Growth Factors, Cytokines, Cell Cycle Molecules

ERK, p38, and Smad Signaling Pathways Differentially Regulate Transforming Growth Factor- β 1 Autoinduction in Proximal Tubular Epithelial Cells

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Transforming growth factor (TGF)- β 1 is a mediator of the final common pathway of fibrosis associated with progressive renal disease, a process in which proximal tubular cells (PTCs) are known to play an important part. The aim of the current study was to examine the mechanism of PTC TGF- β 1 autoinduction. The addition of TGF- β 1 led to increased amounts of TGF- β 1 mRNA and increased *de novo* protein synthesis. The addition of TGF- β 1 led to increased phosphorylation of R-Smads and activation of extracellular signal-regulated kinase mitogen-activated protein (MAP) kinase and p38 MAP kinase pathways. Use of a dominant-negative Smad3 (Smad3 DN) expression vector, Smad3 small interfering RNA, and inhibition of extracellular signal-regulated kinase and p38 MAP kinase pathways with the chemical inhibitors PD98059 or SB203580 suggested that activation of these signaling pathways occurred independently. Smad3 DN expression, Smad3 small interfering RNA, or the addition of PD98059 inhibited TGF- β 1-dependent stimulation of TGF- β 1 mRNA. Furthermore, Smad3 blockade specifically inhibited activation of the transcription factor AP-1 by TGF- β 1, whereas PD98059 prevented TGF- β 1-dependent nuclear factor- κ B activation. In contrast inhibition of p38 MAP kinase inhibited *de novo* TGF- β 1 protein synthesis but did not influence TGF- β 1 mRNA expression or activation of either transcription factor. In summary, in PTCs, TGF- β 1 autoinduction requires the coordinated action of independently regulated Smad and non-Smad pathways. Furthermore these pathways regulate distinct transcriptional and translational components of TGF- β 1 synthesis. (*Am J Pathol* 2006, 169:1282–1293; DOI: 10.2353/ajpath.2006.050921)

inflammation, and diabetes, resulting in end-stage renal failure.^{1,2} With the realization that the degree of interstitial fibrosis is the best correlate with the rate of progression of renal dysfunction,^{3–8} interest has focused on the possible mechanisms that may drive this process.

The most prominent cell type in the renal cortex is the proximal tubular epithelial cell (PTC), responsible in health for the maintenance of fluid and electrolyte balance. We have previously examined the mechanisms that stimulate PTC transforming growth factor (TGF)- β 1 synthesis^{9–13} because it plays a pivotal role in accumulation of extracellular matrix during renal fibrosis and the transition of renal tubular epithelial cells to myofibroblasts.^{14,15} We have demonstrated independent regulatory pathways for TGF- β 1 transcription and translation, with activation at both levels required to increase TGF- β 1 generation. TGF- β 1 positively regulates its own expression in normal and transformed cells.¹⁶ Thus autoinduction of TGF- β 1 at sites of injury may result in a positive feedback loop perpetuating the fibrotic process, thus leading to organ failure. Transcriptional autoinduction of TGF- β 1 has been shown to be dependent on AP-1 in various cell types,¹⁷ and in renal tubular cells, on Smad3.¹⁸ Smad proteins are the specific intracellular effector molecules activated by TGF- β 1. Smad2 and Smad3 are phosphorylated directly by the TGF- β type I receptor kinase, after which they hetero-oligomerize with Smad4, translocate to the nucleus, and together with their binding partners activate or repress their target genes. TGF- β 1 also activates mitogen-activated protein (MAP) kinase signaling pathways, and we have previously demonstrated involvement of both the extracellular signal-regulated kinase (ERK) MAP kinase and p38 MAP kinase pathway in glucose-stimulated TGF- β 1 synthesis.¹² The

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Renal interstitial fibrosis is the common end result caused by diverse clinical entities such as obstruction, chronic

aim of the current study was to characterize the mechanisms involved in TGF- β 1 autoinduction in PTCs. We have sought to define the role of Smad and non-Smad/MAP kinase pathways and to differentiate the contribution that these pathways make to transcriptional and translational activation during TGF- β 1 autoinduction.

Materials And Methods

Materials

Antibodies for Western blot analysis and the final working dilution were as follows: rabbit polyclonal anti-phospho-p38 MAP kinase antibody (dilution, 1:500), rabbit polyclonal anti-p38-MAP kinase antibody (dilution, 1:500), polyclonal rabbit anti-phospho-ERK MAP kinase (dilution, 1:500), rabbit polyclonal anti-ERK-MAP kinase (dilution, 1:500), rabbit polyclonal anti-phospho-Smad3/1 (dilution, 1:500) from Cell Signaling Technology (Beverly, MA); rabbit polyclonal anti-Smad3 (dilution, 1:500), from Zymed Laboratories Inc. (San Francisco, CA); goat anti-rabbit horseradish peroxidase-conjugated secondary antibody from Santa Cruz Biotechnology, Inc. (Wiltshire, UK); and anti-c-Myc polyclonal antibody from Sigma (Poole, UK). For supershift assays, polyclonal rabbit anti-c-fos, c-Jun antibodies, and anti-p50, anti-p52, anti-p65, anti-C-Rel, and anti-Rel-B antibodies were all purchased from Santa Cruz Biotechnology, Inc. For immunoprecipitation of radiolabeled TGF- β 1, we used rabbit polyclonal anti-TGF- β 1 antibody (Santa Cruz Biotechnology, Inc). PD98059, a specific and permeable inhibitor of MAP kinase kinase (MEK), and SB203580, a highly specific cell-permeable inhibitor of p38 kinase were purchased from Calbiochem (Nottingham, UK). 3 H-Amino acid mixture, [γ - 32 P]ATP and [α - 32 P]dTTP were from Amersham (Little Chalfont, UK), and recombinant human TGF- β 1 was from R&D Systems (Oxford, UK).

Cell Culture

HK-2 cells (human renal proximal tubular epithelial cells immortalized by transduction with human papilloma virus 16 E6/E7 genes¹⁹) were cultured in Dulbecco's modified Eagle's medium/Ham's F12 (Gibco BRL, Paisley, UK) supplemented with 10% fetal calf serum (Biological Industries Ltd., Cumbernauld, UK), 2 μ mol/L L-glutamine (Gibco BRL), 20 mmol/L HEPES buffer (Gibco BRL), 5 μ g/ml insulin, 5 μ g/ml transferrin (Sigma), 40 ng/ml hydrocortisone (Sigma), and 5 ng/ml sodium selenite (Sigma). Cells were grown at 37°C in 5% CO₂ and 95% air. Fresh growth medium was added to cells every 3 to 4 days until confluent. With the exception of the cells used for transfection, cells were growth-arrested in serum-free medium for 48 hours before use in experiments. All experiments were performed in serum-free conditions. In all aspects of cell biology that we have studied previously, HK-2 cells respond in an identical manner to primary cultures of human PTCs.^{11,13,20,21} They are therefore a

good model from which general conclusions can be drawn in terms of PTC biology.

Transient Transfection: Reporter Gene Analysis

Transient transfection and reporter gene analyses using HK-2 cells were performed as previously described.¹² Briefly, 80% confluent HK-2 cells were changed to serum-free medium for 4 hours before addition of plasmids and the mixed lipofection agent Fugene 6 (Roche, Lewes, UK) at a ratio of 1 μ g plasmid/3 μ l transfection agent. After an overnight incubation, the medium was replaced with serum-free medium containing additives as necessary for the experiment. Smad-responsive promoter (SBE)₄-Lux (0.9 μ g) (a gift from Aristidis Moustakas, Ludwig Institute for Cancer Research, Uppsala University, Uppsala, Sweden²²) was transfected with 0.1 μ g of pRL-CMV *Renilla* (Promega, Madison, WI) to control for transfection efficiency. After lysis of the cells in reporter lysis buffer (Promega), firefly and *Renilla* luciferase content was quantified using the Dual-Glo assay (Promega).

Inhibition of Smad3: Dominant-Negative Plasmid

The Smad3 dominant-negative expression vector (a gift from Jeffrey Wrana, Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada) was transfected as above, at a ratio of 1 μ g of plasmid/3 μ l of Fugene 6. To confirm adequate transfection efficiency, in separate experiments, cells were transfected with the constitutively active GFP expression vector EGFP-C1 (Clontech, Basingstoke, UK) using Fugene 6 at a ratio of 3 μ l of Fugene/1 μ g of plasmid DNA. Control cells were exposed to mock transfection. Twenty-four hours later, GFP expression was assessed by flow cytometry using channel FL-1 on a FACScalibur flow cytometer (Becton Dickinson, Cowley, UK) to assess transfection efficiency as previously described.²³ Ten thousand cells per group were examined.

Inhibition of Smad3: siRNA

Transfection of HK-2 cells with small interfering RNA (siRNA) was optimized using the GAPDH Silencer II kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. In brief, 9×10^4 cells per 12-well plate well were transfected in suspension with 30 nmol/L siRNA and 5 μ l of siPORT amine (Ambion) in a final volume of 1000 μ l. After 48 hours, cells were lysed and RNA extracted using Triagent, before detection of gene expression using quantitative polymerase chain reaction (PCR) as described below. This protocol was found to give optimal knockdown (reliably 80% or greater reduction in GAPDH mRNA, data not shown). After optimization, the same protocol was followed for Smad3 siRNA transfection (siRNA ID115717; Ambion). Mock-transfected and scrambled siRNA-transfected controls were included in all experiments.

Northern Blot

Northern blotting for TGF- β 1 was performed as previously described.¹¹ After detection of TGF- β 1, blots were stripped and reprobed for GAPDH to confirm approximately equal loading.

Quantitative Reverse Transcriptase (RT)-PCR

After cDNA synthesis as previously described,¹¹ TGF- β 1 mRNA was quantified by free RT-PCR. Primers and probe for TGF- β 1 quantification were designed using Primer Express Software, and purchased from Applied Biosystems (Warrington, UK). The amplification product is intron spanning. Sequences used are; forward primer, 5'-CCT-TTCCTGCTTCTCATGGC-3'; reverse primer, 5'-ACTTC-CAGCCGAGGTCCTTG-3'; probe, 5'-FAM-ACACCACT-ATTGCTTCAGCTCCACGGAGA-3'-TAMRA. PCR was performed on an ABI Prism 7000 (Applied Biosystems) using the machine's standard protocol in a total volume of 25 μ l containing 300 nmol/L forward and reverse primers, 100 nmol/L probe, and 50% v/v 2 $\times$ TaqMan reaction buffer (Applied Biosystems). Smad3 and 18S rRNA expression were quantified using predeveloped TaqMan assay reagent control kits (Applied Biosystems) according to the manufacturer's instructions. Fold changes in expression were calculated using the formula $2^{-(\Delta\text{Ct sample1} - \Delta\text{Ct sample2})}$, where ΔCt is the difference between the amplification threshold for Smad3 or TGF- β 1 and rRNA. *P* values were calculated by analysis of variance using Microsoft Excel.

Analysis of Efficiency of Translation

Polysome analysis was performed as previously described.²⁴ Approximately 1.3×10^7 growth-arrested cells per experiment were trypsinized, pelleted, and extracted in 1 ml of ice-cold lysis buffer [10 mmol/L Tris-Cl, pH 8.0, 150 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 0.5% Nonidet P-40, and 500 U/ml⁻¹ recombinant RNasin (Promega)]. Nuclei were removed by centrifugation at $3000 \times g$ for 2 minutes and the supernatant transferred to a new tube supplemented with 100 μ g/ml cycloheximide, 1 mmol/L phenylmethyl sulfonyl fluoride, 10 mmol/L dithiothreitol, and 0.5 mg/ml heparin then centrifuged at $13,000 \times g$ for 5 minutes to remove mitochondria and membrane debris. The supernatant was layered onto a 10-ml 15 to 40% linear sucrose gradient containing 10 mmol/L Tris-Cl, pH 7.5, 140 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 10 mmol/L dithiothreitol, 10 mmol/L cycloheximide, and 0.5 mg/ml heparin in a Polyallomer centrifuge tube (Beckman, High Wycombe, UK) and centrifuged using an SW41Ti rotor at 36,000 rpm for 2 hours at 4°C. The gradient was fractionated into 22 0.5-ml fractions, each supplemented with 1% sodium dodecyl sulfate (SDS), 10 mmol/L ethylenediaminetetraacetic acid, and 200 μ g/ml proteinase K, and incubated at 37°C for 30 minutes to degrade endogenous nucleases. Subsequently, the fractions were mixed with phenol/chloroform/isoamyl alcohol 24:24:1, and the aqueous layer containing the RNA removed. A 5% aliquot

of each fraction was analyzed by electrophoresis in a 3% agarose gel to ensure that the RNA was not degraded, and that the tRNA and rRNA species were appropriately distributed through the gradient. RNA was precipitated overnight from the remainder of each fraction with 1 ml of 100% ethanol, 50 μ l of 3 mol/L sodium acetate, and 1 μ l of glycogen (Sigma) and washed once with 70% ethanol before air-drying. Fifty percent of samples of each fraction were run as a single large Northern blot, as above. A single 2.4-kb band was detectable on autoradiography, this was quantified by densitometry (Chemi Doc; BioRad, Hemel Hempstead, UK). Data are expressed as percentage of the total TGF- β 1 mRNA for that experiment in each fraction. The actual blot is also shown for comparison.

Assessment of de Novo TGF- β 1 Synthesis

To determine the kinetics of *de novo* TGF- β 1 synthesis we examined incorporation of radioactive amino acids into newly synthesized protein by metabolic labeling and detection by TGF- β 1 immunoprecipitation and autoradiography. Forty μ Ci of ³H-radiolabeled amino acid mixture (1000 μ Ci/ml; Amersham) were added to growth-arrested cells in 25-cm² flasks. Supernatant samples were subsequently collected for TGF- β 1 immunoprecipitation. Before immunoprecipitation supernatant samples were pre-cleared with 25 μ l of protein A-Sepharose beads (Sigma) and 0.25 μ g of normal rabbit immunoglobulin at 4°C for 1 hour with constant mixing. The supernatant was removed from the beads, and 2 μ g of polyclonal anti-TGF- β 1 antibody (Santa Cruz Biotechnology, Inc.) was added to each 1 ml of cleared supernatant and incubated with constant mixing at 4°C for 2 hours. Subsequently, 50 μ l of protein A-Sepharose beads were added, and mixing continued for 12 hours. Samples were centrifuged, the supernatant removed, and the beads washed twice with phosphate-buffered saline. After the final centrifugation and removal of the second phosphate-buffered saline wash, 25 μ l of SDS/ β -mercaptoethanol loading buffer was added, and the samples were heated to 95°C for 10 minutes before SDS-polyacrylamide gel electrophoresis in a 10% gel. Gels were fixed by incubating in 10% acetic acid/40% methanol overnight then soaked in scintillant (Amplify; Amersham Life Science) for 30 minutes and dried before visualization of immunoprecipitated TGF- β 1 by autoradiography.

Western Blot

Immunoblot analysis of lysate samples was performed by standard methodologies. In brief, cell extracts were prepared in SDS sample buffer and boiled for 5 minutes at 95°C. Equal numbers of cells were lysed, and the resultant lysates were loaded onto 10% SDS-polyacrylamide gel electrophoresis and electrophoresis was performed under reducing conditions according to the procedure of Laemmli.²⁵ After electrophoresis the separated proteins were transferred to a nitrocellulose membrane (Amersham). The membrane was blocked with phosphate-buff-

Figure 1. TGF- β 1 increases TGF- β 1 mRNA expression. Confluent monolayers of HK-2 cells were growth-arrested and exposed to recombinant TGF- β 1 (0 to 2 ng/ml) for up to 48 hours. **A:** After RNA isolation TGF- β 1 mRNA was detected as a single 2.4-kb transcript by Northern analysis. Equal mRNA loading was confirmed by stripping the blot and reprobing for GAPDH. In parallel experiments cells were stimulated by the addition of TGF- β 1 (1 ng/ml) for 24 hours. **B:** After RNA isolation and reverse transcription, TGF- β 1 mRNA was quantified by quantitative PCR. Data represent mean $\pm$ SD, $n = 8$.

ered saline containing 5% nonfat powdered milk for 1 hour and then incubated with the appropriate primary antibody (see Materials above) in phosphate-buffered saline containing 0.1% Tween 20 (phosphate-buffered saline-Tween) and then incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Sigma) in Tris-buffered saline-Tween. Proteins were visualized using enhanced chemiluminescence (Amersham) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay

Nuclear protein extraction and electrophoretic mobility shift assays for nuclear factor (NF)- κ B and AP-1 were performed as previously described.²⁶ In brief, cells were harvested in ice-cold phosphate-buffered saline (pH 7.4) and pelleted by centrifugation. Cells were resuspended in ice-cold buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mmol/L MgCl₂, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, and 0.2 mmol/L phenylmethyl sulfonyl fluoride) and incubated on ice for 10 minutes. The cell pellet was collected by centrifugation, resuspended in buffer B (20 mmol/L HEPES-KOH, pH 7.9, 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 0.2 mmol/L ethylenediaminetetraacetic acid, 0.3 mmol/L dithiothreitol, and 0.2 mmol/L phe-

Figure 2. Determination of translational efficiency and *de novo* protein synthesis. Confluent monolayers of HK-2 cells were growth-arrested and exposed to TGF- β 1 (1 ng/ml) for 12 hours before extraction of the cytosol and separation of mRNA on a sucrose gradient as described in Materials and Methods. **A:** Subsequently, a Northern blot of the fractionated mRNA was probed for TGF- β 1. Fractions 1 to 11 represent tRNAs, free ribosomal subunits, monosomes, and untranslated mRNAs in the form of messenger ribonucleoprotein particles. Fractions 12 to 22 represent polysomes of increasing size. **B:** Graphical representation of the polysome distribution of TGF- β 1 mRNA, expressed as percentage of the total TGF- β 1 mRNA detected on a given blot in each fraction, with control shown as shaded area and TGF- β 1-stimulated as the unshaded area. **C:** Incorporation of radioactive amino acids into newly synthesized protein by metabolic labeling was used to assess *de novo* TGF- β 1 synthesis. Cells were stimulated with TGF- β 1 (0 to 5 ng/ml) in the presence of 40 μ Ci of ³H-radiolabeled amino acid mixture (1000 μ Ci/ml; Amersham). Supernatant samples were subsequently collected for TGF- β 1 immunoprecipitation, and radiolabeled TGF- β 1 was detected by autoradiography.

nylmethyl sulfonyl fluoride), and incubated on ice for 20 minutes followed by brief high-speed centrifugation (12,000 $\times g$ for 10 seconds at 4°C), and the resulting supernatants (nuclear extract) were collected. Oligonucleotides containing consensus motifs for NF- κ B (5'-gag-TCCATGGGGAATTCCCC-3' and 3'-AGGTACCCCTTA-AGGGGag-5') were annealed for use in electrophoretic mobility shift assay. These double-stranded fragments were labeled with [α -³²P]dTTP (Amersham-Pharmacia Biotech, Amersham, UK) using the Klenow fragment of DNA polymerase I. The composition of protein/DNA complexes was determined by supershift assays using rabbit

Figure 3. TGF- β 1 mediated activation of signaling pathways. Confluent monolayers of HK2 cells were stimulated by the addition of TGF- β 1 (1 ng/ml) for up to 6 hours. At the time points indicated, total cell extracts were generated, and immunoblot analysis of lysate samples for phosphorylated-Smad/total Smad (A), phosphorylated ERK/total-ERK (B), and phosphorylated p38/total p38 (C) was performed.

polyclonal antibodies specific for NF- κ B p50 (sc-7178), NF- κ B RelB (c-19), NF- κ B c-Rel (n-466), NF- κ B p52 (c-5), and NF- κ B p65 (sc-109) (Santa Cruz Biotechnology, Inc.). Oligonucleotides containing consensus sequences for AP-1 (5'-CGCTTGATGACTCAGGCGGAA-3' and 3'-GCGAACTACTGAGTCGGCCTT-5') were end-labeled using T4 polynucleotide kinase (Promega) according to the manufacturer's instructions in a reaction mix containing 50 pmol of annealed oligonucleotide, 5 μ l of 10 $\times$ buffer, 50 pmol of [γ - 32 P]ATP, and 20 U of T4 polynucleotide kinase.

Statistical Analysis

Unless otherwise specified, statistical analysis was performed using the unpaired Student's *t*-test, with a value of *P* < 0.05 considered to represent a significant difference. The data are presented as means $\pm$ SD of *n* experiments as indicated in figure legends. For each individual experiment, the mean of duplicate determinations was calculated.

Results

Increase in TGF- β 1 mRNA by Quantitative PCR and Northern Analysis

Confluent monolayers of growth-arrested HK2 cells were stimulated with an increasing concentration of recombinant TGF- β 1 for 24 hours. Northern blotting showed in-

Figure 4. Smad activation occurs independently of MAP kinase activation. To determine the role of ERK and p38 MAP kinase in TGF- β 1-mediated Smad activation, cells were stimulated with TGF- β 1 (1 ng/ml) either alone or in the presence of PD98059 (A) or SB203580 (B) for 30 minutes. Subsequently, total cell extracts were generated and immunoblot analysis of lysate samples for phosphorylated Smad/total Smad.

creased expression of a single 2.4-kb TGF- β 1 transcript at all concentrations of TGF- β 1 used within 3 hours of its addition (Figure 1A). Transcriptional regulation was further analyzed by quantitative PCR. This demonstrated a 2.5-fold increase in TGF- β 1 mRNA 24 hours after stimulation with 1 ng/ml TGF- β 1 (Figure 1B).

Increased de Novo Synthesis and Translational Efficiency by Polysome Analysis

We have previously demonstrated independent regulation of TGF- β 1 transcription and translation such that transcriptional up-regulation may be associated with a poorly translated transcript and increased mRNA expression is not sufficient to increase *de novo* synthesis of TGF- β . For this reason, we examined TGF- β 1 mRNA translational efficiency by polysome analysis to assess the number of ribosomes associated with the mRNA and *de novo* TGF- β 1 protein synthesis by incorporation of radioactive amino acids into newly synthesized TGF- β 1 by metabolic labeling.

The results demonstrated that TGF- β 1 mRNA in unstimulated cells is poorly translated. In contrast, 12 hours after addition of TGF- β 1 (1 ng/ml), there was a significant shift of TGF- β 1 mRNA to the region of the gradient corresponding to polysome-associated RNA (fractions 12 to 22) representing well-translated mRNA (Figure 2A). In the control cells, 25% localized to fractions 12 to 22 compared with 61% after TGF- β 1 stimulation (Figure 2B).

Radiolabeling of TGF- β 1-stimulated cells with 3 H amino acids, immunoprecipitation of TGF- β from the cell

Figure 5. MAP kinase activation occurs after expression of Smad dominant-negative expression vector. Inhibition of Smad3 activation was achieved by transient transfection with a *c-myc*-tagged dominant-negative Smad3 expression vector. **A:** Transfection efficiency of the dominant-negative Smad3 expression vector. Histogram of FL-1 fluorescence in EGFP-transfected versus mock-transfected cells. Transfection efficiency, based on the proportion of cells in gated region M1, is estimated at 55%. Efficacy of the expression vector was confirmed by co-transfection of 1.0 μ g of the dominant-negative (DN) expression vector or empty vector (EV) together with 0.9 μ g of the Smad-responsive (SBE)₄-Lux reporter and 0.1 μ g of the *Renilla* luciferase construct using 6 μ l of the mixed lipofection reagent FuGene6. **B:** Twenty-four hours after transfection cells were stimulated with 1 ng/ml TGF- β 1 for 6 hours before quantitation of luciferase content. Results are expressed as ratios of firefly/*Renilla* luciferase and represent mean $\pm$ SD, $n = 3$. In parallel experiments, cells were transiently transfected with 1 μ g of the *c-myc*-tagged dominant-negative Smad3 expression vector using 3 μ l of FuGene6. Twenty-four hours after transfection, cells were stimulated with TGF- β 1 (1 ng/ml) for 30 minutes. Subsequently, total cell extracts were generated, and immunoblot analysis of lysate samples for ERK/total-ERK (**C**) and phosphorylated p38/total p38 (**D**) was performed. **E:** In parallel experiments, overexpression of the vector was confirmed by *c-myc* immunoblot.

culture supernatant, and detection by autoradiography was used to assess *de novo* TGF- β protein synthesis. Increased TGF- β 1 synthesis was demonstrated 48 and 72 hours after the addition of recombinant TGF- β 1 (Figure 2C). Maximal stimulation of *de novo* TGF- β 1 was seen at a dose of 1 ng/ml of recombinant TGF- β 1, which was therefore the dose used for the subsequent experiments.

Activation of Smad, ERK, and p38 MAP Kinase

Activation of Smad and non-Smad signaling pathways was examined by Western blotting. The addition of 1 ng/ml of recombinant TGF- β 1 led to a time-dependent increase in Smad3 phosphorylation, with no change in the expression of total Smad3 (Figure 3A). Likewise, activation of ERK MAP kinase (Figure 3B) and p38 MAP kinase (Figure 3C) pathways was seen within 10 minutes of the addition of TGF- β 1.

Independent Regulation of the Signaling Pathways

Previous studies demonstrate that activation of p38 MAP kinase²⁷ and ERK MAP kinase²⁸ may augment Smad signaling. Activation of p38 MAP kinase may also act downstream of TGF- β 1, for example, mediating TGF- β 1-dependent apoptosis.²⁹ This is further complicated by the finding that in other cell systems, Smad and non-

Smad pathways may act cooperatively in mediating TGF- β 1-dependent events.³⁰ Therefore, having demonstrated activation of both Smad and MAP kinase pathways on stimulation with TGF- β 1, we sought to examine whether activation of these pathways are independent of each other.

Confluent growth-arrested HK-2 cells were stimulated with TGF- β 1 (1 ng/ml) in the presence of either the chemical inhibitor of the ERK MAP kinase pathway PD98059 or the chemical inhibitor of p38 MAP kinase SB203580, as previously described.¹² TGF- β 1-dependent phosphorylation of ERK was inhibited by the PD compound; however, Smad 2/3 phosphorylation was unaffected (Figure 4A). Likewise, TGF- β 1-dependent phosphorylation of Smad 2/3 was unaffected by the SB compound (Figure 4B).

The role of TGF- β 1-dependent Smad phosphorylation in activation of p38 MAP kinase and ERK MAP kinase pathways was examined by transient transfection with a dominant-negative *c-myc*-tagged Smad3 expression vector. Transfection efficiency for HK-2 cells was first demonstrated by transfection with the constitutively active GFP expression vector EGFP-C1 (Clontech) and detection of fluorescence. By this method a transfection efficiency of $\sim$ 55% was achieved (Figure 5A).

Confirmation of the functionality of the dominant-negative expression vector was sought by its co-transfection with the (SBE)₄-Lux reporter (or empty vector) and sub-

sequent stimulation with TGF- β 1 (1 ng/ml) for 6 hours. TGF- β 1 stimulation of cells transfected with the empty vector resulted in a significant increase in luciferase activity. In contrast, no increase in luciferase activity was seen in the cells transfected with the dominant-negative expression vector after TGF- β 1 stimulation (Figure 5B). Subsequently, cells transfected with the Smad3 dominant-negative expression vector were stimulated with recombinant TGF- β 1 and activation of the signaling pathways examined by Western analysis. TGF- β 1-dependent activation of ERK-MAP kinase (Figure 5C) and p38 MAP kinase (Figure 5D) were unaffected by overexpression of the dominant-negative form of Smad3. Confirmation of overexpression of the Smad3 dominant-negative expression vector was sought by *c-myc* immunoblot (Figure 5E).

The role of Smad was further examined by Smad3 gene silencing using Smad3 siRNA. Efficacy of this approach was confirmed by quantitative PCR assessment of SMAD3 mRNA quantitation. Forty-eight hours after transfection, Smad3 mRNA was significantly reduced in cells transfected with Smad3 siRNA compared with the scrambled siRNA (Figure 6A). As with the Smad dominant-negative expression vector, TGF- β 1-dependent activation of ERK-MAP kinase and p38 MAP kinase were unaffected by gene silencing using Smad3 siRNA (Figure 5B). Collectively, these data suggest that TGF- β 1 independently activates Smad, p38, and ERK signaling pathways in PTCs.

Transcription Is Dependent on ERK and Smad

The contribution of the ERK MAP kinase and p38 MAP kinase signaling pathways in transcriptional regulation of TGF- β 1 were determined by stimulation of HK2 cell with TGF- β 1 in the presence of either PD98059 or SB203580. The data demonstrate that TGF- β 1 mRNA autoinduction was inhibited by the ERK MAP kinase inhibitor. In contrast the p38 MAP kinase inhibitor did not affect TGF- β 1 mRNA autoinduction (Figure 7A).

The role of the Smad3 pathway in transcriptional regulation of TGF- β 1 was determined by stimulating HK2 cells transiently transfected with the *c-myc*-tagged Smad3 dominant-negative expression vector with recombinant TGF- β 1 and quantification of TGF- β 1 mRNA by quantitative PCR. The results demonstrate that inhibition of Smad3 activity prevents TGF- β 1 mRNA autoinduction (Figure 7A). Involvement of Smad3 further examined by inhibition of Smad3 expression by gene silencing using Smad3 siRNA and quantitation of TGF- β 1 mRNA. TGF- β 1 stimulation of cells transfected with scrambled siRNA-negative control led to a significant increase in TGF- β 1 mRNA. In contrast, when cells transfected with Smad3 siRNA were stimulated with TGF- β 1, there was no significant increase in TGF- β 1 mRNA (Figure 7B).

Transcription Factor Involvement

Involvement of the transcription factors AP-1 and NF- κ B in the transcriptional component of TGF- β 1 autoinduction was sought by electrophoretic mobility shift assay using

Figure 6. MAP kinase activation occurs after gene silencing of Smad3 by Smad3 siRNA. Cells were transfected with Smad3 siRNA for 48 hours before cell lysis by the addition of Trireagent. **A:** Smad3 mRNA was subsequently analyzed by quantitative PCR. In parallel experiments, cells transfected with Smad3 siRNA were stimulated with TGF- β 1 (1 ng/ml) for 30 minutes. **B:** Subsequently, total cell extracts were generated and immunoblot analysis of lysate samples for ERK/total-ERK and phosphorylated p38/total p38 was performed.

AP-1 or NF- κ B consensus sequence probes. In nuclear extracts from TGF- β 1-stimulated HK2 cells maintained under serum-free conditions, increased binding to both probes was observed. This was abrogated in control experiments in the presence of an excess of unlabeled probe (data not shown).

Enhanced binding of nuclear proteins to AP-1 (Figure 8A) was maximal 10 minutes after stimulation. To identify proteins involved in TGF- β 1-induced AP-1 activation, we performed supershift assays. Inclusion of antibody to c-jun, but not c-fos, led to the presence of a supershifted band (Figure 8B). Binding of AP-1 was dependent on Smad3 signaling because the increased binding after addition of TGF- β for 10 minutes was prevented by transient transfection of the dominant-negative Smad3 expression vector (Figure 8C). In contrast, neither ERK MAP kinase inhibition nor inhibition of p38 MAP kinase had an effect on TGF- β 1-induced protein binding to the AP-1 probe.

Figure 7. Inhibition of ERK and Smad3, but not p38, inhibits autocrine TGF- β 1 transcription. HK2 cell monolayers were stimulated with TGF- β 1 (1 ng/ml) for 24 hours either alone or in the presence of either PD98059 or SB203580 at the concentrations indicated. After RNA isolation and reverse transcription, TGF- β 1 mRNA was quantified by quantitative PCR. Data represent mean $\pm$ SD, $n = 4$. Inhibition of Smad3 was achieved by transient transfection of the Smad3 dominant-negative expression vector (**A**) or by gene silencing using Smad3 siRNA (**B**). Cells were transiently transfected with either the *c-myc*-tagged dominant-negative Smad3 expression vector or Smad3 siRNA. Twenty-four hours after transfection, cells were stimulated with TGF- β 1 (1 ng/ml) for a further 24 hours, and TGF- β 1 mRNA was quantified by quantitative PCR. Data represent mean $\pm$ SD, $n = 4$.

TGF- β 1 also led to protein binding to the NF- κ B consensus probe (Figure 8D). In supershift assays, inclusion of antibody to the p50 subunit led to the presence of a prominent supershifted band, whereas inclusion of antibody to the p65 subunit attenuated binding of nuclear proteins (Figure 8E). Antibodies to c-Rel, Rel-B, and p52 had no effect on binding of nuclear protein to the NF- κ B consensus probe. Enhanced binding was unaffected by transient transfection of the Smad3 dominant-negative expression vector or inhibition of p38 inhibition (Figure 8F). In contrast, inhibition of ERK MAP kinase activation attenuated TGF- β 1 stimulated binding to the NF- κ B consensus probe (Figure 8F).

p38 MAP Kinase Inhibits de Novo Protein Synthesis

Previously, we have demonstrated the involvement of the p38 MAP kinase pathway in TGF- β 1 synthesis. Given the data above, suggesting that inhibition of this pathway did not influence TGF- β 1 mRNA or transcription factor activation, we sought to examine the role of this pathway on *de novo* protein synthesis to reflect translational regulation.

Stimulation of cells with TGF- β 1 in the presence of the p38 MAP kinase inhibitor SB203580 prevented TGF- β 1-stimulated incorporation of radiolabeled amino acids into TGF- β 1 (Figure 9A). In the absence of an effect on the transcriptional component of TGF- β 1 autoinduction, this would suggest a specific role for the p38 MAP kinase pathway in its translation. In parallel experiments, inclusion of the ERK MAP kinase inhibitor PD98059 shown above to inhibit TGF- β 1 mRNA autoinduction also decreased *de novo* TGF- β 1 protein synthesis (Figure 9A). TGF- β 1 is also known to activate the JNK MAP kinase

pathway, the specificity of the effect of inhibition of these MAP kinase pathways was therefore examined by stimulation of cells with TGF- β 1 in the presence of the JNK MAP kinase inhibitor recombinant L-JNK11. In these experiments, inhibition of JNK did not influence TGF- β 1-stimulated autoinduction of *de novo* protein synthesis (Figure 9B).

Discussion

We have previously shown that in PTCs TGF- β 1 synthesis is independently regulated at the levels of transcription and translation. Exposure of PTCs to elevated D-glucose concentrations increases the expression of the poorly translated TGF- β 1 transcript without any associated change in TGF- β 1 protein synthesis.⁹ Platelet-derived growth factor (PDGF) at a low dose does not influence TGF- β 1 transcription but leads to alteration in TGF- β 1 mRNA stability and translation. Without prior glucose-induced increase in the amount of TGF- β 1 transcript, however, PDGF does not stimulate significant TGF- β 1 protein synthesis. Pretreatment of PTCs with elevated glucose concentrations followed by stimulation with PDGF therefore has synergistic effects on TGF- β 1 synthesis.¹¹

Autoinduction of TGF- β 1 is a well-recognized phenomenon described in a variety of cell types and is postulated to be important in amplifying and sustaining its generation.¹⁶ The functional significance of this may be tissue-specific. For example, TGF- β 1 mRNA autoinduction is postulated to have a beneficial role in cardiac wound healing after ischemic injury.³¹ Likewise, TGF- β 1 autoinduction in dermal fibroblasts and keratinocytes is postulated to drive sustained stimulation of extracellular matrix

Figure 8. Transcription factor activation. Mobility shift experiments were performed with nuclear extracts from HK-2 cells cultured for up to 6 hours in the presence of TGF- β 1 (1 ng/ml). Nuclear extract was incubated with probes for AP-1 (**A–C**) and NF- κ B (**D, E**). Identification of proteins involved in AP-1 activation was determined by mobility shift analysis with antibodies to c-fos/c-jun (**B**) and NF- κ B activation using antibodies to p50, p65, cRel, RelB, or p52 as indicated (**E**). The role of Smad3 in either AP-1 (**C**) or NF- κ B (**F**) activation was examined by TGF- β 1 (1 ng/ml) stimulation of HK2 cells transiently transfected with Smad3 dominant-negative expression vector (DN) for 24 hours before performing the mobility shift assay. In the control experiment, cells transiently transfected with the empty vector (EV) were stimulated with TGF- β 1. Likewise, the role of the ERK MAP kinase and p38 MAP kinase pathways in AP-1 activation was examined by TGF- β 1 (1 ng/ml) stimulation of HK2 cells for 10 minutes in the presence of either 8 μ mol/L PD98059 (PD) or 0.8 μ mol/L SB203580 (SB) before analysis by mobility shift assay (**C**), and their role in NF- κ B activation was similarly examined in cells exposed to TGF- β 1 for 6 hours (**F**).

production, needed for granulation and subsequent scar tissue formation during normal cutaneous wound healing.³² In contrast, the role of TGF- β 1-mediating epithelial to mesenchymal transdifferentiation (EMT) points to a disease-promoting effect of TGF- β 1 autoinduction associated with oncogenesis/tumor invasion,³³ and in the formation of renal interstitial myofibroblasts,¹⁵ associated with progressive renal injury.^{34,35} In the kidney, tubulointerstitial fibrosis is the final common result of a variety of progressive renal injuries leading to chronic renal failure, and antagonism of TGF- β 1 has been demonstrated to have therapeutic potential in numerous animal models of renal injury.^{36–39}

Mice lacking Smad3 are protected against tubulointerstitial fibrosis associated with unilateral ureteric obstruction. Significantly, in these studies, the Smad3 pathway was demonstrated to be essential for tubular epithelial cell TGF- β 1 autoinduction.¹⁸ This is consistent with previous work using fibroblasts from Smad2 and Smad3 knockout animals demonstrating that TGF- β 1 autoinduction is Smad3-dependent and does not involve Smad2.⁴⁰

TGF- β 1-mediated transcriptional activation through AP-1 sites may involve a regulated interaction between Smads and AP-1 transcription factors.⁴¹ In the current study, we have shown that in renal proximal tubular epithelial cells of human origin transcriptional autoinduction of TGF- β 1 is dependent on both Smad3 and AP-1. Although the promoter of the human TGF- β 1 gene contains a putative Smad3-binding element, recent studies have failed to demonstrate specific Smad3 binding to this sequence.⁴² This would therefore suggest that Smad3 activates the TGF- β 1 promoter indirectly. Previously, the promoter of the TGF- β 1 gene has been demonstrated to be responsive to autoregulation mediated by binding of AP-1.¹⁷ AP-1 proteins have previously been demonstrated to mediate hyperglycemia-induced activation of the human TGF- β 1 promoter in mesangial cells;⁴³ in the current study, we have demonstrated the importance of Smad3-dependent activation of AP-1 in TGF- β 1 autoinduction in proximal tubular epithelial cells, which is consistent with data showing Smad3-dependent activation of the AP-1 in other cell types.⁴⁴

Figure 9. Inhibition of ERK MAP kinase and P38 MAP kinase inhibits TGF- β 1-stimulated TGF- β 1 *de novo* protein synthesis. **A:** HK-2 cells were stimulated with TGF- β 1 (1 ng/ml) either alone or in combination with PD98059 (8 μ mol/L) or SB203580 (0.8 μ mol/L) for 48 hours. **B:** In parallel experiments, to demonstrate specificity of the kinase inhibitors used, cells were stimulated with TGF- β 1 in combination with the JNK MAP kinase inhibitor recombinant L-JNKI1 (JNKi) at a concentration of 10 μ mol/L. All experiments were performed in the presence of 40 μ Ci of 3 H-radiolabeled amino acid mixture (1000 μ Ci/ml; Amersham). Supernatant samples were subsequently collected for TGF- β 1 immunoprecipitation and radiolabeled TGF- β 1 was detected by autoradiography.

As discussed above, it is apparent that AP-1 activation downstream of Smad3 is necessary for TGF- β 1 autoinduction. However, we have shown that Smad3 activation is not in itself sufficient because inhibition of p38 or ERK MAP kinase pathways prevents *de novo* synthesis of TGF- β 1 without interfering with Smad activation. TGF- β 1 is known to activate NF- κ B downstream of ERK MAP kinase.⁴⁵ We have shown that TGF- β 1 activates NF- κ B via ERK MAP kinase and that this is also required for transcriptional autoinduction of TGF- β .

Smad7 is a regulatory protein, synthesized in response to TGF- β , that inhibits TGF- β 1 signaling in a negative-feedback loop by enhancing degradation of TGF- β receptors and R-Smads. Interestingly, activity of the Smad7 promoter is inhibited by NF- κ B⁴⁶ and enhanced by Smad3.⁴⁷ Furthermore specific Smad3 binding to the promoter of the Smad7 gene has been demonstrated.⁴² Recent studies have demonstrated a competitive interaction between TGF- β 1-activated Smad proteins and NF- κ B that is mediated by the transcriptional co-activator cyclic AMP response element-binding protein (CREB)-binding protein (CBP).⁴⁸ NF- κ B mediates disruption of Smad-CBP interaction and may thus effectively block Smad3 function. NF- κ B may therefore not directly affect the TGF- β 1 promoter itself but may decrease the expression of the inhibitory Smad7, which would result in less binding of this Smad to the type I TGF- β receptor, less antagonism of TGF- β 1, and augmentation of TGF- β 1 autoinduction. This hypothesis is supported by work demonstrating that down-regulation of Smad7 contributes to progressive fibrosis in models of obstructive nephropathy.⁴⁹

Previously cooperative action of MAP kinase and Smad pathways have been implicated in mediating effects of TGF- β 1 on gene targets³⁰ and also in TGF- β 1 autoinduction.⁵⁰ In the current study we have demonstrated that activation of Smad and non-Smad pathways occur independently of each other and act in a complementary way to coordinate TGF- β 1 autoinduction in proximal tubular epithelial cells. In addition to the activation of

the ERK MAP kinase pathway, we have demonstrated activation of the p38 MAP kinase pathway. The lack of effect of inhibition of the p38 MAP kinase on transcriptional responses together with the inhibition of TGF- β 1-dependent TGF- β 1 *de novo* protein by the SB compound suggests that this pathway has a specific effect on TGF- β 1 mRNA translation.

We have demonstrated that after exposure to active TGF- β 1, autoinduction leads to a positive feedback loop of TGF- β 1 synthesis in PTCs, potentially contributing to tubulointerstitial fibrosis. However, enhanced tubulointerstitial TGF- β 1 generation does not always lead to progressive fibrosis and inexorable decline in renal function. After acute renal failure due to acute tubular necrosis, for example, TGF- β 1 is abundantly expressed, but the kidney can recover.⁵¹ An important question is how TGF- β 1 synthesis is limited in the tubulointerstitium of the kidney, given the positive feedback loop that we have demonstrated. One possible explanation is the failure of PTCs to activate TGF- β 1,¹² which contrasts with other cell types such as mesangial cells,⁵² and suggests both that the mechanisms by which TGF- β 1 response is regulated are context- and lineage-specific and that infiltrating macrophages, by activating TGF- β 1 synthesized by PTCs, may play a key role in this process in the tubulointerstitium.

In summary the data demonstrate that in renal proximal tubular epithelial cells, TGF- β 1 autoinduction requires the coordinated action of independently regulated TGF- β 1-activated Smad and non-Smad pathways. Furthermore, these pathways regulate distinct transcriptional and translational components of TGF- β 1 synthesis.

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