ACCELERATED PUBLICATION

The Smad3 linker region contains a transcriptional activation domain

Guannan WANG*†‡§, Jianyin LONG*†‡, Isao MATSUURA*†‡, Dongming HE*†‡ and Fang LIU†‡§¹

*Center for Advanced Biotechnology and Medicine, Rutgers, The State University of New Jersey, 679 Hoes Lane, Piscataway, NJ 08854, U.S.A., †Susan Lehman Cullman Laboratory for Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, 164 Frelinghuysen Road, Piscataway, NJ 08854, U.S.A., ‡The Cancer Institute of New Jersey, 195 Little Albany Street, New Brunswick, NJ 08903, U.S.A., and §Graduate Program in Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, Piscataway, NJ 08854, U.S.A.

Transforming growth factor- β (TGF- β)/Smads regulate a wide variety of biological responses through transcriptional regulation of target genes. Smad3 plays a key role in TGF- β /Smad-mediated transcriptional responses. Here, we show that the proline-rich linker region of Smad3 contains a transcriptional activation domain. When the linker region is fused to a heterologous DNA-binding domain, it activates transcription. We show that the linker region physically interacts with p300. The adenovirus E1a protein, which binds to p300, inhibits the transcriptional activity of the linker region, and overexpression of p300 can rescue the linker-mediated transcriptional activation. In contrast, an adenovirus E1a mutant, which cannot bind to p300, does not inhibit the linker-

mediated transcription. The native Smad3 protein lacking the linker region is unable to mediate TGF- β transcriptional activation responses, although it can be phosphorylated by the TGF- β receptor at the C-terminal tail and has a significantly increased ability to form a heteromeric complex with Smad4. We show further that the linker region and the C-terminal domain of Smad3 synergize for transcriptional activation in the presence of TGF- β . Thus our findings uncover an important function of the Smad3 linker region in Smad-mediated transcriptional control.

Key words: Smad, transcriptional regulation, transforming growth factor- β (TGF- β).

INTRODUCTION

Transforming growth factor- β (TGF- β) regulates a wide variety of biological activities, such as cell proliferation, differentiation, adhesion, motility and apoptosis [1]. It signals through two types of transmembrane serine/threonine kinase receptors [2–6]. The type II receptor is constitutively active. Upon TGF- β binding, the type II receptor phosphorylates the type I receptor, which then plays a major role in specifying downstream events [2–6]. Smad proteins are direct substrates of the TGF- β family type I receptor and can transduce the signals at the cell surface into transcriptional responses in the nucleus, leading to various biological effects [2–8].

On the basis of structural and functional characteristics, the Smad family can be divided into distinct groups [2–8]. One group includes those pathway-specific Smads, termed R-Smads, that are phosphorylated by receptor kinases. For example, Smad2 and Smad3 are phosphorylated by the TGF- β receptor kinase. The second group includes the common Smads, designated Co-Smads, which participate in various TGF- β family-member signalling pathways. The only known member of this group in vertebrates is Smad4. In response to TGF- β treatment, Smad2 and Smad3 are phosphorylated by the activated TGF- β receptor at the SSXS motif in the C-terminal tail, form complexes with Smad4, and together accumulate in the nucleus to regulate transcription of target genes [2–8]. Smads can activate transcription by recruitment of co-activators, such as p300/CBP [cAMP-response-element binding protein (CREB)-binding protein], P/CAF (p300/CBP associated factor), ARC105, MSG1 and SMIF [7–20]. On the other

hand, Smads can also inhibit transcription by recruitment of co-repressors [8,21,22].

The R-Smads and Co-Smads contain conserved N- and C-terminal domains linked by a proline-rich region, termed the linker region, which is divergent in sequence and in length [2–8]. The N-terminal domains of Smad3 and Smad4 possess intrinsic DNA-binding activities [3,4,8]. Although Smad2 is highly homologous with Smad3 [3,4], Smad2 cannot bind to DNA due to the interference of an extra 30 amino acids encoded by a separate exon present immediately before the DNA-binding hairpin [3,4]. Although Smad3 and Smad4 possess DNA-binding activities, their affinities for most natural TGF- β -responsive promoters are not high enough to allow them to bind on their own. Instead, Smads are usually recruited to target promoters through interaction with DNA-binding factors [2–4,7,8]. The C-terminal domains of Smads are responsible for homo-oligomerizations for R-Smads and Co-Smads, and for hetero-oligomerizations between a R-Smad and a Co-Smad [2–8]. Moreover, the C-terminal domains of Smad2 and Smad3 have been shown to participate in transcriptional activation through recruitment of co-activators, such as p300/CBP [9–16]. Smad4 also plays an essential role in Smad-mediated transcriptional regulation [2–8]. This is, at least in part, due to the presence of a SAD (Smad activation domain) in the linker region [18]. The SAD physically interacts with co-activators, such as p300/CBP [17–20]. The function of the linker regions of Smad2 and Smad3 has not been well explored in transcriptional control.

In the present paper, we show that the Smad3 linker region contains a transcriptional activation domain. It physically and

Abbreviations used: CREB, cAMP-response-element binding protein; CBP, CREB-binding protein; DBD, DNA-binding domain; HA, haemagglutinin; SAD, Smad activation domain; TGF- β , transforming growth factor- β .

¹ To whom correspondence should be addressed, at the Center for Advanced Biotechnology and Medicine, Rutgers University (email fangliu@cabm.rutgers.edu).

Figure 1 The Smad3 linker region contains a constitutive transcriptional activation domain

(A) GAL4 (DBD, where DBD refers to DNA-binding domain; 0.8 μ g of DNA) or GAL4-Smad3 (Linker) (0.05, 0.1, 0.2, 0.4 or 0.8 μ g of DNA) were co-transfected with a GAL4 reporter gene (1.6 μ g) into Mv1Lu/L17 cells in 60-mm-diameter dishes, and analysed for luciferase activity. (B) The transcriptional activity of Smad3 linker region is comparable with that of SAD. GAL4 (DBD), GAL4-Smad3 (Linker) or GAL4-SAD were co-transfected with a GAL4 reporter gene into COS cells. Cell lysates were analysed for luciferase activity. Protein expression levels were examined by immunoblotting with an antibody against the GAL4 (DBD). KD = kDa. (C) The transcriptional activity of the Smad3 linker region is constitutive. GAL4 (DBD), GAL4-Smad3 (Linker) or GAL4-Smad3 (Full Length) were co-transfected with a GAL4 reporter gene and analysed for luciferase activity.

functionally interacts with p300. Moreover, we show that the Smad3 linker region co-operates with the C-terminal domain for TGF- β -inducible transcriptional activation.

EXPERIMENTAL

Plasmid constructions

GAL4-Smad3 (Linker), GAL4-Smad3 (C), GAL4-Smad3 (LC), and GAL4-Smad3 (Full Length) were constructed by inserting DNA fragments encoding Smad3 amino acids 142–230, 231–424, 142–424 and 1–424 respectively into the pSG424 vector [23], which encodes the GAL4 DNA-binding domain. Myc-tagged Smad3 (Linker) and Smad3 (Full Length) were constructed in the CS3⁺-6Myc vector [9]. Smad3 (NC) was constructed by inserting DNA fragments encoding Smad3 amino acids 1–141 and amino acids 231–424 into the CS2 vector. GAL4-SAD [18], E1a, E1a(Δ 2–36) and p300-HA (haemagglutinin epitope tag) [10] were as described previously.

Cell culture and antibodies

Mink lung epithelial Mv1Lu/L17 cells were maintained in MEM (minimum essential medium) containing NEAA (non-essential amino acids), 10% (v/v) FBS (dialysed fetal-bovine serum) and histidinol. Human hepatoma HepG2 cells were cultured in MEM containing NEAA and 10% FBS with 1 mM sodium pyruvate. Human HaCaT keratinocytes were maintained in MEM containing NEAA and 10% FBS. COS and HEK-293 cells were cultured in DMEM (Dulbecco's modified Eagle's medium)/10% FBS. All cell-culture media contained 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were grown at 37°C in chambers supplied with 5% CO₂. Antibodies against the GAL4 DNA-binding domain (Upstate Signaling Solutions), the HA epitope (Roche Diagnostics), the Myc epitope (Sigma), the Smad3 linker region (Zymed Laboratories), the Smad3 C-terminal domain (Santa Cruz Biotechnology, Inc.), and the Smad3 C-terminal tail phosphorylation sites (Biosource) were used.

Transfection and reporter gene assay

Mv1Lu/L17, HepG2, HaCaT and COS cells in 60-mm-diameter dishes were transfected by DEAE-dextran. HEK-293 cells were transfected by LIPOFECTAMINE PlusTM reagent. Cells were treated with or without 500 pM TGF- β for 18–24 h, and then analysed for luciferase activity as described previously [24]. Luciferase activities were normalized using the co-transfected *Renilla* luciferase control driven by pRL-TK (Promega). Results represent the means $\pm$ S.D. for at least three independent transfection experiments.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were performed essentially as described previously [25]. In brief, to detect an interaction between the Smad3 linker region and p300, HEK-293 cells were transfected by LIPOFECTAMINE PlusTM reagent. Cells were lysed in TNM buffer [20 mM Tris/HCl (pH 7.6)/150 mM NaCl/3 mM MgCl₂/0.5% Nonidet P40] in the presence of protease and phosphatase inhibitors, and immunoprecipitated using appropriate antibodies. The immunoprecipitates were washed five times each with 1 ml of buffer for 10 min, and then loaded on to a gel and immunoblotted using appropriate antibodies. To detect GAL4 fusion protein expression levels, immunoblotting with an antibody against the GAL4 DNA-binding domain was performed as described previously [24].

RESULTS

The Smad3 linker region has an intrinsic transcriptional activity

To determine whether the Smad3 linker region contains a transcriptional activation domain, we fused the linker region to the GAL4 DNA-binding domain. As shown in Figure 1(A), GAL4-Smad3 (Linker) activates transcription in a dose-dependent manner. GAL4-Smad3 (Linker) has transcriptional activity in various cell lines we examined, including Mv1Lu/L17, HepG2, HaCaT, COS and HEK-293 cells (Figure 1A, and results not shown). When compared with the GAL4-SAD fusion protein, we found

Figure 2 The linker region of Smad3 functionally interacts with p300

(A) E1a inhibits the Smad3 linker-mediated transcriptional activation. Mv1Lu/L17 cells were co-transfected with the GAL4 reporter gene, GAL4 (DBD) or GAL4-Smad3 (Linker) in the absence or presence of wild-type E1a or E1a(Δ2-36), as indicated, and analysed for luciferase activity. (B) p300 can rescue the inhibitory effect of E1a to a large extent. HEK-293 cells were co-transfected with the GAL4 reporter gene, GAL4-Smad3 (Linker), E1a and increasing amounts of p300, and analysed for luciferase activity.

that the Smad3 linker region has a transcriptional activity comparable with that of SAD (Figure 1B). GAL4-Smad3 (Linker) and GAL4-SAD were expressed at comparable levels (Figure 1B). Similar to the SAD [18], the transcriptional activity of Smad3 linker region is also constitutive: TGF- β treatment has little effect on its activity (Figure 1C). This is contrast with the transcriptional activity of GAL4-Smad3 (Full Length), which is significantly induced by TGF- β (Figure 1C). Intramolecular interactions between the N- and C-terminal domains [26] may inhibit the transcriptional activity of both the C-terminal domain and the linker region of Smad3, resulting in a low activity of GAL4-Smad3 (Full Length) at basal state.

The Smad3 linker region functionally interacts with p300

To determine whether the Smad3 linker region functionally interacts with p300, we analysed the effect of the adenovirus E1a protein, which interacts with p300 and inhibits p300-mediated transcriptional activation [27]. As shown in Figure 2(A), wild-type E1a potentially inhibited the transcriptional activity of the GAL4-Smad3 (Linker). In contrast, E1a(Δ2-36), which cannot bind to p300 [27], has little effect. To provide further evidence for the functional requirement of p300 in Smad3 linker-mediated transcription, we asked whether overexpression of p300 can rescue the inhibitory effect of E1a. As shown in Figure 2(B), overexpression of p300 rescued the inhibition in a dose-dependent manner to a large extent. Overexpression of p300 at higher doses did not result in complete rescue (results not shown). Similarly, previous studies found that overexpression of p300 partially restored E1a-mediated repression of GAL4-SAD transcriptional activity [18]. Although it is not clear why it is difficult to achieve complete rescue, the results in Figure 2 suggest that p300 participates in Smad3 linker-mediated transcriptional activation.

The Smad3 linker region physically interacts with p300

We then determined whether the Smad3 linker region physically interacts with p300 by immunoprecipitation/immunoblot assay. Myc epitope-tagged Smad3 linker and HA-epitope-tagged p300

Figure 3 The linker region of Smad3 physically interacts with p300

(A) and (B) HEK-293 cells were co-transfected with Myc-Smad3 (Linker), Myc-Smad3 (Full Length), p300-HA and constitutively active TGF- β type I receptor T β RI (T204D) for TGF- β induction. Cell lysates were immunoprecipitated (IP) by HA antibody, followed by immunoblotting with an antibody against the Myc epitope. Lysate controls are shown for Myc-Smad3 (Linker) and Myc-Smad3 (Full Length) expression levels by Myc immunoblotting, and p300-HA levels by HA immunoblotting. (C) HEK-293 cells were co-transfected with GAL4-Smad3 (Linker), GAL4-Smad3 (Full Length), p300-HA and T β RI (T204D) for TGF- β induction. Cell lysates were immunoprecipitated by HA antibody, followed by immunoblotting with an antibody against the Smad3 linker region. GAL4-Smad3 (Linker) and GAL4-Smad3 (Full Length) expression levels were examined by the antibody against Smad3 linker region. p300-HA levels were examined by HA immunoblotting.

were co-transfected into HEK-293 cells, treated with or without TGF- β . Cell lysates were then immunoprecipitated by the HA antibody. The immunoprecipitates were loaded on to a gel and detected by immunoblotting using an antibody against the Myc epitope. As shown in Figures 3(A) and 3(B), the linker region interacts with p300-HA constitutively, in contrast with TGF- β -induced interaction between full-length Smad3 and p300-HA. To provide further evidence for a physical interaction between the Smad3 linker region and p300, we analysed whether GAL4-Smad3 (Linker) interacted with p300. GAL4-Smad3 (Linker) or GAL4-Smad3 (Full Length) were co-transfected with p300-HA into HEK-293 cells, which were then treated with or without TGF- β . Cell lysates were immunoprecipitated by the HA antibody, followed by immunoblotting with an antibody against the Smad3 linker region. Similar to the results shown in Figures 3(A) and 3(B), GAL4-Smad3 (Linker) interacted with p300-HA constitutively, whereas GAL4-Smad3 (Full Length) interacted with p300-HA in a TGF- β -dependent manner (Figure 3C, and results not shown). Taken together, these results indicate that the linker region of Smad3 physically interacts with p300.

Figure 4 The linker region is necessary for native Smad3 to mediate TGF- β transcriptional activation responses

(A) Smad3 (NC) can be phosphorylated by the TGF- β receptor at the C-terminal tail, and has a significantly increased ability to form a heteromeric complex with Smad4. COS cells were co-transfected with Smad3 (NC) or Smad3 (Full Length), together with Smad4-HA and T β RI (T204D) for TGF- β induction. Cell lysates were analysed for Smad3 C-terminal tail phosphorylation and heteromeric complex formation with Smad4, as indicated. Expression levels of Smad3 (NC), Smad3 (Full Length) and Smad4-HA are also shown. (B) Smad3 (NC) is unable to mediate TGF- β transcriptional activation responses. HepG2 cells were co-transfected with 3TP-Lux, Smad7-Lux or A3-Lux plus FAST-1, together with the CS2 vector, Smad3 (Full Length) or Smad3 (NC), and then treated with or without TGF- β and analysed for luciferase activity.

The linker region is necessary for native Smad3 to mediate TGF- β transcriptional activation responses

To analyse the role of the linker region in the native Smad3 protein, we generated a Smad3 (NC) construct, with the linker region being deleted from full-length Smad3, leaving the N- and C-terminal domains intact and connected with each other. To determine whether Smad3 (NC) retained at least partial structural integrity, we analysed its ability to be phosphorylated by the TGF- β receptor at the C-terminal tail and to form a heteromeric complex with Smad4. Smad3 (NC) or Smad3 (Full Length) were co-transfected with Smad4-HA into COS cells, treated with or without TGF- β . The lysates were directly analysed by immunoblotting with a phosphopeptide antibody that specifically recognized the receptor-phosphorylated serine residues in the Smad3 C-terminal tail. As shown in Figure 4(A) (bottom panel), the C-terminal tail of Smad3 (NC) was phosphorylated to approximately

Figure 5 The linker region and the C-terminal domain of Smad3 synergize for TGF- β -induced transcriptional activation

(A) COS cells were co-transfected with GAL4-Smad3 fusion proteins containing the linker region, the C-terminal domain, or both the linker and C-terminal domain along with the GAL4 reporter gene and T β RI (T204D) for TGF- β induction. Cells were analysed for luciferase activity. (B) GAL4 protein expression levels were analysed by immunoblotting with the antibody against the GAL4 (DBD). (C) Schematic diagram for the structure and amino acid positions for the Linker, C-terminal domain, and both Linker and C-terminal domain of Smad3.

the same extent as that of Smad3 (Full Length). To analyse heteromeric complex formation with Smad4-HA, the lysates were immunoprecipitated with the HA antibody, followed by immunoblotting with an antibody that recognized the Smad3 C-terminal domain. As shown in Figure 4(A) (top panel), Smad3 (NC) has an ability to form a complex with Smad4 that is significantly greater than that of Smad3 (Full Length).

The Smad3 (NC) was then analysed for its ability to activate TGF- β -responsive reporter genes. As shown in Figure 4(B), Smad3 (NC) showed little ability to stimulate transcription of three well-characterized TGF- β -responsive reporter genes: 3TP-Lux, Smad7-Lux and A3-Lux. It even significantly inhibited the activation of the A3-Lux by endogenous Smads, possibly through sequestration of Smad4. Thus the linker region of Smad3 is necessary for TGF- β /Smad-mediated transcriptional activation.

The linker region and the C-terminal domain of Smad3 co-operate for transcriptional activation in the presence of TGF- β

Previous studies have suggested that the C-terminal domain of Smad3 is sufficient for maximal transcriptional activation upon TGF- β treatment; however, the Smad3 C-terminal domain frequently used in previous studies in fact contained one-third of the linker region (e.g. see [10,13]). The results in Figure 4 indicated that the Smad3 linker region is necessary for activation of TGF- β -responsive genes. We therefore determined whether the linker region and the C-terminal domain of Smad3 co-operate with each other for transcriptional activation. Expression plasmids encoding GAL4 fusions containing Smad3 linker region, Smad3 C-terminal domain or both the linker region and the C-terminal domain were co-transfected with the GAL4 reporter gene and treated with or without TGF- β . As shown in Figure 5(A), the linker region and

the C-terminal domain of Smad3 synergize for transcriptional activation in the presence of TGF- β treatment. Immunoblotting with an antibody against the GAL4 DNA-binding domain confirmed that the three GAL4 fusion proteins were expressed at comparable levels (Figure 5B).

DISCUSSION

We have shown in the present paper that the Smad3 linker region has a transcriptional activity. Accordingly, Smad3 plays an important role in the transcriptional control of a number of target genes. Smad3 and Smad2 have overlapping as well as distinct functions [4]. They are highly conserved in the N- and C-terminal domains, but they differ in the linker region [2–8]. Whether the linker region of Smad2 also contains a transcriptional activation domain is not clear. We have generated a GAL4–Smad2 (Linker) plasmid, constructed in a very similar way to that of GAL4–Smad3 (Linker). The resulting fusion had little transcriptional activity. However, this is complicated by the fact that the expression level of GAL4–Smad2 (Linker) was very low (results not shown). Thus far, it is not clear why the expression of GAL4–Smad2 (Linker) was so low. Future studies are necessary to determine whether the Smad2 linker region also contains a transcriptional activation domain.

We have also analysed whether the linker region plays an important role in the activation of several TGF- β /Smad-responsive reporter genes. We generated a Smad3 (NC) construct that contains only the N- and C-terminal domains. Smad3 (NC) has little activity in terms of stimulating transcription of TGF- β -responsive reporter genes. Since Smad3 (NC) can be phosphorylated by the TGF- β receptor and has a significantly increased ability to form a heteromeric complex with Smad4, it is likely that the lack of transcriptional activity in Smad3 (NC) is due to the removal of a necessary activation function in the linker region. Introduction of Smad3 (NC) has distinct effects on different TGF- β -responsive promoters (Figure 4B), which may be due to different configurations of binding sites for Smads and for other transcription factors. Smad3 (NC) markedly inhibited the activation of A3-Lux by endogenous Smads. This may occur through competition with endogenous Smad2 and Smad3 for formation of heteromeric complexes with Smad4. In addition, we made a Smad3 (NL) construct, which encodes only the N-terminal domain and the linker region. The Smad3 (NL) protein has a very low ability to stimulate transcription of several TGF- β /Smad-responsive reporter genes we analysed (results not shown). Smad3 functions as an oligomer, often as a homotrimer in the basal state and in a heterotrimeric complex with Smad4 after TGF- β treatment [28]. Although the Smad3 (NL) construct contains the N-terminal domain for DNA binding and an activation domain in the linker region, it lacks the oligomerization motifs that reside within the C-terminal domain, which provides an explanation for the very low activity of the Smad3 (NL) construct.

We have shown that the linker and the C-terminal domain of Smad3 co-operate for transcriptional activation in the presence of TGF- β . The C-terminal domain was previously shown to have transcriptional activity. It is worth pointing out that the C-terminal domain of Smad3 used in previous studies often contains one-third of the linker region, amino acids 199–230 (e.g. see [10,13]). As shown in the present paper, the C-terminal domain alone has a lower activity when compared with the linker and the C-terminal domain together. How the linker region and the C-terminal domain of Smad3 synergize in the presence of TGF- β is not clear. In a related study [29], the crystal structure of a Smad4 fragment containing the SAD and the C-terminal domain has been solved.

The C-terminal domain of Smad4 is highly homologous with that of Smad2 and Smad3 (50 % identity), except that Smad4 has a unique insert of approx. 35 amino acids, which interact with the C-terminal tail to form a TOWER-like structural extension from the core. The crystal structure suggests that SAD provides transcriptional capability by reinforcing the structural core and coordinating with the TOWER to present the proline-rich surface and a glutamine-rich surface in the TOWER for interaction with transcription partners [29]. It remains to be determined whether the linker region of Smad3 exerts transcriptional activity through a similar mechanism. Since both the linker region and the C-terminal domain of Smad3 can interact with p300, it is possible that presentation of certain surfaces in the linker region and the C-terminal domain engages p300 in an optimal conformation for transcriptional activation.

The Smad3 linker region is proline- and serine-rich. It contains demonstrated, as well as suspected, phosphorylation sites for multiple kinases, such as the cyclin-dependent kinases, ERK (extracellular-signal-regulated kinase) MAP (mitogen-activated protein) kinase, c-Jun N-terminal kinase, p38 MAP kinase and Ca²⁺/calmodulin-dependent kinase II [5,30–35]. Phosphorylation of the linker region by the various kinases may differentially influence Smad3 transcriptional activity in a context-dependent manner. The C-terminal domain of Smad3 is regulated by TGF- β receptor phosphorylation [2–8]. The C-terminal domain of Smad3 is also a protein–protein interaction domain, responsible for homotrimerization, heterotrimerization with Smad4, and also interaction with a number of DNA-binding proteins [2–8,28]. Under different conditions, the linker region and the C-terminal domain may have differing transcriptional activities, leading to distinct biological responses.

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