RACK1 Binds to Smad3 to Modulate Transforming Growth Factor-β1-stimulated α2(I) Collagen Transcription in Renal Tubular Epithelial Cells*

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Although it is clear that transforming growth factor-β1 (TGF-β1) is critical for renal fibrogenesis, the complexity of the involved mechanisms is increasingly apparent. TGF-β1 stimulates phosphorylation of Smad2/3 and activates other signaling molecules as well. The molecular link between these other kinases and Smads is not known. We sought new binding partners for Smad3 in renal cells and identified receptor for activated protein kinase C1 (RACK1) as a novel binding partner of Smad3. The linker region of Smad3 and the tryptophan-aspartic acid repeat 6 and 7 of RACK1 are sufficient for the association. RACK1 also interacts with Smad3 in the human kidney epithelial cell line, HKC. Silencing RACK1 increases transcriptional activity of TGF-β1-responsive promoter sequences of the Smad binding element (SBE), p3TP-Lux, and α2(I) collagen. Conversely, overexpressed RACK1 negatively modulates α2(I) collagen transcriptional activity in TGF-β1-stimulated cells. RACK1 did not affect phosphorylation of Smad3 at the C terminus or in the linker region. However, RACK1 reduced direct binding of Smad3 to the SBE motif. Mutating a RACK1 tyrosine at residue 246, but not at 228, decreased the inhibitory effect of RACK1 on both α2(I) collagen promoter activity and Smad binding to SBE induced by TGF-β1. These results suggest that RACK1 modulates transcription of α2(I) collagen by TGF-β1 through interference with Smad3 binding to the gene promoter.

The importance of transforming growth factor-β (TGF-β) as a mediator of glomerular extracellular matrix (ECM) accumulation in kidney disease has been widely investigated (1, 2). TGF-β family ligands bind to corresponding family receptors, leading to activation of intracellular proteins, the Smads. Stimulation of human mesangial cells (3) and renal tubular cells (4, 5) with TGF-β1 induces phosphorylation of the R-Smads, Smad2 and Smad3, beginning within 5 min of treatment. The phosphorylation causes association of the R-Smads with Smad4. Heteromultimerization and translocation of these Smads into the nucleus leads to transcriptional activation of various genes, including those encoding ECM proteins. The TGF-β1/Smad pathway stimulates the expression of type I and type IV collagen and fibronectin in renal cells (6–8).

Previously, we found that in human mesangial cells, the extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein (MAP) kinases are activated by TGF-β1 within 15–30 min. The ERK MAP kinase pathway, but not the JNK MAP kinase pathway, is critical for TGF-β1-induced α1(I) collagen expression (9). Type I collagen synthesis is enhanced by ERK-dependent phosphorylation at the linker region of R-Smad, whereas the TGF-β receptor directly phosphorylates the C-terminal serine residues of R-Smads. This auxiliary effect of ERK MAP kinase is required for optimal Smad activity in human mesangial cells (10). High glucose conditions stimulate TGF-β expression and enhance ECM production through the ERK MAP kinase pathway in mouse mesangial cells (11) and increase the Smad response to TGF-β1 in human mesangial cells (12). The latter effect is mediated through activation of protein kinase Cθ (13). Phosphatidylinositol 3-kinase also modulates TGF-β1-stimulated collagen expression (14). However, the mechanisms by which these other pathways interact with Smad signaling are poorly understood. TGF-β receptors function as membrane-bound serine/threonine kinases, whereas many of these accessory pathways are activated by receptor tyrosine kinases. This observation suggests that some intracellular mediators exist between Smad signaling and these other pathways.

To identify additional proteins that might bridge these distinct pathways, we employed a yeast two-hybrid assay. Novel proteins were isolated by their ability to interact with human Smad3. We identified several potential binding partners, including a scaffolding protein, receptor for activated protein kinase C1 (RACK1) (15). RACK1 is able to interact with several key molecules, including c-Src, β1-integrin and others (16–18). Through its binding to protein kinase C or c-Src, it may have an effect on ERK or other MAP kinases. Thus, it seemed an ideal candidate to affect cross-talk between ERK MAP kinase and Smad signaling. Here we show that RACK1 affects TGF-β1-
mediated cell signaling via a negative effect on Smad-mediated type I collagen transcription in renal epithelial cells.

**EXPERIMENTAL PROCEDURES**

*Reagents and Antibodies*—Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN) and reconstituted as a 4 μg/ml stock solution in 4 mM HCl with 1 mg/ml bovine serum albumin. For RACK1, a rabbit polyclonal antibody H-187 (Santa Cruz Biotechnology, Santa Cruz, CA) was used for immunoprecipitation (19) and a mouse monoclonal antibody (mAb) from BD Transduction Laboratories was used for immunoblot analyses. Anti-Smad1/2/3 mAb (H-2) and anti-human influenza hemagglutinin (HA) mAb (F-7), were purchased from Santa Cruz Biotechnology. Anti-phosphoserine antibody was obtained from Zymed Laboratories Inc. (South San Francisco, CA). Anti-phosphotyrosine mAb PY20 (BD Transduction Laboratories) was used to immunoprecipitate phosphorylated RACK1 (20). Anti-FLAG M2 mAb was obtained from Sigma, and anti-phospho-Smad3 (Ser-433/Ser-435) antibodies were from Cell Signaling technology (Beverly, MA).

**Plasmid Constructs**—A FLAG-tagged Smad3 in pEXL was obtained from H. F. Lodish and X. Liu (Whitehead Institute, MA). The p3TP-Lux construct was a kind gift from J. Massague (21). The SBE-Luc reporter construct was kindly provided by B. Vogelstein (22). The −376COL1A2-Luc reporter gene was described previously (3). The Gal4-Smad3 construct was kindly gifted from M. P. de Caestecker (23, 24). The Gal4-Smad3 sequence was 5’-AACTTCCACTCTCAGTCTCTAAG-3’.

**Yeast Two-hybrid Screen**—A yeast two-hybrid screen was performed using human Smad3 as bait according to the Clontech MATCHMAKER protocol (Clontech). Total RNA was isolated from human mesangial cells treated with 1 ng/ml TGF-β1 for 15 min as described (26). One hundred ng of total RNA was reverse-transcribed with oligo(dT) primers, and first-strand cDNA was synthesized using random primer from Clontech. Long distance-PCR was employed for amplification of cDNA library. Briefly, an AH109 yeast line (Clontech) was co-transfected with Smad3-pGBKKT7, the cDNA library, and pGADT7-Rec (Clontech). Co-transfection of the double-stranded library DNA and pGADT7-Rec causes homologous recombination within yeast so that the outcome is a fully functional Gal4 activation domain/cDNA expression vector. Transformants of yeast were screened for their ability to grow on a nutrient-deficient selection medium. Yeast can make colonies only when bait protein (Smad3) and library protein interact in the yeast and the association functions as transcriptional activators for the absence essential amino acids to grow on the selection medium. Yeast two-hybrid screen were selected as true positives. The library plasmids were isolated using the YEASTMAKER isolation kit (Clontech).

**Transfection Assays**—HKC cells were transfected with either pEXL-FLAG-Smad3 or empty pEXL using FuGENE 6 (Roche Applied Science). Briefly, 2 × 10^6 cells were seeded in 100-mm dishes. Twenty-four hours later, transfection was performed using 3 μg of plasmid DNA and FuGENE 6 (2 μl/1 μg of plasmid DNA). Five hours later, cells were replaced in fresh media containing 10% HI-NBCS. The next day, the cells were again replaced in serum-free media for an additional 24 h and then treated with 1 ng/ml TGF-β1 for appropriate time periods. For detecting phosphorylated Smad3, 80% confluent HKC cells were transfected with pEXL-FLAG-Smad3 and treated the

**TABLE 1**

Sequences of primers used to generate deletion mutants of Smad3

| Smad3 sequence | 5’ primer | 3’ primer |
|----------------|-----------|-----------|
| WT-(1–426)     | TAGAATTCGGTCCAGCCATGTCG | TCGGATCCTTTACTCCTAGTCTCTAAG |
| ΔMH2-(1–233)   | TAGAATTCGGTCCAGCCATGTCG | GTCGAGCTGCGCTGTTTCPG |
| MH1 alone (1–131) | ACGAATTCCAGAGAGTAGAGACACCA | A7GAGTCCCTCTCAGTCTCTGAGTGTGTCAG |
| Linker alone (128–233) | TCGAATTCGCCATCCTCAGAAG | TCGGATCCTTTACTCCTAGTCTCTAAG |
| MH2 alone (234–426) | TCGAATTCGCCATCCTCAGAAG | TCGGATCCTTTACTCCTAGTCTCTAAG |
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A

|    | WT       | ΔMH2      | MH1-alone | Linker-alone | MH2-alone |
|----|----------|-----------|-----------|--------------|-----------|
| Smad3/pEXL Control/pEXL | IP RACK1 IB Smad3/2/3 |  |  |  |  |

B

FIGURE 1. RACK1 interacts with the linker region of Smad3 in yeast. A, schema of deletion mutants of Smad3 used in yeast two-hybrid screen. B, interaction of Smad3 with RACK1. The pGADT7 containing a partial cDNA for RACK1 (encoding amino acids 253–318) was transfected in the yeast, AH106, together with pGBK7 containing the indicated Smad3 construct or empty pGBK7. Transformants were streaked on the selection medium (SD/- Ade/- His/- Leu/- Trp). After incubation at 30 °C for 4–5 days, colonies were analyzed. Abundant growth of colonies indicates RACK1 interaction with the cloned fragment. C, domain structure of RACK1, which contains seven internal Trp-Asp (WD) repeats. The location of tyrosine residues is shown.

same way with the above. Before harvesting cells, TGF-β1 (1 ng/ml) was added for 30 min.

Protein Extractions, Immunoprecipitations, and Western Blot Analysis—The cells were washed twice with ice-cold phosphate-buffered saline, lysed on ice in radioimmune precipitation buffer following transfection and incubated with anti-RACK1 or anti-α-Smad2/3 mAb for 1 h. After washing, they were incubated with horseradish peroxidase-conjugated donkey anti-mouse IgG (Zymed Laboratories Inc. for RACK1 blots, goat anti-mouse IgG (Santa Cruz Biotechnology) for Smad). Proteins were visualized with enhanced chemiluminescence reagent (Santa Cruz Biotechnology). For detection of the phosphorylation level of RACK1, HKC cells were treated with or without TGF-β1 or serum (10% H1-NBCS). Total cell lysate (0.5 mg) was immunoprecipitated with 1 μg of anti-phosphotyrosine (PY20) mAb (20) and immunoblotted with anti-RACK1 mAb. Anti-FLAG mAb was used for immunoprecipitation of transfected FLAG-Smad3. Transfected HKC cells were lysed in radioimmune precipitation buffer following immunoprecipitation. The samples were resolved by SDS-PAGE and immunoblotted with anti-phosphoserine or anti-phospho-Smad3 antibodies. These membranes were stripped with a stripping buffer (100 mM 2-mercaptoethanol; 2% SDS; 62.5 mM Tris-HCl, pH 6.7) at 55 °C for 30 min and rebotted with anti-Smad1/2/3 mAb to check whether the same amount of FLAG-tagged Smad3 proteins was immunoprecipitated. Total cell lysates were analyzed to know the expression level of overexpressed RACK1 using anti-RACK1 mAb or anti-α-Smad2/3 mAb and the expression level of FLAG-Smad3 using anti-Smad1/2/3 mAb.

Transient Transfection and Luciferase Assay—HKC cells were seeded on 12-well plates at a density of 1 × 10^5/well, and transfection was performed 20 h later with the indicated plasmid DNAs along with CMV-SPORT-β-galactosidase as a control for transfection efficiency. After 5 h of transfection, 1 ng/ml TGF-β1 was added, and the cells were incubated for additional 20 h. Cells were harvested with reporter lysis buffer (Promega, Madison, WI). The luciferase and β-galactosidase activities were measured as described previously (9).

siRNA Transfection—Chemically synthesized, double-stranded RACK1 siRNA or control siRNA was purchased from Santa Cruz Biotechnology. For reporter assay, HKC cells were seeded on
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FIGURE 3. Effect of RACK1 siRNA on COL1A2 and TGF-β1/Smad-mediated transcriptional activity. A, HKC cells were transfected with RACK1 or control siRNA in 6-well plates. After 5 h of transfection, the cells were treated with TGF-β1 and cultured for an additional 20 h. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Smad1/2/3 mAb. B, HKC cells were transfected with RACK1 or control siRNA along with the indicated reporter constructs and β-galactosidase (β-gal) expression constructs. TGF-β1 was added 5 h after transfection for the p3TP-Lux or 20 h after for the COL1A2 and the SBE-Luc promoter constructs. The cells were harvested 24 h later of TGF-β1 treatment. Luciferase activity was measured and normalized to β-galactosidase activity. Values are given as mean ± S.D. of triplicate samples. Similar results were obtained from three independent experiments. *, p < 0.05.

RESULTS

RACK1 as a Novel Binding Partner of Smad3—Yeast two-hybrid interaction trap method and multiple subsequent restreakings identified 64 robust colonies, suggesting that strong interaction with human Smad3 occurs in yeast. One cDNA clone encoded amino acids 251–317 of the scaffolding protein, RACK1. This clone contains one-fifth of the full-length cDNA for RACK1, including its C terminus. To verify bait dependence of the interaction and to map the possible interaction domain of Smad3, the isolated DNA Affinity Precipitation Assay (DAPA)—HKC cells were transfected with either the pcDNA3-HA–RACK1 constructs or the control pcDNA3. After treating with TGF-β1, nuclear extracts (50 μg) were prepared in a standard manner, and equal amounts of protein were incubated with 4 μg of biotin-labeled double-stranded probes (Sigma Genosys) containing the SBE sequence for 3 h at 4 °C. Then samples were incubated with 20 μl of streptavidin-agarose in a 50% slurry (Pierce) for 90 min (27, 28). After centrifugation, the pellet was washed four times with cold phosphate-buffered saline, and bead-bound proteins were eluted with SDS loading buffer followed by SDS-PAGE. After transfer, the membranes were immunoblotted with anti-Smad1/2/3 mAb. Nuclear extracts (15 μg) were analyzed by Western blots using appropriate antibodies.

Statistical Analysis—The data are presented as mean ± S.D. Statistical differences between experimental and control groups were determined by analysis of variance, and a value of p < 0.05 by Student’s t test was considered significant.

RACK1 Interacts with Smad3 in Renal Cells—To examine whether RACK1 and Smad3 interact in mammalian cells, a co-immunoprecipitation assay was performed using whole cell lysate from HKC cells, a renal epithelial cell line. Co-immunoprecipitation with several different commercial antibodies against Smad was technically difficult because of a strong nonspecific band migrating around the anticipated size of RACK1. Instead, polyclonal antibody against RACK1 was used for immunoprecipitation. We were unable to detect interaction between endogenous proteins. HKC cells were then transfected with either the FLAG-Smad3-pEXL construct or the empty plasmid, and nuclear extracts (20 μg) were analyzed by Western blots using appropriate antibodies.

The data are consistent with the yeast two-hybrid results and provide evidence that RACK1 and Smad3 interact in mammalian cells. This interaction may provide a mechanism for regulation of Smad3 activity in response to TGF-β1 signaling.
pEXL construct. The interaction was detected by observation if a band developed with anti-Smad1/2/3/mAb only when the FLAG-Smad3-pEXL was transfected (Fig. 2A). To investigate the effect of TGF-β1 on the interaction, immunoprecipitates were prepared from cells treated with TGF-β1 for different time periods. We also analyzed the same samples by Western blot using anti-RACK1 mAb to check whether equal amounts of RACK1 were immunoprecipitated (Fig. 2B, second panel). Total cell lysates were analyzed to check the level of Smad3 (Fig. 2B, lower panel). Basal interaction was observed without TGF-β1 treatment. In the experiment shown, the level of co-immunoprecipitation slightly increased and slowly decreased subsequently (Fig. 2B, top panel). In other experiments, we observed a similar pattern of interaction, with a slight increase at 10–20 min followed by a decrease over time (data not shown). A similar association between RACK1 and Smad3 was demonstrated in human mesangial cells (data not shown), although the quality of the image was poor, likely due to the low transfection efficiency of the cells.

**RACK1 Inhibits TGF-β1-stimulated Transcriptional Activity**—We performed several experiments to assess the functional significance of RACK1. First, we examined whether silencing RACK1 expression affects transcriptional activity in HKC cells using the SBE-Luc, p3TP-Lux, and 376COL1A2-Luc reporter construct. The interaction was detected by observation if a band developed with anti-Smad1/2/3/mAb only when the FLAG-Smad3-pEXL was transfected (Fig. 2A). To investigate the effect of TGF-β1 on the interaction, immunoprecipitates were prepared from cells treated with TGF-β1 for different time periods. We also analyzed the same samples by Western blot using anti-RACK1 mAb to check whether equal amounts of RACK1 were immunoprecipitated (Fig. 2B, second panel). Total cell lysates were analyzed to check the level of Smad3 (Fig. 2B, lower panel). Basal interaction was observed without TGF-β1 treatment. In the experiment shown, the level of co-immunoprecipitation slightly increased and slowly decreased subsequently (Fig. 2B, top panel). In other experiments, we observed a similar pattern of interaction, with a slight increase at 10–20 min followed by a decrease over time (data not shown). A similar association between RACK1 and Smad3 was demonstrated in human mesangial cells (data not shown), although the quality of the image was poor, likely due to the low transfection efficiency of the cells. **RACK1 Inhibits TGF-β1-stimulated Transcriptional Activity**—We performed several experiments to assess the functional significance of RACK1. First, we examined whether silencing RACK1 expression affects transcriptional activity in HKC cells using the SBE-Luc, p3TP-Lux, and 376COL1A2-Luc reporter construct. The interaction was detected by observation if a band developed with anti-Smad1/2/3/mAb only when the FLAG-Smad3-pEXL was transfected (Fig. 2A). To investigate the effect of TGF-β1 on the interaction, immunoprecipitates were prepared from cells treated with TGF-β1 for different time periods. We also analyzed the same samples by Western blot using anti-RACK1 mAb to check whether equal amounts of RACK1 were immunoprecipitated (Fig. 2B, second panel). Total cell lysates were analyzed to check the level of Smad3 (Fig. 2B, lower panel). Basal interaction was observed without TGF-β1 treatment. In the experiment shown, the level of co-immunoprecipitation slightly increased and slowly decreased subsequently (Fig. 2B, top panel). In other experiments, we observed a similar pattern of interaction, with a slight increase at 10–20 min followed by a decrease over time (data not shown). A similar association between RACK1 and Smad3 was demonstrated in human mesangial cells (data not shown), although the quality of the image was poor, likely due to the low transfection efficiency of the cells.
Luc reporter constructs. The SBE-Luc construct contains four copies of the CTCTAGAC sequence that has been shown to bind recombinant Smad3 and Smad4 (22). Also conventionally utilized in studying TGF-β1/Smad signaling, the p3TP-Lux reporter activity is stimulated by both TGF-β1 and activin through the mediation of their respective receptors and Smad3 (21, 29, 30). RACK1 siRNA decreased RACK1 expression, as demonstrated by Western blot using RACK1 mAb (Fig. 3A). This treatment increased the fold induction of SBE-Luc and p3TP-Lux activity by 1.7 and 2.6 times, respectively (Fig. 3B). The effect of RACK1 siRNA on the COL1A2 promoter was more complex; basal activity was stimulated 3.4-fold, and TGF-β1-stimulated activity was 3.6 times higher. Since decreased RACK1 enhances TGF-β1/Smad3 responsiveness, these data suggest that RACK1 acts as an inhibitor of this signaling pathway.

We then examined the effect of overexpressing RACK1 on COL1A2 promoter activity. As anticipated, RACK1 WT reduced TGF-β1-stimulated transcriptional activity by 70% (Fig. 4A). These results further suggest that RACK1 plays a negative role in TGF-β1/Smad3-mediated signal transduction in renal cells. We next examined the effect of overexpressed RACK1 mutants on type I collagen promoter activity in HKC cells. Two point mutant constructs, pcDNA3-HA-RACK1 Y228F and pcDNA3-HA-RACK1 Y246F, and a double mutant with phenylalanine replacing both Tyr-228 and Tyr-246 residues (RACK1 DM) were employed in the experiment. It has been reported that the tyrosine residues at 228 and 246 in the WD6 domain of RACK1 (Fig. 1C) are critical for regulating Src kinase activity (31). The RACK1 Y228F mutants still had an inhibitory effect on COL1A2 promoter activity. However, the inhibitory effect of RACK1 Y246F mutant was significantly reduced when compared with RACK1 WT and Y228F mutant. Expression of RACK1 DM partially inhibited the promoter activity induced by TGF-β1 (Fig. 4B). This finding suggests that the tyrosine at residue 246, but not 228, of RACK1 has a critical role in regulating collagen transcription in TGF-β1-stimulated renal cells.

To examine the effect of the interaction between RACK1 and Smad3 on COL1A2 transcription, we overexpressed both of these molecules together in HKC cells, and measured reporter activity. Interestingly, overexpression of Smad3 overcame the inhibitory effect of both WT and the two point mutants of RACK1 (Fig. 4C). This result suggests that abundantly expressed Smad3 is sufficient to overcome the inhibitory effect of RACK1.

Phosphorylation of RACK1 and Smad3 in TGF-β1-stimulated HKC Cells—Since the tyrosine residues at 228 and 246 could play a role in COL1A2 transcription in renal cells, we investigated the potential mechanism of this effect. Because phosphorylation of RACK1 is critical for its regulation of Src kinase activity (20), serum-starved HKC cells were treated with TGF-β1, and the total phosphorylation of RACK1 was examined. Even with 24 h of serum starvation, there was basal phosphorylation of RACK1. With brief TGF-β1 treatment, phosphorylation was slightly decreased, whereas serum treatment tested as a positive control dramatically increased it (Fig. 5A). This suggests that TGF-β1 has little effect on phosphorylation of RACK1 or its subsequent Src kinase activity. Next, we investigated whether overexpressed RACK1 affects phosphorylation of Smad3. In a previous report, we found that TGF-β1-activated ERK phosphorylates serine residues, not at the C terminus but rather in the linker region of Smad3 (10). To study only cells that were successfully transfected with RACK1 WT, we examined the effect on phosphorylation of a co-transfected FLAG-Smad3. Overexpression of RACK1 WT affects neither total (Fig. 5B, upper panel) nor C-terminal (Fig. 5B, second panel) phosphorylation of FLAG-immunoprecipitated Smad3. Thus, RACK1 does not affect Smad3 phosphorylation at either the linker region or the C terminus. These results suggest that the inhibitory mechanism(s) of overexpressed RACK1 involves neither tyrosine phosphorylation of RACK1 nor serine phosphorylation of Smad3.

Overexpressed RACK1 Reduces DNA Binding Ability of Smad3—Next, we examined the effect of RACK1 on direct binding of Smad3 to its cognate DNA sequence, the SBE motif. Activation of Smad3 or Smad4 increases SBE-Luc promoter activity (22). Overexpressed RACK1 WT reduced this reporter activity. This suggests that RACK1 acts as an inhibitor of this signaling pathway.
activity, as did the RACK1 Y228F construct. On the other hand, co-expression of RACK1 Y246F had no effect (Fig. 6A). In parallel with the reporter assay, DAPA was performed. RACK1 WT reduced TGF-β1-stimulated binding of Smad3 to SBE oligonucleotides, and mutation at the Tyr-246 residue rescued the binding (Fig. 6B, upper panel). To confirm the specificity of the labeled SBE oligonucleotides, DAPA was performed using samples immunoprecipitated by biotinylated SBE oligonucleotide with increasing concentrations of non-biotinylated SBE oligonucleotide. As the relative amount of non-biotinylated SBE oligonucleotide increased, the intensity of precipitated Smad3 decreased (Fig. 6C). These results suggest that RACK1 decreases direct binding of Smad3 to the SBE motif. In contrast, overexpressed RACK1 WT, and the two mutants did not inhibit the ability of Smad3 to form a transcriptionally active complex in a reporter assay using a Gal4-Smad3 construct and Gal4 binding site fused with luciferase (Fig. 7). We have previously shown that Smad3 transactivation activity in this assay is decreased by inhibitors of protein kinase C. Thus, our data suggest that RACK1 does not suppress the transcriptional activity of Smad3 beyond affecting its ability to bind to the SBE motif.

DISCUSSION

TGF-β1 has been widely implicated in the pathogenesis of tissue fibrosis (2). Multiple signal transduction pathways induced by TGF-β1 have been studied for their ability to regulate ECM in the kidney. Apart from Smad signaling, it is known that TGF-β1 stimulates ERK1/2 MAP kinases (32). Our laboratory showed that ERK1/2 is required for maximal induction of Smad-mediated collagen I expression by TGF-β1 in human mesangial cells (10). Further, TGF-β1 stimulates protein kinase Cδ and phosphatidylinositol 3-kinase/Akt, which in turn enhance TGF-β-stimulated mesangial cell fibrogenesis (13, 14). However, the mechanisms of cross-talk among these distinctive pathways remain unknown.

In the present study, we identified a protein, RACK1, which interacts with Smad3 in both yeast and renal cells. The linker region of Smad3 is critical for this interaction. This is of interest in two respects. One is that R-Smad is phosphorylated at the linker region, not through direct TGF-β receptor kinase activity but through the ERK MAP kinase pathway (10). Another is that the linker region is a binding site for R-Smads to cytoskeletal proteins such as filamin 1 (33) and tubulin (34). RACK1 is a
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36-kDa protein containing seven internal Trp-Asp 40 repeats (WD repeats). The WD repeat sequence of RACK1 is highly conserved in a diverse range of species and homologous to the G protein β subunit, having 42% identity with many conserved amino acid substitutions (35). Our study shows that WD7 and partial WD6 domains are sufficient to support the interaction between RACK1 and Smad3. Each WD repeat forms a propeller blade, which allows RACK1 to associate with several different proteins at one time (15, 35), so it is possible that RACK1 facilitates Smad3 interaction with other signaling molecules. RACK1 does not contain any known Smad3 binding motif. Nonetheless, multiple WD repeats could interact with Smad3.

RACK1 is expressed abundantly in renal cells, and the level of endogenous protein is not affected by TGF-β1. Despite this, it was difficult to show endogenous interaction between RACK1 and Smad3. In part, this may reflect technical difficulties since our Smad3 immunoprecipitates yield a nonspecific band at the size of RACK1. Further, only a limited amount of endogenous Smad3 may be bound by RACK1 since overexpressing Smad3 overcomes RACK1-mediated inhibition. The interaction could be indirect since in yeast two-hybrid screening, it is impossible to state categorically whether the interaction is direct or indirect. Basal interaction between RACK1 and Smad3 is present even after cells are starved in serum-free conditions for 24 h, suggesting that Smad3 belongs to a constitutively binding group that includes the cyclic AMP-specific phosphodiesterase PDE4D5 (17). Phosphorylation of Smad3 is not affected by overexpressed RACK1 WT, and TGF-β1 has little effect on phosphorylation of RACK1 in renal cells. Thus, we concluded that phosphorylation of RACK1 or Smad3 does not play a significant role in the inhibition of Smad3 signaling by RACK1. Importantly, it is still possible that decreasing RACK1 phosphorylation affects other kinase activities, such as that of Src, because dephosphorylation of RACK1 leads to increased Src kinase activity (31). Further, stimulation with serum increases the phosphorylation of RACK1, probably because growth factors in serum activate multiple kinases. In the present series of experiments, we did not examine whether phosphorylation of RACK1 by stimuli other than TGF-β1 could affect RACK1-Smad3 interaction. This possibility would be of interest in dissecting extracellular mechanisms of cross-talk among different growth factors.

Decreasing RACK1 expression with siRNA enhances, and overexpression of the pcDNA3-HA-RACK1 WT effectively reduces, TGF-β1-mediated and Smad-mediated transcriptional activity, implying that RACK1 has a negative role in TGF-β1/Smad signaling. RACK1 siRNA enhanced both basal and TGF-β1-stimulated COL1A2 promoter activity. This suggests that there is a basal level of interaction between RACK1 and Smad3 and that the interaction plays a negative role in transcription of COL1A2 without TGF-β1. Such transcription could reflect partial activation of a more complex promoter by other factors, combined with a basal level of Smad3 activity. Under TGF-β1 stimulation, COL1A2 transcriptional activity is enhanced by silencing RACK1 and decreased to basal levels by overexpressed RACK1 WT, suggesting that RACK1 participates negatively in collagen transcription in renal cells. The inhibition by the RACK1 constructs is diminished by co-transfection of the FLAG-Smad3-pEXL, suggesting that the inhibitory effect is determined by the relative abundance of Smad3 and RACK1.

Overexpressed RACK1 did not affect Smad phosphorylation but decreased Smad binding to a specific DNA sequence (SBE), determined by SBE-Luc reporter assay and DAPA assay. The mechanism of such a role could be novel since our data obviate a major role for phosphorylation of either Smad3 or RACK1. Further, RACK1 showed no inhibitory effect in a Gal4-Smad3 reporter assay. This assay is independent of direct binding of Smad3 to DNA motifs but is useful to measure transactivational activity involving Smad3. Thus, RACK1 does not affect cooperation of Smad3 with other transcription factors in the nucleus. The effects of RACK point mutants were consistent for both COL1A2 promoter activation and Smad binding to the SBE sequence, suggesting that tyrosine 246 of RACK1, but not 228, plays a critical role in the ability of RACK1 to interfere with Smad transcriptional activity via modulating Smad binding to a specific DNA sequence.

Taken together, the results of our yeast two-hybrid, immunoprecipitation, promoter, and DAPA assays suggest that interaction between RACK1 and Smad3 has a negative effect on binding of Smad3 to the DNA motif. However, we cannot definitively exclude the possibility that inhibitory effects of RACK1 are mediated through another protein. Although RACK1 is well established as a scaffold for signaling molecules, it also functions as a component of the ribosomal protein complex (36). In mammals, RACK1 interacts with phosphorylated protein kinase C in the ribosome; activated protein kinase C mediates assembly of a functional ribosome (37). A potential role of
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RACK1 in blocking binding of a transcriptional regulator such as Smad3 to DNA is less established. RACK1 decreases E1A-associated histone acetyltransferase activity in mammalian cells, probably because it inactivates E1A by competitive binding (38). It is possible that RACK1 acts the same way to prevent Smad3 from binding to the SBE motif in renal cells.

The present studies reveal that RACK1 is a novel binding partner of Smad3. The linker region of Smad3 is critical for the interaction, and in addition, the WD7 and/or WD6 of RACK1 are sufficient for the association. We also showed that RACK1 is a negative regulator for TGF-β1/Smad-mediated transcription in renal epithelial cells. This study extends the spectrum of biological activities regulating TGF-β1/Smad signaling and suggests that RACK1 could function as a suppressor of ECM production in the kidney.

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