Mechanical Stretch Modulates the Promoter Activity of the Profibrotic Factor CCN2 through Increased Actin Polymerization and NF-κB Activation*

Received for publication, January 9, 2006, and in revised form, May 15, 2006. Published, JBC Papers in Press, May 16, 2006, DOI 10.1074/jbc.M600214200

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The connective tissue growth factor known as CCN2 is an inducible, profibrotic molecule that becomes aberrantly expressed in mechanical overload-bearing tissues. In this study, we found that CCN2 gene expression is rapidly induced in cyclically stretched bladder smooth muscle cells (SMCs) in vitro and in the detrusor muscle of a mechanically overloaded bladder in a rat model of experimental urethral obstruction. The activity of CCN2 promoter constructs, transiently transfected into cultured SMCs, was increased (up to 6-fold) by continuous cyclic stretching. Molecular analyses of the CCN2 promoter by serial construct deletions, cis-element mutagenesis, and electrophoretic mobility shift assays revealed that a highly conserved NF-κB binding site located within the CCN2 proximal promoter region is responsible for the activation of the promoter by stretch. Chromatin immunoprecipitation assays showed that NF-κB binds to the endogenous CCN2 promoter in both stretched cells and mechanically overloaded bladder tissues. Furthermore, stretch-dependent CCN2 promoter activity was significantly reduced upon inhibition of either phosphatidylinositol 3-kinase, p38 stress-activated kinase, or RhoA GTPase and was completely abolished upon inhibition of actin polymerization. Concordantly, actin polymerization was increased in either mechanically stretched cells or overloaded bladder tissues. Incubation of cultured SMCs with a cell-penetrating peptide containing the N-terminal sequence, Ac-EEED, of smooth muscle α-actin, altered both actin cytoskeleton organization and stretch-mediated nuclear relocation of NF-κB, and subsequently, it reduced CCN2 promoter activity. Thus, mechanical stretch-induced changes in actin dynamics mediate NF-κB activation and induce CCN2 gene expression, which probably initiates the fibrotic reactions observed in mechanical overload-associated pathologies.

The connective tissue growth factor now known as CCN2 is a signaling molecule that belongs to a family of extracellular matrix (ECM)2-associated, cysteine-rich and heparin- and integrin-binding proteins (1, 2). CCN2 functions as a modulator of complex cellular processes, such as cell growth, differentiation, adhesion, and migration (3–5). Studies of diseased tissues from human clinical specimens and animal models established a direct correlation between high levels of the CCN2 protein and excessive accumulation and deposition of ECM proteins in fibrotic tissues, suggesting a pathogenic role for CCN2 in fibroproliferative disorders (6–8). Transforming growth factor-β (TGF-β), a potent fibrogenic molecule, has been shown to be one of the strongest inducers of CCN2, which, in turn, mediates the expression of extracellular matrix proteins, such as fibronectin, type I collagen, and α5-integrin (9, 10). TGF-β-induced CCN2 expression is mediated via a unique TGF-β response element contained in the CCN2 promoter sequence, which, when mutated, abolishes both CCN2 gene induction and the fibrogenic effects of TGF-β (11).

Interestingly, while being minimally expressed in normally functioning quiescent adult tissues, the CCN2 gene is strongly up-regulated in mechanically challenged organ systems resulting from various etiologies (e.g. hypertension, hemodynamics, metabolic injury, and obstruction) (12, 13). In particular, the levels of CCN2 are increased severalfold in atherosclerotic vessels experiencing altered hemodynamic forces compared with normal arteries (14). CCN2 levels are also remarkably elevated in smooth muscle-rich hollow organs, including lung and vasculature in various experimental models of hypertension and in experimental and human glomerulosclerosis (15, 16). In addition, the specialized cases of abnormal scarring (e.g. keloids), which apparently develop in regions of the body that are subjected to relatively higher mechanical strain than others, are lesions highly enriched in CCN2 (17). The scar that persists is itself a tissue under increased mechanical strain and contains abnormally high levels of CCN2. These observations led to our hypothesis that mechanical factors typified by tension, stretch, and hydrostatic pressure might be the primary inducers of the CCN2 gene in these pathological conditions.

This work was supported by NIDDK, National Institutes of Health, Grants R01-DK060572 and R21-DK068483 (to B. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAPK, stress-activated protein kinase; PI, phosphatidylinositol; SMC, smooth muscle cell; NF-κB, nuclear factor-κB; NTSKA, N-terminal skeletal α-actin; NTSMG, N-terminal smooth muscle γ-actin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; ChIP, chromatin immunoprecipitation; DAPI, 4′,6-diamidino-2-phenylindole; JNK, c-Jun N-terminal protein kinase; TGF, transforming growth factor; PIPES, 1,4-piperazinediethanesulfonic acid; SRF, serum response factor.

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Mechanical overload elicits a series of structural and functional changes that culminate in hypertrophy, hyperplasia, and various degrees of fibrosis, particularly in smooth muscle-rich tissues of hollow organs (18, 19). These changes are viewed as adaptational and compensatory responses over the short term but generally lead progressively to altered muscle contractility, physiologic insufficiency, and organ failure, especially when abnormal mechanical stimuli persist. Mechanistically, the transfer of excessive strain results in the activation of multiple signaling cascades culminating into reprogramming of gene expression and the production of various prohypertrophic and fibrotic growth factors, such as CCN2. The CCN2 is likely to induce ECM protein accumulation, which perturbs the structural relationship between and among smooth muscle fibers and alters their contractility (20). However, the molecular mechanisms whereby mechanical forces regulate the expression of this profibrotic factor are, as yet, unresolved.

Many lines of evidence suggest that mechanotransduction pathways, which define the translation of mechanical stimuli into an intracellular biochemical process, involve the actin cytoskeleton (21). The actin cytoskeleton provides, indeed, a continuous and dynamic link between virtually all cellular structures and thus enables internal structures, such as chromatins, to respond directly and immediately to externally applied forces. Actin, which is the major component of the cytoskeleton, is encoded by a gene family of six different isoforms expressed in a tissue-specific fashion: α-smooth muscle, γ-smooth muscle, α-skeletal, α-cardiac, β-cytosplasmic, and γ-cytosplasmic actin. These isoforms share amino acid sequence similarities but have unique N termini that probably determine functional specialization (22). The expression of the α-actin isoform, which is normally restricted to smooth muscle and myofibroblast cytoskeleton, is associated with stress fiber formation and generation of increased contractile force. Thus, it is conceivable that inhibition of α-actin expression and/or actin fiber assembly may interfere with the transmission of tensile forces involved in the induction of force-regulated genes. In agreement with this, inhibition of RhoA GTPase, which promotes actin polymerization, was shown to regulate the promotor activity of several smooth muscle differentiation marker genes, including SM22a and α-actin, and non-smooth muscle specific genes, such as CCN1 (23, 24). Interestingly, we and others have previously shown that increased cytoskeletal actin polymerization suffices to up-regulate the expression of the CCN2 gene and that the physiological state of actin is a critical determinant of CCN2 gene expression (12, 25). This is an important observation, because it suggests that cytoskeletal actin may provide a selective physical link by which mechanical forces may induce a specific pattern of CCN2 gene expression. However, the involvement of dynamic changes between monomer globular actin (G-actin) and polymerized filamentous actin (F-actin) in the mechanical regulation of the CCN2 gene and the underlying molecular mechanisms are unknown and will be investigated in this study.

In addition, whereas extensive evidence suggests that factors influencing cytoskeletal actin polymerization regulate specific gene expression, the biggest challenge is to determine whether such mechanisms operate in native cells and in the whole tissue in response to an altered pattern of mechanical signals. One model that has been studied with respect to the biological effects of mechanical forces in vivo is the mechanically overloaded bladder as a result of experimental urethral obstruction (26). Within the bladder wall, individual cellular elements exist in a complex, mechanically active environment in which smooth muscle cells (SMCs), in particular, are continuously generating and responding to mechanical forces during the filling and emptying processes. Experimentally created urethral resistance changes the normal range of pressure within the system, thus disrupting the normal pattern of mechanical stretch within the bladder wall. The subsequent increase in intravesical pressure leads to an excessive bladder wall distension, which translates into hypertrophic and fibrotic responses (e.g., increased contractile protein gene expression and type I collagen) commonly seen in in vivo pathological conditions (i.e., spina bifida, benign prostatic hyperplasia, etc.) (20). Therefore, this system appears well suited to examine the mechanical regulation of the CCN2 gene in vivo. Accordingly, we designed the present study to determine how mechanical strain regulates the CCN2 gene expression in primary cultures of bladder SMCs and to correlate these mechanisms with those involved in the CCN2 gene activation in a mechanically overloaded bladder tissue. The results indicate a preponderant role for an NF-κB element within the CCN2 promoter and a significant effect of actin dynamics on NF-κB-dependent signal transduction pathways both in vitro and in vivo. Our data support the notion that the actin cytoskeleton integrates mechanical signals to induce transactivation of the profibrotic factor CCN2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stretching**—Primary cultures of SMCs were prepared from the bladder of fetal calves from mid to late gestational stages as previously described (23). Freshly isolated cells were phenotypically characterized using a rabbit polyclonal antibody against smooth muscle α-actin (Abcam, Cambridge, MA) and a mouse antibody against the myosin heavy chain isoforms, SM-1 and SM-2 (Alexis Corp., San Diego, CA). 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. Cells between their third and eighth population doubling level were used for stretch experiments. In a typical experiment, cells were plated on 6-well silicone elastomer plates coated with type I collagen (Biolflex; Flexcell, Hillsborough, NC). Application of cyclic stretch to the cells was performed with the FX-4000™ Flexcell® Tension Plus™ System (Bioflex; Flexcell, Hillsborough, NC). In all experiments, cells were subjected to a maximum of 10–12% strain magnitude at a frequency of 0.3 Hz for selected time periods. This stretch regimen produced optimal conditions for maximum induction of CCN2 in SMCs. Control cells were cultured under static conditions. After completion of the stretch regimen, cells were harvested and processed for both total RNA and protein extraction or other analyses.

**Surgical Induction of Partial Urethral Obstruction**—Partial urethral obstruction was created in Sprague-Dawley female rats (Charles River Laboratories, Wilmington, MA) weighing...
The N-terminal sequence of either smooth muscle CCN2 gene and predeveloped 18 S rRNA (VICTM-dye labeled ITC)-GRQLRIAGRRLRGRSR, Ac-EEETTALVK(AH with fluorescein isothiocyanate (FITC) via an AH molecule into the cells (28). Both nanopeptides were tagged protein were synthesized to a purity of 98% (Pepscan Systems, The Netherlands). The XMTM molecule was used as a delivery agent of the XMTM delivery peptide from the Erns viral surface of the CCN2 Gene Regulation by Mechanical Stretch.

**Cell-penetrating Peptide Synthesis**—Actin-based cell penetrating peptides were constructed based on a model previously reported by Hinz et al. (27). The nanopeptides containing the N-terminal sequence of either smooth muscle α-actin (NTSMA), Ac-EEED, smooth muscle γ-actin (NTSMG), Ac-EEET, or skeletal α-actin (NTSKA), Ac-DEDE, at the N terminus of the XMTM delivery peptide from the E7TS3 viral surface protein were synthesized to a purity of >98% (Pepsan Systems, The Netherlands). The XMTM molecule was used as a delivery vector for its ability to efficiently translocate any attached cargo molecule into the cells (28). Both nanopeptides were tagged with fluorescein isothiocyanate (FITC) via an AHX arm for visualization purposes. The final structure of the NTSMA, NTSMG, and NTSKA chimeras is Ac-EEEDSTALVK(AH-FITC)-GRQLRIAARRLRGRSR, Ac-EEETTALVK(AHX-FITC)-GRQLRIARRLRGRSR, and Ac-DEDETTALVK(AHX-FITC)-GRQLRIAARRLRGRSR respectively. All peptides were used at a concentration of 10 μg/ml.

**RNA Isolation and Quantitative Analysis of mRNA**—Total RNA was extracted from either cells or tissue using TRIzol reagent (Invitrogen). A sample containing 12 μg of total RNA was fractionated by electrophoresis in 1% agarose/formaldehyde gel, transferred to Zeta-Probe nylon filters (Bio-Rad), and hybridized to either a CCN2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) radiolabeled cDNA probe as previously described (25). The filters were analyzed by densitometric scanning, and hybridization signals were quantified to determine the relative amounts of mRNA. The CCN2 mRNA levels were analyzed in duplicate samples and normalized to equivalent values for GAPDH to compensate for variations in loading and transfer.

Quantitative real time reverse transcription-PCR assay was performed using TaqMan technology on an Applied Biosystems 7000 sequence detection system. The Applied Biosystems Assays-on-Demand 20 assay mix of primers and TaqMan™ minor groove binder probes (FAM™ dye-labeled) for rat CCN2 gene and predeveloped 18 S rRNA (VIC™ dye labeled probe) for internal control were used for real time PCR measurements. These assays were designed to span exon-exon junctions so that genomic DNA will not be detected. The same tube for two-step reverse transcription-PCR was used with 50 ng of RNA for both the CCN2 gene and endogenous control using the Reverse Transcript Kit (Invitrogen). The cycling parameters for PCR amplification were as follows: AmpliTaq activation 95 °C for 10 min, denaturation 95 °C for 15 s, and annealing/extension 60 °C for 1 min (40 cycles) on ABI7000. Triplicate CT values were analyzed with Microsoft Excel using the comparative CT (ΔCT) method as described by the manufacturer (Applied Biosystems). The amount of CCN2 transcripts (2-ΔCT) was obtained by normalizing to an endogenous reference (18 S rRNA) relative to a calibrator (one experimental sample). All primers and probe sequences were obtained commercially (Applied Biosystems).

**Western Immunoblot and Immunohistochemical Analyses**—For Western blot analysis, Protein samples (24 μg) were separated by 10% SDS-polyacrylamide gel and transferred to nitrocellulose membrane, and Western blot analysis was performed using anti-human CCN2 antibody. The CCN2 antibody was raised against a 28-synthetic amino acid peptide corresponding to human CCN2 positions 232–259 (29), a gift from Dr. B. Perbal (University of Paris V, France). This peptide shares 100% sequence homology with the deduced amino acid sequence of rat CCN2 (GenBank™ accession number NM022266). Immunodetection was performed using ECL (Amersham Biosciences) according to the manufacturer’s recommendations. For histochemical analyses, the bladder was dissected and immediately fixed in 0.4% formaldehyde, PBS for 2 h followed by an overnight incubation in sucrose solution and frozen in Tissue-Tek Optimal Cutting temperature compound. Ten-micrometer-thick cryostat sections were prepared and stained with hematoxylin-eosin for qualitative assessment. Other sections not necessarily adjacent to the previous ones were incubated for 24 h with either the anti-CCN2 antibody or an anti-NF-κB antibody (Abcam, Cambridge, MA). Immunodetection was performed with either rhodamine- or fluorescein-conjugated anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Sections were washed several times in PBS between incubations. For visualization of actin stress fibers, tissue cross-sections or cells plated on glass coverslips were fixed in 0.4% formaldehyde, PBS for 30 min, permeabilized in 0.1% Triton X-100 at room temperature for 5 min, and stained with rhodamine-phalloidin (Cytoskeleton Inc.).

**G-actin/F-actin in Vitro Assay**—Determination of the amount of filamentous actin (F-actin) content versus free globular actin (G-actin) content was performed using the F-actin/ G-actin in vivo assay kit according to the manufacturer’s instructions (Cytoskeleton, Inc.). Briefly, cell or tissue samples were homogenized in cell lysis and F-actin stabilization buffer (50 mM PIPES, 50 mM NaCl, 5 mM MgCl₂, 5 mM EGTA, 5% glycerol, 0.1% Nonidet P-40, 0.1% Triton X-100, 0.1% Tween 20, 0.1% β-mercaptoethanol, and 0.001% antifoam) and a protease inhibitor mixture, followed by ultracentrifugation for 1 h at 100,000 × g to separate F-actin from G-actin pool. The pellet was resuspended in ice-cold water and incubated in the presence of cytochalasin-D to dissociate F-actin. Aliquots from both supernatant and pellet fractions were separated by Western blot, and actin was quantitated after immunodetection analysis using a rabbit polyclonal anti-actin antibody (Cytoskeleton Inc., Denver, CO) and densitometric scanning. All steps were performed at 4 °C.

**Transient Transfection, Mutagenesis, and Reporter Assay**—For transfection, cultured cells were plated at a density of 1 × 10⁵/cm² in 6-well Bioflex plates and maintained in medium containing 10% serum for 18 h. Transfection was then per-
formed using Fugene 6 transfection reagent in serum-free medium according to the manufacturer’s specifications (Roche Applied Science). The CCN2 promoter-reporter plasmid constructs used in transfection experiments include a 1999-bp fragment of the human CCN2 promoter (from −1999 to +36, GenBank™ accession numbers AL354866 and AF316368) as well as serial deletion of the promoter at positions −736, −634, and −72. These DNA fragments were amplified by PCR from human genomic DNA and cloned into the luciferase reporter vector pGL3-basic. HindIII and Nhel restriction enzyme sites were engineered into 3’ and 5’ primers, respectively, to facilitate directional cloning into the HindIII and Nhel site of the pGL3-basic vector (Promega Corp., Madison, WI). These constructs were a generous gift from Dr. G. Yang (Stanford University, Stanford, CA). The p(−122/+36)-luc and p(−164/+36)-luc construct were prepared by PCR using the p(−1999/+36)-luc as a template. Additional CCN2 promoter/reporter constructs containing mutations to putative cis-acting elements were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The distal AP-1 site was changed from -GATGAGGCAGG- to -AACTAGGCA-, and the core binding site NF-κB was changed from -GGAATGTCCC- to -ATCTGGTTCAT-. These nucleotide mutations abolished AP-1 and NF-κB binding, respectively. Constructs were fully sequenced in both directions to confirm successful mutagenesis before use. Cells were co-transfected with pRL-SV40 vector containing the Renilla luciferase gene to adjust for transfection efficiency. Other co-expression vectors used include those overexpressing dominant negative inhibitors of Fos (A-Fos), ATF-2 (A-ATF) provided by Dr. C. Vinson (NCI, National Institutes of Health, Washington, D. C.), and Egr-1 (CMV-WT1-EGR-1) provided by Dr. F. Rauscher (Wistar Institute, Philadelphia, PA). 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 stretch as described above. Cell lysates were assayed for luciferase activity levels, and firefly luciferase activity was normalized to that of 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.

**Electrophoretic Mobility Shift Assay**—For the study of DNA binding proteins, nuclear protein were extracted as described by Dignam et al. (30). A 102-bp DNA fragment comprising AP-1 and NF-κB binding sites was synthesized by PCR and used in a DNA binding assay. Nuclear protein extracts from control and stretched cells were incubated with 20,000 cpm of the DNA fragment previously labeled with [α-32P]dCTP and incubated in binding buffer containing 10 mM Tris, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol, 1 μg/μl poly(dl-dc) in a final volume of 15 μl. To control for the specificity and nature of the interactions, unlabeled DNA fragments, oligonucleotide with NF-κB consensus sequences (Active Motif Inc., Toronto, Canada), or specific antibodies to the p65 subunit of NF-κB (Upstate Biotechnology, Inc., Lake Placid, NY) were added 10 min before the addition of the labeled DNA fragments. The protein-DNA complexes were electrophoresed through 5% polyacrylamide/Tris borate EDTA and run at 200 V for 2 h. The gel was then dried and autoradiographed.

**Chromatin Immunoprecipitation (ChIP) Assay**—For tissue cultures, cells were either cultured under static conditions or subjected to mechanical stretch for 1 h. Formaldehyde (Fisher) was added at a final concentration of 1% directly to the cell culture medium. 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 collected by centrifugation and rinsed in cold phosphate-buffered saline. Cell pellets were resuspended in swelling buffer (10 mM potassium acetate, 15 mM magnesium acetate, 0.1 M Tris (pH 7.6), 0.5 mM phenylmethylsulfonyl fluoride, and 100 ng of leupeptin and aprotinin/ml), incubated on ice for 20 min, and then Dounce-homogenized. 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 ng 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 samples were sonicated on ice with an Ultrasonics sonicator at setting 10 for three 20-s pulses to reduce chromatin to an average length of ~1,000-bp fragments. The chromatin solution was precleared by centrifugation and 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 NF-κB (Abcam) and those against AP-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoprecipitation, washing, and elution of immune complexes were 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 4–5 h. The samples were then precipitated at −20 °C overnight by the addition of 2.5 volumes of ethanol and then pelleted by microcentrifugation. The samples were resuspended in 100 μl of Tris-EDTA (pH 7.5), 25 μl of 5× proteinase K buffer (1.25% SDS, 50 mM Tris (pH 7.5), and 25 mM EDTA), and 1.5 μl of proteinase K and incubated at 45 °C for 2 h. The pellets were collected by microcentrifugation, resuspended in 30 μl of H2O, and analyzed by PCR. PCR products were fractionated by electrophoresis in agarose gel and stained with ethidium bromide. Primers used in the final PCR amplification of the purified bovine CCN2 DNA were 5′-TTTT-GGTAGCGTGAGGTGTG-3′ and 5′-ACTGGCTGCTCTCC-TCA-3′ (GenBank™ accession number AF309555). For bladder tissue, biopsies were fixed in formaldehyde for 2 h. Tissue was then briefly homogenized (10 s) using an ultraTurax homogenizer in Tris buffer containing a mixture of inhibitors. All of the following steps were identical to those described above for cell cultures. Primers used in the final PCR amplification of the immunoprecipitated DNA were 5′-CTTCCCTGGCCAGCTAAAGTG-3′ and 5′-GTGGGTCTGGCTGCCTTCAG-3′ (GenBank™ accession number: AC127189).

**Statistical Analysis**—Data were expressed as mean ± S.E. A paired Student’s t test was used to analyze differences between
two groups, and \( p \) values of <0.05 or <0.01 were considered significant.

**RESULTS**

Mechanical Stretch Induces CCN2 Gene Expression in Cultured SMCs—As a basis for defining the mechanisms involved in the mechanical regulation of the CCN2 gene, we first determined the effects of mechanical strain on CCN2 gene expression in primary cultures of SMCs. When cells were subjected to 10% of cyclic stretch at 0.3 Hz in multiple experiments, the mRNA levels of CCN2, normalized to those of GAPDH, rapidly increased (3–4-fold) with a peak after 1 h and progressively declined thereafter (Fig. 1, A and B). The minimal strain required to trigger this response was 10%, since no significant and reproducible changes of CCN2 mRNA levels were detected when the strain magnitude was set below 10%, in agreement with previously published data (31). Additionally, CCN2 mRNA levels increased to nearly the same extent when the strain magnitude was varied between 10 and 15% (data not shown), suggesting that beyond 10% maximal elongation, the cells most likely respond to changes in force equilibrium rather than to the magnitude of an externally applied strain. Western blot analysis of cell lysates using a specific CCN2 antibody showed that the CCN2 protein (~37 kDa) expression rapidly increased following the application of stretch (Fig. 1C). The upper protein band (~80 kDa), the levels of which did not change after stretch. Data are mean ± S.E. of three experiments. *, \( p < 0.05 \) versus control nonstretched cells.

![FIGURE 1. Induction of CCN2 gene expression by mechanical stretch in vitro.](image)

**FIGURE 1.** Induction of CCN2 gene expression by mechanical stretch in vitro. A, cultured bladder smooth muscle cells were subjected to mechanical stretch (10% strain, 0.3 Hz) for the indicated time periods. Total RNA was prepared and subjected to Northern blot/hybridization analysis using a specific CCN2 DNA probe. To control for RNA loading, the blot was hybridized with a GAPDH DNA probe. B, graphical representation of CCN2 mRNA levels after densitometric analysis of the hybridization signals. The values are normalized to those of the GAPDH signals and represent mean ± S.E. (\( n = 4 \)). To compare data from different experiments, expression after 1 h of mechanical stimulation was set to 100%. C, CCN2 protein in cyclically stretched cells was detected in cellular lysates by Western blot with an anti-CCN2 antibody. Immunodetection was performed by enhanced chemiluminescence. D, densitometric values of the CCN2 protein band (~37 kDa) were normalized to those of the control upper band (~80 kDa), the levels of which did not change after stretch. Data are mean ± S.E. of three experiments. *, \( p < 0.05 \) versus control nonstretched cells.

Enhanced expression of the CCN2 gene by mechanical overload in vivo.—Mechanical overload-induced tissue distension was imparted to the bladder upon partial obstruction of the urethra in Sprague-Dawley rats. A, the CCN2 mRNA levels were determined by real time PCR after 3 and 7 days postobstruction and normalized to the 18S rRNA levels. Data are given as means ± S.E., \( n = 4 \). **, \( p < 0.01 \) obstructed versus nonobstructed. B, transverse sections from 7-day-obstructed and control nonobstructed bladders were stained with hematoxylin and eosin for qualitative cross-section assessment. Note the increase of the detrusor muscle layer thickness of the obstructed bladder, which is indicative of an adaptational response to mechanical overload. C, immunostaining of cryosections from a control nonobstructed and an obstructed bladder with an anti-CCN2 antibody. The immunostaining was detected with an FITC-conjugated anti-rabbit IgG and visualized by fluorescence microscopy. Magnification: ×40; L, lumen; DM, detrusor muscle.

![FIGURE 2. Enhanced expression of the CCN2 gene by mechanical overload in vivo.](image)
Urethral Resistance-induced Mechanical Overload Enhances CCN2 Expression in Vivo—Real time PCR was carried out to quantify CCN2 transcripts in bladders overloaded mechanically as a result of partial urethral obstruction. As shown in Fig. 2A, CCN2 mRNA levels were significantly elevated (4–5-fold) after 3 days of obstruction and increased to an even greater extent (up to 8-fold) after 7 days of obstruction. Typical histological cross-sections of the bladder wall, representatives of which are shown in Fig. 2B, revealed a remarkable thickening of the bladder wall as a result of hypertrophy/hyperplasia of the detrusor muscle layer. Localization of CCN2 in randomly selected transverse sections from obstructed and nonobstructed bladders was assessed using specific CCN2 antibodies (Fig. 2C). There was a relatively weak immunostaining for CCN2 protein in nonobstructed control. The detrusor smooth muscle layer from obstructed tissues showed relatively strong immunoreactivity to the CCN2 protein, which appeared to localize around cross-sectioned smooth muscle fascicles and along smooth muscle fibers sectioned longitudinally. This immunostaining pattern was not seen when cross-sections had been incubated with a nonspecific IgG antibody (data not shown).

Stretch Activates the CCN2 Promoter through an NF-κB-binding Element—To assess whether mechanical stretch-induced CCN2 gene expression was mediated by cis-elements in the CCN2 gene promoter, we transfected cultured cells with a 1999-bp DNA fragment of the wild type CCN2 promoter and a series of deletion constructs, each ligated to firefly luciferase reporter plasmid (Fig. 3). Twenty-four hours after transfection, cells were either exposed to mechanical stretch for 1 hour or incubated under static conditions. The reporter gene activity was then determined in the cell lysates. All CCN2 promoter-reporter constructs exhibited basal activity levels in cells cultured under static conditions. Application of mechanical stretch resulted in a strong increase of luciferase activity (up to 6-fold) in all promoter/reporter constructs, retaining at least 164 bp upstream of the initiation start site of the CCN2 gene (Fig. 3). The p(-164/+36)-luc construct exhibited the maximum promoter activation by stretch. When the segment length was reduced to 122 bp, which still contains a TATA-like box, the transfected promoter reporter construct lost its stretch inducibility. Its basal expression, however, was not affected (data not shown). Thus, the region between -164

**FIGURE 3.** Cyclic mechanical stretch activates the CCN2 promoter. Cells were transiently transfected with seven CCN2 promoter deletion luciferase (luc) reporter constructs shown schematically on the left. Potential binding sites for transcription factors as revealed by TRANSFAC analysis are marked with black boxes. Twenty-four hours after transfection, cells were incubated in serum-free medium and either subjected to cyclic stretch or kept under static conditions. After completion of the stretch regimen (10% strain, 0.3 Hz, 1 h), the firefly luciferase activity of the reporter gene was measured in the cell lysates and normalized to that of renilla luciferase activity from a co-transfected plasmid as described under “Experimental Procedures.” The graph on the right shows the percentage increase in luciferase activity in response to stretch (black bars) over that of static control cells (white bars) for each construct. Each CCN2 promoter reporter construct was assayed in triplicate transfections in at least two independent experiments. The results are expressed as the mean ± S.E. **, denotes statistical significance at p < 0.01 when compared with control nonstretched.
FIGURE 4. Stretch-induced CCN2 promoter activity is NF-κB-dependent. A, the sequences of the CCN2 promoter that confer stretch responsiveness are compared from several vertebrate animals. Note that the AP-1 and NF-κB sites are remarkably conserved across several mammalian species, including cow, rat, and mouse. B, cells were transfected with either wild type p(-164/+36)-luc construct or the same construct mutated in NF-κB (mut/NF-κB), AP-1 (mut/AP-1), or both NF-κB and AP-1 (mut/AP-1 and NF-κB) binding sites as described under “Experimental Procedures.” Cells were assayed for luciferase activity following the application of cyclic stretch for 1 h. To compare data, the luciferase activity obtained with the native wild type construct in nonstretched cells was set to 100%. Values shown are a representative experiment performed in triplicate. **, p < 0.01. C, cells were transfected with the promoter reporter construct p(-164/+36)-luc along with either an empty vector (pCDNA1), A-Fos, A-ATF-2, or CMV-WT1-EGR-1. Twenty-four hours later, cells were assayed for luciferase activity following the application of cyclic stretch for 1 h. The relative luciferase activity (mean ± S.E.) from a representative transfection experiment performed in triplicate is shown. These experiments were repeated twice using different cell preparations with similar results.
and −122 is required for stretch responsiveness of the CCN2 promoter.

Upon examining the 42-bp segment between −164 and −122 using TRANSFAC cis-element prediction software (33), we found adjacent cis-elements for the transcription factors, AP-1 and NF-κB. A comparison of promoter sequences for CCN2 showed that these elements were remarkably conserved in several vertebrate animals, including mouse, rat, and cow, suggesting that they may have an important regulatory function (Fig. 4A). To determine the individual contribution of these cis-elements to stretch-induced reporter activity, chimeric constructs with site-directed mutagenesis at these putative elements were made and tested in transfection experiments. As shown in Fig. 4B, the construct containing a double mutation at the AP-1 and NF-κB sites (Mut/AP-1 & NF-κB) reduced the reporter activity by greater than 95%. However, the construct bearing only a mutated AP-1 sequence (mut/AP-1) retained a significant stretch inducibility compared with the construct with the double mutation, whereas the construct bearing a mutation at the NF-κB site (mut/NF-κB) was not responsive to stretch. This suggests that this particular NF-κB sequence is involved in stretch responsiveness of the CCN2 promoter. To ascertain the role of potential transcription factors and their variants in the mechanical regulation of the CCN2 promoter, we used vectors expressing the dominant-negative mutant proteins A-Fos, A-ATF-2, and CMV-WT1-EGR-1, which inhibit DNA binding of their corresponding wild-type B-Zip proteins (34, 35). As shown in Fig. 4C, co-transfection of the cells with either A-Fos, A-ATF-2, or CMV-WT1-EGR-1 did not significantly reduce the p(−164/+36)-luc reporter activity in response to cyclic stretch. This rules out the involvement of either AP-1 or Egr-1 in stretch responsiveness.

Interactions between NF-κB and the CCN2 promoter region bearing the NF-κB regulatory sequence were further studied using the electrophoretic mobility shift assay. The molecular probe was derived from the CCN2 promoter, harboring both the NF-κB and AP-1 binding motifs. As shown in Fig. 5, two relatively intense nucleoprotein complexes (I and II) were formed between the CCN2 promoter DNA probe and nuclear proteins from stretched cells as compared with those from non-stretched cells. The formation of these complexes was suppressed when the nucleoproteins were preincubated with a 50-fold molar excess of the cold CCN2 promoter probe. An anti-NF-κB subunit (p65) antibody used at a concentration of either 1 or 2 μg, totally supershifted the nucleoprotein complex I, indicating that the promoter region between −164 and −122 is the site of specific interactions with the NF-κB transcription factor. The nucleoprotein complex II was partially reduced in the presence of the anti-p65 antibodies and may involve other proteins of the NF-κB family, such as Rel, present in the nuclear extract. In contrast, preincubation of the nuclear extract with antibodies to AP-1 altered the migration of neither complex I nor II.

**Mechanical Strain Induces Nuclear Translocation of NF-κB and Its Binding to the CCN2 Promoter within Intact Chromatin**—In control unstretched cells, NF-κB, which is a constitutively expressed protein, was mostly localized within the cytoplasm in cultured SMCs as determined by histochemical analysis (Fig. 6A). Application of mechanical stretch to the cells induced a rapid translocation of NF-κB to the nucleus, which is identified by 4',6-diamidino-2-phenylindole (DAPI) counterstaining. To address directly whether NF-κB is active in the context of chromatin, we performed a ChIP assay in which DNA-binding proteins were covalently linked to genomic DNA by treatment of the cells with formaldehyde. Cross-linked chromatin was fragmented and immunoprecipitated with either specific or irrelevant antibodies (e.g. anti-NF-κB, anti-AP-1, and a nonspecific mouse IgG). The precipitated chromatin DNA was then purified and amplified by PCR with specific primers for the CCN2 promoter region harboring the putative stretch-responsive element. As shown in Fig. 6B, a PCR product was successfully obtained when the DNA/protein adducts were immunoprecipitated with an anti-NF-κB antibody from mechanically stretched cells. Conversely, immunoprecipitation of chromatin with AP-1 antibody did not yield PCR products after amplification, suggesting that interactions between AP-1 and the CCN2 promoter did not occur in stretched cells. Similarly, PCR products were obtained neither from control unstretched cells nor when an irrelevant mouse IgG antibody was used for chromatin immunoprecipitation. Thus, NF-κB interacts with the CCN2 promoter within the context of chromatin in mechanically stretched cells.

Next, we sought to extrapolate these cell culture data to in vivo conditions by examining the tissue localization of NF-κB and its interaction with the CCN2 promoter in mechanically overloaded tissue. As shown in Fig. 6C, cross-sections from partially obstructed bladders showed intense nuclear staining for NF-κB. 

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**FIGURE 5. Cyclic stretch increases the binding of NF-κB to the proximal region of the CCN2 promoter.** Nuclear extracts were isolated from control and stretched cells, and an electrophoretic mobility shift assay was performed using a radioactively labeled DNA fragment (102 bp) bearing the stretch-responsive region of the CCN2 promoter. Competition experiments were carried out with 50-fold molar excess of cold probe. The nucleoprotein complexes (I and II) formed between the DNA probe and the nuclear extracts from stretched cells were analyzed by supershift analysis using anti-NF-κB (1 and 2 μg) and anti-AP-1 (2 μg) antibodies (Ab). I and II, nucleoprotein complexes I and II; SS, supershifted band. Free probe is indicated.
within detrusor smooth muscle fascicles. Immunostaining for NF-κB overlaid most cells with nuclear staining with DAPI. Formaldehyde cross-linking was also performed on whole bladder tissue from sham control and partially obstructed bladder biopsies. Nuclear chromatin was subjected to ChIP assay in which the final PCR amplification was performed with a set of primers flanking the NF-κB and AP-1 cis-elements in the rat CCN2 gene. As shown in Fig. 6D, there was an inducible interaction between the NF-κB and the CCN2 promoter in mechanically overloaded bladder tissue. This probably accounts for enhanced CCN2 gene transactivation as a result of altered bladder wall stretch. However, interaction of the promoter region with the AP-1 transcription factor was detected as well. Thus, whereas interactions between AP-1 and CCN2 promoter do not take place in monolayer cultures of bladder SMCs, they seem to occur in the bladder tissue as a result of an altered pattern of mechanical stretch. The coexistence in vivo of various cell types and stimuli may account for these discrepancies between in vitro and in vivo conditions, which are further discussed under “Discussion.”

Mechanical Stretch-induced CCN2 Promoter Activity Is Inhibited by Phosphatidylinositol (PI) 3-Kinase, p38 Stress-activated Protein Kinase (SAPK), RhoA GTPase, and Actin Polymerization Inhibitors—Previous studies have established that mechanical forces are mediated biochemically through various protein kinase and monomeric GTP-binding protein signaling pathways, including p42/p44, JNK, SAPK p38, PI 3-kinase, and RhoA GTPase (36). To delineate the mechanotransduction pathways involved in the activation of the CCN2 promoter, we transiently transfected SMCs with the p(-164/+36)-luc construct, treated the cells with pharmacological inhibitors of known signaling molecules, and monitored changes in luciferase activity in stretched and nonstretched cells (Fig. 7). Induction of luciferase activity was not altered when the cells were treated with specific inhibitors for protein kinase A and JNK mitogen-activated protein kinase. Inhibition of Erk1/2 did not affect stretch-dependent promoter activity either (data not shown). In contrast, stretch-induced luciferase activity was reduced by 40, 43, and 113% in the presence of SB-203580, a specific inhibitor for the SAPK p38, wortmannin, a specific inhibitor of PI 3-kinase, and latrunculin B, which inhibit cytoskeletal actin polymerization, respectively. The efficiency of the pharmacological inhibitors used in our experiments was previously confirmed by testing their ability to prevent the activation of authentic substrates of their targeted kinases (23). The effects of latrunculin B on actin polymerization was the most drastic on the reporter gene activity, since it affected not only
stretch-induced but also basal CCN2 promoter activity. In addition, treatment of the cells with Y-27632, a specific inhibitor of RhoA-associated kinase, which regulates, at least in part, actin polymerization, reduced the luciferase activity by 38%.

These data support a critical role of cytoskeletal actin-dependent pathways in the mechanical regulation of the CCN2 gene. Mechanical Forces Regulate Actin Polymerization in Vitro and in Vivo—Since the CCN2 promoter activity is sensitive to cytoskeletal actin dynamics, it is important to determine whether the polymerization of actin is affected by mechanical forces, since this may be one condition where a physiological stimulus affects gene expression. As shown in Fig. 8A, phalloidin staining of stress fibers, which are predominantly composed of F-actin, is more intense in cells stretched for up to 60 min and contains more branching than in cells cultured under static conditions. To further analyze actin dynamics under these conditions, we determined the G- to F-actin ratio in stretched versus control nonstretched SMCs. As shown in Fig. 8B, application of cyclic stretch for either 10 or 30 min induced a significant decrease (up to 35%) in the G- to F-actin ratio as compared with static conditions. This indicates that more G-actin in stretched cells is recruited into polymerized F-actin microfilaments, leaving less free soluble G-actin. The G- to F-actin ratio was not significantly affected after 60 min of cyclic stretch, probably due to the moderate sensitivity of the methodology used. To determine whether changes of actin dynamics occur in vivo as well, we analyzed the F-actin stress fibers and G- to F-actin ratio in mechanically overloaded bladder tissue. As shown in Fig. 8C, a relatively dense staining was observed in phalloidin-stained cross-sections from mechanically overloaded bladders versus sham controls. Intense staining was seen throughout the bladder wall but predominantly in the thick muscular layer. Quantitative analysis of G- and F-actin pools showed a significant decrease in the G- to F-actin ratio (−33% and −27%) in mechanically overloaded tissue after 3 and 7 days, respectively, as compared with that in sham control tissue (Fig. 8D). Therefore, a larger fraction of the total actin pool exists as filamentous actin in mechanically overloaded bladders as well.

Role of Actin Polymerization in the Mechanosensitive Activation of the CCN2 Promoter—Actin isoforms have unique N termini, which probably confer to each isoform functional specialization mediated through specific interactions with other proteins. Previous studies have shown that when the N-terminal sequence of smooth muscle α-actin, Ac-EEED, was delivered to cultured myofibroblasts, it altered both stress fiber formation and myofibroblast contractility (27). Based on these observations and since significant proportions of both α- and γ-actin isoforms contribute to total actin in bladder SMCs, we postulated that their
N-terminal regions have the potential to interfere with actin polymerization and stretch-induced CCN2 promoter activation. To test this hypothesis, we examined the effects of synthetic N-terminal peptides for smooth muscle α-actin (NTSMA) and smooth muscle γ-actin (NTSMG) on stress fiber formation in cultured bladder SMCs. Each peptide was chemically conjugated to another peptide carrier to facilitate its transmembrane transport as described under “Experimental Procedures.” As a control, we used a similarly constructed synthetic peptide containing the NTSA peptide. All peptide chimeras were FITC-labeled for visualization purposes. When incubated with cultured bladder SMCs for 1 h and upon visualization of the peptide fluorescence by fluorescence microscopy, all peptide chimeras appeared to localize intracellularly, indicating that they successfully translocate across the membrane (Fig. 9, A–C). Rhodamine-conjugated phalloidin staining of the cells showed a substantial alteration of stress fiber formation and organization in cells exposed to the NTSMA peptide as compared with those exposed to NTSA (Fig. 9A). Cell treatment with NTSMG appeared to reduce somewhat stress fiber formation but did not completely abolish their formation (Fig. 9B). However, NTSMA-treated cells displayed a nearly stress fiber-free pattern, indicating that this peptide caused dramatic loss of smooth muscle α-actin from stress fibers (Fig. 9C). Quantification of the G- to F-actin ratio showed no significant differences between NTSA- and NTSMG-treated cells (0.241 ± 0.031 versus 0.295 ± 0.041 (n = 3), respectively), but a significant increase is seen in NTSMA-treated cells (0.398 ± 0.09, p < 0.05, n = 3). The NTSMA-treated cells contain at least 65% less filamentous actin than NTSA-treated cells. This suggests that NTSMA induces rapid disassembly of actin filaments, perhaps by interfering with putative partners of smooth muscle α-actin, preventing its incorporation into stress fibers. The inability of NTSMG to alter actin cytoskeleton organization and stress fiber formation favors a longstanding idea that different actin isoforms have different functions in the cytoskeleton.

Next, we tested the effects of these peptides on mechanical stretch-induced CCN2 promoter activity. Cells were transfected with the p(−164/+36)-luc construct and exposed for up to 6 h to either NTSA or NTSMA peptide chimeras. Cells were assayed for luciferase activity following the application of cyclic stretch for 1 h. To compare data, the luciferase activity obtained with p(−164/+36)-luc construct in nonstretched cells was set to 100%. Values shown are representative of three separate experiments. **, p < 0.01 versus stretch in the absence of actin peptide.
The observation that CCN2 is up-regulated in collagen gel-embedded fibroblasts that are under extreme tensional forces and show large cell spanning stress fibers supports the notion of mechanical regulation of CCN2 by actin (17, 37). This is also consistent with our previous studies that showed a superinduction of the CCN2gene in the presence of actin polymerization-inducing drugs (12, 25). In our present study, the role of actin in stretch up-regulation of the CCN2 gene is demonstrated by the use of the NTSMA chimera, an Ac-EEED-containing peptide that reduces the incorporation of actin into stress fibers. Intracellular delivery of the Ac-EEED peptide was previously shown to decrease the tension exerted by myofibroblasts on a deformable substrate and to reduce granulation wound tissue contraction in vivo (27). It is conceivable that this peptide may interfere with the binding of putative partners of smooth muscle α-actin (i.e. myosin, cofilin, and gel-
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solin), preventing the incorporation of actin into stress fibers (38). Of note is that the effects of the NTSMG peptide chimera on actin organization and stretch-induced CCN2 gene are not unique to SMCs from the bladder wall, but they are also observed in SMCs cultured from other beds, such as the pulmonary arteries, in which α-actin is the dominant isoform (data not shown). Interestingly, incubation of bladder SMCs with the NTSMG peptide harboring the N-terminal sequence of smooth muscle γ-actin neither dramatically altered the stress fiber staining by rhodamine-phalloidin nor significantly reduced stretch-induced CCN2 promoter-reporter gene activation. These observations were somewhat unexpected, since the γ-actin isoform, which is considered to be a marker of fully differentiated bladder SMCs (39), has been shown to contribute 37–63% of total actin in bladder smooth muscle, depending on species and pathological state of the bladder (40, 41). It is noteworthy that the homogeneity and differentiation state of primary cultures have been recurrent issues in various studies of mechanosensing and force generation mechanisms (42). The recent development of transgenic mice from which pure and homogenous populations of γ-actin-expressing SMCs from the bladder wall can be isolated and studied is an important step to address issues related to the specialized functions of actin isoforms in mechanotransduction (43). However, although α- and γ-actin isoforms are highly conserved proteins, there is no compelling evidence that they contribute equally to stress fiber formation and smooth muscle contractility and/or have similar functions in the mechanosensing mechanisms. This hypothesis is supported by two observations. 1) Differences in interaction of various actin isoforms with some actin-binding proteins have been previously reported (44). This isoform specificity supports the idea that various actin isoforms have different functions in the cytoskeleton. 2) Actin isoforms can partially substitute for but cannot functionally compensate each other (45, 46). In particular, smooth muscle α-actin was partially substituted by skeletal α-actin in smooth muscle α-actin-deficient mice, and ectopic overexpression of enteric smooth muscle γ-actin rescued the heart of mice lacking cardiac α-actin. However, up-regulation of other actin isoforms was not sufficient to render normalcy and maintain full organ functionality and resulted often in altered muscle contractility in those mice. Meanwhile, with specific relevance to our current study, Zimmerman et al. (41) reported that the bladder from smooth muscle α-actin-null mice generates significantly less force than bladder from wild-type mice in response to electrical field stimulation. This supports a preponderant role for smooth muscle α-actin in force sensing and transfer within bladder SMCs. A detailed study is currently under way to dissect the functional significance of individual actin isoform expression in the response of bladder SMCs to externally imposed forces.

We and others have shown that NF-κB regulates the expression of various stretch- and shear-stress-sensitive genes, including the platelet-activating factor receptor, brain natriuretic peptide, and monocyte chemoattractant protein-1 genes (47–50). Thus, mechanical activation of NF-κB appears as a consistent theme linking mechanical stimuli to activation of gene expression. Our data further extends those studies by demonstrating that NF-κB senses mechanical stretch-induced changes in the actin cytoskeleton and converts them into increased promoter activity of the CCN2 gene. This seems to be a highly selective process, since stretch-induced AP-1 activation was neither affected by cytoskeletal actin alterations nor involved in stretch-induced CCN2 promoter activity. However, the precise mechanisms involved remain obscure. From a consideration of the structural and functional features of the actin cytoskeleton, several potential mechanisms may be considered. The most plausible one is based on the cytoskeletal control of nuclear import of NF-κB. NF-κB is a dimeric transcription factor that is sequestered in the cytoplasm of unstimulated cells by binding to IκB proteins. NF-κB-activating stimuli activate the IκB kinase signalosome that phosphorylates IκB, leading to its ubiquitination and degradation and liberating NF-κB dimers to translocate into the nucleus and regulate target gene transcription. The stability of IκB in resting cells depends on its anchor- age to the actin cytoskeleton, possibly via its ankyrin repeat domain (51). Interestingly, stretch-dependent activation of the CCN2 promoter was not only altered by the Ac-EEED containing peptide but also by the RhoA-associated kinase inhibitor, Y-27632, which has been shown both to alter the actin network and to inhibit NF-κB binding activity by inducing cytosolic stabilization of IκBα (52, 53). This suggests the existence of a regulatory cascade in which inhibition of RhoA-actin signaling prevents IκB kinase activation, which, in turn, stabilizes IκB and inhibits NF-κB translocation and CCN2 promoter transactivation. Thus, stretch-mediated activation of the CCN2 promoter is likely to be spatially coupled to dynamic rearrangement of the actin cytoskeleton associated with IκB destabilization.

However, our observations are at variance with prior findings by Abraham and colleagues, who showed that TNF-α, a potent NF-κB-activating stimulus, suppresses TGF-β-induced CCN2 gene expression and proposed that this inhibition may be directly or indirectly mediated by NF-κB activation (54). These results, while intriguing in their own right, are in disagreement with both our data and those of Tian et al. (55), who showed that ectopic expression of constitutively active NF-κB/Rel A up-regulates the expression of a gene network that includes the CCN2 gene, suggesting that NF-κB binding is sufficient to transactivate the CCN2 promoter. We have no straightforward explanation for the differences between results from different laboratories, but they may be due to cell type-specific, tissue-specific, or species variations. Precisely, the involvement of additional cell type or tissue-specific actin-sensitive transcriptional co-activators or co-repressors in the regulation of the CCN2 promoter cannot be excluded. Indeed, aside from the spatial sequestration of NF-κB by the cytoskeleton, G-actin was reported to either activate or inhibit transcription factors through interaction with cofactors required for their activation (56). This property is particularly illustrated in the model of regulation of serum response factor (SRF) activity and SRF target genes, including CCN2. In this regard, it has been shown that MAL, known also as myocardin-related SRF coactivator, is a G-actin-associated SRF co-activator, which is redistributed from the cytoplasm to the nucleus as a result of serum-induced actin polymerization (57). SRF-MAL interaction activates several gene promoter targets in response to serum stimulation.
Whether MAL and/or other transcriptional co-factors are involved in stretch-induced CCN2 gene expression and what regulatory function, if any, they may have in this process remain to be investigated. Recent studies have shown that MAL is sensitive to mechanical stretch and that MAL-D, a homologue of MAL in Drosophila, is particularly critical for cells that perform force-driven processes, such as migration (58–60). This suggests that MAL plays an important role in the mechanosensing mechanisms. Further studies are needed to assess the potential involvement of these or other co-factors in the mechanical regulation of the CCN2 gene.

Meanwhile, signaling mechanisms via p38 and PI 3-kinase are also required for stretch-induced activation of the CCN2 promoter. The upstream signaling molecules that feed into the p38 and PI 3-kinase signaling pathways are diverse and cell type-specific. Inhibition of PI 3-kinase, which can be either upstream or downstream of p38, depending on the system, was as effective as the inhibition of p38 in reducing CCN2 promoter activity. When the cells were pretreated with both PI 3-kinase and p38 inhibitors, the effects on CCN2 promoter activation were nearly additive (data not shown). Therefore, PI 3-kinase and p38 are probably involved in distinct signaling pathways that could merge/overlap downstream. Interestingly, a key downstream component of either p38 or PI 3-kinase pathway is the actin cytoskeleton. In particular, recruitment of PI 3-kinase by polymerized F-actin has been shown to be crucial for amplification of chemotactic signals (61). Similarly, p38 was shown to bind to α-actin upon application of mechanical forces to the cells, which, in turn, exhibit enhanced force-induced activation of p38 when they express abundant smooth muscle α-actin (62). The actin cytoskeleton plays the role of a docking surface that is important for the activation of various signaling molecules and for sustained signaling between cell surface and nucleus. Reversibly, activation of signaling molecules, such as p38 and PI 3-kinase, may affect reorganization of the actin cytoskeleton and gene expression through either their effects on α-actin expression or their interaction with Rho proteins. A detailed study of the complex interactions among these signaling molecules and with the actin cytoskeleton in mechanically challenged cells is in progress in our laboratory.

Another interesting outcome of our study design is that the mechanosensing mechanisms responsible for CCN2 promoter activation in vitro appear to take place, at least in part, in vivo, since increased bladder wall distension was accompanied by high levels of polymerized actin, binding of NF-κB to CCN2 promoter and transactivation of the CCN2 gene. However, our ChIP analysis of the endogenous CCN2 gene in overloaded tissue revealed that an additional interaction between AP-1 and its binding site adjacent to that of NF-κB in the CCN2 promoter takes place as well. There are several potential explanations to these findings. First, it is possible that AP-1 interaction with the CCN2 promoter in vivo occurs in a separate distinct “wave” and not simultaneously with NF-κB. Indeed, both NF-κB and AP-1 sites are in close proximity to one another, and steric encumbrance should hinder their simultaneous interaction with the CCN2 promoter. Second, the AP-1-CCN2 promoter interaction may occur in a different cell type. In fact, although smooth muscle represents the major cellular component of the bladder wall, additional constitutive cell types include urothelial cells of the lining epithelium, the lamina propria fibroblasts, and vascular endothelial cells. Each of these cell types may respond in a unique fashion to mechanical stimuli. Third, AP-1 may interact with the CCN2 promoter in response to distinct stimuli that co-exist with mechanical overload within the tissue (e.g. humoral factors, neurotransmitters, etc.). Mechanical distension of the bladder wall in isolated organ baths is an alternative to our in vivo experiments and will be carried out in future studies to tease out the effects of mechanical distension in isolation from systemic and local factors.

Functionally, CCN2 has been shown to be mitogenic to NRK cells and chemotactic to NIH 3T3 cells and induces extracellular matrix synthesis such as type I collagen in connective tissue type cells (63, 64). It has previously been proposed that CCN2 integrates its activities on SMCs, fibroblasts, endothelial cells, and macrophages to regulate angiogenesis, inflammation, and ECM protein remodeling in the context of wound healing (9). Although our study was not designed to evaluate the profibrotic and proangiogenic effects of CCN2 in mechanically overloaded bladders, there is evidence that CCN2 may exert such effects in mechanically overloaded bladders based on its currently known biological activities. Interestingly, it has been shown that incubation of cultured myofibroblasts with a cell-penetrating peptide containing the Ac-EEED sequence of smooth muscle α-actin significantly reduced the amount of type I collagen produced by these cells, suggesting a link between collagen expression and actin cytoskeleton organization (27). On the basis of the data reported here, it is likely that CCN2 plays an important role in this process. Delineation of the underlying mechanisms is fundamentally important to fully understanding the manifold effects of mechanical forces on gene expression. This should open new avenues of targeted pharmacological intervention to interfere with the deleterious effects of the CCN2 protein.

Acknowledgment—We thank Dr. J. Rushbrook for critical reading of the manuscript.

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