Regulation of Plasminogen Activator Inhibitor-1 Expression by Transforming Growth Factor-β-induced Physical and Functional Interactions between Smads and Sp1*

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Members of the transforming growth factor-β (TGF-β) superfamily mediate a broad range of biological activities by regulating the expression of target genes. Smad proteins play a critical role in this process by binding directly to the promoter elements and/or associating with other transcription factors. TGF-β up-regulates several genes transcriptionally through Sp1 binding sites; however, the mechanism of TGF-β induction of gene expression through Sp1 sites is largely unknown. Here we report the identification of a novel 38-base pair TGF-β-responsive element in the human plasminogen activator inhibitor-1 (PAI-1) promoter, which contains two Sp1 binding sites, and is required for TGF-β-induced Smad-dependent transcriptional activation. Three canonical Sp1 binding sites also support strong transcriptional activation by TGF-β and Smads from a minimal heterologous promoter. TGF-β induction of PAI-1 and p21 is blocked by the Sp1 inhibitor mithramycin, implicating Sp1 in the in vivo regulation of these genes by TGF-β. We show that the association between endogenous Sp1 and Smad3 is induced by TGF-β in several cell lines; however, Smad4 shows constitutive interaction with Sp1. These data provide novel insights into the mechanism by which TGF-β up-regulates several gene expression by activating Sp1-dependent transcription through the induction of Smad/Sp1 complex formation.

Extracellular matrix (ECM) remodeling plays an important role in several biological processes ranging from cell proliferation and differentiation to cell adhesion, migration, and tissue morphogenesis (1). ECM is a complex and dynamic meshwork of several proteoglycans and proteins, and its degradation is inhibited by TGF-β. TGF-β stabilizes ECM by down-regulating the expression of the ECM proteases and by stimulating the expression of some ECM protease inhibitors, including the plasminogen activator inhibitor (PAI-1) (2). PAI-1 is the primary inhibitor of both tissue-type and urokinase-type plasminogen activator. The induction of PAI-1 mRNA and protein by TGF-β has been observed in several types of cultured cells (3). Recent work has identified potential consensus Smad3-Smad4 DNA binding motifs in the distal region of the PAI-1 promoter, AG(C)/ACAGACA (4) and AGACAAGTGTG (5), which contain the 4-base pair AGAC element in common and have been shown to mediate TGF-β-transcriptional induction.

Information about the mechanism of TGF-β-mediated activation of transcription of target genes is increasing rapidly (6). Although several studies demonstrate that Smad3-Smad4 binding sites are required for TGF-β-mediated promoter regulation, they do not completely explain the broad spectrum of TGF-β responsiveness in activating target genes. Biochemical studies, under conditions of overexpression, have suggested that a Smad3-Smad4 complex and an AP-1 complex synergize in the transcriptional activation from the c-Jun promoter (7). Cooperation between Smad2- or Smad3-Smad4 complexes and other sequence-specific DNA-binding proteins such as FAST1 and FAST2 has been demonstrated to regulate TGF-β-responsive promoters (8-10). Although no physical association between the basic helix-loop-helix protein TFE3 and Smad3/Smad4 has been shown, all three proteins interact with a segment of PAI-1 promoter to induce transcription in response to TGF-β (11). AML-Smad complexes target the IgA promoter in response to TGF-β (12). Several reports demonstrate that Smads can also activate transcription from specific promoters in response to TGF-β by interacting with non-DNA-binding proteins (6).

Functional analysis of the promoters of some TGF-β target genes has led to the identification of putative cis-acting TGF-β response elements and transacting factors associated with those elements. TGF-β1 activates the transcription of several cell cycle regulatory genes, including the cyclin-dependent kinase inhibitors p21 and p15 genes (13–15) or genes involved in extracellular matrix formation including the α2(I) collagen, fibronectin, and PAI-1 genes (2, 3, 16–18). Some of these studies have provided convincing evidence that Sp1 consensus sites in several promoters are required to support TGF-β-mediated activation. However, it is not known how Sp1 binding sites in those promoters mediate the TGF-β transactivating signal or how TGF-β signaling and the transcription factor Sp1 is linked to the TGF-β-regulated expression of various genes. In the present study, we elucidate a novel mechanism by which TGF-β up-regulates gene expression through Sp1 binding sites. We have identified a 38-base pair element containing two Sp1 binding sites in the proximal region of PAI-1 promoter, which is required, in part, for TGF-β-mediated induction of this promoter. Mithramycin, an inhibitor of Sp1-DNA binding, blocks TGF-β stimulation of PAI-1 and p21 proteins. Here, we show that Sp1 interacts with Smad3 and Smad4 in vivo and that the
association between endogenous Smad3 and Sp1 proteins is induced by TGF-β in several cell lines. Our results indicate that TGF-β-induced physical and functional interactions between Sp1 and Smads may explain how TGF-β induces gene expression through Sp1 binding sites.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Mv1Lu, HepG2, COS-1, 293, NIH 3T3, and rat intestinal epithelial (RIE) cells were obtained from the American Type Culture Collection and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Mv1Lu and HepG2 cells were supplemented with nonessential amino acids. Cells were transfected as described previously (19).

**Immunoprecipitation and Immunoblot Analyses**—COS-1 cells were transfected with expression constructs as indicated. After 40 h cells were washed, scraped, and solubilized in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5 mM dithiothreitol, 5 mM sodium fluoride, 0.5 mM sodium orthovanadate, 1.0 mM phenylmethylsulfonl fluoride, 2 µg (each) of leupeptin, pepstatin, and aprogin per ml). Cleared cell lysates were incubated with anti-FLAG M2 monoclonal antibody (Sigma), anti-HA polyclonal antibody (T11, Santa Cruz Biotechnology), or anti-Sp1 polyclonal antibody (Santa Cruz Biotechnology) for 2 h at 4 °C, followed by incubation with protein G-Sepharose (Sigma) for 1 h. Precipitates were analyzed by immunoblotting as described previously (19). For in vivo interaction assays, anti-Smad3 polyclonal antibody (Zymed Laboratories Inc.), anti-Smad4 monoclonal and polyclonal antibodies, anti-Sp1 polyclonal antibody, and anti-HDAC1 polyclonal antibody (Santa Cruz Biotechnology) were used for immunoprecipitation and immunoblotting. Anti-Polyclonal antibody (Torrey Pines Biologals) and anti-p21 polyclonal antibody (Santa Cruz Biotechnology) were used for detecting endogenous proteins after treating the cells with TGF-β for 6 h and mithramycin for 24 h before lysis in low serum.

**Transcriptional Response Assays**—Sp1-luc, PAI-1 promoter constructs, 806 PAI Luc, and 806 PAI Δb1+Δb2+Δb3 Luc have been described (4, 20, 21). HepG2 and Mv1Lu cells were transiently transfected with various constructs as indicated and pCMV-β-gal using the calcium phosphate and DEAE-dextran method, respectively. In each experiment equal amounts of total DNA were transfected. Twenty hours after transfection, cells were incubated in medium containing 0.2% fetal bovine serum with 200 µM TGF-β, mithramycin (150 nM), and trichostatin A (400 nM) as indicated. Luciferase activity and β-galactosidase activity were measured as described previously (19). Luciferase activity was normalized to β-galactosidase activity to control for differences in transfection efficiency.

**RESULTS**

Previous studies have shown that the Sp1 binding sites present in several promoters are required for TGF-β-induced gene expression (13–16). To better understand how TGF-β activates target genes through Sp1 binding sites, we first examined the effect of TGF-β and Smads on a promoter containing three Sp1 consensus sites placed upstream of a luciferase gene (Fig. 1A). Transient transfection of this reporter into HepG2 cells resulted in a low basal level of transcription, which was less effective than the wild type version in inducing promoter activity (Fig. 1B). Transfection of either Smad2 or Smad4 had little effect on the Sp1-luc promoter activity, either in the absence or presence of TGF-β. Coexpression of Smad2 or Smad4 with Smad3 did not potentiate Smad3-dependent transcriptional activation. TGF-β induces phosphorylation of serine residues at the carboxyl terminus of Smad3, which is essential for signaling (22). Smad3(S-A), a receptor-mediated phosphorylation incompetent mutant, was unable to activate wild type Smad3-dependent promoter activity (Fig. 1B). We observed similar effects of TGF-β and Smads on other heterologous promoters containing one or two Sp1 sites (data not shown). These data suggest that Smad3 is required for TGF-β-mediated activation of Sp1 sites independent of Smad DNA binding activity.

To determine whether Sp1-dependent transactivation is involved in the TGF-β stimulation of PAI-1 gene expression, we carried out mutational analyses of the PAI-1 promoter. A schematic diagram of the PAI-1 promoter 5'-flanking region and a detailed picture of major regulatory elements in the proximal promoter is shown in Fig. 1A. Previous analyses have shown that the sequence between -791 and -187 contains three Smad binding CAGA box elements involved in the induction of PAI-1 promoter in response to TGF-β (4, 5, 11). There are four recognized regulatory elements in the proximal region: the −82 and −40, including a C-box (cAMP response element), a T-box (weak affinity AP-1 site), and two Sp1 sites (21). Four base substitutions in either Sp1-A or Sp1-B abolishes binding of Sp1 (data not shown and Ref. 21) to those respective sites. HepG2 cells were transiently transfected with these deleted/mutated promoter constructs and Smad3 expression vector and assayed for responses to TGF-β and Smad3. Two pro-
moter constructs pGL 1.3k and pGL 884, which contain three CAGA box elements, exhibited strong induction by TGF-β and/or Smad3 as expected (Fig. 1C). Deletion of the promoter sequences from −1.3 kilobases to −85 bases resulted in a 46% decrease in basal promoter activity, and importantly this deleted promoter was still strongly activated by TGF-β and Smad3 (22-fold), suggesting the presence of TGF-β-responsive elements within this proximal region. The mutant pGL 85 mSp1A, which contains a four-base substitution in the first Sp1 site (Sp1-A), showed little decrease in promoter activity in the absence of TGF-β and retained partially (5-fold) the ability to be induced by TGF-β and Smad3. A substitution mutation in the second Sp1 site (Sp1-B) resulted in significant loss of promoter activity (Fig. 1C). However, this mutant was significantly activated by TGF-β and Smad3 (12-fold). Mutations in both Sp1 sites resulted in a complete loss of promoter activity both in the absence and presence of TGF-β and Smad3. Taken together, these data suggest that both Sp1 sites in the proximal region (−77 to −40) are required, in part, for full induction of the PAI-1 promoter in response to TGF-β.

The wild type human PAI-1 promoter contains three Smad binding CAGA box elements in the distal region (4). To confirm the importance of the Sp1 consensus sites in the proximal region in conferring transcription inducibility of the PAI-1 promoter by TGF-β, we used wild type PAI-1 promoter construct (806 PAI Luc) and a mutant promoter construct (806 PAI Δb1+Δb2+Δb3 Luc), where all three Smad binding sites are mutated. The wild type promoter, containing intact distal and proximal regions, was induced by TGF-β both in absence (8.7-fold) and presence of Smad3 (18-fold) (Fig. 1D). When all three Smad sites were mutated, the mutant promoter was still capable of being induced by TGF-β in the absence (3-fold) and presence of Smad3 (6.5-fold). Together with the promoter deletion analysis, these data demonstrate that in addition to the Sp1 binding sites, Sp1 consensus sites in the proximal region are also important for the mediation of TGF-β induction of the PAI-1 gene.

To further investigate the involvement of Smad proteins in the TGF-β-induced regulation of PAI-1 promoter through Sp1 sites in the proximal region, HepG2 cells were cotransfected with the reporter pGL 85 along with expression vectors for Smad proteins and the dominant negative type II receptor (DNIIR). TGF-β induced the promoter activity by 3.6-fold. Co-expression of Smad3 alone resulted in a dramatic increase in the luciferase activity, both in the presence and absence of TGF-β (Fig. 2A). In contrast, only a slight induction of the promoter activity by either Smad2 or Smad4 was observed. Cotransfection of Smad2 and/or Smad4 with Smad3 did not have any significant effect on Smad3-induced promoter activity. Activation of Smad3 through TGF-β-induced phosphorylation was required in the induction of the minimal PAI-1 promoter, because mutations in the COOH-terminal serine residues (Smad3(S-A)) blocked the ability of Smad3 to transactivate the promoter in presence or absence of TGF-β. Smad7 or DNIIR, which inhibits TGF-β signaling, abrogated TGF-β-mediated induction of transcription from the minimal (pGL 85) promoter. These data, in combination with the results discussed above, indicate that Smad3 is essential in the transactivation of PAI-1 promoter by TGF-β through Sp1 binding sites. Moreover, our results strongly support the notion that within this context, TGF-β-mediated transcriptional activation of the reporters does not depend on the DNA binding function of Smads, but rather on the ability of Smads to act through Sp1 sites as transcriptional coactivators.

To determine whether Sp1 binding to its DNA element was required for TGF-β and Smad-dependent activation of PAI-1 promoter, pGL 85 or Sp1-luc was cotransfected into HepG2 cells with Smad3. Cells were then treated with TGF-β and mithramycin, which inhibits Sp1-DNA binding by modifying GC-rich sites (16). Mithramycin blocked the activation of both reporters by TGF-β and Smad3 (Fig. 2B). Sp1 is known to repress transcription through its binding site by recruiting HDAC1 (23). It is tempting to hypothesize that TGF-β might induce Sp1-dependent transcription by dissociating the complex between Sp1 and HDAC1. But this does not appear to be the case, because: (a) TGF-β and Smad-mediated induction of the promoter activities were not enhanced by trichostatin A (TSA), a HDAC inhibitor (Fig. 2B); (b) the association between Sp1 and HDAC1 in vivo was not affected by TGF-β in HepG2, Mv1Lu, and 293 cells (Fig. 4D, third panel). Our findings demonstrate that Smad proteins transactivate the PAI-1 promoter by functionally interacting with Sp1 via the proximal −77/−40 region that contains two Sp1 binding sites. Previous studies showed that Sp1 binding sites are required for TGF-β activation of the p21 promoter (13, 14). To pinpoint the importance of Sp1-dependent transcription through binding to its consensus sites in inducing endogenous PAI-1 and p21 expression by TGF-β, we performed immunoblotting experiments with lysates from RIE cells after treating with TGF-β and mithramycin as indicated. TGF-β induced the expression of PAI-1 and p21 proteins as reported previously (3, 13). The induction of PAI-1 protein was partially inhibited by mithramycin (Fig. 3A, lanes 3 and 4). The lack of complete inhibition is likely because PAI-1 promoter is induced by TGF-β not only...
through Sp1 sites in the proximal region (our data) but also through the distal region where Smads can bind to the CAGA box elements directly (4, 5, 11). In contrast, induction of p21 by TGF-β, Smad4 strongly induced the promoter activity both in the presence or absence of TGF-β. A, COS-1 cells were transiently transfected with Sp1 expression constructs either alone or together with HA- or FLAG-tagged Smad constructs as indicated. Cell lysates were subjected to anti-FLAG or anti-HA immunoprecipitation (IP), and coprecipitated Sp1 was detected by immunoblotting (Blot) with anti-Sp1 antibody (first panel) from top). In the second panel, total lysates were immunoprecipitated using a Sp1-specific antibody and then immunoblotted with either an anti-HA or an anti-FLAG antibody. Expression of Smad proteins was monitored (third panel). B, COS-1 cells were transfected with HA-tagged Smad3 or Smad4 either alone or together with FLAG-tagged full-length Sp1 (wild type, W) or Sp1(1–612) (mutant, M) expression constructs. Cell lysates were immunoprecipitated with anti-FLAG antibody and then immunoblotted with anti-HA antibody (first panel). Expression of transfected plasmids was monitored as indicated (second and third panels). C, lysates from 293 or NIH 3T3 cells were subjected to immunoprecipitation (IP) with control rabbit IgG, anti-Sp1, anti-Smad3, or anti-Smad4 antibody as indicated. Coprecipitated proteins were subjected to anti-Sp1 antibody immunoblotting (first left and right panels). In the second panel, total lysates from 293 cells were immunoprecipitated with control rabbit IgG, anti-Sp1, or anti-Sp1 peptide (anti-Sp1 antibody was preincubated with the immunizing peptide). Coprecipitating Smad4 was detected by immunoblotting with anti-Smad4 monoclonal antibody. D, lysates from Mv1Lu, 293, or HepG2 cells, either untreated or treated for 1 h with 400 pM TGF-β, were subjected to immunoprecipitation with either anti-Smad3 (first left panel from top) or anti-Smad4 antibody (first right panel) as indicated. Coprecipitated proteins were analyzed by immunoblotting with anti-Sp1 antibody. The same blots were sequentially used for immunoblotting with an anti-HDAC1 antibody (third panel). The expression of Sp1 and Smad3 was not affected by TGF-β (fourth and fifth panels from top).

Our combined data demonstrated a functional interaction between Smads and Sp1 to induce TGF-β-mediated transcription through Sp1 binding sites in response to TGF-β. We observed similar results in HepG2 cells using another TGF-β-responsive reporter p3TP-Lux (26) (data not shown). These results suggest that Sp1 does not regulate Smad3/Smad4-dependent transcription through Smad binding elements in response to TGF-β.

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Smad2, which showed a barely detectable interaction. To examine which part of the Sp1 protein was involved in the interaction with Smads, we coexpressed FLAG-Sp1(1–778) (wild type, W) or FLAG-Sp1(1–612) (mutant, M) with HA-Smad3 or HA-Smad4 in COS-1 cells. In these experiments, Smad3 or Smad4 was coimmunoprecipitated with wild type Sp1 (lanes 3 and 6), but not with mutant Sp1, Sp1(1–612) (Fig. 4B, lanes 4 and 7), suggesting the involvement of the carboxyl terminus of Sp1 in binding with Smad3 and Smad4. We also tested for interaction between endogenous Sp1 and Smad3 or Smad4. For this, Smad3 and Smad4 were immunoprecipitated with their corresponding antibodies from 293 and NIH 3T3 cell lysates (Fig. 4C, first panel). Sp1 was detected by immunoblotting anti-Sp1, anti-Smad3, and anti-Smad4 immunoprecipitates. In reciprocal experiments, Smad4 was detected in the anti-Sp1 antibody immune complex precipitated from 293 cell lysate using an anti-Smad4 antibody (Fig. 4C, second panel). Under similar conditions, no Smad4 was detected when the anti-Sp1 antibody was preincubated with the immunizing peptide. In an attempt to determine whether the association between endogenous Sp1 and Smads is regulated by TGF-β, Smad3 or Smad4 was immunoprecipitated from Mv1Lu, 293, or HepG2 cells treated with or without TGF-β. Coprecipitated Sp1 was increased significantly in the Smad3 immune complexes from TGF-β-treated cells (Fig. 4D, left first panel, lanes 1–6), although the levels of Sp1 and Smad3 were not changed in response to TGF-β (fourth and fifth panels from top). In contrast, coprecipitation of Sp1 with Smad4 was not affected following TGF-β treatment (first right panel, lanes 7–10). TGF-β treatment of the cells increased the association of Smad3 with Smad4 as described previously (second left panel) (6). Since Sp1 has been shown to function as a transcriptional repressor by recruiting HDAC1 (23), we investigated whether TGF-β could increase Sp1-dependent transcription by dissociating the complex between Sp1 and HDAC1. We did not observe any change in the coprecipitation of HDAC1 with Sp1 under these conditions (Fig. 4D, third panel). Thus, the association between Sp1 and Smad3, but not between Sp1 and Smad4, is induced by TGF-β. These results suggest a mechanism for TGF-β-induced transcription of several genes through the Sp1 binding sites in their promoters.

**DISCUSSION**

The ability of Smads to induce specific transcriptional programs in response to TGF-β results from a functional cooperativity with other transcription factors in multiprotein complexes in the nucleus (6). TGF-β modulates extracellular proteolytic activity, which may be linked to the regulation of matrix formation by the coordinated regulation of plasminogen activators and their inhibitors, including PAI-1. For induction of the PAI-1 promoter by TGF-β, several promoter elements in the distal region have been identified, which require direct Smad3 and Smad4 binding (4, 5, 11). Here, we have identified a promoter element in the proximal region (~77 to ~40), which contains two Sp1 binding sites that are required for TGF-β-induced and Smad-dependent transcriptional activation of the PAI-1 promoter. The requirement of Sp1-dependent transcription in TGF-β-induced gene expression is confirmed by the fact that the expression of endogenous PAI-1 and p21 is decreased dramatically by mithramycin, an inhibitor of Sp1-DNA binding. We demonstrate that Smads function as transcriptional coactivators for TGF-β-mediated induction of PAI-1 promoter through Sp1 sites.

Sp1 consensus sites are required in the regulation of numerous genes, including many inducible genes, housekeeping genes, and cellular proto-oncogenes (13–16, 24, 25). The specificity of the gene regulation by Sp1 is most probably determined by cofactors or activators that interact with Sp1 or by the combinatorial effects of Sp1 sites and other regulatory elements within the promoter. Although Sp1 is thought to be necessary for the basal transcription of many genes, this work supports the hypothesis that Sp1 can also play a key role in the regulation of certain genes in response to specific signals. There could be several possible mechanisms by which TGF-β activates gene expression through the transcription factor Sp1: 1) Sp1 might activate Smad3/Smad4-dependent transcription through Smad binding sites in the TGF-β-responsive promoters by interacting with Smads and by functioning as coactivator, 2) the DNA binding activities of Sp1 might be altered by TGF-β, 3) the repressive effect of Sp1 through the recruitment of HDAC1 might be relieved by TGF-β, or 4) the transactivating activity of Sp1 through its own binding to the GC-rich elements in the promoter might be regulated by TGF-β signal through specific interaction between Sp1 and Smads.

The first possibility of TGF-β-mediated activation through Sp1 was not supported by our data. We did not see any significant regulation of Smad3/Smad4-dependent transcription through Smad binding to the promoter by Sp1 in response to TGF-β, as measured by the pSFP-Lux (data not shown) and (CAGA)₉ MLP-Luc reporters (Fig. 3C). Our data indicate that both Sp1 sites in the proximal PAI-1 promoter are required, in part, for TGF-β- and Smad-mediated transcriptional activation. Mutation of both Sp1 sites in the proximal promoter abrogated TGF-β responsiveness. Our work (data not shown) and previous work (21) indicated that Sp1 does not bind to these mutated sites. Although both Sp1 and Sp3 are ubiquitously expressed and bind to the same GC boxes, the possibility of Sp3 being involved in this TGF-β-mediated induction is ruled out by previous reports (15, 24), demonstrating that Sp3 is generally a transcriptional repressor, and that Sp1, but not Sp3, mediates the responsiveness to TGF-β.

We have shown that mithramycin blocks TGF-β- and Smad-induced Sp1 site containing promoter activity, including PAI-1 promoter (Fig. 2B), and partially inhibits TGF-β stimulation of endogenous PAI-1 (Fig. 3A). In contrast, TGF-β induction of p21 is almost completely inhibited by mithramycin (Fig. 3B). This differential inhibition by mithramycin is likely because the PAI-1 promoter is induced by TGF-β significantly through Smad binding sites in the distal region (4, 5, 11), and partially through proximal region containing Sp1 sites (our data and 18), whereas p21 promoter is induced by TGF-β through the Sp1 sites (13, 14). Since mithramycin inhibits Sp1-DNA binding by modifying GC-rich sites, it could be argued that Sp1 binding to its consensus sequence in several promoters is required for TGF-β-mediated transcriptional activation through Sp1 binding sites. Previous studies demonstrated that TGF-β treatment of cells did not change either the DNA binding activities of Sp1 or overall phosphorylation of Sp1 (15). Our findings that the association between Sp1 and HDAC1 is not affected by TGF-β, and that the HDAC inhibitor trichostatin A has no effect on Sp1-dependent transcription in response to TGF-β, suggest that relieving HDAC1-mediated repression is not involved in TGF-β-induced Sp1-dependent transcription. We demonstrate that Smad3 and Smad4 bind Sp1 in vivo and that the association between endogenous Smad3 and Sp1 is induced by TGF-β in several cell lines. Smad3 strongly induces the minimal PAI-1 promoter, pGL 85, and the heterologous promoter containing three Sp1 sites in the presence or absence of TGF-β, but the phosphorylation incompetent mutant of Smad3, Smad3(S/A), was unable to induce transcription. Thus, the PAI-1 promoter is induced by TGF-β primarily by two mechanisms: 1) this promoter is strongly activated by TGF-β-induced binding of a Smad3/Smad4 containing nuclear complex to CAGA boxes in...
the distal region; 2) the proximal promoter, containing two Sp1 sites, mediates moderate induction through TGF-β-induced interaction between Smad3 and Sp1, independent of Smad/DNA interactions, but dependent upon Sp1/DNA interactions. Both these mechanisms are required for full induction of the PAI-1 promoter. In conclusion, our results suggest a model for how Smads mediate TGF-β-induced transcription at Sp1 binding sites in mammalian promoters through physical and functional interaction with Sp1, and this represents a novel mechanism of gene regulation by TGF-β.

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REFERENCES
1. Matrisian, L. M. (1990) Trends Genet. 6, 121–125
2. Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
3. Westerhausen, D. R., Jr., Hopkins, W. E., and Billadello, J. J. (1991) J. Biol. Chem. 266, 1092–1100
4. Dennler, S., Itoh, S., Vivien, D., ten Dijke, P., Huët, S., and Gauthier, J. M. (1998) EMBO J. 17, 3091–3100
5. Song, C.-Z., Siok, T. E., and Gelehrter, T. D. (1998) J. Biol. Chem. 273, 29287–29290
6. Massague, J., and Wolsten, D. (2000) EMBO J. 19, 745–754
7. Wong, C., Rougier-Chapman, E. M., Frederick, J. P., Datto, M. B., Liberati, N. T., Li, J.-M., and Wang, X.-F. (1999) Mol. Cell. Biol. 19, 1821–1830
8. Chen, X., Wiesberg, E., Fridmacher, V., Watanabe, M., Naco, G., and Whitman, M. (1997) Nature 389, 85–89
9. Labbé, E., Silvestri, C., Hoodless, P. A., Wrana, J. L., and Attisano, L. (1998) Mol. Cell 2, 109–120
10. Zhou, S., Zawel, L., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Mol. Cell 2, 121–127
11. Hua, X., Liu, X., Ansari, D. O., and Lodish, H. F. (1998) Genes Dev. 12, 3084–3095
12. Pardali, E., Xie, X. Q., Tsapogas, P., Itoh, S., Arvanitidis, K., Heldin, C. H., ten Dijke, P., Grundstrom, T., and Sideras, P. (2000) J. Biol. Chem. 273, 3552–3560
13. Datto, M. B., Yu, Y., and Wang, X.-F. (1995) J. Biol. Chem. 270, 28623–28628
14. Moustakas, A., and Kardassis, D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6733–6738
15. Li, J.-M., Datto, M. B., Shen, X., Hu, P. P., Yu, Y., and Wang, X.-F. (1998) Nucleic Acids Res. 26, 2449–2456
16. Greenwell, P., Inagaki, Y., Hu, W., Walsh, M., and Ramirez, F. (1997) J. Biol. Chem. 272, 19738–19745
17. Ignoffo, R. A., and Massague, J. (1986) J. Biol. Chem. 261, 4337–4345
18. Keeton, M. R., Curriden, S. A., Zonneveld, A. V., and Loskutoff, D. J. (1991) J. Biol. Chem. 266, 23048–23052
19. Datta, P. K., and Moses, H. L. (2000) Mol. Cell. Biol. 20, 3157–3167
20. Slansky, J. E., Li, Y., Kaelin, W. G., and Farnham, P. J. (1993) Mol. Cell. Biol. 13, 1610–1618
21. Chen, Y.-Q., Su, M., Walia, B. K., Hane, Q., Covington, J. W., and Vaughan, D. E. (1998) J. Biol. Chem. 273, 8225–8231
22. Liu, X., Sun, Y., Constantinescu, S. N., Karam, E., Weinberg, R. A., and Lodish, H. F. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10669–10674
23. Doetzlhofer, A., Rotheneder, H., Lagger, G., Koranda, M., Kunter, V., Brosch, G., Wintersberger, E., and Seiser, C. (1999) Mol. Cell. Biol. 19, 5504–5511
24. Lanini, L., Majello, B., and Luca, P. D. (1997) Int. J. Biochem. Cell Biol. 29, 1313–1323
25. Azizkhan, J. C., Jensen, D. E., Pierce, A. J., and Wade, M. (1993) Crit. Rev. Eukaryotic Gene Expression 3, 229–254
26. Wieser, R., Wrana, J. L., and Massague, J. (1995) EMBO J. 14, 2199–2208
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