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A Smad Transcriptional Corepressor

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Summary

Following TGFβ receptor–mediated phosphorylation and association with Smad4, Smad2 moves into the nucleus, binds to target promoters in association with DNA-binding cofactors, and recruits coactivators such as p300/CBP to activate transcription. We identified the homeodomain protein TGIF as a Smad2-binding protein and a repressor of transcription. A TGFβ-activated Smad complex can recruit TGIF and histone deacetylases (HDACs) to a Smad target promoter, repressing transcription. Thus, upon entering the nucleus, a Smad2–Smad4 complex may interact with coactivators, forming a transcriptional activation complex, or with TGIF and HDACs, forming a transcriptional repressor complex. Formation of one of these two mutually exclusive complexes is determined by the relative levels of Smad corepressors and coactivators within the cell.

Introduction

Smad proteins are central mediators of the transcriptional effects of the transforming growth factor β (TGFβ) family (Heldin et al., 1997; Massagué, 1998). Smads are directly phosphorylated and activated by type I TGFβ family receptors. TGFβ and activin receptors phosphorylate Smad2 and Smad3 (Baker and Harland, 1996; Macias-Silva et al., 1996; Zhang et al., 1996a; Nakao et al., 1997a), whereas bone morphogenetic protein (BMP) receptors phosphorylate Smads 1, 5, and 8 (Graff et al., 1994; Hoodless et al., 1996; Liu et al., 1996; Kretzschmar et al., 1997a; Macias-Silva et al., 1998). Receptor-activated Smads oligomerize with a member of a separate subfamily, Smad4 (Lagna et al., 1996), and these complexes enter the nucleus, where they activate transcription (Baker and Harland, 1996; Hoodless et al., 1996; Liu et al., 1996). The gene responses activated by Smads underlie many developmental and proliferative events, including dorsalization and organogenesis in fly embryos (Sekelsky et al., 1995; Wisotzkey et al., 1998), gastrulation, mesoderm formation, and polarity in vertebrates (Baker and Harland, 1996; Graff et al., 1996; Nomura and Li, 1998; Sirard et al., 1998; Waldrip et al., 1998), and antiproliferative responses in mammalian cells (Lagna et al., 1996; Zhang et al., 1996a), which, when lost due to mutations in *Smad2* or *Smad4*, contribute to cancer (Eppert et al., 1996; Hahn et al., 1996; Takaku et al., 1998).

Activation of specific genes by Smads is brought about by interaction with specific DNA-binding proteins. The *Xenopus* protein Fast1 is the prototypic Smad-recruiting DNA-binding factor (Chen et al., 1996). Fast1, which contains a "winged helix" DNA-binding domain, binds to the activin response element (ARE) and is absolutely required for activation of the *Mix.2* gene in response to activin or TGFβ. Fast1 bound to DNA alone does not activate transcription. However, recruitment of an activated Smad2–Smad4 complex to the ARE by Fast1 results in activation of *Mix.2* expression (Chen et al., 1997; Liu et al., 1997). In the transcriptional complex, the Smads contact DNA via their conserved N-terminal domain (known as the MH1 domain) (Kim et al., 1997; Shi et al., 1998; Zawel et al., 1998). The C-terminal domain (or MH2 domain) of Smads, which in the cytoplasm mediates Smad–receptor interaction (Lo et al., 1998) and Smad oligomerization (Hata et al., 1997; Shi et al., 1997), in the nucleus mediates association with DNA-binding cofactors (e.g., Fast1 in the case of Smads 2 and 3) (Chen et al., 1997) and transcriptional activation (Liu et al., 1996, 1997). Recently, association of various Smads with the coactivators p300 or CBP via the MH2 domain has been demonstrated (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998).

p300 and CBP have histone acetyl transferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996). The acetylation state of core histones plays a critical role in transcription (Struhl, 1998). Acetylation of histone amino-terminal tails alters nucleosomal structure, increasing accessibility to the transcriptional machinery (Lee et al., 1993; Vettese-Dadey et al., 1996). The recruitment of HAT activity by promoter-bound factors may be a general feature of transcriptional activation. In contrast, histone deacetylases (HDACs) (Taunton et al., 1996; Yang et al., 1997) play a role in the repression of transcription, causing tighter nucleosomal packing resulting in decreased transcription factor accessibility (Wolffe, 1996). The interaction of HDACs with corepressors results in HDAC recruitment to target genes, facilitating specific transcriptional repression (Alland et al., 1997; Hassig et al., 1997; Heinzel et al., 1997; Kadosh and Struhl, 1997; Laherty et al., 1997; Nagy et al., 1997; Zhang et al., 1997). Repressors can maintain genes in a transcriptionally inactive state, or they can play a role in the specific downregulation of a gene response (Gray and Levine, 1996).

Here, we demonstrate that Smad2 interacts with TGIF, a ubiquitously expressed homeodomain protein (Bertolino et al., 1995). The homeodomain, an approximately 60–amino acid region, is present in a diverse group of transcriptional regulators with roles in transcriptional activation and repression (Gehring et al., 1994). In addition to having DNA binding ability, homeodomains can act in transcriptional regulation via interactions with general transcription factors (Zhang et al., 1996b; Li and Manley, 1998). The function of TGIF has until now been

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unclear. Here, we show that the interaction of TGIF with Smad2 results in the recruitment of TGIF to TGFβ-responsive genes and the repression of TGFβ-induced transcription. We show that this repression is mediated by the interaction of TGIF with HDACs. These results provide evidence for the existence of a Smad corepressor.

Results

Smads Interact with TGIF

To identify proteins that participate in TGFβ signaling, we screened a two-hybrid library with a LexA fusion encoding the conserved carboxy-terminal MH2 domain, the linker region, and part of the amino-terminal MH1 domain of Smad2. This fusion, LexA/Smad2(100–467), interacts with Smad4 in yeast and has a relatively low background activity in the yeast two-hybrid assay. Of 40 positive clones isolated by screening a HeLa cDNA library (Zervos et al., 1993), eight encoded fusions to Smad4, which verifies the effectiveness of the screening, and two encoded fusions to the homeodomain protein TGIF (Figure 1A). One clone encoded the carboxy-terminal half of TGIF (amino acids 138–272) fused in-frame to the B42 activation domain; the second contained the entire coding sequence of TGIF. Further two-hybrid analysis revealed that TGIF was able to interact with LexA fusions to Smad2 and Smad3, but not Smad4 or LexA alone (data not shown).

To confirm the interaction of Smad2 with TGIF, we tagged TGIF at its amino terminus with a single Flag epitope and expressed this tagged protein together with various Smad proteins in COS-1 cells. Smad2 was detectable in Flag immunoprecipitates from cells transfected with Flag–TGIF and Smad2, and this interaction was increased by the addition of TGFβ for 6 hr prior to cell lysis (Figure 1B). A similar TGFβ-inducible interaction of TGIF with Smad3 was observed (data not shown). The Smad2–TGIF interaction was maximal after 1–2 hr of TGFβ treatment (data not shown). A TGIF–Smad1 interaction was detected following cell stimulation with BMP, but this interaction was weaker than the TGIF–Smad2 interaction. Thus, TGIF–Smad interactions are specific and dependent on Smad activation. These interactions most likely take place upon Smad translocation into the nucleus, as immunofluorescence assays revealed that Flag–TGIF is exclusively localized in the nucleus (Figure 1D).

To identify the Smad-interacting domain of TGIF, a series of Flag-tagged TGIF carboxy-terminal deletion constructs was created and tested for interaction with Smad2 in COS-1 cells. Deletion of the carboxy-terminal 80 amino acids did not affect Smad2 interaction (Figure 1C). Together with the fact that we isolated a TGIF two-hybrid fusion encoding amino acids 138–272, this result places the Smad-interacting domain (SID) between amino acids 138 and 192. To confirm this, we created a small internal deletion, removing amino acids 149–176. No interaction of Smad2 with this construct was observed, suggesting that this deletion overlaps the SID (Figure 1C).

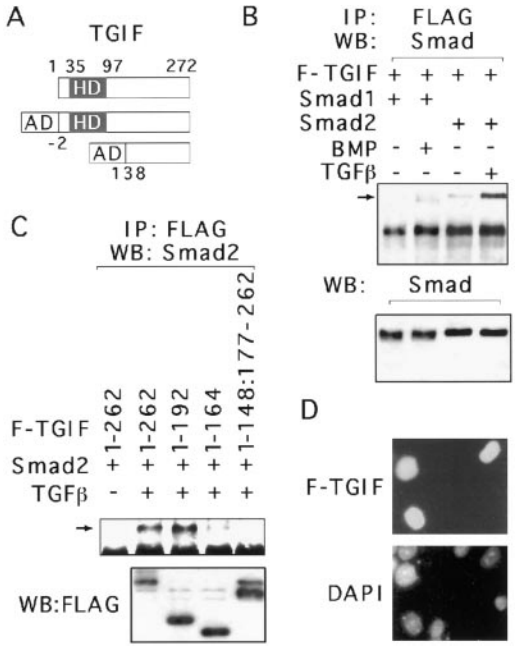

Figure 1. TGIF Interacts with Smad2

(A) TGIF and two activation domain (AD) fusions to TGIF isolated by yeast two-hybrid screening are shown schematically. One fusion contained the carboxy-terminal 135 amino acids of TGIF; the other was fused in-frame seven bases upstream of the TGIF initiation codon. The TGIF homeodomain (HD) is shown in black; amino acid numbers are indicated.

(B) COS-1 cells were transfected with Flag–TGIF and the indicated full-length Smad expression constructs. TGFβ (200 pM) and BMP-4 (5 nM) were added for 6 hr prior to lysis as indicated. Interactions were analyzed by immunoblotting of Flag immunoprecipitates using a mix of Smad1- and Smad2-specific antisera. Expression of transfected DNA was monitored by direct immunoblotting using Smad1- or Smad2-specific antisera. Bands corresponding to coprecipitating proteins are indicated by arrows. The nonspecific band in the upper panels corresponds to Ig heavy chain.

(C) Flag-tagged TGIF deletion constructs encoding the indicated amino acids were transfected into COS-1 cells. Flag immunoprecipitates were subjected to immunoblotting using a Smad2-specific antiserum. Expression of Flag–TGIF constructs was assessed by direct Western blot.

(D) TGIF is nuclear. COS-1 cells transfected with Flag–TGIF were analyzed by indirect immunofluorescence using a Flag monoclonal antibody. DAPI staining of DNA is shown for comparison.

TGIF Decreases TGFβ- and Smad-Dependent Transcription

To begin to investigate the role of TGIF in TGFβ signaling, we examined the effects of ectopically expressed TGIF on TGFβ- and Smad-dependent transcriptional responses. L17 mink lung epithelial cells were transiently transfected with TGIF and the 3TP-lux reporter (Cárcamo et al., 1995), which contains TGFβ response elements from *plasminogen activator inhibitor-1* (*PAI-1*) and *collagenase* and has been widely used to monitor TGFβ and Smad signaling. Coexpression of increasing amounts of TGIF resulted in a dramatic inhibition (up to 20-fold) of TGFβ-induced 3TP-lux activity, while having little effect on the basal activity in the absence of TGFβ (Figure 2A). Significant repression was observed with even a relatively low level of TGIF expression vector.

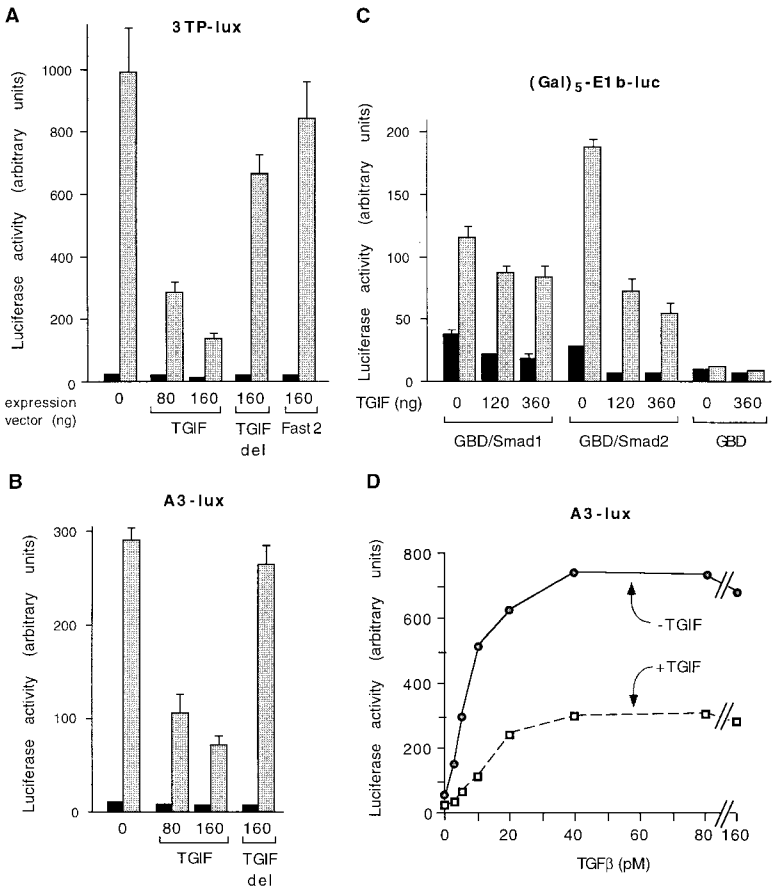

Figure 2. Repression of TGFβ Stimulated Transcription by TGIF

(A) L17 cells were transfected with the 3TP-lux reporter (160 ng/well) together with the indicated amount of plasmids encoding either TGIF, the deletion construct TGIF(1–148:177–262) (del), or Fast2. Cells were treated with (gray bars) or without (black bars) TGFβ for 24 hr prior to lysis and then analyzed for luciferase activity.

(B) L17 cells were transfected with the A3-lux reporter and Fast2, and then assayed as in (A).

(C) L17 cells were transfected with a Gal-luc reporter containing five copies of a Gal4p-binding site upstream of a minimal TATA element. Transcription was activated by Gal4 DNA–binding domain (GBD) fusions to Smad1 or Smad2 or by GBD alone in the presence of cotransfected constitutively active TGFβ type I receptor (GBD/Smad2 and GBD) or BMP type I receptor (GBD/Smad1). Luciferase activity with increasing amounts of cotransfected TGIF expression construct is shown.

(D) L17 cells were transfected with A3-lux, Fast2, and either TGIF or a control vector. Cells were treated without or with TGFβ at the concentrations indicated. Results are shown as mean and SD of triplicate transfections (A–C); in (D) the SD, omitted for ease of presentation, was less than 12% for each point.

Coexpression of TGIF similarly repressed TGFβ-induced 3TP-lux activity in the human keratinocyte line HaCaT (data not shown). The TGIF(1–148:177–262) deletion mutant, which does not interact with Smad2, had little effect on the 3TP-lux response (Figure 2A). An unrelated Smad2-interacting protein, Fast2 (see below), also had little effect (Figure 2A). Thus, the transcriptional inhibitory effect was specific to TGIF and dependent on its ability to interact with Smad2.

Xenopus Fast1 in association with Smads 2 and 4 is required for activation of the *Mix.2* gene via a TGFβ/activin response element (ARE) (Chen et al., 1996, 1997; Liu et al., 1997). Binding to the ARE is jointly mediated by the DNA binding activities of Fast1 and Smad4 (Chen et al., 1996, 1997; Liu et al., 1997), whereas transcriptional activation is mediated by p300/CBP bound to Smads in the complex (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998). Fast1 and a recently identified mouse homolog, Fast2, can mediate activation of an ARE reporter construct (A3-lux) in transient transfection assays (Labbé et al., 1998; Liu et al., 1999). Since the molecular mechanism of the TGFβ response of the A3-lux reporter is more clearly understood than that of 3TP-lux, we examined the effect of TGIF on A3-lux activity. Coexpression of TGIF with Fast2 and the A3-lux reporter resulted in a marked reduction in TGFβ-stimulated luciferase activity (Figure 2B). Inhibition by TGIF of the *Mix.2* natural promoter was also observed (data not shown). No repression of A3-lux activity by TGIF(1–148:177–262), which is unable to interact with Smad2, was observed (Figure 2B).

Fusion of Smad1 or Smad2 to the Gal4p DNA–binding domain (GBD) creates agonist- (BMP or TGFβ) inducible transcriptional activators, which can be targeted to a reporter gene by multiple Gal4-binding sites. To determine whether the effect of TGIF on Smad-dependent transcription was due to interference with the DNA binding function of Smad or Fast proteins, we determined the effect of TGIF on GBD/Smad fusions. Coexpression of TGIF repressed TGFβ-activated transcription by GBD/Smad2 (Figure 2C), demonstrating that TGIF can repress transcriptional activation by Smad2 when bound to DNA via a heterologous DNA-binding domain. Repression of the transcriptional activity of a GBD/Smad3 fusion was also observed (data not shown). TGIF had only a limited inhibitory effect on GBD/Smad1-mediated transcription, which is consistent with the weak interaction between TGIF and Smad1. Thus, TGIF appears to act as a selective repressor of TGFβ-induced Smad-dependent transcription.

TGIF repressed A3-lux activity at all concentrations of TGFβ tested (Figure 2D). Little repression of basal A3-lux activity was observed; however, a similar degree of repression by TGIF was observed at all concentrations of TGFβ. A3-lux activity appeared to plateau after 40 pM TGFβ, in the presence or absence of TGIF, but

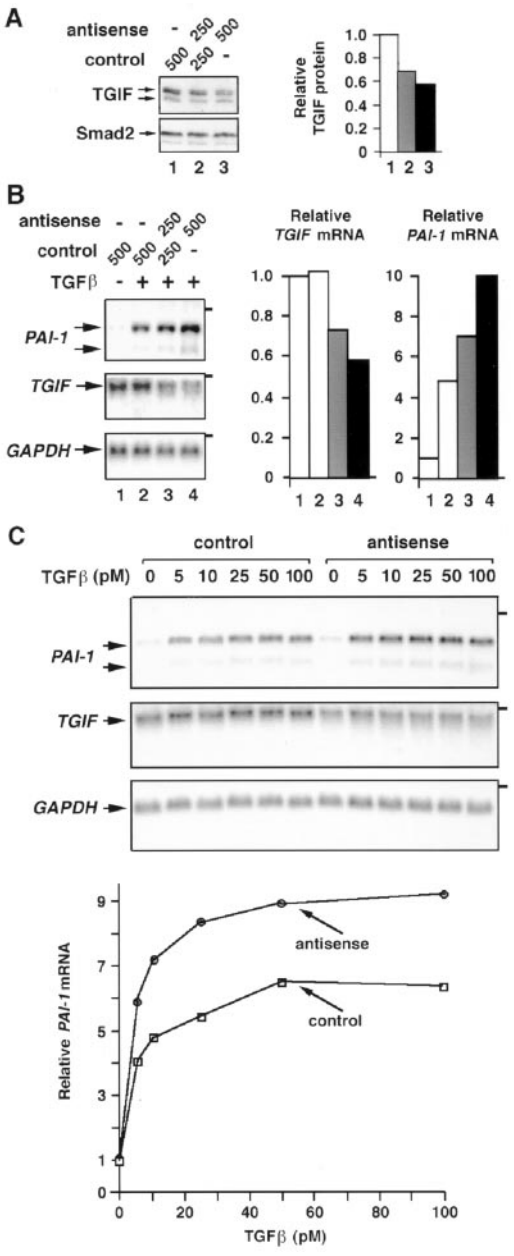

Figure 3. Reduction of Endogenous TGIF Activity

(A) A549 cells were treated with TGIF antisense or mismatched control oligonucleotides at the indicated nanomolar concentrations for 14 hr. Lysates from equal numbers of cells were fractionated by SDS-PAGE, and TGIF protein levels were visualized by Western blotting. Smad2 levels were assessed with a Smad2-specific antiserum as a control for loading. A densitometric quantitation of the relative expression level of TGIF is shown on the right.

(B) A549 cells were treated with the indicated concentrations (nM) of antisense or mismatched control oligonucleotides. TGFβ (40 pM) was added as indicated for 4 hr prior to RNA isolation. The levels of *TGIF* and *PAI-1* mRNAs and *GAPDH*, as a loading control, were assessed by Northern analysis. The relative levels of *TGIF* and *PAI-1* mRNAs (normalized to *GAPDH* levels) for each of the four treatments are shown. The level of *TGIF* and *PAI-1* mRNA in cells treated with control oligonucleotide alone has been set equal to 1.

(C) A549 cells were treated with 500 nM antisense or control oligonucleotide, as in (A), and TGFβ was added to the indicated concentration. An analysis of *PAI-1*, *TGIF*, and *GAPDH* mRNA levels is shown, with the relative levels of *PAI-1* (normalized to *GAPDH*) shown graphically below. The level of *PAI-1* in cells treated with control oligonucleotide in the absence of TGFβ has been set equal to 1. In this experiment, *TGIF* mRNA levels were decreased by 30%–40% by addition of antisense oligonucleotide. Bars on the right of each gel indicate the positions of the 18S (*TGIF* and *GAPDH*) and 28S (*PAI-1*) ribosomal RNAs.

TGIF significantly reduced the level of this plateau, suggesting that TGIF may act to set a cap on TGFβ-activated transcription.

Together, these results suggest that in addition to its ability to repress transcription when tethered to DNA (D. W. et al., unpublished data), TGIF can act as a repressor when recruited by Smad proteins.

Reduction of TGIF Levels Increases the TGFβ Response

To determine whether the endogenous TGIF protein acts as a repressor of TGFβ-activated transcription, we attempted to reduce the level of TGIF using antisense oligonucleotides directed against *TGIF* mRNA. Human A549 lung epithelial cells were chosen because they express a relatively high level of *TGIF* (see Figure 4A) and are TGFβ responsive. Twelve oligonucleotides were tested for their ability to affect the level of TGIF protein as assessed by Western blotting. As shown in Figure 3A, one antisense oligonucleotide (ASO3) resulted in a reduction of TGIF protein by up to 45% when compared to the addition of a mismatched control oligonucleotide. To determine whether this reduction in TGIF had any effect on TGFβ-induced transcriptional activation, we examined the *PAI-1* response to TGFβ. *PAI-1* expression has been shown to be induced severalfold by TGFβ in A549 cells (Keski-Oja et al., 1988 and Figure 3). Addition of ASO3 resulted in a clear increase in TGFβ-activated *PAI-1* expression (Figure 3B). In the presence of ASO3 and TGFβ, *PAI-1* levels were almost double those in cells treated with TGFβ and a control oligonucleotide. In contrast, hybridization of the same blot with a probe for *TGIF* revealed a decrease of up to 40% in the level of *TGIF* mRNA compared to cells treated with a control oligonucleotide. This decrease in *TGIF* mRNA was similar to the decrease observed in TGIF protein expression. No effect of ASO3 on expression of a housekeeping mRNA (*GAPDH*) was observed (Figures 3B and 3C). To further examine the effect of TGIF on *PAI-1* expression, cells were transfected with antisense or control oligonucleotides and treated with a range of concentrations of TGFβ (Figure 3C). In this experiment, the level of *TGIF* mRNA in cells treated with antisense oligonucleotide was reduced by 30%–40% compared with control cells. Little effect of ASO3 on *PAI-1* expression was observed in the absence of TGFβ. However, at all concentrations of added TGFβ, a significant increase in *PAI-1* mRNA was observed (Figure 3C). Thus, reducing the level of endogenously expressed TGIF results in a concomitant increase in the expression of a known transcriptional target of TGFβ. This effect on *PAI-1* mRNA occurs primarily in the presence of TGFβ signaling, even though TGIF levels are decreased irrespective of TGFβ addition, suggesting that the effect of TGIF on *PAI-1* expression is Smad dependent.

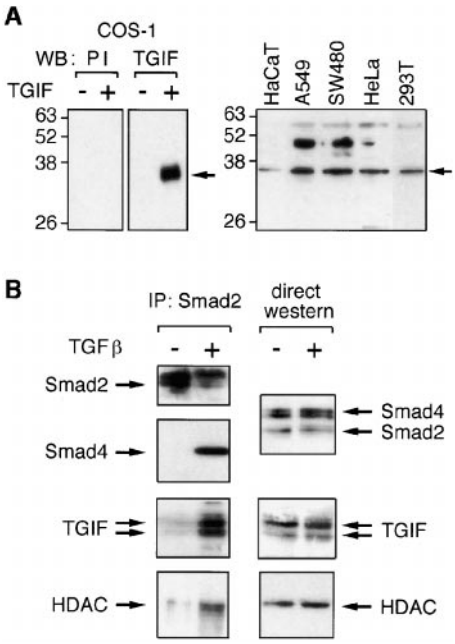

Figure 4. Analysis of Endogenous Smad2-Associated Proteins

(A) A TGIF-specific polyclonal antiserum was generated in rabbits. Western analysis of COS-1 cells transfected with TGIF or a control vector is shown (left). Membranes were probed with the TGIF-specific antiserum or with a similar dilution of the preimmune serum (PI). Lysates from several cell lines were subjected to Western analysis using the TGIF-specific antiserum. Bands corresponding to TGIF are indicated by arrows.

(B) Proteins were precipitated from A549 cells, and A549 cells were treated with TGFβ for 1 hr using a Smad2-specific antiserum. Precipitated proteins were analyzed by Western blotting with polyclonal antisera specific for Smad2, Smad4, TGIF, or HDAC1. For comparison, a portion of the cell lysates was probed with the same antisera (right).

It appears from these results that the effect of TGIF is to set a maximum level for TGFβ-induced expression of *PAI-1*. Reducing TGIF results in an increase in the maximal TGFβ response, whereas increasing TGIF levels (Figure 2D) results in a decrease in the maximal response to TGFβ. Thus, TGIF may act to set a ceiling on TGFβ-induced transcriptional responses.

Interaction of Endogenous TGIF and Smad2 Proteins

To determine whether the interaction of Smad2 and TGIF occurred with physiological levels of these proteins, we first raised antibodies to TGIF. Six-histidine-tagged TGIF protein (amino acids 35–272) was expressed in bacteria, purified on nickel resin, and used to raise a polyclonal TGIF antiserum. As shown in Figure 4A, this antiserum specifically recognized TGIF protein from several cell lines, as well as recombinant TGIF expressed in COS-1 cells. TGIF, whether transfected or endogenous, often appeared as a doublet. In contrast, no proteins of the expected size for TGIF were detected with the preimmune serum.

Smad2-associated proteins were precipitated with a purified Smad2-specific polyclonal antiserum from A549 cells and from A549 cells treated with TGFβ for 1 hr. As shown in Figure 4B, Smad2 was precipitated from both treated and untreated cell extracts, whereas Smad4 was present only in immunocomplexes from TGFβ-treated cells. In addition, TGIF was clearly detectable in Smad2 immunocomplexes following TGFβ treatment. Since TGIF has been shown to act as a transcriptional repressor that is capable of repressing Smad-activated transcription (Figure 2; D. W. et al., unpublished data), we wanted to know whether TGIF was able to interact with a histone deacetylase. As shown in Figure 4B, a TGFβ-inducible interaction of HDAC1 with Smad2 was observed, suggesting that TGIF may recruit HDAC1 to TGFβ-activated Smad complexes. Together, these results demonstrate that an interaction of Smad2 with a complex of repressor proteins is induced on addition of TGFβ.

A Fast–Smad–TGIF Complex

It is not clear from the analysis of endogenous Smad-containing protein complexes whether the interaction of HDAC with Smad2 is direct or dependent on TGIF. To analyze the makeup of the Smad repressor complex, COS-1 cells were transfected with expression vectors encoding components of this complex and treated with TGFβ. Cotransfection of either Smad4 or Fast2 with Flag–TGIF and Smad2 resulted in an increase in the amount of Smad2 present in Flag–TGIF immunocomplexes (Figure 5A), suggesting that both Smad4 and Fast2 can stabilize Smad2–TGIF complexes and that TGIF may bind to the Smad2–Smad4–Fast complex. Similarly, TGIF stabilized the Smad2–Fast2 interaction (Figure 5A). As in the case of endogenous TGIF (see Figure 4B), exogenous TGIF migrated as a doublet. Preliminary evidence suggests that the upper band is a phosphorylated form of the faster migrating form (D. W. et al., unpublished data).

Cotransfection of COS-1 cells with Myc–Fast2, Flag–TGIF, and Smad2 revealed that TGIF was clearly detectable in complexes precipitated via the Myc epitope present on Fast2. This interaction of TGIF with Fast2 was dependent on Smad2, as no TGIF coprecipitated with Fast2 from cells not cotransfected with Smad2 (Figure 5B). Similar results were obtained when Myc-Fast1 was used instead of Myc-Fast2 (data not shown). Flag immunocomplexes from cells cotransfected with Flag–TGIF, Myc–Fast2, and Smad2 were eluted using Flag peptide and reprecipitated with a Myc-specific antibody. As shown in Figure 5C, Smad2 was clearly present in eluted and reprecipitated immunocomplexes only when all three components were cotransfected. Thus, both Fast2 and TGIF can coexist in the same Smad2-containing complex.

To determine whether TGIF was able to recruit an HDAC to Smad2-containing complexes, TGIF–HA, Smad2, and Flag–HDAC1 were cotransfected into COS-1 cells. Following immunoprecipitation via the Flag epitope on HDAC1, complexes were analyzed for the presence of Smad2. As shown in Figure 5D, Smad2 coprecipitated with HDAC1 in the presence of cotransfected TGIF. No Smad2–HDAC1 interaction was observed in the absence of TGFβ treatment or coexpressed TGIF. Additionally, the TGIF deletion construct that is unable to interact

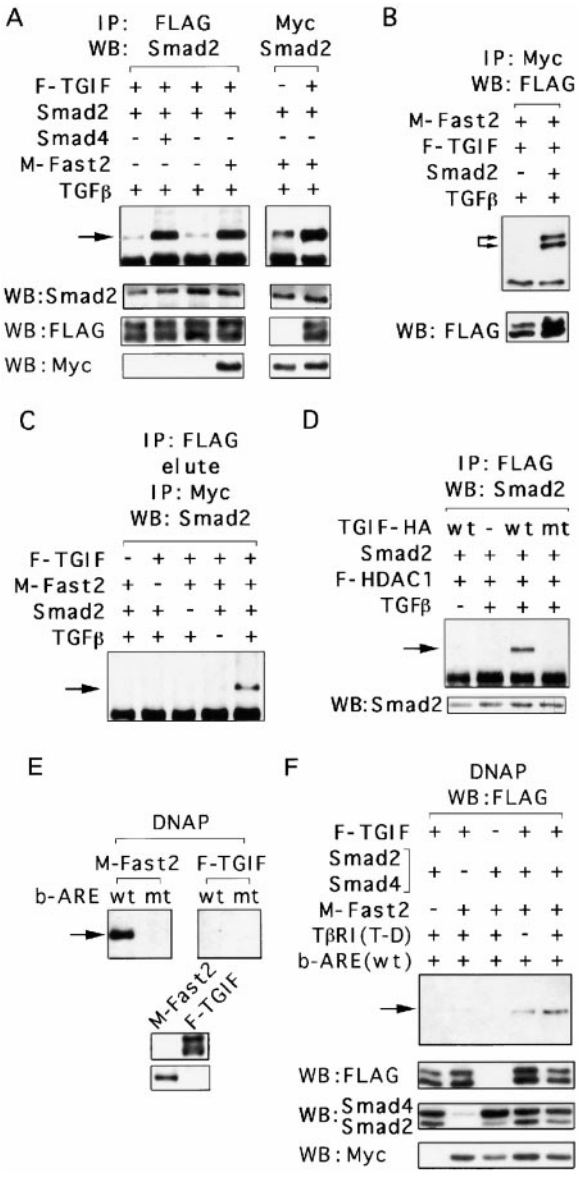

Figure 5. TGIF Is Present in a Smad2 Complex Together with Fast2

Analysis of TGIF-containing complexes by coimmunoprecipitation.

(A) Enhancement of the TGIF–Smad2 interaction by coexpression of Smad4 or Fast2. Increased Smad2–Fast2 interaction is also observed with overexpression of TGIF. The presence of Smad2 in Flag or Myc immunoprecipitates was detected by Western immunoblotting with a Smad2-specific antiserum. Cells were transfected with Flag–TGIF and Smad2, together with Smad4 or Myc–Fast2, as indicated.

(B) Myc immunoprecipitates from COS-1 cells transfected with Myc-Fast2 and Flag–TGIF in the presence or absence of coexpressed Smad2 were analyzed for the presence of TGIF by Western immunoblotting with a Flag antibody.

(C) Cells were transfected with the indicated combinations of Flag–TGIF, Smad2, and Myc–Fast2. Following immunoprecipitation with a Flag-specific antibody, proteins were eluted with Flag peptide, reprecipitated with a Myc-specific antibody, and analyzed by Western immunoblotting with a Smad2-specific antiserum.

(D) COS-1 cells were transfected with Smad2, Flag–HDAC1, and HA-tagged wild-type TGIF (wt) or TGIF(1–148:177–262) (mt). TGFβ (100 pM) was added, as indicated, and cell lysates were subjected to immunoprecipitation with a Flag antibody. Coprecipitation of Smad2 was detected by immunoblotting of the precipitates with a Smad2 antiserum.

with Smad2 was unable to bridge the Smad2–HDAC1 interaction (Figure 5D), suggesting that the interaction of Smad2 with HDAC1 is dependent on the presence of TGIF. Thus, it is likely that Smad2 is able to recruit TGIF and a histone deacetylase to the DNA-binding protein Fast2.

Recruitment of TGIF to the ARE

The interaction of Fast2, Smads, and TGIF suggests that TGIF may be brought to the ARE via this complex. Transfection of COS-1 cells with Fast2, followed by DNA affinity precipitation (DNAP) using a biotinylated ARE oligonucleotide revealed that the transfected Fast2 specifically bound the ARE, but not a mutant ARE probe (Figure 5E). In contrast, no binding of transfected TGIF to either wild-type or mutant ARE probes was observed. To test whether the Fast2–Smad complex was able to recruit TGIF to the ARE, similar experiments were carried out using extracts from COS-1 cells transfected with TGIF, Smad2, Smad4, and Fast2. Precipitation of Flag–TGIF bound to the ARE was observed only when all components of the complex were present (Figure 5F). In this DNAP experiment, the faster migrating form of TGIF appeared to be preferentially incorporated within the Fast–Smad complex. Additionally, the amount of TGIF recruited to the ARE was enhanced by TGFβ receptor signaling (Figure 5F). Thus, TGIF can be brought to a Smad-responsive element via the interaction of TGIF with Smad2 and the recruitment of activated Smad complexes by a DNA-binding protein such as Fast2.

Requirements for Repression of Smad-Dependent Transcription

TGIF contains two separable transcriptional repression domains, one overlapping the amino-terminal homeodomain and the other at the extreme carboxyl terminus of the protein (D. W. et al., unpublished data). To localize the regions of the TGIF protein required for the repression of Smad-dependent transcription, we used the A3-lux assay. Coexpression of a series of TGIF deletion constructs (Figure 6A) with the A3-lux reporter revealed that deletion of the SID prevents repression of TGFβ-induced A3-lux activation (Figure 6A). Amino-terminal truncation up to amino acid 63 or 108, which removes the N-terminal repressor domain leaving the SID and the C-terminal repressor domain intact, abolished the

(E) COS-1 cells were transfected with either Fast2 or TGIF expression constructs. Cell lysates were divided in two and incubated with either wild-type (wt) or mutant (mt) biotinylated double-stranded ARE oligonucleotides. DNA-bound proteins were precipitated by streptavidin-agarose and the presence of TGIF or Fast2 detected by Western blotting. Protein expression was monitored by direct Western blotting of the two transfected cell lysates (lower panel).

(F) COS-1 cells were transfected with Fast2, Smad2 and Smad4, TGIF, and constitutively active TGFβ receptor type I [TβRI(T-D)], as indicated. Complexes were precipitated with a biotinylated wild-type ARE oligonucleotide and streptavidin-agarose and analyzed for the presence of TGIF. Expression of transfected proteins was assessed by direct Western blotting. TGIF appears as a doublet; however, in (F), the faster migrating form of TGIF was coprecipitated preferentially. Bands corresponding to coprecipitating proteins are indicated by arrows.

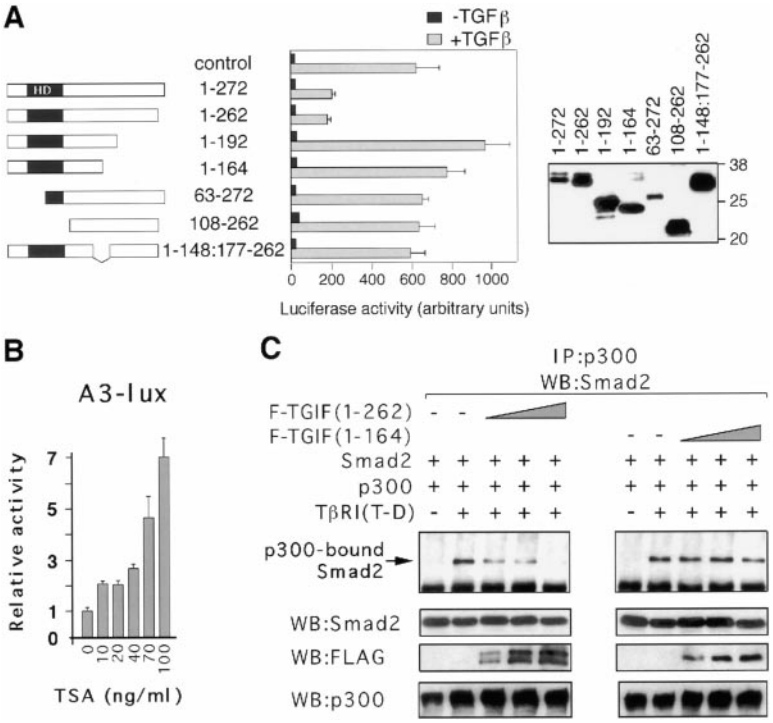

Figure 6. Requirements for Repression of Smad2-Dependent Transcription by TGIF

(A) L17 cells were transfected with the A3-lux reporter, together with the indicated TGIF expression constructs. Transfections were incubated without or with TGFβ (100 pM) for 24 hr prior to analysis of luciferase activity; results are mean + SD of triplicate transfections. COS-1 cells were transfected with the indicated Flag-tagged TGIF expression constructs, and expression levels were assessed by Western immunoblotting of cell lysates with Flag antibody (right).

(B) L17 cells were transfected with the A3-lux reporter and either a control plasmid or a TGIF expression vector. Twenty-four hours prior to analysis, TGFβ (100 pM) and the indicated concentrations of TSA were added. Luciferase activity was assayed and is presented as the increase in activity induced by TSA in cells transfected with TGIF relative to cells transfected with the control plasmid (mean + SD of triplicate transfections). The activity obtained in the absence of TSA has been set equal to 1.

(C) 293T cells were transfected with Smad2, p300, constitutively active TGFβ receptor type I [TβRI(T-D)], and either TGIF(1–262) or TGIF(1–164), as indicated. The presence of Smad2 in p300 immunoprecipitates was assessed using a Smad2-specific antiserum. Expression of transfected proteins was monitored by direct Western blotting.

ability of TGIF to repress A3-lux activation. Carboxy-terminal truncation to amino acid 192, which removes the C-terminal repressor domain leaving the SID and the N-terminal repression domain, abolished repression of A3-lux, resulting in a small but reproducible enhancement of A3-lux activity (Figure 6A). Further truncation to amino acid 164, which eliminates both the SID and the C-terminal repressor domain, also abolished repression (Figure 6A). These results suggest that, in addition to the SID, two other regions of TGIF are required for repression of A3-lux activity. The location of these domains appears to correlate with the locations of two separable repression domains identified within TGIF (D. W. et al., unpublished data), both of which appear to be required for repression of A3-lux transcription.

The repression by TGIF of Smad2-dependent transcription may be, at least in part, due to the ability of TGIF to recruit a histone deacetylase to Smad complexes. We, therefore, tested the possibility that repression of A3-lux by TGIF is dependent on the deacetylase activity recruited into the complex by TGIF. L17 cells cotransfected with A3-lux, Fast2, and either TGIF or a control plasmid were incubated with TGFβ and increasing concentrations of the inhibitor of histone deacetylase activity, trichostatin A (TSA). The luciferase activity in the presence of cotransfected TGIF relative to that obtained with a control plasmid was calculated for each concentration of TSA. Increasing TSA resulted in a small increase (up to 2- to 3-fold) in the basal activity of A3-lux in the absence of TGFβ (data not shown). In TGFβ-treated cells transfected with TGIF, A3-lux activity increased by up to 7-fold with the highest concentration of TSA (Figure 6B). Thus, the interaction of a deacetylase with TGIF appears to be important for the repression of A3-lux by TGIF, suggesting that recruitment of HDAC activity into Smad–TGIF complexes may play a role in the silencing of TGFβ transcriptional responses.

Competition between p300 and TGIF for Binding to Smad2

The interaction of Smad2 with both TGIF and with coactivators such as p300 suggests that these two transcriptional modulators may compete for interaction with activated Smad complexes. To test this possibility, 293T cells were transfected with Smad2 and p300 expression vectors, together with increasing amounts of TGIF or a TGIF deletion mutant that is unable to interact with Smad2. Complexes were precipitated with a p300-specific antiserum, and the presence of Smad2 was detected by Western blotting. A TGFβ-inducible interaction of Smad2 with p300 was clearly detectable. However, the interaction of p300 with Smad2 was greatly reduced on coexpression of increasing amounts of TGIF (Figure 6C, left). In contrast, expressing increasing amounts of TGIF(1–164) had no effect on the observed p300–Smad2 interaction (Figure 6C, right). These results suggest that modulation of the level of TGIF within the cell can alter the balance of Smad–coactivator or Smad–corepressor complexes formed. Thus, it appears that TGIF- and p300-containing Smad complexes are mutually exclusive and that TGIF and p300 may compete for interaction with activated Smad complexes as they enter the nucleus.

Discussion

A Smad Corepressor

We have isolated TGIF as a protein that interacts with Smads 2 and 3 and represses TGFβ-induced transcription. TGIF was originally isolated as a ubiquitously expressed homeodomain protein that can bind to a retinoid X receptor (RXR) response element (Bertolino et al., 1995). TGIF belongs to the TALE class of homeodomain proteins, which have a three–amino acid insertion between helices 1 and 2 of the homeodomain (Bertolino et al., 1995; Burglin, 1997). Transcriptional repression has been attributed to various homeodomain proteins, including a member of the TALE superfamily, Prep1 (Berthelsen et al., 1998a, 1998b). Prep1 is able to participate in both transcriptional activation and repression, depending on the other proteins with which it associates. Binding of TGIF to the RXRE has been shown to inhibit RXR-mediated transcriptional activation by competitive DNA binding to overlapping sites. However, the role of TGIF in retinoid-responsive transcription remains unclear. Analysis of the transcriptional activity of TGIF has demonstrated that TGIF contains transcriptional repression domains that repress both basal and activated transcription (D. W. et al., unpublished data). Here, we demonstrate that by associating with TGFβ-activated Smads, TGIF generates a Smad-dependent transcriptional repressor complex.

TGIF Represses Transcription by Associating with HDAC

The acetylation state of core histones plays an important role in the regulation of transcription (Struhl, 1998). Interaction of transcription factors and coactivator proteins with histone acetyl transferase (HAT) activity appears to be a general requirement for transcriptional activation. The ability of histone deacetylases (HDACs) to act on a specific gene depends on their recruitment to the promoter, via gene-specific repressors or corepressors. The balance between transcriptional activation and repression is, in part, due to differential recruitment of histone acetylases and deacetylases.

Recent evidence has implicated p300 and CBP, which have HAT activity, in transcriptional activation by Smad complexes (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998). We demonstrate the recruitment of HDAC into a TGFβ-activated Smad complex via TGIF. Repression by TGIF of a TGFβ- and Smad-responsive *Mix.2* gene reporter (A3-lux) is dependent on the presence of both repression domains of TGIF and HDAC activity. The activated Smad2 is likely able to recruit HDAC activity to Smad-responsive genes via its interaction with TGIF. Thus, TGFβ-responsive transcription can be repressed by deacetylase recruitment via a Smad complex that can alternatively recruit a HAT. The interaction of Smad2 with either HDACs (via TGIF) or p300/CBP suggests that Smad complexes regulate transcription in part by remodeling of the chromatin template.

Functional Implications

The observed repression by TGIF of TGFβ-induced A3-lux and 3TP-lux reporter activities supports a model in which TGIF is recruited to Smad target genes, leading to repression of these genes. TGIF could act to set either a threshold level or a ceiling for TGFβ-mediated transcriptional activation. If TGIF sets a threshold, a low level of TGFβ signaling would be rendered ineffective by the presence of TGIF, whereas at higher concentrations of TGFβ, TGIF would have no effect. However, our data (Figures 2D and 3C) argue against this, rather suggesting a model in which the presence of TGIF sets a maximum level of TGFβ-activated transcription. Thus, a reduction in the level of endogenously expressed TGIF increases maximal TGFβ-activated transcription, whereas increasing TGIF expression by transfection lowers the maximal TGFβ response. This ceiling would be expected to differ among different cell types and might vary during development, depending on the level of TGIF expressed. In this context, it should be noted that, although TGIF is expressed in a wide range of tissues and cell lines, its expression level varies significantly (Bertolino et al., 1995, Figure 4A, and unpublished results). In addition, the action of TGIF might allow for modulation of the magnitude of TGFβ responses in response to other signaling inputs.

Smad signaling can be limited by mechanisms such as the MAP kinase phosphorylation of Smads, which prevents Smad accumulation in the nucleus (Kretzschmar et al., 1997b, 1999). Smad signaling can also be limited by the antagonistic Smads, Smad6 and Smad7, which act as interfering decoys in receptor–Smad or Smad4–Smad interactions (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997b; Hata et al., 1998; Ulloa et al., 1999). Thus, interaction with the receptor, interaction with Smad4, and nuclear translocation can be blocked. In addition, the zinc finger protein Evi-1 has recently been suggested to inhibit TGFβ signaling by inhibiting Smad3 binding to DNA (Kurokawa et al., 1998). The transcriptional repressor TGIF could also be considered to be blocking TGFβ signaling. However, the action of TGIF in TGFβ signaling differs in one important aspect. Not only can TGIF interfere with TGFβ-mediated transcriptional activation by competing with p300 for Smad interaction, but once recruited to receptor-activated Smad complexes, TGIF provides a specific activity, namely transcriptional repression. The function of TGIF appears to be different from that of the *Drosophila* repressor *brinker*, which has been shown to play a role in modulating Dpp signaling (Campbell and Tomlinson, 1999; Jazwinska et al., 1999). TGIF is specifically recruited to Smad target genes, which are activated by TGFβ signaling, whereas repression by *brinker* is likely to be relieved by Smad activation.

We propose that upon entering the nucleus and becoming tethered to a target gene via proteins such as Fast1 or Fast2, an incoming Smad2/3–Smad4 complex has two choices (see Figure 7): to interact with p300/CBP, forming a transcriptional activation complex as previously reported (Feng et al., 1998; Janknecht et al., 1998; Pouponnot et al., 1998), or to interact with TGIF, resulting in the recruitment of HDAC and the formation of a transcriptional repressor complex. The outcome of this competition is dependent in part on the relative levels of Smad corepressors and coactivators present in the cell. Extracellular signals that regulate the activity of either the corepressor or the coactivator would be

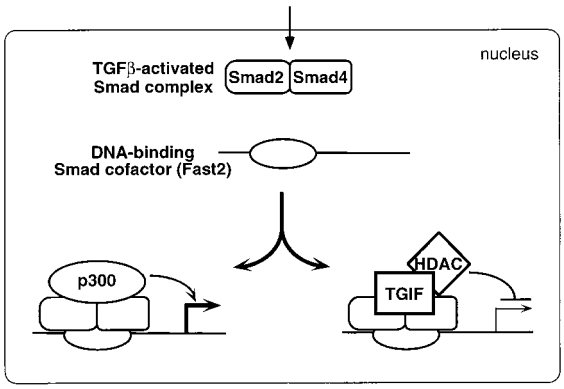

Figure 7. A Model Depicting Smad-Dependent Transcriptional Activation and Transcriptional Repression Complexes

The ARE bound by Fast1 or Fast2 has been taken as a model Smad-responsive element. The possible mode of assembly of TGFβ/activin-induced transcriptional complexes on a Fast-like cofactor is shown. Fast-bound Smads can recruit either p300/CBP histone acetyl transferases, activating transcription as previously described, or TGIF and a histone deacetylase (HDAC), repressing transcription as described in the present work.

likely to affect this balance. It will be of interest to determine the possible effects on TGIF activity of signals that oppose TGFβ signaling. Such signal-regulated modulation of Smad interactions with corepressors and coactivators may allow precise regulation of the level of transcriptional activation by TGFβ. In summary, the balance between coactivators and corepressors, such as TGIF, with which Smad complexes interact in the nucleus is likely to play a central role in TGFβ/Smad regulation of gene expression.

Experimental Procedures

Two-Hybrid Screening

LexA/Smad fusions were created in pALA-17 (D. W. and J. M., unpublished data), and the HeLa cDNA library was present in pJG4-5 (Zervos et al., 1993). Library screens were carried out using a *HIS3* biosynthetic reporter within the strain yGI-12a (D. W. and J. M., unpublished data). Briefly, yGI-12a cells were tranformed with LexA/Smad2 and 100 μg of library and plated to glucose-containing media. Colonies were replica plated to galactose media, and positives were picked after 3–5 days. After testing on media with glucose and galactose, PCR products from positives that were dependent on expression of the library fusion were sequenced using a Sequenase PCR sequencing kit (Amersham).

Cell Culture and Transfection

Mink lung epithelial L17 cells and COS-1 cells were maintained as previously described, and L17 cells were transfected in six-well plates using DEAE-dextran as described (Hata et al., 1997). COS-1 cells were transfected using LipofectAMINE (GIBCO-BRL), according to the manufacturer's instructions. Human 293T and A549 cells were maintained in DME supplemented with 10% fetal bovine serum and glutamine (2 mg/ml). 293T cells were transfected by standard calcium phosphate precipitation. A pCMV5-GFP or pCMV5-β-galactosidase expression vector was used to keep amounts of DNA transfected for coimmunoprecipitation assays constant. For analysis of TGFβ signaling, L17 cells were transfected with an expression construct encoding the TGFβ type I receptor.

Antisense Oligonucleotide Treatment

Phosphorothioate-modified oligonucleotides complementary to the TGIF mRNA sequence were synthesized on an ABI 392 and purified on OPC columns (ABI). Oligonucleotides were delivered to A549 cells with 30 μg/ml Lipofectin (GIBCO-BRL) in OptiMEM (GIBCO-BRL). Following overnight incubation, cells were transferred to DME with 0.2% serum for 2 hr prior to addition of TGFβ. RNA was isolated after 4 hr of incubation with TGFβ. The sequence of the oligonucleotides used are as follows: ASO3, CACAGCCGACTCTCCCGTAAC; control, ACACCGGCCACTCTCCTGAAC.

Northern Analysis

RNA was isolated using Ultraspec (Biotecx), fractionated through 1% agarose, and transferred to Hybond N^+ nylon membranes (Amersham). Probes were labeled by random priming and hybridized for 1 hr at 68°C in ExpressHyb (Clontech). Following washing, signals were detected and quantitated with a Storm 840 phosphorimager (Molecular Dynamics). Membranes were cut to allow probing with *TGIF* and *PAI-1* separately; following stripping of hybridized *TGIF* probe, the lower half of the membrane was rehybridized to a *GAPDH* probe.

Reporter Assays

The A3-lux and 3TP-lux reporters have been described (Cárcamo et al., 1995; Huang et al., 1995; Liu et al., 1996). Five Gal4p-binding sites are present upstream of the E1b TATA box in $(Gal)_5$ E1b-luc. The control plasmids used in transfections for luciferase assays were either pCMV5 lacking an insert, or a pCMV5-farnesyl transferase expression construct. Luciferase assays were carried out using the Promega luciferase assay kit and a Berthold luminometer. A CMV-Renilla luciferase plasmid (Promega) was included to control for transfection efficiency. Renilla luciferase activity was assayed in 25 mM Tris (pH 7.5), 100 mM NaCl, with 0.09 μM coelenterazine (Biosynth) from a 0.09 μM stock in 20 mM HCl in methanol. Cells were incubated with 100 pM TGFβ, as indicated, in media containing 0.2% serum for 24 hr. Trichostatin A, in 100% ethanol, was added to culture media for 24 hr as indicated.

Immunoprecipitation Assays and Western Blotting

TGIF expression constructs were created within a modified pCMV5, containing an initiation codon together with either a Flag epitope or two copies of an HA epitope. Untagged Smad1 and Smad2 were expressed from within pCMV5, or pCS2. Myc-Fast2 was expressed from pCS2 and contained the entire coding sequence of Fast2 (a kind gift of Dr. E. Lai). Flag-tagged HDAC1 is as described (Yang et al., 1997). Twenty-four hours post transfection, COS-1 cells were treated with the desired ligand for 5–6 hr, when necessary, under reduced serum. COS-1 cells were then washed, resuspended in LSLD (50 mM HEPES [pH 7.4], 50 mM NaCl, 0.1% Tween-20, 10% glycerol) supplemented with protease and phosphatase inhibitors, and lysed by sonication. The lysates were then subjected to immunoprecipitation using M2 Flag monoclonal antibody (Kodak) or anti-cMyc (9E10) monoclonal antibody (Santa Cruz Biotechnology, Inc). HA-tagged proteins were detected with an HA-specific monoclonal antibody (12CA5; Boehringer Mannheim). 293T cells were lysed in TNMG (20 mM Tris-HCl [pH 8], 50 mM NaCl, 5 mM $MgCl_2$, 0.5% N-P40, 10% glycerol) with protease and phosphatase inhibitors. p300 was both immunoprecipitated and detected using mixed mouse monoclonal antibodies (Upstate Biotechnology). Smad proteins were detected with rabbit polyclonal antisera raised against the corresponding GST-Smad fusion. A fraction of the lysates was subjected to direct immunoblotting to monitor transfection efficiency. For detection of endogenous Smad2-associated proteins, three 24.5 × 24.5 cm plates of A549 cells at 70%–80% confluence were washed in PBS and lysed by sonication in 12 ml LSLD with 20% glycerol, and protease and phosphatase inhibitors. DNA was sheared and cellular debris removed by centrifugation at 10,000 × g for 20 min. Lysates were incubated with 3 μg purified Smad2-specific antiserum for 14 hr at 4°C. Protein complexes were collected with 25 μl of protein A-Sepharose, loaded into a 2 ml disposable column, and washed with 10 ml of LSLD. Bound proteins were eluted twice with 200 μl LSLD containing 2 M $MgCl_2$. Proteins were transferred to PVDF membranes (Immobilon-P, Millipore). Proteins were detected using chemiluminescence (ECL; Amersham).

DNA Affinity Precipitation

COS-1 cell lysates prepared as for immunoprecipitation assays were precleared with ImmunoPure streptavidin-agarose (Pierce), then incubated at 4°C (in 1 ml) with 200 ng of biotinylated double-stranded oligonucleotides and 2 μg of poly(dI-dC).poly(dI-dC) for 1 hr. DNA-bound proteins were precipitated with streptavidin-agarose for 1 hr, washed extensively, and detected by Western blotting. The sequence (upper strand) of the ARE oligonucleotide (wt) was TATCTGC TGCCCTAAAATGTGTATTCCATGGAAATGTCTGCCCTTCTCTC CACGTCTAGCGAATTCGGATCC and the mutant, TATCTGCTGCCC TAA**T**AT**C**TGTATTCCATGGA**T**AT**C**TCTGCCCTTCTCTCCACGTC TAGCGAATTCGGATCC. Changes in the mutant ARE are in bold; the oligonucleotides were biotinylated at the 5′ end of the lower strand.

Immunofluorescence

Twenty-four hours after transfection, COS-1 cells, transfected with Flag–TGIF, were transferred to chamber slides (Nunc). TGFβ (1 nM) was added for 1 hr prior to immunostaining. Cells were processed as described (Harlow and Lane, 1988) and incubated with 1 μg/ml M2 Flag monoclonal antibody (Kodak) for 1 hr, followed by FITC-conjugated goat anti-mouse antibody (Pierce). Prior to visualization, DNA was stained with DAPI.

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