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CELL SIGNALING

Smad proteins and transforming growth factor-β signaling

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Smad proteins and transforming growth factor-β signaling. It is now generally accepted that transforming growth factor-β (TGF-β) has an important role in the pathogenesis of both acute and chronic forms of renal disease. Although TGF-β's potent fibrogenic activity is considered a major factor in chronic progression of renal disease, this cytokine participates in the control of several fundamental cellular responses in the kidney including inflammation, programmed cell death, cell growth, cell differentiation, and cellular hypertrophy. Recent identification of Smad proteins as intracellular mediators of TGF-β signaling has provided important insights into mechanisms that may determine the specificity of TGF-β action in different renal and inflammatory cells. Thus, Smads are characterized by an astonishingly complex array of molecular and functional interactions with other signaling pathways. These emerging patterns of signaling cross talk involving Smad proteins suggest a dynamic profile of positive or negative transmodulation of TGF-β signaling, depending on the cellular context. Understanding the interplay between these signaling cascades is an important field of investigation that will ultimately reveal new targets for precise and selective modulation of TGF-β's diverse actions in renal diseases.

MULTIPLE ROLES FOR TGF-β IN THE KIDNEY

Renal development and acute tubular injury

Organogenesis of the metanephric kidney is a precisely orchestrated process including inductions of an epithelial structure, the ureteric bud, and a surrounding mesenchyme, the metanephric blastema. Addition of transforming growth factor-β (TGF-β) to cultured metanephroi inhibits tubulogenesis and anti-TGF-β antibodies enhance nephron formation in cultured metanephroi [1]. Together with immunolocalization of the TGF-β signaling system in the ureteric bud, these results suggest that TGF-β may impair induction of tubulogenesis in metanephric blastema by the ureteric bud. The process of restoration of renal tubular integrity after acute tubular injury is similar to that of kidney development in that each is characterized by cellular proliferation, migration, and differentiation associated with the expression of "developmentally regulated" genes [2]. Interestingly, following acute experimental ischemic injury induced by renal artery clamping, TGF-β expression and activation was enhanced in proximal tubular papillary proliferations, indicating a role for TGF-β in the final reorganization of the regenerating tubule [3].

Development, reactivity, and pathophysiology of blood vessels

Members of the TGF-β family have diverse chronic and acute effects in the cardiovascular system. During embryogenesis, TGF-βs have prominent roles in de novo development of blood vessels [4], and in the kidney, precisely timed TGF-β signals direct formation of the capillary bed in developing glomeruli by steering growth, migration, and differentiation of glomerular endothelial cells [5]. In vitro and in vivo studies demonstrate important roles for TGF-β1 in both hypertensive blood vessel remodeling and in the formation of atherosclerotic lesions primarily through its effects on vascular smooth muscle cell growth and phenotype [6]. Several observations support a role for TGF-β in the acute regulation of vascular tone. TGF-β induces the vasoconstrictor endothelin 1 (ET-1) and suppresses inducible nitric oxide synthase (iNOS) resulting in decreased secretion of the vasodilator NO in endothelial cells and vascular smooth muscle cells. In addition, studies by Sharma and coworkers demonstrate that TGF-β may limit the extent of vasoconstrictor stimulation in smooth muscle and mesangial cells by down-regulating type I inositol 1,4,5-triphosphate receptors (IP_3R) thus limiting intracellular calcium mobilization (see elsewhere in this Supplement and [7]). These studies point to a role for TGF-β in control of acute and chronic hemodynamic regulation.

Inflammatory and immune-mediated renal disease

Relative to its profibrotic activities in renal disease, TGF-β's potent immunosuppressive effects in the kidney

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are underappreciated, considering that TGF-β is more potent by a factor of 10^4 than cyclosporine as an immunosuppressive agent [8]. In this regard, several recent investigations attempted to explore whether the immunosuppressive activity of TGF-β in immune-mediated renal disease models is essential to limit the expression of immune cell-associated mediators that mediate renal injury. Together, these studies indicate that a primary function of increased TGF-β in immune-mediated renal injury may be to limit the inflammatory response and that blocking TGF-β expression can exaggerate inflammatory responses and renal damage [9, 10].

Glomerulosclerosis and interstitial fibrosis

Several investigations in many model systems focusing on the powerful profibrogenic activities of TGF-β [11–13] have led to the paradigm that TGF-β plays a central role in the pathogenesis of chronic renal lesions such as glomerulosclerosis and interstitial fibrosis. These insights stem primarily from correlative examination of mesangioproliferative glomerulonephritis induced by the anti-Thy1.1 antiserum [14] or Habu snake venom [15], aminonucleoside nephrosis [16], obstructive nephropathy [17], and diabetic nephropathy [18]. These in vivo results are consistent with numerous in vitro observations of the profibrotic mechanisms induced by TGF-β. Finally, the functional significance of TGF-β as a central mediator of renal fibrosis is demonstrated by the glomerulosclerosis and interstitial fibrosis phenotype that develops spontaneously in TGF-β1 transgenic mice [19]. In addition, several studies indicate that TGF-β may mediate the profibrotic effects of angiotensin II [20], cyclosporine [21], and compounds in the "diabetic milieu," that is, glucose [22] and glycated albumin (see elsewhere in this supplement; and [23]).

Understanding the mechanisms of TGF-β signal transduction will hold the key to a new research field focusing on selective modulation of TGF-β actions in the kidney

Discussion in the previous section highlights the importance of new research into a molecular understanding of the intracellular signaling mechanisms that specify diverse cellular responses to TGF-β in renal and inflammatory cells. Recent identification of the Smad family of intracellular signaling proteins has led to new concepts of the mechanisms that may control the biologic specificity of TGF-β's action in a physiologic or pathophysiologic context. An emerging theme now indicates the importance of several interactive signaling pathways in addition to Smad signaling pathways downstream from TGF-β receptors. For example, TGF-β stimulates protein kinase A (PKA) pathways [24] and mitogen-activated protein (MAP) kinases [25] in mesangial cells, and directly activates MAP kinase (MAPK) pathway signaling through the extracellular signal-regulated kinases 1 and 2 (ERK1/2) in macrophage cultures [26] (see also elsewhere in this supplement). In this report, we focus on the function of Smads in TGF-β signaling and discuss how other signaling inputs may affect the TGF-β/Smad pathway.

THE SMAD FAMILY: A BRIEF INTRODUCTION TO MEMBER PROTEINS AND FUNCTIONS

Smad signaling pathways have been conserved throughout evolution. Although Smad-related genes *Mad* and *sma* were first discovered using genetic screens in *Drosophila* [27] and *Caenorhabditis elegans* [28], respectively, we concentrate our discussion here on vertebrate Smads that are defined by two Mad protein homology domains located at the N-terminal (MH1) or C-terminal (MH2) ends, respectively, and a less well-conserved linker region [29]. Nine distinct vertebrate Smad proteins can be classified in three groups [30] (Fig. 1).

Receptor-activated Smads (R-Smads)

R-Smads are defined by a SxS serine motif on the C-terminal end where R-Smads are phosphorylated and activated by type I receptor kinases. The highly similar Smad2 and Smad3 interact with TGF-β type I receptor or activin type I B receptor, whereas Smad1, Smad5, and Smad8 interact with type I receptors of the bone morphogenetic protein (BMP) receptor subfamily. This pathway specificity is determined by the so-called L3-loop in the MH2 domain of R-Smads [31] (Fig. 1).

Common partner Smad4

Following type I receptor induced phosphorylation on serines in the SxS motif, R-Smads form heteromeric complexes with the common partner Smad4 (Co-Smad) and these complexes translocate to the nucleus (Fig. 2). Smad4 was first described as *Deleted in Pancreatic Carcinoma Locus 4 (DPC4)*, a candidate tumor suppressor gene [32] initially thought to be an essential component in all TGF-β signaling responses [33]. Because Smad4 forms complexes with both, R-Smads in the TGF-β/activin and BMP pathways, it acts as a convergent node and its principal function is to regulate transcription rather than to transmit signals [34]. Smad4 shares with both Smad2 and Smad3 transcriptional activator function localized in the MH2 domain, and, like Smad3, directly interacts with DNA consensus binding sequences (CAGA) [35] through a β-hairpin structure in the MH1 domain (Fig. 1). Another shared function of the MH1 domains in Smad2, 3, and 4 are autoinhibition by preventing ligand-independent heteromerizations [36]. Smad–Smad protein interactions require the MH2 domains, respectively. Unlike Smad3 and Smad4, Smad2 has no direct DNA-binding capacity

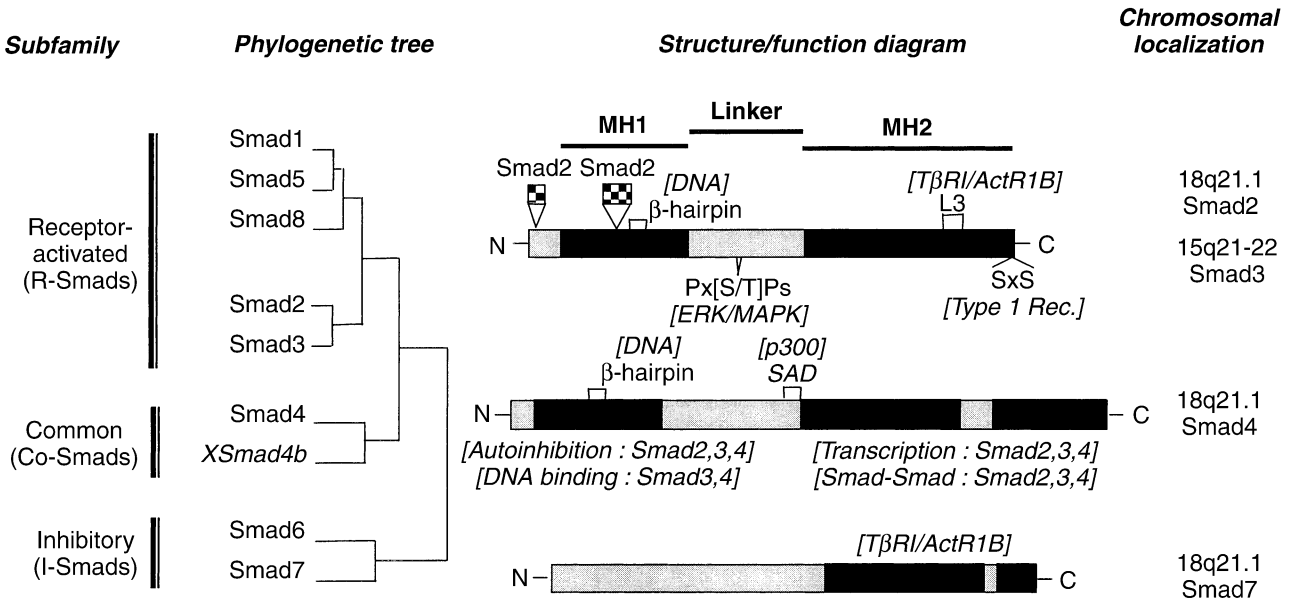

Fig. 1. The Smad protein family. Phylogenetic tree of vertebrate Smads (except for Xenopus Smad4b [XSmad4b]). Schematic structure/function diagram illustrates receptor-activated Smads (R-Smad) (example shown here Smad2, 3), common-partner Smads (Co-Smad) (example here Smad4), and inhibitory Smads (I-Smads)(example here Smad7). MH1and MH2 indicate Mad homology region 1 and 2, respectively. MH1 region mediates autoinhibition of Smad2, 3, and 4 and DNA binding of Smad3 and 4. MH2 regions include transcriptional activation domains and Smad–Smad protein interactions in Smad2, 3, and 4, respectively. Phosphorylation sites for Erk/MAPK (Px[S/T]Ps) and type I TGF-β receptors (SxS) are shown in Smad2/Smad3. Checkered boxes (Smad2) indicate extra exons in Smad2 compared with Smad3. The β-hairpin domain indicates DNA binding motif in Smad2, 3, and 4. SAD indicates Smad4 activation domain [59]. L3 indicates domain for specificity in type I receptor interaction (example shown here for TGF-β and activin type I receptors (TβRI/ActR1B)). Right-hand column shows chromosomal map positions for human Smad2, Smad3, Smad4, and Smad7.

because of the presence of an extra exon in the MH1 domain (Fig. 1). Contrary to earlier studies, recent observations have identified a number of Smad4-independent TGF-β actions [37, 38]. This raises the possibility that as yet unidentified Co-Smads could mediate certain TGF-β responses in the absence of Smad4. Indeed, a second Co-Smad (XSmad4b) has recently been identified in *Xenopus* [39] and several groups are searching for a mammalian othologue of XSmad4b.

Inhibitory Smads

Smad signaling systems are notable for an autoinhibitory feedback loop that involves so-called "inhibitory" Smads (I-Smads). The I-Smads, Smad6 and Smad7, were first identified in a screen for shear-stress-regulated genes in endothelial cells [40] and lack the MH1 domains of R-Smads and Co-Smads (Fig. 1). These Smads act to oppose R-Smads by forming stable interactions with activated type I receptors, thus preventing the phosphorylation/activation of R-Smads [41, 42]. At physiologic concentrations, Smad6 appears to inhibit BMP signaling by interacting with BMP type I receptors and Smad7 inhibits TGF-β/activin signaling by interaction with TGF-β/activin type I receptors, which suggests that the principal functions of Smad6 and Smad7 are to regulate BMP or TGF-β/activin pathways, respectively.

Signaling through Smad pathways is controlled by positive and negative interactions with independent signaling pathways and dynamic changes of cellular structure

Evidence suggests that Smads are devoid of enzymatic activity and that Smad signaling is thus not amplified. Therefore, regulation of target genes and Smad-induced cellular responses may be sensitive to small changes of protein levels of Smads, of their subcellular and spatial compartmentalization, and of their interactions with independent pathways (Fig. 2). A rational hypothetical model of the control of signaling flow through the Smad pathway can be developed.

Cytoskeletal sequestration of Smads and their recruitment to receptors

A recent report shows that unphosphorylated Smad2, 3, and 4 are bound to intact microtubules in the unactivated cellular state. Both TGF-β stimulation and disruption of the microtubular structure induced by cytoskeletal alterations result in dissociation from β-tubulin and ligand-dependent or ligand-independent phosphorylation of Smad2 or Smad3 and activation of TGF-β transcriptional responses [43]. It is possible that after release from cytoskeletal complexes, the R-Smads Smad2 and Smad3 are then recruited to the TGF-β or activin recep-

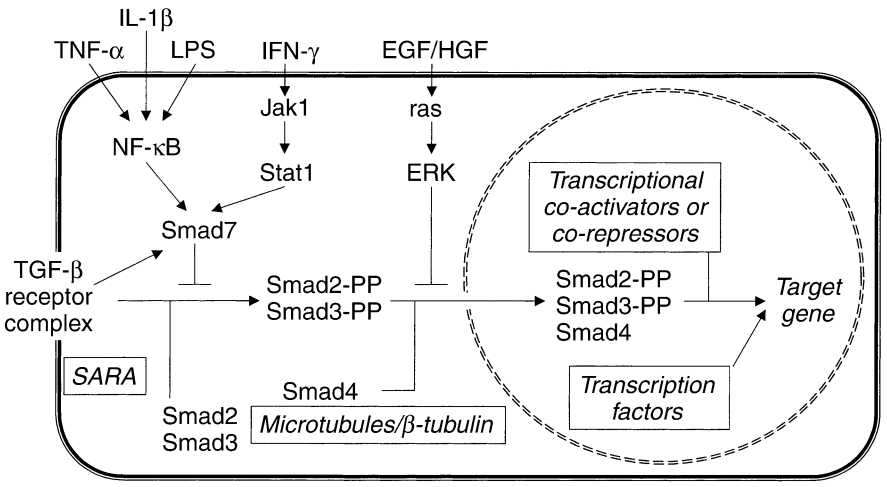

Fig. 2. Signaling in the TGF-β/Smad pathway is positively and negatively regulated by interactions with structural cellular elements and converging signaling pathways. Smad2, Smad3, and Smad4 transmit signals from TGF-β or activin receptor complexes to the nucleus where they control transcription of target genes. Critical features of regulation of the activity of this pathway include: (a) sequestration of Smads by intact microtubules in unactivated cells; (b) release of Smad2 and Smad3 to membrane and receptor anchored molecules such as SARA in response to disruption of microtubules and/or TGF-β signal; (c) recruitment of Smad2 and Smad3 to activated receptor complexes by SARA and phosphorylation of R-Smads on C-terminal serines (Smad2-PP and Smad3-PP); (d) negative regulation of R-Smad phosphorylation by competitive interaction of Smad7 and activated receptor complexes in response to signaling pathways with opposing activities (NF-κB and Stat1), and negative autofeedback (not shown); and (e) negative regulation by activated Erk MAP kinase signaling in response to growth factors (EGF and HGF) or oncogenic Ras. These "cross-talking" interplays depend on the cellular context and modulate the "dosage" of R-Smad/Co-Smad (Smad2-PP, Smad3-PP, and Smad4) signaling complexes that translocate to the nucleus in response to TGF-β. In the nucleus, R-Smad/Co-Smad complexes interact with different transcriptional activators and/or repressors either independent of or dependent on Smad binding at specific DNA consensus sequences in target genes (for an excellent review, see ten Dijke et al. [30]).

tor complex by interaction with the membrane-associated protein Smad-anchor for receptor activation (SARA) [44] (Fig. 2). SARA is able to present Smad2 and Smad3, but not Smad4, to the activated type I receptors by binding cooperatively to nonphosphorylated Smads and the receptor complex [44]. An additional level of control of accessibility of R-Smad proteins to activated receptor complexes is exerted by the regulated synthesis of inhibitory Smad7. Work by several groups including our own shows that Smad7 competes with R-Smads for access to activated TGF-β or activin type I receptors, thereby preventing their phosphorylation [41, 45, 46].

Inhibition of phosphorylation/activation of R-Smads by opposing signaling pathways

We have demonstrated recently that well-documented transmodulation between major opposing signaling pathways and the TGF-β/Smad pathway can be mediated through NF-κB dependent activation of inhibitory Smad7 [46]. In our report, we show that the NF-κB subunit p65/RelA is required for transcriptional activation of Smad7 by lipopolysaccharides (LPS), and the proinflammatory cytokines interleukin-1β and tumor necrosis factor-α (TNF-α) (Fig. 3). Smad7 induced by TNF-α suppresses TGF-β/Smad signaling through its direct interaction with the type I receptor on TGF-β ligand-receptor binding. Increased occupancy of activated TGF-β receptor complexes by Smad7 results in inhibition of phosphorylation, nuclear translocation, and DNA binding of R-Smad/Co-Smad [46] (Fig. 2). Because it is well documented that TNF-α inhibits induction of type I collagen synthesis by TGF-β [47], our observations may provide an important molecular mechanism for negative control of profibrotic actions of TGF-β in glomerulosclerosis and interstitial fibrosis in the kidney.

Interestingly, a recent report demonstrates that inhibition of TGF-β/Smad signaling by interferon (IFN)-γ may also be mediated through increased binding of inhibitory Smad7 and TGF-β receptors, following induction of Smad7 expression by the Jak1/Stat1 pathway [48] (Fig. 2). Taken together, these observations suggest that Smad7 may function as a general negative regulator of TGF-β receptor signaling, inasmuch as Smad7 is capable of mediating both autoinhibitory feedback and down-regulation of TGF-β signaling strength by major opposing pathways including the Jak1/Stat1 and the NF-κB pathway (see schematic in Fig. 2). We propose that inhibition of TGF-β

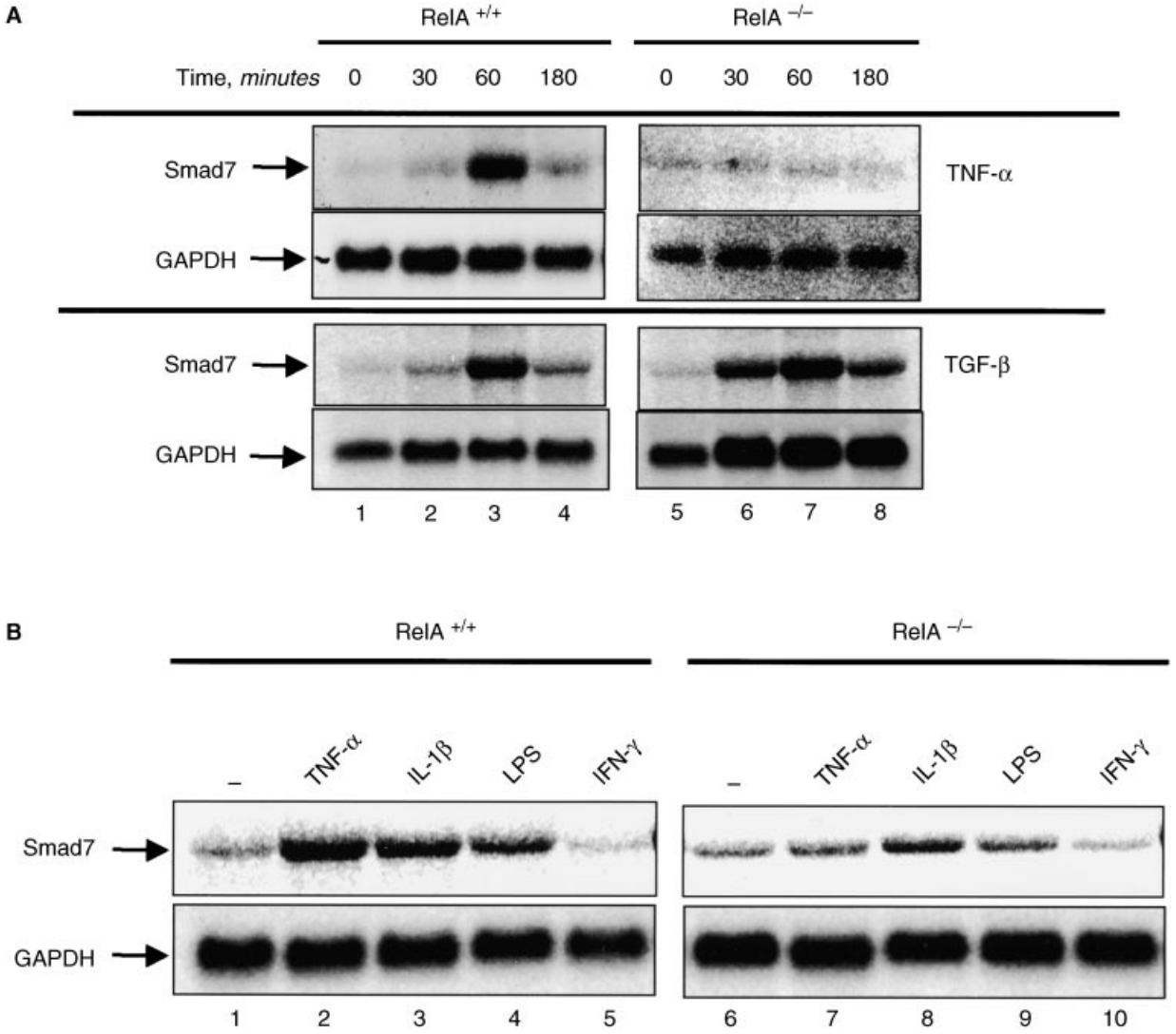

Fig. 3. Transcriptional activation of Smad7 by TNF-α, IL-1β, and LPS requires NF-κB/RelA. (*A*) Northern blot analysis of Smad7 transcript levels in RelA$^{+/+}$ and RelA$^{-/-}$ fibroblasts treated with TNF-α (10 ng/ml) or TGF-β (1 ng/ml) for the indicated time periods. (*B*) RelA$^{+/+}$ and RelA$^{-/-}$ fibroblasts were incubated with TNF-α (10 ng/ml), IL-1β (1 ng/ml), LPS (10 μg/ml), and IFN-γ (250 U/ml) for 1 h, respectively. Blots were probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for RNA loading (with permission to reprint [46]).

receptor signaling function by inhibitory Smad7 may represent an effective and general mechanism to alter the balance between signals with opposing effects on complex cellular responses including inflammation and cell proliferation/cell death.

Inhibition of nuclear translocation of Smad complexes by Erk mitogen-activated protein kinases

Reports by several groups indicate a direct interaction between R-Smads and the Erk mitogen-activated protein (MAP) kinase pathway [49–51]. Activation of Erk MAP kinases by epidermal growth factor (EGF) or oncogenic Ras can lead to phosphorylation of middle linker regions of R-Smads at [S/T]P or Px[S/T]P motifs, resulting in inhibition of nuclear translocation of these Smads and suppression of TGF-β/Smad signaling (Fig. 2). These observations may provide a mechanism of suppression of TGF-β/Smad signaling by hyperactivated Ras that may be operative during tubular regeneration after acute tubular injury. In contrast, a different study finds Erk-dependent phosphorylation of Smad2 at a different motif resulting in increased nuclear translocation and cooperative signaling with TGF-β/Smad signaling [52].

Regulation of Smad protein synthesis and degradation

We and others find that protein levels of Smad2 and Smad4 are not regulated by extracellular signals, whereas Smad3 is down-regulated following treatment with TGF-β in some epithelial and mesangial cells [53, 54; Schiffer and Böttinger, unpublished observations]. Recent reports

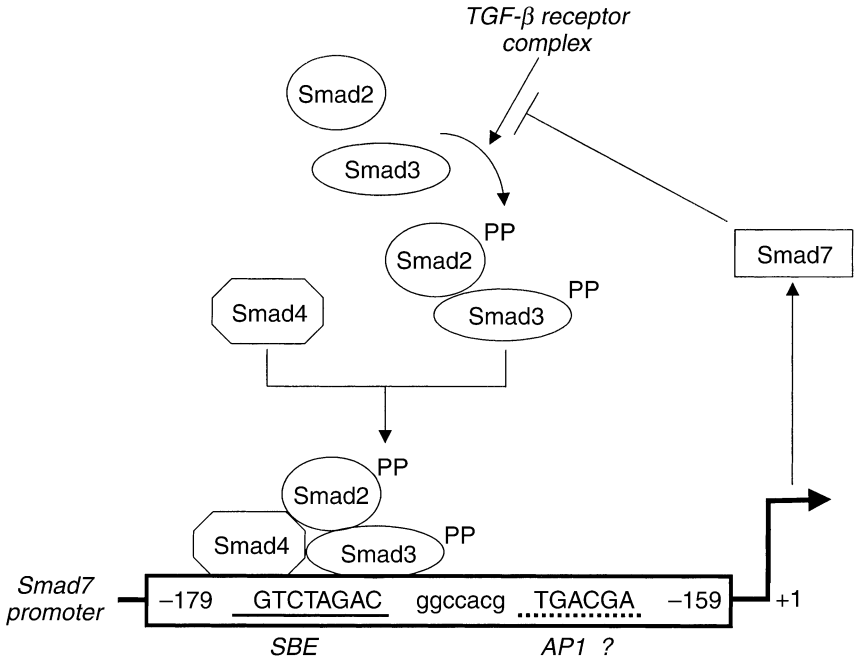

Fig. 4. Molecular mechanism of an intracellular TGF-β autoinhibitory loop. On ligand-binding and activation of TGF-β receptor complexes, R-Smads Smad2 and Smad3 become phosphorylated by type I receptor on C-terminal serines and form heterooligomeric complexes with Co-Smad Smad4. After nuclear translocation, Smad2-PP/Smad3-PP/Smad4 complexes bind to a functional GTCTAGAC Smad binding element between nucleotides −179 and −172 of the human Smad7 promoter and mediate transcriptional activation of Smad7 by TGF-β. An adjacent imperfect putative AP-1 protein binding site may interact with AP-1 protein, but its functional relevance remains to be determined. TGF-β/Smad-induced synthesis of Smad7 results in increasing intracellular levels of Smad7 and enhanced negative regulation of activated TGF-β receptor complexes by Smad7 [57].

have examined mechanisms and regulation of turnover/degradation of both cytoplasmic and nuclear Smads. For example, the cytoplasmic E3 ubiquitin-ligase SMURF1 has been shown to target Smad1 in the BMP-signaling pathway for proteosomal degradation in a ligand-independent manner [54]. It is possible that analogous mechanisms of control of protein degradation of R-Smads exist in the TGF-β/activin pathways. However, ubiquitin-dependent degradation of R-Smads is not only restricted to cytoplasmic Smads but may also limit the extend of binding and transcriptional activity of Smad complexes bound to specific DNA sequence motifs in target gene promoters [55]. Understanding of the mechanisms that regulate the ubiquitination-pathways of Smad proteins may offer future opportunities to modulate Smad signaling activity in a cell or target gene specific manner.

Transcriptional regulation of target gene expression by Smads involves direct binding of Smads to DNA and recruitment of transcriptional (Co-) activator and (Co-) repressor complexes, respectively

A rapidly evolving field has been investigating the mechanisms by which Smad proteins direct expression of target genes of TGF-β signaling. It is now accepted that a variety of different mechanisms may cooperate to achieve regulated transcription, including direct DNA binding of R-Smad/Smad4 complexes and interactions with a growing number of DNA-binding or non-DNA-binding activators and repressors of transcription (Fig. 2). This extensive topic is beyond the scope of this review. It has recently been discussed in an excellent review by ten Dijke and colleagues [30].

GENE REGULATION OF INHIBITORY SMAD7

Given that Smad7 is an inducible intracellular inhibitor of TGF-β signaling that is transcriptionally regulated as an immediate-early gene by several signaling pathways of major importance in renal diseases [40, 46, 49, 56], we cloned the promoter of the human *SMAD7* gene and have begun to characterize *cis*-acting elements and transcription factors that control the expression of this important gene [57]. The human *SMAD7* gene consists of 4 exons distributed over approximately 25 kilobases and is located on chromosome 18q21.1 (Susztak and Böttinger, unpublished observations; [58]). Within a −303 to +672 region relative to a major transcription start site, we identified a palindromic GTCTAGAC Smad binding element (SBE) [35] between nucleotides −179 and −172 that mediates induction of a Smad7 promoter reporter gene by TGF-β. The SBE is located in close proximity to a putative activator protein (AP)-1 protein binding site. The functional significance of the putative AP-1 element remains unclear, although preliminary mobility shift assays indicate that it interacts at least with AP-1 family transcription factors (von Gersdorff and Böttinger, unpublished observations). Our biochemical and functional characterization of the SBE demonstrates that TGF-β treatment of cells in culture rapidly induces binding of an endogenous complex of nuclear proteins to the SBE that contains Smad2, Smad3, and Smad4 (Fig. 4). In cells that lack either Smad3 or Smad4, no binding of protein complexes to the SBE is detectable. These results are consistent with our data obtained from transcriptional reporter gene assays showing that the

induction of the Smad7 promoter requires Smad3 and Smad4, but not Smad2. A functional role of Smad2 in the regulation of Smad7 transcription has not yet been determined.

Thus, we have identified a molecular mechanism that may have a central role in negative autoregulation of TGF-β/Smad signaling. We propose that ligand-dependent activation of TGF-β receptor complexes stimulates binding of Smad2, Smad3, and Smad4 transcription factor complexes to a consensus SBE in the Smad7 promoter. Mutation of the SBE by site-directed mutagenesis, or deletion of either Smad3 or Smad4, but not Smad2, abolishes the ability of TGF-β to activate the Smad7 promoter. We conclude that transcriptional regulation of Smad7 by TGF-β itself is mediated through rapid and direct Smad3-and Smad4-dependent signaling and transcriptional activation. Our observations are consistent with a report indicating that Smad3 and Smad4 interact with and activate the murine Smad7 promoter [56].

Interestingly, our studies indicate that TNF-α and IFN-γ may regulate the *SMAD7* gene through *cis*- and *trans*-acting factors that are distinct from the SBE and Smads, respectively. Thus, we provide evidence for a model in which the overall level of *Smad7* gene expression may be determined by combined activation of distinct regulatory elements in the Smad7 promoter in response to distinct signaling pathways. Ongoing investigations in our laboratory focus on the localization of functional Stat1 and NF-κB/RelA regulatory elements in the *Smad7* gene to map the molecular determinants of transcriptional activation of *SMAD7* by TNF-α and IFN-γ. We anticipate that the continued investigation of Smad7 and related molecules may lead to novel approaches in the design of inhibitors of TGF-β signaling for therapeutic use in chronic progressive kidney diseases characterized by prominent glomerulosclerosis and interstitial fibrosis.

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