Specificity protein 1 and Smad-dependent regulation of human heme oxygenase-1 gene by transforming growth factor-β1 in renal epithelial cells

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Traylor A, Hock T, Hill-Kapturczak N. Specificity protein 1 and Smad-dependent regulation of human heme oxygenase-1 gene by transforming growth factor-β1 in renal epithelial cells. Am J Physiol Renal Physiol 293: F885–F894, 2007. First published June 13, 2007; doi:10.1152/ajprenal.00519.2006.—Excess transforming growth factor-β1 (TGF-β1) in the kidney leads to increased cell proliferation and deposition of extracellular matrix, resulting in progressive kidney fibrosis. TGF-β1, however, stabilizes and attenuates tissue injury through the activation of cytoprotective proteins, including heme oxygenase-1 (HO-1). HO-1 catabolizes pro-oxidant heme into substances with anti-oxidant, anti-apoptotic, anti-fibrogenic, vasodilatory and immune modulatory properties. Little is known regarding the molecular regulation of human HO-1 induction by TGF-β1 except that it is dependent on de novo RNA synthesis and requires a group of structurally related proteins called Smads. It is not known whether other DNA binding proteins are required to initiate transcription of HO-1 and, furthermore, the promoter region(s) involved in TGF-β1-mediated induction of HO-1 has not been identified. The purpose of this study was to further delineate the molecular regulation of HO-1 by TGF-β1 in human renal proximal tubular cells. Actinomycin D and nuclear run-on studies demonstrate that TGF-β1 augments HO-1 expression by increased gene transcription and does not involve increased mRNA stability. Using transient transfection, mithramycin A, small interfering RNA, electrophoretic mobility shift assays, and decoy oligonucleotide experiments, a TGF-β1-responsive region is identified between 9.1 and 9.4 kb of the human HO-1 promoter. This ~280-bp TGF-β1-responsive region contains a putative Smad binding element and specificity protein 1 binding sites, both of which are required for human HO-1 induction by TGF-β1.

gene regulation; fibrosis; renal proximal tubule cell

TRANSFORMING GROWTH FACTOR-β1 (TGF-β1) is a regulatory cytokine linked to the pathogenesis and progression of numerous kidney diseases due to its biological properties which result in increased extracellular matrix deposition and fibrosis (reviewed in Refs. 5, 54). TGF-β1 levels are increased in human and animal models of progressive kidney disease (9, 17, 55, 56). Transgenic mice overexpressing TGF-β1 develop progressive kidney fibrosis and die from renal failure (25). Paradoxically, TGF-β1 is released in response to injury to regulate homeostasis and suppress inflammation through mechanisms that are not clearly understood. One mechanism may involve its ability to upregulate a cytoprotective protein, heme oxygenase-1 (HO-1) (20, 27, 37), an inducible 32-kDa enzyme that modulates adaptive responses to tissue injury in several pathophysiological states (reviewed in Refs. 1, 31, 36). HO-1 catalyzes the rate-limiting step in the degradation of pro-oxidant heme yielding iron, biliverdin, and carbon monoxide (CO), a vasodilator, with anti-apoptotic and immunomodulatory effects (31, 38). Biliverdin is transformed by biliverdin reductase into bilirubin, an antioxidant that can scavenge lipid peroxides (11, 49). Recent studies demonstrated that HO-1, via bilirubin and CO, also displays anti-fibrogenic properties (16, 29, 32, 51, 53, 59).

The molecular mechanisms of HO-1 induction by TGF-β1 have not been entirely elucidated. TGF-β1 signals through two receptor serine-threonine kinases (type I and type II) to initiate downstream signaling events through intracellular effector substrates, which include a group of structurally related proteins called Smads (reviewed in Refs. 12, 34). Activated TGF-β receptors phosphorylate receptor-regulated Smads (Smad2, Smad3) which in turn form complexes with the common mediator Smad (Smad4) (12, 34). The Smad complex translocates to the nucleus and activates transcription by directly binding to a Smad binding element (SBE), a β hairpin with the major groove of the sequence GTCT and CAGA, or by association with other DNA binding proteins (12, 48, 57). Smad3 and Smad4 have also been shown to bind to GC-rich sequences (12, 15, 23). Smads can interact with diverse transcriptional coactivators including the Jun family of proteins, CCATT enhancer binding protein, and specificity protein 1 (Sp1) as well as others (reviewed in Ref. 12).

What is known regarding the molecular mechanism of HO-1 upregulation by TGF-β1 is that HO-1 induction by TGF-β1 is dependent on de novo RNA synthesis and is attenuated by overexpression of inhibitory Smad, Smad7, in human renal epithelial cells (20). Although it has been demonstrated that HO-1 induction by TGF-β1 requires Smads (20, 26), it is not known whether other DNA binding proteins are required and, if so, which one(s) to initiate transcription of HO-1. Furthermore, the promoter region(s) involved in TGF-β1-mediated activation of HO-1 has not been identified. It has been previously demonstrated that TGF-β1-mediated HO-1 induction requires sequences outside the ~4.5-kb promoter region responsive to hemin, cadmium, triterpenoid, and nerve growth factor (19, 20, 30, 44). In addition, we previously described an internal enhancer in the human HO-1 gene that, together with the 4.5-kb promoter, is responsible for maximal induction following heme and cadmium stimulation but it is not responsive to all stimuli, including TGF-β1 (19). The purpose of this study was to identify the TGF-β1-responsive region(s) in the human HO-1 promoter and further characterize the molecular mechanism(s) of TGF-β1-mediated HO-1 induction.

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EXPERIMENTAL PROCEDURES

Reagents. Tissue culture media, serum and supplements, Hank’s balanced saline solution, Lipofectamine 2000, LTX, Oligofectamine, OptiMEM, Klenow, and restriction enzymes were obtained from Invitrogen (Carlsbad, CA). Recombinant human TGF-β1 was from R & D Systems (Minneapolis, MN). Anti-HO-1 antibody (SPA 896) was from Stressgen (Vancouver, BC, Canada). Anti-actin antibody, actinomycin D, and mithramycin A were from Sigma (St. Louis, MO). Anti-Sp1 antibody was obtained from Upstate Cell Signaling Solutions. The peroxide-conjugated goat anti-rabbit IgG antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA) and SiGENOME SMARTpool, a mixture of four different sequences designed to target Sp1, was purchased from Dharmacon (Lafayette, CO). Adenovirus containing Smad4 (AdSmad4) was a generous gift from Dr. W. Samuel, National Institutes of Health (Bethesda, MD).

Cell culture. An immortalized human renal proximal tubule epithelial cell line from normal adult kidney, transduced with HPV-16 (45), HK-2 cells (American Type Culture Collection, Manassas, VA), was grown in keratinocyte serum-free medium (Invitrogen) supplemented with 5 ng/ml recombinant epidermal growth factor, 40 μg/ml bovine pituitary extract, and 1% antibiotic-antimycotic solution (In-vitrogen). MDA-MB-468 cells (American Type Culture Collection), a human breast cancer cell line deficient in Smad4, were kindly provided by Dr. W. Samuel and were grown in Leibovitz’s L-15 medium with 2 mM L-glutamine, 10% FBS, and 1% antibiotic-antimycotic solution. All cells were grown in a humidified incubator at 37°C, 95% air-5% CO2.

Expression constructs for Smad2, Smad3, Smad4, and Smad7 were generously provided by Takeshi Imamura and Kohei Miyazono and were transformed in DH10B competent cells and purified by Qiagen DNA isolation kits. All constructs were verified for orientation by sequencing and restriction digestion analysis.

Northern and immunoblot analysis. After treatments, total RNA and protein were collected and Northern and immunoblot analyses were performed as described previously (20). HO enzyme activity. Confluent HK-2 cells were treated with vehicle (BSA/HCl), hemin (5 μM), or TGF-β1 (10 ng/ml) for 6.5 h. HO enzyme activity was measured by previously described methods (2, 4). HO activity is expressed as picomoles of bilirubin formed per hour per milligram of protein.

Nuclear run-on assay. HK-2 cells were grown to confluency in 150-mm cell culture plates and treated with vehicle (BSA/HCl) or TGF-β1 (10 ng/ml) for 2 h. The transcription rate of the HO-1 gene by TGF-β1 was measured by nuclear run-on assay as previously described (3). Radiolabeled RNA was hybridized to a hybrid membrane containing 3.5 μg of human HO-1 and GAPDH cDNAs in the form of linearized plasmids (HO-1/pCDNA3.1 and GAPDH/pCR2.1TOPO, respectively).

Plasmid constructs. The construction of the 4.5-, 9.1-, and 11.6-kb promoter fragments in a luciferase vector (pHOGL3/4.5, pHOGL3/9.1, pHOGL3/11.6) have been described previously (19, 21). Additional HO-1 promoter deletion constructs (pHOGL3/9.7, pHOGL3/9.4, pHOGL3/8.5) were prepared by excising the 11.6-kb promoter fragment from pHOGL3/11.6, with Sac2 and MluI followed by exonuclease digestion with 3 units of Bal-31. The ends were repaired with Klenow and then digested with NheI, leaving the 5’-end of the promoter fragments blunt ended and the 3’-end with an NheI overhang. The pHOGL3/11.6 vector was linearized, by digestion with MluI, filling in the overhang with Klenow, followed by digestion with NheI. This resulted in the pGL3 vector with 3.5 kb of the HO-1 promoter with an NheI overhang and the other end of the vector containing a blunt end. The linear vector containing the 3.5-kb HO-1 promoter was treated with calf intestinal alkaline phosphatase and then ligated with the Bal-31-digested inserts. The ligation products were transformed in DH10B competent cells and purified by Qiagen DNA isolation kits. All constructs were verified for orientation by sequencing and restriction digestion analysis.

Expression constructs for Smad2, Smad3, Smad4, and Smad7 were generously provided by Takeshi Imamura and Kohei Miyazono and have been described previously (22). The Sp1 expression vector was kindly provided by Kun-Sang Chang (MDACC South Campus) and has been described elsewhere (52).

Transfection and measurement of luciferase activity. HK-2 cells (80–90% confluent) were grown in 100-mm tissue culture dishes and were transfected with equimolar amounts of promoter reporter plasmids using Lipofectamine 2000 or LTX, according to the manufacturer’s instructions. In some experiments, promoter-reporter plasmids were cotransfected with equimolar amounts of Smad2, Smad3, Smad4, Smad7, or Sp1 expression vectors and pCDNA3 empty vector. Three to six hours posttransfection of promoter-reporter plasmids, cells were passaged into 6- or 12-well tissue culture dishes to generate several sets of cells from the same transfection batch [a batch transfection protocol as previously described (19)]. Transfected cells were allowed to recuperate for 24 h before stimulus treatments with vehicle (BSA/HCl) or TGF-β1 (5–10 ng/ml) for 16 h. Luciferase activity was monitored using the luciferase reporter assay system (Promega) according to the manufacturer’s instructions using a Sirius Luminometer (Berthold Detection Systems, Pforzheim, Germany).

For Sp1 RNA interference, cells were transfected with a mixture of four different sequences designed to target Sp1 (siGENOME SMARTpool, 400 nM) or a nontargeting oligonucleotide as a control for nonsequence-specific effects (mock: AAUGGAAGACCCUC-CACCCUC, 400 nM), using Oligofectamine reagent. Oligofectamine without oligonucleotides was used as control. Cells were allowed to recover overnight before transfection with the promoter-reporter plasmids, treatment, and luciferase activity measurements as described above. Some cells were analyzed for Sp1 protein, HO-1 protein, or HO-1 RNA, by immunoblot or Northern analysis, as described above.

Nuclear extract and EMSA protocol. Nuclear extracts were prepared from HK-2 cells treated with TGF-β1 for 30 min. Cells were harvested in 30-ml conical tubes, pelleted, and resuspended in 300 μl of a hypotonic buffer containing 10 mM HEPES (pH 7.9), 0.75 mM spermidine, 0.75 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 10 mM KCl and incubated on ice for 15 min. Fifteen microliters of 10% NP-40 were added and cells were vortexed for 10 s. The tube was centrifuged at 2,000 rpm, 4°C for 15 s to pellet the nuclei. The supernatant was removed and the nuclear pellet was resuspended in 118.6 μl of a nuclear resuspension buffer containing 20 mM HEPES (pH 7.9), 0.75 mM spermidine, 0.75 mM spermine, 0.2 mM EDTA, 0.2 mM EGTA, 25% glycerol, 2 mM DTT; and 21.4 μl of 4 M KCl was added to the tube and was rocked vigorously on ice for 20 min. Forty-six microliters of cold resuspension buffer were added to the tube and mixed thoroughly. The nuclear extract was centrifuged at 10,000 rpm, 4°C for 15 min and the supernatant was transferred to a new tube and stored at −80°C.

The Sp1 and mutated Sp1 probes consisted of the oligonucleotides described in Fig. 7A annealed to their respective complements. Nuclear extracts (4 μg) were incubated with oligonucleotide inhibitor (20-fold molar excess) or Sp1 antibody (1 μg) in a 20-μl reaction containing 20% glycerol, 5 mM MgCl2, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris (pH 7.5), and 50 μg poly (dl)DC for 20 min at room temperature with 0.1 pmol of an end labeled probe. The resulting protein-DNA complexes were analyzed by electrophoresis in a 5% (w/v) nondenaturing gel. The gels were fixed in methanol (10%)/acetic acid solution (10%), then dried, and exposed to film.

Decoy oligonucleotide assays. The sequences of the forward strand for the wild-type, SBE-mutated, and Sp1-mutated decoy oligonucleotides are presented in Fig. 8A. Double-strand oligonucleotides were prepared by combining equal amounts of forward and reverse strand oligonucleotides in annealing buffer [10 mM Tris (pH 8), 1 mM EDTA, and 50 mM NaCl], boiling for 10 s, and slow cooling overnight at room temperature. The double-strand oligonucleotides were cotransfected in HK-2 cell cultures together with the pHOGL3/9.4 reporter construct, using 5- to 200-fold (3.25–130 pmol) greater molar equivalents of the double-strand decoy oligonucleotides vs. the
HO-1 INDUCTION BY TRANSFORMING GROWTH FACTOR-β1

induction of HO-1 by TGF-β1 occurs by direct increases in de novo transcription.

Since nitric oxide-mediated HO-1 induction occurs, at least in part, through increased HO-1 mRNA stability (6), the effects of TGF-β1 on HO-1 mRNA abundance and stability were explored by measuring the half-life of HO-1 mRNA in HK-2 cells. As shown in Fig. 2B, the half-life of HO-1 mRNA following TGF-β1 stimulation was ~2 h and was similar in the presence or absence of additional TGF-β1 exposure. Similar results were obtained in the primary renal proximal tubular cell culture (data not shown). These data suggest that, similar to most other inducers of HO-1 including heme, mRNA stability is not involved in TGF-β1-mediated HO-1 mRNA induction.

A cis-acting region between −9.1 and −9.4 kb of the human HO-1 promoter is responsible for TGF-β1-mediated HO-1 induction. To identify promoter elements in the human HO-1 gene that mediate its transcription following stimulation with TGF-β1, we performed transient transfection experiments in HK-2 cells using equimolar amounts of 4.5-, 8.5-, 9.1-, 9.4-, 9.7-, and 11.6-kb promoter fragments of the HO-1 gene cloned into a promoterless luciferase vector (pGL3). Previously, we showed that the 4.5-kb construct demonstrates significant reporter activity with heme, cadmium, and triterpenoids but not with TGF-β1 or oxidized LDL, implying that the mechanism of the upregulation of HO-1 differs depending on the inducer (3, 19, 20, 30). As shown in Fig. 3A, the 4.5- and 8.5-kb promoter fragments were not responsive to TGF-β1 (5 ng/ml, report construct, treated with TGF-β1, and assayed for luciferase activity as described above.

Data analysis. All results are derived from at least two to four independent experiments. Unpaired t-test with Welch correction was used to compare two groups. For multiple group comparisons, ANOVA and Student-Newman-Keuls posttest were used. Data are represented as means ± SE and considered significant at P < 0.05.

RESULTS

TGF-β1 induces HO-1 mRNA, protein expression, and HO activity in an immortalized human renal proximal tubule (HK-2) cell line. HK-2 cells, an immortalized renal proximal tubular epithelial cell line, were examined for TGF-β1 responsiveness and steady-state levels of HO-1 mRNA and protein were analyzed. Similar to a primary human renal proximal tubular cell line (20), HK-2 cells treated with TGF-β1 exhibited a significant induction in HO-1 mRNA and protein over vehicle-treated control (Fig. 1). Maximum HO-1 mRNA induction (~5.4-fold) was observed at 4 h (Fig. 1A) and maximum induction of HO-1 protein was observed at 4–8 h (~3.1-fold, Fig. 1B). HO enzyme activity was ~1.5-fold higher in TGF-β1-treated HK-2 cells than vehicle-treated cells [10.17 ± 0.12, 15.77 ± 0.47, and 73.93 ± 10.75 pmol of bilirubin formed h⁻¹·mg protein⁻¹ for vehicle-, TGF-β1-, and hemin (positive control)-treated cells, respectively, n = 2).

Transcriptional activation of HO-1 by TGF-β1. The importance of de novo transcription was assessed by nuclear run-on analysis following stimulation with TGF-β1. HK-2 cells were treated with media containing vehicle (control) or TGF-β1 (10 ng/ml) and nuclei were collected after 2 h. Nuclei were then allowed to transcribe RNA in the presence of radiolabeled 32P-UTP as described in EXPERIMENTAL PROCEDURES. As shown in Fig. 2A, TGF-β1 augmented HO-1 gene transcription by about twofold compared with control cells. Therefore, the idea that TGF-β1 induces HO-1 transcription by a mechanism that is distinct from heme treatment.

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Fig. 1. Heme oxygenase-1 (HO-1) mRNA and protein are upregulated by transforming growth factor-β1 (TGF-β1) in HK-2 cells. A: confluent HK-2 cells were treated with TGF-β1 (5 ng/ml) for 2–24 h and TGF-β1 (3.0–7.0 ng/ml) for 4 h. RNA was collected and Northern analysis was performed using 32P-labeled human HO-1 and GAPDH cDNA probes as described in EXPERIMENTAL PROCEDURES. B: HK-2 cells were treated with TGF (5 or 10 ng/ml) for 4–24 h. For immunoblot analysis, total cell lysates were collected and separated as described in EXPERIMENTAL PROCEDURES. Membranes were incubated with anti-HO-1 (1:5,000) followed by a 1:10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG antibody (1:10,000). Vehicle (V) for TGF-β1 (BSA/HCl) was used as control. Representative Northern immunoblots of n = 3.

Fig. 2. TGF-β1-mediated HO-1 induction occurs at the transcriptional level and not by stabilization of HO-1 mRNA. A: confluent HK-2 cells were treated with V (BSA/HCl) or TGF-β1 (10 ng/ml) for 2 h and nuclei were collected. Nuclei were allowed to transcribe RNA in the presence of ATP, CTP, GTP, and radiolabeled 32P-UTP as described in EXPERIMENTAL PROCEDURES. Radio-labeled RNA was purified and hybridized to a hybond membrane containing 3.5 μg of HO-1 and GAPDH cDNA in the form of linearized plasmids (HO-1/pcDNA3.1 and GAPDH/pCR2.1TOPO, respectively). Representative of n = 2. B: confluent HK-2 cells were all exposed to TGF-β1 (5 ng/ml) for 4 h, the time at which maximal induction is observed, except V (BSA/HCl)-treated control (lane 1). Cells were then washed with HBSS and fresh media was added containing actinomycin D (Act. D; 4 μM) with or without additional TGF-β1 (5 ng/ml). RNA was collected at 0 (no additional treatment) and various time points up to 8 h. Northern analysis was performed as described in EXPERIMENTAL PROCEDURES. Representative of n = 3.
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induction of HO-1 by TGF-β1

1. Computer analysis reveals that each demonstrated increased activity (1.5-fold) and the 9.4-, 9.7-, and 11.6-kb fragments (regions 9.1 and 9.4 kb of the human HO-1 promoter are required for TGF-β1-mediated HO-1 induction in renal proximal tubular epithelial cells, EMSA was performed using end-labeled dsDNA probes containing the putative Sp1-binding site (Fig. 7A). Protein-DNA complexes were competed out with 20-fold molar excess of unlabeled wild-type probe (Fig. 7B, lane 3). To show specificity, supershift experiments were performed with an anti-Sp1 antibody, which produced a reduction in the upper band shift intensity and a small supershift (Fig. 7B, lane 4). When the GT/GC-box was mutated in the 9.4-kb promoter fragment of the HO-1 gene (pHOGL3/9.4), containing the ~280-bp TGF-β1-responsive region with its putative Sp1 sites was assayed for luciferase activity after exposure to TGF-β1 in the presence or absence of mithramycin A (100 nM). Interruption of GC-binding suppressed TGF-β1-inducible promoter activity as well as basal promoter activity (Fig. 5A). In addition, mithramycin A also blocked endogenous HO-1 mRNA and protein expression induced by TGF-β1 in HK-2 cells (Fig. 5, B and C).

Effect of Sp1 on the transcriptional activity of the 9.4-kb HO-1 promoter. Cotransfections were performed in HK-2 cells with the pHOGL3/9.4 construct and an Sp1 expression vector or siRNA targeted against Sp1. Overexpression of Sp1 enhanced basal HO-1 promoter-reporter activity by ~2.5-fold (Fig. 6A), whereas siRNA-based inhibition of Sp1 reduced basal reporter activity by ~28% compared with mock siRNA control and prevented TGF-β1-mediated activation (Fig. 6B). As shown in 6C, siRNA against Sp1 reduced Sp1 proteins levels by ~76% which was sufficient to reduce reporter activity, but not TGF-β1-mediated increases in endogenous HO-1 mRNA or endogenous HO-protein (data not shown).

EMS indicates that Sp1 interacts with the HO-1 promoter in vitro. To further examine the role of Sp1 in TGF-β1-mediated HO-1 induction in renal proximal tubular epithelial cells, EMSA was performed using end-labeled dsDNA probes containing the putative Sp1-binding site (Sp1 probe, Fig. 7A). Obvious protein-DNA complexes were observed with the wild-type Sp1 oligonucleotide probe (Fig. 7B, lane 2 and Fig. 7C, lane 1). Protein-DNA complexes were competed out with 20-fold molar excess of unlabeled wild-type probe (Fig. 7B, lane 3). To show specificity, supershift experiments were performed with an anti-Sp1 antibody, which produced a reduction in the upper band shift intensity and a small supershift (Fig. 7B, lane 4). When the GT/GC-box was mutated in the Sp1 probe, there was no formation of the upper protein-DNA complex (lanes 2–5). Interestingly, when Mut2 and Mut4 (regions 5’ of the GT-box/GC-box also mutated) were used as probes (Fig. 7C, lanes 3 and 5), there was no formation of either protein-DNA complex. Taken together, Sp1 appears to bind to putative Sp1-binding sites in the HO-1 promoter.
Effect of decoy oligonucleotides on TGF-β1-mediated HO-1 induction. The region between 9.1 and 9.4 kb of the HO-1 promoter is very GC rich, including a sequence of 22 G’s and/or C’s between the GT-box/GC-boxes of the SBE. Attempts to make mutations in the pHOGL3/9.4 based on PCR were limited by several factors such as the size of the plasmid, difficulty in designing unique primers, and most notable, the string of G’s and C’s. In fact, in several experiments all elongation ceases at the GC box located at 9.19 kb. Thus we performed studies using a decoy oligonucleotide method that has been well described (10, 14, 24). Double-strand decoy oligonucleotides were cotransfected with the HO-1 promoter construct in HK-2 cell cultures to interfere with Smad and/or Sp1 binding to respective cis-acting elements within the 9.4-kb HO-1 promoter-reporter vector. Figure 8A lists the top strand sequences of wild-type and mutated oligonucleotides used for these decoy studies. To establish the amount of decoy oligonucleotides required, HK-2 cells were batch transfected with the pHOGL3/9.4 construct (0.65 pmol) and 5- to 200-fold greater (3.25–130 pmol) wild-type double-strand oligonucleotide bearing the putative SBE and the Sp1 cis-sequence. HO-1 promoter transcription was determined after TGF-β1 (5 ng/ml, 16 h) or vehicle treatment. When the reporter construct was cotransfected with 100- and 200-fold more wild-type decoy oligonucleotide, TGF-β1-induced transcription was inhibited (Fig. 8B). Using 200-fold greater Sp1 and/or SBE mutated decoy oligonucleotides, TGF-β1-induced reporter activities were restored (Fig. 8C). Thus both the SBE and Sp1-binding sites are required for transcriptional activation of the human HO-1 promoter by TGF-β1.

DISCUSSION

TGF-β regulates a broad range of cellular activities that are often cell type and context dependent, including proliferation, differentiation, apoptosis, adhesion, motility, and extracellular matrix deposition (reviewed in Ref. 33). While the Smad pathway is the best-characterized mechanism of TGF-β signaling, alternate pathways also play key roles in modulating TGF-β responsiveness. In fact, combinatorial signaling of a diversity of DNA sequence-binding transcription factors and the Smad pathway has been shown to modulate the wide range of different responses to TGF-β (reviewed in Refs. 8, 12, 47). Smads have been shown to interact with several transcription factor members of the bHLH, the bZip, the forkhead, the nuclear receptor, the Runx, the zinc finger protein families as well as others (reviewed in Refs. 12, 33). In this way, TGF-β can elicit different gene responses based on the availability of...
cofactors within a cell type. Thus identifying Smad DNA-binding cofactors is important for delineating cell-specific responses of TGF-β action. One such regulatory mechanism for several TGF-β-responsive genes, including amyloid-β peptide, a cyclin-dependent kinase inhibitor (p15Ink4B), and others, involves the cooperation between Smads and the transcription factor Sp1, a member of the zinc finger protein family, to regulate gene expression (7, 10, 13, 28, 39, 42, 46, 58). Here, we identify a TGF-β-responsive region between 9.1 and 9.4 kb of the human HO-1 promoter that contains a putative SBE and several putative Sp1-binding sites.

Fig. 5. GC-binding inhibitor, mithramycin A, prevents TGF-β1-mediated HO-1 promoter activity as well as HO-1 mRNA and protein induction. A: HK-2 cells were transiently transfected with pHOGL3/9.4 (~9.4-kb HO-1 promoter). Five to seven hours posttransfection, cells were split into 12-well trays and allowed to recover for 24 h. Cells were exposed to mithramycin A (MA; 100 nM) or V for MA (VMA; methanol) for 3 h before TGF-β1 (5 ng/ml) or V for TGF-β1 (BSA/HCl) for 16 h. Cell lysates were collected and luciferase activity was measured (n = 3, with 12 replicates/group/experiment, *P < 0.001 vs. V-treated pHOGL3/9.4). B and C: HK-2 cells were treated with MA (10 or 100 nM) or VMA (methanol) for 16 h and then TGF-β1 (5 ng/ml) or V for TGF-β1 (BSA/HCl) for 4 h for Northern analysis (B, representative of n = 3) or 8 h for immunoblot analysis (C, representative of n = 3) as described in EXPERIMENTAL PROCEDURES.

Fig. 6. Effects of overexpression of Sp1 or siRNA targeted against Sp1. A: HK-2 cells were cotransfected with pHOGL3/9.4 (~9.4-kb HO-1 promoter) and an Sp1 expression vector or empty vector (pcDNA3). Five to seven hours posttransfection, cells were split into 12-well trays and allowed to recover for 40 h (n = 3, with 12 replicates/group/experiment, *P < 0.0001 vs. empty vector control). B: HK-2 cells were transfected with siRNA against Sp1 (siSp1, 400 nM) or mock (400 nM) and allowed to recover for 24 h before transfected with pHOGL3/9.4. Five to seven hours posttransfection, cells were split into 12-well trays and allowed to recover for 24 h before treatment with TGF-β1 (5 ng/ml) or V for TGF-β1 (BSA/HCl) for 16 h (n = 2, with 12 replicates/group/experiment, †P < 0.001 vs. all other groups, ‡P < 0.05 vs. mock control). C: HK-2 cells were transfected with oligofectamine alone (control), 400 nM mock control (mock), or 400 nM siSp1. Protein was collected 28 h later and immunoblot analysis of Sp1 was performed. Top: immunoblot (representative of n = 5). Bottom: graphical representation of arbitrary units of Sp1/actin densitometry. ‡P < <0.05 vs. oligofectamine control.
a putative SBE in close proximity to several GC-rich potential Sp1-binding sites (Fig. 3B). TGF-β1 has been previously shown to increase the association between Sp1 and Smads in human mesangial cells and human renal proximal tubular epithelial cells using a biotinylated DNA precipitation assay and immunoprecipitation studies (42, 58). Herein, we demonstrate that mithramycin A, a G-C-specific DNA binding antibiotic that inhibits RNA synthesis by preventing binding of regulatory proteins such as Sp1 to G-C-rich sequences (42, 43, 58), prevented TGF-β1-mediated HO-1 mRNA, protein expression, and promoter activity. In EMSA, a protein-DNA

Fig. 7. EMSA showing DNA-protein interaction with Sp1-binding site. A: sequences of the forward strand for the oligonucleotides containing the Sp1 site just 3’ proximal to the SBE site of the human HO-1 promoter (boxed oligonucleotide region in Fig. 3B) used for EMSA probes are shown. The Sp1 core sequence is underlined, and mutated base pairs are shown in larger font. B: nuclear extracts were prepared from TGF-β1-treated HK-2 cells and incubated with the Sp1 probe (lane 2). The arrow and arrowhead indicate the shifted DNA-protein complexes. A 20-fold excess of cold competitor (cc) was added as indicated consisting of the cold probe itself (lane 3). Specific Sp1 antibody (Ab) was added and the supershift marked with an * (lane 4). C: nuclear extracts were prepared from TGF-β1-treated HK-2 cells and incubated with the Sp1 probe (lane 1) or mutated probes (Mut1-Mut4, lanes 2–5, respectively). The arrow and arrowhead indicate the shifted DNA-protein complexes. Representative EMSAs of n = 3, using different nuclear extracts each time.

Fig. 8. Both the SBE and Sp1-like sequences are required for TGF-β1-mediated HO-1 induction. A: sequences of the forward strand for the wild-type, SBE-mutated, and Sp1-mutated decoy oligonucleotides are shown. The SBE and Sp1 consensus sequences are underlined, and mutated base pairs are in larger font. B: pHOGL3/9.4 (0.65 pmol): wild-type (WT) decoy oligonucleotide (3.25–130 pmol) dose response; each bar represents n = 12 replicates/group. C: wild-type and mutated decoy oligonucleotides (expression vector/decoy equal to 1:200), each bar represents n = 2–8 independent experiments with 12 replicates/group/experiment. *P < 0.001, †P < 0.01 compared with respective expression vector/decoy, V-treated control.
complex that is formed using a probe containing the Sp1-binding sequence within the 280-bp TGF-β1-responsive region is supershifted by Sp1 antibody. When the Sp1-binding sequence is mutated, binding of the upper protein-DNA complex is lost. Furthermore, siRNA targeted against Sp1 reduced both basal and TGF-β-mediated promoter activity while overexpression of Sp1 enhanced HO-1 promoter activity.

Overexpression of Smad2, Smad3, and/or Smad4 increased basal promoter activity, with Smad3 having the largest effect on activation of the human HO-1 promoter. Smad2 and Smad3 have been shown to have differential roles in TGF-β1-induced cellular responses. TGF-β1/Smad2 signaling is a key mediator of TGF-β-induced profibrotic outcomes in renal and nonrenal cells (26, 41). Recently, it has been suggested that early TGF-β1 responses that initiate tubulointerstitial fibrosis (induction of connective tissue growth factor, downregulation of E-cadherin) are critically dependent on Smad3; however, delayed responses involved in the development of tubulointerstitial fibrosis (induction of matrix metalloproteinase-2) require Smad2 (40). HO-1 induction has been shown to be antifibrogenic and perhaps its upregulation by TGF-β1 may serve as a counterbalance to the profibrotic effects observed in early events (16, 18, 29, 32, 35, 51, 53, 59).

HO-1 induction by TGF-β1 is dependent on the Smad pathway and was first observed by overexpression of the inhibitor Smad, Smad7, in human renal proximal tubular cells (20). Here, we show that overexpression of Smad7 inhibits basal and TGF-β1-mediated HO-1 promoter/reporter activity. Further corroboration of the involvement of Smads was provided using a Smad4-deficient breast cancer cell line to reveal that Smad4 is required for TGF-β1 but not hemin-mediated HO-1 induction. These observations are consistent with a report by Kretscher et al. (26) who used antisense oligonucleotides to knockdown Smad2, Smad3, and Smad4 expression to demonstrate that the presence of all three Smads is required for the induction of HO-1 by TGF-β in HaCaT cells, a human keratinocyte cell line.

The critical TGF-β1-responsive elements within the HO-1 promoter were further explored using an oligonucleotide decoy assay as described in numerous other studies, including the transcriptional regulation of amyloid-β precursor protein by TGF-β1 (10, 14, 24). In this approach, transcription factors interact with wild-type decoy oligonucleotides instead of the natural regulatory motifs (reviewed in Ref. 14). A 33-bp wild-type decoy oligonucleotide containing the SBE and the proximal Sp1 consensus sequences located in the human HO-1 promoter prevented TGF-β1-mediated activity of the HO-1 promoter-reporter vector, implicating the importance of both the SBE and Sp1-binding sites. When the SBE site or the Sp1 site in the decoy oligonucleotide was mutated, the decoy no longer functioned and TGF-β1-mediated HO-1 promoter/reporter activity was restored. Thus both the Smads and Sp1 are required for TGF-β1-inducible HO-1 promoter-reporter activity. HO-1 upregulation by TGF-β1 may therefore follow a model similar to that described by Feng et al. (13) regarding TGF-β1-mediated upregulation of p15Ink4b, where upon TGF-β1 treatment, the Smad complex undergoes nuclear translocation and interacts directly with an SBE adjacent to DNA-bound Sp1 to increase transcription. Conversely, TGF-β1-mediated induction of HO-1 may resemble a more complex model similar to that described for TGF-β-mediated erythropoietin upregulation. Sanchez-Elsner et al. (46) reported that TGF-β stimulation causes a region of the upstream erythropoietin promoter and a 3′ downstream enhancer to become in physical contact through Sp1 and Smad3, which occurs through binding of the intervening sequences. Sp1 reinforces the promoter/enhancer contact, while Smad3 stabilizes the complex by interacting with hypoxia-inducible factor-1/Sp1/HNF-4 and the coactivator CBP/p300 (46).

In summary, a region between 9.1 and 9.4 kb of the human HO-1 promoter, Smads, and Sp1 are required to activate transcription of HO-1 in response to TGF-β1 in renal proximal tubular cells. This is a molecular mechanism that differs from that of other known inducers of human HO-1. We speculate that TGF-β1 release after tissue injury promotes healing and restoration of organ function through a balanced activation of signaling systems which counteract excessive fibrosis development such as HO-1 with its protective and antifibrogenic properties.

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REFERENCES


12. Dore S, Takahashi M, Ferris CD, Hester LD, Guastella D, Snyder SH, Bilirubin, formed by activation of heme oxygenase-2, protects neurons...


