Alternatively Spliced Variant of Smad2 Lacking Exon 3

COMPARISON WITH WILD-TYPE Smad2 AND Smad3

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An alternatively spliced variant of Smad2 with a deletion of exon 3 (Smad2Δexon3) is found in various cell types. Here, we studied the function of Smad2Δexon3 and compared it with those of wild-type Smad2 containing exon 3 (Smad2(wt)) and Smad3. When transcriptional activity was measured using the p3TP-lux construct, Smad2Δexon3 was more potent than Smad2(wt), and had activity similar to Smad3. Transcriptional activation of the activin-responsive element (ARE) of Mix.2 gene promoter by Smad2Δexon3 was also similar to that by Smad3, and slightly less potent than that by Smad2(wt). Phosphorylation by the activated transforming growth factor-β type I receptor and heteromer formation with Smad4 occurred to similar extents in Smad2Δexon3, Smad2(wt), and Smad3. However, DNA binding to the activating protein-1 sites of p3TP-lux was observed in Smad2Δexon3 as well as in Smad3, but not in Smad2(wt). In contrast, Smad2(wt), Smad2Δexon3, and Smad3 efficiently formed ARE-binding complexes with Smad4 and FAST1, although Smad2(wt) did not directly bind to ARE. These results suggest that exon 3 of Smad2 interferes with the direct DNA binding of Smad2, and modifies the function of Smad2 in transcription of certain target genes.

Members of the transforming growth factor-β (TGF-β) superfAMILY transduce signals through two different types of serine/threonine kinase receptors, known as type II and type I receptors (1). In the TGF-β receptor system, ligand binds to the TGF-β type II receptor (TβR-II), which has a constitutively active kinase. TGF-β type I receptor (TβR-I) is then recruited into the TGF-β/TβR-II complex, and phosphorylated mainly at the glycine-serine-rich domain (GS domain), which results in the activation of TβR-I kinase (2). The TβR-I kinase transduces intracellular signals by activation of various proteins, including Smad proteins. TβR-I thus acts as a downstream component of TβR-II. Mutation in Thr-204 of TβR-I to aspartic acid (TβR-I(D)) results in the constitutive activation of the TβR-I kinase, which has signaling activity in the absence of TGF-β and TβR-II (3).

Smad proteins have recently been shown to comprise a family of proteins that mediate signals for members of the TGF-β superfamily (4–6). Thus far, eight mammalian Smad proteins have been identified, termed Smad1 through Smad8. Smads are classified into three subgroups based on their structure and function, i.e. pathway-restricted Smads, common mediator Smads, and inhibitory Smads. Pathway-restricted Smads can be further subdivided into those involved in the TGF-β and activin signaling pathways, and those activated by the bone morphogenetic protein pathway. Smad2 and Smad3 serve as pathway-restricted Smads for the TGF-β/activin signaling pathways. Smad1, Smad5, and possibly Smad8/MADH6 are activated by bone morphogenetic protein receptors. Smad4 is a common mediator Smad; thus far only one common mediator Smad has been identified in mammals. Smad6 and Smad7 are inhibitory Smads.

Pathway-restricted Smads are phosphorylated by serine/threonine kinase receptors, and form hetero-oligomers with Smad4. Phosphorylation of pathway-restricted Smads occurs at the C-terminal Ser-Ser-X-Ser motif (7–9). The heteromers then translocate into the nucleus and activate the transcription of various target genes. Smad proteins have been shown to interact with DNA-binding proteins, such as winged-helix transcription factors, Xenopus, and human FAST1 and mouse FAST2 (10–12), and also to directly bind to specific DNA sequences (13–17).

Smads have conserved N- and C-terminal regions known as Mothers against decapentaplegic (Mad) homology domain-1 (MH1) and -2 (MH2), respectively, which are linked by a linker region of variable length and amino acid sequence. The MH2 domain is a functional domain, which has transactivation activity when fused to the Gal4-DNA binding domain (18, 19). The MH2 domain also plays important roles in interaction with type I receptors (20), homo- and hetero-oligomerization by Smad proteins (21, 22), interaction with a transcription factor, FAST1 (23, 24), and association with transcriptional co-activators, p300/CBP (25–28). The MH1 domain has been shown to physically interact with the MH2 domain, and thereby inhibits the activity of the latter (29). However, it has been shown that the MH1 domain has an intrinsic function in signal transduction, i.e. direct binding to specific DNA sequences. Mad in Drosophila was shown to bind to the quadrate enhancer of vestigial gene (13). Smad3 and Smad4 have also been shown to bind to specific DNA sequences through their MH1 domains (13–17).
Although Smad2 and Smad3 are 91% identical in amino acid sequence, they have certain differences in biological activity. In contrast to Smad3 and Smad4, Smad2 does not directly bind to DNA (12–17). Binding to a transcriptional regulator, Evi-1, is observed for Smad3 but not for Smad2 (30). A functional difference between Smad2 and Smad3 has also been suggested in the effects of TGF-β and activin on the HaCaT keratinocyte cells (31). TGF-β and activin inhibit the growth of HaCaT cells, but TGF-β is much more potent than activin. TGF-β induces the phosphorylation of both Smad2 and Smad3, whereas activin preferentially activates Smad3 (31).

Smad2 has a region with 30 amino acid residues, which is not found in Smad3 or other Smads in mammals. The Smad2 gene is composed of 11 exons, and this short 30-amino acid region is translated by exon 3. Recently, we found that a Smad2 transcript which lacks exon 3 is present in certain tissues and cells, although the amount of the transcript is about 1/10 of that containing exon 3 (32). Here we studied the function of Smad2 without exon 3 (Smad2Δexon3) and compared it with those of wild-type Smad2 (Smad2wt) and Smad3. In the transcriptional activation assay using the p3TP-lux construct, Smad2Δexon3 was more potent than Smad2wt, and had activity almost similar to Smad3. Phosphorylation by TβR-I and heteromer formation with Smad4 did not differ between Smad2Δexon3 and Smad2wt. However, Smad2Δexon3, but not Smad2wt, was able to bind to the activating protein (AP)-1 sites from p3TP-lux. In contrast, both Smad2wt and Smad2Δexon3 could form the activin-responsive factor (ARF) with FAST1 and Smad4, and transactivate the Xenopus Mix.2 gene promoter.

EXPERIMENTAL PROCEDURES

cDNA Constructs—The original constructions of Flag-pcDNA3, Myc-pcDNA3, HA-pcDNA3, TβR-I(TD)-HA, Smad2wt, and Smad3 have been described previously (33, 34). Smad2Δexon3 was prepared by inserting the 330-bp polymerase chain reaction (PCR) product of Smad2Δexon3 into Smad2wt. In order to obtain efficient expression levels of proteins, some constructs were subcloned into another expression vector, pcDEF3 (35). Constructs for GST-fused Smad2wtΔMH2, Smad2Δexon3, and Flag-tagged Smad2Δexon3ΔMH2 lacking the MH2 domains were obtained by subcloning the corresponding cDNAs into pGEX-4T-1 (Amersham Pharmacia Biotech). Xenopus FAST1 cDNA (provided by M. Whitman) was subcloned into HA-pcDEF3. All of the PCR products were sequenced.

Reverse Transcriptase-PCR of Smad2—Poly(A)+ RNAs were prepared from various cell lines and reverse-transcribed. The resulting cDNAs were amplified by PCR using primers 2A (5'-TTT TCC TAG CTC GGC TTG-3') and 4A (5'-TCA GAG AGT TGA GAC ACC AG-3') under conditions as described (32). The PCR products were subjected to the second-round PCR using primers 2B (5'-GAA GAG AGT ACG ACC AG-3') and 4B (5'-CAA GCC GAT TGA CCC GAG ACA TGC-3') and 20 ng of each primer. The PCR fragment was run on a 2% agarose gel, and a 330-bp product was cloned using a TA cloning kit (Promega).

Cell Culture and cDNA Transfection—Cells were obtained from American Type Culture Collection. R mutant MvLuI cells were provided by J. Massagué. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml gentamycin. For transient transfection, 60–80% confluent cells in six-well plates or 10-cm culture dishes were transfected using FuGENE6 transfection reagent (Boehringer Mannheim) following the manufacturer's protocol.

Luciferase Assay—R mutant MvLuI cells were transiently transfected with p3TP-lux or pAR3-lux (provided by J. L. Wrana) (36) in the presence of various combinations of Smad constructs, TβR-I(TD), and FAST1. For normalization of transfection efficiency, the Renilla luciferase reporter gene in the pRL-CMV vector (Promega) was co-transfected in each transfection. After transfection, cells were incubated for 36 h, and luciferase activity in the cell lysates was determined with a dual luciferase assay system (Promega) using a luminometer (Lumat LB 9501, EG & G Berthold) according to the manufacturer's recommendations. The luciferase activities of p3TP-lux and pAR3-lux constructs were measured as luminescence of firefly luciferase. As an internal control, Renilla luciferase was measured immediately afterward by quenching the firefly luminescence.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting have been described previously (33). Briefly, COS7 cells were transfected with expression constructs for Smads, TβR-I(TD), and FAST1. Forty-eight hours after transfection, the cells were solubilized, and the cell lysates were incubated with the anti-Flag M2 (Eastman Kodak Co.) or anti-Myc 9E10 antibodies (Santa Cruz Biotechnology), followed by incubation with protein G-Sepharose beads. The immunocomplexes were then eluted by boiling for 3 min in the SDS sample buffer containing 10 mM dithiothreitol and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Aliquots of the cell lysates were subjected to SDS-PAGE. Proteins were electrotransferred to polyvinylidene difluoride membrane (Prolimmunoblot membranes, Applied Biosystems) and immunoblotted with the anti-Flag M2, anti-Myc 9E10, anti-HA 3F10 (Boehringer Mannheim), or anti-phosphoserine antibodies (Zymed Laboratories Inc.) and developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For re-blotting, the polyvinylidene difluoride membranes were stripped following the manufacturer's protocol.

Gel-mobility Shift Assay—Gel-mobility shift assay was performed as described previously (14). Briefly, whole cell extracts were prepared from the COS7 cells transfected with Smad, TβR-I(TD), and FAST1 expression constructs. Cells were lysed in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na2VO4, 20 μM dithiothreitol, and proteinase inhibitors. Glutathione S-transferase (GST) fusion proteins were prepared as described (16). For supershift analysis, anti-Flag and/or anti-Myc antibodies (1–2 μl each) or antisera against Smad2 or Smad3 (2 μl each, provided by P. ten Dijke) (33) were added to the whole cell lysates or GST-fused Smad proteins. The probe containing AP-1 sites (77 bp) was created by digestion of the p2TP-lux with NdeI and SphI, and that containing ARE was prepared by digestion of the pAR3-lux with Acc I and BsmHI. The probes were then subjected to [γ-32P]ATP KLlenow labeling. Whole cell lysates (3 μl, containing 3 μg of protein) or GST fusion proteins (150 ng each) were added with a premix solution (13.4 μl) containing 1 μg of poly(dI-dC) and 1 μl of the probe labeled to an activity of 2.0 × 104 cpm/μl (37). The final concentration of NaCl in the samples was adjusted to 110 mM by hypotonic and hypertonic lysis buffers. Complexes were then resolved on 4% polyacrylamide gel and analyzed by autoradiography.

RESULTS

Construction of Smad2Δexon3 Plasmid—Smad2 has a 30-amino acid region in the middle of the MH1 domain (amino acid 79–108), which is not found in other Smads in mammals (Fig. 1A). mRNAs were prepared from various human cell lines, i.e. the HaCaT keratinocyte cell line, HEL human erythroleukemia cell line, U937 human monocytic leukemia cell line, and Molt-4 human T cell leukemia cell line. PCR was performed using primers (2A and 4A) corresponding to the sequences in exons 2 and 4, and the PCR products were subjected to second-round PCR using primers 2B and 4B. Similar to previous results obtained with mRNAs from placenta and HaCaT keratinocyes (32), we observed two bands in HaCaT and Molt-4 cells, i.e. major bands of 420 bp and faint bands of 330 bp (Fig. 1B). The 330-bp bands were only very weakly detected in the other two cell lines. By DNA sequencing, we confirmed that the 330-bp products correspond to Smad2 lacking exon 3. By inserting the 330-bp product into Smad2wt at PvuII-RsrI sites, we prepared a construct for Smad2Δexon3.

The Smad constructs were transfected into COS7 cells, and the expression of proteins was analyzed by immunoblotting. N-terminally Flag-tagged Smad2Δexon3 was efficiently expressed in COS7 cells, with protein levels similar to those with Smad2wt and Smad3 (data not shown). The size of Smad2Δexon3 is smaller than that of Smad2wt, and larger than that of Smad3, consistent with their estimated molecular weights.

Smad2Δexon3 Has Transcriptional Activity Different from That of Smad2wt—Next, we studied the transcriptional activity of Smad2Δexon3 and compared it with those of Smad2wt and Smad3 using the p3TP-lux promoter reporter construct, which contains the promoter region of plasminogen.
activator inhibitor (PAI)-1 and three tandemly linked AP-1 sites (38) (Fig. 2A). The R mutant Mv1Lu cells, which lack functional TβR-I, were used to determine the transcriptional activity of Smads. In the presence of small amounts of TβR-I(TD) plasmid, a slight increase in the transcriptional activity on p3TP-lux was observed. Smad3 efficiently induced transcription even in the absence of exogenous Smad4, while Smad2(wt) was less efficient in transcriptional activation than Smad3. Co-expression of Smad4 led to higher transcriptional activity of Smad2(wt), although it was less than that of Smad3. Interestingly, Smad2Δexon3 had transcriptional activity similar to that of Smad3 in the absence or presence of Smad4, which was more potent than that of Smad2(wt).

The transcriptional activity of Smad2Δexon3 was also tested in the reporter gene pAR3-lux containing the ARE of Mix.2 promoter in Xenopus (36) in the presence or absence of the specific DNA-binding protein, Xenopus FAST1 (10). In contrast to the results obtained using p3TP-lux, Smad2 was slightly more potent than Smad3 in inducing luciferase activity of pAR3-lux (Fig. 2B). The luciferase induction by Smad2Δexon3 was similar to that by Smad3, and slightly less effective than that by Smad2.

**Phosphorylation by TβR-I and Complex Formation with Smad4**—In order to understand mechanism of the higher transcriptional activity in p3TP-lux of Smad2Δexon3 than of Smad2(wt), we examined the phosphorylation of Smad2 and Smad3 by TβR-I. Flag-tagged Smad constructs were transfected into COS7 cells together with various amounts of TβR-I(TD), and immunoprecipitated with anti-Flag antibody, followed by immunoblotting using anti-phosphoserine antibodies (Fig. 3A). Although Smad2Δexon3 and Smad3 were more potent than Smad2(wt) in inducing transcriptional activity on p3TP-lux, there was no significant difference between the levels of phosphorylation in Smad2Δexon3, Smad2(wt), and Smad3.

Heteromer formation with Smad4 was also studied using Smad2Δexon3, Smad2(wt), and Smad3 (Fig. 3B). Weak interaction with Smad4 was observed in Smad2Δexon3 and Smad2(wt) even without TβR-I(TD), and was correlated with the weak phosphorylation of the Smad2 proteins. Strong heteromer formation was induced in all Smad2 and Smad3 constructs by TβR-I(TD), and we found no differences in between Smad2Δexon3, Smad2(wt), and Smad3.

Smad2Δexon3 but Not Smad2(wt) Binds to the AP-1 Sites of p3TP-lux—We then studied the formation of DNA-binding complexes by Smad2Δexon3 at the AP-1 sites of p3TP-lux. It was previously shown that Smad3 and Smad4, but not Smad2, bind to probe prepared from p3TP-lux DNA (13). Whole cell extracts were prepared from COS7 cells transfected with the indicated Smad constructs and TβR-I(TD), and subjected to gel-mobility shift analysis (Fig. 4A). None of the Smad proteins formed DNA-binding complexes in the absence of TβR-I(TD) (data not shown). In the presence of TβR-I(TD), Smad3 and
Smad2 lacking exon 3

We next examined whether Smad2Δexon3ΔMH2 can directly bind to the AP-1 sites derived from p3TP-lux. Smad3 lacking the MH2 domain was previously shown to directly bind to DNA (12, 14–17). GST-fused Smad2(wt)ΔMH2, Smad3ΔMH2, and Smad2Δexon3ΔMH2 were transfected into COS7 cells in the presence of TβR-I(TD)-HA. Whole cell lysates were prepared, and binding to the 32P-labeled probe containing the AP-1 sites was analyzed by gel mobility shift assay. For supershift analysis, whole cell lysates were incubated with the anti-Flag (F) and/or anti-Myc (M) antibodies, and subjected to gel-shift assay. B, binding of purified GST-Smad proteins to DNA. GST-fused Smad2(wt)ΔMH2, Flag-Smad2Δexon3ΔMH2, and Smad3ΔMH2 lacking MH2 domains were prepared, and gel shifts were performed using the probe containing the AP-1 sites. For supershift analysis, the antisera to Smad2 (2) or Smad3 (3), or anti-Flag (F) antibody was used. For competition (C) of the DNA binding, excess amounts of cold probe was added.

Smad2Δexon3, but not Smad2(wt), formed DNA-binding complexes. The complexes supershifted in the presence of the anti-Flag antibody. Smad4 alone did not form a DNA-binding complex in the presence of TβR-I(TD), but it did participate in DNA-binding complexes in the presence of Smad2Δexon3 or Smad3, but not of Smad2(wt). The bands shifted in the presence of anti-Myc antibody, although they were weak compared with those obtained with the anti-Flag antibody. Moreover, addition of both anti-Myc and anti-Flag antibodies led to shift with a slower mobility, indicating that the DNA-binding complexes contained Smad4. These results indicate that by deletion of exon 3, Smad2 acquired the ability to participate in DNA-binding complexes.

**Fig. 3.** Phosphorylation of Smad2(wt), Smad2Δexon3, and Smad3 and heteromer formation with Smad4. A, phosphorylation of Smad proteins by TβR-I(TD). COS7 cells were transfected with Smad constructs together with increasing amounts of the TβR-I(TD) plasmid. Cell lysates were immunoprecipitated with the anti-Flag antibody, followed by immunoblotting using anti-phosphoserine (anti-P-serine) antibody. Aliquots of the cell lysates were directly subjected to SDS-PAGE and immunoblotted with the anti-Flag or anti-HA antibody to detect the expression of Smad proteins and TβR-I(TD), respectively. B, complex formation of Smad2/3, and Smad4 with Smad4. COS7 cells were transfected with Smad2/3 and Smad4 constructs with or without TβR-I(TD). Cell lysates were immunoprecipitated with the anti-Myc antibody followed by immunoblotting with the anti-Flag antibody. In order to detect the phosphorylation of Smad2/3, aliquots of the cell lysates were subjected to immunoprecipitation using the anti-Flag antibody followed by immunoblotting using the anti-phosphoserine antibodies, as described in A. Expression of Flag-Smad2/3 was detected after stripping the membrane and immunoblotting using the anti-Flag antibody. Expression of TβR-I(TD) was detected by immunoblotting using the anti-HA antibody.

**Fig. 4.** Smad2Δexon3 and Smad3 bind to the AP-1 sites derived from p3TP-lux. A, Flag-Smad2(wt), Flag-Smad2Δexon3, or Flag-Smad3 and Myc-Smad4 were transfected into COS7 cells in the presence of TβR-I(TD)-HA. Whole cell lysates were prepared, and binding to the 32P-labeled probe containing the AP-1 sites was analyzed by gel mobility shift assay. For supershift analysis, whole cell lysates were incubated with the anti-Flag (F) and/or anti-Myc (M) antibodies, and subjected to gel-shift assay. B, binding of purified GST-Smad proteins to DNA. GST-fused Smad2(wt)ΔMH2, Flag-Smad2Δexon3ΔMH2, and Smad3ΔMH2 lacking MH2 domains were prepared, and gel shifts were performed using the probe containing the AP-1 sites. For supershift analysis, the antisera to Smad2 (2) or Smad3 (3), or anti-Flag (F) antibody was used. For competition (C) of the DNA binding, excess amounts of cold probe was added.
was slightly more potent in inducing transcriptional activity on pAR3-lux than Smad3 or Smad2Δexon3; however, complex formation with Xenopus FAST1 was observed similarly in between Smad2(wt), Smad2Δexon3, and Smad3 in the presence and absence of TβR-I(TD) (Fig. 5A). We, therefore, examined whether there are any differences in the DNA-binding abilities of the ARF complexes containing different Smads. ARF containing Smad2(wt), Smad4, and FAST1 efficiently bound ARE in response to TGF-β receptor activation, similar to those containing Smad3 or Smad2Δexon3 (Fig. 5B). Addition of anti-Flag, anti-Myc, or anti-HA antibodies led to shifts of the bands, indicating that the DNA-binding ARF complexes contained Smad2, Smad3, or Smad2Δexon3, together with Smad4 and FAST1. FAST1 was shown to interact with Smad2 through the MH2 domain (23, 24). These findings indicate that the presence of exon 3 of Smad2 does not interfere with the formation of DNA-binding ARF complex containing FAST1.

We further investigated the ability of different GST-fused Smad proteins to directly bind to DNA containing the ARE sequence in the absence of FAST1. Similar to binding to the AP-1 sites (Fig. 4A), Smad3ΔMH2 and Smad2Δexon3ΔMH2 recognized the ARE sequence, but Smad2(wt)ΔMH2 did not (Fig. 5C). The bands shifted in the presence of Smad antisera (lanes 6 and 8). Shift of the bands was more remarkable with the anti-Flag antibody (lane 9), probably because of the higher affinity of the anti-Flag antibody and possible induction of oligomerization. These data indicate that in addition to indirectly binding to DNA as ARF complexes, Smad3 and Smad2Δexon3, but not Smad2(wt), have abilities to directly bind to ARE.

**DISCUSSION**

Smad proteins have MH1 and MH2 domains, which are linked by a linker region. Inhibitory Smads have MH2 domains, but have divergent MH1-like regions. The MH2 domain plays important roles in various functions of Smads, i.e. interaction with type I receptors, homo- and hetero-oligomer formation, association with DNA-binding proteins, and interaction with transcriptional coactivators, e.g. p300/CBP (4–6, 25–28). Smad2, which lacks the MH1 domain, is constitutively located in the nucleus and activates target genes (39). The MH2 domain interacts with type I receptors, and the L3 loop, a 17-amino acid region in the MH2 domain, plays a critical role in this interaction (20). In addition, α-helix 2 of the MH2 domain has recently been shown to determine the binding specificity to DNA-binding proteins such as FAST1 (40).

The MH1 domain plays an important role as a repressor of the function of the MH2 domain. In addition, the MH1 domain has intrinsic activity, i.e. binding to specific DNA sequences.
Drosophila Mad, an ortholog of mammalian Smad1 and 5, directly binds to the quadrant enhancer of vestigial gene (13). Smad3 and Smad4, but not Smad2, have been shown to bind to the AP-1 sites of p3TP-lux (14, 41). More recently, Smad3 and Smad4 have been shown to bind to specific DNA sequences, and luciferase reporter constructs containing multiple copies of these specific DNA sequences have been shown to be activated by Smad3 and Smad4 (15–17).

Smad2 and Smad3 are structurally very similar, and serve as pathway-restricted Smads in the TGF-β and activin signaling pathways. However, functional differences between Smad2 and Smad3 have been suggested. In addition to the differences between them in DNA-binding ability, Smad3, but not Smad2, binds to a transcriptional regulator Evi-1, which consequently suppresses the activity of Smad3 (30). In the HaCaT keratinocyte cell line, TGF-β induces phosphorylation of both Smad2 and Smad3, while activin A preferentially activates Smad3 (31). Since TGF-β and activin have different activities in the growth inhibition and differentiation of this cell type, the difference in phosphorylation between Smad2 and Smad3 may be, at least in part, responsible for their distinct biological effects.

Smad2 differs from Smad3 in having a 30-amino acid region in the middle of the MH1 domain. We have shown in the previous report (32) and in the present study that mRNA lacking exon 3 is present in various tissues and cells. Amounts of the Smad2 transcript lacking exon 3 appear to differ between cell types. It would be of great interest to determine whether the mRNA encoding Smad2Δexon3 is preferentially induced under certain physiological and pathological conditions.

In the present study, we have shown that Smad2 lacking exon 3 has higher transcriptional activity on p3TP-lux than does Smad2Δexon3. The MH1 domain represses the function of the MH2 domain. Mutation of Arg-133 in Smad2 resulted in increase of the affinity between MH1 and MH2 domains, and this mutation was less active than the wild-type Smad2 (29). Similar results were reported for Smad4 with mutation at Arg-100 (29). In the present study, however, deletion of exon 3 of Smad2 did not lead to increase in phosphorylation by TβR-I, or heteromer formation with Smad4, suggesting that exon 3 is not directly involved in the repressor activity of the MH1 domain.

Smad2Δexon3 is able to bind to DNA containing the AP-1 sites, a finding not observed for Smad2Δexon3. These findings strongly suggest that the direct binding of Smad2Δexon3 is a crucial step in the transcriptional activation of the p3TP-lux reporter. In whole cell extracts, DNA-binding complex formation was observed in both the presence and absence of transfected Smad4. Direct DNA binding of Smad3 and Smad2Δexon3 was detected when GST-Smad proteins lacking MH2 domains were used. These findings together with those of previous reports (12–17) suggest that the interaction may occur through the MH1 domain, when repression by the MH2 domain is released. Since Smad2Δexon3 has higher transcriptional activity than Smad2Δexon3, direct DNA binding of Smads may positively regulate their transcriptional activity on p3TP-lux promoter. However, since Smad2Δexon3 can, to some extents, induce transcriptional activation of p3TP-lux, Smad proteins can partially activate target genes through a mechanism which does not require the direct DNA binding of Smads.

In contrast to the possible role of DNA binding of Smad2Δexon3 in the transcriptional activation of p3TP, transcription of the Mix.2 gene appeared to be less strongly affected by direct DNA binding of Smad2Δexon3. The Mix.2 promoter contains a sequence similar to the Smad-binding element (15, 17), to which Smad3, and possibly Smad2Δexon3, may bind. Smad2Δexon3, which failed to directly bind the Mix.2 promoter, was efficient in inducing transcriptional activation of p3TP-lux. Since the binding of Smad2 to FAST1 occurs through the MH2 domain, differences in the structure of the MH1 domain may have less important effects on the transcription of the Mix.2 gene. Interestingly, Smad2 activated the goosecoid promoter together with Smad4 and mouse FAST2, a winged-helix transcription factor related to Xenopus FAST1, whereas Smad3 strongly suppressed transcription-activation of this promoter (12). Smad3 and Smad4, but not Smad2, directly bound to the GC-rich sequences of the goosecoid promoter. These findings suggest that direct DNA binding of Smads through the MH1 domain as well as indirect binding via other DNA-binding factors, e.g. FAST1 and FAST2, may cooperatively regulate the transcription of target genes. Direct DNA binding of Smads may play important roles in transcription of certain target genes, such as PAI-1, while it is less critical or acts negatively for other genes, i.e. Mix.2 and goosecoid.

Expression profile of Smad3 appears to be limited compared with that of Smad2 (32). In certain cell types that lack the expression of Smad3, Smad2Δexon3 may function as a Smad3-like molecule. The region like exon 3 is observed only in Smad2 but not in other Smads in mammals. Exon 3 of Smad2 may play a crucial role in modulating the function of Smad2 by interfering with the direct DNA binding to target genes.

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