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The MAD-Related Protein Smad7 Associates with the TGFβ Receptor and Functions as an Antagonist of TGFβ Signaling

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Summary

TGFβ signaling is initiated when the type I receptor phosphorylates the MAD-related protein, Smad2, on C-terminal serine residues. This leads to Smad2 association with Smad4, translocation to the nucleus, and regulation of transcriptional responses. Here we demonstrate that Smad7 is an inhibitor of TGFβ signaling. Smad7 prevents TGFβ-dependent formation of Smad2/Smad4 complexes and inhibits the nuclear accumulation of Smad2. Smad7 interacts stably with the activated TGFβ type I receptor, thereby blocking the association, phosphorylation, and activation of Smad2. Furthermore, mutations in Smad7 that interfere with receptor binding disrupt its inhibitory activity. These studies thus define a novel function for MAD-related proteins as intracellular antagonists of the type I kinase domain of TGFβ family receptors.

Introduction

Members of the transforming growth factor β (TGFβ) superfamily play a critical role in regulating cellular growth and differentiation in a wide range of biological systems. These polypeptide growth factors signal through heteromeric complexes of type I and type II transmembrane Ser/Thr kinase receptors (Attisano and Wrana, 1996; Weis-Garcia and Massague, 1996). Activation of the receptor complex occurs when the type II receptor kinase transphosphorylates the GS domain of the type I kinase (Wrana et al., 1994). This activates the type I kinase, which transiently associates with and phosphorylates a unique family of intracellular signaling molecules related to the Drosophila gene *Mothers against dpp* (*Mad*; Derynck and Zhang, 1996; Massagué, 1996; Wrana and Attisano, 1996).

The MAD-related (MADR) family includes the *sma-2*, *sma-3*, and *sma-4* genes from C. elegans and six vertebrate members, Smad1–6 (Wrana and Attisano, 1996; Hoodless and Wrana, 1997). These proteins are highly conserved across species, but have no known structural motifs and bear no resemblance to components of other signaling pathways. All members of the family share certain structural domains including the highly conserved amino and carboxy-terminal regions termed the MH1 and MH2 domains, respectively. Smad6 is the exception to this, and encodes a truncated protein that possesses only the MH2 domain. Smads are critical intracellular mediators of Ser/Thr kinase receptor signaling and play a role in specifying biological responses to ligands. Thus, Smad1 and 5 function in BMP signaling pathways, while Smad2 and 3 mediate TGFβ and activin signaling (Baker and Harland, 1996; Eppert et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Kretzchmar et al., 1997; Lechleider et al., 1996; Liu et al., 1996; Macías-Silva et al., 1996; Thomsen, 1996; Yingling et al., 1996; Zhang et al., 1997).

Smads are regulated by receptor-dependent phosphorylation that is highly specific. Smad2 and Smad3 interact with TGFβ receptors (Macías-Silva et al., 1996; Zhang et al., 1996), while Smad1 is targeted by BMP pathways (Hoodless et al., 1996; Kretzchmar et al., 1997). Phosphorylation of Smads is mediated directly by the activated type I kinase domain and occurs on serine residues within a conserved SSXS motif at the C terminus of the protein (Macías-Silva et al., 1996; Kretzchmar et al., 1997). Phosphorylation of these receptor-regulated Smads results in formation of a heteromeric complex with another MAD-related protein, Smad4 (Kretzchmar et al., 1997). Thus, Smad1, 2, 3, and 5 appear to play specific roles in these pathways, while Smad4 fulfills a common function (Lagna et al., 1996; Zhang et al., 1997). Phosphorylation of Smads is required for their accumulation in the nucleus (Macías-Silva et al., 1996; Kretzchmar et al., 1997) where the proteins can function as transcriptional activators through their interaction with DNA-binding proteins. For instance, in Xenopus, Smad2 interacts with the forkhead-containing DNA-binding protein, FAST1, to activate the activin responsive element in the *mix.2* gene (Chen et al., 1996). This signaling pathway thus provides a direct link between the receptor kinase and the transcriptional machinery required to mediate the biological response to ligand.

The biochemical and functional characterization of Smad1–5 as downstream mediators of TGFβ superfamily signaling is yielding a comprehensive view of how this signaling pathway may function. However, novel Smads with unknown functions have been identified in the expressed sequence tag (EST) database or in screens unrelated to TGFβ signaling. We now investigate the MAD-related protein Smad7 which is induced by laminar fluid shear stress and is expressed at elevated levels in the vascular endothelium (Topper et al.,

1996, 1997). We demonstrate that expression of Smad7 in TGFβ-responsive cells suppresses ligand-dependent signaling. Smad7 directly interferes with TGFβ-mediated activation of Smad2 by preventing its phosphorylation, interaction with Smad4, and nuclear accumulation. Smad7 functions by associating stably with the activated TGFβ type I receptor to block access and phosphorylation of Smad2. Thus, Smad7 defines a novel class of MAD-related proteins that inhibit Ser/Thr kinase receptor signaling by acting as intracellular antagonists of the type I receptor kinase domain.

Results

Smad7 Inhibits TGFβ Signaling

The MAD-related protein Smad7 was isolated in a screen to identify genes that are induced when endothelial cells are placed under laminar fluid shear stress (Topper et al., 1996) and is represented in the expressed sequence tag database. Smad7, which possesses MH1 and MH2 domains, lacks an SSXS motif at the C terminus (Topper et al., 1997), suggesting that the protein is not a target for receptor phosphorylation and may fulfill an undescribed function in Ser/Thr kinase receptor signaling.

We first tested the potential role of Smad7, by investigating the effects of its expression on TGFβ signaling. For these studies, we utilized HepG2 and Mv1Lu cells, both of which possess receptors and are TGFβ responsive. Initially, we focused our analyses on p3TP-Lux, which contains elements from the PAI-1 promoter, is TGFβ responsive, and drives expression of a luciferase reporter gene (Wrana et al., 1992). Transient transfection of p3TP-Lux into either HepG2 or Mv1Lu cells resulted in low basal levels of transcription in untreated cells, which was strongly induced by stimulation with 100 pM TGFβ (Figure 1). In cells cotransfected with Smad7, there was little effect on basal transcription; however, we observed a strong suppression of TGFβ-dependent induction of the 3TP promoter. TGFβ signaling can be initiated by expression of a constitutively active version of the TGFβ type I receptor, TβRI, which contains a substitution of threonine residue 204 to aspartate and signals TGFβ responses in the absence of ligand and the type II receptor (Wieser et al., 1995). In both HepG2 and Mv1Lu cells, transfection of activated TβRI led to strong stimulation of the 3TP promoter that was potently inhibited by coexpression of Smad7 (Figure 1). Since our subsequent biochemical analyses involved versions of Smad7 that were epitope tagged at the amino and carboxyl termini with myc and HA epitopes, respectively, we also tested the activity of the modified proteins. These versions of Smad7 were indistinguishable from untagged proteins in blocking TGFβ-dependent signaling in all of the assays that we employed (data not shown). Together, these data suggest that Smad7 can function to block TGFβ signaling downstream of its receptors.

To determine whether Smad7 inhibitory activity was a direct effect on TGFβ-signaling pathways, we developed an immediate early response assay for TGFβ signaling. In Xenopus, activin signaling leads to the specific association of Smad2 with the forkhead-containing

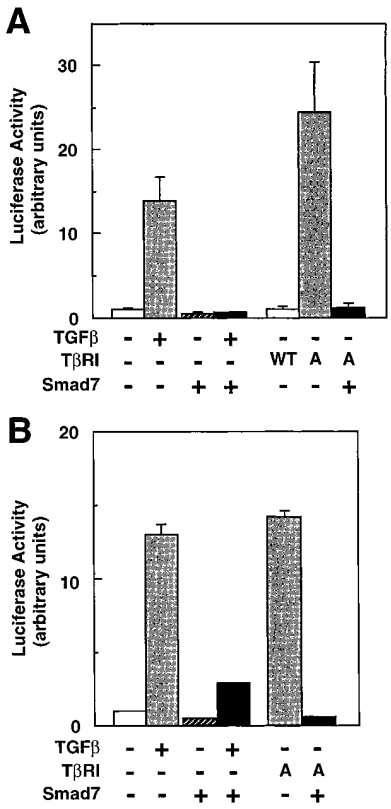

Figure 1. TGFβ-Induced Transcriptional Activation of the 3TP Promoter Is Inhibited by Smad7

HepG2 (A) or Mv1Lu (B) cells were transiently transfected with p3TP-Lux alone or with wild-type (WT) or activated (A) TβRI in the absence or presence of Smad7. Cells were incubated overnight in the absence or presence of 100 pM of TGFβ, and the relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase activity and is expressed as the mean ± SD of triplicates from a representative experiment.

DNA-binding protein, FAST1, and activation of transcription from an activin-responsive element (ARE; Chen et al., 1996). Since Smad2 functions in both TGFβ- and activin-signaling pathways (Baker and Harland, 1996; Eppert et al., 1996; Graff et al., 1996; Macías-Silva et al., 1996), we utilized this system to investigate the role of Smad7 in blocking immediate early responses to TGFβ. To establish that this assay functions in HepG2 cells, we constructed pAR3-lux, which contains a luciferase reporter gene under the control of three AREs linked to a basic TATA box–containing promoter. This construct had minimal basal activity in HepG2 cells, but when it was cotransfected with FAST1, we observed a strong TGFβ-dependent induction of the reporter gene (Figure 2A). These data indicate that HepG2 cells contain the necessary components to activate transcription from an ARE, but are missing the DNA-binding component, FAST1. To determine whether Smad7 can block activation of pAR3, HepG2 cells were cotransfected with pAR3-Lux, FAST1, and Smad7. In contrast to the stimulation observed in cells receiving only FAST1, TGFβ-dependent activation was suppressed in cells cotransfected with Smad7 (Figure 2A).

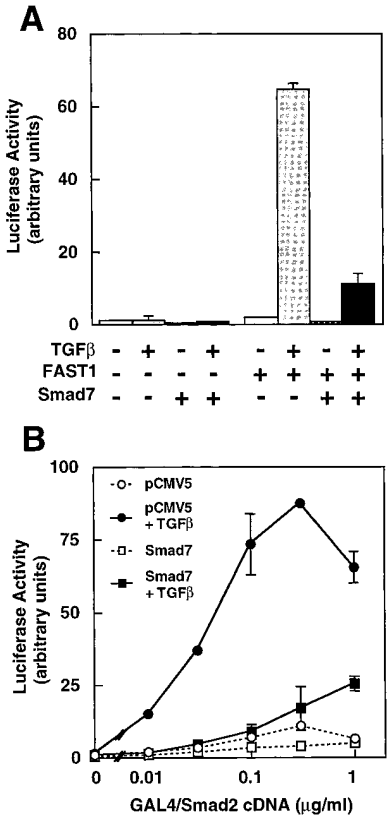

Figure 2. Smad7 Interferes with Activation of TGFβ-Signaling Pathways

(A) Smad7 blocks an immediate-early response to TGFβ. HepG2 cells were cotransfected with the reporter gene (AR3-lux) alone or with FAST1 in the absence or presence of Smad7. Cells were incubated overnight with or without 100 pM of TGFβ, and the relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase activity and was expressed as the mean ± SD of triplicates from a representative experiment.

(B) TGFβ-dependent activation of GAL4/Smad2 is inhibited by Smad7. HepG2 cells were cotransfected with the reporter gene (pG5-E1b-lux) alone or with the indicated dose of GAL4/Smad2 in the absence (circle) or presence (square) of Smad7. Cells were incubated overnight in the absence (open symbols) or presence (closed symbols) of 100 pM of TGFβ, and the relative luciferase activity was measured in cell lysates. Luciferase activity was normalized to β-galactosidase activity and was expressed as the mean ± SD of triplicates from a representative experiment.

Since Smad2 is centrally involved in mediating TGFβ signaling, we investigated whether Smad7 might specifically disrupt ligand-dependent activation of Smad2 transcriptional activity. To test this, we employed a heterologous transcriptional activation assay (Liu et al., 1996), in which Smad2 is fused to the DNA-binding domain of the yeast transcription factor GAL4. Expression of this fusion protein in HepG2 cells mediated TGFβ-dependent induction of the heterologous promoter construct, pG5-E1b-lux, which contains five GAL4 elements linked to an E1b TATA box and a luciferase reporter gene. To determine if Smad7 disrupts this response, pG5-E1b-lux was transfected into HepG2 cells together with increasing amounts of GAL4/Smad2 expression vector either in the presence or absence of Smad7. In untreated cells, basal transcription from the reporter gene was low and was unaffected by cotransfection with Smad7. However, treatment of cells with 100 pM TGFβ resulted in a strong induction of the promoter in cells transfected with GAL4/Smad2 alone. In contrast, cotransfection with Smad7 resulted in almost complete abrogation of TGFβ-dependent activation of the promoter that was partially reversed when higher doses of GAL4/Smad2 were transfected into the cells. Together, these data suggest that Smad7 may block TGFβ responses by interfering directly with the signaling pathways.

Smad7 Blocks Activation of Smad2

TGFβ signaling through Smad2 is initiated when the type I receptor kinase, activated by receptor II–mediated transphosphorylation, directly phosphorylates Smad2 on the C-terminal serine residues (Macías-Silva et al., 1996). This phosphorylation results in dissociation of Smad2 from the receptor and is required for subsequent interactions with Smad4 and nuclear translocation of the protein (Macías-Silva et al., 1996; Kretzschmar et al., 1997). To investigate whether Smad7 interferes with any of these signaling events, we determined if it could block either Smad2 association with Smad4 or its nuclear accumulation. To investigate heteromeric complex formation, HepG2 cells were transiently transfected with Smad2 and Smad4 either in the presence or absence of Smad7. In cells expressing Smad2 and Smad4, no association between these two proteins could be detected in the absence of TGFβ signaling. However, coexpression with an activated type I receptor resulted in formation of Smad2/Smad4 heteromeric complexes, similar to previous observations (Figure 3A; Lagna et al., 1996). In contrast, in cells coexpressing Smad7, we observed almost a complete block in TGFβ-dependent association of Smad2 and Smad4.

We also investigated whether Smad7 could disrupt the nuclear localization of Smad2 in response to TGFβ signaling. HepG2 cells were transiently transfected with Flag/Smad2 in the presence or absence of Smad7/HA and either wild-type or activated TβRI. The subcellular localization of Flag/Smad2 was determined by immunofluorescence and confocal microscopy using a mouse anti-Flag antibody. Similar to our previous observations, Smad2 was localized throughout the cell in unstimulated cultures, and activation of TGFβ signaling resulted in nuclear accumulation of the protein. However, in the presence of Smad7, the nuclear accumulation of Smad2 in response to TGFβ signals was blocked (Figure 3B). For these studies, we simultaneously analyzed the expression of Smad7/HA using a rabbit anti-HA antibody and confirmed that Smad2 and Smad7 were coexpressed in the same cells (data not shown). Together, these data suggest that Smad7 inhibits TGFβ signaling by preventing Smad2 association with Smad4 and nuclear accumulation of the complex.

To explore the mechanism of Smad7 function, we first tested if it could engage Smad2 in nonproductive heteromeric complexes. However, we were unable to detect any physical association of Smad7 with Smad2

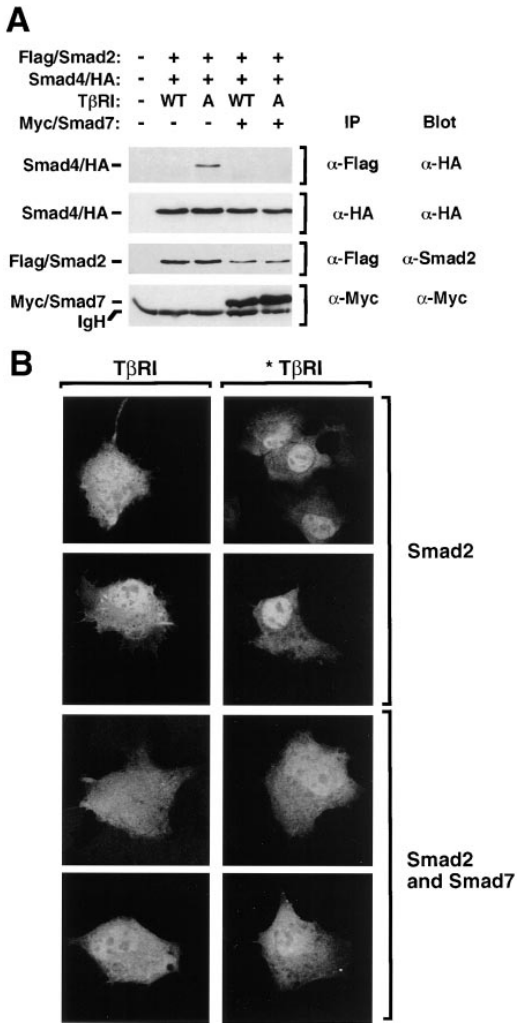

Figure 3. Smad7 Blocks Smad2 Activation in Response to TGFβ Signaling

(A) Smad7 disrupts TGFβ-induced interaction of Smad2 with Smad4. HepG2 cells were transiently transfected with empty vector (−) or Flag/Smad2 and Smad4/HA together with wild-type (WT) or activated (A) TβRI either in the absence or presence of myc/Smad7. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag M2 antibody (α-Flag) and then immunoblotted (blot) using anti-HA antibody (α-HA). Expression of Smad2 was monitored by reprobing the same membrane with a rabbit anti-Smad2 polyclonal antisera (α-Smad2). To confirm equivalent levels of Smad4 expression, aliquots of total lysates were immunoprecipitated using an anti-HA antibody and then immunoblotted with anti-HA antibody (α-HA). Expression of Smad7 was measured in a similar manner using an anti-myc monoclonal antibody (α-myc).

(B) Smad7 blocks the nuclear accumulation of Smad2 in response to TGFβ signaling. HepG2 cells were transfected with Flag/Smad2 together with wild-type (TβRI) or activated (*TβRI) TGFβ type I receptors, either in the absence (top four panels) or presence (bottom four panels) of Smad7. Flag/Smad2 was detected by immunofluorescence using anti-Flag M2 antibody and FITC-conjugated secondary antibody and analyzed by confocal microscopy. Smad2 is localized prominently in the nucleus in the presence of activated TβRI. Coexpression with Smad7 blocks this nuclear accumulation, resulting in a diffuse staining pattern that is similar to controls.

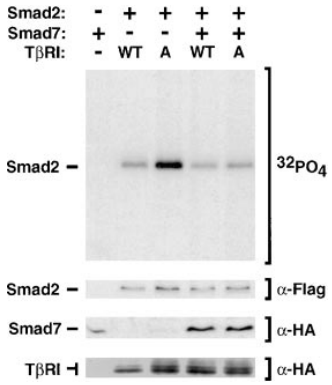

Figure 4. Smad7 Prevents Receptor-Dependent Phosphorylation of Smad2

COS-1 cells were transiently transfected with the indicated combinations of Flag/Smad2, Smad7/HA, and wild-type (WT) or activated (A) TβRI. Cells were labeled with [^{32}P]phosphate, and Flag/Smad2 was purified by immunoprecipitation with anti-Flag M2 antibody and analyzed by SDS–PAGE and autoradiography ($^{32}PO_4$). The expression of Flag/Smad2, Smad7/HA, and TβRI/HA proteins was determined by immunoblotting total cell lysates using anti-Flag M2 antibody (α-Flag) or anti-HA antibody (α-HA) as indicated. The migration of each protein is indicated on the left.

or Smad4 in either yeast or mammalian cells (data not shown). Since phosphorylation of Smad2 by the TGFβ type I receptor is required for its activation and downstream functions, we investigated whether Smad7 interferes with phosphorylation of the protein. To test this, Smad2 was expressed in COS cells along with wild type or constitutively activated TβRI, and phosphorylation of the protein was analyzed by metabolic labeling with [^{32}P]phosphate. As described previously, coexpression of Smad2 with activated TβRI resulted in a strong increase in phosphate incorporation into the protein that is due to receptor-mediated phosphorylation at the C-terminal serine residues (Figure 4; Macías-Silva et al., 1996). In contrast, in the presence of Smad7, we observed little change in Smad2 phosphorylation (Figure 4). We also investigated the phosphorylation of Smad7 itself. Although Smad7 was expressed abundantly in these cells, we detected only very low levels of basal phosphorylation that were unaffected by TGFβ signaling (data not shown). These results indicate that Smad7 prevents TβRI-dependent phosphorylation of Smad2 in intact cells but is not itself a target for TGFβ-regulated phosphorylation.

Smad7 Interacts Stably with the TGFβ Receptor

Phosphorylation of Smad2 in response to TGFβ signaling is mediated by the activated TGFβ type I receptor. To test whether the inhibition of Smad2 phosphorylation occurs via interference with receptor function, we determined whether Smad7 directly interacts with the TGFβ type I receptor. For this, we utilized a similar approach to our previous studies on Smad2 (Macías-Silva et al., 1996). Briefly, COS cells were transfected with wild-type or kinase-deficient versions of TβRII or TβRI, together with Smad7 tagged at the carboxyl terminus with the HA epitope. Receptors were affinity-labeled using [^{125}I]TGFβ, and receptors coprecipitating with Smad7/

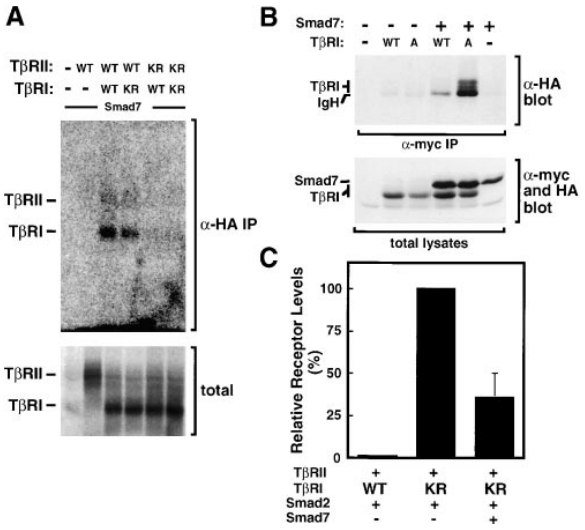

Figure 5. Smad7 Interacts Stably with Activated TβRI and Blocks Smad2 Association with the Receptor

(A) Interaction of Smad7 with TGFβ receptors. COS-1 cells were transiently transfected with Smad7/HA alone or with the indicated combinations of wild-type (WT) or kinase-deficient (KR) TβRI and TβRII. The cells were affinity labeled with 200 pM [^{125}I]TGFβ, and lysates were subjected to immunoprecipitation with anti-HA antibody (α-HA IP). Affinity-labeled receptor complexes coprecipitating with Smad7/HA were visualized by SDS–PAGE and autoradiography. Similar levels of receptor expression were confirmed by analyzing aliquots of total cell lysates by SDS–PAGE and autoradiography (bottom panel, total).

(B) Smad7 interacts preferentially with constitutively active TβRI. COS-1 cells were transfected with combinations of myc-tagged Smad7 and either wild-type (WT) or activated (A) HA-tagged TβRI as indicated. Cell lysates were subjected to anti-myc immunoprecipitation using a monoclonal antibody followed by immunoblottng using anti-HA Y-11 polyclonal antibody (upper panel, α-HA blot). Equivalent expression of Myc/Smad7 and TβRI/HA proteins was confirmed by immunoblotting total cell lysates using anti-myc and anti-HA monoclonal antibodies (bottom panel, α-myc and HA blot).

(C) Smad7 blocks association of Smad2 with the TGFβ receptor complex. COS-1 cells were transfected with Flag/Smad2 and the indicated combinations of TβRII, Smad7, and wild-type (WT) or kinase-deficient (KR) TβRI. Cells were affinity labeled with [^{125}I]TGFβ as described in (A) and lysates were subjected to anti-Flag immunoprecipitation. Receptor complexes coprecipitating with Smad2 were quantitated using a gamma counter. Data are expressed as a percentage of the counts in anti-Flag immunoprecipitates obtained from affinity-labeled cells transfected with TβRII, kinase-deficient TβRI, and Flag/Smad2 and represent the mean (±SD) of three independent experiments.

HA were analyzed in anti-HA immunoprecipitates. In cells cotransfected with wild-type TβRII and TβRI, receptor complexes could clearly be detected coprecipitating with Smad7, and this interaction was comparable to levels obtained from cells expressing kinase-deficient TβRI (Figure 5A). Furthermore, we detected minimal interaction between Smad7 and receptor complexes assembled in the presence of kinase-deficient TβRII(KR). Since this mutation prevents transphosphorylation and activation of TβRI, these results indicate that Smad7 preferentially binds to activated receptor I. During the course of these experiments, we consistently observed a low level of interaction between receptors and Smad7 in cells transfected with TβRII. This may reflect binding to the endogenous receptors that are expressed in these cells (Figure 5A; lower panel).

To confirm that Smad7 associates directly with the type I receptor, we investigated interactions between Smad7 and either wild-type or constitutively active TβRI. COS-1 cells were transfected with myc-tagged Smad7 together with wild-type or activated TβRI/HA. Cell lysates were then subjected to immunoprecipitation with an anti-myc monoclonal antibody and the immunoprecipitates analyzed by immunoblotting using a rabbit anti-HA polyclonal antibody. In cells cotransfected with wild-type TβRI, a low level of interaction between Smad7 and the receptor could be detected (Figure 5B). However, in cells expressing activated TβRI, we observed a strong increase in the amount of type I receptor that coprecipitated with Smad7. Together, these data demonstrate that Smad7 associates stably with the TGFβ receptor complex via the activated type I kinase domain.

Since Smad7 binds to the receptor and blocks phosphorylation of Smad2, we examined whether Smad7 alters association of Smad2 with the TGFβ receptor. As described previously, stable interaction between wild-type Smad2 and the receptor requires a kinase-deficient TβRI to trap the Smad2 substrate (Macías-Silva et al., 1996). Thus, we expressed Smad2 together with TβRII and TβRI(KR), either in the presence or absence of Smad7. Analysis of controls showed that affinity-labeled receptor complexes coprecipitated with Smad2. However, in the presence of Smad7 expression, there was a reduction in the amount of receptors that bound to Smad2. This decrease was specific for Smad7, since Smad4, which does not interact with the TGFβ receptor, had no effect in this assay (data not shown). These results suggest that Smad7 inhibits TGFβ signaling by preventing Smad2 access to the receptor.

A Nonfunctional Mutant of Smad7 Does Not Interact with the TGFβ Receptor

To gain further evidence that Smad7 inhibits TGFβ signaling through its associations with the TGFβ receptor, we investigated the function of a mutant version of Smad7. Previous studies have demonstrated that a truncation mutant of Smad4, identified in a pancreatic cancer, inactivates its function in TGFβ signaling (Hahn et al., 1996; Lagna et al., 1996; Zhang et al., 1996). We constructed an analogous mutant of Smad7 by introducing a stop codon after amino acid residue 408 (Figure 6A). To investigate the activity of this mutant, we transfected HepG2 cells with p3TP-Lux and increasing amounts of wild-type Smad7 or Smad7(Δ408). Similar to our previous observations, Smad7 inhibited TGFβ-dependent induction of the 3TP promoter in a dose-dependent fashion (Figure 6B). However, Smad7(Δ408), which was expressed efficiently (data not shown and Figure 6C), had little or no ability to block TGFβ signaling even at the highest doses of transfected DNA.

To investigate how this truncation disrupts Smad7 activity, we examined whether Smad7(Δ408) interacts with TGFβ receptors. For these studies, wild-type or mutant Smad7 was expressed in COS cells together with TβRII and TβRI. Receptors were affinity-labeled using [^{125}I]TGFβ and complexes coprecipitating with

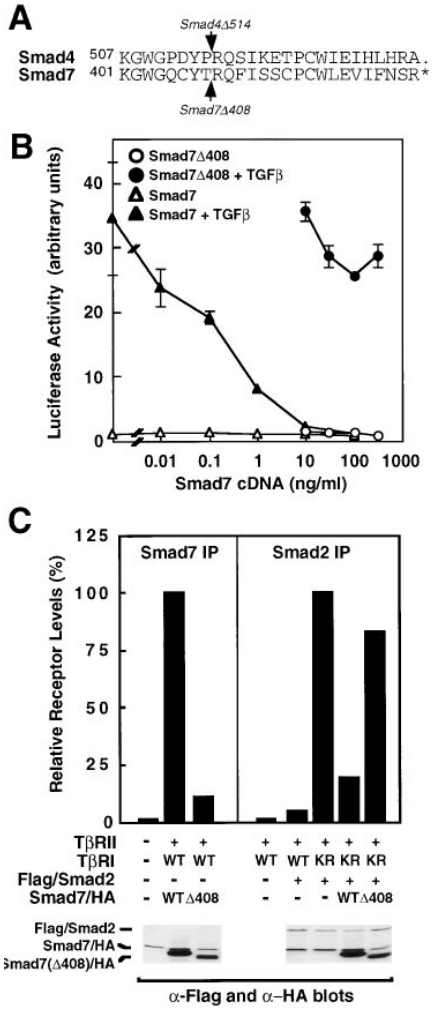

Figure 6. Truncation of Smad7 Blocks Its Inhibitory Activity and Disrupts Interaction with the TGFβ Receptor

(A) The C-terminal amino acid sequence of Smad4 and Smad7 is shown. The location of the premature stop codon of a mutant of Smad4 (D514; Hahn et al., 1996) and the corresponding truncation in Smad7 (Δ408) are indicated.

(B) TGFβ-induced transcriptional activation of the 3TP promoter is inhibited by wild-type but not truncated Smad7. HepG2 cells were transiently transfected with p3TP-Lux, and increasing amounts of either wild-type Smad7 (triangles) or the truncated version (Smad7Δ408; circles), and cells were incubated overnight in the presence (closed symbols) or absence (open symbols) of 100 pM of TGFβ. The relative luciferase activity was measured in cell lysates and is expressed as the mean ± SD of triplicates from a representative experiment.

(C) Smad7(Δ408) does not associate with the TGFβ receptor and does not block interaction of Smad2 with the receptor complex. COS-1 cells were transfected with indicated combinations of Flag/Smad2, TβRII, wild-type (WT), or kinase-deficient (KR) TβRI and wild-type (WT) or truncated (Δ408) Smad7/HA. Cells were affinity labeled with [^{125}I]TGFβ as described in Figure 5A, and lysates were subjected to anti-HA or anti-Flag immunoprecipitation. Receptor complexes coprecipitating with Smad7 (left panel) or Smad2 (right panel) were quantitated using a gamma counter. Data are expressed as amount relative to immunoprecipitates obtained from cells transfected with wild-type TGFβ receptors for Smad7 (left panel) or receptor complexes containing TβRI (KR) for Smad2 (right panel). Expression of Flag/Smad2, Smad7/HA and Smad7(Δ408)/HA was assessed by immunoblotting total cell lysates from the transfected cells with anti-Flag and anti-HA antibodies (bottom panel).

Smad7/HA were analyzed quantitatively in a gamma counter. In cells expressing wild-type Smad7, receptor complexes labeled with [^{125}I]TGFβ were readily detected coprecipitating with Smad7 protein (Figure 6C). However, in cells expressing Smad7(Δ408), there was a 10-fold reduction, to near background levels, in the amount of receptors that coprecipitated with the protein. We also determined directly whether Smad7(Δ408) could interfere with Smad2 association with the TGFβ receptor. In contrast to wild-type Smad7, the mutant, Smad7(Δ408), was unable to block association of Smad2 with the TGFβ receptor (Figure 6C). Together, these data indicate that the stable interaction of Smad7 with the receptor is critical for its inhibitory activity in TGFβ signaling and strongly suggest that Smad7 is an intracellular antagonist of the TGFβ type I receptor kinase domain.

Discussion

TGFβ superfamily signaling is initiated when ligand induces formation of a heteromeric complex of type II and type I Ser/Thr kinase receptors (Attisano and Wrana, 1996; Weis-Garcia and Massague, 1996). Within this complex, the type II receptor transphosphorylates receptor I in the GS domain, thus activating the type I kinase domain that targets downstream substrates. Numerous biochemical and genetic studies on the TGFβ superfamily have revealed that the MAD-related family of signaling molecules are critical downstream mediators of these receptor complexes (Hoodless and Wrana, 1997). The direct phosphorylation of these proteins by Ser/Thr kinase type I receptors followed by heteromeric complex formation and nuclear accumulation has emerged as a central theme in this signaling pathway. Thus, Smad1 and 5 are specifically regulated by BMP signaling, while Smad2 and 3 function in TGFβ/activin pathways. Smad4 forms heteromeric complexes with these receptor-regulated Smads and thus may fulfill a common function in both signaling pathways. In this report, we investigate the role of Smad7 in TGFβ signaling. We demonstrate that Smad7 is an inhibitor of TGFβ signaling, preventing heteromeric complex formation between Smad2 and Smad4 and the nuclear accumulation of Smad2 in response to TGFβ signaling. Smad7 functions by associating stably with the activated TGFβ type I receptor to block the interaction and phosphorylation of Smad2. Furthermore, a truncation mutant of Smad7 that fails to associate with the receptor does not inhibit TGFβ signaling. These studies thus define a class of Smads that inhibit TGFβ signaling by acting as intracellular antagonists of the type I kinase domain of Ser/Thr kinase receptors.

Smad7 Is an Antagonist of the TβRI Kinase Domain

In TGFβ signaling, the interaction of Smad2 with the TGFβ receptor results in its phosphorylation by the type I receptor, dissociation from the receptor complex, and nuclear accumulation (Macías-Silva et al., 1996). In contrast to these transient interactions, we have shown that Smad7 associates stably with the activated TGFβ type

I receptor. Previous studies on Smad2 showed that dissociation from the receptor required phosphorylation of the protein on the C-terminal serine residues (Macías-Silva et al., 1996). Analysis of the sequence of Smad7 reveals that while the protein possesses both an MH1 and MH2 domain, it does not contain the C-terminal SSXS motif, which is the target for receptor-mediated phosphorylation of other Smads (Macías-Silva et al., 1996; Kretzchmar et al., 1997). Consistent with this, we were unable to detect any phosphorylation of Smad7 in response to TGFβ signaling, suggesting that this receptor-associated Smad is not a substrate of the receptor. In this regard, Smad7 functions similarly to the phosphorylation site mutant of Smad2 characterized previously. This mutant, Smad2(3SA), which is not phosphorylated, interacts stably with the receptor and blocks TGFβ signaling to endogenous MADR2 protein (Macías-Silva et al., 1996). Thus, Smad7 represents a naturally occurring receptor kinase antagonist that functions to inhibit TGFβ signaling by preventing access of Smad2 to the receptor kinase domain.

Determinants within the MH2 Domain Are Required for Interaction of Smad7 with the TGFβ Receptor

Receptor-regulated Smad proteins interact transiently with the type I receptors. This may reflect an association that is restricted to the phosphotransferase reaction between the receptor kinase and its C-terminal peptide substrate (Kretzchmar et al., 1997). However our observation that Smad7, which lacks the C-terminal region, associates stably with the receptor suggests an alternative model in which the MH2 domain is the primary determinant that mediates interaction of Smads with the receptor. In agreement with this, truncation of the MH2 domain of Smad7 disrupts binding to the receptor.

The potential role of the MH2 domain in mediating receptor interactions suggests that determinants within this domain may also be critical for establishing specificity. Biochemical and functional studies indicate that Smad1 and 5 function in BMP signaling and that Smad2 and 3 signal TGFβ/activin responses. Thus, the molecular basis for this high degree of specificity may reside in the MH2 domain as opposed to the C-terminal tail. Of note, numerous mutations in the MH2 domain of receptor-regulated Smads have been identified in human cancers and in the *sma* and *Mad* genes from C. elegans and Drosophila, respectively (Eppert et al., 1996; Sekelsky et al., 1995; Savage et al., 1996). Many of these mutations have been shown to block receptor-mediated phosphorylation of the protein (Eppert et al., 1996; Hoodless et al., 1996), and it will be interesting to determine whether these MH2 domain mutants function by disrupting interaction of these proteins with the type I receptor kinase.

Smad7 Functions at an Early Step to Inhibit TGFβ Signaling

Our studies have defined Smad7 as an inhibitor that functions at a very early step in the TGFβ signaling pathway. As a direct antagonist of the receptor kinase, Smad7 prevents the phosphorylation of Smad2, its association with Smad4, and the nuclear accumulation of the protein. Thus, Smad7 is likely to be a general inhibitor of phosphorylated Smad2 function, as opposed to modulating subsets of Smad2-mediated responses. These conclusions are consistent with the general block that we observed in all of the transcriptional assays we employed to study activation of Smad2 in response to TGFβ signaling. Interestingly, Smad3, which also functions in TGFβ signaling, is over 90% identical to Smad2 in the MH2 domain and interacts with the TGFβ receptor. Although this has not yet been directly investigated, it is likely that Smad7 may function similarly to prevent association and activation of Smad3 by the TGFβ receptor.

Our studies indicate one potential function for Smad7 as an inhibitor of TGFβ signaling. However, they do not rule out the possibility that this protein may have other functions independent of its inhibitory role. The lack of an SSXS motif suggests this protein is unlikely to function as a direct substrate of Ser/Thr kinase receptors. Smad7 was initially cloned based on its induction in response to fluid shear stress in endothelial cells (Topper et al., 1996, 1997). It will be interesting to determine whether, in addition to suppressing TGFβ signaling, it may fulfill other functions in the normal physiology of the vasculature.

Negative Regulation of TGFβ Signaling

Members of the TGFβ superfamily are potent regulators of the growth and differentiation of a wide range of cell types during development. Thus, the spatial and temporal control of their activity is critical to normal development and homeostasis. A wide range of mechanisms exist to control the activity of these proteins, including a range of extracellular antagonists that bind the ligands and prevent activation of the signaling receptors. Our studies now define a novel intracellular mechanism that can function to suppress TGFβ signaling. Cell type–specific regulation of intracellular receptor antagonists could allow autonomous control of the response of cells to extracellular ligands in complex tissues. This could provide for a precision in the control of TGFβ signaling not afforded by secreted molecules. In particular, TGFβ could differentially regulate cellular responses in human vasculature, where Smad7 is expressed selectively in the endothelial layer (Topper et al., 1997).

In numerous tumors, resistance to TGFβ is associated with inactivating mutations that can occur either in the receptors or in components of the signaling pathways, such as Smad2 or Smad4 (Eppert et al., 1996; Hahn et al., 1996; Markowitz and Roberts, 1996). Here we have identified a Smad protein that functions as an antagonist of TGFβ signaling. This suggests that amplified expression of these proteins, as opposed to inactivation, might act to suppress TGFβ signals in tumors. Thus, it will clearly be important in the future to investigate whether elevation of Smad7 expression may contribute to the progression of human cancer.

Experimental Procedures

Construction of Expression Vectors

Human Smad7 was cloned from human umbilical vein endothelial cells (HUVECs) isolated from multiple segments of normal-term

umbilical cords by differential display analysis as described previously (Topper et al., 1996) and is represented in the expressed sequence tag database (clone numbers: EST54659, EST53587, and EST24567). Myc/Smad7 was generated by introducing a Myc epitope tag at the amino terminus of Smad7 by standard PCR-based protocols and was subcloned into pN8e-Myc/Smad7. Myc/Smad7 was then subcloned into pCMV5 using HindIII/XhoI sites to create pCMV5-Myc/Smad7. pCMV5-Smad7 was generated by subcloning the open reading frame of Smad7 into pCMV5 and was epitope tagged at the carboxyl terminus with the HA epitope, using standard approaches. The truncation mutant, pCMV5-Smad7(Δ408)/HA, was constructed from pCMV5-Smad7 by introducing an HA epitope tag and stop codon after amino acid residue 408. The construction of pCMV5B-Flag/Smad2 has been described previously (Eppert et al., 1996). Mouse Smad4, cloned as described previously (Macías-Silva et al., 1996), was tagged with the HA epitope at the carboxyl terminus by PCR. The pAR3-lux construct, containing the luciferase reporter gene, was generated by subcloning a HindIII/XhoI insert of pSP72-A3-CAT, (a kind gift from M. Whitman; Chen et al., 1996) into EcoRI/XhoI of pGL2-basic. The pG5-E1b-lux construct, containing the luciferase reporter gene downstream of the DNA-binding elements of GAL4 and the E1b TATA box, was generated by subcloning an XhoI/EcoRI insert of pG5-E1b-CAT (a kind gift from A. Bonni) into XhoI/HindIII of pGL2-Basic. pCMV5B-GAL4/Smad2, consisting of Smad2 fused to the DNA-binding domain of GAL4 (amino acids 1–147), was constructed by subcloning the GAL4/Smad2 insert from pGBT9-Smad2 into pCMV5B using HindIII/XbaI sites. pCMV5B-GAL4 was generated from pCMV5B-GAL4/Smad2 vector by digestion with EcoRI/XbaI. All constructs were confirmed by sequencing.

Cell Lines and Transfections

Mv1Lu and HepG2 cells were maintained in minimal essential media containing 10% fetal calf serum and nonessential amino acids, and COS-1 cells were grown in high glucose-Dulbecco's modified Eagle's media supplemented with 10% fetal calf serum. For transient transfections, HepG2 cells were seeded at 25% confluency in 24-well plates and transfected overnight with 0.5 μg of DNA per well using the calcium phosphate-DNA precipitation method as described previously (Hoodless et al., 1996). For transfections in COS-1 and Mv1Lu, cells were seeded at 50%–70% confluency and were transfected with DEAE-dextran as described previously (Attisano et al., 1996). Expression of Smad2, Smad4, Smad7, and TβRI proteins were determined by immunoblotting total cell lysates or immunoprecipitates using appropriate antibodies.

Phosphate Labeling, Immunoprecipitations, and Immunoblotting

For [^{32}P]phosphate labeling, transiently transfected COS-1 cells were washed and preincubated with phosphate-free media containing 0.2% dialyzed fetal calf serum. The cells were then incubated with media containing 1 mCi/ml [^{32}P]phosphate for 2 hr at 37°C, followed by lysis and immunoprecipitation as described previously (Wrana et al., 1994).

For anti-Flag or anti-Myc immunoprecipitations, cell lysates were subjected to immunoprecipitation with anti-Flag M2 antibody (IBI, Eastman Kodak) or anti-Myc 9E10 antibody, respectively, followed by adsorption to protein G–Sepharose (Pharmacia) as described previously (Hoodless et al., 1996). For anti-HA immunoprecipitations, cell lysates were subjected to immunoprecipitation with anti-HA polyclonal antibody (Y11, Santa Cruz Biotechnology) or monoclonal antibody (12CA5), followed by adsorption to protein A–Sepharose (Pharmacia). Immunoprecipitates were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by autoradiography. For determination of total protein levels, cell lysates were separated by SDS–PAGE and assayed by immunoblotting or alternatively were subjected immunoprecipitation and Western blotting, as indicated.

Affinity Labeling

COS-1 cells transfected with DEAE dextran or calcium phosphate were affinity labeled using 200 pM [^{125}I]TGFβ in media containing 0.2% fetal calf serum at 37°C for 30 min, and the receptors were cross-linked to the ligand as described previously (Macías-Silva et al., 1996). Cell lysates were then subjected to anti-HA or anti-Flag immunoprecipitation as described above. Receptor complexes in the immunoprecipitates and in total cell lysates were visualized by separation on SDS–PAGE gels and autoradiography.

Transcriptional Response Assay

For TGFβ-inducible luciferase reporter assays, HepG2 cells were transiently transfected with the reporter plasmid (p3TP-Lux), pCMVβ-gal and the indicated constructs or with empty vector alone. To induce the luciferase reporter, cells were treated overnight in 100 pM TGFβ. Luciferase activity in cell lysates was measured using the luciferase assay system (Promega) in a Berthold Lumat LB 9501 luminometer.

For TGFβ-inducible immediate early response gene transcription assays, HepG2 cells were transiently transfected with the reporter plasmid (pAR3-lux), pCMVβ-gal, and the indicated constructs or with empty vector alone. Cells were treated overnight with or without 100 pM TGFβ, and luciferase activity in cell lysates was measured.

For GAL4/Smad2 transcriptional assays, HepG2 cells were transiently transfected with the reporter plasmid (pG5-E1b-lux), pCMVβ-gal, and the GAL4/Smad2 constructs or with empty vector alone. Cells were treated overnight with or without 100 pM TGFβ, and luciferase activity in cell lysates was measured. To determine transfection efficiency in each assay, β-galactosidase activity assay was performed as described (Sambrook et al., 1989), and luciferase assay results were normalized for β-galactosidase activity.

Immunofluorescence

HepG2 cells plated in Permanox chamber slides (Nunc) were transiently transfected with various combinations of Smad7/HA, Flag/Smad2 and wild-type or activated TβRI. Cells were fixed in 4% paraformaldehyde and permeabilized as described previously (Hoodless et al., 1996). Cells were blocked in 10% goat serum and incubated overnight at 4°C with anti-HA polyclonal antibody, Y11 (1 μg/ml; Santa Cruz Biotechnology) and anti-Flag monoclonal antibody, M2 (10 μg/ml). Cells were washed in PBS and incubated with Texas Red (TR)–conjugated goat anti-rabbit IgG and fluorescein isothiocynate (FITC)–conjugated goat anti-mouse IgG (1:200 dilution; Jackson Immunologicals) as secondary antibodies (Hoodless et al., 1996). Images were obtained using a Leica confocal microscope.

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