Sp1 and Smad Proteins Cooperate to Mediate Transforming Growth Factor-β1-induced α2(I) Collagen Expression in Human Glomerular Mesangial Cells*

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The mechanism(s) by which Smads mediate and modulate the transforming growth factor (TGF)-β signal transduction pathway in fibrogenesis are not well characterized. We previously showed that Smad3 promotes α2(I) collagen gene (COL1A2) activation in human glomerular mesangial cells, potentially contributing to glomerulosclerosis. Here, we report that Sp1 binding is necessary for TGF-β1-induced type I collagen mRNA expression. Deletion of three Sp1 sites (GC box) between −376 and −268 or mutation of a CAGA box at −268/−260 inhibited TGF-β1-induced α2(I) collagen promoter activity. TGF-β1 inducibility was also blocked by a Smad3 dominant negative mutant. Chemical inhibition of Sp1 binding with mithramycin A, or deletion of the GC boxes, inhibited COL1A2 activation by Smad3, suggesting cooperation between Smad3 and Sp1 in the TGF-β1 response. Electrophoretic mobility shift assay showed that Sp1 and Smad3 form complexes with −283/−250 promoter sequences. Comimonoprecipitation experiments demonstrate that endogenous Sp1, Smad3, and Smad4 form complexes in mesangial cells. In a Gal4-LUC reporter assay system, Sp1 stimulated the TGF-β1-induced transcriptional activity of Gal4-Smad3, Gal4-Smad4 (266–552), or both. Using the transactivation domain B of Sp1 fused to the Gal4 DNA binding domain, we show that, in our system, the transcriptional activity of this Sp1 domain is not regulated by TGF-β1, but it becomes responsive to this factor when Smad3 is coexpressed. Finally, combined Sp1 and Smad3 overexpression induces marked ligand-independent and ligand-dependent promoter activity of COL1A2. Thus, Sp1 and Smad3 proteins form complexes and their synergy plays an important role in mediating TGF-β1-induced α2(I) collagen expression in human mesangial cells.

Glomerulosclerosis is a scarring process involving extracellular matrix (ECM)† accumulation and obliteration of glomerular capillaries. It is considered to be the final pathway leading to the progressive loss of renal function in several kidney diseases. Mechanical factors such as hyperfiltration and intraglomerular hypertension, as well as a variety of mediators including cytokines, growth factors, and eicosanoids derived from circulating or glomerular cells, have been implicated in initiating or maintaining sclerosis (1). However, little information is available regarding the cellular mechanisms by which these factors affect matrix turnover. Our laboratory has been studying the mechanisms by which transforming growth factor (TGF)-β stimulates ECM accumulation. Previously, we determined that the Smad pathway plays a role in activating type I collagen gene expression in human glomerular mesangial cells.

The Smads are a series of proteins that function downstream from the serine/threonine kinase receptors of the TGF-β family to transduce signal to the nucleus (2–4). Following TGF-β binding to its receptors, the receptor-regulated Smads (R-Smads), Smad2 and Smad3, are phosphorylated by the type I receptor and associate with the common partner, Smad4. The resulting heteromultimer translocates to the nucleus where it regulates expression of TGF-β target genes (2–4). The inhibitory Smads, Smad6 and Smad7, may participate in a negative feedback loop to control TGF-β responses by competitive interaction with the type I receptor (2–4).

Smad3 and Smad4 are able to directly bind to DNA through their N-terminal MH1 domain (5, 6). Although these studies described two different consensus sequences for Smad binding (TGTCTAG, called Smad binding element or SBE; and AGC/ A/CAGACAC, called CAGA box), both sequences contain the core motif AGAC, which represents the optimal binding sequence for Smad3 and Smad4 (6, 7). This motif is present in the regulatory regions of several TGF-β target genes, including those for PAI-1 (5, 8) JunB (9), c-Jun (10), type VII collagen (11), Smad7 (12, 13), gelsolin lgo (14, 15), and PDGF-B (16). Several of these studies showed that mutations of these CAGA boxes in the context of the promoter abrogate TGF-β stimulation. Conversely, multiple copies of this sequence support ligand inducibility, demonstrating the importance of Smad3/Smad4 binding sites in mediating TGF-β responsiveness. However, not all target genes of the TGF-β family contain a canonical SBE in their regulatory region (17, 18). Taken together with the fact that only multiple copies of SBE confer TGF-β inducibility (6) and that binding of a Smad MH1 to SBE is of low affinity (7), these observations suggest that the CAGA sequence alone is not sufficient to support maximal TGF-β-mediated transcriptional activation of target genes. Indeed, several studies indicate that, in response to TGF-β/activin, Smads can cooperate with other DNA-binding proteins such as poly(adenosine diphosphate) polymerase chain reaction; bp, base pair(s).
members of the AP-1 family (10, 19, 20), PEBP2/CFB/AML (14, 15), Fast proteins (18, 21), and TFE3 (8). R-Smads and Smad4 also have been shown to interact with the coactivators p300/CBP (22–28).

We previously showed that a construct containing the sequence from –376 to +58 of the human α2(I) collagen gene (COL1A2) promoter is responsive to TGF-β in human mesangial cells and that overexpression of Smad3 stimulates COL1A2 promoter activity (29). The TGF-β response element of the α2(I) collagen gene has been mapped to the sequences located between –340 and –183 from the transcription start site (30). This region contains three Sp1 binding sites and one AP-1 consensus sequence. Different groups have implicated either Sp1 or AP-1 as the mediator of TGF-β stimulation of α2(I) collagen gene in dermal fibroblasts (30–32). Between the Sp1 and AP-1 binding sites lies a CAGA box. In gel shift experiments, a transcriptional complex containing Smad proteins in nuclear extracts from TGF-β-treated fibroblasts binds to this CAGA box (33). Multiple copies of this motif conferred TGF-β inducibility to a heterologous promoter in HepG2 cells and in fibroblasts (34).

In the present study, we sought to further delineate the region responsible for TGF-β stimulation of COL1A2 promoter in human mesangial cells and to address the controversy concerning the role of AP-1, Sp1, and Smad proteins in TGF-β-induced α2(I) collagen gene expression.

**EXPERIMENTAL PROCEDURES**

**Materials**—Reagents were purchased from the following vendors: active, human recombinant TGF-β1 from R&D Systems; goat polyclonal anti-Smad2/3 IgG (N-19), mouse monoclonal anti-Smad1/2/3 IgG (H-2), mouse monoclonal anti-Smad4 IgG (B-8), rabbit anti-Sp1 IgG (P4P-2), normal goat IgG, normal mouse IgG, normal rabbit IgG, anti-goat IgG-horseradish peroxidase, anti-mouse IgG-horseradish peroxidase antibody, Sp1 consensus binding site, AP-1 consensus binding site, Smad3/4 binding site (CAGA), and mutated CAGA oligonucleotide from Santa Cruz Biotechnology; rabbit anti-phospho-Smad2 IgG from Upstate Biotechnology Inc.; recombinant protein G-Sepharose and rabbit polyclonal anti-phosphoserine antibody from Zymed Laboratories Inc.; anti-rabbit IgG-horseradish peroxidase, luciferase, and β-galactosidase assay systems from Promega; P60 DNA polymerase and rapid DNA ligation kit from Roche Molecular Biochemicals; T4 polynucleotide kinase from Life Technologies, Inc.; mithramycin A and curcumin from Sigma.

**Cell Culture**—Human mesangial cells were isolated from glomeruli by differential sieving of minced normal human renal cortex obtained from anonymous surgery or autopsy specimens. The cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium, supplemented with 20% heat-inactivated fetal bovine serum (FBS), glutamine, penicillin/streptomycin, sodium pyruvate, Hepes buffer, and 8 µg/ml insulin (Life Technologies, Inc.) as described previously (35) and were used between passages 5 and 8.

**RNA Isolation and Northern Blot**—Cells were plated in 100-mm culture dishes (3–5 × 10^5 cells/dish). The next day, they were preincubated with mithramycin A (100 µm) for 17 h or curcumin (20 µM) for 30 min before addition of 1 ng/ml TGF-β1 or control vehicle for 24 h. These conditions are similar to the ones showing efficient inhibition of Sp1 binding by mithramycin A and of AP-1 activation by curcumin (32). Total RNA was analyzed by Northern blot as described previously (36). Autoradiograms were scanned with an Arcus II Scanner (AGFA) in transparency mode, and densitometric analysis was performed using the NIH Image 1.61 program for Macintosh. The same blots were successively rehybridized with additional probes after confirming complete stripping. CDNAs for human α1(I) (clone H677; Ref. 37) and α2(I) collagen (clone H1313; Ref. 38) chains were obtained from Dr. Y. Yamada (National Institutes of Health, Bethesda, MD). The cDNA for the human tissue inhibitor of metalloproteinase (TIMP)-1 (39) was obtained from Dr. D. Carmichael (Synergex, Boulder, CO). The signals were immobilized by hybridization with these probes were corrected for loading using the signal obtained with a bovine cDNA for 28 S ribosomal RNA provided by Dr. H. Sage (University of Washington, Seattle, WA).

**Transient Transfection and Luciferase Assay**—The day before the transfaction, 6.5–8 × 10^4 cells were seeded in six-well plates. Eighteen hours later, cells were switched to 1% FBS medium and transfected with the indicated constructs along with 0.5 µg of CMV-SPORT-β-galactosidase (Life Technologies, Inc.) as a control of transfection efficiency. Transfection was performed with the FuGene6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer’s instructions. After 3 h, 1 ng/ml TGF-β1 or control vehicle was added to the cells. In some experiments, the transfected cells were pretreated for 2 h with mithramycin A before adding TGF-β1. Twenty-four hours later, the cells were harvested in 300 µl of reporter lysis buffer (Promega). Luciferase and β-galactosidase activities were measured as described previously (29). Luciferase assay results were normalized for transfection efficiency. Densitometric points were realized in triplicate in at least two independent transfections.

**Plasmid Constructs**—The 376COL1A2-LUC construct containing 376 bp of the α2(I) collagen gene (COL1A2) promoter and 58 bp of the transcribed sequence fused to the luciferase (LUC) reporter gene was described previously (29). The 5′ deletion constructs were generated by PCR amplification using the plasmid pMS-3.5/CAT (40) as a template and the following pairs of primers: 5′-GGGAGTCCATGCAGACAGACAGGAGTCCAC3′ or 5′-CAGGATCCAGTGCAAGCTTTCCC-3′ with 5′-CCTCCATGACCGGGTGCTCGAGTA-5′. The PCR reactions were carried with the proofreading Pfu DNA polymerase. After digestion by BamHI and SacI, the PCR products were extracted from an agarose gel with the QiAquick gel extraction kit (Qiagen). The purified fragments were cloned into the BamHI vector (41), which was used to rescue reporter gene without a promoter. The resulting constructs were called 268COL1A2-LUC (region from –268 to +50) and 258COL1A2-LUC (region from –258 to +50). Point mutations were introduced into the potential Smad recognition site (at –268/–260) of the 376COL1A2-LUC construct using the QuickChange site-directed mutagenesis kit (Stratagene), according to the manufacturer’s instructions with the following modification: 1 cycle of 1 min at 94 °C, 90 cycles of 1 min at 94 °C, 1 min at 52 °C, 7 min at 72 °C; 1 cycle of 15 min at 72 °C. The primers used were 5′-AGGGCGGAGTAGTTATGATACACACGGTAGCAG-3′ and 5′-CCTCTAGCTGCTGTATATACACTACCCGCCC-3′. The resulting clone was named 376mCACA-LUC. Mutation and deletion constructs were verified by sequencing. The vectors expressing the indicated Smad2 variants (42) were kindly provided by Drs. H. F. Lodish and X. Liu. The expression vector for Sp1 (43) was kindly provided by Dr. T. Shenk. The Gal4-Smad constructs (28) were kindly provided by Dr. M. P. de Caestecker. The Gal4-Sp1 constructs (44) were kindly provided by Dr. R. Tjian.

**Preparation of Nuclear Extracts and Electrophoretic Mobility Shift Assays** (**EMSA**)—Nuclear extracts were prepared from control and TGF-β1-treated cells as described by Schreiber et al. (45). Double-stranded oligonucleotides were labeled with [γ-32P]ATP and T4 polynucleotide kinase. Five µg of nuclear extracts were incubated for 30 min at 4 °C with 40,000 cpm of 32P probe in 10 µl Hepes, pH 7.9, 5% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 µg of poly(dI-dC)poly(dI-dC). For competition and supershift assays, nuclear extracts were preincubated with a 100-fold molar excess of cold oligonucleotide or 1 µg of a specificity control competitor 30 min or 1 h before addition of the labeled probe. The DNA-protein complexes were separated on a 5% polyacrylamide gel in 0.5× TBE buffer and visualized by autoradiography.

**Preparation of Cell Lysates, Immunoprecipitation, and Western Blot Analysis**—Cells plated in 150-mm dishes (0.75–1 × 10^6 cells/plate) were cultured for 3 days. The cells were switched to medium containing 1% FBS and then treated with 1 ng/ml TGF-β1 for different time periods leading up to simultaneous harvest. The cells were lysed at 4 °C in lysis buffer (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidot P-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 µg/ml aprotonin) and phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 40 µM β-glycerophosphate). Lysates were clarified by centrifugation at 18,000 × g for 10 min. The protein content was determined by Bradford protein assay (Bio-Rad). Three milligrams of lysate protein were immunoprecipitated overnight at 4 °C with 6 µg of anti-Smad2/3 antibody (N-19) or anti-Sp1, followed by precipitation with 100 µl of protein G-Sepharose for 90 min at 4 °C. After four washes with complete lysis buffer, the immunoprecipitates were eluted by boiling for 5 min in 60 µl of 2× Laemmli sample buffer. The resulting immunoprecipitates were electrophoresed through a 15% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and immunoblotted with anti-Smad1/2/3, anti-Smad2/3, anti-Smad4, anti-Sp1, or anti-phospho-Smad2 antibody (0.2 µg/ml), or anti-phosphosmokinase antibody (1 µg/ml). The blots were developed with chemiluminescence reagents according to the manufacturer’s protocol (Santa Cruz Biotechnology). Quantification of the bands on autoradiograms was performed...
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RESULTS

Sp1 Binding Is Necessary for TGF-β-induced Type I Collagen Expression—The 5′-flanking region of the human COL1A2 gene contains several GC boxes (Sp1 binding sites) and an AP-1 recognition sequence between nucleotides −340 and −230 (Fig. 1A). This region has been shown to mediate TGF-β responses in fibroblasts, although contradictory data exist concerning the role of these sites for basal activity and TGF-β inducibility of the α2(I) collagen promoter (30–32, 46). Moreover, a CAGA box, which can interact with recombinant Smad3 and Smad4, is present between the Sp1 and AP-1 binding sites, at the location −268/−260. Here, we investigated the role of these transcription factors in mediating TGF-β1-induced type I collagen expression in human mesangial cells. Cells were pretreated with mithramycin A, an inhibitor of Sp1 binding (47), or with curcumin, an inhibitor of c-Jun N-terminal kinase pathway and AP-1 activation (48, 49), before adding TGF-β1 for 24 h. Type I collagen mRNA expression was analyzed by Northern blot. Mithramycin A inhibited both basal and TGF-β1-induced α1(I) and α2(I) collagen mRNA levels, whereas curcumin did not significantly affect type I collagen expression (Fig. 1B). These results are consistent with previous findings from other groups using human fibroblasts (32, 50). The inhibitory effect of mithramycin A was specific since transcription of other genes such as TIMP-1 was not blocked by this chemical antagonist (Fig. 1B). These data suggest that Sp1, but not AP-1, is involved in induction of type I collagen transcription by TGF-β1.

To further support these findings and delineate the TGF-β1-responsive region of COL1A2 in mesangial cells, we performed transient transfection experiments with a series of 5′-deletion mutants of the α2(I) collagen promoter. The construct 376COL1A2-LUC, containing the sequence from −376 to +58 of the human α2(I) collagen promoter in front of the luciferase reporter gene, was stimulated about 2-fold by TGF-β1 (Fig. 2). Deletion of the sequence between −376 and −268, containing three GC boxes, blocked the TGF-β1 stimulation of COL1A2 promoter activity. However, basal expression was not affected. Further deletion, removing the CAGA box but leaving the AP-1 motif intact, slightly decreased basal activity and still inhibited TGF-β1 inducibility. These data indicate that the three GC boxes are necessary for TGF-β1-induced α2(I) collagen promoter activity while the CAGA motif in the context of the promoter is not sufficient to support ligand stimulation in mesangial cells. They also confirm that the AP-1 site is not involved in the activation of the α2(I) collagen gene by TGF-β1.

Involvement of Sp1 Binding in Smad3-mediated TGF-β1 Induction of COL1A2—The contribution of Smad3 to TGF-β1-induced COL1A2 promoter activity was examined. As shown in Fig. 3A, the 376COL1A2-LUC construct was stimulated by TGF-β1 when mesangial cells were cotransfected with the empty expression vector, pEXL (2.07 ± 0.22-fold induction, n = 7). Overexpression of Smad3 increased both basal (11.09 ± 2.82-fold, n = 7) and TGF-β1-induced luciferase activity (a further 3.90 ± 0.47-fold, n = 7). In contrast, a Smad3 dominant negative mutant construct in which the three C-terminal serine residues are replaced by three alanines (Flag-N-Smad3; Ref. 42) completely inhibited the effect of TGF-β1 (1.08 ± 0.08-fold induction, n = 7). These results are similar to our previous findings in transient transfection experiments in the presence of serum (29). However, addition of TGF-β1 to cells cotransfected with Smad3 in complete culture medium led only to a modest induction over untreated cells (1.44 ± 0.23, n = 4, compared with 3.90 ± 0.47-fold induction, n = 11, in low serum-containing medium). The effect of Smad3 overexpression on COL1A2 promoter activity increased in dose-dependent manner (Fig. 3B). To further examine the role of Smad proteins in COL1A2 gene transcription, mutations were introduced into the CAGA box at −268/−260. These substitutions almost com-
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Fig. 3. Smad3 is involved in TGF-β1-induced α2(I) collagen promoter activity. A, cells were cotransfected with 0.5 µg of the 376COL1A2-LUC construct and either 0.5 µg of the vector encoding wild-type Smad3 (Flag-N-Smad3), mutated Smad3 (Flag-N-Smad3A), or the empty expression vector (pEXL), along with 0.5 µg of CMV-β-galactosidase vector. The transfected cells were treated with 1 ng/ml TGF-β1 for 24 h. Luciferase activity was normalized to β-galactosidase activity. The results from at least seven independent experiments are expressed as fold induction over untreated cells. B, cells were transfected with 0.5 µg of the 376COL1A2-LUC and the indicated amount of Flag-N-Smad3. The total amount of DNA was maintained constant by cotransfection of empty expression vector. Relative activity of untreated cells cotransfected with the empty expression vector for wild-type Smad3 or the empty expression vector, along with 0.5 µg of either 376COL1A2-LUC or 376mCAGA-LUC. The results from three transfection experiments are shown as fold induction.

We next investigated whether Sp1 binding is required for Smad3-mediated α2(I) collagen promoter activity. Cells were transfected with the 376COL1A2-LUC construct and the Smad3 expression vector. Then, they were incubated with mithramycin A for 2 h prior to treatment with TGF-β1. Mithramycin A slightly decreased basal and TGF-β1-induced 376COL1A2-LUC activity in cells cotransfected with an empty expression vector (Fig. 4). However, it significantly inhibited basal and ligand-induced effects of Smad3 on type 1 collagen promoter activity. In the presence of mithramycin A, COL1A2 promoter activity induced by overexpression of Smad3 was reduced to levels similar to basal COL1A2 promoter activity, suggesting that ligand-independent stimulation of α2(I) collagen expression by Smad3 requires Sp1 binding.

Similar to the pharmacological inhibition of Sp1 binding, deletion of the three Sp1 sites (268COL1A2-LUC) decreased the effect of overexpressed Smad3 (Fig. 5). Deletion of the CAGA box, leaving the AP-1 motif intact (283COL1A2-LUC), inhibited TGF-β1 inducibility, an effect that could not be completely overcome by coexpressing Smad3. Point mutations of the CAGA box, leaving the Sp1 sites intact (376mCAGA-LUC), also inhibited the TGF-β1 response. However, overexpression of Smad3 increased promoter activity of this construct. Addition of TGF-β1 further enhanced luciferase activity, although this induction was not as strong as for the wild-type construct. The Sp1 binding sites lying between −376 and −283 are thus necessary for Smad3 to induce COL1A2 activity in a ligand-independent fashion. Taken together, these results suggest cooperation between Smad3 and Sp1 in the TGF-β1 response.

Sp1 and Smad Proteins Bind to the COL1A2 Promoter—We next sought to determine whether Sp1 and Smads were able to interact with the COL1A2 promoter. EMSAs were performed with nuclear extracts from control and TGF-β1-treated mesangial cells and a fragment of DNA spanning the COL1A2 promoter sequences from −283 to −250 (WT). This region contains a GC and a CAGA box, and an AP-1 site. Four DNA/protein complexes were detected (Fig. 6A, complexes a–d), and their intensity slightly increased with 10 min of TGF-β1 treatment (compare lanes 2 and 3). This increase was also observed with nuclear extracts from cells treated with TGF-β1 for 30 or 90 min (data not shown). Competition experiments with unlabeled probe markedly decreased the intensity of all four complexes (lanes 4 and 5). Point mutations of the Sp1 site (Mut) abolished its ability to compete with complexes a and b but not c and d (lanes 6 and 7), whereas an oligonucleotide containing a Sp1 consensus binding site was only able to compete with complexes c and d (lanes 8 and 9). To further demonstrate the presence of Sp1 in complexes c and d, nuclear extracts were preincubated with anti-Sp1 antibody (Fig. 6B). In these conditions, a slower migrating complex was observed while formation of both complexes c and d was partially inhibited (lanes 4 and 5). In contrast, normal rabbit IgG did not affect formation of any of these complexes (lanes 6 and 7). Thus, Sp1 binds to −283/−250 of the COL1A2 promoter and this binding does not
Deletion of the three Sp1 sites between −376/−268 decreases the effect of overexpressed Smad3. Cells were cotransfected with 0.5 µg of the indicated reporter construct, 0.5 µg of Flag-N-Smad3 or pEXL, and 0.5 µg of CMV-β-galactosidase. Luciferase and β-galactosidase activities were measured 24 h after addition of TGF-β1. The data represent the means of a least three independent experiments. The inset shows the TGF-β1 stimulation for each reporter construct in cells cotransfected with the empty expression vector pEXL.

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FIG. 5. Deletion of the three Sp1 sites between −376/−268 decreases the effect of overexpressed Smad3. Cells were cotransfected with 0.5 µg of the indicated reporter construct, 0.5 µg of Flag-N-Smad3 or pEXL, and 0.5 µg of CMV-β-galactosidase. Luciferase and β-galactosidase activities were measured 24 h after addition of TGF-β1. The data represent the means of at least three independent experiments. The inset shows the TGF-β1 stimulation for each reporter construct in cells cotransfected with the empty expression vector pEXL.

Functional Interaction between Sp1 and Smad Proteins Is Stimulated by TGF-β1—The above experiments indicate that endogenous Smads and Sp1 can form complexes in human mesangial cells. We then investigated whether their interaction can lead to transcriptional activation using the Gal4-LUC assay system. A reporter construct containing three Gal4 binding sites in front of the luciferase gene (pFR-LUC) was cotransfected with the Gal4 DNA binding domain fused to full-length Smad3 (Gal4-Smad3) or Smad4 (266–552) (Gal4-Smad4(ΔN)), and with a vector expressing Sp1 or the control empty vector. TGF-β1 stimulated transcriptional activity of Gal4-Smad3, Gal4-Smad4(ΔN), or both by 1.75-, 4.29-, and 2.66-fold, respectively (Fig. 9A). Overexpressed Sp1 enhanced Smad3-mediated transcription (2.27-fold increase over activity in control cells transfected with empty expression vector). This effect was further increased after treatment with TGF-β1. In contrast to Smad3, the basal activity of Gal4-Smad4 (266–552) was not increased by Sp1. However, overexpressed Sp1 enhanced responses to TGF-β1. Ligand-independent and ligand-dependent stimulation by Sp1 was markedly increased when cells were cotransfected with a combination of Gal4-Smad3 and Gal4-Smad4(ΔN). Neither TGF-β1 nor Sp1 was able to activate the reporter construct cotransfected with the Gal4 DNA binding domain alone. Thus, the interaction between Sp1 and Smad3 observed in Figs. 7 and 8 correlates with the functional cooperation between those proteins. Moreover, the TGF-β1-induced association leads to increased transcriptional cooperativity between Smads and Sp1.

The functional interaction between Smad3 and Sp1 was further examined by cotransfecting human mesangial cells with the pFR-LUC reporter construct along with a plasmid encoding for the glutamine-rich transactivation domain B of Sp1 fused to the Gal4 DNA binding domain (Gal4-Sp1(B)), residues 263–542) along with a Smad3 expression vector or the control empty construct. Additionally, the transcriptional activity of two other fusion proteins were studied: the Gal4-Sp1(B-n), containing residues 421–542 of Sp1, which was shown to activate transcription in Drosophila SL2 and HeLa cells; and the Gal4-Sp1(B-n), containing residues 263–424, which is inactive (44). Gal4-Sp1(B) and Gal4-Sp1(B-n) stimulated activity of the pFR-LUC construct by 5–11-fold (Fig. 9B). TGF-β1 had no effect, suggesting that, in our system, this factor does not directly stimulate transcriptional activity of the B domain of Sp1. Cotransfected Smad3 enhanced transcription by Gal4-Sp1(B) and Gal4-Sp1(B-n) only in the presence of TGF-β1 (−2-fold increase over untreated cells), while the phosphorylation-deficient Smad3 mutant had no effect (data not shown). Only low levels of reporter activity were detected with the Gal4 DNA binding domain alone or Gal4-Sp1(B-n), and these levels were not af-
Enhanced TGF-β transcription in a ligand-dependent manner, and suggest that Sp1 functionally cooperates between Sp1 and Smad3 to induce transcription in response to TGF-β1.

Expression—Stimulation of COL1A2 by TGF-β1 or Smad3: We have shown that Sp1 and Smad3 can form complexes in human mesangial cells and that these proteins functionally interact in a Gal4 transactivation assay system. To evaluate whether this interaction is involved in a2(I) collagen gene expression, mesangial cells were cotransfected with the TGF-β1-responsive collagen construct, 376COL1A2-LUC, or with 5' deletion mutants and expression vectors for Smad3, Sp1, both, or the corresponding empty vectors. TGF-β1 increased 376COL1A2-LUC activity by 2–3-fold in cells cotransfected with the empty expression vectors, whereas it did not stimulate the shorter COL1A2-LUC constructs (Fig. 10A). Overexpression of Sp1 did not affect basal or ligand-induced expression of 376COL1A2-LUC. Sp1 also had no effect on 268COL1A2-LUC or 258COL1A2-LUC activity. On the other hand, Smad3 increased 376COL1A2-LUC activity in the absence or presence of TGF-β1 by 11.09 ± 2.82- and 16.32 ± 1.68-fold, respectively, over cells transfected with empty expression vector (n = 12). Similar to results presented in Fig. 5, overexpressed Smad3 had a limited effect on TGF-β1-induced luciferase activity of the 5' deletion constructs. These results suggest that Sp1 is saturated for expression of the a2(I) collagen promoter in mesangial cells, while Smad3 is the limiting factor. Indeed, when Sp1 and Smad3 were overexpressed together, they induced marked ligand-independent and ligand-dependent transcriptional activity of 376COL1A2-LUC (Fig. 10B). When sequences between −376 and −268, containing three Sp1 sites, or between −376 and −258, containing three Sp1 sites and one CAGA box, were deleted, responsiveness to TGF-β1 could be rescued only partially by coexpressing Smad3 and Sp1 together (Fig. 10A). These results suggest that, when Smad3 and Sp1 are overexpressed, they could form complexes able to bind to more proximal regulatory regions of less affinity, while endogenous levels of these proteins would not be able to interact with these sequences to stimulate transcription in response to TGF-β1.

FIG. 6. Sp1 and Smad binding to the COL1A2 promoter. A, nuclear extracts from control cells (−) or cells treated with 1 ng/ml TGF-β1 for 10 min (+) were incubated with a labeled oligonucleotide spanning COL1A2 promoter sequences between −283 and −250 (WT). For competition experiments, extracts were preincubated with antibody for 1 h before addition of labeled WT oligonucleotide. An arrow indicates the supershifted complex. B, supershift experiments were performed by preincubating the extracts with antibody for 1 h before addition of labeled WT oligonucleotide. An arrow indicates the supershifted complex. C, nuclear extracts were incubated with labeled COL1A2 oligonucleotide mutated at the Sp1 site (Mut) or WT oligonucleotide. Unlabeled oligonucleotides used in competition experiments are indicated.

Effect of TGF-β1 or Smad3 treatment on COL1A2 expression: In dermal fibroblasts, the deletion of sequences between −376 and −265, encompassing three Sp1 consensus sites, decreased basal COL1A2 expression (31, 46). However, one study showed that deletion of the two upstream Sp1 sites reduced basal promoter activity by about 50%, while deletion of the third site had no effect. The other study showed that the deletion of all three sites was necessary to alter the promoter expression. Our data indicate that the region between −376 and −265 does not play a role in basal expression of COL1A2 in mesangial cells. Mithramycin A, an inhibitor of Sp1 binding, decreased basal α1(1) and α2(1) collagen mRNA expression by ~50% (Fig. 1B). In contrast, in transient transfection experiments, this biochemical antagonist only slightly inhibited basal activity of the construct containing the promoter regulatory sequences from −376 to +58 (Fig. 4). These results suggest that further upstream and/or downstream regions are involved in basal COL1A2 gene transcription. Indeed, a positive regulatory region for fibroblast expression has been identified between −3500 and −772 (31, 40). Ihn and collaborators (55) have also identified positive cis-elements at −128/−123 and

DISCUSSION

In the present study, we show that Smad proteins and Sp1 interact together to stimulate human α2(I) collagen gene expression in response to TGF-β1 in glomerular mesangial cells. While this manuscript was in revision, several manuscripts from other groups, which have been published or accepted for publication, showed cooperation between Sp1 and Smad proteins in mediating p15ink4b, β3 integrin subunit, COL1A2, and PAI-1 gene expression (51–54). Our data agree with these findings and extend them by showing direct evidence of interaction among endogenous Sp1 and Smad3 and Smad4 in non-transformed cells. In addition, our experiments show that this interaction leads to ligand-induced activation and cooperation of these proteins. These results provide strong support for the concept that this mechanism contributes to pathophysiological collagen accumulation in human renal disease, which has been associated with increased TGF-β1 activity.
- 84/- 80. Mutations of either site led to ~90% decrease in basal promoter activity in fibroblasts. The Sp family members, Sp1 and Sp3, were shown to bind to the TCCCTCC motif at -128/-123 (56). These regions might also play a role in mesangial cell COL1A2 gene expression.

Our transient transfection experiments with 5’ deletion of the COL1A2 promoter and analysis of steady-state levels of mRNA from cells treated with mithramycin A indicate that Sp1 binding is required for TGF-β1-induced α2(I) collagen gene transcription in human mesangial cells. We have defined a TGF-β1-responsive element in the COL1A2 promoter that contains three Sp1 binding sites and is located between -376 and -268 upstream from the initiation site (Fig. 2). A similar region, lying between -330 and -255, confers TGF-β responsiveness to a heterologous promoter in human dermal fibroblasts (30). In the same study, the authors also showed that optimal response to TGF-β1 depends on the structural integrity of the Sp1 sites. In contrast, Chung et al. (31) showed that deletion of the sequence between -376 and -265 did not affect TGF-β1-induced COL1A2 promoter activity in fibroblasts while the region between -265 and -241, containing an AP-1 site, was essential for ligand induction. There is no obvious explanation for these discrepancies in fibroblasts except for the experimental conditions and/or the TGF-β isoforms used. However, our data exclude that AP-1 binding is required for the TGF-β1 stimulation of COL1A2 in human mesangial cells, in agreement with data obtained in fibroblasts by Greenwel et al. (32). Mutation of the CAGA box (at -268/-260) decreased TGF-β1 responses in mesangial cells (Fig. 3C) as well as in fibroblasts. Six tandem repeats of this sequence are sufficient to induce TGF-β1 responsiveness to a minimal promoter (30, 34). However, our deletion experiments also indicate that, without the upstream Sp1 binding sites, the CAGA box is not able to confer TGF-β1 inducibility. This suggests that, in the context of the COL1A2 promoter, a single CAGA sequence requires the presence of Sp1 regulatory element(s) to support the TGF-β1 response. Further support of the importance of Sp1 binding for TGF-β1/Smad-mediated stimulation of COL1A2 comes from experiments in which human mesangial cells were cotransfected with 5’ deletion mutants of the COL1A2 promoter and Smad3 expression vector. In the absence of the Sp1 binding sites located between -376 and -268, overexpressed Smad3 cannot fully stimulate COL1A2 promoter activity (Fig. 5). In contrast, when the CAGA box between -268/-260 is mutated, overexpressed Smad3 is still able to stimulate COL1A2 transcription in a ligand-independent manner, suggesting that the effect of Smad3 overexpression on promoter activity is mediated through the Sp1 binding sites. Thus, our results indicate that both Sp1 binding sites and the CAGA box located between -376 and -258 play a role in TGF-β1-induced α2(I) collagen promoter activity in human mesangial cells.

Overexpression of Sp1 alone does not affect basal or TGF-β1-induced activity of the 376COL1A2-LUC construct, whereas Smad3 overexpression enhances basal expression and TGF-β1 responsiveness in a dose-dependent manner (Figs. 3B and 10A). These data indicate that Smad3 is the limiting factor for TGF-β1-induced α2(I) collagen expression. When Sp1 and Smad3 are overexpressed together, they cooperate to increased COL1A2 promoter activity. The low levels of stimulation of COL1A2 by Sp1 and TGF-β1-activated Smad3 in the absence of
the GC and CAGA sequences between −376 and −258 suggest that, when overexpressed, Sp1 and Smad3 could bind to more proximal regions of the promoter. Indeed, the TCCTCC motif at −128/−123, which was found to constitute a Sp1 binding site and to play a role in basal COL1A2 expression in fibroblasts (56), is separated by 3 bp from a motif similar to the Smad3/4 response element described by Dennler et al. (5). These regions could support TGF-β1 inducibility when Sp1 and Smad3 are overexpressed. This hypothesis is under investigation.

Binding of Sp1 to the COL1A2 promoter sequences between −283 and −250 was demonstrated by gel shift experiments. The presence of Smads in nucleoprotein complexes with the same promoter elements was also suggested by specific competition experiments. However, we were unable to detect Smad binding with a specific anti-Smad1/2/3 antibody. The same antibody recognized the interaction of endogenous Smads with an oligonucleotide containing three repeats of a CAGA box, as reported similar findings in NIH3T3 cells. The failure of the antibody to induce a supershift of Smad proteins in complex with the COL1A2 promoter could be due to the relatively low amount of Smads in these nucleoprotein complexes. Indeed, Smad binding to the TGF-β1-responsive element in the COL1A2 promoter could only be demonstrated in NIH3T3 cells when both Smad3 and Smad4 were overexpressed (53). Another possible explanation is that Sp1 and/or other nuclear factors could impair recognition by the anti-Smad antibody used in these experiments.

To our knowledge, this is the first report showing formation of complexes between endogenous Smad proteins with a member of the Sp family in nontransformed cells. Sp1 and Smad3/4 associate in the absence of TGF-β1, suggesting that Sp1 and Smad3/4 can interact in the absence of Smad3 phosphorylation. There is increased interaction upon ligand stimulation. This effect is maximal between 10 and 90 min after adding TGF-β1, similar to Smad3 phosphorylation (Figs. 7 and 8). However, the amount of Smad3/Sp1 complex detected remains higher than in control cells for up to 24 h. These results suggest that this minimal association between Sp1 and Smad3/4 might play a role in the sustained response to TGF-β1. In the coimmunoprecipitation experiments, Smad3 and Smad4, but not Smad2, were detected in Sp1 immunoprecipitates, suggesting that, in human mesangial cells, Sp1 preferentially associates with endogenous Smad3 and Smad4. However, we could not completely exclude association with Smad2, since the antibody used for detection of Smad2 and Smad3 recognizes Smad2 with less affinity. Moreover, in the Gal4-LUC assay system, TGF-β1-induced Gal4-Smad2 transcriptional activity is increased in the presence of Sp1, indicating that when overexpressed these two proteins can interact. Pardali et al. (57) have shown, by glutathione S-transferase pull-down analysis, that Smad2, Smad3, and Smad4 can directly interact with Sp1. Coimmunoprecipitation of tagged proteins transiently expressed in COS-7 cells showed that there is constitutive association between Sp1 and Smad3 and a weaker association with Smad2. Activation of the TGF-β signaling pathway by means of a constitutively active type I receptor increased these interactions. These data are similar to our results obtained with endogenous Smad and Sp1 proteins from human mesangial cells. Of note, only the 105-kDa form of Sp1 is detected in coimmunoprecipitation with Smad3. Since this species results from phosphorylation of the 95-kDa form and this modification occurs in vitro only when DNA is present in the reaction (58), this would argue that association of endogenous Sp1 and Smad3 occurs when Sp1 is bound to DNA.

Our experiments show that formation of Sp1 and Smad3/4 complexes (Figs. 7 and 8) leads to functional activation of COL1A2 transcription.
transcription (Figs. 9 and 10). Sp1 cooperates with Smad3 or Smad4 to stimulate transcription in a Gal4-LUC reporter assay system (Fig. 9A). The ligand-independent stimulation of Gal4-Smad3 by Sp1 is in agreement with the observation of Sp1/Smad3 complexes in the absence of TGF-β1. The transcriptional cooperation between Sp1 and Gal4-Smad3 or Gal4-Smad4(DN) is further increased when both chimeric proteins are expressed together, suggesting that Sp1, Smad3, and Smad4 synergistically interact to stimulate transcription. Using the same Gal4-LUC assay system with Gal4-Sp1 chimeric proteins, we showed that the C-terminal part of the B domain of Sp1 functionally cooperates with ligand-activated Smad3 to stimulate gene transcription (Fig. 9B). The B domain of Sp1 is able to activate transcription from a heterologous Gal4 promoter independently of TGF-β1 stimulation. Thus, in our system, the transcriptional activity of the B domain of Sp1 is not regulated by TGF-β1. In contrast, Li et al. and Feng et al. (51, 59) showed that TGF-β1 could stimulate the transactivating activity of the B domain of Sp1 in HaCaT cells. These differences might reflect cell-specific responses to TGF-β1. TGF-β1 could regulate Sp1 activity by modulating the activity of Sp1-associated proteins that are not present or are present in too low concentration in human mesangial cells compared with HaCaT cells. Indeed, when Smad3 is coexpressed, the Gal4-Sp1(B) and Gal4-Sp1(B-c) constructs become responsive to TGF-β1. TGF-β1 could also modulate the activity of Sp1 domains other than the transactivating domain B. In fact, Sp1 DNA binding activity has been shown to be modulated by several kinases including extracellular signal-regulated kinase 2 and protein kinase A activity (60, 61). Since we and others have shown that TGF-β1 stimulates extracellular signal-regulated kinase 1/2 and protein kinase A activity in mesangial cells (62, 63), these kinases could contribute to modulation of Sp1 activity in response to TGF-β1. Recently, Moustakas’ group showed that Smad3 increased activity of Gal4-LUC induced by chimeric proteins of Gal4 linked to various domains of Sp1 in HepG2 cells (57, 64). However, the effect of TGF-β1 was not
examined. Our experiments show that Smad3 has no significant inducing activity on Gal4-Sp1(B) or Gal4-Sp1(B-c) in the absence of TGF-β1, perhaps reflecting a cell-specific response. In summary, our results suggest that ligand-activated Smad3 either acts as a cofactor to Sp1 transcriptional activity or recruits other coactivators to modulate Sp1 transactivating activity.

Sp1 has been shown to interact directly with components of the TFIIID complex, TAF29110 and TAF29130, via the C-terminal part of its B domain (44, 65) and those interactions are thought to participate in the recruitment and/or stabilization of the preinitiation complex at the promoter in eukaryotes. Smad3 and Smad4 have been shown to interact with the coactivators CBP/p300 to the initiation transcription complex. Smad3 and Smad4 could enhance levels of transcription in response to TGF-β1 by interacting together and with TAFs and recruiting coactivators such as CBP/p300 to the initiation transcription complex.

In summary, we showed that Sp1 binding is required for Smad3 stimulation of COLIA2 promoter activity and is essential for the human glomerular mesangial cell responsiveness to TGF-β1. Sp1 and Smads form protein complexes, and their synergistic cooperation plays an important role in mediating TGF-β1-induced α(1)I collagen expression.

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