Synergism between Transcription Factors TFE3 and Smad3 in Transforming Growth Factor-β-induced Transcription of the Smad7 Gene*

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Xianxin Hua‡, Zachary A. Miller1, Hassina Benchabane§, Jeffrey L. Wrana** and Harvey F. Lodish‡‡
From the ‡Whitehead Institute for Biomedical Research, Cambridge, Massachusetts 02142 and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 and §Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada

Activation of transforming growth factor-β (TGF-β) receptors triggers phosphorylation of Smad2 and Smad3. After binding to Smad4, the complex enters the nucleus and interacts with other transcription factors to activate gene transcription. Unlike other Smads, Smad7 inhibits phosphorylation of Smad2 and Smad3, and its transcription is induced by TGF-β, suggesting a negative feedback loop. Here, we show that TFE3 and Smad3 synergistically mediate TGF-β-induced transcription from the Smad7 promoter by binding to an E-box and two adjacent Smad binding elements (SBEs), respectively. A precise 3-base pair spacer between one SBE and the E-box is essential. Previously, we showed that the fully functional TGF-β element in the Smad7 promoter also requires two SBEs following TGF-β stimulation (10–14). Interestingly, expression of the Smad7 gene is induced by TGF-β, suggesting the existence of a negative feedback loop in TGF-β signaling (4, 5).

Smad7 is involved in the regulation of a variety of physiological and pathological processes such as shear stress in the vascular epithelium (6), apoptosis (7), lung fibrosis (8), and tumorigenicity (9). TGF-β-induced transcription of Smad7 is mediated through two palindromic repeats of a Smad binding element (SBE) in the Smad7 promoter; Smads 2, 3, and/or 4 bind to these SBEs following TGF-β stimulation (10–14). However, the SBEs to which the Smads bind are only 4 bp in length, and Smads are thought to partner with other transcription factors to induce transcription of specific genes (15–21).

Both the AP-1 and Sp1 binding sites in the Smad7 promoter were shown to be important in TGF-β-induced transcription (12). We showed previously that transcription factor TFE3 and Smads cooperate to mediate TGF-β-induced transcription of plasminogen activator inhibitor-1 (PAI-1) gene (22). In the PAI-1 promoter, the E-box, which is the binding site for TFE3, and one SBE are separated by a 3-base pair spacer (22, 23). An intact E-box is essential for basal as well as TGF-β-induced PAI-1 transcription, and two of the three SBEs are essential for TGF-β-induced, but not basal transcription. Most strikingly, the spacer between the SBE and the E-box must be precisely 3 bp. Here, we show that the fully functional TGF-β-inducible element in the Smad7 promoter also requires two SBEs followed by precisely 3 bp and an intact E-box. Thus, TFE3-Smad3 complex may mediate TGF-β-dependent induction of a group of gene targets.

EXPERIMENTAL PROCEDURES

Cloning of the Smad7 Promoter—Genomic DNA containing the mouse Smad7 promoter was isolated from a 129Sv female kidney DNA library (Stratagene) using as probe a 296-bp EST clone spanning the 5’-untranslated region of the cDNA sequence. Positive clones containing a 5.5-kilobase pair BamHI fragment with the Smad7 promoter were isolated and subjected to promoter analysis (10).

Plasmid Construction—Standard molecular biology techniques were used (24). To construct luciferase reporter genes driven by an intact Smad7 promoter, the 285-bp KpnI/StuI fragment of the Smad7 promoter was cloned into the Smal site of the pGL3 vector (Promega) to generate pS7-Luc. The E-box sequence CACGTG in this 285-bp promoter was mutated by designing mutated oligonucleotides followed by polymerase chain reaction amplification to aacGac to generate pS7-eE-Luc. To construct luciferase reporter genes driven by SB7, a TGF-β-inducible 33-bp fragment from the Smad7 promoter, we designed wild type or various mutant oligonucleotides, which span this region of the promoter as indicated in Fig. 3B. The annealed oligonucleotides were inserted into the KpnI/StuI site of PE2.1-Luc (23) to generate the resulting reporter genes as shown in Fig. 3. Constructs for in vitro translation of TFE3 (pET-TFE3) (22) or for expression of the GST-Smad3 fusion protein (pGEK6-Smad3) in Escherichia coli were described previously (23). The mutations in all of the plasmids and all of the mutations were sequenced to verify that they were correct.

Cell Culture, Cell Transfection, and Luciferase Assay—HepG2 cells were cultured in Dulbecco’s Modified Eagle’s medium containing 10% fetal calf serum as described (22). Cells were transfected by the calcium
Activation of Smad7 Transcription by TFE3 and Smad Proteins

RESULTS

An Intact E-box Is Essential for Basal and TGF-β-induced Transcription of the Smad7 Promoter—The Smad7 promoter contains one E-box (CACCGT, −254 to −259, Fig. 1A). Just upstream are two palindromic SBEs (GTCT; AGAC in reverse), and 3 bp separate the E-box from the adjacent SBE. In the PAI-1 promoter, TFE3 binds to the E-box; this sequence is essential for basal as well as TGF-β-induced transcription of the PAI-1 gene. Fig. 1B shows that the same results were obtained with the Smad7 promoter. The 285-bp wild type Smad7 promoter (S7) mediated robust transcription induced by TGF-β. However, point mutations in the E-box markedly decreased both the basal and TGF-β-inducible promoter activities (left panel). To further dissect the TGF-β-inducible promoter, we studied a 33-bp fragment from S7 (SS7, −254 to −286) that includes the critical E-box as well as two SBEs. TGF-β mediated a 4-fold increase in SS7 promoter activity (Fig. 1B, right panel). However, mutations in the SS7 promoter completely abolished both basal and TGF-β-induced transcription from the SS7 promoter. In the same experiment, both the basal and TGF-β-induced transcription from SS7 were about 5% of the activity of the full-length Smad7 promoter (note the different scales in the left and right panels in Fig. 1B), and thus other elements in the Smad7 promoter must also regulate its expression. Nevertheless, Fig. 1C shows that the promoter activity of SS7 is higher than that of PE2, a well-characterized small promoter element from the TGF-β-inducible PAI-1 gene, which also contains an essential E-box (22). Together, these results indicate that the E-box, which can bind TFE3 specifically, plays a critical role in TGF-β induction of the Smad7 promoter, but other transcription factors must also play a role to increase overall transcription induced by TGF-β.

TFE3 and Smad3 Synergistically Activate the Smad7 Promoter—To test whether TFE3 participates in the TGF-β-induced transcription from the Smad7 promoter, and if so, whether it does so through the E-box sequence, we cotransfected cells with various luciferase reporter genes together with either TFE3 or Smad3 (Fig. 2). Panel A shows that the full-length S7 Smad7 promoter mediates robust induction of transcription by TGF-β, and overexpression of either TFE3 or Smad3 hardly affects basal or TGF-β-induced transcription. However, cotransfection of both TFE3 and Smad3 dramatically increased both basal and TGF-β-induced transcription from the Smad7 promoter. Importantly, mutations in the E-box sequence decreased both basal and TGF-β-induced transcriptions whether or not TFE3 and Smad3 were overexpressed (Fig. 2A). Fig. 2B shows that a similar situation is obtained with the SS7 promoter fragment, which includes the critical E-box as well as two SBEs. Cotransfection of either TFE3 or Smad3 did not markedly affect either basal or TGF-β-inducible transcription. In contrast, a combination of TFE3 and Smad3 dramatically increased basal transcription from SS7; recall that, when over-
expressed, Smad3 is able to activate gene transcription even in the absence of TGF-β (25, 26). Cotransfection of TFE3 and Smad3 also caused an increase in TGF-β-induced SS7 activity. Importantly, an SS7 promoter with a mutation in the E-box was completely inactive even when TFE3 and Smad3 were overexpressed, clearly demonstrating that TFE3 and Smad3 require a functional E-box in the Smad7 promoter to mediate TGF-β-induced transcription. Taken together, these results clearly demonstrate that TFE3 and Smad3 cooperatively mediate TGF-β-induced transcription from the Smad7 promoter, and this cooperation requires a functional TFE3 binding site, the E-box.

A 3-bp Spacer between the SBE and E-box Is Essential for TGF-β Induction of the SS7 Smad7 Promoter Fragment—We previously reported that two essential SBEs followed by an E-box are essential for TGF-β-induced transcription from the PE2 fragment of the PAI-1 promoter. Importantly, the spacer between the SBE and the E-box must be precisely 3 bp, although the sequence of the spacer is not important (23). Fig. 3A shows that the mouse, human, and rat Smad7 promoters all contain two palindromic SBEs separated by a 3-bp spacer, GCC, though the sequence of the spacer seems immaterial (23). Fig. 3B shows that, as expected, after transfection into HepG2 cells, SS7 mediates robust transcription induced by TGF-β. Either insertion (SS7 +1) or deletion (SS7 −1) of one base in the spacer region abolished TGF-β-induced transcription from the SS7 promoter. Mutations in either the first (SS7 mS1) or the second SBE (SS7 mS2) also completely abrogated TGF-β-induced transcription from the SS7 promoter, and confirming the data from Fig. 1C, mutations in the SS7 E-box abolished both basal and TGF-β-induced transcription. These results strongly suggest that the length of the spacer is important for the cooperation of TFE3 and Smad proteins in TGF-β-induced gene transcription. Thus, TGF-β-induced transcription from the PAI-1 and Smad7 promoters are similar in that they require two SBEs and a precise 3 bp spacer between one SBE and the E-box.

Alteration of the Length of the 3-bp Spacer Does Not Change the Ability of Either TFE3 or Smad3 to Bind to the SS7 Promoter—We tested whether insertion or deletion of a single base pair impairs the ability of either TFE3 or Smad3 to bind the Smad7 promoter. To this end, we first incubated 32P-labeled 33-bp wild type or mutant SS7 DNA fragments with TFE3 synthesized in vitro by a reticulocyte lysate followed by a gel shift assay (Fig. 4A). The reticulocyte lysate contains some protein(s) able to bind to this DNA (lane 1); this species probably binds to the E-box because mutations in the E-box abolished the binding (lane 2). Lysates containing in vitro translated TFE3 exhibited a new band in the gel shift assay (lane 5). As expected, this TFE3 protein did not bind to the SS7 DNA containing a mutant E-box (lane 6) but did bind normally to SS7 DNAs with altered spacing between the SBE and E-box (lanes 7 and 8). Furthermore, the T7 epitope-tagged TFE3 was supershifted by an anti-T7 epitope antibody (lanes 9, 11, and 12). These results suggest that insertion or deletion of a single base pair in the spacer region did not change the binding affinity between TFE3 and the E-box sequence.

Similar gel shift assays showed that GST-Smad3 bound to the 32P-labeled wild type SS7 DNA fragment (Fig. 4B, lane 2). It bound equally well to SS7 DNAs containing a mutant E-box (lane 3) and to SS7 DNAs with altered spacing between the SBE and E-box (lanes 4 and 5). As a control, GST did not bind to the wild type SS7 DNA fragment (lane 1). Thus, an insertion or deletion in the 3-bp spacer region does not decrease the ability of Smad3 to bind the promoter DNA.

DISCUSSION

Here, we dissected the Smad7 promoter and elucidated the sequences required for transcriptional induction by TGF-β. In doing so, we uncovered striking similarities with the sequences in the PAI-1 promoter and the transcription factors essential for induction by TGF-β. Both the Smad7 and PAI-1 promoters contain a SBE separated by precisely 3 bp from an E-box (23). Both promoters also contain a second SBE upstream of the first, although the spacing between the two SBEs is different in the two cases. In both minimal promoters, SS7 for Smad7 and PE2 for PAI-1, mutation of the E-box sequence completely abolished basal and TGF-β-induced transcription. In both the PE2 and SS7 promoters, mutation of either of the two SBEs abolished TGF-β-induced transcription but did not affect basal gene expression. Most strikingly, in both the PE2 and SS7 promoters, the addition or deletion of 1 base pair to the 3-base pair spacer also abolished TGF-β-induced transcription but did not affect basal gene expression.

Both the Smad7 promoter (Fig. 2) and the PAI-1 promoter (22) were synergistically activated by TFE3 and Smad3. As with the PAI-1 promoter, cooperation between TFE3 and
Smad3 in TGF-β-induced activation of the Smad7 promoter requires the TFE3 binding site, the E-box, because point mutations of the E-box sequence markedly reduced TGF-β-dependent transcription from the full-length Smad7 promoter S7 (Fig. 2). These point mutations in the E-box sequence completely abolished the transcription driven by S7, a minimal Smad7 promoter, in the presence or absence of TGF-β (Fig. 2B), suggesting the critical role of TFE3 in TGF-β-induced transcription of the Smad7 gene as well as in its basal transcription.

Whereas point mutations in the full-length Smad7 promoter S7 still allowed about 20% of the wild type level of TGF-β-induced transcriptional activity (Fig. 1B), the same mutation in the E-box of the 33-bp minimal Smad7 promoter, SS7, completely abrogated inducibility by TGF-β (Fig. 1B, right panel). These results suggest that other transcription factors binding other sites in the full-length Smad7 promoter play a role in increasing the overall transcription induced by TGF-β. These results are consistent with a recent report by Brodin et al. (12) showing that AP-1 and Sp1 binding sites downstream of the SBE repeats in the Smad7 promoter are functionally important for basal as well as TGF-β-induced transcription from the promoter (12). Recently, the E-box sequence downstream of the SBEs was also shown to be important for TGF-β-induced gene transcription (13). Together, these results strongly argue that multiple transcription factors, including TFE3, AP-1, and Sp1, may cooperate with Smad proteins in mediating TGF-β-induced Smad7 transcription.

The model we proposed for TGF-β-induced transcription of the PAI-1 promoter thus applies to the Smad7 promoter. In brief, TGF-β induces phosphorylation of Smad3, and phosphorylated Smad3 binds to TFE3. TFE3 may also bind to Smad4 in the E-box of the Smad7 promoter, completely abolished the transcription driven by SS7, a minimal Smad7 promoter, such that 3 base pairs separate the E-box from one of the two essential Smad binding sites. Following binding to DNA, the Smad3-TF3-TE3 complex likely recruits transcription coactivators such as p300 and CBP (27–31), leading to enhanced transcription. This model suggests a critical role for combinations of at least two SBEs and binding sites for other transcription factors as well as a requirement for a fixed spacer between the two types of binding sites in achieving high specificity of TGF-β-induced gene transcription.

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