Phosphorylation regulation of the interaction between Smad7 and activin type I receptor

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Abstract Signal transduction of activin, one of the members in the transforming growth factor-\(\beta\) superfamily, is initiated by ligand binding with two distinct membrane receptors (type II and type I) followed by activation of Smad2 or Smad3. We report here that activin-induced signaling is negatively regulated by another Smad molecule, Smad7. When expressed in Chinese hamster ovary cells, Smad7 inhibited the transcriptional response induced by either activin treatment or a constitutively active activin type I receptor (ALK-4). In addition, Smad7 also inhibited mouse FAST-2-mediated transactivation of the Xenopus Mix.2 promoter stimulated by the constitutively active ALK-4. Smad7 was able to directly associate with ALK-4 and this association was dependent on the phosphorylation of the type I receptor in its GS domain by activin type II receptors. Expression of kinase defective activin type II receptors decreased the association of Smad7 with ALK-4. Correspondingly, Smad7 bound poorly to a mutant ALK-4 bearing serine to alanine substitutions in four putative phosphorylation sites in its GS domain. These studies not only illustrated the counter regulatory function of Smad7 on activin signaling, but also indicated the involvement of phosphorylation at activin type I receptor in the inhibitory action of Smad7.

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1. Introduction

Signal transduction by transforming growth factor-\(\beta\) (TGF-\(\beta\)) family members, including TGF-\(\beta\)-s, activins and bone morphogenetic proteins (BMPs) is initiated by the interaction of a ligand, in the form of a dimerized peptide, with two types (type I and type II) of transmembrane serine kinase receptors kinases [1]. TGF-\(\beta\)-s and activins first interact with their cognate type II receptors and the ligand/type II receptor complex subsequently recruits the type I receptors into a heteromeric complex on the cell surface. In the heteromeric ligand/receptor complex, the type II receptor phosphorylates the type I receptor within a glycase- and serine-rich GS domain and initiates downstream signaling. Mutations of the putative phosphorylation sites in the GS domain of the activin type I receptor, ALK-4, to neutral amino acids abrogates ALK-4-mediated signaling [2,3]. On the other hand, a mutation of a threonine 206 in ALK-4 or 204 in TGF-\(\beta\)-I type I receptor (ALK-5) to an acidic amino acid generates a constitutively active type I receptor that can signal in the absence of ligand and type II receptors [3,4]. The activated type I receptor is thus the transducer responsible for the propagation of the signal to downstream targets.

The Smads comprise a family of proteins that function as intracellular signaling mediators and modulators of the TGF-\(\beta\) family members [5,6]. Functional and structural considerations allow subdivision of Smads into three subfamilies: pathway-specific, common mediator and inhibitory Smads. Pathway-specific Smads are activated by type I receptor serine kinases in a ligand and type II receptor-dependent manner. This subfamily includes Smad1, 2, 3, 5 and 6 [6]. Smad1 and 5 mediate signaling by BMP2 and 4 [7–10], Smad2 and 3 mediate signaling by TGF-\(\beta\)-s and activins [11–14], and Smad8 mediates signaling by the receptor serine kinase, ALK-2 [15]. The second Smad subfamily is represented by Smad4 [16], which serves as a common signaling mediator. Activation of the pathway-specific Smads by their individual receptors induces an association of these Smads with Smad4 and this association is critical for nuclear translocation and proper downstream signaling [17].

Smad6, Smad7 and the Drosophila DAD (daughters against dpp) comprise the third Smad subfamily and have been reported to function as negative regulators of receptor serine kinases of TGF-\(\beta\) family members [18–23]. Both Smad6 and Smad7 have been shown to inhibit signal transduction by the TGF-\(\beta\) receptor, ALK-5 [18,19]. In addition, Smad7 was able to inhibit activin signaling [24], while Smad6 was reported to inhibit BMP signaling [20]. A Xenopus Smad7 homolog was also reported recently to antagonize activin signaling in frog embryo [22,23]. Another Drosophila Smad, DAD was shown to inhibit signaling by the fly BMP2/4 ortholog, dpp, in Drosophila and BMP2 or 4 in the Xenopus embryo [21]. To further elucidate the regulation of activin signaling by Smad7, we isolated a rat Smad7 homolog and studied its effects on signal transduction by the activin receptor complex and how phosphorylation is involved in the regulatory effect of Smad7.

2. Materials and methods

2.1. Cell culture, cell transfection and plasmids

Chinese hamster ovary cells (CHO-K1) and human embryonic kidney 293 cells (HEK293) were cultured in Dulbecco’s modified Eagle’s...
medium containing 10% fetal bovine serum (FBS). Cell transfection was performed by the calcium phosphate method as previously described [14]. For plasmids used here, the mouse FAST-2 has been previously described by us [25]. The myc-tagged and Smad7 were generated by in-frame fusion of the 5’-ends of rat Smad7 with CS2-MT [26], that contains six repeats of the myc epitope sequence.

2.2. Cloning of a rat Smad7 homolog

Two degenerate primers were designed according to the sequence of human Smad7 [18] and used in polymerase chain reaction (PCR) with a cDNA reverse transcribed from mouse total brain RNA. The PCR products were subcloned and their sequences were determined (Sequenase version 2.0, USB). Sequence comparison with human Smad7 indicated that one of the PCR products represented the mouse Smad7 homolog. This mouse PCR fragment was then used to screen a rat brain cDNA library at low stringency. Six independent clones were isolated and all of them encoded the same rat Smad7 homolog. The sequence of Smad7 was determined by sequencing both strands. The full-length cDNA insert of Smad7 was subcloned into pCS2+ vector for expression in cultured cells.

2.3. Promoter assay

For the luciferase assay, about 1 × 10^5 cells in a 6-cm dish were transfected with 0.5 µg each of 3TP-Lux and pCMV-β-gal, and pcDNA3 (Invitrogen) was used to make up a total of 5 µg DNA for each transfection. About 36 h after transfection, the cells were changed to a medium with 0.2% FBS and cultured for another 12 h. In the activin treated groups, cells were treated with activin A (25 ng/ml) during this 12 h period. The cells were then harvested and used in luciferase and β-gal assays as described before [14].

2.4. Immunoprecipitation and immunoblotting analyses

For immunoprecipitation studies, the cell lysate was prepared in NP-40 lysing buffer [17] and incubated with rabbit anti-ALK-4 polyclonal antibodies (~2 µg/ml) in the presence of protein-A Sepharose beads (Sigma). After incubation at 4°C for 2 h, the Sepharose beads were washed with the lysis buffer and the immunoprecipitate was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to nitrocellulose membrane, blotted with 9E10 anti-myc antibody to detect the myc-tagged proteins. The membranes were then incubated with a secondary horseradish peroxidase-conjugated antibody (Bio-Rad), and detected by Chemiluminescence Reagent Plus (NEN). For Western blotting, the cell lysate, equivalent to about 1/30 of the amount used for immunoprecipitation, were resolved on 8% SDS-PAGE and detected by the mouse 9E10 anti-myc or polyclonal anti-ALK-4 antibodies to examine the expression level of related proteins.

3. Results

3.1. Isolation of a rat Smad7 homolog

Recent studies have revealed a subclass of Smads that are able to antagonize the signaling of TGF-β family members [18–22,24]. To further investigate the molecular mechanism of the inhibitory action of Smad7, we isolated a rat Smad7 homolog from a rat brain cDNA library by low stringency hybridization with a PCR probe generated by degenerate primers on the basis of the human Smad7 sequence [18]. At the amino acid level, rat Smad7 is 98% identical to the mouse ortholog [19], 97% to the human ortholog [18] and 80% to the presumptive Xenopus ortholog [22,23]. Smad7 shares considerable similarity to Smad6, consistent with the functional studies showing that both Smad6 and Smad7 are able to inhibit TGF-β signaling [18–20].

3.2. Inhibition of activin-induced transactivation activity by Smad7

To determine if activin signaling is affected by Smad7, we expressed Smad7 in CHO-K1 cells and tested its effect on activin-induced 3TP-Lux activity. We found earlier that CHO-K1 cells possess endogenous activin receptors and were able to respond to activin treatment [27]. The 3TP-Lux promoter is a fusion construct with multiple TPA-responsive elements and the plasminogen activator inhibitor promoter. It has been extensively used as a promoter element to test the activity of TGF-β and activin signaling [28]. As shown in Fig. 1A, activin treatment of CHO-K1 cells transfected with vector (pcDNA3), 3TP-Lux (0.5 µg), pCMV-β-gal (0.5 µg) and Smad7 (2.5 µg) as indicated. The fold change of luciferase activity normalized by β-galactosidase activity was compared to the value of the vector-transfected and unstimulated cells (set to 1) and shown as mean±S.D. B: CHO-K1 cells were transfected with 3TP-Lux (0.5 µg), pCMV-β-gal (0.5 µg), CA-ALK-4 (1 µg) and Smad7 (0.25, 0.75 and 2.5 µg) as indicated. The 3TP-Lux assay was performed and presented as in A. C: HEK293 cells were transfected with vector (pcDNA3), AR3-lux (0.2 µg), pCMV-β-gal (0.2 µg), mouse FAST-2 (0.6 µg), constitutively active ALK-4 (0.4 µg), constitutively active ALK-5 (0.4 µg) and Smad7 (0.2, 1.0 and 4.0 µg). The luciferase assay was performed as in A.

The current model of TGF-β family signaling indicates that the type I receptor is responsible for transducing the signals to downstream targets. A constitutively active form of the activin type I receptor ALK-4 (CA-ALK-4) bearing a threonine to aspartic acid substitution at position 206 (T206D) has been found to stimulate activin signaling in the absence of ligand.
stimulation [2]. To test if Smad7 also antagonizes activin type I receptor-mediated signaling, CA-ALK-4 was used to stimulate 3TP-Lux activity in CHO-K1 cells and the effect of Smad7 was studied (Fig. 1B). When the cells were transfected with CA-ALK-4 alone, 3TP-Lux activity was increased to about five-fold as compared to the vector control. However, co-expression of Smad7 with CA-ALK-4 led to a dose-dependent decrease of 3TP-Lux stimulation by CA-ALK-4. At the highest concentration, Smad7 completely blocked CA-ALK-4-induced 3TP-Lux activation. These findings suggest that the block of activin signaling by Smad7 can occur after receptor activation.

Recent studies have suggested that a group of forkhead transcription factors (FAST-1 in *Xenopus* and human, FAST-2 in mouse) are involved in the regulation of Mix.2 and goosecoid promoters [25,29–31]. We examined if Smad7 was also able to inhibit the FAST-2-mediated transcriptional regulation on the *Xenopus Mix.2* promoter, AR3-lux, that contains three tandem repeat of activin-responsive element in the front of a luciferase reporter [18]. In the presence of mouse FAST-2, both CA-ALK-4 and TGF-β type I receptor (CA-ALK-5) was able to stimulate the AR3-lux activity to over 40-fold (Fig. 1C). However, co-expression of Smad7 led to a dose-dependent inhibition of the active ALK-4 and ALK-5-mediated stimulation of AR3-lux (Fig. 1C). These data further suggest that Smad7 is able to antagonize the signaling of activin receptor.

### 3.3. Association of Smad7 with ALK-4

To address the possibility that Smad7 blocks activin signal-

- ALK-4 - - wt wt wt CA KD CAKD
- ActRIIB - + + + + + + +
- myc/Smad7 - + - + + + + +
- Activin - - - - 15' 60' - - -

**Fig. 2.** Association of Smad7 with ALK-4. HEK293 cells were transfected with activin type IIB receptor and a myc-tagged Smad7 with different forms of ALK-4 including wild-type (wt), a constitutively active T206D mutant (CA), a kinase defective mutant (KD) and a double mutant of T206D with defective kinase (CA/KD). Cells were treated with activin (25 ng/ml) for 15 or 60 min as indicated. The transfected cells were lysed 48 h after transfection and the ALK-4 was immunoprecipitated with a polyclonal anti-ALK-4 antibody and the immunoprecipitate was blotted with an anti-myc antibody (upper panel). The crude cell lysate was also blotted with anti-myc and anti-ALK-4 antibodies to determine the protein expression level of the tagged Smad7 and ALK-4 (lower panels). The possible phosphorylation state of ALK-4 is labeled as pALK-4 for hypophosphorylated receptor and ppALK-4 for hyperphosphorylated receptor.

- ALK-4 - wt wt wt
- ActRIIB - wt KD -
- myc/Smad7 + + + +

**Fig. 3.** Kinase defective activin type II receptors inhibit association of Smad7 with ALK-4. HEK293 cells were transfected with ALK-4, a myc-tagged Smad7 (myc/Smad7), a wild-type (wt) activin type IIB receptor, a kinase defective (KD) activin type IIB or type II receptors as indicated. The cell lysate was immunoprecipitated with an anti-ALK-4 antibody and blotted with an anti-myc antibody (upper panel). The crude cell lysate was also blotted with anti-myc and anti-ALK-4 antibodies to determine the protein expression level of Smad7 and ALK-4 (lower panels). The possible phosphorylation state of ALK-4 is labeled as pALK-4 for hypophosphorylated receptor and ppALK-4 for hyperphosphorylated receptor.
ing by association with the activin type I receptor, we expressed a myc-tagged Smad7 with different forms of ALK-4 in HEK293 cells and studied their possible interactions by a co-immunoprecipitation experiment. As shown in Fig. 2, Smad7 did interact with ALK-4 in the absence of ligand stimulation when both activin type I and type II receptors were overexpressed (lane 4). Activin treatment for 15 or 60 min in these cells had no apparent effect on the association between Smad7 and ALK-4 (lanes 5 and 6). The Smad7/ALK-4 association was not dependent on the kinase activity of the type I receptor because the kinase defective ALK-4 mutant generated by changing lysine to arginine at position 234 [27] was also able to associate with Smad7 at a level similar to that of wild-type ALK-4 (lane 8). Interestingly, the constitutively active ALK-4 (T206D) mutant [2,32] showed 3-4-fold more association with Smad7 than did the wild-type ALK-4 (lane 7). This elevated Smad7 association also occurred to the ALK-4 double mutant that bears the T206D and the kinase defective mutation (lane 9). We also noted that the mobility of the T206D mutant protein was less than that of wild-type ALK-4 in SDS-PAGE gel (lanes 7 and 9, lower panel), perhaps due to an increase in negative charge.

3.4. Phosphorylation of ALK-4 by the activin type II receptor regulates association with Smad7

Our observation that the T206D mutation mimicking phosphorylation of ALK-4 could enhance its binding to Smad7 (Fig. 2) suggests that the interaction of Smad7 with the receptor might be affected by the phosphorylation state in the GS domain of ALK-4. To address this question, we tested if the association of ALK-4 with Smad7 is dependent on the kinase activity of the activin type II receptors. It has been shown that overexpression of both activin type I and type II receptors together is able to mediate activin signaling in a ligand independent manner [2]. This finding was interpreted to indicate that overexpression of the type II receptor is able to force formation of a complex with the type I receptor and activation of the type I receptor by phosphorylating its GS domain in the absence of ligand. We co-expressed a myc-tagged Smad7 and ALK-4 in HEK293 cells and examined the association of Smad7 with ALK-4 in the presence of a wild-type type II receptor or kinase defective type II receptors. The kinase defective activin type IIIB receptor was made by mutation of lysine to arginine at position 214 [2] and the kinase defective activin type II receptor was generated by mutating the GS domain. We also noted that the mobility of the T206D mutant protein was less than that of wild-type ALK-4 in SDS-PAGE gel (lanes 7 and 9, lower panel), perhaps due to an increase in negative charge.

Fig. 4. Association of Smad7 with ALK-4 is dependent on the phosphorylation in the GS domain by activin type II receptors. A: Schematic representation of ALK-4 and the ALK-4 4SA mutant. The extracellular domain (ECD), transmembrane domain (TM), GS domain, serine kinase domain and the position of mutations are indicated. B: Co-expression of the activin type IIB receptor does not increase association of ALK-4 4SA with Smad7. HEK293 cells were transfected with the wild-type ALK-4 (wt), the serine to alanine mutant of ALK-4 (4SA), an activin type IIB receptor and a myc-tagged Smad7 (myc/Smad7). The cell lysate was immunoprecipitated with an anti-ALK-4 antibody and blotted with an anti-myc antibody (upper panel). The crude cell lysate was blotted with anti-myc and anti-ALK-4 antibodies to determine the protein expression level of Smad7 and ALK-4 (lower panels).
tation of lysine to glutamate at position 200 [27]. As shown in Fig. 3, ALK-4 bound strongly to Smad7 when a wild-type activin type II receptor was co-expressed (lane 2). However, co-expression of either a kinase defective activin type IIB (lane 3) or a kinase defective activin type II receptor (lane 4) could not facilitate association of ALK-4 with Smad7. From these results, we infer that phosphorylation of the activin type I receptor by type II receptor is required for the association of ALK-4 with Smad7. Because it is the kinase activity of the type II receptor that effects the phosphorylation of the activin type I receptor, the phosphorylation status of ALK-4 as well as its ability to associate with Smad7 should not be affected by the kinase defective ALK-4 (Fig. 2, lane 8). Likewise, the forced phosphorylation of ALK-4 by overexpressed type II receptor may also mask the ligand-induced phosphorylation of ALK-4 [2] and therefore results in our observation (Fig. 2, lane 5 and 6) that activin treatment did not lead to further increase of receptor association with Smad7.

We also noted that the phosphorylation state of ALK-4 might be reflected by protein mobility in the immunoblotting with the anti-ALK-4 antibody (Fig. 3, lower panel). Co-expression of the wild-type activin type IIIB receptors might lead to hyperphosphorylation of ALK-4, manifested as a slower mobility on the gel due to its increased negative charge (Fig. 3, lane 2, lower panel). However, co-expression of the kinase defective activin type II receptors failed to phosphorylate ALK-4 and led to a lack of hyperphosphorylated ALK-4 with slower mobility (Fig. 3, lanes 3 and 4, lower panel).

The type II receptor-mediated ALK-4 phosphorylation has been proposed to occur in the serine and threonine residues in the GS domain. Mutations of these putative phosphorylation sites into neutral amino acids can lead to loss of the downstream signaling [2,3]. If the association of ALK-4 with Smad7 is dependent on the phosphorylation in the GS domain, we hypothesized that such mutations would abrogate the association. Based on this consideration, we examined the ability of one of these mutants, 4SA (Fig. 4A) to associate with Smad7. This mutant has been reported to be functionally deficient in mediating activin-induced 3TP-Lux activation [3]. As shown in Fig. 4B, wild-type ALK-4 weakly associated with Smad7 in the absence of the activin type IIB receptor (lane 2) and this association is greatly enhanced by co-expression of the activin type IIB receptor (lane 3). The ALK-4 4SA mutant was also able to weakly associate with Smad7 (lane 4) and this modestly elevated binding was apparently due to increased protein expression judged by the Western blotting (Fig. 4B, the lower panel). However, co-expression of the activin type IIB receptor did not further elevate the association of ALK-4 4SA with Smad7 (Fig. 4B, lane 5), consistent with our hypothesis that the association of Smad7 with ALK-4 is dependent upon its phosphorylation in the GS domain by the type II receptors. The phosphorylation state of ALK-4 might also be revealed by the change of mobility in SDS-PAGE gel (Fig. 4, lower panel). Co-expression of activin type IIB receptors led to an increase in the amount of hyperphosphorylated ALK-4 (lane 2, lower panel), but failed to hyperphosphorylate the ALK-4 4SA mutant (lane 4, lower panel).

4. Discussion

We present evidence that Smad7 is able to negatively modulate signal transduction by the activin receptor serine kinase complex. One of the molecular mechanisms underlying the antagonization of TGF-β superfamily member signaling by Smad7 appears to be related to the association of Smad7 with the type I receptors. Smad7 was reported to weakly bind the wild-type TGF-β type I receptor and the association was greatly elevated with a constitutively active TGF-β type I receptor [18]. We found here that Smad7 can associate with ALK-4, the type I activin receptor, and that the association of Smad7 with a constitutively active ALK-4 mutant is stronger than to the wild-type receptor. Our data also suggest that the association of Smad7 with ALK-4 is regulated by the phosphorylation of the type I receptor in the GS domain by the type II receptor. This is further supported by our observation that co-expression of the activin type IIB receptor did not further elevate the association of ALK-4 4SA mutant with Smad7 (Fig. 4), although we could not rule out the possibility that the 4SA mutation may change the overall structure of ALK-4 and its affinity for Smad7. Nevertheless, our results suggest that Smad7 may interfere with signal transduction from the type I receptor to Smad2 or Smad3 proteins by binding to the activated type I receptor. This binding may displace the ligand-induced transient association of the pathway-specific Smads with the receptor and block the phosphorylation of these pathway-specific Smads by the receptor. This is evidenced by the finding that Smad7 is able to block the transient association of Smad2 and Smad3 with activin receptor complex upon ligand stimulation [33]. Another proposed mechanism of action for the inhibitory Smads involves a direct interaction with pathway-specific Smads. Smad6 was reported to directly associate with Smad1 forming an inactive complex and consequently disrupting the Smad1/Smad4 association induced by BMP receptor activation [34]. Such a mechanism seems unlikely to apply to the inhibition of activin signaling by Smad7 because we could not detect any association of Smad7 with Smad2 by co-immunoprecipitation assay (data not shown).

It is noteworthy that the association of Smad7 with ALK-4 is dependent on the phosphorylation in the GS domain of ALK-4 and on the kinase activity of its corresponding type II receptors. These observations are also consistent with the findings that the mimicked phosphorylation in the constitutively active activin and TGF-β type I receptors could lead to a stronger association with Smad7 than do the wild-type receptors. This increased association of Smad7 with activated type I receptors may promote termination of receptor signaling. Future studies of the molecular mechanisms by which the phosphorylation of the type I receptors by the type II receptors affects the Smad7 binding would undoubtedly help to elucidate the structure/function relationships of the Smad/receptor interactions. These studies may also reveal new ways to develop therapeutic approaches to modulate the signaling by TGF-β superfamily members.

Activin and its cognate receptor serine kinases are involved in various biological processes including reproduction, erythropoiesis, neuronal differentiation and early development [35]. Two extracellular regulators, inhibin and follistatin, have been identified previously to antagonize the activity of activin [35]. Inhibin shares a common subunit with activin and exerts its inhibitory effect by interacting with betaglycan, a specific inhibin receptor [36]. Follistatin inhibits activin function by forming an inactive complex with activin [37]. Our studies here, together with recent reports from other groups, has in-
dicated another mode of regulation of activin function by Smad7, which may participate in an intracellular negative feedback pathway that serves to counter-regulate activin receptor serine kinase stimulation.

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