Upstream Stimulatory Factors Regulate Aortic Preferentially Expressed Gene-1 Expression in Vascular Smooth Muscle Cells*

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The phenotypic modulation of vascular smooth muscle cells (VSMC) plays a central role in the pathogenesis of arteriosclerosis. Aortic preferentially expressed gene-1 (APEG-1), a VSMC-specific gene, is expressed highly in differentiated but not in dedifferentiated VSMC. Previously, we identified an E-box element in the mouse APEG-1 proximal promoter, which is essential for VSMC reporter activity. In this study, we investigated the role of upstream stimulatory factors (USF) in the regulation of APEG-1 transcription via this E-box element. By electrophoretic mobility shift assays, recombinant USF1 and USF2 homo- and heterodimers bound specifically to the APEG-1 E-box. Nuclear extracts prepared from primary cultures of rat aortic smooth muscle cells exhibited specific USF1 and USF2 binding to the APEG-1 E-box. To investigate the binding properties of USF during VSMC differentiation, nuclear extracts were prepared from the neural crest cell line, MONC-1, which differentiates into VSMC in culture. Maximal USF1 and USF2 protein levels and binding to the APEG-1 E-box occurred 3 h after the differentiation of MONC-1 cells was initiated. Co-transfection experiments demonstrated that dominant negative USF repressed APEG-1 promoter activity, and USF1, but not USF2, transactivated the APEG-1 promoter. Our studies demonstrate that USF factors contribute to the regulation of APEG-1 expression and may influence the differentiation of VSMC.

Vascular smooth muscle cells (VSMC), the major cell type in blood vessel walls, exhibit a spectrum of phenotypes that change in response to environmental cues (1). The conversion of VSMC from a quiescent to a proliferative phenotype contributes to the pathogenesis of arterial restenosis, hypertension, atherosclerosis, and its related complications (2). The development of VSMC is highly specific and selective gene expression is only beginning to be elucidated (4). Thus, identifying these mechanisms may enhance our understanding of VSMC phenotypic regulation and provide potential therapeutic or preventive targets for vascular proliferative syndromes, including atherosclerosis.

Aortic preferentially expressed gene-1 (APEG-1) (5) is a VSMC-specific gene, which is expressed in differentiated, but not in dedifferentiated, VSMC. Its encoded protein may be a marker for SMC lineage, similar to smooth muscle (SM) α-actin, calponin, SM-22α, desmin, and SM-myosin heavy chain (1). However, little is known about the precise transcriptional mechanisms regulating the APEG-1 promoter in VSMC.

Using deletion and mutation analysis, we previously identified an E-box motif (CAGCTG) located in exon 1 of the APEG-1 gene (bp +39/+44) as a major positive regulatory element (6). In addition to APEG-1, several SMC-specific marker genes contain E-box elements in their promoters including SM α-actin, SM-22α, and SM-myosin heavy chain (7–9). Basic helix-loop-helix (bHLH) and bHLH leucine zipper transcription factor families bind to E-box motifs (10). Previously, we did not detect E12 and E47 binding to the APEG-1 E-box (6), suggesting that other E-box binding proteins are present in these complexes. Upstream stimulatory factors 1 and 2 (USF1 and -2), originally identified as activators of the adenovirus major late promoter (11), are ubiquitously expressed transcription factors (12). USF1 and USF2 recognize and bind to DNA with an E-box motif as either homodimers or heterodimers. The USF family members regulate the expression of several genes (13, 14), including SMC-expressed genes (15), and they are known to bind to E-boxes similar to that found in the APEG-1 promoter (16). In this report, we wanted to further elucidate the nuclear proteins binding to the APEG-1 E-box and to investigate their role in the regulation of APEG-1 expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Rat aortic smooth muscle cells (RASMC), harvested from the thoracic aorta of adult male Sprague-Dawley rats by enzymatic dissociation (17), were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal bovine serum (HyClone, UT), 4 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C and 5% CO2. Drosophila cells (D.Mel-2; Invitrogen) were cultured in Drosophila SFM at 28 °C. The mouse neural crest cell line MONC-1 was previously provided to our laboratory by Dr. D. Anderson (Pasadena, CA). The culture and differentiation of MONC-1 cells were as described (18).

Plasmid Constructs and DNA Probes Used—The luciferase reporter plasmids APEG-1 pT−355/+76, pT−122/+76, and pT−122/+76E-mut were generated by subcloning these fragments into the pGL3-Basic
vector (Promega) as described (6). The 0.9-kb USF1 cDNA fragment was cloned by reverse transcription-PCR from mouse aortic SMC RNA. The 1-kb USF2 cDNA fragment from psvUSF2 was provided by Dr. M. Sawadogo (Houston, TX). The Drosophila expression plasmids, pPAC and phsp82LacZ, were provided by Dr. T. Maniatis (19). The expression vectors of USF1 and USF2 were constructed by subcloning the USF1 and USF2 inserts into pcDNA3.l(+) (Invitrogen) and pPAC. To generate a dominant negative USF expression construct, we amplified a fragment of the USF1 cDNA (encoding amino acids 213–310) by PCR. This fragment was cloned into the pCMV/myc/nuc vector (Invitrogen) that contains a nuclear localization signal and subsequently into pcDNA3 (Invitrogen). All constructs were confirmed by sequencing.

Preparation of Nuclear Extracts and In Vitro Translated Products—Nuclear extracts were prepared from cultured cells as described (20). Protein concentrations of nuclear extracts were measured by the Bradford dye-binding method (21) with the Bio-Rad protein assay reagent. In vitro translated products were prepared using the TNT T7 Quick Coupled Transcription/Translation system (Promega).

Electrophoretic Mobility Shift Assays (EMSA)—Two 18-bp oligonucleotide probes, containing the E-box sequence, 5′-GGGCGTGACCTGGTCAG-3′, or mutated E-box, 5′-GGGCGTGACGAGTCAG-3′, were synthesized according to the mouse APEG-1 exon 1 sequence (6). USF E-box consensus oligonucleotide was synthesized in the context of the APEG-1 promoter, 5′-GGGCTCACTGGTCAG-3′. After annealing, the double-stranded oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs) and purified with G-50 spin columns (Amersham Pharmacia Biotech). The binding reaction contained 20,000 cpm of DNA probe, 2 μg of poly(dI-dC), 25 mM HEPES (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and either 10 μg of nuclear protein or in vitro translated products. In cold competition assays, a 100-fold molar excess of unlabeled double-stranded oligonucleotides, including APEG-1 E-box, APEG-1 E-mut, and E-box consensus oligonucleotides, were added to the binding reaction 30 min before the addition of radiolabeled probe. For mobility supershift experiments, 2 μg of USF1 (C-20) and USF2 (N-19) polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to the binding reactions and incubated at room temperature for 30 min before the addition of radioactive probe.

Western Blot Analysis—Nuclear proteins (30 μg) were separated on 6% SDS-PAGE and transferred to nitrocellulose filters (22). The blot was probed with USF1 or USF2 polyclonal rabbit antibodies. Signal was detected on Western blots using enhanced chemiluminescence (SuperSignal West Pico kit; Pierce).

RNA Extraction and Northern Blot Analysis—Total RNA from cultured cells was extracted using a MINI RNA isolation kit (Qiagen). Total RNA (10 μg) was denatured, fractionated on 1.3% formaldehyde–agarose gels, and subsequently transferred to NitroPure filters (Osmonics). The filters were then hybridized with random primed, [α-32P]dCTP-labeled APEG-1, USF1, USF2, and SM-α-actin cDNA probes. To correct for the differences in RNA loading, blots were subsequently hybridized to a 32P-labeled oligonucleotide probe complementary to 28 S rRNA. The blots were exposed to a phosphoscreen and x-ray film. The radioactivity was measured on a PhosphorImager by using a Molecular Dynamics, Inc., Sunnyvale, CA).

RESULTS

USF1 and USF2 Bind to an E-Box Element in Exon 1 of the Mouse APEG-1 Gene—We previously identified an E-box element within exon 1 of the APEG-1 gene (bp +39/+44), which was essential for APEG-1 promoter activity in VSMC (6). We wanted to test the hypothesis that USF proteins, which are capable of binding E-box motifs (CAGCTG) (15), may be important for the regulation of APEG-1 in VSMC. To determine whether USF1 or USF2 proteins could bind to the APEG-1 E-box, we performed EMSA using in vitro translated products of USF1 and USF2 incubated with radiolabeled APEG-1 E-box, APEG-1 E-mut, or E-box consensus oligonucleotides as probes. USF1 and USF2 homodimers and USF1/USF2 heterodimers bound to APEG-1 E-box and E-box consensus (CAGCTG) probes (Fig. 1, left and right panels, respectively). USF proteins did not bind to the mutated APEG-1 E-box oligonucleotide (CAGCAC) (Fig. 1, middle panel). The migration of USF1 homodimer is faster than USF2, and the migration of USF1/USF2 heterodimer is intermediate between these two homodimers.

USF Binding Activity in VSMC—To determine whether USF proteins bind to the APEG-1 E-box in VSMC, we prepared nuclear extracts from RASMC. EMSA using APEG-1 E-box (Fig. 2, left panel) and consensus E-box oligonucleotide probes (Fig. 2, right panel) revealed the presence of strong DNA-protein complexes (a and b) when incubated with nuclear extracts from RASMC. There was a faint supershifted band after USF1-specific antibody was added (○), and two supershifted bands after USF2-specific antibody or a combination of USF1/USF2 antibodies were added (•). Moreover, the intensity of band a decreased with use of USF1 and USF2 antibodies. The specificity of the DNA-protein complex was also assessed by adding an unlabeled E-box competitor in the binding reaction. As shown in the right lane of each gel, bands a and b were competed away by the 100-fold molar excess of E-box cold competitor. Our results indicated that USF1 and USF2 specifically bound to the APEG-1 E-box in RASMC (band a).

Early Induction of USF Binding Activity and Protein Expression during MONC-1 Differentiation—The MONC-1 neural crest cell line differentiates into smooth muscle cells in culture (18, 23). We used this model to study USF binding and expression during the differentiation of MONC-1 into smooth muscle cells. We harvested total RNA and nuclear extracts from undifferentiated MONC-1 cells and from cells in differentiation medium for 3, 6, 9, 12, 24, 48, 96, and 144 h. There were three major DNA-protein complexes detected in MONC-1 cell nuclear extracts (arrows a, b, and c in Fig. 3).
In the present study, recombinant USF (Fig. 1) or USF in RASMC nuclear extracts do not bind to the APEG-1 E-box (6). To investigate the specificity of binding, we added unlabeled competitor (APEG-1 E-box, APEG-1 E-mut, and E-box consensus oligonucleotides) to the binding reactions. Unlabeled APEG-1 E-box oligonucleotide competed away DNA-protein complexes (a, b, and c) (Fig. 3C). The E-box consensus oligonucleotide mainly competed away complex a, with some diminishment in complexes (b) and (c). In contrast, APEG-1 E-mut competed away only the fastest migrating complex (c). These data suggest that complexes a and b are specific and that complex c contains the USF proteins. In contrast, it is likely that complex c is binding to the flanking sequence outside of the APEG-1 E-box site.

We next determined the mRNA and protein levels of USF1 and USF2 during SMC differentiation. By Northern blot analysis, we found there was no significant change in USF1 or USF2 mRNA levels in MONC-1 during differentiation (Fig. 4A). The levels of APEG-1 and SM α-actin mRNA increased throughout differentiation. Moreover, binding of USF to the APEG-1 promoter (3-h peak) preceded APEG-1 mRNA induction (as early as 6 h) after the initiation of MONC-1 differentiation. By Western blot analysis, we found that the expression of USF proteins reached their peaks 3 h after the initiation of MONC-1 cell differentiation (Fig. 4B), which correlated with the peak USF binding activity (Fig. 3A).

**Dominant Negative USF Represses the Activity of the APEG-1 Promoter in RASMC—** RASMC exhibit high levels of endogenous USF binding activity (Fig. 2 and data not shown); thus, we wanted to inhibit USF binding activity to determine its role in regulating the APEG-1 promoter. To perform these experiments, we designed a dominant negative USF expression construct that encodes a protein lacking the basic DNA-binding domain but retains the dimerization domain (24). In addition, this construct contains a nuclear targeting sequence. We confirmed the nuclear localization of dominant negative USF by immunofluorescent staining of RASMC transfected by this construct (data not shown). Using *in vitro* translated products, dominant negative USF suppressed the binding of USF1 and USF2 to APEG-1 E-box in a dose-dependent manner (Fig. 5A). Co-transfection of dominant negative USF with the APEG-1 promoter construct in RASMC resulted in a 50% reduction in APEG-1 promoter activity (Fig. 5B). These results confirm that endogenous USF proteins have a role in the regulation of the APEG-1 promoter.

**USF1 Participates in Activation of the APEG-1 Promoter—** To examine the role of USF1 and USF2 transcription factors in regulating the APEG-1 promoter, we used *Drosophila* cells (D.Mel-2 cells), which do not contain functional USF (25). *Drosophila* cells were transfected with APEG-1 promoter p(−122/+38) reporter construct, USF1 and USF2 expression constructs, and phsp82lacZ to normalize for transfection efficiency. We previously showed that APEG-1 promoter construct (−122/+38) contains nearly full promoter activity (6). The overexpression of USF1 produced a dose-dependent increase in luciferase activity with a maximum of −9-fold (Fig. 6). Interestingly, USF2 homodimers and USF1/USF2 heterodimers did not activate the APEG-1 promoter in *Drosophila* cells (Fig. 6). These data suggest that USF2 is not an activator of the APEG-1 promoter, and when in a heterodimer with USF1, USF2 represses APEG-1 promoter activity. In addition, the USF1 expression construct did not activate the mutated APEG-1 E-box construct, APEG-1 p(−122/+76)E-mut, indicating that USF1 transactivation is through this site.

**DISCUSSION**

We have demonstrated previously that high levels of APEG-1 gene expression in VSMC require the E-box motif in exon 1 (6). In the present study, we showed that USF transcription factors bind to this E-box and contribute to the regulation of APEG-1 gene expression, in a positive or a negative manner, depending on the USF family member.

USF proteins belong to the group B bHLH transcription factors (26, 27). They bind preferentially to the CACGTG E-box (28), which is different from the core CAGCTG E-box motif in the APEG-1 gene. The group A bHLH proteins, such as the ubiquitously expressed E2A proteins E12 and E47, recognize and bind as heterodimers to the CAGCTG type of E-box site (28). We have previously shown that E2A proteins from RASMC nuclear extracts do not bind to the APEG-1 E-box (6).

In the present study, recombinant USF (Fig. 1) or USF in RASMC nuclear extracts (Fig. 2) bound to the APEG-1 E-box and regulated APEG-1 promoter activity. These results further support the idea that USF factors can recognize and regulate promoters through this type of E-box site.

E-box elements and their associated bHLH transcription factors, such as MyoD, are known to be important in the regulation of striated muscle-specific gene expression and differ-
entiation of striated muscle cells in a tissue-specific manner (29, 30). However, no such tissue-restricted bHLH family mem-
ber has been identified in mature arteries; nor have they been
shown to regulate VSMC-expressed genes. While it is not likely
that ubiquitously expressed bHLH family members can solely
account for expression of VSMC-restricted genes, Johnson and
Owens (15) have previously demonstrated that USF factors
contribute to the regulation of SM α-actin, a SMC differentiation
marker gene. One may hypothesize that a ubiquitously ex-
pressed factor, such as USF, heterodimerizes with a smooth
muscle-specific factor to promote cell type-restricted gene ex-
pression. Alternatively, USF factors may be incorporated into a
complex of transcription factors, including co-activators, whose
stereospecific assembly allows smooth muscle cell-restricted
gene expression. Consistent with this hypothesis, Qyang et al.
(12) proposed that the ability of USF to activate a given pro-
moter was dependent on cell-specific co-activators.

To study the role of USF in the regulation of APEG-1 during
the differentiation of VSMC, we used an in vitro MONC-1 differ-
entiation system (18). We found that in MONC-1 cells,
USF1 and USF2 protein levels and their APEG-1 E-box bind-
ing activity peaked very early after differentiation was initi-
ated (Fig. 3). Moreover, expression and binding of USF factors
to the APEG-1 E-box preceded expression of the APEG-1 gene
(Fig. 4). Therefore, the increased amounts and binding activity
of USF proteins may contribute to APEG-1 promoter regulation
during VSMC differentiation. These data were supported by
functional studies showing that overexpression of a dominant
negative USF (that prevents wild-type USF protein binding)
decreased APEG-1 promoter activity (Fig. 5).

APEG-1, like SM α-actin that is regulated by USF factors, is
rapidly down-regulated in injured blood vessels or in dediffer-
entiated VSMC in culture (5). We now show that USF1 is able
to transactivate the APEG-1 promoter; however, USF2 is un-

Fig. 3. Binding activity of USF1 and USF2 to APEG-1 E-box during
MONC-1 cell differentiation into smooth muscle cells. A, nuclear ex-
tracts prepared from MONC-1 cells 0–96 h after the initiation of differentia-
tion were incubated with a radioactive APEG-1 E-box probe. The arrows point to
the DNA-protein complexes (a, b, and c), detected in these nuclear extracts. B, for
supershift reactions, nuclear extracts were incubated with rabbit polyclonal an-
tibodies directed toward USF1 and USF2. USF1- and USF2-supershifted complexes
were indicated by dots and asterisks, respectively. C, in competition assays, the
unlabeled APEG-1 E-box, APEG-1 E-mut, and E-box consensus oligonucleotide
probes were used as competitors. Hours, the duration of time after differentiation
was initiated.

Fig. 4. The mRNA and protein levels of USF1 and USF2 in the
differentiating MONC-1 cells. A, total RNA extracted from MONC-1
cells at different time points of differentiation was analyzed by North-
ern blot analysis using radiolabeled probes for USF1, USF2, APEG-1,
and SM α-actin. RNA loading was verified by hybridization to a radio-
labeled oligonucleotide complementary to the 28 S ribosomal RNA. B, for
Western blotting, the nuclear extracts prepared from MONC-1 cells
at different time points of differentiation were resolved, transferred,
and hybridized with antibodies against USF1 or USF2.
able to increase APEG-1 promoter activity (Fig. 6). Even more importantly, co-transfection of an identical amount of USF2 expression plasmid was able to suppress USF1 transactivation of the APEG-1 promoter. These data suggest that USF2 may be acting as a repressor, as described previously in the regulation of plasminogen activator inhibitor-1 promoter (31). Taken together, our data suggest that the relative amounts of USF1 and USF2 and the way in which they interact as heterodimers may determine the level of APEG-1 expression. This effect of USF2 on APEG-1 is different from that described for the SM α-actin promoter, in which both USF1 and USF2 caused promoter transactivation (15). Nevertheless, both studies demonstrate that the USF factors modulate the expression of genes. APEG-1 and SM α-actin, that are acutely regulated by the differentiation status of VSMC. We propose that USF factors, by altering the expression of key genes, may play an important role in the differentiation capability of VSMC.

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