Regulation of the Versican Promoter by the β-Catenin-T-cell Factor Complex in Vascular Smooth Muscle Cells*

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The proteoglycan versican is pro-atherogenic and central to vascular injury and repair events. We identified the signaling pathways and promoter elements involved in regulation of versican expression in vascular smooth muscle cells. Phosphatidylinositol 3-kinase inhibitor, LY294002, significantly decreased versican-luciferase (Luc) promoter activity and endogenous mRNA levels. We further examined the roles of protein kinase B and glycogen synthase kinase (GSK)-3β, downstream effectors of phosphatidylinositol 3-kinase, in the regulation of versican transcription. Co-transfection of dominant negative and constitutively active kinase B constructs with a versican-Luc construct decreased and increased promoter activity, respectively. Inhibition of GSK-3β activity by LiCl augmented accumulation of β-catenin and caused induction of versican-Luc activity as well as versican mRNA levels. β-Catenin has no DNA binding domain, therefore it cannot directly induce transcription of the versican promoter. Software analysis of the versican promoter revealed two potential binding sites for T-cell factors (TCFs), proteins that confer transcriptional activation of β-catenin. Electrophoretic mobility shift and supershift assays revealed specific binding of human TCF-4 and β-catenin to oligonucleotides corresponding to a potential TCF binding site in the versican promoter. In addition to binding assays, we directly assessed the dependence of versican promoter activity on TCF binding sites. Site-directed mutagenesis of the TCF site located ~492 bp relative to the transcription start site markedly diminished versican-Luc activity. Co-transfection of TCF-4 with versican-Luc did not increase promoter activity, but addition of β-catenin and TCF-4 significantly stimulated basal versican promoter activity. Our findings suggest that versican transcription is predominantly mediated by the GSK-3β pathway via the β-catenin-TCF transcription factor complex in smooth muscle cells, wherein such regulation contributes to the normal or aberrant formation of provisional matrix in vascular injury and repair events.

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1 The abbreviations used are: PG, proteoglycan; ECM, extracellular matrix; GSK, glycogen synthase kinase; Luc, luciferase; MCDB, molecular and cellular developmental biology-131; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; SMC, smooth muscle cell; TCF, T-cell factor; LEF, lymphoid enhancer factor; PDGF, platelet-derived growth factor; DN-PKB, dominant negative mutant of PKB1 construct; CA-PKB, constitutively activated mutant of PKB1; CtBP, C-terminal binding protein; wt-PTEN, wild-type PTEN; mut-PTEN, mutant PTEN; EMSA, electrophoretic mobility shift assay.

Findings from our laboratory and others indicate that the proteoglycan (PG) versican is one of several extracellular matrix (ECM) molecules that accumulates in vascular lesions (1–3). Versican is generally considered to be pro-atherogenic because of its ability to trap cholesterol-rich lipoproteins (4–6) in addition to its crucial role in regulation of cell adhesion, survival, proliferation, and migration and ECM assembly, all fundamental processes involved in vascular disease (4–7).

The complete versican gene structure has been elucidated in humans (8, 9) and the mouse (10). The human and murine genes prove to be remarkably conserved in genomic organization. Both versican genes extend for ~90 kb and contain 15 exons that align in an identical manner with the protein subdomains. Naso et al. (9) report that the human versican gene has one transcription start site. Meanwhile, sequence analysis reveals potential binding sites for several transcription factors in addition to the TATA box. Transient expression assays of reporter constructs driven by an 876-bp (~632/+240 relative to the transcriptional start site) piece of the versican promoter in HeLa cells and IMR-90 embryonic lung fibroblasts have shown significant expression. These results indicate that the human versican 5′-flanking sequence contains promoter and enhancer elements able to drive reporter gene expression in cells derived from epithelial or mesenchymal tissues (9).

Various growth factors and mediators influence the expression of versican. Studies using arterial smooth muscle cells (SMCs) have demonstrated that transforming growth factor β1 and platelet-derived growth factor (PDGF)-AB increase versican mRNA levels, core protein synthesis, and glycosaminoglycan chain length (11). Similarly, normal human gingival fibroblasts respond to treatment with either transforming growth factor β or PDGF-BB by increasing versican mRNA levels (12, 13). In contrast, the pro-inflammatory cytokine interleukin-1β appears to decrease the synthesis of this PG in human gingival fibroblasts and arterial SMCs (14). Data from recent investigations suggest that versican synthesis by mesenchymal cells can be regulated by physical stimuli, including cell density and mechanical strain (15). In vitro experiments using monkey arterial SMCs have shown that PDGF-BB stimulates versican core protein expression; this signaling apparently occurs through a receptor tyrosine kinase.
Regulation of Versican Promoter by β-Catenin/TCF Signaling

kinase-dependent, protein kinase C-independent pathway (16). Angiotensin II-mediated stimulation of SMC versican expression is regulated by epidermal growth factor receptor-dependent tyrosine kinase pathways (17).

The canonical Wnt-wingless signaling pathway regulates various biologic processes including early embryogenesis and neoplasia by increasing the stability and transcriptional activity of a key mediator, β-catenin (18–20). In the absence of Wnt ligand, GSK-3 promotes the phosphorylation of β-catenin at key serine/threonine residues, targeting it for degradation through the ubiquitin-ligase pathway (21). In response to Wnt, the GSK-3-binding protein inhibits GSK-3 activity (22). Some growth factors can regulate GSK-3 activity by mediating its phosphorylation at serine 9 independent of Wnt ligand. Although serine 9 phosphorylation of GSK-3 is associated with its inactivation, Wnt ligand does not necessarily regulate this phosphorylation (23). GSK-3 inactivation leads to β-catenin stabilization and translocation into the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (LEF) family proteins to form a transcription factor complex that activates target genes such as the matrix metalloproteinase-7, fibronectin, vascular endothelial growth factor, cyclin D1, and c-myc (24).

A variety of mitogenic stimuli including Wnts, insulin, epidermal growth factor, and PDGF result in catalytic inactivation of GSK-3. The catalytic inactivation of GSK-3 induced by most polypeptide mitogens is reversible by treatment with serine/threonine-specific phosphatases (25). The inactivation event has been demonstrated to be due to phosphorylation of serine 21 and serine 9 of GSK-3α and GSK-3β, respectively (26–28). These residues are specific targets for several protein-serine kinases, including PKB, pp90rsk, and cyclic AMP-dependent protein kinase A (29, 30). The inactivating biochemical consequence of phosphorylation by all three enzymes is identical; what differs is the initiating signal. Thus, activation of the phosphatidylinositol 3-kinase (PI3K) pathway (usually via receptor tyrosine kinase activation) results in stimulation of PKB. Inactivation of GSK-3 in response to many mitogens can be inhibited by antagonists of PI3K such as LY294002 inhibitor (25). These mechanisms are all independent of Wnt-induced regulation of GSK-3. Most protein kinases are induced by cellular stimuli, whereas GSK-3 is shut down. In addition, the enzyme has a broad variety of target proteins, most of which are inactivated by phosphorylation of GSK-3. Thus, inhibition of this one enzyme will tend to induce the functions of a diverse array of targets including transcription factors and other regulatory molecules (31).

Despite the importance of versican in vascular pathophysiology, the function and regulation of expression of this versatile molecule in vitro and in vivo are unknown. Our results suggest that a signaling molecule activated by 3-phosphoinositides, and GSK-3 promotes the phosphorylation of β-catenin at key serine/threonine residues, targeting it for degradation through the ubiquitin-ligase pathway (21). In response to Wnt, the GSK-3-binding protein inhibits GSK-3 activity (22). Some growth factors can regulate GSK-3 activity by mediating its phosphorylation at serine 9 independent of Wnt ligand. Although serine 9 phosphorylation of GSK-3 is associated with its inactivation, Wnt ligand does not necessarily regulate this phosphorylation (23). GSK-3 inactivation leads to β-catenin stabilization and translocation into the nucleus, where it binds to T-cell factor/lymphoid enhancer factor (LEF) family proteins to form a transcription factor complex that activates target genes such as the matrix metalloproteinase-7, fibronectin, vascular endothelial growth factor, cyclin D1, and c-myc (24).

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Despite the importance of versican in vascular pathophysiology, the function and regulation of expression of this versatile molecule in vitro and in vivo are unknown. Our results suggest that a signaling molecule activated by 3-phosphoinositides, namely PKB, plays a critical role in serum-stimulated versican transcription. Furthermore, we provide evidence that phosphorylation and inhibition of GSK-3β by PKB and subsequent activation of the β-catenin-TCF complex are essential for transcription from the versican promoter.

**Experimental Procedures**

Isolation and Primary Culture of Rat Aortic SMCs—A rat aortic SMC culture was established by a modification of the enzymatic dispersion technique (32, 33). Briefly, four adult male Fisher rats (275–350 g) were killed by cervical dislocation and tracheal occlusion, and the thoracic aorta was removed and immediately washed in MCDB-131 medium (Sigma–Aldrich). Enzyme I (0.5 mg/ml collagenase II; Worthington Biochemical Corp., Freehold, NJ) was applied to the exposed media, and the tissue was incubated for 20 min at 37 °C to loosen the media from the underlying adventitia. Medial strips were removed with sterile forcesps, taking care not to reach the adventitial layer, transferred to a 35-mm tissue culture dish containing 500 μl of Enzyme II (0.5 mg/ml collagenase II, 0.2 mg/ml elastase; Worthington Biochemical Corp.), and minced. Medial tissue from the aortas of all four rats was pooled, additional Enzyme II solution was added to the dish, and the tissue was incubated at 37 °C for 2.5 h with pipetting at regular intervals to disperse cells. Liberated cells were subsequently pelleted at 1000 rpm for 5 min, resuspended in 1 ml of MCDB-131 containing 20% newborn calf serum, and seeded into a 35-mm tissue culture dish. Cells were grown to confluence, released by trypsinization, and subcultured at a density of 1.0 × 10^5 cells/cm^2 in MCDB-131 supplemented with 5% newborn calf serum.

***Generation of Promoter Reporter, Mutant, and Deletion Constructs***—A 752-bp versican promoter (−654/+118) and a shorter fragment (−438/+118) corresponding to the versican promoter sequences (43). During these constructs were generated by PCR from human genomic DNA and used with appropriate sets of primers (available upon request). These inserts were cloned into a pGL3 basic vector (Promega) by standard molecular biology techniques and called wt-versican-Luc and −438del-versican-Luc as depicted in Fig. 5A. The putative TCF binding sites (TCCCTTTCAGG and TCCTTCTTCTGAT) at positions −546 and −492 bp, respectively, were mutated in the wt-versican-Luc as denoted in Fig. 5A. The wild-type-directed mutagenesis using a QuickChange mutagenesis kit from Stratagene as described previously (35). The mutated inserts were generated by PCR and then inserted into the promoterless luciferase vector pGL3-Basic. All constructs were verified by sequencing.

**Plasmid Constructs**—The DN-PKB (Upstate Biotechnology), CA-PKB (Upstate Biotechnology), and empty vector control were used in transient and stable transfection of SMCs and HeLa cells, respectively. The expression vectors, pCMV-β-galactosidase (Promega), pCMV-wt-PTEN and pCMV-PTEN were kindly provided by J. Dixon (Department of Pharmacology, University of California, San Diego, CA) and subcloned into pXJ41-neo (Stratagene) to generate expression vectors containing the human PTEN gene. The expression vectors TCF-1E and TCF-4E harboring the wild-type human TCF-4 gene and chimeric TCF-1 construct lacking C-terminal binding protein (CiBP) structural domain, respectively, were a gift from A. Hecht (Max-Planck-Institute of Immunobiology, Freiburg, Germany) (36). The myc-tagged dominant negative TCF-4 (ΔNTCF-4) was kindly provided by H. Clevers (Department of Immunobiology, University Medical Center Utrecht, The Netherlands) (37). Wild-type GSK-3β was a kind gift from G. Cooper (Department of Pathology, Harvard Medical School, Boston, MA) (38).

**Transfection and Luciferase Activity Assays**—Starved SMCs were transiently transfected in 6-well plates using up to 2 μg of plasmid DNA and FuGENE 6 reagent (Roche Applied Science) according to the procedure recommended by the manufacturer. In brief, a 3:1 ratio of FuGENE 6 reagent (in microliters) to plasmid (in micrograms) was incubated for 30 min at room temperature in incomplete medium before addition to 70–80% confluent cells in medium containing the mediator of interest or in complete medium for the period of time indicated in the figure legends. After the indicated incubation period, cells were lysed, and luciferase activities were measured with a kit from Promega according to the manufacturer’s protocol. Protein concentrations were measured with a Bradford protein assay kit from Bio-Rad, and luciferase values were normalized to the obtained protein concentrations. In some transfection experiments, normalization was done by LacZ reporter (Promega), and β-galactosidase assays were performed according to the manufacturer’s protocol.

**Cytoskeletal Staining**—SMCs were grown in 6-well plates. Cells were lysed with 200 μl of lysis buffer. For PKB, lysis buffer contained 50 mm HEPES (pH 7.6), 1 mm EDTA, 5 mm EGTA, 10 mm MgCl, 50 mm β-glycerophosphate, 1 mm NaVO, 10 mm NaF, 30 mm sodium pyrophosphate, 2 mm dithiothreitol, and 1 mm 4-(2-aminoethyl)-benzenesulfonyl fluoride. For GSK-3, lysis buffer contained 20 mm Tris (pH 7.5), 25 mm β-glycerophosphate, 100 mm NaCl, 1 mm NaVO, 2 mm EGTA, 2 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μM 4-(2-aminoethyl)-benze-
nesulfonyl fluoride. Samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were probed with anti-phospho-PKB (Ser-473; Cell Signaling Technology) or anti-phospho-GSK-3 (Ser-9; Oncogene Research Product) antibodies. Horse-radish peroxidase-conjugated goat anti-rabbit or anti-mouse antibodies were used as secondary antibodies. The levels of wt-PTEN and mutant-PTEN were determined 48 h post-transfection of PC3 cells by Western blot analysis using antibody against PTEN (Santa Cruz Biotechnology). The bound secondary antibody was detected by enhanced chemiluminescence (Amersham Biosciences).

**RNA Extraction and cDNA Synthesis—**Total RNA was isolated from treated and untreated SMCs using the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). All preparations were treated with RNase-free DNase (Qiagen) to remove genomic DNA. RNA (0.5–1 μg) was reverse transcribed in a total volume of 20 μl in the presence of 200 units of SuperScript RNase H- Reverse Transcriptase (Invitrogen), 40 units of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen), and 0.5 μg of Oligo(dT) Primer (Invitrogen) according to the manufacturer's instructions.

**SYBR Green Quantitative Real-time Reverse Transcription-PCR—**Quantitative two-step real-time reverse transcription-PCR was performed using a LightCycler (Roche Applied Science) in order to assess versican mRNA expression in SMCs. β-Actin was used as a housekeeping gene. Primer pairs were designed to flank an intron-containing sequence. PCR conditions used included 3 mM MgCl₂, 0.3 μM forward and reverse primers, and 2 μl of LightCycler FastStart DNA Master SYBR Green 1 Mix (Roche Applied Science) in a final volume of 20 μl. The reactions were loaded in the LightCycler glass capillaries, closed, centrifuged, and placed in the LightCycler rotor. The cycling program consisted of 10 min of initial denaturation at 95 °C, 45 cycles of 95 °C for 5 s, 55 °C for 5 s, and 72 °C for 20 s, and single detection for 1 s with a single fluorescence acquisition (ramp rates, 20 °C/s). The analytical melting program was 95 °C for 0 s and 65 °C for 15 s, increasing to 95 °C at a ramp rate of 0.2 °C/s, with continuous fluorescence acquisition. Each sample was run in triplicate. A standard curve was included in each run. Standards were prepared by cloning the target sequence into plasmid DNA. The data were analyzed by using the second-derivative maximum of each amplification reaction and relating it to its respective standard curve. The results from the quantitative PCR were expressed as the ratio of the mean target gene measurements to the mean housekeeping gene value for a given sample (target/reference).

**Electrophoretic Mobility Shift Assay—**Individual oligonucleotides were 5′-end [γ-32P]dideoxyadenine triphosphate-labeled with T4 polynucleotide kinase. Labeled oligonucleotides (100 ng) were annealed to equimolar amounts of their complementary strands (unlabeled) by heating to 95 °C for 5 min in Tris-EDTA supplemented with 50 mM NaCl and slowly cooling to room temperature. Double-stranded oligonucleotide probes were purified on a 5% (w/v) polyacrylamide, 0.5× Tris-borate EDTA non-denaturing gel; eluted in 500 μl of elution buffer (0.6 mM NH₄OAc, 0.1% (v/v) SDS, and 1 mM EDTA); and ethanol precipitated prior to use in electrophoretic mobility shift assays (EMSAs). Nuclear extracts used in EMSAs were isolated from SW480 human colorectal carcinoma cells, using a modified version of the method of Dignam et al. (39). Final nuclear protein preparations were collected in buffer C (400 mM NaCl, 20 mM HEPEs (pH 7.4), 25% glycerol, 1.5 mM MgCl₂, 0.2 mM EDTA, and 0.5 mM dithiothreitol). For band shift experiments, 20,000 cpm of labeled oligoduplex probes were added to 5 μl of nuclear extracts in 40 μl of DNA binding buffer (10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 60 mM KCl). To prevent nonspecific binding of nucleic acids, 2 μl of poly(dI-dC) was added to a concentration of 100 μg/ml, and the binding reaction was incubated at room temperature for 10 min prior to electrophoresis. For supershift experiments, 1 μg of goat anti-TCP-4 polyclonal antibody (N-20; 0.4 μg/ml; Santa Cruz Biotechnology) was added to the binding reaction and incubated for 10 min at room temperature prior to electrophoresis. For EMSA interference experiments, 0.2 and 0.4 μg of goat anti-β-catenin polyclonal antibody was used (H-102; 0.4 μg/ml; Santa Cruz Biotechnology). For both supershift and interference experiments, antibody control reactions contained an equivalent mass of normal mouse IgG, and antibody negative reactions were supplemented with an equal volume of phospho-bluffered saline. Protein-DNA complexes were separated from unbound DNA using a 5% (w/v) polyacrylamide, 0.5× Tris-borate EDTA non-denaturing gel run at a constant voltage of 400 V for 1 h. The oligonucleotides corresponding to the TCP/LEF site from versican promoter used for EMSA were as follows: 5′-ACTTCCTTTTGTAGG-GACAG-3′ and 5′-CTGTCCCCATCAAAGGAGGA-3′.

**RESULTS**

The 3-Phosphoinositide-dependent Signaling Mediates Versican Transcription in Vascular SMCs—We examined the role of the PI3K-PKB pathway in induction of versican transcription as a result of serum stimulation in vascular SMCs. First, the levels of activated PKB were determined by Western blot analysis using phosphospecific antibody, after a 30-min pre-incubation of quiescent SMCs with different concentrations of LY294002, a pharmacological inhibitor of PI3K, followed by serum stimulation. LY294002 inhibited activation of downstream PKB at all concentrations tested (Fig. 1 A). Next we examined whether activation of PI3K and subsequent genera-
Regulation of Versican Promoter by β-Catenin/TCF Signaling

PKB Is a Downstream Effector of PI3K in Serum Stimulation of Versican Transcription in SMCs—PKB was suggested to be a major downstream mediator of the PI3K in gene regulation in SMCs (41). To investigate whether it is also involved in the serum stimulation of versican transcription, we co-transfected expression vectors for either a DN-PKB plasmid that is incapable of being phosphorylated in response to different stimuli or a CA-PKB expression plasmid with versican-Luc into SMCs. Empty vector was used as a control plasmid. As shown in Fig. 3, A and B, DN-PKB expression significantly decreased versican promoter activity, and CA-PKB significantly increased versican promoter activity, as compared with the corresponding empty vector control. To further study the contribution of PKB to versican regulation, we used HeLa cells stably transfected with DN-PKB, CA-PKB, or empty vector control plasmids. As expected, the DN-PKB mutant blocked PKB1 phosphorylation and activation in transfected HeLa cells when compared with control cells as determined by measuring the phosphorylation of PKB1 and GSK-3β/β, a PKB-regulated downstream protein (Fig. 3F). In contrast, HeLa cells stably transfected by CA-PKB significantly augmented phosphorylation of PKB1 and GSK-3β/β as compared with control cells containing empty vector (Fig. 3E). We transiently transfected a versican-Luc reporter construct into HeLa cells stably expressing CA-PKB, DN-PKB, and corresponding empty vectors (Fig. 3, C–E). The results were similar to those observed in transient transfections; versican promoter activity was significantly increased and decreased in HeLa cells stably transfected with CA-PKB and DN-PKB, respectively. The effects of DN-PKB and CA-PKB expression on versican-Luc activity are similar to those seen when PI3K is inhibited by LY294002 treatment or on co-transfection of the mut-PTEN expression construct. Overall, these data suggest that PKB is a downstream mediator of PI3K in the serum regulation of versican transcription in SMCs.

Cytoplasmic Accumulation of β-Catenin via GSK-3β Inhibition Stimulates Transcription of Versican in SMCs—We have used various approaches to show the role of GSK-3β in versican regulation (Fig. 4A). To assess whether GSK-3β is involved in up-regulation of versican expression via β-catenin, SMCs were transfected with the versican-Luc promoter construct and treated with 30 mM LiCl, an inhibitor of GSK-3β. LiCl is an inhibitor of GSK-3 that induces accumulation of dephosphorylated β-catenin and increases the activity of synthetic TCF-dependent reporter constructs. Addition of LiCl resulted in a more than 2-fold increase in versican promoter activity (Fig. 4A). Up-regulation of versican mRNA was also evidenced in SMCs after treatment with 30 mM LiCl (Fig. 4B). To confirm the involvement of GSK-3β in versican activation, SMCs were co-transfected with increasing amounts of GSK-3β expression vector, and this was shown to dose-dependently decrease versican promoter activity (Fig. 4C). These findings are consistent with our results using another inhibitor of GSK-3B, SB415286, which caused a similar increase in versican-Luc promoter activity and versican mRNA levels (data not shown). Our results support the notion that GSK-3β regulates versican transcription in SMCs via accumulation of β-catenin.

TCF/LEF DNA Binding Is Involved in β-Catenin-induced Versican Transcription—β-Catenin contains no DNA binding domain and therefore cannot regulate versican promoter activity directly. The proximal versican promoter was searched for transcription factor binding sites that would confer β-catenin transactivation. A search for consensus TCF binding sites in the versican promoter using the MatInspector program (42) revealed the presence of two potential TCF/LEF binding sites.
The Versican Promoter Is Activated by the β-Catenin-TCF Complex—The presence of possible TCF/LEF binding motifs in the versican promoter prompted us to examine the effects of β-catenin and TCF-4 expression on versican promoter activity. Co-transfection of versican-Luc with a β-catenin expression vector significantly and dose-dependently increased versican promoter activity (Fig. 6A). These results demonstrate that the accumulation of cytosolic β-catenin results in increased versican promoter activity.

In humans, there are four known isoforms of TCF, LEF-1, TCF-1, TCF-3, and TCF-4; the most conserved portions among these transcription factors are the β-catenin binding domain and the high mobility group box, the latter of which facilitates DNA binding. Some isoforms of TCF do not possess the β-catenin binding domain or contain domains that function as repressors such as CtBP binding structural domains and can therefore act in a dominant negative fashion. These isoforms are incapable of activating transcription and thought to ordinarily function as tumor suppressors. These differences are thought to contribute to the bimodal behavior of TCFs, through which they are known to be capable of both repression and activation. We co-transfected the versican-Luc reporter vector with the TCF-4 expression vector containing the CtBP binding domain in the absence or presence of a β-catenin expression plasmid.
The results showed that TCF-4 inhibited versican promoter activity in the absence of β-catenin but was able to induce versican promoter activation in the presence of β-catenin (Fig. 6B). In contrast, co-transfection of the versican-Luc reporter with a TCF-1 chimeric construct (TCF-1E) that does not contain a CtBP domain does not inhibit promoter activity, and versican promoter activity significantly increased, regardless of the co-transfection of β-catenin (Fig. 6C). These findings support a repressor role for CtBP in β-catenin-mediated versican promoter activation by TCFs. To assess whether versican transcriptional activation by TCF-4-dependent, we performed a co-transfection of the β-catenin expression vector with a dominant negative mutant (ΔNTCF-4) devoid of β-catenin binding domains. The ΔNTCF-4 mutant inhibited β-catenin-driven transactivation (Fig. 6D).

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TCF-4 Binds to the Versican TCF/LEF Site—In the next set of experiments, we analyzed the binding of nuclear extract protein to the TCF/LEF site in the versican promoter by gel shift analysis (Fig. 7). Oligonucleotides corresponding to the indicated DNA elements were labeled with 32P and incubated with nuclear extract derived from the SW487 human colorectal cancer cell line. The versican TCF site element showed a strong double shift consistent with previously observed TCF4 and TCF4-β-catenin complexes and a supershift on addition of anti-TCF4 antibodies (43, 44) (Fig. 7A). Addition of anti-β-catenin antibodies to the shift experiment resulted in interference of the formation of the larger complex, which is consistent with antibody binding preventing the TCF4/β-catenin interaction (Fig. 7B).

DISCUSSION

In the present study, we found that PI3K and its downstream effector PKB mediate serum stimulation of versican transcription in SMCs. Furthermore, we showed that cytoplasmic accumulation of β-catenin via inhibition of GSK-3β stimulates transcription of versican. Finally, we demonstrated that a TCF/LEF binding site in the versican promoter is involved in β-catenin-induced versican transcription and that at least one of the members of TCF family of transcription factors, TCF-4, binds to the TCF/LEF site in the human versican promoter. These observations may support a new concept for the pathophysiological role of PI3K/PKB/GSK-3β signaling through the β-catenin-TCF transactivation complex, modulating the gene expression patterns observed in atheromatous lesions and provisional matrix formation involved in normal and aberrant vascular injury and repair processes (1–7, 45).

Numerous studies have demonstrated that after mechanical arterial injury, vessel walls follow a response to injury pattern of wound healing leading to restenosis secondary to the neointimal accumulation of SMCs and ECM, with the ECM accounting for >90% of the neointimal volume (46, 47). The PG versican is one of several ECM molecules that accumulate in vascular lesions (1, 2). Versican is generally considered to be pro-atherogenic because of its ability to trap cholesterol-rich lipoproteins (4, 6) in addition to its role in regulation of fundamental events involved in vascular disease (4, 6, 7). The dynamic process of vascular injury and repair involves molecular signaling cascades that govern arterial SMC migration, differentiation, proliferation, and fate (48–50). The canonical Wnt-wingless signaling pathway regulates various biologic processes including early embryogenesis, neoplasia, and healing by increasing the stability and transcriptional activity of a key mediator, β-catenin (51–53). Upon its stabilization and accumulation (51–53), β-catenin translocates to the nucleus and activates the TCF/LEF family of transcription factors (53). Evidence suggests a potentially important role for the β-catenin/TCF signaling in vascular remodeling events (54–57). The present study suggests that upon activation of growth factor receptors and Wnt signaling, β-catenin increases and activates TCF target genes, including versican, which, in turn, retains therogenic lipoproteins and contributes to lesion development. The production of versican also influences cellular functions and survival directly or indirectly. Our finding may suggest one of the molecular mechanisms by which versican influences vascular injury and repair events.

In vitro experiments using monkey arterial SMCs show that PDGF-stimulated versican core protein expression apparently occurs through a receptor tyrosine kinase-dependent, protein kinase C-independent pathway (16). The concentration- and time-dependent increase in mRNA levels by AT1 receptor-expressing cells in response to angiotensin II is inhibited by the AT1 receptor antagonist losartan, the epidermal growth factor

<Fig. 4. GSK-3b inhibition stimulates transcription of versican in SMCs. A, lithium chloride, a GSK-3b inhibitor, increased versican promoter luciferase activity. SMCs were transfected with wt-versican-Luc reporter (2 μg/well). After 3 h, the DNA-liposome mixture was removed, and cells were kept in serum-free medium containing 0.2% bovine serum albumin for 24 h. Twenty-four hours post-transfection, the cells were incubated in the presence or absence of LiCl (30 mM) for 24 h. Cell extracts were obtained, and luciferase activity was assayed. Transfection efficiency was monitored by co-transfecting the cells with 0.2 μg/well Rous sarcoma virus/β-galactosidase construct as described under “Experimental Procedures.” The values are representative of three independent experiments, each performed in duplicate or triplicate, and are shown relative to the activity of luciferase of cells treated with vehicle. Values are mean ± S.D. B, lithium chloride increased versican mRNA levels. Confluent serum-starved (24 h) SMCs were incubated in the presence or absence of LiCl (30 mM) for 24 h. Total RNA was isolated, and the versican mRNA level was quantified by real-time reverse transcription-PCR as described under “Experimental Procedures.” Expression was normalized with respect to β-actin RNA level in the same samples and presented relative to mRNA level from untreated cells. Results shown are the mean ± S.D. of two cDNA samples analyzed in duplicates representative of two independent experiments. Asterisk denotes a significant difference (p < 0.05) between versican expression in cells treated with LiCl and those treated with vehicle. C, GSK-3b inhibited versican promoter activity. SMCs were co-transfected with the versican-Luc reporter and wild-type GSK-3b construct or with the empty vector, incubated for 24 h in the presence of 5% serum, and assayed for luciferase activity. The values are representative of three independent experiments, each performed in duplicate or triplicate, and are shown relative to the luciferase activity in cells transfected with empty plasmid. Values are mean ± S.D.

The production of versican also influences cellular functions and survival directly or indirectly. Our finding may suggest one of the molecular mechanisms by which versican influences vascular injury and repair events. In vitro experiments using monkey arterial SMCs show that PDGF-stimulated versican core protein expression apparently occurs through a receptor tyrosine kinase-dependent, protein kinase C-independent pathway (16). The concentration- and time-dependent increase in mRNA levels by AT1 receptor-expressing cells in response to angiotensin II is inhibited by the AT1 receptor antagonist losartan, the epidermal growth factor
receptor inhibitor AG1478, and the mitogen-activated protein kinase inhibitor PD98059. The increase in versican gene expression was not inhibited by the protein kinase C inhibitors chelerythrine and staurosporine, indicating that angiotensin II-mediated stimulation of SMC versican expression is regulated by receptor tyrosine kinase-dependent mechanisms (17).

**FIG. 5.** The TCF/LEF binding sequence at position −492 is crucial for activation of the versican promoter by the β-catenin-TCF complex. A, schematic representation of the sequence for TCF/LEF binding sites in wild-type human versican promoter and mutant constructs. Two putative TCF/LEF binding sites are localized at position −546 and −492 bp from the transcription start site. Mutations known to abolish TCF binding were introduced into the TCF/LEF recognition sites to make the mutant constructs −546mut-versican-Luc, −492mut-versican-Luc, and −546/−492mut-versican-Luc in the context of the wt-versican-Luc promoter. A deletion construct lacking the TCF/LEF binding sites, −438del-versican-Luc, was also generated as described under "Experimental Procedures." B, mutation at position −492 of the versican promoter inhibits versican luciferase promoter activity. SMCs were transfected with wt-versican-Luc or mutant versican promoter luciferase vectors and harvested 24 h post-transfection. The values are representative of three independent experiments, each performed in duplicate, and are shown relative to the activity of luciferase at cells transfected with wt-versican-Luc construct. C, mutation at position −492 of the versican promoter inhibited the response of luciferase promoter activity to LiCl treatment and β-catenin transactivation in SMCs. SMCs containing normal endogenous β-catenin were transiently transfected with 0.5 μg of wt-versican-Luc and treated with 30 mM LiCl. The cells were co-transfected with 0.8 μg of β-catenin expression vector or an equal amount of empty vector and harvested 24 h after transfection. Total amounts of plasmid DNA were kept constant by adding the empty pCDNA3.1 vector. All experiments were performed in duplicate and repeated at least three times. Values represent mean ± S.D. D, a deletion construct lacking the TCF/LEF binding sequences showed no β-catenin-mediated versican promoter transactivation. SMCs were transfected with wt-versican-Luc or −438del-versican-Luc reporter, along with β-catenin (0.8 μg) or empty vector, as indicated in the figure. Total amounts of plasmid DNA were kept constant by adding the empty pCDNA3.1 vector. All experiments were performed in duplicate and repeated at least three times. Values represent mean ± S.D.
Regulation of Versican Promoter by β-Catenin/TCF Signaling

Our data, for the first time, suggest that PI3K/PKB signaling is involved in the serum regulation of versican transcription in SMCs. We established this regulatory sequence of events in a series of experiments. First, we showed that the pharmacological inhibitor of PI3K, LY294002, inhibited activation of downstream PKB at all concentrations tested and resulted in significant inhibition of versican promoter reporter activity and a reduced versican mRNA level in SMCs. Furthermore, consistent with previous results, expression of wt-PTEN and mut-PTEN reduced and increased versican promoter function, respectively. PTEN antagonizes the diverse downstream signaling effector pathways activated by PI3K-derived phospholipids, which supports our previous finding regarding the role of PI3K in versican promoter regulation. PKB was suggested to be a major downstream effector of PI3K-related gene regulation in SMCs. Finally, the results of our experiments demonstrate that versican promoter activity of versican-Luc was significantly increased and decreased in transient co-transfection with CA-PKB and DN-PKB constructs, respectively. Overall, these results suggest that reduced production of 3-phosphoinositides inhibits serum-induced PKB signaling stimulation of versican transcription in SMCs.

GSK-3 inactivation leads to β-catenin stabilization and translocation into the nucleus, where β-catenin binds to TCF/LEF family proteins to form a transcription factor complex that activates target genes such as matrix metalloproteinase-7, fibronectin, vascular endothelial growth factor, cyclin D1, and c-myc (24). Recent work suggests a role for the Wnt/β-catenin signaling pathway in pathophysiological remodeling within the cardiovascular system (21, 22, 59, 60). There is also evidence that differential inhibition of GSK-3β in intimal tissue following vascular injury acts as a critical signal mediating SMC survival (21). Finally, Mao et al. (56) recently demonstrated altered expression of the Frizzled receptor family in SMCs in response to vascular injury.

There is a recent report that the versican gene was identified as a target gene of Wnt signaling using microarray technology to analyze human embryonic carcinoma cells stimulated with active Wnt protein (61). The promoters of nearly all the target genes identified include putative TCF binding sites including the versican promoter. Kishimoto et al. (58) also showed that co-culture of cells expressing a secreted Wnt3a protein maintains the expression of green fluorescent protein placed under the control of a fragment of the human versican promoter. In this study, we have observed that versican gene expression is up-regulated by β-catenin. We show that endogenous versican mRNA is induced in SMCs by LiCl treatment, which inactivates GSK-3 and therefore stabilizes wild-type β-catenin. This regulation occurs at the transcriptional level because the versican promoter responded to β-catenin in the presence or absence of co-transfected TCF-4 expression plasmid, whereas dominant negative TCF-4 expression inhibited these effects.
Our data demonstrate that a single consensus TCF/LEF binding sequence located 492 bp from the transcriptional start site is critical for β-catenin responsiveness, thus identifying versican as a direct target of the β-catenin-TCF complex. We also show a potential repressor function of some TCF isoforms on versican transcriptional activity by co-transfecting a versican promoter reporter and a vector expressing a TCF-4 isoform containing a CtBP structural domain in the presence or absence of β-catenin expression plasmid. The results showed the CtBP domain of TCF-4 mediated inhibition of versican promoter activation in the absence of β-catenin but was able to induce versican promoter activity in the presence of β-catenin. In contrast, co-transfection of the versican promoter with a TCF chimeric construct that does not contain CtBP domains did not repress promoter activity (data not shown). These findings support the hypothesis that certain TCF isoforms containing the CtBP structural domain can repress TCF target genes such as versican. Taken together, these data suggest that the level of different isoforms of TCF transcription factors containing different repressor or co-activator domains determines the repression activity of TCF on target genes in SMCs.

In conclusion, we suggest that the PI3K/PKB pathway plays a critical role in serum-stimulated versican transcription. Furthermore, we provide evidence that phosphorylation and inhibition of GSK-3β by PKB and subsequent β-catenin-TCF complex formation are essential for versican transcription (Fig. 8). The roles of versican in cell growth, motility, adhesion, and angiogenesis strongly indicate that this PG of the ECM is an important downstream effector of the Wnt pathway during developmental and pathological processes. We also suggest that β-catenin, through regulation of this versatile PG versican and other candidate target genes, augments the establishment of a provisional ECM, induces proliferation and survival of vascular cells, and modulates adhesive, migratory, and angiogenic processes that lead to normal or aberrant vascular injury and repair.

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FIG. 7. EMSA of a potential TCF-4 binding site in the versican promoter. A double-stranded oligonucleotide corresponding to a potential TCF-4 binding site within the versican promoter was radiolabeled and incubated with nuclear extracts derived from the SW480 cell line. The indicated antibodies were added to the DNA binding reaction prior to electrophoresis. Anti-TCF-4 antibodies generated a supershifted complex (A), whereas anti-β-catenin antibodies interfered with the formation of the higher molecular weight complex present in the control reactions (B).

FIG. 8. Hypothetical model of versican promoter regulation via PI3K/PKB signaling and β-catenin-TCF transcription factor complex. In the resting cells, under unstimulated conditions, cytoplasmic β-catenin is associated with GSK-3β and other scaffolding proteins, and GSK-3β is active and phosphorylates β-catenin in the cytoplasm and renders it susceptible to degradation via the ubiquitin-proteasome pathway. Activation of PI3K or Wnt signaling leads to phosphorylation of GSK-3β but induces inactivation of GSK and destabilizes the destruction complex, and β-catenin accumulates in the cytoplasm and subsequently translocates to the nucleus. Nuclear accumulation of β-catenin leads to complexing with TCF/LEF transcription factors and transactivation of TCF/LEF target genes. Our findings support the model that versican transcription is predominantly mediated by activation of PI3K/PKB and subsequent phosphorylation/inhibition of the GSK-3β pathway via β-catenin-TCF transcription factor complex at the versican promoter in vascular SMCs.
