Transforming Growth Factor β Up-regulates Cysteine-rich Protein 2 in Vascular Smooth Muscle Cells via Activating Transcription Factor 2*

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CRP2 (cysteine-rich protein) is a vascular smooth muscle cell (VSMC)-expressed LIM-only protein. CRP2 associates with the actin cytoskeleton and interacts with transcription factors in the nucleus to mediate smooth muscle cell gene expression. Using Csrp2 (gene symbol of the mouse CRP2 gene)-deficient mice, we previously demonstrated that an absence of CRP2 enhances VSMC migration and increases neointima formation following arterial injury. Despite its importance in vascular injury, the molecular mechanisms controlling CRP2 expression in VSMC are largely unknown. Transforming growth factor β (TGFβ), a key factor present in the vessel wall in the early phases of arterial response to injury, plays an important role in modulating lesion formation. Because both CRP2 and TGFβ are mediators of VSMC responses, we examined the possibility that TGFβ might regulate CRP2 expression. TGFβ significantly induced CRP2 mRNA and protein expression in VSMCs. Promoter analysis identified a conserved CRE-responsive element (CRE)-like site (TAACGTCA) in the Csrp2 promoter that was critical for basal promoter activity and response to TGFβ. Gel mobility shift assays revealed that mainly ATF2 bound to this CRE-like element, and mutation of the CRE sequences abolished binding. TGFβ enhanced the activation of ATF2, leading to increased phospho-ATF2 levels within the DNA-protein complexes. Furthermore, ATF2-transactivated Csrp2 promoter activity and TGFβ enhanced this activation. In addition, a phosphorylation-negative ATF2 mutant construct decreased basal and TGFβ-mediated Csrp2 promoter activity. Our results show for the first time in VSMC that TGFβ activates ATP2 phosphorylation and Csrp2 gene expression via a CRE promoter element.

Vascular smooth muscle cells (VSMCs) of the arterial wall play a critical role in the development of occlusive vascular lesions. In normal vessels, VSMCs are quiescent, differentiated, and contract and regulate vascular tone and blood pressure. In response to arterial injury, VSMCs undergo a phenotypic transition whereby they migrate and proliferate from the media into the intima, contributing to vascular lesion formation and arteriosclerosis (1).

The LIM domain is a double zinc finger structure that functions as a modular protein-binding interface that mediates protein-protein interactions (2–6). By binding to target proteins and serving as an adapter molecule in the assembly of multi-protein complexes, LIM proteins participate in diverse biological processes (6, 7). The LIM-only cysteine-rich protein (CRP) family, which includes CRP1 (8, 9), CRP2/SmLIM (10), and CRP3/MLP (muscle LIM protein) (11), is characterized by two tandem LIM domains, each followed by a short glycine-rich repeat (12, 13). CRP1 is expressed in several cell types, including vascular and visceral smooth muscle cells (SMCs) (8, 14), whereas CRP2 is mainly expressed in VSMCs, particularly arterial SMC (10, 15, 16). CRP3 is specifically expressed in cardiac and skeletal muscle (11, 17). CRPs are involved in promoting protein assembly along the actin-based cytoskeleton (7, 13, 18). CRPs associate with the actin cytoskeleton via interacting with the actin-cross-linking protein α-actinin and adhesion plaque protein 2y2n (4, 13, 19). In addition, CRPs also interact with transcription factors to activate gene transcription in the nucleus (20, 21). Given their prominent cytoskeletal association and presence in the nucleus, CRPs have both a major cytoskeletal function and a role controlling gene expression (6). A cytoskeletal function of CRP3 was demonstrated in mice lacking CRP3; those mice developed dilated cardiomyopathy with hypertrophy and heart failure after birth (17). Ultrastructural analysis revealed dramatic disruption of cardiomyocyte cytoarchitecture (17). Further supporting a function of CRP3 in maintaining the cytoarchitecture of striated muscle cells, the Droso-phiola homolog of muscle LIM protein, Mlp84B, was recently

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§ The abbreviations used are: VSMC, vascular smooth muscle cell; CRP, cysteine-rich protein; SMC, smooth muscle cell; TGFβ, transforming growth factor β; CRE, CAMP-responsive element; CREB, CAMP-responsive element-binding protein; p-CREB, phosphorylated CREB; PDGF, platelet-derived growth factor; AcD, actinomycin D; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation.

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demonstrated to cooperate with α-titin to maintain muscle structural integrity (22).

We recently identified a key role for the LIM domain-containing protein, CRP2, in the development of arteriosclerosis (16). Following femoral artery wire injury, CRP2 expression persisted in the first few days and then decreased but was not abolished in the vessel wall by 14 days (16), implicating a role for CRP2 in vascular injury. By gene deletion experiments, we demonstrated that a lack of CRP2 increased neointima formation following arterial injury in mice (16). Importantly, the increased intimal thickening in Csrp2 (gene symbol of the mouse CRP2 gene)-deficient mice correlated with enhanced VSMC migration into the intima (16). This migratory role of CRP2 in arterial SMC emphasizes a cytoskeleton-associated function of CRP2 protein.

In response to injury, many cytokines and growth factors are released within injured blood vessels (1, 23, 24). These mediators in turn alter gene expression patterns of vascular cells, including SMC marker genes, leading to phenotypic modulation of VSMC and neointima formation. Despite its important function in the pathogenesis of arteriosclerosis, the molecular mechanisms controlling CRP2 expression in the context of vascular injury remain largely unknown. We showed previously that one of these factors, PDGF-BB, down-regulates CRP2 mRNA expression (10). Given that an absence of CRP2 promotes VSMC migration both in vitro and in vivo (16), induction of CRP2 by factors present in the injured vessels might serve as a protective mechanism and reduces VSMC migration and subsequent neointima formation.

In the current study, we sought to identify factors, present in the context of vascular injury, that up-regulated CRP2. CRP2 regulation by these factors might ultimately protect against intimal thickening. TGFβ, a key factor present in the vessel wall in the early phases of the arterial response to injury plays an important role in modulating lesion formation. We identified that TGFβ induces CRP2 protein and mRNA expression. Analysis of the Csrp2 promoter by reporter gene transfection experiments revealed that a conserved CRE-like motif located at bp −461 to −454 of the Csrp2 gene was required for both basal and TGFβ-induced activation of the Csrp2 promoter. We further demonstrated that TGFβ activates ATF2 phosphorylation and Csrp2 gene expression via a CRE promoter element.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Primary VSMCs were isolated from mouse or rat aortas and cultured in Dulbecco’s modified Eagle’s medium as described previously (16, 25). Cells were passaged every 3–5 days, and experiments were performed on cells 6–8 passages from primary culture. Actinomycin D and cycloheximide were purchased from Sigma.

Western Blot Analysis—Approximately 1 × 10⁶ VSMCs were plated on 100-mm cell culture dishes and incubated overnight. Cells were treated with human recombinant TGFβ (10 ng/ml; PeproTech, Rocky Hill, NJ) for various times and lysed in extraction buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 2% Nonidet P-40, and 0.2% SDS) containing protease inhibitors (Complete™; Roche Applied Science). Cell extracts were cleared by centrifugation and subjected to SDS-PAGE using Novex gels (Invitrogen). Protein concentrations were determined by BCA protein assay (Pierce), and 20 μg of protein were loaded per lane. Separated proteins were then transferred to Protran nitrocellulose membranes (Whatman Schleicher & Schuell), followed by immunoblotting (26). Membranes were incubated with CRP2 (91–98) antisem (16) and monoclonal α-tubulin antibody (Sigma) to verify equivalent loading. To assess phosphorylation of ATF2 and CREB, cells were plated, incubated overnight, serum-starved (0.5% fetal bovine serum in Dulbecco’s modified Eagle’s medium) for 24 h, and then stimulated with TGFβ. Protein extracts were prepared in extraction buffer containing protease inhibitor Complete™ and phosphatase inhibitor mixture 1 and 2 (Sigma) at the indicated time points. Membranes were incubated with antibodies for phospho-ATF2 (Thr71) (phosphorylated Thr of human ATF2; Cell Signaling Technology, Danvers, MA) that recognizes both Thr⁵¹/Thr⁵³ dually phosphorylated and Thr⁵¹ singly phosphorylated mouse ATF2, total CREB, and 2 (Sigma). Membranes were incubated with CRP2-(91–98) antibody (Affinity BioReagents, Golden, CO) to verify equivalent loading. To assess phosphorylation of ATF2 and CREB, cells were plated, incubated overnight, serum-starved (0.5% fetal bovine serum in Dulbecco’s modified Eagle’s medium) for 24 h, and then stimulated with TGFβ. Protein extracts were prepared in extraction buffer containing protease inhibitor Complete™ and phosphatase inhibitor mixture 1 and 2 (Sigma) at the indicated time points. Membranes were incubated with antibodies for phospho-ATF2 (Thr71) (phosphorylated Thr of human ATF2; Cell Signaling Technology, Danvers, MA) that recognizes both Thr⁵¹/Thr⁵³ dually phosphorylated and Thr⁵¹ singly phosphorylated mouse ATF2, total ATF2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-CREB that recognizes phosphorylated Ser¹³ of CREB (Upstate Cell Signaling Solutions, Charlotteville, VA), or total CREB (Santa Cruz Biotechnology).

Northern Blot Analysis and Real Time Quantitative Polymerase Chain Reaction—Total RNA was isolated from VSMCs using the RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA), and Northern blot analysis was performed as described (26). The filters were hybridized with a random primed 3²p-labeled mouse CRP2 cDNA open reading frame probe (15). To correct for loading differences, the filters were hybridized to a 3²P-labeled oligonucleotide complementary to 18 S ribosomal RNA. The signal intensity was measured on a PhosphorImager using ImageQuant software (GE Healthcare). In cytocheximide experiments, 1 μg of RNA was reverse transcribed to cDNA using the SuperScript III first strand synthesis system (Invitrogen). Real time quantitative PCR was performed with the 7500 real time PCR system (ABI) using 2× SYBR Green PCR master mix (ABI) and 1% of the starting 1 μg of RNA with the following primers for CRP2: forward, 5′-CGACTGAAAGAAAGGCGAATA-3′; reverse, 5′-TGTCGTTCATTCAGTA-GTGA-3′. β-Actin was used as the internal control with the following primers: forward, 5′-GAGAGGTACCGTACCCCTG-AAAG-3′; reverse, 5′-TGATCTGGGTGACCTTTTTCAGG-3′. Quantification was performed by the comparative C⁰ method.

Migration Assays—To assess migration, wild type and Csrp2 null (Csrp2−/−) VSMCs (16) were serum-starved (0.5% fetal bovine serum in Dulbecco’s modified Eagle’s medium) for 24 h and treated with or without TGFβ (10 ng/ml) for 12 h, and then migration assays were performed as described (16). Cells were placed in the upper chamber of 6-well transwell plates (8-pore size; Costar) in triplicate (300,000 cells/well). The bottom chambers were filled with 0.5% fetal bovine serum medium containing PDGF-BB (10 ng/ml) (Peprotech) as a chemoattractant. Cells migrating through the filters after 2 h were quantified and normalized to the cell number of wild type without TGFβ treatment.

Luciferase Reporter and Expression Plasmid Constructs—The mouse Csrp2 luciferase reporter plasmid −795Csrp2-luc was described previously (27). To generate a series of 5′-dele-
tion constructs, Csrp2 promoter deletion fragments were generated by PCR with the use of Pfu polymerase (Stratagene, La Jolla, CA) and cloned into the luciferase reporter pGL2-Basic (Promega, Madison, WI). These constructs share a common 3′-end at bp +40 but differ at the 5′-end at bp −573, −549, −480, and −438. With the −795Csrp2-luc construct as a template, site-directed mutagenesis was performed using Pfu polymerase to create an internal deletion of bp −480 to −438 to generate the −795Δ(480/438)Csrp2-luc construct. Site-directed mutagenesis was performed to mutate the CRE site from −461TAACGTCA to CACCGTAA (mutated bases are underlined) to generate the −795CREmutCsrp2-luc construct. All constructs were confirmed by DNA sequencing. The mouse ATF2 expression plasmid pCMV-SPORT6-ATF2 was purchased from Invitrogen. The open reading frame of ATF2 was amplified with Pfu polymerase using pCMV-SPORT6-ATF2 as a template and cloned into pFLAG-CMV5 vector (Sigma) to generate pFLAG-CMV5-ATF2. Site-directed mutagenesis was then performed using Pfu polymerase to mutate the three potential phosphorylation sites (Thr51, Thr53, and Ser72) to alanines (T51A, T53A, and S72A) (28) to generate phosphorylation-negative mutant pFLAG-CMV5-ATF2-AAA, which also functions as a dominant negative mutant (28).

**Transient Transfection Assays**—Approximately 1.6 × 10⁵ VSMCs were plated onto each well of 6-well plates and allowed to attach overnight. Cells were then transfected using FuGENE 6 reagent (Roche Applied Science) (21). To correct for differences in transfection efficiency, 500 ng each of the luciferase plasmids and pCMVβ (Clontech) were cotransfected into cells. Two hours following transfection, cells were treated with or without TGFβ (10 ng/ml). Luciferase and β-galactosidase activities were measured 24 h later; luciferase activity was normalized to β-galactosidase activity. For cotransfection experiments, −795Csrp2-luc (0.33 μg/well) and expression plasmid (0.67 μg/well) pCMV-CREB (Clontech), pCMV-SPORT6-ATF2, or empty vector were cotransfected into VSMCs. Similarly, for phosphorylation-negative ATF2 experiments, −795Csrp2-luc and pFLAG-CMV5-ATF2-AAA or empty vector pFLAG-CMV5 were cotransfected into VSMCs. For dominant negative Smad3 experiments, −795Csrp2-luc or 3TP-Lux (29) was cotransfected with pR5K-Smad3ΔC, which lacks C-terminal phosphorylation and activation sites (30) or empty pR5K vector into VSMCs. Two hours following transfection, the indicated wells were treated with or without TGFβ. Because pCMVβ contains a consensus CRE site for potential ATF2 binding (data not shown), we omitted pCMVβ for transfection efficiency control and normalized luciferase activity to total protein content as described by Owens and co-workers (31). Each construct was transfected at least three times, and each transfection was performed in triplicate.

**Electrophoretic Mobility Shift Assays**—Nuclear extracts were prepared from VSMCs, and DNA-binding assays were performed as described (27). Complementary oligonucleotides from the Csrp2 promoter bp −467 to −448 containing the CRE-like sequence (in boldface type), 5′-TTTCTCATA-ACGTCAATTGTGTT-3′ or three bases mutated (underlined) in the core sequence 5′-TTTCTCAACCGTAATTGTGTT-3′, and the CRE consensus sequence 5′-ATTGCCCTGACGTCA-

GAGAGC-3′ (32) were synthesized. Additional mutant oligonucleotides with one or two bases mutated (underlined) in the core sequence (in boldface type) were also synthesized: mut1, 5′-TTTTGACAAACGTCTTTTGTGTT-3′ and mut2 5′-TTTTC-

AAGGACGTATTGTGTT-3′. Annealed complementary oligonucleotides were end-labeled with [γ-³²P]ATP and T4 polynucleotide kinase (New England Biolabs). To determine the specificity of the DNA-protein complexes, competition assays were performed with a 200-fold molar excess of unlabeled double-stranded oligonucleotides. To characterize the specific DNA-binding proteins, nuclear proteins were incubated with antibodies to ATF-2, CREB, or c-Jun (Santa Cruz Biotechnology). To detect phosphorylated proteins in the DNA-protein complexes, nuclear proteins were incubated with antibodies to phospho-ATF2 (Thr71) (Cell Signaling Technology) or phospho-

CREB (Ser¹⁴³) (Upstate Cell Signaling Solutions).

**Chromatin Immunoprecipitation Assays**—Mouse VSMCs were cultured in 150-mm dishes and fixed with formaldehyde as described in the instructions of the EZ ChiP chromatin immunoprecipitation kit (Upstate Cell Signaling Solutions). The fixed cells were harvested and prepared for chromatin immunoprecipitation according to the manufacturer’s instructions. Following DNA fragmentation by sonication, aliquots of the samples equivalent to 2 × 10⁶ cells were used in each reaction and subsequently incubated with 5 μg of CREB antibody (Santa Cruz Biotechnology), phospho-CREB (Ser¹⁴³) antibody (Upstate Cell Signaling Solutions), or normal rabbit IgG (Upstate Cell Signaling Solutions). Quantitative PCR (as described under “Northern Blot Analysis and Real Time Quantitative Polymerase Chain Reaction”) was subsequently performed using immunoprecipitated DNA. Aliquots of samples equivalent to 1% of initial cell lysate for each reaction were processed, and DNA was purified to use as input DNA control. The primers used to amplify a 165-bp fragment of the mouse Csrp2-CRE were 5′-TGAGTTTTCTTCCCTCCCTCAG-3′ (forward) and 5′-CAGGATAGTCTGGTCAGAATC-3′ (reverse), and the primers used to amplify a 92-bp fragment of the mouse cyclin D1-CRE were 5′-CTGCCCGGCTTT-GATCTCT-3′ (forward) and 5′-CTCTGGAAGCTGCAG-GAC-3′ (reverse). As an additional negative control, a 164-bp fragment within intron 1 of the Csrp2 gene was also amplified by quantitative PCR with the following primers: forward, 5′-TTTCCCTACCTGCGGTGTCTCTC-3′; reverse, 5′-ACAC-ATATCCTGGGGGCTGAAG-3′.

**RESULTS**

TGFβ Induces Csrp2 Expression in Vascular Smooth Muscle Cells—TGFβ, a key factor present in the vessel wall in the early phases of arterial response to injury, plays important roles in modulating lesion formation (33). Because CRP2 also has a significant role in modulating this response, we examined the possibility that TGFβ might regulate CRP2 expression. We treated VSMCs with TGFβ and evaluated CRP2 protein levels by Western blot analysis. The physiological concentrations of TGFβ have been reported to be ∼2–3 ng/ml in plasma and cells (34, 35). However, a higher concentration of 10 ng/ml has been routinely used in cultured cells (34, 36, 37). To mimic the likely higher concentrations of cytokines released at the injured arte-
tein extracts were harvested at the indicated time points. Total protein was
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FIGURE 1. TGFβ Increases CRP2 Expression in VSMCs. A, TGFβ increases CRP2 protein expression. VSMCs were exposed to TGFβ (10 ng/ml), and protein extracts were harvested at the indicated time points. Total protein was
determined with enhanced chemiluminescence and exposed to film. Blots were
subsequently probed for CRP2-(91–98) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. The blot was visualized with a polyclonal primary antiserum specific for CRP2 and a
18S ribosomal RNA probe. The normalized intensities were expressed relative to control without TGFβ stimulation at 0 h. Values are mean ± S.E. of three experiments. B, up-regulation of CRP2 mRNA by TGFβ. VSMCs were treated with TGFβ (10 ng/ml) for the indicated times. Northern analysis revealed that in the absence of
actinomycin D, TGFβ substantially induced CRP2 mRNA expression, whereas actinomycin D blocked induction of CRP2 mRNA (Fig. 2A). Given that TGFβ can regulate mRNA levels of many target genes by altering mRNA stability and half-life (38–40), we measured the half-life of CRP2 mRNA in VSMCs stimulated with or without TGFβ. In the absence of TGFβ, the half-life of CRP2 mRNA was ~14 h (Fig. 2B). The half-life of CRP2 mRNA in TGFβ-treated cells was also ~14 h, indicating that TGFβ has no effect on CRP2 mRNA half-life (Fig. 2B).

CRP2 Induction by TGFβ Does Not Require New Protein Synthesis—To further examine whether CRP2 mRNA induction by TGFβ required protein synthesis, we preincubated VSMCs with protein synthesis inhibitor cyclohexamide for 30 min and then treated for 4 h with or without TGFβ. Real time
also extracted from unstimulated control cells. Western blot analyses were
performed using 20 μg of total protein/lane. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with a polyclonal primary antiserum specific for CRP2 (91–98) and a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody. The blot was visualized with enhanced chemiluminescence and exposed to film. Blots were
subsequently probed for α-tubulin for normalization. CRP2 protein induction is expressed relative to control without TGFβ stimulation at 0 h. Values are mean ± S.E. of three experiments. B, up-regulation of CRP2 mRNA by TGFβ. VSMCs were treated with TGFβ (10 ng/ml) for the indicated times. Northern blot analysis was performed with 10 μg of total RNA/lane. After electrophoresis, RNA was transferred to nitrocellulose filters and hybridized with a random primed 32P-labeled mouse CRP2 cDNA probe that hybridized to a 1.0-kb CRP2 message. The blots were subsequently hybridized with a 32P-labeled 18 S oligonucleotide to verify loading. The signal intensity of each RNA sample hybridized to the CRP2 probe was divided by that hybridized to the 18 S probe. The normalized intensities were expressed relative to control without TGFβ stimulation at 0 h. Values are mean ± S.E. of 3–5 experiments. C, TGFβ treatment reduces wild type but not Csrp2−/− VSMC migration in response to PDGF-BB. Wild type (+/+ ) and Csrp2−/− (−/−) VSMCs were serum-starved for 24 h, treated without or with TGFβ (10 ng/ml) for 12 h and then plated in triplicate in 6-well transwell plates for migration assays using PDGF-BB (10 ng/ml) as a chemoattractant. Cells migrating through the filters after 2 h were quantified and normalized to the cell number of wild type without TGFβ treatment. Values are mean ± S.D. of two experiments.

TGFβ Does Not Alter the Half-life of CRP2 mRNA—To begin to elucidate the mechanisms underlying CRP2 mRNA induction, we performed experiments with the transcriptional inhibitor actinomycin D. VSMCs were preincubated with vehicle or actinomycin D for 30 min and then treated for 4 h with or without TGFβ. Northern analysis revealed that in the absence of actinomycin D, TGFβ substantially induced CRP2 mRNA expression, whereas actinomycin D blocked induction of CRP2 mRNA (Fig. 2A). Given that TGFβ can regulate mRNA levels of many target genes by altering mRNA stability and half-life (38–40), we measured the half-life of CRP2 mRNA in VSMCs stimulated with or without TGFβ. In the absence of TGFβ, the half-life of CRP2 mRNA was ~14 h (Fig. 2B). The half-life of CRP2 mRNA in TGFβ-treated cells was also ~14 h, indicating that TGFβ has no effect on CRP2 mRNA half-life (Fig. 2B).

CRP2 Induction by TGFβ Does Not Require New Protein Synthesis—To further examine whether CRP2 mRNA induction by TGFβ required protein synthesis, we preincubated VSMCs with protein synthesis inhibitor cyclohexamide for 30 min and then treated for 4 h with or without TGFβ. Real time
quantitative PCR analysis showed that cyclohexamide did not prevent CRP2 mRNA induction by TGFβ (Fig. 2C), demonstrating that transcriptional activation of Csrp2 by TGFβ does not require new protein synthesis.

The Csrp2 Promoter Region bp −480 to −438 Is Important for TGFβ Induction—To determine whether elements responsible for TGFβ induction were present in the Csrp2 promoter, we transiently transfected VSMCs with luciferase plasmid Csrp2-luc containing 795 bp of the Csrp2 promoter. We demonstrated previously that this region is sufficient to drive lacZ reporter gene expression in VSMCs of blood vessels in transgenic mice (21). TGFβ increased 795Csrp2 promoter activity by 4-fold (Fig. 3A), indicating that the −795 proximal
promoter contained TGFβ-responsive elements for the induction of CRP2. To identify the elements, a series of Csrp2 5′-deletion promoter-luciferase constructs were generated and transiently transfected into VSMCs. Similar to the −795 construct, the −573 construct showed a 3.8-fold induction by TGFβ (Fig. 3A). Deletion of the 5′-sequences to bp −549 retained TGFβ inducibility (Fig. 3A). Additional deletion to bp −480 only slightly diminished the responsiveness to TGFβ. Significantly, further deletion to bp −438 abolished the promoter induction by TGFβ (Fig. 3A), indicating that the region between bp −480 and −438 was required for the TGFβ stimulation of Csrp2 promoter activity. To determine the functional importance of this region in the Csrp2 promoter, we generated a −795Δ(480/438)Csrp2-luc construct that had a 43-bp internal deletion from bp −480 to −438 within the context of −795Csrp2-luc construct. Transient transfection experiments revealed that deletion of this region reduced basal promoter activity by >50% (Fig. 3B, white bars). Furthermore, internal deletion of this region also abolished the TGFβ induction (Fig. 3B, filled bars), consistent with the 5′-deletion studies (Fig. 3A).

The CRE-like Element Is Critical for Basal and Inducible Activity of the Csrp2 Promoter—Comparison of the sequences from the mouse promoter bp −485 to −435 with human and rat promoters (GenBankTM accession numbers U95017 and NW04774, respectively) revealed that this region is highly conserved across species (Fig. 4A). Through transcription factor data bases (BCM Search Launcher; TRANSFAC) searches and closer examination of the sequences, we identified a putative CRE-like element (TAACGTCA) (Fig. 4A, boldface type). To test the potential function of this conserved CRE-like site, which is one base divergent from the consensus CRE site (TGACGTCA), we generated a luciferase construct, −795CREmutCsrp2-luc, with three bases mutated (underlined) within the putative CRE site (CACCG-TAA). Compared with the control, the −795CREmutCsrp2-luc construct substantially reduced basal promoter activity, similar to that of the internal deletion construct, −795Δ(480/438)Csrp2-luc (Fig. 4B, white bars). Most importantly, mutation of this site abrogated TGFβ-mediated induction of the Csrp2 promoter (Fig. 4B, black bars).

Nuclear Proteins Binding to the CRE-like Site of the Csrp2 Promoter—Given the functional importance of the CRE-like site in regulating Csrp2 promoter activity, we were interested in characterizing the transcription factors that bound to this site. We first tested whether nuclear proteins from control VSMCs (i.e. not treated with TGFβ) bound to the Csrp2-CRE site by gel mobility shift assays. Oligonucleotide sequences used in the gel shift assays are indicated (Fig. 5A). Incubation of VSMC nuclear extracts with an oligonucleotide probe encoding bp −467 to −448 of the Csrp2 promoter resulted in two prominent DNA-protein complexes, a dominant upper complex I and a minor lower complex II (Fig. 5A, lane 2). These two complexes were specific, because they were competed by excess unlabeled identical probe (Fig. 5A, lane 3) but not by an unrelated probe (data not shown). The mutant oligonucleotides (Fig. 5A, mut) with three bases mutated (CACCG-TAA) failed to compete away the binding complexes (Fig. 5A, lane 4). Conversely, no specific complex formation was observed when the mutant was used as a probe (Fig. 5A, lane 7), further indicating that nuclear factors bound specifically to this CRE-like site. The consensus CRE oligonucleotides also competed away the complexes (Fig. 5A, lane 5), indicating that this is a bona fide CRE site. When 32P-labeled consensus CRE was used as a probe, the intensity of complex I relative to complex II was reduced compared with the Csrp2 probe (Fig. 5A, lane 9 versus lane 2). The identical unlabeled CRE competitor abolished the complexes, confirming the specificity of the two complexes (Fig. 5A, lane 4). Intriguingly, the Csrp2 competitor primarily abolished binding of complex I and to a lesser degree with complex II (Fig. 5A, lane 11). Taken together, these results suggest that nuclear factors that bound to Csrp2-CRE differ from that bound to consensus CRE. To examine whether less severe mutations are sufficient to disrupt nuclear protein binding, we performed additional gel shift assays using oligonucleotides with one or two bases mutated in the Csrp2-CRE core sequence (Fig. 5B). Consistently, incubation of VSMC nuclear extracts with Csrp2 oligonucleotide probe resulted in complex I and II formation (Fig. 5B, lane 2). Complexes I and II were abolished by the addition of unlabeled identical (Fig. 5B, lane 3)
CRP2 Induction by TGFβ

A Csrp2 -467 TTTCAAAACGTCATTGGT -448
mut -467 TTTCAACACGTCATTGGT -448
CRE 5' ATGGCCTGACGTCAGAGGC 3'

Probe Csrp2 mut CRE
Competitor - - Csrp2 - - CRE
NE - + + -

II

ns

lane # 1 2 3 4 5 6 7

B Csrp2 -467 TTTCAAAACGTCATTGGT -448
mut1 -467 TTTCAACACGTCATTGGT -448
mut2 -467 TTTCAACACGTCATTGGT -448

Probe Csrp2 mut1 mut2
Competitor - - Csrp2 - - mut1 - - mut2 - - - -
NE - + + + + + +

II

lane # 1 2 3 4 5 6 7

C NE - Control TGFβ
Ab - - ATF2 CREB c-Jun - - ATF2 CREB c-Jun

II

lane # 1 2 3 4 5 6 7

FIGURE 5. Nuclear proteins binding to the CRE-like element of Csrp2 promoter. A, oligonucleotide sequences used in the electrophoretic mobility shift assays (EMSAs) are shown. The core sequence of CRE-like and consensus CRE sites are in boldface type, and mutated sequence is underlined. EMSAs were performed with double-stranded oligonucleotides corresponding to bp −467 to −448 of the Csrp2 promoter. The addition of nuclear extracts (10 μg) from VSMCs to the 32P-labeled Csrp2 probe resulted in two major retarded DNA-protein complexes, designated I and II (arrows on left) (lane 2). A nonspecific band (ns) is indicated. Complexes I and II were abolished by the addition of unlabeled identical (Csrp2, lane 3) or consensus CRE (lane 5) oligonucleotides as competitors but not by the addition of three bases mutated (mut) oligonucleotides (lane 4). Conversely, EMSAs using 32P-labeled mutated oligonucleotides did not result in specific complex formation (lane 7). As a comparison, EMSAs using 32P-labeled CRE oligonucleotides were performed. The addition of nuclear extracts to the CRE probe resulted in the formation of complexes I and II (lane 9), which were competed away by identical unlabeled oligonucleotides (CRE; lane 10). The addition of unlabeled Csrp2 mainly abolished complex I and to a lesser degree complex II (lane 11). B, oligonucleotides used in the EMSAs are indicated. Csrp2 oligonucleotides contain the core sequence of CRE-like site (in boldface type), whereas mut1 has a one-base mutation (underlined) in the core and mut2 has two bases mutated (underlined). As in A, complex I and II were abolished by the addition of unlabeled identical (Csrp2, lane 3) oligonucleotides as competitors, mut1 partially competed away the complexes (lane 4), whereas mut2 did not compete away the complexes (lane 5). EMSAs using 32P-labeled mut1 oligonucleotides resulted in low intensity complex I and II formation (lane 6), whereas 32P-labeled mut2 oligonucleotides did not result in specific complex formation (lane 7). C, nuclear extracts from control (lanes 2–5) or TGFβ treated for 15 min (lanes 6–9) VSMCs were incubated with 32P-labeled Csrp2 probe without the addition of antibodies (lanes 2 and 6) or antibodies specific to ATF2 (lanes 3 and 7), CREB (lanes 4 and 8), or c-Jun (lanes 5 and 9). ATF2 antibody completely supershifted complex I to an upper band (⋆, lanes 3 and 7), whereas CREB antibody supershifted complex II to an upper complex (⋆, lanes 4 and 8). Incubation with c-Jun antibody did not produce supershifted bands (lanes 5 and 9).
CRP2 Induction by TGFβ

| TGFβ (min) | 0   | 5   | 10  | 15  | 30  | kDa |
|-----------|-----|-----|-----|-----|-----|-----|
| p-ATF2    | 70  |     |     |     |     |     |
| ATF2      | 70  |     |     |     |     |     |
| p-CREB    | 43  |     |     |     |     |     |
| CREB      | 43  |     |     |     |     |     |

FIGURE 6. TGFβ increases the phosphorylation of ATF2 but not CREB. VSMCs were stimulated with TGFβ (10 ng/ml) and activation of ATF2 and CREB was determined using cell lysates harvested at the indicated time points by Western blot analysis. Phosphorylation of ATF2 and CREB was detected by using phospho-ATF2 (p-ATF2) and p-CREB antibodies, respectively. To verify equal loading, the blots were probed with total ATF2 and CREB antibodies. A representative of three independent experiments is shown.

nuclear extracts (Fig. 5C, lanes 2–5). Using nuclear extracts from VSMCs treated with TGFβ for 30 min also showed similar DNA-protein complex patterns as controls (data not shown).

TGFβ Increases Phosphorylation Levels of ATF-2 But Not CREB—CREB/ATF2 can bind to CRE in an unphosphorylated and transcriptionally inactive form (42–44). Phosphorylation of ATF2 or CREB within the activation domain leads to their activation of gene expression (45, 46). Thus, we hypothesized that TGFβ might increase the phosphorylation of ATF2 and CREB in VSMCs. To investigate this possibility, VSMCs were treated with TGFβ and protein extracts harvested 5–30 min after treatment. Western blot analysis revealed that TGFβ increased ATF2 phosphorylation as early as 5 min after stimulation but did not alter total ATF2 levels (Fig. 6). In contrast, TGFβ did not affect either phosphorylated CREB levels, which were present at baseline, or total amounts of CREB (Fig. 6). To test the hypothesis that ATF2 and its phosphorylation levels might be responsible for TGFβ induction of CRP2, we performed supershift assays using antibodies specific for phosphorylated ATF2 or phosphorylated CREB. Phospho-ATF2 antibodies partially supershifted complex I (Fig. 7A, lane 3) when control nuclear extracts were used. When nuclear extracts from VSMCs treated with TGFβ were used in the reactions, most of complex I was supershifted (Fig. 7A, lane 6). TGFβ increased the ratio of phospho-ATF2 to unphosphorylated ATF2 within complex I from 1.35 ± 0.06 of controls to 3.09 ± 0.29 (n = 3), indicating that TGFβ enhanced phosphorylation of ATF2 within the DNA-protein complex. Phospho-CREB antibodies appeared to disrupt complex II from both the control and TGFβ-treated nuclear extracts (Fig. 7A, lanes 4 and 7); however, the results were not conclusive. To investigate further whether CREB and p-CREB bound to Csrp2-CRE in the intact native chromatin, we performed quantitative ChIP assays. The CRE of the cyclin D1 promoter with the core sequence identical to that of Csrp2-CRE has been shown mainly to bind CREB (46); thus, mouse cyclin D1-CRE was used as a positive control. CREB binding to the cyclin D1 promoter was substantially higher than to the Csrp2 promoter (52 and 6%, respectively, Fig. 7B), indicating much lower levels of CREB bound to Csrp2-CRE than cyclin D1-CRE. ChIP assays using p-CREB antibodies revealed that, compared with 2% of p-CREB bound to cyclin D1-CRE, p-CREB binding to Csrp2 was barely detectable (Fig. 7B). Taken together, the results of gel shifts and ChIP assays suggested that low levels of CREB bound to Csrp2-CRE; however, the bound CREB were probably not phosphorylated.

Transcriptional Activation of the Csrp2 Promoter by ATF2 via the CRE Site—To evaluate the functional role of CREB and ATF2 in the induction of CRP2, we cotransfected −795Csrp2-luc construct with expression plasmids in transient transfection assays. CREB did not increase Csrp2 promoter activity (Fig. 8A). In contrast, ATF2 expression increased Csrp2 promoter activity by 3-fold (Fig. 8A), and TGFβ further enhanced the transcriptional activation by ATF2 7-fold (Fig. 8A). To further confirm the function of ATF2 and the importance of its phosphorylation, we cotransfected −795Csrp2-luc with a phosphorylation-negative ATF2 mutant (ATF2-AAA), in which the three important phosphorylation residues Thr51, Thr53, and Ser72 were mutated to alanines; thus, it also func-
Smad3 contributes to the transcriptional regulation of Csrp2 by TGFβ—Given the role of Smad3 in mediating numerous TGFβ signaling pathways (30, 47, 48), we examined the significance of Smad3 in the regulation of Csrp2 by TGFβ. We cotransfected −795Csrp2-luc with Smad3ΔC, a dominant-negative Smad3 expression plasmid that lacks C-terminal phosphorylation and activation sites (30). In transient transfection assays, Smad3ΔC decreased Csrp2 promoter activity by 43% in the absence of TGFβ (Fig. 8A). Furthermore, Smad3ΔC reduced TGFβ induction from 350 to 139% (Fig. 8A). As a positive control, we used TGFβ/Smad3-inducible 3TP-Lux reporter construct, which contains three copies of the TRE and a TGFβ-responsive PAI-1 promoter (29) in transfections. Smad3ΔC reduced 3TP-Lux activity to a similar degree as the Csrp2 promoter in the absence or presence of TGFβ (Fig. 9B). Together, these data indicate that Smad3 also contributed to the transcriptional regulation of Csrp2 by TGFβ.

DISCUSSION

We recently demonstrated a key role for CRP2 in the development of arteriosclerosis (16). An absence of CRP2 enhances intimal thickening following arterial injury, implicating a protective function of CRP2 against neointima formation. In line with a vascular protective role of CRP2, by microarray analysis Watanabe et al. (49) showed that estrogen, which has been proposed to have atheroprotective effects, up-regulates CRP2/Smlim in the vascular wall. In the current study, we sought to identify factor(s) that could potentially up-regulate CRP2 expression in the context of vascular injury, which might serve as a protective mechanism against intimal thickening. We demonstrated that TGFβ up-regulated CRP2 protein and mRNA expression. Furthermore, we identified a conserved CRE-like element that was critical for basal and TGFβ-induced activation of the Csrp2 promoter. In addition, TGFβ increased phospho-ATF2 binding to the Csrp2-CRE element. More impor-
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FIGURE 9. Dominant negative Smad3 decreases Csrp2 promoter activity in the absence and presence of TGFβ. A, VSMCs were transiently cotransfected with luciferase reporter plasmids −795Csrp2-luc and empty vector pRK5 or expression plasmid pRK5-Smad3ΔC. Two hours following transfection, cells were treated with or without TGFβ (10 ng/ml) for 24 h. Cells were then harvested for luciferase activity and total protein assays. Csrp2 promoter activity was plotted as percentage of activity of −795 with empty vector without TGFβ. Values are mean ± S.E. of three experiments. B, VSMCs were transiently cotransfected with 3TP-Lux reporter plasmid and empty vector pRK5 or expression plasmid pRK5-Smad3ΔC as in A. 3TP-Lux luciferase activity was plotted as fold induction compared with activity of vector without TGFβ. Values are mean ± S.E.

Csrp2 gene and can enhance SMC differentiation (48, 52). Despite numerous studies, the precise role of TGFβ in vascular injury remains controversial (53, 54). Although TGFβ has been suggested to promote lesion formation (33, 51), interestingly, disruption of a TGFβ signaling pathway in Smad3 null mice enhances neointimal hyperplasia in response to injury. This latter study indicates an inhibitory and protective role for TGFβ in vascular lesion formation (53).

By 5′-promoter deletion experiments, we identified a region at bp −480 to −438 important for TGFβ induction of the Csrp2 promoter (Fig. 3). The sequence conservation of this region across mouse, human, and rat promoters further indicated a functional importance (Fig. 4A). Indeed, internal deletion of this region in the context of the larger −795 promoter construct abolished TGFβ induction (Fig. 3B). Surprisingly, it also reduced the basal promoter activity by >50% (Fig. 3B). Mutation of a conserved CRE-like motif (TAACGTC/CA), which has one base divergent from the consensus CRE motif (TGACGTCA), within this region at bp −461 to −454 of the mouse Csrp2 promoter also abolished TGFβ induction and reduced basal promoter activity (Fig. 4B). These complementary experiments demonstrated an essential role of the CRE motif in conferring Csrp2 basal and TGFβ-inducible promoter activity in VSMCs. Supporting a critical role of CRE in regulating VSMC gene expression, the homeobox transcription factor Hex modulates CRE-dependent transcription of Smemb/non-muscle myosin heavy chain-B gene in VSMCs (55). Additionally, a consensus CRE located in the intronic enhancer of the Csrp1 gene, a member of the CRP family, binds CREB and was important for CRP1 expression in VSMCs (56). Taken together, it appears that CRE elements might regulate several functionally significant genes in VSMCs.

Members of the ATF/CREB family of transcription factors form homodimers or heterodimers with the Fos/Jun family member, leading to transcriptional activation of target genes via CRE elements (TGACGTCA) (42, 57). The heterogeneity in dimer composition contributes to the functional diversification of ATF/CREB complexes (57). In addition, variations within the CRE core sequences affect binding affinities of these complexes (58). Interestingly, our results showed that predominantly ATF2 bound to the Csrp2-CRE site (Fig. 5C). Although ATF2 homodimers and ATF2/c-Jun heterodimers have identical affinities for the CRE (59), ATF2 probably bound to the Csrp2-CRE as a homodimer, because an ATF2-specific antibody completely supershifted complex 1, but c-Jun antibody did not (Fig. 5C). This binding pattern differs from the consensus CRE (Fig. 5A) or Csrp1-CRE complexes (56), where CREB was the predominant binding factor. The differential transcription factor binding might be due to one base divergence of the core sequence (from G to A in the second base). However, the CRE of the cyclin D1 promoter with the identical core sequence with Csrp2-CRE has been shown to mainly bind CREB and activate cyclin D1 promoter in rat insulinoma INS-1 cells (46). ChIP assays using VSMCs demonstrated that in contrast to cyclin D1-CRE, much lower levels of CREB bound to Csrp2-CRE (Fig. 7B), suggesting that flanking sequences might confer the specificity for transcription factor binding.

The functional role of ATF2 in activating the Csrp2 promoter was confirmed by transiently overexpressing ATF2 in transfection experiments (Fig. 8A). Although ATF2 can bind to CREs in the unphosphorylated state (60), TGFβ enhanced ATF2 phosphorylation (Fig. 6) and increased phospho-ATF2 levels binding to Csrp2-CRE (Fig. 7A), implicating the importance of ATF2 phosphorylation in mediating TGFβ induction of CRP2.
expression. The significance of post-translational modification rather than new protein synthesis in TGFβ-mediated transcriptional activation of Csrp2 was supported by the findings that protein synthesis inhibitor cyclohexamide did not prevent Csrp2 mRNA induction by TGFβ (Fig. 2C). A phosphorylation-negative mutant, which also functions as a dominant negative mutant, reduced basal and TGFβ-mediated Csrp2 promoter activity (Fig. 8B), further demonstrating the critical importance of ATF2 phosphorylation in regulating Csrp2 expression. In contrast to ATF2, CREB probably played a minimal role in regulating Csrp2 gene expression, given its very low levels of binding (particularly p-CREB) to Csrp2-CRE both in vitro and in vivo and that cotransfection of CREB did not increase Csrp2 promoter activity (Fig. 8A). Interestingly, TGFβ signaling molecule Smad3 also contributed to the transcriptional regulation of Csrp2 (Fig. 9). However, further studies are needed to investigate the underlying mechanisms.

In conclusion, we identified a conserved CRE element (TAACGTCA) in the Csrp2 promoter that was critical for basal promoter activity and responsiveness to TGFβ. Our results show for the first time in VSMC that TGFβ activates ATF2 phosphorylation and Csrp2 gene expression via a CRE promoter element, which may represent a previously unrecognized mechanism of VSMC gene expression. Understanding the transcriptional activation of Csrp2 may help to elucidate the molecular mechanisms that control VSMC gene expression in vascular disease.

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