

Regulation of *Cyr61/CCN1* gene expression through RhoA GTPase and p38MAPK signaling pathways

Role of CREB and AP-1 transcription factors

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Cysteine-rich protein 61 (*Cyr61/CCN1*) is an angiogenic factor and a member of a family of growth factor-inducible immediate-early genes with functions in cell adhesion, proliferation and differentiation. We investigated the regulatory mechanisms and signaling pathways involved in *Cyr61/CCN1* gene activation in smooth muscle cells. Treatment of these cells with sphingosine 1-phosphate (S1P), a bioactive lysolipid, increased rapidly but transiently the expression of the *Cyr61/CCN1* gene at both the mRNA and protein levels. *Cyr61/CCN1* mRNA stability was not altered but the transcription rate of the *Cyr61/CCN1* gene was increased fivefold in isolated nuclei from S1P-stimulated cells indicating that the level of control is primarily transcriptional. Transfection experiments showed that a 936-bp promoter fragment of the human *Cyr61/CCN1* gene is functional and induces a reporter gene activity in S1P-treated cells. Using a combination of *cis*-element mutagenesis and expression of dominant negative inhibitors of transcription factors, we

showed that both a CRE and AP-1 site and their cognate transcription factors, cAMP response element binding protein (CREB) and AP-1, were responsible for the promoter activity in S1P-stimulated cells. Furthermore, by using either pharmacological inhibitors or active forms of known signaling molecules, we showed that inducible *Cyr61/CCN1* gene expression occurs through RhoA GTPase and that additional signaling through the p38 pathway is required. In particular, p38 seems to regulate *Cyr61/CCN1* promoter activity through modulation of phosphorylation of CREB and the CREB kinase, MSK1. These findings demonstrate the transcriptional regulation of the *Cyr61/CCN1* gene and provide clues to the signaling molecules and transcription factors involved in such regulation.

Keywords: AP-1; CREB; CTGF/CCN2; *Cyr61/CCN1*; p38 MAP kinase; RhoA GTPase; signal transduction; transcription factors.

The cysteine-rich protein 61 (*Cyr61/CCN1*) is encoded by a nontranscription factor immediate early gene whose expression is rapidly and transiently induced in response to growth and stress stimuli [1,2]. *Cyr61/CCN1* is a \approx 40-kDa cysteine-rich and heparin-binding protein that either localizes intra-

cellularly or associates with extracellular matrix and cell surfaces and belongs to the CCN family of genes that includes, in addition to *Cyr61/CCN1*, another immediate early gene, connective tissue growth factor (*CTGF/CCN2*), nephroblastoma overexpressed (*Nov/CCN3*) and Wnt induced secreted protein 1–3 (*WISP1-3/CCN4-6*) [3,4]. These proteins exhibit a highly conserved structural organization but a distinct expression profile and tissue distribution both *in vivo* and *in vitro*. In addition, their biological functions may vary in a cell-type and cell-context specific manner.

At the functional level, *Cyr61/CCN1* recombinant protein was reported to activate a repertoire of genes that regulate angiogenesis, inflammation, extracellular matrix remodeling and cell–matrix interactions [5]. The *Cyr61/CCN1* protein activities are potentially mediated through interactions with membrane proteins such as heparan sulfate proteoglycans, other growth factor receptors, integrins and/or through other incompletely characterized nonintegrin receptors [6,7]. The *Cyr61/CCN1* protein also exhibits a remarkable expression profile during development as it was reported to induce vascularization, and to participate in chondrogenesis, skeletogenesis and pathological disorders [8,9]. In particular, *Cyr61/CCN1* has been described as a pro-hypertrophic/pro-hyperplastic protein by virtue of its strong and sustained expression in hypertrophied detrusor smooth muscle cells in partially obstructed bladders and during proliferative restenosis in the media

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Abbreviations: *Cyr61/CCN1*, cysteine-rich protein 61; *CTGF/CCN2*, connective tissue growth factor; S1P, sphingosine 1-phosphate; SRE, serum response element; SRF, serum response factor; SMC, smooth muscle cell; ERK, extracellular-regulated kinase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; GAPDH, glyceraldehyde phosphate dehydrogenase; CAT, chloramphenicol N-acetyltransferase; AP-1, activator protein-1; CRE, cAMP-responsive element; CREB, CRE-binding protein; CBP, CREB-binding protein; B-Zip, basic leucine zipper; PKA, protein kinase A; MSK, mitogen- and stress-activated kinase; SAPK, stress-activated protein kinase; BIM, bis-indolyl maleimide.

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and neointima muscle layers following vascular injury [10,11]. Therefore, understanding the mechanisms regulating *Cyr61/CCN1* gene expression could be of great advantage for the purpose of identifying reaction sites that are amenable to pharmacological modulation in disease states involving *Cyr61/CCN1* metabolism.

The amount of information regarding the molecular mechanisms involved in the regulation of the *Cyr61/CCN1* gene is still somewhat limited. The mouse *Cyr61/CCN1* promoter has been studied in cultured fibroblasts in transient transfection assays [12]. It was found that a serum response element (SRE), located ≈ 2 kb upstream of the transcription start site, is necessary and sufficient to confer inducibility by serum and serum growth factors. Additionally, we have shown that this SRE is also involved in the regulation of the *Cyr61/CCN1* gene during neuronal cell death [13]. However, even though the SRE contains a CarG box sequence element implicated in muscle-specific gene expression, further *in vivo* studies revealed the nonessential nature of the *Cyr61/CCN1* SRE for its expression in smooth muscle [14,15]. In agreement with this, based on TRANSFAC analyses, the human *Cyr61/CCN1* promoter lacks SRE-like sites, which indicates that transcription factors, other than serum response factor (SRF), regulate growth factor-induced and muscle-specific expression of the *Cyr61/CCN1* gene.

Sphingosine 1-phosphate (S1P) is a bioactive polar lysolipid metabolite produced in a wide variety of cell types in response to diverse stimuli including growth factors, cytokines, G-protein coupled-receptor agonists, antigens, etc. (reviewed in [16,17]). Either smooth muscle or endothelial cells are targets for S1P and can be exposed to significant levels of S1P *in vivo* [18]. In primary cultures of smooth muscle cells (SMCs), S1P stimulates proliferation, contraction and regulates cell migration. Once produced, S1P acts as a local hormone or autacoid under certain physiological and pathological conditions. The extracellular effects of S1P are mediated via plasma membrane G-protein-coupled receptors originally known as endothelial differentiation gene receptors. In the short term, S1P receptor activation is coupled differentially via G_i , G_q , $G_{12/13}$ and Rho to multiple effector systems, including adenylate cyclase, phospholipases C and D, extracellular-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein (MAP) kinase and nonreceptor tyrosine kinases [19,20]. These signaling pathways are linked to transcription factor activation, cytoskeletal proteins, adhesion molecule expression, caspase activities, etc. Therefore, S1P action is likely connected to cytoplasmic and nuclear events.

In the present study, we provide evidence that the *Cyr61/CCN1* gene is a downstream target of S1P signaling in primary cultures of SMCs and its regulation occurs at the promoter level. We investigated the nature of the intracellular signaling pathways that link S1P signaling to *Cyr61/CCN1* gene expression and showed that the activation of RhoA GTPase and p38 MAP kinase pathways is required for *Cyr61/CCN1* gene induction. Additionally, we showed that pathways connecting these signaling molecules to nuclear events such as activation of the CREB and AP-1 transcription factors

are implicated in S1P-induced promoter activation of the *Cyr61/CCN1* gene.

Materials and methods

Materials

Modified Eagle's medium referred to as M199 was obtained from Life Technologies, Inc. S1P were obtained from Avanti (Alabaster, AL, USA). Chemical inhibitors were purchased from CalBiochem Corp. All other chemicals used were of reagent grade. Y-27632 inhibitor was kindly provided by T. Kondo (Welfide Corp., Osaka, Japan). Anti-*Cyr61/CCN1* and anti-CTGF/CCN2 Igs were described elsewhere [2,21]. Anti-phospho-Erk1/2, anti-total Erk1/2, anti-c-jun, anti-total p38, anti-HA and anti-Myc Igs were from Santa Cruz Biotech. Anti-phospho-p38, anti-phospho-MSK1 and anti-phospho-CREB, anti-phospho-c-jun, anti-Cdc42, anti-Rac1 Igs were from New England Biolabs. Anti-CREB Ig was from Geneka (Toronto, Canada), anti- $\phi 10$ (T7-Tag) Ig was from Novagen (Madison, WI), anti-RhoA Ig was from Upstate Biotechnology (Charlottesville, VA), anti-glyceraldehyde phosphate dehydrogenase (GAPDH) and anti-c-fos Igs were from Oncogene (Boston, MA, USA). Radioactive materials such as [α - 32 P]UTP, [α - 32 P]dCTP, [γ - 32 P]ATP and [14 C]chloramphenicol were from NEN Life Science Products.

Cell culture and drug treatments

Primary cultures of smooth muscle cells were prepared from bladders of mid to late gestational fetal calves as described previously [22,23]. Freshly isolated cells were phenotypically characterized using muscle specific antibodies against smooth muscle actin. Cells were maintained in M199 supplemented with 10% fetal bovine serum and antibiotics in a humidified atmosphere containing 5% CO₂ in air at 37 °C. Cells from passages 2 through 8 were used for the experiments. For most experiments cells were grown to subconfluence either in 25 cm² culture flasks or in 35 mm 6-well plates. Twenty-four hours later, cells were washed with M199 to remove traces of serum and placed in serum-free M199 with or without exogenous S1P as indicated in the text. To test the effects of specific inhibitors on signal transduction pathways, the cells were left in the presence of a given inhibitor for at least 30 min followed by the addition of S1P for 1 h. Stock solutions of each inhibitor were made in either aqueous solution, dimethyl sulfoxide or chloroform and diluted to a working concentration in serum-free medium. For control conditions, cells were treated with equal amounts of the corresponding solvent (i.e. dimethyl sulfoxide or chloroform).

RNA isolation and Northern blot analysis

Total RNA was extracted from cells using TRIzol Reagent from Invitrogen. A sample containing 12 μ g total RNA was fractionated by electrophoresis in 1% agarose/formaldehyde gel, transferred to Zeta-Probe nylon filters (Bio-Rad, Richmond, CA) and hybridized to *Cyr61/CCN1* radiolabeled cDNA probe as described previously [2]. A specific probe for CTGF/CCN2 was radiolabeled also, and

hybridized to the filters that were stripped according the manufacturer's instructions (Bio-Rad). Total RNA loading and transfer were evaluated by probing with a GAPDH cDNA probe. The filters were analyzed by phosphorimaging and hybridization signals were quantified to determine the relative amounts of mRNA (Molecular Dynamics, CA, USA). The mRNA levels were analyzed in duplicate samples and normalized to equivalent values for GAPDH to compensate for variations in loading and transfer.

Messenger RNA stability assay

Cells were cultured in tissue culture flasks as described above and treated with chemical stimuli for 1 h. The culture medium was then replaced with serum-free M199 containing $10 \mu\text{g}\cdot\text{mL}^{-1}$ actinomycin D and the cells were harvested after 0, 0.5, 1, 2 and 4 h. Total RNA was purified and analyzed by Northern blot and phosphorimaging densitometry. The relative amounts of normalized messenger RNA were plotted as a function of time and the slope of this curve was used to calculate the interval period of time within which half of the original amount of mRNA had decayed.

Immunoblotting and immunodetection with phosphospecific antibodies

For Western blot analyses, cells were cultured in 35-mm dishes under normal cell culture conditions. Treatment with SIP was performed as described in the text. The cells were then washed twice with NaCl/P_i and cell lysates were prepared by harvesting the cells in 0.1% Triton X-100 lysis buffer. Protein concentration was determined by using the Bradford protein assay (Bio-Rad). Protein samples (20 μg) were separated by SDS/PAGE (10% acrylamide), transferred to nitrocellulose membranes and Western blot analysis performed using either Cyr61/CCN1 or CTGF/CCN2 Igs. Immunodetection was performed by enhanced chemiluminescence (Amersham Bioscience Inc.). For immunodetection of phosphorylated proteins, SDS sample buffer was added directly to the cells that were subsequently scraped off the plate and subjected to denaturing SDS/PAGE under reducing conditions.

Rho-GTP pull down assay

Measurement of GTP-bound Rho was performed using the Rho activation assay kit (Upstate Biotechnology), according to the manufacturer's instructions. Briefly, the RhoA-binding domain of Rhotekin, a downstream effector of RhoA, was used to affinity precipitate GTP-bound Rho from cells lysed in 50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM MgCl_2 , and a cocktail of protease inhibitors (Roche). Precipitated Rho-GTP was then detected by immunoblot analysis, using a polyclonal anti-Rho (-A, -B, -C) antibody. Total RhoA in each lysate was determined by Western blotting analysis in the protein lysate of each sample.

Nuclear run-on assay

Subconfluent smooth muscle cells were stimulated with SIP for 1 h. Cells were then washed twice with NaCl/P_i ,

trypsinized and centrifuged at 4 °C. The cellular pellet was resuspended in buffer containing 10 mM Tris/HCl pH 7.4, 10 mM NaCl, 3 mM MgCl_2 , and 0.5% Nonidet P-40 allowing swelling and lysis of the cell membrane. The lysate was recentrifuged at 300 *g* at 4 °C and the resulting nuclear pellet was resuspended in 150 μL of buffer containing 20 mM Tris/HCl pH 8.0, 75 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol and 50% glycerol. *In vitro* transcription was then performed with the suspended nuclei at 30 °C for 30 min in a buffer containing 10 mM Hepes pH 8.3, 5 mM MgCl_2 , 300 mM KCl, 50 mM EDTA, 1 mM dithiothreitol, 0.1 mM rCTP, rATP, rGTP and 250 μCi [α - ^{32}P]UTP. The radiolabeled RNA was extracted from the nuclei as described above. Equal amounts (2.5 μg) of Cyr61/CCN1, CTGF/CCN2 and GAPDH cDNA probes as well, as a linearized pCRII vector, were vacuum transferred onto a Zeta-probe nylon membrane using a slot blot apparatus (Biorad). The membrane was UV-irradiated and prehybridized as described above for Northern blotting. Equal amounts of the purified radiolabeled transcripts (10^6 c.p.m.) were resuspended in hybridization solution. Hybridization with the slot-blotted DNA probes was carried out for 48 h at 42 °C. The membranes were then washed under stringent conditions before phosphorimager scanning of the hybridization signals.

Transient transfection, co-expression and reporter assays

All Cyr61/CCN1 promoter constructs were cloned into the chloramphenicol acetyltransferase (CAT) reporter vector pGL3 basic (Promega). A 936-bp construct was obtained by amplification of genomic DNA from the clone RP-11653 obtained from the Sanger Institute (London, UK). Smaller constructs were obtained by PCR cloning utilizing the *Kpn*I and *Xho*I sites of the pGL3 basic. Identity and orientation of the constructs were verified by sequencing of the obtained promoter–vector constructs. Cultured smooth muscle cells were plated at a density of $1 \times 10^5\text{cm}^{-2}$ in 24-well tissue culture plates and maintained in medium containing 10% serum for 18 h. Transfection was then performed using Fugene 6 Transfection Reagent (Roche Diagnostics) in serum-free medium according to the manufacturer's specifications. In addition to specific chimeric Cyr61/CCN1 promoter–CAT plasmid constructs, the cells were cotransfected with constitutively expressed β -galactosidase reporter plasmid constructs (RSV- β -gal) to adjust for transfection efficiency. Coexpression experiments were carried out by including 0.25 μg empty vector or vector overexpressing constitutively active forms of either RhoA (Ca-RhoA), Cdc42 (Ca-Cdc42), or Rac (Ca-Rac). These expression plasmids were a generous gift from A. Hall (University College, London, UK). Other coexpression vectors used include those overexpressing active forms for MKK3 (Ca-MKK3) and MKK6 (Ca-MKK6) both provided by J.H. Han (The Scripps Institute, San Diego, CA, USA). Dominant negative inhibitors of CREB (K-CREB) from J.E.-B. Reusch (University of Colorado, Denver, CO, USA), fos (A-fos) and ATF-2 (A-ATF) provided by C. Vinson (NCI, Washington DC) were also used in our experiments. The Fugene6: DNA mixtures plus serum-free medium were left on cells for 3 h. The cells were allowed to recover in fresh medium containing 10% serum. The next

day, the experimental treatments were performed as described in the text. Cells were then washed three times with ice-cold NaCl/P_i and lysed in 1 × Reporter Lysis Buffer (Promega) for analysis of reporter gene expression. CAT activity was assayed by incubation for 3 h in the presence of 0.25 μCi [¹⁴C]chloramphenicol (100 mCi·mmol⁻¹) and 200 μg·mL⁻¹ butyryl-CoA in 0.25 M Tris/HCl pH 7.4. Labeled butyrylated products were extracted with a mixture of xylene and 2,6,10,14-tetramethyl-pentadecane (1 : 2) and counted. Each experiment was performed three times in duplicate and all experiments included negative (promoterless pCAT) controls. The latter served as a baseline indicator of CAT activity and the activity of each promoter construct was expressed relative to the promoterless activity (fold induction). Transfection efficiency was evaluated using fluorescence microscopy in cells cotransfected with plasmid containing the green fluorescent protein gene (pEGFP-N1; CLONTECH). The transfection efficiency, using 1 μg of pEGFP-N1 per 10⁵ cells, varied between 35 and 45%.

Site directed mutagenesis

Mutations to putative *cis*-acting elements were made using the QuickChange Site-Directed Mutagenesis protocol from Stratagene following the manufacturer's specifications. The distal AP-1 site was changed from -TGACTCAG- to -GCTCACAG- and the core binding site CRE3 was changed from -CGACGTC- to -CTAAACCA-. These nucleotide mutations were previously shown to disrupt AP-1 and CRE function and abolish binding to specific nuclear proteins [24,25]. Constructs were fully sequenced in both directions to confirm successful mutagenesis before use.

Statistical analysis

Data were expressed as mean ± SEM. A paired Student's *t*-test was used to analyze differences between two groups, and *P*-values of < 0.05 were considered significant.

Results

Effects of S1P on *Cyr61/CCN1* gene expression

Cyr61/CCN1 is not constitutively expressed in resting smooth muscle cells. First, we sought to determine and characterize the kinetic parameters of its induction by the lysolipid S1P, which has been shown to form in the cells in response to and mimic the effects of diverse stimuli including cytokines, growth factors, receptor-tyrosine kinase and G-protein-receptor agonists and vitamin D3 [16]. As shown in Fig. 1, exposure of cultured SMCs to S1P stimulates the expression of the *Cyr61/CCN1* at both the mRNA and protein levels. The increase in *Cyr61/CCN1* mRNA levels was detectable within 30 min, maximal by 1 h and returned progressively to baseline levels after 4 h. The *Cyr61/CCN1* protein levels were increased after 1 h of exposure and thus being coordinated with the changes in the mRNA levels. The mRNA levels of *CTGF/CCN2* peaked after 1 h of incubation with S1P and decayed progressively thereafter. These experiments revealed a stronger and earlier increase of *Cyr61/CCN1* mRNA levels than those of

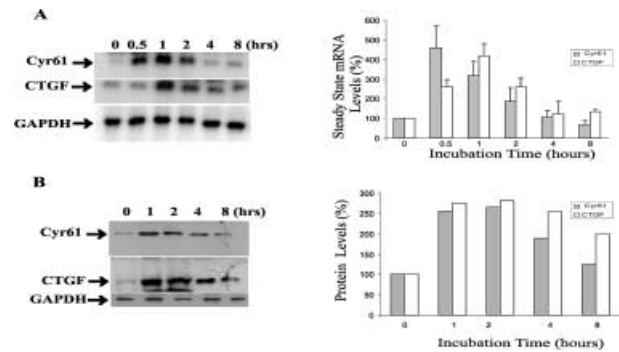


Fig. 1. Stimulation of *Cyr61/CCN1* gene expression by S1P. (A) Relative mRNA levels of *Cyr61/CCN1* in cells treated with S1P. Cells were treated with S1P (10 μM) for the indicated time periods. Total RNA was isolated and analyzed by Northern blot hybridization using a specific DNA probe for *Cyr61/CCN1*. The same blots were stripped and re-probed with specific DNA probes for *CTGF/CCN2* and *GAPDH*. The latter was used to control for unequal RNA loading. Representative autoradiograms are shown in the left panels while a graphical representation of the hybridization signals as quantified by phosphorimager scanning is shown in the right panel. To compare mRNA expression from different experiments, mRNA levels of control cells were set to 100%. Data represent means ± SEM (*n* = 4). (B) Treatment of the cells with S1P increases *Cyr61/CCN1* and *CTGF/CCN2* protein levels. Twenty μg of total proteins from cell lysates were used for Western blot to determine the protein levels of *Cyr61/CCN1* and *CTGF/CCN2* using primary anti-*Cyr61/CCN1* and anti-*CTGF/CCN2* Igs, respectively. *GAPDH* was used as a loading control. Immunodetection was performed by enhanced chemiluminescence. The left panels show representative autoradiograms and the right panel shows the protein levels as measured by densitometric scanning of the intensity of the protein bands. To compare data from different experiments, protein expression in control cells was set to 100%. Data represent means of two independent experiments.

CTGF/CCN2 mRNA whereas only minimal differences are seen between the increased levels of *Cyr61/CCN1* and *CTGF/CCN2* proteins. After 2 h of stimulation with S1P, *CTGF/CCN2* protein levels decreased at a slower rate than those of *Cyr61/CCN1* suggesting that *CTGF/CCN2* may be, in part, regulated by protein stability. The micromolar concentration of S1P used in our experiments were within the range reported to occur either physiologically or in serum [16,26]. Lower concentrations (in the nanomolar or picomolar range) did not induce either *Cyr61/CCN1* or *CTGF/CCN2* gene expression (data not shown). Higher concentrations were not used to avoid potential nonspecific and/or toxic effects of S1P.

Transcriptional regulation of the *Cyr61/CCN1* gene

To determine whether S1P increased *Cyr61/CCN1* mRNA accumulation by increasing the rate of its synthesis or decreasing that of its degradation, SMCs were incubated either in the presence or absence of S1P for 1 h and then incubated further with actinomycin D (10 μg·mL⁻¹) to inhibit transcriptional activity. As shown in Fig. 2, the half-life (*t*_{1/2} < 1.5 h) of *Cyr61/CCN1* mRNA was not affected by stimulation with S1P. In comparison, the *CTGF/CCN2* mRNA decay curve was steeper in S1P-stimulated cells

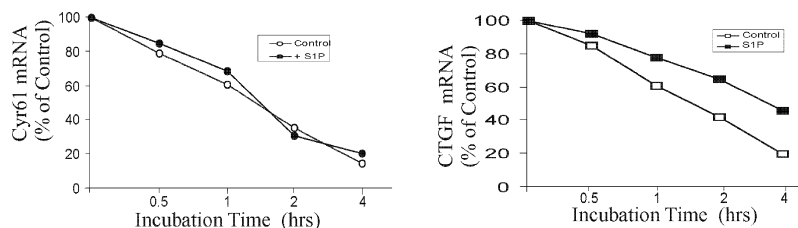


Fig. 2. Effects of S1P on the *Cyr61/CCN1* and *CTGF/CCN2* mRNA decay in transcriptionally blocked cells. Cells were treated with either control vehicle or S1P for 1 h and were further incubated with actinomycin D ($10 \mu\text{g mL}^{-1}$) for the indicated time periods. For each time point, total RNA was prepared and analyzed by Northern blot-hybridization. Each point is the mean of two separate experiments.

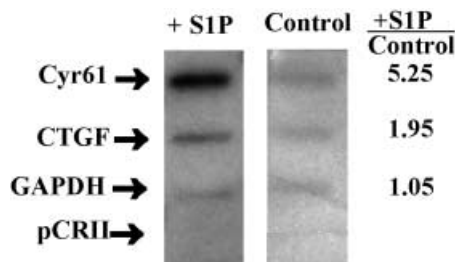


Fig. 3. Nuclear run-on assay showing the effects of S1P on *Cyr61/CCN1* and *CTGF/CCN2* gene transcription. Nuclei were prepared from either control nontreated or S1P-treated cells for 1 h. The mRNA was radiolabeled, isolated and hybridized to *Cyr61/CCN1*, *CTGF/CCN2* and GAPDH cDNA probes and a plasmid vector, which had been slot-blotted on nylon membranes. The hybridization signals were measured and standardized between S1P-treated and control cells. The blots shown are representative of two independent experiments with similar results. Specificity of the hybridization signals is demonstrated by lack of signal with pCRII, an unrelated plasmid DNA.

($t_{1/2} < 2$ h) than in control cells ($t_{1/2} < 2.5$ h) suggesting that post-transcriptional regulation of *CTGF/CCN2* mRNA occurs in the stimulated cells i.e. *CTGF/CCN2* mRNA has a longer half-life. Furthermore, to establish the transcriptional activation of the *Cyr61/CCN1* gene, we performed nuclear run-on experiments using nuclei from control nonstimulated and S1P-stimulated cells. There was a fivefold increase of *Cyr61/CCN1* gene transcription rate in nuclei from S1P-stimulated cells compared with those from control cells demonstrating enhanced *de novo* synthesis of *Cyr61/CCN1* mRNA (Fig. 3). Moreover, the transcription rate of *CTGF/CCN2* was nearly twofold higher in nuclei from S1P-stimulated cells than in those from control cells indicating that a relatively modest transcriptional regulation of the *CTGF/CCN2* gene occurred as compared to that of the *Cyr61/CCN1* gene. Specificity of these hybridization signals was established by lack of hybridization signals to the pCRII insertless vector. Transcription of the GAPDH gene served as an internal control.

Regulation of *Cyr61/CCN1* gene promoter

According to TRANSFAC analysis [27], the promoter of either the human or mouse *Cyr61/CCN1* gene (GenBank Accession Number AL162256 and X56790, respectively) contains several response elements, including sequences which bind the transcription factors CREB, AP-1,

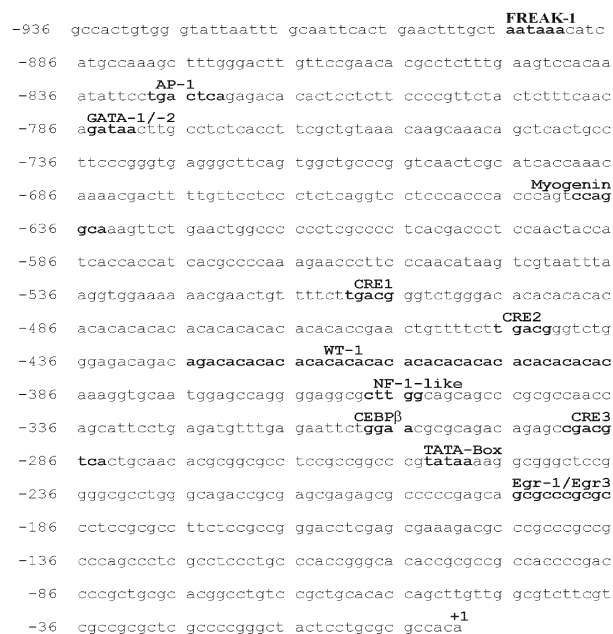


Fig. 4. The human *Cyr61/CCN1* promoter and its regulatory elements as cloned in the pGL3-CAT vector. Potential nucleotide sequences corresponding to the TATA box and some transcription factor binding sites revealed by TRANSFAC analysis are marked. The numbering is based on the start of transcription (+1). This DNA fragment represents a continuous region of high homology between human and mouse promoter of the *Cyr61/CCN1* gene.

GATA-2, Wt-1 and egr-1. To assess the molecular basis for *Cyr61/CCN1* gene promoter activity in SMCs, we have cloned a 936-bp 5' flanking sequence upstream of the transcription start site of the *Cyr61/CCN1* gene by PCR using the clone RP-1165 harboring a portion of the human chromosome 1 as a template. The PCR obtained product was then cloned into a promoterless CAT reporter vector pGL3-basic. The sequence of the cloned DNA fragment is shown in Fig. 4 and the transcription initiation site, the TATA box and some of the putative transcription factor binding elements are indicated. Additionally, to identify sequences important for the promoter activity, other 5' deletion constructs were made by PCR-cloning using the previous 936-bp fragment as a template. All constructs obtained were cloned into the promoterless CAT pGL3-basic. These constructs are represented diagrammatically in Fig. 5A.

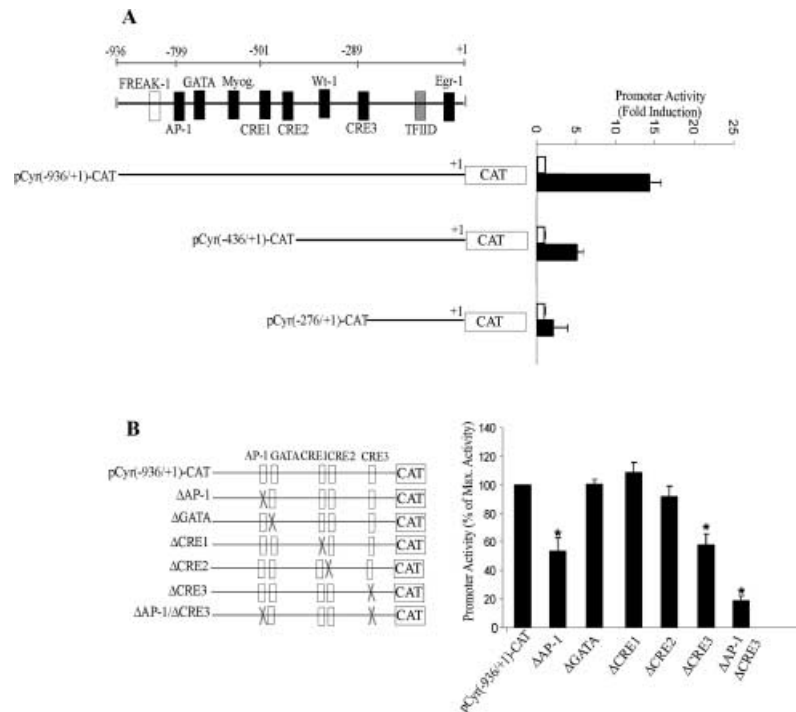


Fig. 5. Induction of the *Cyr61/CCN1* promoter upon stimulation by S1P and mapping of the responsive elements in the *Cyr61/CCN1* promoter sequence. (A) Cells were transiently transfected with constructs containing various segments of the *Cyr61/CCN1* promoter fused to the CAT reporter gene (a schematic diagram of which is shown in the left panel) as described in Materials and methods. Twenty-four hours later, cells were incubated in either serum-free medium or S1P-containing serum-free medium for 1 h, lysed and assayed for CAT activity. Each *Cyr61/CCN1* promoter–reporter construct was assayed in triplicate transfections in at least two independent experiments. Values indicate the magnitude (100% = 1 × fold) of *Cyr61/CCN1* promoter–reporter induction over basal expression obtained with a promoterless reporter construct. The results are expressed as the means ± SEM. (B) Mutational analysis of the *Cyr61/CCN1* promoter was performed by mutating specific *cis*-acting elements of the *Cyr61/CCN1* promoter as shown in the schematic diagram in the left panel. Cells were transfected with the mutated constructs and assayed for CAT activity upon stimulation with S1P as described previously. To compare data, the native nonmutated construct was set to 100%. Values shown are a representative experiment performed in triplicate. *Denotes statistical significance at $P < 0.05$ when compared with the control.

To identify possible transcriptional elements promoting *Cyr61/CCN1* gene induction, we performed transfection experiments with the *Cyr61/CCN1* promoter–reporter constructs obtained. After transfection, cells were subsequently treated with S1P, lysed and assayed for CAT activity as described in Materials and methods. As shown in Fig. 5A, S1P treatment of cells transfected with the pCyr61/CCN1(-936/+1)-CAT reporter construct resulted in a nearly 15-fold induction of CAT activity as compared to nontreated cells. Transfection with the shorter promoter construct, pCyr61/CCN1(-436/+1)-CAT conferred only a sixfold induction of CAT activity upon S1P stimulation whereas transfection with the construct pCyr61/CCN1(-276/+1)-CAT resulted in a further decreased reporter gene activity, suggesting that the promoter region between -936 and -436 contains regulatory elements indispensable for the *Cyr61/CCN1* promoter activity and that the region between -476 and -276 contains additional element(s) that further augment the promoter activity. Potential transcription factor binding elements in this region include two CRE elements (CRE1 and CRE2), AP-1 and GATA-2, located at nucleotides -336, -396, -651 and -756, respectively (Fig. 4). To determine the individual contribution of these *cis*-elements to S1P-induced reporter

gene expression, we mutated these *cis*-element sequences and tested the reporter gene activity of the mutated constructs. Mutations in the GATA-2 and either CRE1 or CRE2 sites did not significantly affect the promoter activation of the reporter gene (Fig. 5B). In contrast, mutation of the distal AP-1 element reduced the promoter–reporter construct activity by nearly 45%. In addition, mutations within the third CRE site (CRE3), located in the shorter promoter that was relatively poorly inducible, reduced the induction by one-third. A construct containing a double mutation at the distal AP-1 and the proximal CRE3 sites reduced the reporter activity by more than 75%. This suggests that the distal AP-1 and the proximal CRE3 sites mediate S1P regulation of the reporter gene by the *Cyr61/CCN1* promoter.

Regulation of the *Cyr61/CCN1* promoter by the AP-1 and CREB transcription factors

The AP-1 refers to the DNA binding activity specific for the palindromic sequence 5'-TGAGTCAG-3'. Transcription factors of the basic leucine zipper (B-Zip) family composed of heterodimers of jun–fos or homodimers of jun–jun recognize the AP-1 consensus site while heterodimers like

jun-ATF recognize CRE-like sites. Studies have shown that some substitutions in the consensus sequence are tolerated with only a modest reduction in affinity [28]. The AP-1 like element in the *Cyr61/CCN1* gene promoter is a variant of the AP-1 consensus sequence in which a single-base substitution of the center nucleotide has occurred (5'-TGACTCAG-3'). Moreover, CREB is also a transcription factor of the B-Zip family that binds to CRE-like elements. The consensus CRE is 5'-TGACGTCA-3'. This DNA sequence may be bound by various homodimer or heterodimer combinations of B-Zip transcription factors including CREB homodimers, CREB-ATF heterodimers and dimers consisting of other ATF transcription factors. In addition, there are other structurally related *cis*-elements consisting of at least the same half site (NNNNGTCA) two of which are located within the *Cyr61/CCN1* promoter (CRE1 and CRE2). The CRE3 site sequence in the *Cyr61/CCN1* promoter is 5'-CGACGTCA-3'. The latter is similar to the CRE consensus sequence with the first nucleotide of the first dyad deleted resulting in a pseudopalindromic site.

To further determine the role of AP-1, CREB and their variants in the regulation of the *Cyr61/CCN1* promoter, we used their dominant-negative mutants termed A-fos, K-CREB and A-ATF-2. The potency and efficiency of these dominant-negative mutants to inhibit DNA binding of wild-type B-Zip proteins has been compellingly proven [29,30]. As shown, in Fig. 6A, cotransfection of the cells with A-fos or K-CREB significantly reduced the pCyr61(-936/+1)-CAT promoter-reporter construct induction by SIP while A-ATF-2 had no significant effect. Western blot analyses were performed from parallel experiments to establish whether these dominant negative inhibitors were effectively expressed in the transfected cells. As shown in Fig. 6B, both A-fos and A-ATF-2 proteins were detected with T7-Tag antibody directed against the epitope leader sequence tagged to either A-fos or A-ATF-2 confirming the actual expression of these proteins in the transfected cells. Endogenous c-fos protein levels seem to be elevated in cells treated with SIP as compared to nontreated cells consistent with the inducible immediate early gene pattern of the *c-fos* gene, while endogenous levels of ATF-2 seem unchanged in cells treated with SIP vs. nontreated cells consistent with the constitutive expression pattern of ATF-2. Immunodetection of the dominant negative inhibitor K-CREB was achieved using an anti-CREB antibody although K-CREB is undistinguishable from the endogenous form. However, cells transfected with K-CREB show a stronger CREB protein signal than those transfected with an empty vector. The expression levels of GAPDH show a relatively equal protein loading, indicating that enhanced CREB signal in K-CREB transfected cells is likely the result of the effective expression of K-CREB. Treatment of untransfected cells with SIP had no effect on CREB protein levels (data not shown). Taken together, these data clearly implicate both AP-1 and CREB in the regulation of the *Cyr61/CCN1* promoter activity.

Characterization of signal transduction pathways involved in *Cyr61/CCN1* gene activation

Previous studies have established that the biochemical actions of sphingolipid-derived messengers such as SIP were

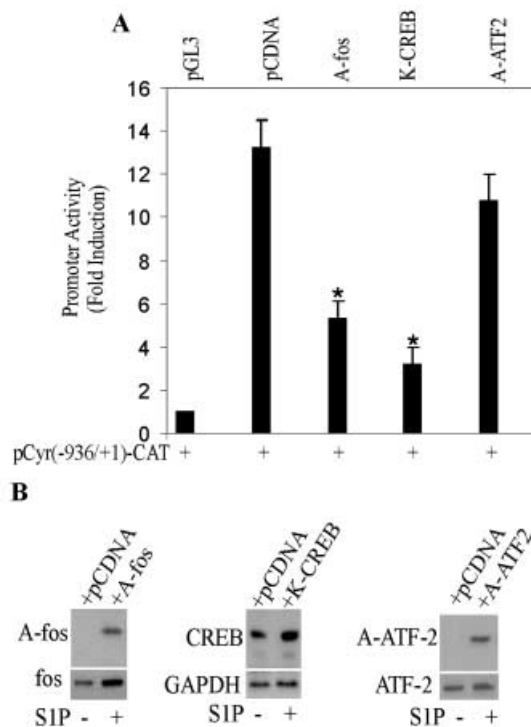


Fig. 6. Selective inhibition of SIP-induced *Cyr61/CCN1* promoter-reporter construct by dominant negative inhibitors of the AP-1 and CREB transcription factors. (A) Cells were transfected in serum-free medium with the promoter-reporter construct pCyr(-936/+1) CAT along with either empty vector (pRC-CMV), A-fos, K-CREB or A-ATF-2 constructs. Twenty-four hours later, cells were treated with or without SIP (10 μ M) for 1 h and assayed for CAT activity. The values indicate the relative CAT activity (means \pm SEM) from a representative transfection experiment performed in triplicate. (B) Expression of the proteins encoded by A-fos, K-CREB and A-ATF-2 constructs as shown by Western blot analysis of cells transfected with the corresponding vectors. Cells were transfected with the indicated vectors and incubated with SIP as described in (A). Cell lysates were prepared and analyzed by Western blotting. Immunodetection of A-fos and A-ATF-2 was achieved by probing the blots with anti- ϕ 10 Ig directed against the 12-amino-acid ϕ 10 leader sequence tagged to A-fos and A-ATF-2 proteins. Immunodetection of endogenous c-fos and ATF-2 was performed with anti-c-fos and anti-ATF-2 Igs, respectively, using the same cell lysates. Detection of K-CREB and endogenous CREB was achieved by using an anti-CREB Ig and equal protein loading is shown by probing the same blot with GAPDH Ig.

mediated through various protein kinase and monomeric GTP-binding protein signaling pathways including MAP kinases and Rho GTPases [18,20,31]. To determine the signal transduction pathways that couple SIP to *Cyr61/CCN1* gene induction, we treated SMCs with pharmacological inhibitors of known signaling molecules. Northern blots of RNA derived from SIP-treated and nontreated cells were hybridized with a *Cyr61/CCN1* DNA probe and hybridization signals were normalized to those of GAPDH (Fig. 7A). Induction of *Cyr61/CCN1* gene expression by SIP was not altered when the cells were treated with specific inhibitors for either protein kinase C, PI 3-kinase or p42/p44 MAP kinase. Similarly, a specific protein kinase A (PKA)

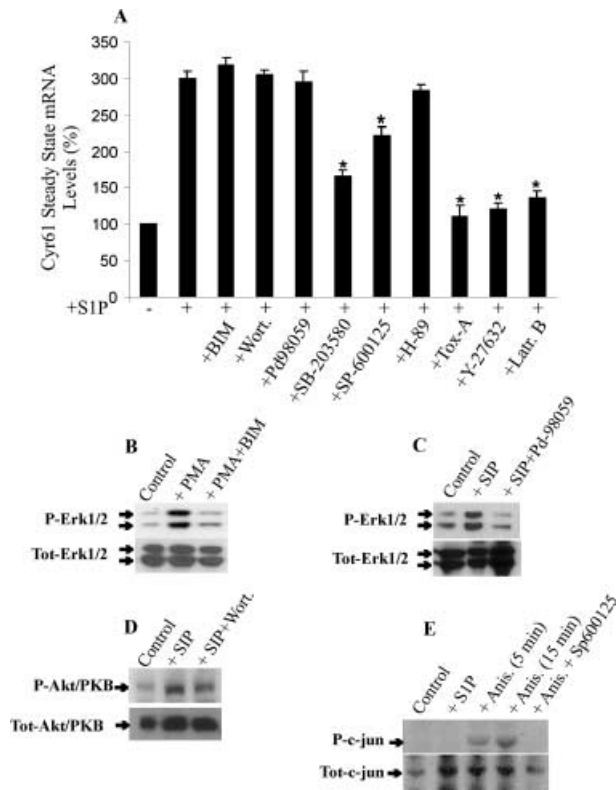


Fig. 7. Pharmacological inhibition of *Cyr61/CCN1* gene expression in S1P-treated cells. (A) Cells were pretreated for 1 h with the indicated pharmacological inhibitors followed by stimulation with S1P (10 μ M) for an additional hour. The inhibitory drugs used were: BIM (10 μ M) for PKC, wortmanin (100 nM) for PI-3 kinase, Pd98059 (20 μ M) for ERK1/2, SB-203580 (10 μ M) for p38, SP-600125 (20 μ M) for JNK, H-89 (1 μ M) for PKA, toxin A (5 ng·mL⁻¹) for Rho GTPases, Y-27632 (10 μ M) for RhoA kinase and latrunculin B (10 nM) for RhoA-mediated actin polymerization. Northern blot analyses of RNA derived from control nontreated and S1P-treated cells were performed to assess the transcript levels of *Cyr61/CCN1* as described in Materials and Methods. Shown is the percentage of the relative increase in mRNA levels. The values are the means \pm SEM ($n = 3$). (B–D) Inhibitory profiles of the pharmacological inhibitors BIM, Pd98059 and wortmanin. Cells were incubated with the indicated inhibitors as described in (A) and further incubated with either PMA (10 μ M) or S1P (10 μ M) for 15 min. P-Erk1/2 and Tot-Erk1/2 refer to phosphorylated and total Erk1/2, respectively. P-Akt/PKB and Tot-Akt/PKB refer to phosphorylated and total Akt/PKB, respectively. (E) Inhibitory profile of SP600125 as shown by its inhibition of JNK-mediated c-jun phosphorylation in cells treated with anisomycin (10 μ g·mL⁻¹) used as a positive control. S1P did not affect JNK activation as shown by the absence of its effects on c-jun phosphorylation. Total c-jun protein is shown as well.

inhibitor did not significantly affect *Cyr61/CCN1* gene expression. In contrast, SB-203580, a specific inhibitor for the stress-activated protein kinase (SAPK) p38, induced a 38% decrease of *Cyr61/CCN1* mRNA levels in cells treated with S1P. The recently developed inhibitor of JNK, SP-600125, reduced the *Cyr61/CCN1* mRNA levels by 25% in S1P-treated cells [32]. Moreover, treatment of the cells with either toxin A, a general inhibitor of Rho proteins,

or Y-27632, a specific inhibitor of RhoA-associated kinase, nearly abrogated *Cyr61/CCN1* gene expression induced by S1P indicating a preponderant role of RhoA signaling in *Cyr61/CCN1* gene expression. Similarly, treatment of the cells with latrunculin B, a specific agent that disrupts the actin cytoskeleton, significantly reduced the *Cyr61/CCN1* mRNA levels, which is consistent with the role of RhoA in cytoskeletal rearrangement. The efficiency of the pharmacological inhibitors used in our experiments was confirmed by testing their ability to prevent the activation of authentic substrates of their targeted kinases. As expected, exposure of the cells to bis-indolyl maleimide (BIM) prevented phorbol 12-myristate 13-acetate (PMA)-induced Erk1/2 activation (Fig. 7B). Inhibition of S1P-induced Erk1/2 phosphorylation by Pd98059 indicated the effectiveness of this drug while decreased S1P-induced Akt/PKB phosphorylation in wortmanin-treated cells confirmed the selective inhibitory effect of wortmanin (Fig. 7C and D). In contrast, treatment of the cells with S1P did not affect c-jun phosphorylation which is mediated via JNK but seemed to increase the total amount of c-jun protein (Fig. 7E). Treatment of the cells with anisomycin, a well-known activator of JNK, induced c-jun phosphorylation. The latter was completely abrogated in the presence of SP600125, a specific JNK inhibitor.

Next, we sought to determine if the apparent regulation of *Cyr61/CCN1* gene expression through RhoA and SAPK p38 cascades is associated with the actual activation of these pathways or merely a result of nonspecific side-effects of the pharmacological inhibitors used. The activity of RhoA was determined using an activation state-specific binding protein, rhotekin, that forms a complex with the GTP-bound activated form of RhoA only. As shown in Fig. 8A, treatment of the cells with S1P induced a rapid increase in the amount of the active GTP-bound form of RhoA culminating in a sixfold increase after 5 min. S1P effects on RhoA activation was sustained for up to 15 min and did not alter the cellular levels of total RhoA. We also analyzed p38 and JNK phosphorylation status by Western blot and immunodetection analysis with antibodies against their phosphorylated forms, that are determinant of their activation. Our data showed enhanced p38 phosphorylation in S1P-treated cells (Fig. 8B). The maximal extent of activation was achieved within 10 min and was sustained for at least 30 min. In contrast, S1P treatment was, without effects, on JNK phosphorylation consistent of the lack of S1P effects on c-jun phosphorylation. Therefore, the effects of the JNK inhibitor SP-600125 on *Cyr61/CCN1* gene expression are unrelated to the JNK pathways and are likely the result of partial inhibition of the p38 pathway by this inhibitor as reported previously [32].

Interestingly, one of the ways in which these signaling molecules produce gene activation is by the phosphorylation and activation of transcription factors either directly or indirectly by other kinases that they activate. One such transcription factor is CREB that appears to be required for S1P-induced *Cyr61/CCN1* gene expression. Activation of CREB requires phosphorylation at serine 133 and is catalyzed by either PKA, commonly associated with cyclic AMP-elevating agents, or by protein kinases activated by members of the mitogen-activated protein (MAP) kinase family [33–35]. Potential CREB kinases include

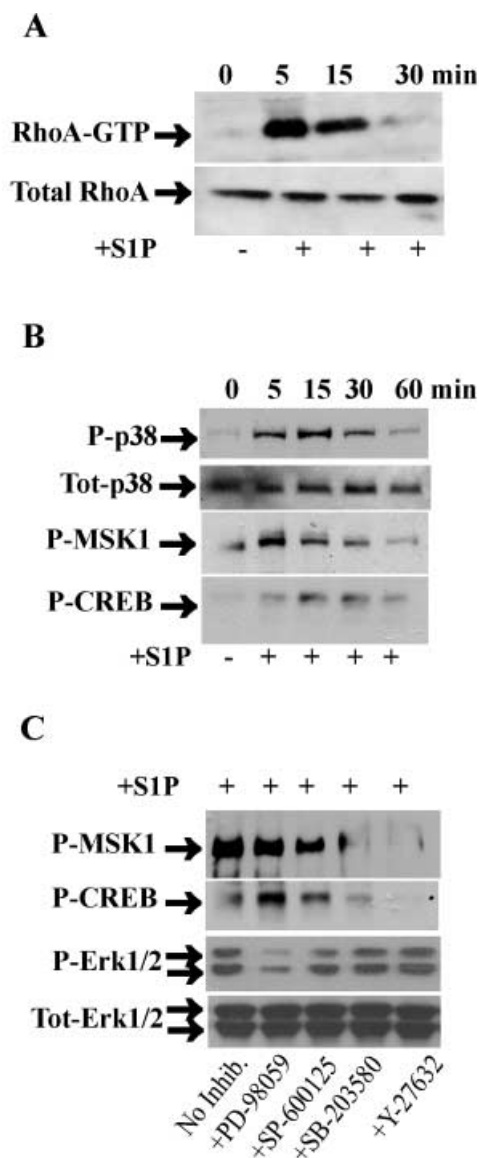


Fig. 8. Immunoblot analyses of RhoA activation and p38 MAP kinase, CREB and MSK1 phosphorylation in SIP-stimulated cells. (A) Cells were stimulated with (10 μ M) SIP for the indicated time periods and the amount of GTP-loaded RhoA (active form of RhoA) was determined by pull-down assay as described in Materials and methods. Total amount of RhoA in the same samples was determined by Western blot and immunodetection analyses. (B) Cells were treated for the indicated time periods with SIP, lysed and 20 μ g of each protein lysate were subjected to SDS/PAGE, transferred to nitrocellulose membrane and immunoblotted with specific antibodies against phosphorylated p38 (P-p38), total p38 (Tot-p38), phosphorylated CREB (P-CREB) and phosphorylated MSK1 (P-MSK1). (C) Cells were pretreated with various pharmacological inhibitors for 1 h followed by incubation with SIP for 15 min. Cell lysates were prepared and resolved by SDS/PAGE and subsequent immunoblotting with monoclonal Igs for either P-CREB, P-MSK1, P-Erk1/2 or Tot-Erk1/2. The blots are representative of at least three separate experiments with similar results.

MAPK-activated kinase 1 (MAPKAP-K1, also called RSK) which is activated by ERK1/2 and mitogen- and stress-activated protein kinase (MSK) which is activated by either ERK1/2 or p38. As SIP-induced *Cyr61/CCN1* gene expression is not mediated through either PKA- or ERK1/2-signaling pathways, we further explored the role of MSK1 in SIP-induced CREB activation. As shown in Fig. 8B, SIP induced both CREB and MSK1 phosphorylation. The latter was increased in a time-dependent manner with peaks at 5 and 15 min of incubation and a progressive decrease thereafter. The phosphorylation of both MSK1 and CREB was blocked by SB-203580 and Y-27632 that inhibit p38 and RhoA kinase activation, respectively (Fig. 8C). In contrast, the phosphorylation of CREB and MSK1 was not depleted by Pd-98059 and SP-600125 that inhibit ERK1/2 and JNK, respectively, consistent with the absence of effects of these signaling molecules on *Cyr61/CCN1* gene expression. Exposure of the cells to Pd98059 inhibited Erk1/2 activation confirming the effectiveness of this drug. These data indicate a prominent role of RhoA and p38 signaling in the activation of CREB via MSK1.

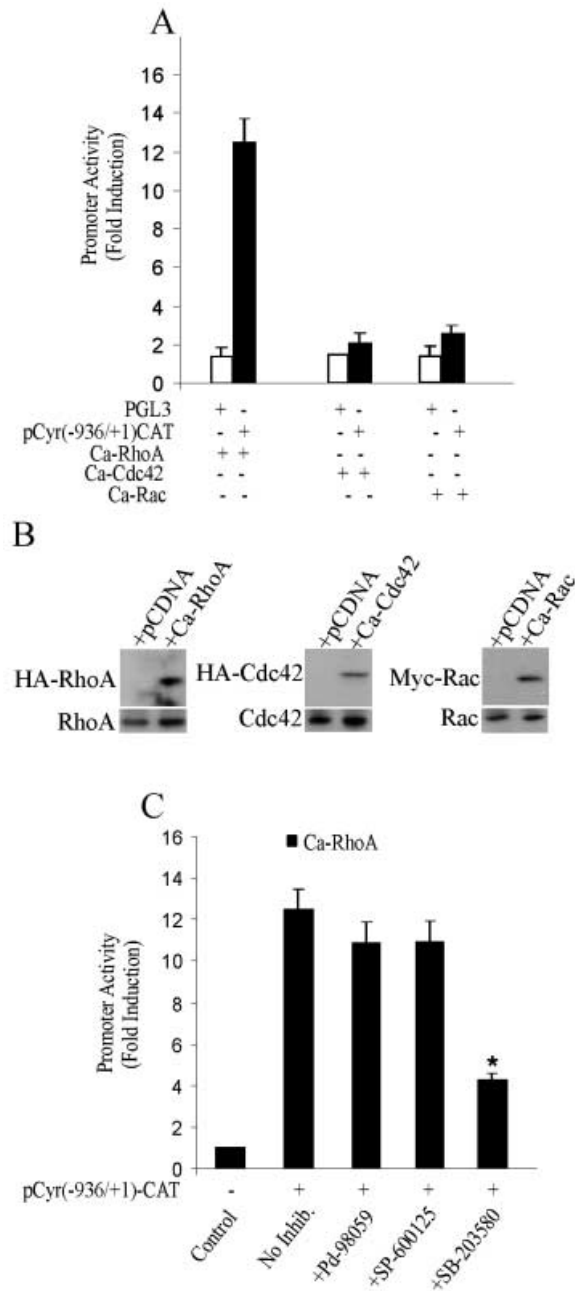
Role of RhoA and p38 kinase in the activation of *Cyr61/CCN1* promoter

To test whether the promoter activity of the *Cyr61/CCN1* gene was dependent on activated RhoA and/or activated p38, we examined the ability of representative Rho proteins such as RhoA, Cdc42 and Rac, to stimulate the reporter gene driven by the *Cyr61/CCN1* promoter. We performed coexpression experiments by transfecting SMCs with the CAT reporter construct driven by the *Cyr61/CCN1* promoter [pCyr-(936/+1)-CAT] along with an expression vector over-expressing constitutively active (Ca) forms of either RhoA, Cdc42 or Rac. As shown in Fig. 9A, Ca-RhoA induced a 13-fold increase of *Cyr61/CCN1* promoter activity whereas Ca-Cdc42 and Ca-Rac had a minimal effect. Western blot analyses were performed from parallel experiments to establish whether the transfected Ca-RhoA, Ca-Cdc42 and Ca-Rac were effectively expressed in the cells. As shown in Fig. 9B, the constitutively active forms of these proteins appear to be expressed in the transfected cells although their expression levels seem relatively lower than the corresponding endogenous proteins. The protein band intensity of Ca-RhoA, Ca-Cdc42 and Ca-Rac is largely dependent on the transfection efficiency and/or the efficiency of their immunodetection with antibodies against the epitope peptide tagged to these proteins. Nonetheless, the effective expression of these proteins in the transfected cells further demonstrates the specificity of RhoA effects. Moreover, the ability of Ca-RhoA to stimulate the *Cyr61/CCN1* promoter was significantly decreased when the cells were treated with the p38 inhibitor, SB-20589 (Fig. 9C). These data confirm the observation that this GTPase signals to *Cyr61/CCN1* gene expression, at least in part, through the SAPK p38 pathway.

The effect of p38 kinase on *Cyr61/CCN1* promoter activation was also established in coexpression experiments using expression vectors encoding either Ca-MKK6 or Ca-MKK3 that function as upstream activators for the p38 MAP kinase. As shown in Fig. 10A, either Ca-MKK6 or

Fig. 9. Regulation of *Cyr61/CCN1* promoter through RhoA signaling.

Cultured SMCs were transfected with the *Cyr61/CCN1* promoter–CAT reporter construct along with either the empty vector pCDNA3, or Ca-RhoA, Ca-Cdc42 or Ca-Rac constructs. The Svbgl plasmid was included in the transfection mixture to normalize for transfection efficiency. Twenty-four hours later, cells were incubated in serum-free medium for 6 h and their lysates were assayed for CAT activity. The latter was expressed relative to the control CAT activity of a promoterless pGL3–CAT construct. Values are the means \pm SEM of triplicate samples from a typical experiment. Nearly identical results were obtained in three separate experiments. (B) Expression of the proteins encoded by Ca-RhoA, Ca-Cdc42 and Ca-Rac constructs as shown by Western blot analysis of cells transfected with the corresponding vectors. Immunodetection of Ca-RhoA and Ca-Cdc42 proteins was achieved by probing the blots with a HA-Tag Ig while that of Ca-Rac protein was achieved by using a Myc-Tag Ig. Endogenous RhoA, Cdc42 and Rac were detected in the same cell lysates using anti-RhoA, anti-Cdc42 and anti-Rac Igs, respectively. (C) Cells were transfected with the *Cyr61/CCN1* promoter–CAT–reporter construct along with Ca-RhoA. After 24 h, cells were incubated in serum-free medium with the pharmacological inhibitors Pd-98059 (20 μ M), SP600125 (20 μ M) or SB-203580 (10 μ M) for 6 h. CAT activity was further measured and expressed as described in (A).



Ca-MKK3 increased the promoter activity by seven- to ninefold. Western blot analyses from parallel experiments showed an increased p38 phosphorylation in cells transfected with either Ca-MKK3 or Ca-MKK6 indicating that the transfected Ca-MKK3 and Ca-MKK6 constructs express the active forms of MKK3 and MKK6 (Fig. 10B). Furthermore, incubation of the transfected cells with p38 inhibitor, SB203580, significantly decreased the promoter–reporter activity by 65 and 55% when the cells were cotransfected with Ca-MKK6 and Ca-MKK3, respectively, indicating that p38 MAP kinase intervenes downstream of MKK3 and MKK6 (Fig. 10C). Taken together, these data link the *Cyr61/CCN1* promoter activity to the activation of the SAPK p38 pathway.

Discussion

The present work has focused on investigating the molecular mechanisms whereby the *Cyr61/CCN1* gene is activated in SMCs exposed to S1P, a bioactive lysolipid and G-protein-coupled receptor agonist. The *Cyr61/CCN1* gene, which is expressed at a quasi-undetectable level in nonstimulated SMCs, is markedly induced in a time-dependent manner, at the mRNA and protein levels. We compared the expression profile of the *Cyr61/CCN1* gene to that of the *CTGF/CCN2* gene and showed that S1P coordinately regulates the expression of both *Cyr61/CCN1* and *CTGF/CCN2* but the final level of control is unequivocally transcriptional for *Cyr61/CCN1* and possibly transcriptional and post-transcriptional, albeit to different extents, for *CTGF/CCN2*. The difference between *Cyr61/CCN1* and *CTGF/CCN2* gene regulation may lie within their respective mRNA sequences that contains within it the information needed to determine their stability within the cells. Interestingly, Kondo *et al.* have identified, in the 3'-untranslated region of the *CTGF/CCN2* gene a 91-nucleotide fragment that may act as a *cis*-acting element

forming a stable secondary structure that interacts with proteins involved in either mRNA stabilization or destabilization [36,37]. Such a regulatory element was not found in the *Cyr61/CCN1* gene. Effectors like S1P may, in all likelihood, induce stabilization of *CTGF/CCN2* mRNA through post-translational modifications of pre-existing destabilizing proteins that reduce their RNA binding affinity. Additionally, the relative decrease of *CTGF/CCN2* protein levels appeared to be slower than that of *Cyr61/CCN1* protein indicating a potential increase of the *CTGF/CCN1* protein stability as well. Upon its secretion, *CTGF/CCN2* protein was shown to be internalized from the cell surface in endosomes and accumulates in juxta-nuclear organelles from which it translocates into the cytosol and the nucleus [38].

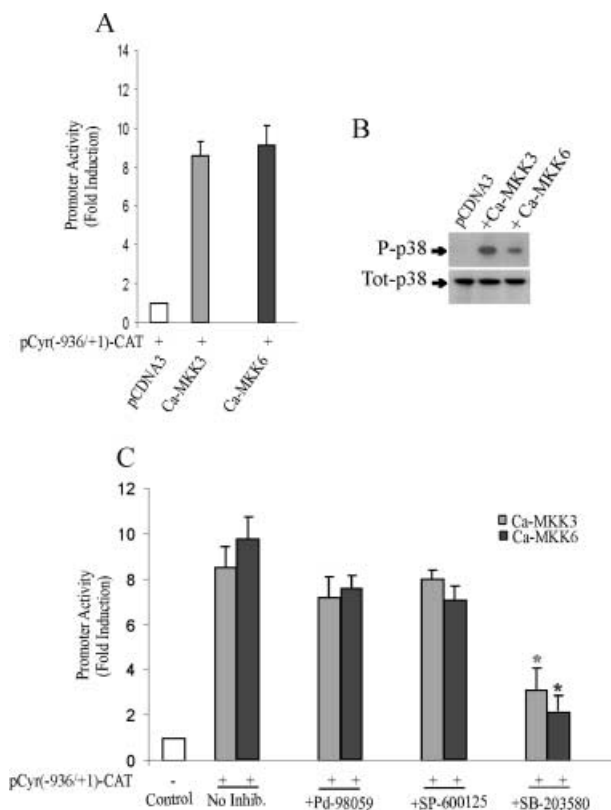


Fig. 10. Regulation of Cyr61/CCN1 promoter through p38 MAP kinase signaling. Cultured SMC were transfected with the Cyr61/CCN1 promoter–CAT reporter construct along with either the empty vector pCDNA3, or the same vector that expresses the constitutively active (Ca) form of either MKK3 or MKK6. The Svβgal plasmid was included in the transfection mixture to normalize for transfection efficiency. Twenty-four hours later, cells were incubated in serum-free medium for 6 h and their lysates were assayed for CAT activity that was expressed relative to the control CAT activity of the promoterless pGL3–CAT construct. Values are means \pm SEM of triplicate samples from a typical experiment. Nearly identical results were obtained in three separate experiments. (B) Western blot analysis of p38 phosphorylation in cells transfected with either pCDNA3, Ca-MKK3 or Ca-MKK6. The same blot was probed with total p38 as an indication of total protein loading. (C) Cells were transfected with the Cyr61/CCN1 promoter–CAT–reporter construct along with either Ca-MKK3 or Ca-MKK6. After 24 h, cells were incubated in serum-free medium together with the pharmacological inhibitors PD098059 (20 μ M), SP600125 (20 μ M) or SB-203580 (10 μ M) for 8 h. CAT activity was further measured and expressed as described in (A).

Data from our transfection experiments showed that a 936-bp DNA fragment of the human Cyr61/CCN1 promoter was functional and inducible in cells exposed to S1P. This promoter fragment represents a continuous region of high homology between human and mouse with conserved transcription factor-binding sites. Using a combination of *cis*-element mutagenesis and the expression of dominant-negative inhibitors of transcription factors that bind to these *cis*-elements, we provided evidence that a proximal CRE and distal AP-1 *cis*-elements are critical for the activity of Cyr61/CCN1 promoter. Mutation of both the CRE and the

AP-1 sites caused an additive reduction in the promoter activity suggesting that transcription factors bound to these two sites independently regulate Cyr61/CCN1 promoter activity.

Both CREB and AP-1 promote gene transcription through association with their specific DNA binding sites in the promoters of their targeted genes [39–41]. CREB transactivation is stimulated through phosphorylation at serine 133, which increases its association with transcriptional adapter proteins like CREB-binding protein (CBP) or other transcription coactivators that interact with the basal transcriptional machinery and increase the rate of transcription. Perhaps, activation of CREB in S1P-treated cells leads to recruitment of coactivators such as CBP/P300 that physically interact with AP-1 and increase gene transcription. Indeed, a ‘cross talk’ between the CBP and AP-1 components, *c-fos* and *c-jun*, was previously reported [42,43]. Meanwhile, CREB’s serine 133 phosphorylation state is determined by the level of activity of a myriad of signaling cascades that leads to the activation of CREB kinases such as PKA, RSK, calmodulin kinase and MSK1/2. It was suggested that the group of genes that is activated by CREB may depend on the kinase phosphorylating CREB through, yet, unknown mechanisms [44]. In our pharmacological studies, we found that the induction of *Cyr61/CCN1* gene expression was PKA-independent and was not mediated through the ERK1/2 MAP kinase pathway that activates the CREB kinase, RSK. Treatment of the cells with forskolin, which increases cAMP production and induces PKA activation, did not affect the expression of *Cyr61/CCN1* gene (data not shown). We found that RhoA GTPase and p38 MAP kinase pathways predominantly mediate *Cyr61/CCN1* gene induction. Consistent with these data, specific inhibitors of p38 and RhoA alter the phosphorylation state of both CREB and the CREB kinase, MSK1 in S1P-treated cells indicating that S1P-induced *Cyr61/CCN1* gene expression involves RhoA and p38 activation of CREB through MSK1. Compared with other CREB kinases, MSK1 was reported to have a far higher affinity for CREB, indicating that MSK1 might have a primary function in regulating CREB activity [45].

Perhaps, the ability of a CREB target gene to respond to one signal (e.g. MSK1) but not to another (e.g. PKA), despite comparable serine 133 phosphorylation of CREB, could reflect differences in occupancy of the CRE site over the promoter or the ability of CREB to recruit the transcriptional apparatus [46,47]. It has been shown that CREB phosphorylation, induced by a signaling cascade other than that involving PKA, is not sufficient for gene induction, and recruitment of additional transcription factors is required. For instance, *c-fos* promoter activation by UV radiation involves MSK1/2-dependent phosphorylation of CREB [48,49]. However, only 50% of *c-fos* induction by UV radiation can be blocked by a dominant-negative form of CREB. Mutation of the CRE site in the *c-fos* promoter caused a 50% reduction in *c-fos* and the remaining 50% was unaffected by a dominant-negative CREB indicating that *c-fos* promoter can be transcribed independently of CRE site and CREB [48]. It was suggested that induction of *c-fos* likely results from the direct p38- and ERK-catalyzed activation of the transcription factor TCF that binds to the SRE in the *c-fos* promoter. Similarly the

transcription of *junB* has been reported to be controlled by both SRE and CRE-like sequences which are located at 5' and 3' flanking regions of the gene [50,51]. Correspondingly, *Cyr61/CCN1* gene activation in response to S1P requires additional promoter-bound factors such as AP-1 that further augments the effects of CREB. In agreement with this, treatment of the cells with curcumin, a specific inhibitor of AP-1, significantly diminished S1P-induced endogenous *Cyr61/CCN1* gene expression (data not shown).

Transcriptional activation of AP-1-regulated genes is mediated by fos-jun heterodimers and is highly dependent upon c-fos protein expression. Expression of the c-jun and c-fos genes does not require newly synthesized proteins and involves mainly post-translational modification of pre-existing proteins [52]. The search for molecules regulating the activity of c-fos and c-jun revealed the existence of an intricate network of biochemical routes involving one or more cytoplasmic kinase cascades acting on the MAP kinase family [53]. In particular, the GTP-binding protein RhoA can stimulate the expression of either c-fos or c-jun. Studies have shown that activated RhoA stimulates the c-fos promoter through recruitment by DNA-bound SRF of an, as yet, unidentified accessory factor [54]. The regulation of c-jun is complex and may involve an increase in the levels of c-jun protein and/or phosphorylation of specific serines (63 and/or 73) by JNK. Stimulation of our cells with S1P, while it induced an increase in the c-jun protein levels, did not seem to promote JNK activation or c-jun phosphorylation. Similarly, it has been reported that c-jun regulation of cell cycle progression was independent of its phosphorylation [55].

Moreover, activated RhoA was also found to stimulate c-jun expression and c-jun promoter activity. RhoA activation can initiate a linear kinase cascade involving PKN, a Rho effector molecule, the p38 MAP kinase, and the consequent stimulation of transcription factors such as ATF-2 and MEF-2 which act on the c-jun promoter through AP-1 and MEF-2 response elements [56]. Using activated forms of Rho GTPases, we have demonstrated that RhoA specifically enhances the promoter activity of *Cyr61/CCN1*. Similarly, S1P-induced *Cyr61*-promoter-reporter activity was significantly reduced in cells transfected with a dominant negative form of RhoA but was unaffected in cells transfected with a dominant negative form of either Cdc42 or Rac (data not shown). Furthermore, the stimulatory effect of RhoA was significantly diminished when the distal AP-1 site was mutated and further reduced when both distal AP-1 and proximal CRE sites were simultaneously mutated (data not shown). Taken together, these observations indicate that activated RhoA regulates the *Cyr61/CCN1* promoter activity through the AP-1 and CREB transcription factors.

The molecular steps between RhoA and *Cyr61/CCN1* gene expression seem to involve p38 activation as inhibiting p38 MAP kinase pathway with SB-203580 partially blocked transactivation by constitutively active RhoA, indicating that p38 activation is downstream of RhoA. Previous studies using a variety of complementary approaches, have shown that RhoA stimulates the activity of endogenous p38. Cell surface receptors that stimulate RhoA, such as lysophosphatidic acid, can effectively stimulate p38 and the inhibition of Rho proteins, by the use of C3 toxin, inhibits

this response selectively [56]. Additionally, the ability of constitutively active forms of the upstream activators of p38, MKK3 and MKK6, to transactivate the *Cyr61/CCN1* promoter-reporter construct provided additional evidence supporting the involvement of the p38 pathway in the activation of the *Cyr61/CCN1* gene. In agreement with this, the *Cyr61/CCN1* promoter-reporter construct, mutated in the proximal CRE3 and/or the distal AP-1 sites, were less responsive to coexpression of the active forms of MKK6 and MKK3 supporting the finding that both the CREB and AP-1 transcription factors are downstream targets of the p38 pathway (data not shown).

Previous studies have suggested that the effects of RhoA on gene transcription may be secondary to its actions on the actin cytoskeleton [54,57]. Activation of RhoA is known to cause the bundling of actin filaments in stress fibers, thus, RhoA likely plays a regulatory role whenever filamentous actin is used to drive cellular processes. Inhibiting actin polymerization with latrunculin B, blocked S1P-induced *Cyr61/CCN1* gene expression, which supports a model in which RhoA-mediated increase in filamentous actin regulates gene transcription. Our previous observation that jasplakinolide, an actin polymerizing drug, solely activates *Cyr61/CCN1* gene transcription, is also consistent with this model [2]. Such a regulatory mechanism has been demonstrated for a subset of serum-response factor target genes such as SRF and vinculin [54]. However, the precise mechanisms by which actin dynamics affect gene transcription are currently unknown. The role of the actin cytoskeleton in gene transcription may simply reflect the importance of the cytoskeleton components in relaying signals between signaling molecules. Another potential explanation suggests a model whereby, in the absence of Rho-induced actin polymerization, G-actin inhibits transcription factors either directly or by sequestering cofactors required for their activation [54,58]. Further investigation of the signaling pathway coupling the actin cytoskeleton and *Cyr61/CCN1* gene expression is required to better understand the interactions between actin dynamics and genetic programming in the cells.

Within this study, we have achieved our initial objective, which was to identify cytoplasmic and nuclear events that could activate *Cyr61/CCN1* gene expression. We have demonstrated that S1P stimulates *Cyr61/CCN1* gene expression through RhoA GTPase and that additional signaling, through the p38 MAP kinase pathway, is critical for such regulation. Similarly, we have found that activation of serpentine receptors through lysophosphatidic acid regulates the *Cyr61/CCN1* gene in a RhoA- and p38 pathway-dependent manner (data not shown). Therefore, RhoA and/or p38 activation may serve as a convergence point for the various chemical and physical factors known to regulate both *Cyr61/CCN1* gene expression and RhoA and/or p38 activation. In particular, mechanical stretch and contractile agonists such as thrombin are well-known regulators of both *Cyr61/CCN1* gene expression and RhoA GTPase activation. Whether these chemical and physical stimuli regulate *Cyr61/CCN1* gene expression via RhoA, p38 and/or changes in actin dynamics will be important to investigate in future studies.

In summary, both CREB and AP-1 seem to be important determinants of *Cyr61/CCN1* promoter activity and the

activation of RhoA GTPase and p38 MAPK appears to be required for CREB- and AP-1-mediated Cyr61/CCN1 promoter activation. It is possible that both factors collaborate functionally to elicit activation of the promoter. Further studies should be directed towards gaining further insights into this aspect of *Cyr61/CCN1* gene regulation.

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