Cooperative Binding of Smad Proteins to Two Adjacent DNA Elements in the Plasminogen Activator Inhibitor-1 Promoter Mediates Transforming Growth Factor β-induced Smad-dependent Transcriptional Activation

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Transforming growth factor β (TGFβ) activates transcription of the plasminogen activator inhibitor type-1 (PAI-1) gene through a major TGFβ-responsive region (~740 and ~647) in the PAI-1 promoter. This process requires the Smad family of signaling molecules. Upon phosphorylation by the TGFβ receptors, Smad2 and Smad3 homologimerize and heterologimerize with Smad4, translocate to the nucleus and activate transcription of TGFβ-responsive genes. Smad3 and Smad4 have been shown to bind to various sites in the PAI-1 promoter. To determine the number of Smad-binding sites within the 94-base pair major TGFβ-responsive region and the mechanism of Smad-mediated transactivation, we systematically mapped the Smad-binding sites and show that Smad4 and Smad3 bind cooperatively to two adjacent DNA elements in this region. Both elements were required for TGFβ-induced, Smad3- and Smad4-dependent activation of PAI-1 transcription. Contrary to previous reports, transactivation of the PAI-1 promoter was mediated by the amino- but not carboxyl-terminal domains of the Smads. Furthermore, oligomerization of Smad3 markedly enhanced its binding to the two binding sites. Finally, a Smad4 mutation identified in a human pancreatic carcinoma that inactivates Smad4 signaling abolished Smad4 DNA binding activity, hence preventing transactivation of TGFβ-responsive genes. These results underscore the importance of the Smad4 DNA binding activity in controlling cell growth and carcinogenesis.

Transforming growth factor-β (TGFβ) is a multipotytkine that elicits many biological functions including inhibition of the growth of cells of epithelial, endothelial, and lymphoid origins, production of extracellular matrix components, and regulation of differentiation of many cell types (1). These activities are mediated by the cell surface types I and II TGFβ receptors, TβRI and TβRII, which are receptor serine/threonine kinases (2–4). In the absence of ligand, while the TβRI kinase is inactive, the TβRII kinase is constitutively active and the receptor is autophosphorylated (2, 5). Binding of TGFβ1 to TβRII results in the formation of a heteromeric complex containing TβRI and TβRII, followed by transphosphorylation of TβRI by the TβRII kinase (6). Phosphorylation of TβRI by TβRII is thought to activate the TβRI kinase activity, allowing it to phosphorylate and activate downstream Smad2 (7–10) and Smad3 proteins (11, 12).

The Smad family proteins are critical components of the TGFβ signaling pathway. Depending on their mechanisms of action, the Smads can be divided into three classes: pathway-restricted Smads, common-mediator Smads, and inhibitory Smads (13). All Smad proteins share considerable homology in their primary sequence and most contain two highly conserved Mad homology domains: MH1 in the amino-terminal half and MH2 in the carboxyl-terminal half separated by a diverse proline-rich linker. Upon stimulation by TGFβ1, the pathway-restricted Smads, Smad2, and Smad3, interact with the TGFβ receptor complex, and become phosphorylated on three serine residues located at the carboxyl termini of the molecules (9, 10). Phosphorylated Smad2 and Smad3 then form a heteromeric complex with the common mediator Smad4 (14–16), translocate into the nucleus, and activate transcription of TGFβ-responsive genes. As the common mediator, Smad4 plays a central role in downstream signal transduction by the TGFβ receptor family members. By forming heteromeric complexes with various pathway restricted Smads, Smad4 participates in the activation of multiple signaling pathways initiated by TGFβ family members and may be directly involved in transcriptional activation of many downstream genes (14, 16). As such, it plays a key role in the control of cell growth and differentiation. Indeed, mutations that inactivate Smad4 function have been detected in many types of human cancer, including cancers of the pancreas, colon, breast, neck, and stomach (17–21). Importantly, many of these Smad4 mutants are defective in transcriptional activation.

The mechanisms by which Smad proteins activate transcription of specific genes are not well understood. The COOH-terminal MH2 domains of Smad1 have been shown to act as transcriptional activators when fused to a heterologous DNA-binding domain (22). Smad2, Smad3, and Smad4 have been shown to interact with sequence-specific DNA-binding proteins such as FAST-1 and FAST-2 and participate in the regulation of transcriptional activation of specific promoters (23–25).
binding partner is required to bring the MH2 domain of the Smads into close proximity of a TGFβ responsive promoter in order to activate transcription. In agreement with this model, overexpression of the MH2 domains of Smad2, Smad3, or Smad4 is sufficient to activate transcription of the p3TPlux luciferase reporter construct in a breast cancer cell line (14, 15). Smad3 may cooperate with general transcription factors such as Sp1 (26), AP1 (27), and CBP/p300 (28, 29, 50) to activate transcription.

Recently, the N-terminal MH1 domains of Drosophila Mad and human Smad3 and Smad4 were shown to possess sequence-specific DNA binding activity (30–33, 49). Interestingly, although Smad2 shares 91% sequence identity with Smad3, it does not bind DNA. Using a PCR-based selection procedure, an 8-bp palindromic DNA sequence (GTCTTAGAC) was selected from a random pool of oligonucleotides as the optimal Smad-binding element (SBE) for both Smad3 and Smad4 (33). The crystal structure of the MH1 domain of Smad3 bound to SBE determined a minimal Smad box (GTCT) required for binding to Smad3 (34), although a high affinity interaction may require additional sequences. These results suggest that direct DNA binding of Smad3 and Smad4 may play a role in transcriptional activation by TGFβ. However, since the involvement of the MH1 domains of Smads in TGFβ-induced transcriptional activation has not been demonstrated, the exact role of the DNA binding activity of Smads in transcription is not clear.

The plasminogen activator inhibitor type-1 (PAI-1) promoter is the best characterized TGFβ-inducible promoter. Activation of PAI-1 gene expression occurs mostly at the level of transcription with fast kinetics (<30 min) and can reach up to 68-fold over the basal level (35, 36). Deletional analysis suggested that there are multiple TGFβ responsive elements present in the PAI-1 promoter that, together, mediate optimal activation by TGFβ. Among these elements, a 94-bp region between −740 and −647 upstream of the initiation site was found to be the major TGFβ-responsive element that can mediate up to 50-fold activation of PAI-1 transcription (35, 36). Several AP1-like sequences within this region have been proposed to play an important role in mediating TGFβ responsiveness (35). However, it is not clear how TGFβ induces transcriptional activation through this element. Recently, Denller et al. (32) reported identification of a CAGA sequence found in three different locations in the promoter of the PAI-1 gene that, when consecutively three times, can mediate direct binding of the Smad3 and Smad4 MH1 domains. Only one of the CAGA containing sequences is located within this 94-bp major TGFβ responsive element. Song et al. (37) reported that a 12-bp sequence which overlaps with the above described CAGA sequence can mediate Smad binding. We decided to carry out a detailed analysis of this major TGFβ responsive region to determine how many binding sites there are in this region of the PAI-1 promoter and whether the Smad-binding sites can mediate transcriptional activation in response to TGFβ. We have identified two specific Smad-binding sites in this region which mediate cooperative binding of Smad3 and Smad4 and are required for TGFβ-induced transcriptional activation of the PAI-1 promoter. These two sites mediate a much stronger binding to Smads than the previously reported CAGA containing sequences. A point mutation identified in human pancreatic cancer that inactivates Smad4 function was found to destroy the DNA binding activity of Smad4. Finally, we showed that oligomerization of the Smad proteins are not required for DNA binding.

**Experimental Procedures**

**Con structs—**Flag-tagged human Smad2 and Smad4 cDNA in pCMV5B as well as Smad4 in pGEX-4T were kindly provided by J. Wrana and L. Attisano (9). Human Smad3 cDNA was a generous gift from R. Derynck (11). To express full-length Smad3 and Smad3 and Smad4 mutants in *Escherichia coli*, the Smad3 and Smad4 mutants were PCR amplified and cloned into pGEX-4T-1. N- and COOH-terminal Smad mutants included the following amino acid residues: Smad3N, 1–201; and Smad3C, 202–230. Mutations were generated using a PCR based approach and verified by sequencing. p3TPlux was kindly provided by J. Massague (38). To generate 4 × wild-type PAI-1 (−740/−647), a DNA fragment containing sequences between −647 and −740 from the PAI-1 promoter (39) was PCR amplified with a BamHI site added at the 5′ end and a BglII site added at the 3′ end. The PCR product was digested with BamHI and BglII and self-ligated in the same orientation. A fragment containing four copies of the PAI-1 promoter sequence in the same orientation was then cloned into pGL2-promoter vector (Promega) at the BglII site. PAI-1 promoter sequences containing various point mutations were generated by PCR, and multimerized similarly as described for the wild-type promoter constructs.

**Cells and Antigens—**293T cells were kindly provided by W. Pears (40) and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Hep3B, a human hepatoma cell line (ATCC), was maintained in minimum essential media supplemented with 10% fetal bovine serum. TGFβ1 is a kind gift from R&D systems. The monoclonal antibody against the Flag tag (M2) was purchased from Kodak and Sigma. The antibody against Smad2/3 (E-20) was purchased from Santa Cruz Biotechnology. A polyclonal antibody against a NH2-terminal peptide sequence of Smad4 was kindly provided by X. Liu.

**Purification of GST Fusion Proteins—**GST fusion proteins were expressed in and purified from *E. coli* as described (41). Thrombin cleavage was performed with proteins bound to glutathione-Sepharose (41). After elution, glycerol was added to 15% and proteins were stored at −80°C.

**Transfection and Luciferase Assays—**293T cells were transiently transfected using the calcium phosphate co-precipitation method (5). Hep3B cells were transfected using a LipofectAMINE protocol (Life Technologies, Inc.). For luciferase assay, a total of 2 μg of DNA (1 μg of luciferase reporter construct and 1 μg of expression vectors for Flag- and HA-tagged Smad proteins) were isolated by immunoprecipitation from transfected 293T cells as described (42). Briefly, 48 h after transfection, cells were lysed in lysis buffer (50 mM Hepes pH 7.8, 500 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 3 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) and Flag- or HA-tagged proteins isolated by immunoprecipitation with anti-Flag M2 affinity gel (Sigma) or anti-HA affinity beads. Proteins were eluted from the antibody column with the elution solution containing 1 mg/ml Flag or HA peptide (Sigma) and quantified by Western blotting.

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**Electrophoresis Mobility Shift Assays (EMSA)—**For EMSA assays, various amounts of recombinant proteins were incubated with 2 × 104 cpm 32P-labeled probes for 15 min at room temperature in binding buffer (25 mM Tris-CI, pH 7.5, 80 mM NaCl, 35 mM KCl, 5 mM MgCl2, 10% glycerol, 1 mM dithiothreitol, 10 μg/ml poly(dI-dC), 300 μg/ml bovine serum albumin, and 2% Nonidet P-40). The protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 0.5% TBE. Double-stranded oligonucleotide probes used in the EMSA assay were: Probe A (PAI-1 promoter region, −688/−660): wild-type: 5′-GGACACGTGGGCATTAAGCCGAG-3′, M1 5′-GAGACTCTGGGACAGTGCTGACAGACC-3′; M2 5′-GAGACTCTGGGACAGTGCTGACAGACC-3′; Probe B (PAI-1 promoter region, −675/−647): wild-type 5′-GGACACGTGGGCATTAAGCCGAG-3′, M4 5′-CGTGGGCGCTTATTACGCGCG-3′; M5 5′-CGTGGGCGCTTATTACGCGCG-3′; Probe C (PAI-1 promoter region, −732/−708): wild-type 5′-GGACACGTGGGCATTAAGCCGAG-3′; M6 5′-CGTGGGCGCTTATTACGCGCG-3′; M7 5′-CGTGGGCGCTTATTACGCGCG-3′

**Single-stranded oligonucleotides were end labeled using T4 polynucleotide kinase, annealed, and gel-purified (43). The complete PAI-1 −740/−647 region was digested from p3TPlux using BamHI and end labeled using T4 polynucleotide kinase.**
Transactivation by Smads through Direct DNA Binding

9433

PAI-1 Promoter -740/647

Probes: FL FL

GST-Smad3

GST-Smad4

Probes: FL FL

A B C D

RESULTS

Smad3 and Smad4 Bind to Two Adjacent DNA Elements in the PAI-1 Promoter in a Cooperative Manner—EMSA were used to determine whether Smad3 and Smad4 directly bind to the major TGFβ-responsive region between −740 and −647 upstream of the initiation site in the PAI-1 promoter. Recombinant full-length Smad3 and Smad4 were purified from E. coli and incubated with the 32P-labeled 94-bp DNA fragment (probe FL). As shown in Fig. 1, both GST-Smad4 and GST-Smad3 bound to this DNA fragment and induced a shift in the mobility of the labeled probe (lanes 1 and 2). To further pinpoint the region that mediates Smad binding, four overlapping oligonucleotide probes that cover the entire 94-bp region were synthesized and tested in the EMSA assay (Fig. 1). Among the four probes, probe A mediated strongest binding to Smad4 (lane 3, Fig. 1), while the other three probes all bound weal (lanes 4–6, Fig. 1). Therefore, the sequences between −688 and −660 covered by probe A contain the major binding sites for Smad4. Similar results were obtained for Smad3 (data not shown). The CAGA containing sequence that was previously reported to mediate Smad3 binding is covered by probes C and D. The 12-bp region that was reported by Song et al. (37) to bind Smad3 was also included in probes C and D. Neither probes displayed a significant affinity for Smads in our experiment (Fig. 1).

A careful examination of the DNA sequences between −688 and −660 revealed two potentially important sequence motifs: one is similar to the SBE identified previously as the optimal Smad-binding sequence (33) with a single A to G change at position 5 (SBE, GTCTAGAC; PAI-1 −684/−677, GTCTGGAC), but the minimal Smad box is intact (34). Six base pairs 3’ to the SBE-like element is an AP1-like motif which contains a single base pair change from the consensus AP1 site (AP1, TGA(g/c)TCA; PAI-1 −670/−664, GGAGTCA) (Fig. 2A). This AP1-like site has been implicated in mediating TGFβ-induced transactivation of the PAI-1 gene (35) and the type I collagen gene (44). The last four nucleotides in this AP1-like motif is also homologous to the Smad box (GTCT) (34) with 1-bp mismatch (GTCA). To determine whether the two motifs are required for interaction with Smad4, oligonucleotide probes containing point mutations in either of the two elements were generated (Fig. 2A) and their ability to complex with Smad4 was tested by EMSA (Fig. 2B). Mutation of either the SBE-like site or AP1-like site significantly reduced binding by GST-Smad4 (lanes 2 and 3, Fig. 2B), while mutation of both site I and site II completely abolished binding of Smad4 (lane 4, Fig. 2B). This suggests that there are two Smad4-binding sites in probe A and that both sites are important for interaction with Smad4. Between the two sites, site I is the predominant one since mutation of this site (probe M2) resulted in a greater decrease in Smad4 interaction (lane 3, Fig. 2B). Interaction of Smad4 with site II, although weaker, was also specific (see below and Fig. 3). As a control, GST alone did not bind to probe A (lane 10, Fig. 2B). Furthermore, cleavage of GST by thrombin resulted in a Smad4 that bound to probe A in a manner indistinguishable from that of the GST-fusion proteins (lanes 5–8, Fig. 2B), confirming that the observed DNA binding activity was not due to the presence of GST.

The amount of Smad4 complexed with wild-type probe A was 4- or 5-fold more than the amount complexed with probe M1 and M2 added together, suggesting that the two binding sites act in a cooperative manner to mediate binding of Smad4 (lanes 1–3 and 5–7, Fig. 2B). A careful titration experiment using various amounts of Smad4 in EMSA further confirmed the cooperative action between the two sites (Fig. 2D). Mutation of site I or site II also resulted in a decreased mobility shift, suggesting that Smad4 occupies both binding sites on the same DNA probe simultaneously. The presence of multiple shifted bands in EMSA assay (lanes 2, 3, 6, and 7, Fig. 2B) could be due to the different oligomeric states of Smad4 caused by GST fusion or homooligomerization of Smad4.

Thus, recombinant Smad4 binds to two adjacent sites in the PAI-1 promoter in a cooperative manner. Similarly, the two sites also mediated binding of GST-Smad3, albeit with a reduced affinity (lanes 1–4, Fig. 2C): at least 8 times more GST-Smad3 was required to shift a similar amount of the DNA probe. The cooperativity between the two sites in binding to Smad3 was also less prominent than that observed in binding to Smad4 (Fig. 2, C and E, and data not shown). Therefore, Smad3 and Smad4 displayed different binding properties for this region of the PAI-1 promoter.

Consistent with previously published studies, the N domains of Smad3 and Smad4 (GST-Smad3N and GST-Smad4N) were capable of binding to DNA (lanes 11–14, Fig. 2B, and lanes 5–8, Fig. 2C). Neither GST-Smad3C nor GST-Smad4C bound DNA (lane 9, Fig. 2, B and C).

To examine whether Smad3 and Smad4 isolated from mammalian cells also interact with the PAI-1 promoter with a similar affinity, Flag-tagged Smad3 and Smad4 were isolated by immunoprecipitation from transiently transfected 293T cells, eluted with Flag peptides, and subjected to EMSA analysis. The amount of Smad proteins prepared this way was quantified by comparing to recombinant GST-Smad proteins using Western blotting analysis (Fig. 2E).
FIG. 2. Smad3 and Smad4 bind to two adjacent DNA regions in the PAI-1 promoter in a cooperative manner. A, nucleotide sequence of wild-type and mutant probe A. SBE and AP1-like motifs are capitalized and underlined. Point mutations are indicated by lowercase, bold-typed letters. B, EMSA assay using 32P-labeled wild-type and mutant probe A. 0.4 μg of Smad4 and Smad4N domain (including the MH1 domain and proline-rich linker region) purified from E. coli either as a GST fusion protein or cleaved with thrombin were incubated with the indicated probes, and the protein-DNA complexes resolved on a 5% polyacrylamide gel as described under “Experimental Procedures.” The position of unbound DNA probe is indicated. The exposure time was 1 h for lanes 11–14 and 4 h for the rest. Lanes 1–4, GST-Smad4 incubated with wild-type and mutant probe A; lanes 5–8, full-length Smad4 produced by thrombin cleavage of GST-Smad4 incubated with wild-type and mutant probe A; lane 9, GST-Smad4N incubated with wild-type probe A; lane 10, GST incubated with wild-type probe A; lanes 11–14, GST-Smad4N incubated with wild-type and mutant probe A. C, 1.6 μg of GST-Smad3 (lanes 1–4), GST-Smad3N (lanes 5–8), and GST-Smad3C (lane 9) were incubated with wild-type and mutant probe A as indicated, and analyzed in an EMSA assay as described. The gel was exposed for approximately 10 h. D, cooperative binding of Smad4 to the two Smad-binding sites. Indicated amounts of Smad4 produced by thrombin cleavage of GST-Smad4 were incubated with wild-type or mutant probe A and analyzed in an EMSA assay. The intensity of the DNA-protein complex was quantified. E, Western blotting analysis of Flag-tagged Smad proteins. Flag-tagged Smad proteins were isolated by immunoprecipitation from transfected 293T cells, eluted with Flag peptide, and quantified by Western blotting analysis using the antibodies indicated. The Flag-Smad3 or Flag-Smad4 were compared with a known quantity of recombinant Smad3 or Smad4 as indicated. F, left panel, 0.4 μg of Flag-tagged full-length and truncated Smad4 proteins isolated from transfected 293T cells were incubated with 32P-labeled probe A, and the ability of Flag-tagged wild-type and mutant Smad4 to bind to DNA was tested by EMSA (lanes 1–6). Antibody supershift was performed using 1 μg of anti-Flag (M2) antibody (lane 4). The gel was exposed for 1 h. Right panel, approximately 1 μg of Flag-tagged full-length and truncated Smad3 proteins isolated from transfected 293T cells were subjected to EMSA assay with 32P-labeled probe A (lanes 7–11). The gel was exposed for 3 h. Lane 12, a longer exposure (12 h) of lane 9.
As with bacterially expressed Smad3 and Smad4, Flag-Smad3 and Flag-Smad4 isolated from transfected 293T cells bound to both site I and site II in the PAI-1 promoter in a cooperative manner and with affinities similar to those of the bacterially expressed proteins (lanes 1–3, 7–9, and 12, Fig. 2F). Addition of an anti-Flag antibody to the reaction induced a complete supershift of the Smad4-DNA complex (lane 4, Fig. 2F), confirming that the observed DNA-protein complex contained Flag-Smad4. Also similar to recombinant Smad proteins, the N domains of Flag-Smad3 and Flag-Smad4 were responsible for DNA binding (lanes 5 and 10, Fig. 2F), while the C domains failed to bind (lanes 6 and 11, Fig. 2F). The N domain of Flag-Smad3 or GST-Smad3N bound probe A with a higher affinity than the full-length protein (compare lane 7 with lane 10, Fig. 2F, and lane 1 with lane 5, Fig. 2C), suggesting that the Smad3 C domain may inhibit DNA binding by the N domain. However, in contrast to Smad3, the N domain of Flag-Smad4 bound no better or even weaker than the full-length Smad4 (compare lane 1 with lane 5, Fig. 2F). It is difficult to estimate the amount of GST-Smad4N used in the EMSA assay due to the presence of many shorter protein fragments co-purified with the full-length Smad4N (lane 4, Fig. 2E). Since some of these fragments may contain DNA binding activity, it is difficult to compare the DNA binding activity between GST-Smad4N and GST-Smad4.

Taken together, our results suggest that Smad4 and to a lesser extent, Smad3, purified either from bacteria or from mammalian cells, bind specifically to two adjacent DNA elements in the PAI-1 promoter in a cooperative manner, and this interaction is mediated by the NH₂-terminal domains of Smad3 and Smad4.

Fig. 2—continued
ping with the AP1-like Site Located at −670/−664 in the PAI-1 Promoter—Some AP1 or AP1-like sites contain overlapping Smad boxes and have been shown to mediate TGFβ responsiveness (45). The TGFβ-responsive region in the PAI-1 promoter between −670 and −653 contains two AP1-like sites (35). We have shown that efficient binding of Smad3 and Smad4 to probe A requires sequences in the first AP1-like site. This AP1-like site contains a degenerate Smad box (GTCA) with a single base pair mismatch. The second AP1-like site located 3′ to site II also contains a degenerate Smad box (GTAT) (34). To determine whether Smad proteins could recognize this sequence, a second set of probes (probe B) were synthesized and used to test binding to the 32P-labeled probes as indicated. Binding of Flag-Smad3 and Flag-Smad4 to wild-type and mutant PAI-1 promoter −740/−647 region and to wild-type and mutant probe D. EMSA was performed as described using 0.4 μg of Flag-Smad4 and 1 μg of Flag-Smad3 isolated from transfected 293T cells. Probes used: lanes 1 and 3, full-length PAI-1 promoter −740/−647 region (FL); lanes 2 and 4, PAI-1 promoter −740/−647 region containing point mutations in sites I and II (M); lanes 5 and 8, Flag-Smad4 and Flag-Smad3 binding to probe A; lanes 6 and 9, Flag-Smad4 and Flag-Smad3 binding to wild-type probe D; and lanes 7 and 10, Flag-Smad4 and Flag-Smad3 binding to mutant probe D.

**Additional Binding Sites within the 94-bp TGFβ Responsive Region of the PAI-1 Promoter**—To confirm that sites I and II are indeed the major Smad-binding sites in the 94-bp region, a full-length probe containing mutations in both site I and site II (probe M) was tested for binding to Smad3 and Smad4 in the EMSA assay. Mutations of both site I and site II together greatly reduced binding by Smad4 (lane 2, Fig. 3B) and Smad3 (lane 4, Fig. 3B), confirming that sites I and II are the predominant Smad-binding sites, but that additional sequences outside the region covered by probe A may also mediate a low affinity binding to Smads. Since probes C and D showed a weak binding to Smads (Fig. 1), and the region covered by both probes contains the CAGA sequence (−732 to −725) described by Dennler et al. (32) and the 12-bp sequence by Song et al. (37) that interacts with GST-Smad3N and GST-Smad4, this CAGA sequence is likely responsible for the residual low affinity binding. Indeed, mutation of the CAGA site in probe D completely abolished binding to Smad3 (lane 10, Fig. 3B) and Smad4 (lane 7, Fig. 3B). Compared with probe A, binding of Flag-Smad3 and Flag-Smad4 to probe D was weaker by at least 10-fold (lanes 5, 6, 8, and 9, Fig. 3B). Thus, although the CAGA sequence can mediate a low affinity binding to Smads, sites I and II are the major sites recognized by Smad3 and Smad4. Taken together,
these data indicate that the 94-bp PAI-1 promoter region contains three Smad-binding sites. This is consistent with a previous observation that this region of the PAI-1 promoter contains multiple TGFβ response elements (35).

*The Two Smad-binding Sites in the PAI-1 Promoter Are Essential for TGFβ-induced, Smad3-, and Smad4-dependent transcriptional activation*—To determine whether the two Smad-binding sites we identified are necessary for TGFβ-induced activation of PAI-1 gene expression, luciferase reporter constructs that contained four copies of the wild-type or mutant PAI-1 promoter DNA sequences were transfected with 2 µg of luciferase reporter constructs listed in A and stimulated with 100 µM TGFβ1 for 16 h. Luciferase activity was measured as described under “Experimental Procedures.” The experiment was repeated 5 times and one representative set of results are shown. C, Hep3B cells were co-transfected with the 4x wild-type luciferase reporter construct and various Flag-tagged Smad proteins. Luciferase activity was measured 48 h after transfection in the absence of TGFβ1. The addition of TGFβ1 did not further increase the level of transactivation. D, Hep3B cells were co-transfected with wild-type or mutant 4x wild-type luciferase reporter constructs together with Flag-Smad3 and Flag-Smad4. Luciferase activity was assayed 48 h after transfection in the absence of TGFβ1.

**Fig. 4.** The two Smad-binding sites in the human PAI-1 promoter are required for TGFβ-induced, Smad3- and Smad4-dependent transcriptional activation. A, luciferase reporter constructs used in the transactivation experiments contain four copies of the wild-type or mutant PAI-1 promoter DNA sequences. B, Hep3B cells were transfected with 2 µg of luciferase reporter constructs listed in A and stimulated with 100 µM TGFβ1 for 16 h. Luciferase activity was measured as described under “Experimental Procedures.” The experiment was repeated 5 times and one representative set of results are shown. C, Hep3B cells were co-transfected with the 4x wild-type luciferase reporter construct and various Flag-tagged Smad proteins. Luciferase activity was measured 48 h after transfection in the absence of TGFβ1. The addition of TGFβ1 did not further increase the level of transactivation. D, Hep3B cells were co-transfected with wild-type or mutant 4x wild-type luciferase reporter constructs together with Flag-Smad3 and Flag-Smad4. Luciferase activity was assayed 48 h after transfection in the absence of TGFβ1.
greatly reduced binding of Smad3 and Smad4, and the mutant promoter was unable to respond to TGFβ1 (M3, Fig. 4B). These results indicate that the two Smad-binding sites in the PAI-1 promoter we identified were necessary for TGFβ-induced transcriptional activation.

To examine whether transactivation mediated by the two Smad-binding sites are dependent on Smad3 and Smad4, we co-expressed various Smad proteins together with the luciferase reporter construct containing four copies of the wild-type PAI-1 promoter sequence. Consistent with their DNA binding activity, overexpression of Smad3 and Smad4 in Hep3B cells activated PAI-1 promoter activity (Fig. 4C). Smad2 failed to bind to this promoter sequence (data not shown), and it did not activate PAI-1 transcription (Fig. 4C). Since the N domains of Smad3 and Smad4 are responsible for DNA binding, we next examined whether they can activate transcription from this PAI-1 promoter region. Expression of Smad3N or Smad4N alone (data not shown), or both together (Fig. 4C), activated PAI-1 transcription to the same extent as did the full-length Smad3 and Smad4. In contrast, expression of the COOH-terminal domains of Smad3 and Smad4 did not activate PAI-1 transcription, consistent with the lack of DNA binding activity by these C domains (Fig. 2).

To determine whether Smad3- and Smad4-induced transactivation is dependent on the two Smad-binding sites, we co-expressed Smad3 and Smad4 with various mutant PAI-1 promoters in Hep3B cells (Fig. 4D). Mutation of site II alone resulted in a 60% reduction in transactivation while alteration of site I, or both site I and site II abolished the ability of Smad3 and Smad4 to activate PAI-1 transcription (Fig. 4D). Therefore, direct binding of Smad3 and Smad4 to the two Smad-binding sites in the PAI-1 promoter through their NH2-terminal domains is required for transactivation.

Homologimerization of Smad3 Enhances Binding of Smad3 to the Two Smad-binding Sites—Upon ligand stimulation, Smad3 can be phosphorylated by the activated TβRI and form homo- as well as heterologomers. To investigate whether activation by TβRI affects the DNA binding activity of Smad3, Flag-Smad3 was co-transfected with or without the constitutively active TβRI(T204D), and the ability of Smad3 to bind to 32P-labeled probe A was examined by EMSA. Co-transfection of the active TβRI significantly enhanced binding of Smad3 to DNA (lanes 1 and 2, Fig. 5A), suggesting that phosphorylation and oligomerization of Smad3 may increase the affinity of Smad3 for DNA. To investigate the effects of homo- and heterologimerization of Smad proteins on DNA binding, Smad3 mutants containing point mutations changing Arg-268 to Cys, Val-277 to Asp, or Asp-408 to Glu were generated. These amino acid residues localize at the trimer interface and may mediate homologimerization and are conserved among Smad2, Smad3, and Smad4 (46). Point mutations of these three residues in Smad2 and Smad4 were originally identified in human cancer and may abolish both homo- and heterologimerization of Smad proteins (46).

The ability of Smad3 containing the Arg (R268C), Val (V277D), or Asp (D408E) mutation to bind DNA was tested next by EMSA with labeled probe A (Fig. 5A). Mutant Smad3 proteins isolated from co-transfected 293T cells (lanes 3–5, Fig. 5A) exhibited much weaker binding to probe A than wild-type Smad3 (lane 2, Fig. 5A), suggesting that the wild-type DNA-Smad3 complex is due to the activity of the Smad3 homologimers and that oligomerization of Smad3 greatly increases the affinity of Smad3 for DNA. Consistent with the decreased binding activity, the ability of the R268C and V277D mutants to mediate transscriptional activation was also reduced (Fig. 5C).

Similar Arg, Val, and Asp mutations were introduced into Smad4. These mutations were found previously to abolish both homo- as well as heterologimerization of the Smad4 C domain (15). As expected, we found that these mutant Smad4 were defective in heterologimerization with Smad3 (right panel, Fig. 5B). However, they did not disrupt homologimerization of the full-length Smad4 (Fig. 5B), probably because the N domain of Smad4 can also mediate oligomerization (15). These mutant Smad4 proteins bound to probe A in a manner indistinguishable from that of wild-type Smad4 (lanes 6–10, Fig. 5A), suggesting that heterotrimerization of Smad4 with Smad3 is not essential for Smad4 to bind DNA. However, since these mutants still form homologimers, it is not clear whether homologimerization is required for Smad4 to bind DNA. In transactivation assays, these two mutants displayed a slightly reduced transactivation activity than did the wild-type Smad4 (Fig. 5C). Therefore, heterologimerization of Smad4 with Smad3 does not affect direct binding to DNA, but may be required for optimal transactivation in vivo.

A Mutation in Smad4, R100T, Identified Originally in a Human Pancreatic Carcinoma Abolished the DNA Binding Activity of Smad4—Mutations in Smad4 have been detected in many types of human cancer. One mutation, R100T, found in a pancreatic carcinoma, is located in the N domain and was reported to inactivate Smad4 function through an autoinhibitory mechanism (47). We asked whether the R100T mutation affects the ability of Smad4 to bind DNA. As shown in Fig. 6A, Flag-tagged, full-length as well as the NH2-terminal domain of Smad4 containing the R100T mutation isolated from transfected 293T cells failed to bind DNA (lanes 2 and 4, Fig. 6A). The lack of DNA binding could be due to disruption of the Smad4 DNA binding activity by the point mutation or due to the autoinhibitory effect, e.g. blocking of the Smad4 N domain by the interacting Smad4 C domain or by other interacting molecules present in the transfected 293T cells. To distinguish between these two possibilities, the recombinant N domain of Smad4(R100T) was purified from E. coli and was found to display no DNA binding activity (lane 8, Fig. 6A), suggesting that this point mutation directly destroyed the DNA binding activity of Smad4. Consistent with the DNA binding data, Smad4(R100T) failed to activate transcription from the PAI-1 promoter (Fig. 5C). Our results were consistent with those reported by Song et al. (37) and Kim et al. (30) that the R100T mutation destroys the DNA binding activity of Smad4, but did not agree with that reported by Shi et al. (34).

**DISCUSSION**

We have identified two major Smad-binding sites in the major TGFβ-responsive region of the PAI-1 promoter which interact with Smad3 or Smad4 in a cooperative manner and are required for transactivation in a TGFβ-induced, Smad3 and Smad4-dependent manner. One of the sites (site I) contains a SBE-like element (GTCTGGAC) with a perfect Smad box and a single base pair mismatch (underlined nucleotide). This single nucleotide mismatch results in a significant decrease in Smad binding (33). Interestingly, the second Smad-
The effects of homo- and heteroligomerization of Smad3 and Smad4 on binding to the two Smad-binding sites. 

A. EMSA assay was performed with 32P-labeled probe A and an equal amount of wild-type and mutant Smad3 isolated from singly (lane 1) or co-transfected 293T cells (lanes 2–5), and wild-type and mutant Smad4 (lanes 6–10). B, homo- and heteroligomerization of Smad3 and Smad4 mutants. HA- or Flag-tagged Smad3 or Smad4 proteins were isolated by immunoprecipitation from co-transfected 293T cells and analyzed by Western blotting as indicated. C, Hep3B cells were co-transfected with the 4x wild-type luciferase reporter construct and wild-type or mutant Smad3 (left panel) and Smad4 (right panel). Luciferase activity was measured 48 h after transfection.
binding site we identified (site II) is part of an AP1-like motif. This AP1-like site has been suggested to play a role in mediating TGFβ-induced transactivation of both the PAI-1 gene (35) and type I collagen gene (44). The Smad box in this site contains a mismatch that should decrease its affinity for the Smad proteins significantly (34). Indeed, compared with site I, the binding of Smad3 and Smad4 to site II was markedly weakened. Although the affinity of Smads to either site I or site II alone is not optimal, the cooperative effect mediated by multiple sites present in close proximity may increase the affinity significantly. Interestingly, although another degenerate Smad box (GTAT) is present in another AP1-like site located 3′ to site II, this sequence did not mediate Smad binding. Another degenerate Smad box (AGCC) located immediately 3′ to site II was not recognized by Smad proteins either, since a mutant probe (M4) containing this sequence but lacking site II did not bind Smad proteins (Fig. 3A). Therefore, sequences surrounding the core Smad box also play an important role in the recognition of Smad proteins.

Dennler et al. (32) have identified three CAGA containing sequences in the PAI-1 promoter that mediate binding to Smad3N and Smad4, and are required for TGFβ-induced transcriptional activation of the PAI-1 promoter. One of the three CAGA sequences is located in this major TGFβ responsive region of the PAI-1 promoter. Song et al. (37) also reported a 12-bp sequence overlapping with the CAGA sequence that mediates binding of Smad3 and Smad4. Compared with the two Smad-binding sites we identified, however, Smad binding mediated by these motifs is much weaker. Therefore, under our experimental conditions, the major Smad binding activity is mediated by sites I and II.

Our result that the two Smad-binding sites acted cooperatively to mediate binding of Smad4 and to a lesser extent, Smad3, differs from the report by Shi et al. (34) that two repeats of the Smad box did not display cooperativity in mediating binding of the MH1 domain of Smad3. The apparent difference could be due to the different Smad protein preparations used in the EMSA assays. First of all, only part of the MH1 domain of Smad3 (amino acid residues 1–145) was tested by Shi et al. (34) while our experiments used full-length Smad4 and Smad3. We found that the cooperativity of the two sites in mediating binding of Smads is more prominent with full-length Smad proteins and much less so with the MH1 domains alone (Fig. 2, B and C, and data not shown). This cooperativity requires the C domains of the Smads and could be due to homoligomerization of full-length Smad proteins. Consistent with this hypothesis, disruption of oligomerization of Smad3 by mutation resulted in a decreased affinity for the two sites and a reduced mobility shift. These mutants also exhibited a much decreased ability to activate transcription.

**Direct DNA Binding by Smads Is Critical for Transcriptional Activation by TGFβ**—Although several reports have demonstrated that bacterially produced Smad3 and Smad4 can bind DNA directly (30–33, 49), it is not clear whether this binding activity is critical for transcriptional activation. Our findings that the NH2-terminal domains of Smad3 and Smad4 directly bound to two adjacent DNA sequences in the PAI-1 promoter and activated transcription in response to TGFβ directly link the DNA binding activity of Smads with their ability to transactivate. The C domains of Smad3 and Smad4 did not bind DNA, and failed to activate transcription from the two Smad-binding sites (Fig. 4C). Our result is consistent with a previous report that the N domain of Smad4 possessed transactivation activity both in yeast and in mammalian cells (15). The N domain of Smad3 was found to interact with c-Jun (27) and the N domain of Smad4 was shown to interact with CBP/p300 (28). These interactions may play a role in transactivation by the N domains of Smad proteins. The C domains of Smad3 and Smad4 have been shown by many studies to contain transactivation activity. The C domain of Smad3 has been shown to interact with general transcription factors including Sp1 (26), AP1 (27), and CBP/p300 (28, 29). The Smad3 C domain may cooperate with these proteins to activate transcription. The N domains of Smad proteins could activate transcription by a mechanism different from that used by the C domains.

Multiple mechanisms may be involved in TGFβ-induced ac-
activation of gene expression. The PAI-1 promoter contains many regions that can mediate TGFβ responses. In addition to the three CAGA sequences (32) and the two Smad-binding sites we identified here, an E box region that interacts with the transcription factor TFE3 and Smad3 was also shown to mediate TGFβ responses (48). Therefore, it is possible that Smad proteins may be present in multiple transcription complexes on different regions of the promoter and activate transcription through different mechanisms.

We have shown that the two Smad-binding sites in the PAI-1 promoter are necessary for TGFβ-induced transactivation. Consistent with the DNA binding activity, mutation of either one of the two sites decreases TGFβ-induced transcriptional activation, while mutation of both sites abolished this activity completely. Although the two Smad-binding sites act cooperatively to mediate binding of Smad proteins, no cooperativity in transactivation is apparent. It is possible that binding of Smad proteins to DNA is not the rate-limiting step during transcriptional activation, and interaction of Smad proteins with other cellular factors may stabilize their binding to DNA in vivo.

We have found that a point mutation in Smad4, R100T, originally identified in a human pancreatic carcinoma, abolished DNA binding activity of Smad4, and this mutant Smad4 cannot mediate activation of PAI-1 transcription. Since this residue is not directly involved in contacting DNA (34), R100T mutation is likely to affect the conformation of the DNA-binding domain of Smad4, rendering it incapable of binding to DNA. Partial proteolytic analysis revealed changes in sensitivity to protease digestion (data not shown), suggesting that the R100T mutation caused conformational changes in Smad4. Our result is consistent with those reported by Song et al. (37) and Kim et al. (30), but not with that by Shi et al. (34). Taken together, these results provide a good correlation between DNA binding activity and the ability to transactivate Smads.

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