, SNAP oligonucleotide; lane 2, scrambled sequence oligonucleotide; lane 3, no oligonucleotide.

Fig. 6. Differential subcellular localization of NFATc3 between HEK293 and A7r5 cells. A, representative immunofluorescence confocal microscopic images of endogenous NFATc3 in HEK293 and A7r5 cells. White pixels represent co-localization of NFATc3 (red) and nuclei (SYTOX green staining). Scale bar represents 50 μm. B, EGFP fluorescence in EGFP-NFATc3-transfected HEK293 and A7r5 cells monitored at 37 °C using 440DF20/480DF30 excitation/emission filters (Lambda 10-2; Sutter Instruments, Novato, CA) with the aid of METAFLUOR 4.01 analysis software (Universal Imaging, Media, PA). EGFP-NFATc3 exhibits both nuclear and cytosolic localization in SMCs; HEK293 cells show only cytosolic/perinuclear fluorescence. C, representative images of an A7r5 cell showing EGFP-NFATc3 nuclear expression before and 15 min after treatment with 1 μM FK506 and 1 μM CsA at 37 °C. Inset images correspond to endogenous NFATc3 expression detected by immunofluorescence in A7r5 cells under the same treatment conditions.

Fig. 7. Calcineurin/NFAT pathway inhibition reduces α-actin expression in aorta-derived smooth muscle cells (A7r5). Cells were seeded in 96-well plates and grown in Dulbecco’s modified Eagle’s medium/10% fetal bovine serum. After 24 h, medium was changed to Dulbecco’s modified Eagle’s medium/1% fetal bovine serum in the absence (Control) or presence of 1 μM FK506 and 1 μM CsA (FK + CsA) for 48 h. SM α-actin and β-actin were detected by in-cell Western blot (Odysssey Technology). Results are expressed as the ratio of integrated α- or β-actin staining intensity to integrated intensity of DNA staining. *, p < 0.0001 (n = 48). NS, nonsignificant (n = 18).

DISCUSSION

We have found that a 56-bp region of the mouse SM α-actin first intron that contains a highly conserved SRF/NFAT/AP-1 (SNAP) composite binding site sequence is capable of acting as an enhancer element, increasing reporter activity in the context of a minimal TK promoter. These results are consistent with a previous report showing that a larger (180-bp) segment of the corresponding region of the human α-actin first intron, cloned upstream or downstream of a minimal SV40 promoter, exhibits enhancer activity (13) and further support more recent results showing that the corresponding intronic region of the rat α-actin gene is required for robust in vitro and in vivo reporter expression in the context of a construct that includes 5’-regulatory elements and the first exon (10). More importantly, our results indicate that the previously unrecognized NFAT site and the overlapping SRF-binding CARG element are both absolutely required for the elevated basal activity in SMCs and induced activity in HEK293 cells. The SNAP region simultaneously binds both SRF and NFAT in HEK293 cells under conditions in which activity is induced but binds only SRF in the absence of stimulation, providing the first evidence that NFAT and SRF may interact to cooperatively regulate α-actin transcription.

In HEK293 cells transfected with the SNAP-Luc construct, reporter expression in the absence of stimulation was signifi-
cantly increased over that in cells transfected with a control luciferase reporter plasmid containing only a minimal TK promoter. The basal level of expression in HEK293 cells, however, was ~8-fold lower than that in SNAP-Luc-transfected SMCs, consistent with a potential SMC-selective role for this element. Basal expression in HEK293 cells was sensitive to disruption of the CArG box but unaffected by mutations in the NFAT site or the AP-1 site. This is in sharp contrast to Io/PMA-stimulated HEK293 cells (or unstimulated SMCs), in which mutations in NFAT or SRF sites were equally effective in abrogating reporter activity and a mutation to the AP-1 site partially inhibited activity. Thus, in non-smooth muscle HEK293 cells, SRF alone appears capable of mediating a low basal level of transcription from the SNAP enhancer element, whereas the induced activity in these cells and the much higher basal activity in SMCs require both NFAT and SRF.

Interestingly, in SMCs, stimulation of the calcineurin/NFAT pathway with Io/PMA failed to increase the reporter activity of the SNAP-Luc construct beyond the already elevated basal level. The absence of inducible activity in SNAP-Luc-transfected SMCs, as well as the profound difference in basal enhancer activity between SMCs and HEK293 cells, may be partially accounted for by differences in the subcellular distribution of the NFATc3 isoform between these two cell types. Although NFAT is normally presumed to localize to the cytosol of unstimulated cells, we found that a preponderance of endogenous as well as exogenously expressed NFATc3 is constitutively localized to the nucleus of A7r5 cells. In HEK293 cells, both endogenous and exogenously expressed NFATc3 is localized to the cytosol under basal conditions. Thus, a virtual absence of nuclear NFATc3 in unstimulated HEK293 cells is associated with low level, NFAT-independent SNAP enhancer activity. Stimulation of HEK293 cells with Io/PMA increases nuclear NFATc3 (data not shown) and SNAP enhancer activity. In SMCs, in which NFATc3 is constitutively nuclear, SNAP activity is high, NFAT-dependent, and unresponsive to Io/PMA treatment. Although these results do not necessarily indicate that SNAP preferentially binds the NFATc3 isoform, they are consistent with the interpretation that sufficient nuclear NFAT in the form of NFATc3 is present in SMCs to drive maximal enhancer activity under basal conditions.

The constitutively nuclear NFATc3 observed in SMCs is redistributed to the cytosol following inhibition of calcineurin with FK506 and CsA (Fig. 6C). This result suggests that NFAT import mechanisms may be constitutively elevated in these cultured SMCs, an observation that could be explained by elevated calcineurin activity. Alternatively, mechanisms that act to promote nuclear export may be less active under basal conditions. Recent work from our laboratory has shown that regulation of NFATc3 nuclear export may be central to the activation state of NFAT in smooth muscle (reviewed in Ref. 44). In unpressurized arteries, basal NFATc3 nuclear export is high as a result of elevated JNK2-mediated export; stimuli that induce a net nuclear accumulation of NFATc3 are those that not only elevate intracellular Ca2+ but also result in a decrease in JNK2-mediated export (45). In response to a normotensive pressure stimulus, JNK2 activity in native smooth muscle is reduced via a nitric oxide/protein kinase G-dependent pathway, and NFATc3 is predominantly localized to the nucleus (35). In this respect, A7r5 may exist in a state similar to that of pressurized arteries, with reduced JNK2-mediated export activity and constitutively nuclear NFATc3.

SMCs are known to express higher levels of SRF than other cell types (46, 47), so it is conceivable that the difference between the levels of basal and induced enhancer activity between SMCs and HEK293 cells may also reflect limiting SRF levels in HEK293 cells. Although HEK293 cells do contain sufficient SRF to drive low basal levels of enhancer activity and to detect in a complex with the SNAP element, it may be that higher levels of SRF are required for efficient recruitment of NFAT and maximal enhancer activity.

Our reporter assay data showing that basal SNAP enhancer activity in SMCs and induced activity in HEK293 cells are dependent on both intact SRF and NFAT sites suggest a cooperative mechanism of action between SRF and NFAT. This idea is supported by the binding of both NFATc3 and SRF to the SNAP element under conditions in which the NFAT pathway is activated in HEK293 cells. The presence of SRF in NFATc3 immunoprecipitates provides compelling evidence that SRF and NFATc3 interact. The fact that associated SRF is increased in the presence of SNAP is most consistent with the interpretation that simultaneous binding of SRF and NFATc3 to their respective sites facilitates NFATc3/SRF interactions. SRF binding alone is insufficient to promote enhancer activity under NFAT stimulated conditions because a mutation at the NFAT binding site does not substantially inhibit SRF binding to the overlapping CArG box but almost completely abrogates induced reporter activity.

SRF, a member of the MADS family of transcription factors (5), has been shown to bind to the REL homology domain of nuclear factor-κB (48) to enhance nuclear factor-κB transcriptional activity. NFAT, which also contains a REL homology domain, has been previously shown to functionally interact with the MADS family member Mef2 (21). This is the first report, however, to suggest that NFAT and SRF may interact with one another to function as transcriptional co-activators. The SRF and NFAT domains that may be responsible for mediating protein-protein interactions in the putative SRF-NFAT complex, as well as the role that the individual NFAT and/or SRF transactivation domains may play in mediating interactions with the transcriptional machinery, are currently unknown. Our data also suggest that AP-1, which is a common NFAT cofactor (43) that has also been implicated in SRF signaling (49), contributes to NFAT/SRF enhancer efficacy, although the molecular details of this involvement remain to be defined.

SRF has been previously shown to interact with other transcription factors, such as ETS domain-containing transcription factors (e.g. Elk-1), the homeodomain proteins Barx1b and Nks3.2, and the zinc finger protein GATA6 (6). In smooth muscle, the transcription factor myocardin has been shown to regulate SRF-dependent transcription of a −2.6/−2.8-kb α-actin promoter/intronic construct through interactions that exhibit a combinatorial dependence on CArG elements, primarily involving CArG B and CArG A boxes (50). The apparent preferential interaction of myocardin with proximal promoter CArG boxes suggests that NFAT action at the intronic SNAP element may operate in parallel with myocardin activity, with both pathways required for full transcriptional activity. Considered in the context of the demonstrated Ca2+ dependence of smooth muscle differentiation marker gene expression (51), it is possible that the NFAT pathway may contribute to the Ca2+ sensitivity component of SMC gene expression, serving to integrate Ca2+ signals at regulatory elements of smooth muscle-specific genes, including α-actin.

In addition to the molecular evidence supporting a role for NFAT in the regulation of α-actin expression, this study shows that inhibition of the calcineurin/NFAT pathway in cultured rat SMCs decreases α-actin protein expression. This result is in accord with a recent report showing that blockade of calcineurin/NFAT in primary cultures of human vascular smooth muscle is associated with a reduction in both α-actin
and SM MHC protein expression (33). Another recent study has also implicated NFAT in the regulation of α1 integrin and caldesmon expression in cultured visceral SMCs, showing that calcineurin activity is required for the expression of these smooth muscle markers and maintenance of the contractile smooth muscle phenotype (32). The proposed interactions between NFAT and SRF at the α-actin intronic composite binding site may provide a molecular framework to help explain the apparent NFAT dependence of α-actin expression in SMCs, but it appears unlikely that this interaction can be invoked as a general mechanism to account for the NFAT dependence of other smooth muscle-specific genes. For example, although the regulation of SM MHC expression in smooth muscle exhibits similarities to that of α-actin regulation in that dual 5′ CArG boxes and an intronic CArG box are all required for smooth muscle-specific expression, neither the 5′ nor intronic CArG box is associated with NFAT binding sites. Instead, NFAT appears to regulate SM MHC expression through an association with GATA at unidentified sites in the 5′-flanking sequence of the SM MHC gene (33). The single 5′-upstream CArG box of α1 integrin that is required for expression in differentiated SMCs (52) likewise lacks an associated NFAT site. In addition to the conserved intronic NFAT site characterized here, the first intron of α-actin is rich in potential NFAT binding sites, many of which are phylogenetically conserved and might be expected to contribute to NFAT-sensitive expression. To the extent that NFAT is involved in regulating the expression of SMC-specific genes, it is likely that it does so through multiple mechanisms involving interactions with different cofactors.

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