Mechanical Regulation of the Proangiogenic Factor CCN1/CYR61 Gene Requires the Combined Activities of MRTF-A and CREB-binding Protein Histone Acetyltransferase*

Mary Hanna, Haibo Liu, Jawaria Amir, Yi Sun, Stephan W. Morris, M. A. Q. Siddiqui, Lester F. Lau, and Ibrahim Chaqour

From the 6Department of Cell Biology, State University of New York Downstate Medical Center, Brooklyn, New York 11203, the 7Department of Pathology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105, and the 8Department of Biochemistry and Molecular Genetics, University of Illinois College of Medicine, Chicago, Illinois 60607

Smooth muscle-rich tissues respond to mechanical overload by an adaptive hypertrophic growth combined with activation of angiogenesis, which potentiates their mechanical overload-bearing capabilities. Neovascularization is associated with mechanical strain-dependent induction of angiogenic factors such as CCN1, an immediate-early gene-encoded matricellular molecule critical for vascular development and repair. Here we have demonstrated that mechanical strain-dependent induction of the CCN1 gene involves signaling cascades through RhoA-mediated actin remodeling and the p38 stress-activated protein kinase (SAPK). Actin signaling controls serum response factor (SRF) activity via SRF interaction with the myocardin-related transcriptional activator (MRTF)-A and tethering to a single CArG box sequence within the CCN1 promoter. Such activity was abolished in mechanically stimulated mouse MRTF-A−/− cells or upon inhibition of CREB-binding protein (CBP) histone acetyltransferase (HAT) either pharmacologically or by siRNAs. Mechanical strain induced CBP-mediated acetylation of histones 3 and 4 at the SRF-binding site and within the CCN1 gene coding region. Inhibition of p38 SAPK reduced CBP HAT activity and its recruitment to the SRF-MRTF-A complex, whereas enforced induction of p38 by upstream activators (e.g. MKK3 and MKK6) enhanced both CBP HAT and CCN1 promoter activities. Similarly, mechanical overload-induced CCN1 gene expression in vivo was associated with nuclear localization of MRTF-A and enrichment of the CCN1 promoter with both MRTF-A and acetylated histone H3. Taken together, these data suggest that signal-controlled activation of SRF, MRTF-A, and CBP provides a novel connection between mechanical stimuli and angiogenic gene expression.

The cellular components of the cardiovascular, digestive, and urinary systems elicit adaptive responses to mechanical/pressure overload acutely by retooling their cytoskeletal structures (i.e. increasing actin polymerization rate and contractile protein levels) and chronically by remodeling their extracellular environment. These compensatory responses are associated with the activation of angiogenesis to meet increased metabolic demands and improve tissue perfusion (1, 2). In the absence of angiogenesis, hypertrophic growth increases diffusion distance around microvessels resulting in reduced oxygen supply and hypoxia, altered muscle contractility, and organ failure. Clearly, an unsatisfactory/insufficient vasculization is an important restraint on the adaptive capabilities of mechanically overloaded tissues.

Neovascularization in mechanically challenged smooth muscle-rich organs in particular is promoted by various mechanoresponsive angiogenic factors including CCN1, formerly known as cysteine-rich protein 61 (Cyr61), a multifaceted matricellular protein that appears in the extracellular environment particularly during development and pathological states (3, 4). The CCN1 protein acts either independently or in concert with vascular endothelial growth factor to drive sprouting and branching of new blood vessels and provides protection against oxidative stress (5, 6). Essentially, the CCN1 protein enhances angiogenesis by providing structural integrity to blood vessels, supplying necessary growth factors for endothelial and perivascular cells, and modulating extracellular matrix synthesis and degradation. Targeted disruption of the CCN1 gene leads to early or perinatal lethality in mice due to impaired vessel formation and/or branching (7). The CCN1 proangiogenic properties have further been demonstrated in different models of angiogenesis including rabbit ischemic hind limb and rat cornea models in which CCN1 improves angiogenesis and collateral blood flow to an even larger extent than vascular endothelial growth factor (8, 9).

Mechanical strain typified by tension, stretch, shear, and pressure largely controls the expression of the CCN1 gene, although little is known about the molecular mechanisms involved (10). CCN1 was induced in mechanically stimulated myocardial fibroblasts and in smooth muscle cells (SMCs)2 2 h

2 The abbreviations used are: SMC, smooth muscle cell; SRE, serum response element; SRF, serum response factor; MRTF, myocardin/myocardin-related transcription factor; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; HAT, histone acetyltransferase; DN, dominant negative; ChIP, chromatin immunoprecipitation; Erk, extracellular signal-regulated kinase; JNK, Janus N-terminal kinase; LMA, leptomycin A; PCAF, p300/CBP-associated factor; TSA, trichostatin A; siRNA, small interfering RNA.

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after angioplasty, in arterial and bladder smooth muscle in a hypertensive rat model, and in pressure-overloaded heart and bladder (11–14). Previous in vitro studies demonstrate that selective inhibition of RhoA GTPase, best known for regulating the physiological state of actin, suppresses mechanical strain-induced CCN1 expression in SMCs in vitro (15, 16). Similarly, actin polymerization inhibitors suppress CCN1 gene induction by mechanical stretch, and treatment of the cells with actin polymerization-inducing drugs (i.e. jasplakinolide, swinholide A) suffices to induce CCN1 gene expression (15). Mechanical strain-dependent activation of Rho GTPase increases both de novo actin polymerization and filament stabilization and concomitantly decreases the pool of monomeric G-actin in the cells (17). Interestingly, the relative concentration of F- and G-actin regulates serum response factor (SRF) activity, an important determinant of immediate-early gene expression including that of CCN1 (18, 19). However, the spectrum of genes activated by SRF is dictated by its affinity to CArG box sequences and its association with a variety of positive and negative co-factors, many of which are cell type-specific and cell signal-regulated (20, 21). SRF can be activated by at least two families of co-activators, p62TCF and myocardin/myocardin-related transcription factor (MRTF)-A and -B (22). Binding of these factors to the same region of SRF is mutually exclusive. Additionally, the p62TCF and p67SRF proteins are direct targets for distinct signal transduction pathways: p62TCF is at the nuclear end point of the Ras/MAPK pathway, and p67SRF is targeted by the Rho/Rac small G-proteins (23). Whether SRF-dependent gene expression involves other regulatory molecules and/or is cell type-specific is unknown.

In this study, we investigated the mechanisms whereby tensile force regulates CCN1 gene induction in smooth muscle of the bladder both in vivo and in cultured cells in vitro. We showed that mechanical strain-induced Rho-actin signaling promotes SRF-MRTF-A interaction and binding to an SRF-binding site in the CCN1 promoter. Furthermore, strain-mediated p38 signaling induced activation and recruitment of CREB-binding protein (CBP) to the SRF-MRTF-A complex and acetylation of nucleosomal histones surrounding the SRF-binding site, which culminates into a strong induction of the CCN1 gene. Thus, chromatin remodeling catalyzed by covalent histone modifications by CBP acts as a nucleator that unfolds chromatin and promotes SRF-MRTF-A transactivation of the angiogenic CCN1 gene in response to mechanical strain.

EXPERIMENTAL PROCEDURES

Cell Culture and Mechanical Force Stimulation—Primary cultures of human bladder SMCs were obtained from Cambrex/Lonza (Allendale, NJ). Cells were cultured in SmGM®-2 containing 5% fetal bovine serum and a supplement of growth factors and cytokines packaged in the SingleQuots® kit (Cambrex). These cells maintain differentiated properties in culture after 4–5 passages. Mechanical stimulation of cultured cells was performed as described previously (2, 17). Briefly, cells were plated on 6-well silicone elastomer-bottomed culture Flex plates coated with type I collagen and incubated for 24 h. The medium was then replaced with SmGM®-2 containing 0.5% fetal bovine serum and a 10% concentration of growth factor supplement, and cells were incubated for an additional 24 h. For experimental plates, cyclic strain was applied to the cells using the FX-4000™ Flexercell® Tension Plus™ system (Bioflex; Flexcell, Hillsborough, NC). Cells were subjected to a maximum of 12% strain magnitude at a frequency of 0.3 Hz for selected periods of time. This strain regimen produced optimal conditions for the maximum induction of the CCN1 gene in SMCs. For control, cells were kept under static conditions in the same culture medium. After completion of either the strain regimen or incubation time periods under static conditions, cells were pooled from each 6-well plate and processed for further analyses. Stretch and control experiments were carried out simultaneously and analyzed identically.

Culture of MRTF-A-deficient SMCs—Generation and characterization of MRTF-A-deficient mice was described previously (24). Primary cultures of SMCs were isolated from the bladder of 14-day-old wild-type and MRTF-A-deficient mice. Briefly, under sterile conditions, the detrusor muscle layer of each bladder was scraped from the urothelium-lamina propria layers, minced into small pieces, and digested with bacterial collagenase (100 units/ml). After inactivation of the enzyme with fetal bovine serum, cells were allowed to attach to a plastic tissue culture flask overnight. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO2 in air at 37°C. Freshly isolated SMCs were phenotypically characterized using muscle-specific antibodies against smooth muscle α-actin.

Transient Transfection, Mutagenesis, and Reporter Assay—For transfection, cultured cells were plated at a density of 1 × 10^5/cm² in 6-well Bioflex plates and maintained in serum-containing medium for 18 h. Transfection was then performed using FuGENE 6 transfection reagent in serum-free medium according to the manufacturer’s specifications (Roche Diagnostics). The CCN1 promoter-reporter plasmid construct p-luc (−2398/+1) used in transfection experiments includes a 2398-bp promoter fragment of CCN1 gene amplified by PCR from human genomic DNA clone RP11-290M5 (obtained from the BAC-PAC Resource Center at Children’s Hospital Oakland Research Institute, Oakland, CA) and cloned into the luciferase reporter vector pGL3-basic. Identity and orientation of the construct were verified by sequencing. Additional CCN1 promoter-reporter constructs containing mutations to putative Ets box and CArG box were generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The distal Ets box was changed from -CAGGCT- to -AGATTC-, and the CArG box was changed from -CCAAA- to -AGATCC-. These nucleotide mutations abolished Ets and p62SRF-binding sites. Constructs were fully sequenced in both directions to confirm successful mutagenesis before use. Other co-expression vectors used include the myocardin expression vector myc-Q-(1–935) (25); MRTF-A and MRTF-B expression vectors (23, 26); the dominant negative (DN) form for SRF (DN-SRF) encoding the SRF deletion mutant lacking the C-terminal SRF transcription activation domain subcloned into pcDNA3 (27); DN-CBP, which lacks C-terminal domain (CBP-(1–1097)); CBP-ΔHAT, which lacks histone acetyltransferase (HAT) activity; and DN-p300.
(Δ851–1045) (28). Cells were co-transfected with pRL-SV40 vector containing the Renilla luciferase gene to adjust for transfection efficiency. The FuGENE 6:DNA mixture plus serum-free medium was left on cells for 3 h. The cells were allowed to recover in fresh medium containing 10% serum. The next day, cells were incubated in serum-free medium and subjected to cyclic strain as described above. Cell lysates were assayed for luciferase activity levels, and firefly luciferase activity was normalized to that of the Renilla luciferase. Each experiment was performed at least three times in duplicate, and all experiments included negative (promoterless pGL3-basic) controls. The latter served as a base-line indicator of luciferase activity.

Co-expression experiments were carried out by with 0.25 μg of either empty vector or vector overexpressing constitutively active forms of FLAG-tagged MRTF-A (29) or active forms of MKK3, MKK4, and MKK6 (30, 31). The cells were allowed to recover in fresh medium containing 10% serum. The next day, experimental treatments were performed as described above and luciferase activity was measured.

**RNA Isolation and Real-time PCR**—Total RNA was extracted from cells using RNeasy kit (Qiagen), and the integrity of the RNA was verified by gel electrophoresis. A quantitative real-time reverse transcriptase-PCR assay was performed using the Master Mix SYBR Green I kit (SABiosciences, Baltimore, MD) in an Applied Biosystems 7000 sequence detection system. The primers used for each amplified sequence were designed to span exon-exon junctions so that genomic DNA would not be detected. Samples were combined with one-step master mix in a 20-μl reaction volume. The cycling parameters for PCR amplification were: AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (40 cycles). Triplicate Ct values were analyzed with Microsoft Excel using the comparative Ct (ΔCt) method as described by the manufacturer (Applied Biosystems). The amount of amplified sequences (2^−ΔΔCt) was obtained by normalizing to an endogenous reference (18S rRNA) relative to a calibrator (one experimental sample).

**Nuclear Protein Extraction, Immunoprecipitation, and Western Immunoblotting**—Nuclear proteins were extracted as described by Dignam et al. (32). Briefly, cells were collected, pelleted by centrifugation, and resuspended in ice-cold sucrose buffer containing 0.1% Triton X-100 to lyse the cells. The preparations were then washed several times with lysis buffer to remove the cytoplasm and debris. The purity of nuclei preparations was verified by their microscopic examination. The purified nuclear fractions were further extracted and subjected to immunoprecipitation and/or immunoblotting analysis. For immunoprecipitation, 150 μg of nuclear proteins was incubated overnight at 4 °C with antibody against either CBP (Santa Cruz Biotechnology), SRF (Santa Cruz Biotechnology), or MRTF-A or MRTF-B (a generous gift from Dr. R. Prywes) and then exposed to a 50% slurry of protein A/G Plus-agarose beads (Santa Cruz Biotechnology) for 2 h. After two washes in buffer (0.1% Tween 20 phosphate-buffered saline), the immunoprecipitated proteins were collected and separated by gel electrophoresis. Protein samples were separated by 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and Western blot analysis was performed using the appropriate antibody. Immunodetection was performed using enhanced chemiluminescence (ECL, Amersham Biosciences) according to the manufacturer’s recommendations.

**Chromatin Immunoprecipitation (ChIP) Assay**—ChIP was performed as described previously with modifications allowing for a quantitative analysis of protein-DNA interactions (17). DNA-protein interactions were fixed by directly adding formaldehyde (1%) to the cell culture media. Fixation proceeded at 22 °C for 10 min and was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were pelleted and resuspended in swelling buffer (10 mM potassium acetate, 15 mM magnesium acetate, 0.1 mM Tris, pH 7.6) containing a mixture of protease inhibitors. The nuclei were collected by microcentrifugation and then resuspended in sonication buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 0.5 mM phenylmethylsulfonyl fluoride, and 100 μg of leupeptin and aprotinin/ml) and incubated on ice for 10 min. Prior to sonication, 0.1 g of glass beads was added to each sample. The chromatin solution was precleared by centrifugation, incubated with 1 μg of affinity-purified rabbit polyclonal antibody or no antibody, and rotated at 4 °C for 12–16 h. Antibodies used included those against acetylated histone H3 (Abcam, Cambridge, MA) or MRTF-A. Immunoprecipitation, washing, and elution of immune complexes was carried out. Prior to the first wash, 20% of the supernatant from the reaction with no primary antibody for each time point was saved as total input chromatin and was processed with the eluted immunoprecipitates beginning at the cross-link reversal step. Cross-links were reversed by the addition of NaCl to a final concentration of 200 mM, and RNA was removed by the addition of 10 μg of RNase A per sample followed by incubation at 65 °C for 4 to 5 h. The samples were precipitated at −20 °C overnight with ethanol and then pelleted by microcentrifugation. Samples were treated with proteinase K for 2 h at 45 °C. The pellets were collected by microcentrifugation and resuspended in 30 μl of H2O. Real-time PCR was performed on 1 ng of genomic DNA from the ChIP experiments. Two pairs of real-time PCR primers were designed to flank the 5′-CAG elements of the CCN1 promoter region and the first exon coding region. Quantification of the protein-DNA interaction/enrichment was determined as follows: 2^−(Ct(Cont)−Ct(IP))−2^−(Ct(Cont)−Ct(no antibody control)) (where Ct is cycle threshold, Cont is control, and IP is the immunoprecipitated). Alternatively, PCR products were fractionated by electrophoresis on 2% agarose gel and quantified by densitometry.

**Histone Acetyltransferase Activity Assay**—Histone acetyltransferase activity was determined using a commercially available kit (Biovision, Mountain View, CA) according to the manufacturer’s instruction. Briefly, 200 μg of nuclear protein in 500 μl of lysis buffer was precleared with 50 μl of protein G-agarose bead slurry at 4 °C for 30 min and then immunoprecipitated with antibody against CBP, MRTF-A, SRF, or a nonspecific IgG at 4 °C for 2 h. Histone acetyltransferase activity was determined by monitoring the fluorescence associated with a histone H4 peptide.

**Surgical Induction of Partial Urethral Obstruction**—Partial urethral obstruction was created in Sprague-Dawley female rats (Charles River Laboratories, Wilmington, MA) weighing ~200 g. After anesthetic induction, a lower midline abdominal...
incision was made to expose the bladder and the proximal urethra. A size 2-0 USP silk ligature then was tied loosely around a length of a plastic rod placed parallel to the urethra. Once the ligature had been secured, the plastic rod was removed by sliding the rod out from beneath the ligature, thus ensuring that the lumen diameter was constrained to ~1 mm. In the sham rats, the bladder and urethra were exposed, but no ligature was applied. Animals were placed under a warm lamp and allowed to recover. At the end of the period of obstruction, rats were anesthetized, and their bladders were excised rapidly and processed for molecular and immunohistochemical analyses.

Immunohistochemistry—For immunohistochemical analyses, tissue was dissected and immediately fixed in 0.4% formaldehyde/phosphate-buffered saline for 2 h followed by an overnight incubation in sucrose solution and frozen in Tissue-Tek OCT. Ten-μm-thick cryostat sections were prepared and stained with hematoxylin-eosin for qualitative assessment. Other sections were incubated for 24 h with anti-MRTF-A antibodies. Immunodetection was performed with rhodamine-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Sections were washed several times in phosphate-buffered saline between incubations.

For visualization of actin stress fibers in cultured cells, cells plated either on flexible membrane or glass cover slips were fixed in 0.4% formaldehyde-phosphate-buffered saline for 30 min, permeabilized in 0.1% Triton X-100 at room temperature for 5 min, and either stained with rhodamine-phalloidin (Cytoskeleton Inc.) or incubated with primary antibody. Immunostaining was visualized using rhodamine-conjugated anti-rabbit IgG.

Statistical Analysis—Data are expressed as mean ± S.E. Statistical significance among various groups was confirmed with a one-way analysis of variance when appropriate. Statistical significance between specific groups was determined by a post-hoc multiple comparison Student-Newman-Keuls test (p < 0.05).

RESULTS

Mechanical Strain Activates the CCN1 Promoter via CArG Box of Serum Response Element (SRE)—The 5’ flanking region of the CCN1 gene contains a remarkably conserved SRE sequence that comprises an Ets-like box, which binds p62TCF, and the CArG box, which binds p67SRF (Fig. 1, upper panel). Utilization of a promoter-reporter assay showed that CCN1 promoter activity was regulated in an SRF/CArG box-dependent manner (Fig. 1, lower panel). Strain-dependent activation of the CCN1 promoter was attenuated by either co-transfection with DN-SRF or utilization of a CArG box-mutated CCN1 promoter. The Ets box-mutated CCN1 promoter remained fully responsive to cyclic strain.

Specific Regulation of CCN1 Promoter Activity by MRTF-A in Response to Cyclic Strain—Mechanical induction of the CCN1 gene was tested in SMCs derived from mouse bladder harboring a loss-of-function mutation in MRTF-A, a major transcriptional co-activators of p67SRF. MRTF-A-null cells appeared smaller and less spread than wild-type cells (Fig. 2A). They also exhibited a decreased proportion of stress fibers and filamentous actin, which largely localized cortically. In these cells, cyclic strain induced a mere 2.5-fold increase in CCN1 transcript levels, whereas in wild-type cells, mechanical stimulation induced a 9-fold increase in CCN1 gene transcript levels (Fig. 2B). Similarly, promoter-reporter assay showed a 60-fold increase in CCN1 promoter activity in wild-type cells but only a 3-fold increase in MRTF-A-null cells (Fig. 2C). Ectopic expression of MRTF-A significantly increased the CCN1 promoter activity in mechanically stimulated MRTF-A-null cells, whereas co-expression of the MRTF-A-related family members, MRTF-B and myocardin, was ineffective in restoring CCN1 promoter sensitivity to mechanical strain.

Mechanical Strain-induced Actin-dependent Translocation of MRTF-A into the Nuclei and Binding to the CCN1 Promoter—We had previously reported that mechanical strain-induced CCN1 gene expression involved Rho GTPase-dependent enhancement of actin polymerization in mechanically stimulated cells (16). Concordantly, strain-induced CCN1 promoter-reporter activity was blocked upon pretreatment of the cells with latrunculin B, C3 toxin, or Y27632 inhibitor, which interfere with actin polymerization, Rho GTPases, and RhoA-associated kinase activity, respectively (Fig. 3A). Pharmacological blockade of the p38 SAPK by SB-203580 induced a 40% decrease of the promoter activity, whereas inhibition of MAPK Erk1/2 and JNK by Pd-098059 and SP-600125, respectively, had no effect on CCN1 promoter activation by strain. Furthermore, pretreatment of the cells with a cell-penetrating peptide chimeric containing the N-terminal sequence of smooth muscle α-actin, Ac-EEED, which induces the rapid disassembly of F-actin (17, 33), completely abolished CCN1 promoter responsiveness to mechanical strain. Clearly, strain-induced CCN1 promoter activity is independent of ternary complex factors.
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FIGURE 2. MRTF-A controls sensitivity of the CCN1 promoter to SRF action in response to mechanical strain. A, cells explanted from the detrusor muscle of the bladder from wild-type and MRTF-A-deficient mice were stained with rhodamine-conjugated phalloidin. B, effects of mechanical strain on CCN1 mRNA levels in wild-type and MRTF-A-deficient mouse cells. Data are given as mean ± S.E., n = 4, **, p < 0.01 versus control nonstimulated cells. C, MRTF-A-dependent activation of the CCN1 promoter is independent of MRTF-B and myocardin expression. CCN1 promoter-reporter activity was determined in MRTF-A-null cells co-transfected with empty vector, pCDNA1, MRTF-A, MRTF-B, or myocardin. Cells were assayed for luciferase activity following the application of cyclic strain for 1 h. The relative luciferase activity (mean ± S.E.) from a representative transfection experiment performed in triplicate is shown. **, p < 0.01 versus control nonstimulated cells.

that are targeted by MAPK Erk1/2 signaling and relies rather on Rho signaling, which promotes filamentous actin (F-actin) accumulation and stabilization and concomitant globular actin (G-actin) depletion.

Next, we examined whether and how mechanical strain regulates MRTF-A expression and/or activity. Application of cyclic strain to the cells did not increase MRTF-A mRNA levels, as determined by real-time PCR, but induced a 2.5-fold increase in MRTF-B transcripts (Fig. 3B). This suggests that the expression of MRTF-A and MRTF-B is not coordinately regulated by mechanical stimuli and that MRTF-A activity could be achieved only through changes in cell signaling. Because nuclear localization of MRTF-A depends on globular and filamentous actin dynamics (34), we examined changes in the cellular localization of MRTF-A in response to mechanical strain. Fig. 3C shows that MRTF-A localized within both the cytoplasm and nucleus in cells cultured under static conditions, whereas MRTF-B localized largely within the cytoplasm. Application of cyclic strain for 30 min induced translocation of MRTF-A into the nucleus, whereas MRTF-B remained largely cytoplasmic and/or perinuclear. Thus, the inability of MRTF-B to substitute for MRTF-A in MRTF-A-null cells is due potentially to the largely cytoplasmic localization of MRTF-B.

Cyclic strain markedly augmented nuclear MRTF-A protein levels as determined by Western immunoblotting analysis of nuclear protein extracts (Fig. 3D). Pretreatment of the cells with Erk1/2 inhibitor did not reduce the nuclear translocation of MRTF-A, whereas either C3 toxin or Ac-EEED peptide chimera substantially decreased nuclear MRTF-A levels. A quantitative ChIP assay showed that cyclic strain induced a nearly 4-fold enrichment of MRTF-A in the CCN1 promoter region encompassing the CarG box sequence. Binding of MRTF-A to other portions of the CCN1 was not detected (data not shown). However, enrichment of the CarG box region with MRTF-A was diminished upon pretreatment of the cells with either C3 toxin or Ac-EEED chimera peptide. Erk1/2 inhibition had no dramatic effects on CCN1 promoter enrichment with MRTF-A. These data underscore the importance of actin-dependent signaling for the mechanical regulation of both CCN1 promoter and MRTF-A activities.

Nuclear Translocation of MRTF-A Is Necessary but Not Sufficient for Mechanical Strain-dependent Activation of the CCN1 Promoter—Because nuclear import of MRTF-A is a key step in strain-induced CCN1 gene expression, we examined the signaling requirement for MRTF-A nuclear import in the absence of export. Cells transfected with FLAG-tagged MRTF-A exhibited either a cytoplasmic or diffused nucleocytoplasmic localization of FLAG-MRTF-A (Fig. 4A). Application of cyclic strain and/or treatment with leptomycin A (LMA), an inhibitory drug of the exportin CRM1, induced nuclear accumulation of the ectopically expressed MRTF-A indicating that MRTF-A continuously shuttles between the cytoplasm and nucleus and uses the CRM1 pathway for nuclear export. Furthermore, ChIP analysis showed that mechanical strain and/or LMA treatment induced ~2.5-fold enrichment of the CCN1 promoter region with MRTF-A as compared with nontreated cells (Fig. 4B). However, LMA treatment neither activated the CCN1 promoter nor did it have additive or synergistic effects with cyclic strain. Clearly, nuclear import of MRTF-A is necessary but not sufficient for CCN1 gene expression.

Role of Chromatin Acetylation by CBP in the Regulation of CCN1 Promoter Activity in Response to Cyclic Strain—Recent studies have shown that mechanical stimuli such as shear stress regulate gene expression by inducing epigenetic modification of histones and activation of transcription complexes bearing HAT activity (35, 36). Therefore, we examined the potential involvement of epigenetic modifications of histones in
Mechanical strain-induced actin signaling regulates nuclear translocation and binding of MRTF-A to the CCN1 promoter. A, mechanical strain-induced CCN1 promoter-reporter activity was determined in cells left untreated or pretreated for 30 min with one of the following pharmacological inhibitors: Pd-098059 (20 µM), SP-600125 (20 µM), SB-203580 (10 µM), C3 toxin (5 ng/ml), Y27632 (10 µM), or Ac-EEED chimera peptide (10 ng/ml). Luciferase activity after 1 h of mechanical stimulation was set to 100%. Values shown (data not shown).

To determine whether and which HAT-containing co-activator is involved in mechanical regulation of the CCN1 gene, cells transfected with the CCN1 promoter-reporter construct were pretreated prior to the application of cyclic strain with either garcinol, which inhibits p300 and p300/CBP-associated factor (PCAF) or curcumin, a known inhibitor of p300/CBP but not PCAF. As shown in Fig. 5C, pretreatment with either curcumin or garcinol decreased mechanical strain-induced CCN1 promoter activity by 50 and 80%, respectively. Pretreatment with trichostatin A (TSA) alone, a known inhibitor of histone deacetylase, induced a significant increase (7-fold) in the promoter activity. The observed effects of TSA alone were not surprising, inasmuch as TSA has the capacity to open up epistolal structures by inducing hyper-acetylation of histones and potentially recruiting co-activators to the promoter region of the CCN1 gene. Furthermore, the combined effects of TSA and mechanical strain were neither additive nor synergistic, suggesting that their effects could not translate into changes beyond a functional threshold.

We further examined the proper effects of CBP and p300 as potential regulators of the CCN1 promoter activity. As shown in Fig. 5D, co-expression of either CBP or the p300 construct increased CCN1 promoter activity by more than 2-fold. Co-expression of a HAT-deficient CBP mutant instead of the wild-type CBP was unable to stimulate the CCN1 promoter, suggesting that CBP activity involves acetylation-dependent transcription regulation.

Mechanical Strain-induced Transactivation of the CCN1 Promoter Involves p38 SAPK-mediated Activation of CBP and Its Recruitment to the SRF-MRTF-A Complex—CBP is a nuclear phosphoprotein that is activated by various kinases such as protein kinase A, calcium/calmodulin-dependent kinase N, and MAPKs such as Erk1/2 and p38 SAPK, which aids CBP-mediated transcription activation through yet unknown mechanisms (38). To identify potential interactions between CBP and SRF-MRTF-A complex and determine the upstream signals responsible for CBP activity, co-immunoprecipitation assays were performed on protein extracts from cells treated with LMA or subjected to cyclic strain using antibodies to SRF, MRTF-A, CBP, or FLAG. The HAT activity of the immunoprecipitated complexes was measured using a HAT fluorescence assay.

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FIGURE 4. Nuclear translocation of MRTF-A is necessary but not sufficient for mechanical strain-dependent activation of the CCN1 promoter. A, immunolocalization of MRTF-A in cells exposed to cyclic strain and/or LMA. Cells were transfected with FLAG-tagged MRTF-A and left untreated, treated with LMA (5 μM), or exposed to cyclic strain in the presence and absence of LMA. Immunostaining was performed with an anti-FLAG antibody. B, cells were treated as described in A, and chromatin enrichment with MRTF-A was analyzed using ChIP assay. Data are means ± half-range of two experiments. In parallel experiments, cells were transfected with the CCN1 promoter-reporter construct and left untreated, treated with LMA, or exposed to cyclic strain in the presence and absence of LMA. To facilitate comparisons among different experiments, the luciferase activity was set to 100% in cells subjected to mechanical stimulation for 1 h. The values represent the average of determinations ± S.E. from an experiment performed in triplicate. Experiment was repeated twice with nearly identical results. **, p < 0.05 versus static control.

As shown in Fig. 6A, MRTF-A and SRF complexes from cyclically strained cells possessed HAT activity 9- and 13-fold higher than those of the immunoprecipitates from cells cultured under static conditions. Immunoprecipitated CBP complexes exhibited a 26-fold higher activity in mechanically stimulated cells compared with control cells. Conversely, LMA treatment alone did not significantly increase the HAT activity of MRTF-A-containing complexes. Thus, MRTF-A is involved in transcriptional complexes containing CBP HAT activity in mechanically stimulated cells. Pretreatment of cells with Y27632 and SB025856, which inhibit Rho kinase and p38, respectively, reduced HAT activity associated with MRTF-A complexes, indicating that p38 is an upstream activator of CBP.

To ascertain the composition of the protein complexes, co-immunoprecipitation assays were performed from cells transfected with FLAG-tagged MRTF-A. Western blotting of anti-CBP immunoprecipitates from mechanically stimulated cells showed that CBP complexes contained both MRTF-A and SRF proteins (Fig. 6B). Interestingly, the levels of MRTF-A associated with CBP complexes decreased in the presence of either Y27632 or SB025856 as compared with the levels of CBP in the same samples. The effects of the inhibitor Y27632 are, at least in part, due to decreased MRTF-A import to the nucleus as shown in Fig. 3D. Because the p38 SAPK is a potential upstream activator of CBP, we examined whether p38 inhibition by SB025856 regulates CBP binding/activity in mechanically stimulated cells. As shown in Fig. 6C, enforced induction of p38 by ectopic expression of dual specificity upstream activators, MKK3, MKK4, or MKK6 (39), induced a 12.8-, 4.4-, and 13.4-fold increase in the CCN1 promoter activity, respectively. As expected, MKK4, which activates the JNK rather than the p38 SAPK pathway, induced much lower activity. None of these p38 upstream activators was able to stimulate the activity of a CAκG box-mutated CCN1 promoter.

To examine specifically the role of p38 as an upstream activator of CBP, CCN1 promoter-reporter activity was measured upon cell transfection with a CBP siRNA. As shown in Fig. 6C, CBP siRNA significantly reduced both MKK3- and MKK6-induced CCN1 promoter activity. A non-silencing RNA did not affect CCN1 promoter activity under conditions identical to those used for CBP siRNA (data not shown). These data suggest that p38-mediated CBP activation is critical for CBP recruitment to the SRF-MRTF-A complex and subsequent CCN1 gene activation.

Regulation of the CCN1 Gene by Mechanical Overload in Vivo—Real-time PCR was carried out to determine changes in CCN1 gene expression in mechanically overloaded rat bladders as a result of partial urethral obstruction. As shown in Fig. 7A, CCN1 mRNA levels were significantly elevated (4–5-fold versus control) after 3 days and remained elevated (up to 8-fold versus control levels) after 7 days of obstruction. The CCN1 protein localized essentially within both the detrusor muscle and lamina propria layers of obstructed bladders, but the protein was undetected in sham controls (data not shown). To correlate the mechanisms of CCN1 gene regulation in vitro to in vivo conditions, we examined the tissue distribution of MRTF-A and its potential interaction with the CCN1 promoter in mechanically overloaded bladders. Western blot analysis of nuclear proteins showed that MRTF-A was robustly expressed in the detrusor muscle of obstructed/overloaded tissue and may represent posttranslationally modified forms of MRTF-A (40). Cross-sections of detrusor muscle from mechanically overloaded tissue showed an extensive nuclear staining for MRTF-A, which are largely overlaid with nuclear staining with 4,6-diamidino-2-phenylindole (Fig. 7C). A mixed nucleocytoplasmic immunostaining to MRTF-A was observed in the detrusor muscle from sham controls.

Nuclear chromatin from the detrusor muscle of obstructed and nonobstructed bladders was subjected to a ChIP assay using MRTF-A antibodies. As shown in Fig. 7D, the CCN1 promoter region containing the SRE site was highly enriched with MRTF-A, particularly in tissue homogenates from mechanically overloaded bladders versus sham controls. This suggests a potential role of MRTF-A in mechanical overload-induced CCN1 gene in vivo as well.

Mechanical Overload-induced Histone Acetylation at the SRE Site of the CCN1 Promoter in Vivo—The effect of mechanical overload on histone modifications was examined by Western blot analysis. As shown in Fig. 8A, obstruction-induced overload resulted in phosphorylation of H3 on serine 9 and its hyperacetylation on lysine 14. H4 was acetylated as well as a result of mechanical overload which corroborates the in vitro
data. ChIP assay was performed to determine the acetylation status of the H3 in the context of the CCN1 gene. As shown in Fig. 8B, ChIP signal was detected in the sequence surrounding the SRE site of the CCN1 gene and was stronger than that in the sham control. ChIP signals of IgG were less than 0.1% relative to the input signal (data not shown). Mechanical overload induced a 2-fold increase in CCN1 enrichment with acetylated H3 after 3 and 7 days post-obstruction, which is consistent with sustained up-regulation of the CCN1 gene in vivo. Clearly, epigenetic modifications play a critical role in the mechanical regulation of the CCN1 gene in vivo as well.

**DISCUSSION**

In this study, we have examined the molecular mechanisms whereby mechanical strain regulates the expression of the proangiogenic gene encoding CCN1 both in vitro and in vivo. Several novel findings were uncovered including (i) the requirement of actin-dependent signaling pathway of RhoA GTPase and p38 SAPK for SRF-mediated CCN1 promoter activation in mechanically challenged cells; (ii) recruitment of MRTF-A, a G-actin-binding SRF-co-activator, and CBP, a downstream target of p38 SAPK, to the CCN1 promoter; (iii) mechanical stimulus-dependent stimulation of chromatin acetylation of the CCN1 gene by CBP; and (iv) physical interaction among SRF, MRTF-A, and CBP within the context of the CCN1 promoter in response to mechanical stimulation. Because previous studies and data presented in Fig. 7 have shown that MRTF-A and CBP interact directly with SRF. We concluded that CBP acts as a nucleator that unfolds chromatin and increases SRF-MRTF-A transcriptional activity. Additionally, data shown in Fig. 8 demonstrate that SRF and MRTF-A were bound to the SRE-CArG box in nonstimulated cells without significantly increasing the promoter activity of the CCN1 gene, suggesting that formation of SRF-MRTF-A complexes within the context of the CCN1 gene is not sufficient for gene transcription activation. This is consistent with previous studies showing that in some cell types, expression of MRTF-A target genes remain silent despite the constitutive expression of MRTF-A in the nucleus (41). Clearly, additional signals and co-factors combine to enable or disable the transcriptional activity in SRF target gene promoters. In this context, our data showed that neither SRF nor MRTF-A has an intrinsic histone acetylase activity, but they may either directly or indirectly promote remodeling of chromatin through nucleosome reorganization, which then facilitates the recruitment of p300/CBP that covalently modify histones. MRTF-A interaction with a hydrophobic groove on the SRF DNA-binding domain has been shown to be facilitated by SRF-induced DNA bending, which argues in favor of SRF-MRTF-A-mediated localized nucleosomal remodeling (42). Similarly, Brg1, a major component of the SWI/SNF ATP-dependent chromatin remodeling complex, has been shown to interact directly with MRTF-A, forming a complex with weakly bound SRF at the promoter of muscle-specific genes (43). This leads to chromatin remodeling, which further enhances tight binding of SRF complexes and subsequent transcription activation. This partnership between SRF and other co-factors may vary within the context of each promoter and may not be interchangeable. However, the question of what determines the specificity of SRF co-factors recruitment remains outstanding.

MRTF-A-dependent regulation of the CCN1 gene by mechanical strain was evidenced by the inability of MRTF-A<sup>−/−</sup> SMCs to up-regulate the CCN1 gene in response to mechanical strain. MRTF-A-null cells exhibited dramatic morphological and cytoskeletal changes, suggesting that MRTF-A deficiency results in cytoskeletal protein defects. This is consistent with data from comparative genomic analyses that predict that SRF-
MRTF-A and CBP-dependent Regulation of CCN1 by Strain

FIGURE 6. Mechanical strain-induced p38 SAPK is required for activation and interaction of CBP with SRF-MRTF-A complexes and activation of the CCN1 promoter. A, HAT activity levels of protein complexes formed in response to cyclic strain. Cells were either treated with LMA (5 μM) or subjected to cyclic strain in the presence or absence of SB-203580 or Y27632. Protein complexes were immunoprecipitated using antibodies to FLAG, MRTF-A, SRF, or CBP, and HAT activity levels of the immunocomplexes were determined using a colorimetric assay. Data are means ± S.E. from a representative experiment performed in triplicate. *, p < 0.01 versus anti-FLAG control; **, p < 0.01 versus anti-MRTF-A (+ strain). B, interactions among MRTF-A, SRF, and CBP are dependent on Rho and p38 signaling pathways. Cells were transfected with FLAG-tagged MRTF-A and either left untreated or pretreated with the SB-203580 or Y27632 inhibitor prior to the application of cyclic strain. Nuclear proteins were immunoprecipitated (IP) with CBP antibodies. Immunodetection was performed with antibodies to FLAG, SRF, or CBP. WB, Western blot. C, SAK p38 signaling regulates CBP activation and recruitment to nuclear complexes. Cells were co-transfected with either wild-type or CArG box-mutated (mut-CArG) CCN1 promoter-reporter construct along with pCDNA, MKK3, MKK4, or MKK6 construct in the presence and absence of a CBP siRNA. The values represent the average of determinations of luciferase activity ± S.E. from three experiments. ***, p < 0.01 versus pCDNA; ***, p < 0.05 versus no siRNA.

responsive genes, potentially dependent on the MRTF-A pathway, include those of cytoskeletal proteins and their transcriptional regulators, e.g. SRF itself, SM22a, Jun B, α-actin, and vinculin (44). Interestingly, the sensitivity of the CCN1 promoter to cyclic strain in MRTF-A-null cells was, at least in part, restored by ectopic expression of MRTF-A, whereas MRTF-B and myocardin were ineffective. Additionally, a dominant negative form of either myocardin or MRTF-B was unable to significantly reduce strain-induced CCN1 gene expression (data not shown). Mouse MRTF-A and MRTF-B share >60% amino acid homology and exhibit several common structural domains especially in their basic and Q-rich domains (42). However, our data show that although the bulk of MRTF-A localized in the nucleus in mechanically stimulated cells, MRTF-B remained essentially cytoplasmic and/or in perinuclear localization. This is consistent with the finding by Kuwahara et al. (29), showing that even though nuclear localization of both MRTF-A and MRTF-B may be regulated through actin-dependent mechanisms, the nuclear accumulation of MRTF-B is much lower than that of MRTF-A. MRTF-B nuclear import and export appeared to be extremely slow and, perhaps, stimulus-dependent. This does not exclude the possibility that MRTF-B may have cytoplasmic functions and/or associated only with its capacity to heterodimerize with MRTF-A through its conserved leucine zipper domain. 3) The C terminus of myocardin contains a transcriptional activation domain that functions with heterologous promoters but shares only low level sequence identity with the transcriptional activation domains of MRTF-A and MRTF-B (20, 45). Additionally, myocardin stability and transactivity require posttranslational modifications (e.g. phosphorylation/dephosphorylation, interaction with heat shock proteins) that may or may not occur in mechanically stimulated cells (49). Finally, mice harboring loss-of-function mutations in myocardin, MRTF-A, and MRTF-B, respectively, display distinct phenotypes, including cell autonomous defects in vascular smooth muscle cell, and myoepithelial cell differentiation and function (24, 50, 51). Thus, myocardin and MRTFs have distinct target genes/functions, and their functional redundancy may depend on the context within each of their targeted gene promoters.

Meanwhile, our data have shown that CBP HAT activity is an early necessary event in mechanical strain-induced CCN1 gene expression. SRF-MRTF-A complexes that were not associated with CBP (i.e. they were immunoprecipitated from either control non-stretched or LMA-treated cells) contained significantly less HAT activity than complexes containing CBP. Func-
tionally, p300/CBP converts chromatin to an open state by catalyzing histone acetylation of multiple sites in the core histone tail (52). Acetylation creates a surface that facilitates protein-DNA recognition, which is consistent with the observation that many transcription factors have enhanced DNA-binding activity upon acetylation. However, CBP HAT activity was nearly abolished upon p38 SAPK inhibition. In addition, the data shown in Fig. 6 demonstrate that MKK3/6-dependent phosphorylation of p38 is an important signal for chromatin recruitment of CBP. Previous studies analyzing the composition of the transcriptome assembled on the chromatin of muscle genes in response to the activation of the p38 kinase have shown that the p38α/β isoforms promote the assembly of a transcription-competent transcriptosome by recruiting the chromatin-remodeling SWI/SNF complex, AKT1, and AKT2, which induce the association of MyoD with CBP and PCAF acetyltransferases via direct phosphorylation of CBP (53). Phosphorylation of CBP aids CBP-mediated transcriptional activation, although the critical phosphorylation sites on CBP remain to be identified.

Another interesting outcome of our study design is that the control mechanisms of the CCN1 gene in vitro are physiologically relevant in vivo as well. Using the rat model of bladder outlet obstruction, we found that obstruction-induced overdistension of the bladder was associated with nuclear localization of p38 SAPK and increased polymerized F-actin levels (15, 17). However, our observations are at variance with prior findings by Grote et al. (54) who showed that mechanical stretch-induced expression...
of the CCN1 gene is dependent on the inducible expression of Egr-1 after aortic banding. Differences between results from the Grote et al. (54) study and ours may be due to the experimental conditions used as well as cell type- and tissue-specific and/or species variations. However, the finding by these authors that expression of the Egr-1 and CCN1 genes is independent of novel protein synthesis (10, 55). Furthermore, the expression of the Egr-1 gene involves Erk1/2-dependent signaling pathways, which are not involved in mechanical strain-induced CCN1 gene expression in bladder SMCs (56). Thus, mechanical regulation mechanisms of the CCN1 gene may differ in SMCs from different tissue beds.

Our findings are highly significant in that they reinforce the notion that the adaptive responses (e.g. hypertrophy and angiogenesis) to mechanical overload share common regulatory factors. Indeed, transcription of immediate-early genes encoding angiogenic proteins, as well as multiple genes affecting muscle function, is regulated by SRF through a Rho GTPase-induced actin signaling pathway (2). The SRF protein is constitutively localized to the nucleus and bound to SRF sequences, and no direct modifications of the protein are required for its regulation (57). Therefore, the association of SRF with transcriptional co-activators and repressors provides an efficient transcriptional control mechanism of functionally unrelated genes, e.g. muscle- and angiogenesis-specific genes. The factors that govern additional co-factors recruitment (e.g. CBP) and how cells integrate SRF-co-factor recruitment into a specific transcriptional gene activation in response to mechanical stimulation are not well understood. Our studies have shown that such specificity may be dictated, at least in part, by the plasticity and reversibility of epigenetic changes in DNA and chromatin. As shown in Fig. 8, mechanical overload was associated with a rapid and sustained acetylation of H3 and H4. In the heart, overexpression of CBP/p300 was sufficient to induce hypertrophy and left ventricular remodeling in transgenic mice, mimicking mechanical overload-induced cardiac remodeling (58). Proteins with HAT activity such as p300 and CBP tend to be large and to act as scaffolding that can bridge between basal transcription factors and various transactivators. Thus, CBP/p300 is an essential co-factor for a distinct subset of genes that initiate the adaptive/compensatory responses to mechanical overload. The reversibility of epigenetic alterations as a limiting step in the control of gene expression makes chromatin changes attractive therapeutic targets in controlling the adaptive and plasticity responses to mechanical overload.

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MRTF-A- and CBP-dependent Regulation of CCN1 by Strain

Mechanical Regulation of the Proangiogenic Factor CCN1/CYR61 Gene Requires the Combined Activities of MRTF-A and CREB-binding Protein Histone Acetyltransferase
Mary Hanna, Haibo Liu, Jawaria Amir, Yi Sun, Stephan W. Morris, M. A. Q. Siddiqui, Lester F. Lau and Brahim Chaqour

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