Roles for the MH2 Domain of Smad7 in the Specific Inhibition of Transforming Growth Factor-β Superfamily Signaling* [S]

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Signals by cytokines of the transforming growth factor-β (TGF-β) superfamily are negatively regulated by inhibitory Smads (I-Smads). Smad7 inhibits signaling by both TGF-β and bone morphogenetic proteins (BMPs), whereas Smad6 inhibits TGF-β signals less effectively. I-Smads have amino-terminal N domains and carboxyl-terminal Mad homology 2 (MH2) domains. The N domains are essential for specific inhibition of TGF-β signaling by Smad7, whereas the MH2 domains of I-Smads are involved in the inhibition of TGF-β superfamily signals through interaction with type I receptors. Here, we have identified four basic amino acid residues (Lys-312, Lys-316, Lys-401, and Arg-409) in the basic surface of the Smad7 MH2 domain that play important roles in interaction with type I receptors. Mutations of the four basic amino acid residues to acidic residues (K312E, K316E, K401E, and R409E) abolished the interaction of Smad7 with TGF-β type I receptors, inhibition of Smad2 phosphorylation and transcriptional responses induced by TGF-β, and induction of target genes of endogenous activin/Nodal signals in Xenopus early embryos. The K401E and R409E mutants of Smad7 were also unable to interact with BMP type I receptors (BMPR-I), repress the Smad5 phosphorylation and transcription induced by BMP, and effectively inhibit endogenous BMP signals in Xenopus early embryos. However, the K312E and K316E mutants were able to interact with BMPR-I and retained the ability to inhibit BMP signaling. Thus, the MH2 domain of Smad7 plays important roles in specific inhibition of TGF-β superfamily signals through differential interaction with type I receptors.

Transforming growth factor-β (TGF-β)† belongs to a family of cytokines that regulate proliferation, apoptosis, and differentiation of many different types of cells. TGF-β superfamily members, which include TGF-βs, activins, Nodal, bone morphogenetic proteins (BMP), growth/differentiation factors, and Müllerian inhibiting substance, share structural similarities and have been found to play critical roles during embryogenesis and in maintenance of tissue homeostasis. Deregulation of TGF-β superfamily signals has been implicated in various developmental disorders and human diseases including fibrosis, autoimmune diseases, and multiple types of cancer (1).

Members of the TGF-β superfamily bind to two different types of serine/threonine kinase receptors termed type I and II receptors. The type II receptor, containing a constitutively active kinase in its intracellular domain, trans-phosphorylates and activates the type I receptor, which in turn phosphorylates the intracellular signaling components, Smad proteins (2–4). Based on structural and functional features, Smads have been classified into three subclasses. The first two subclasses include receptor-regulated Smads (R-Smads, Smad1, 2, 3, 5, and 8 in vertebrates) and common partner Smad (Co-Smad, Smad4 in vertebrates). “Activated” TGF-β/activin family type I receptors (activin receptor-like kinase-4, 5, and 7) directly phosphorylate Smad2 and 3, whereas BMP family type I receptors (activin receptor-like kinase-2, 3, and 6) phosphorylate Smad1, 5, and 8. Phosphorylated R-Smads form oligomeric complexes with Co-Smad, then translocate into the nucleus, where they regulate the transcription of target genes.

The third subclass of Smads comprises inhibitory Smads (1-Smads), consisting of Smad6 and 7 in vertebrates. I-Smads inhibit TGF-β superfamily signals through stable binding to activated type I receptors and competing with R-Smads for receptor activation (5–8). Smad6 and 7 also recruit E3 ubiquitin ligases, Smad ubiquitin regulatory factor (Smurf)1 and 2, to type I receptors, leading to ubiquitin-dependent degradation of the receptor complexes (9–11). Smad6 also forms a complex with Smad1 to compete with Smad4 for oligomer formation (12) and with Smad1/5 to promote their degradation (11). Moreover, Smad6 has been reported to interact with homeobox (Hox) c-8 and 9 as a transcriptional corepressor, inhibiting BMP signaling in the nucleus (13). Smad6 was also shown to recruit CtBP and inhibit the transcription of genes in the nucleus (14). Expression of I-Smads is induced by various signals including TGF-β and BMP (15–18) suggesting that I-Smads act as negative feedback components of TGF-β superfamily signals.

Smad6 and 7 have been reported to show significant differences in the inhibition of TGF-β superfamily signals. BMP homology; DMZ, dorsal marginal zone; E3, ubiquitin-protein isopeptide ligase; VMZ, ventral marginal zone; gsc, goosecoid; N-CAM, neural cell adhesion molecule.
signals are inhibited by both Smad6 and 7, whereas TGF-β/activin signals are inhibited more potently by Smad7 (12, 19–21). Therefore, Smad7 has been shown to antagonize the growth inhibition, matrix formation, apoptosis induction, and embryonic lung morphogenesis induced by TGF-β or activin (21, 22). Additionally, in Xenopus embryos, both Smad6 and 7 block endogenous BMP signals, as shown by their ability to induce secondary dorsal axes when ectopically expressed ventrally, whereas Smad7 inhibits additional activin/Nodal signals and causes spina bifida when ectopically expressed dorsally (23). Structural differences between Smad6 and 7 have been implicated in their functional differences (24).

R-Smads and Co-Smads have highly conserved amino- and carboxyl-terminal regions termed Mad homology 1 (MH1) and MH2 domains, respectively, that are linked by linker regions with variable lengths and sequences. The MH2 domain plays important roles in receptor recognition, interaction with transcription factors, and homo- and hetero-oligomerization among R-Smads and Co-Smad. Additionally, most Smad mutations found in tumors map to the MH2 domain. The MH1 domain exhibits sequence-specific DNA binding activity and negatively regulates the functions of the MH2 domain through physical interaction. This physical interaction between MH1 and MH2 domains is released upon receptor activation (25).

I-Smads have conserved MH2 domains, but their amino-terminal domains (N domains) are highly divergent from the MH1 domains and linker regions of R-Smads and Co-Smads. Moreover, amino acid sequences of the N domains are only partially conserved between Smad6 and 7 (36.7%). It has been shown that the MH2 domains were responsible for the inhibition of both TGF-β and BMP signaling by I-Smads but that the isolated MH2 domain of Smad7 was less potent than the full-length Smad7 in inhibiting TGF-β signals (24). The isolated N domain of Smad7 physically interacted with the MH2 domain of Smad7 and, unlike the MH1 domain of other Smads, enhanced the inhibitory activity of the Smad7 MH2 domain through facilitating interaction with TGF-β type I receptors. Thus, N domains of I-Smads were implicated in the specific

**Fig. 1.** Identification of amino acids involved in the basic surface of the Smad7 MH2 domain. A, inspected three-dimensional structure of the Smad7 MH2 domain. The surfaces with negative electrostatic potentials are colored in red, whereas positive ones are represented in blue. The highly basic surface patch (blue) is conserved with Smad2 and thought to contribute to the interaction with TβR-I. B, four amino acids that are located in the basic surface patch are represented (Lys-306 is not shown in the figure). C, sequence alignment of MH2 domains of Smad7, Smad2, Smad4, and Smad6. Five amino acids in the basic surface of Smad7 MH2 domain are highlighted. The L3 loop is boxed.
inhibition of TGF-β superfamily signals (24).

These findings prompted us to ask whether the MH2 domain of Smad7 may also take part in the specific inhibition of TGF-β superfamily signals. We introduced mutations to the basic amino acids within the Smad7 MH2 domain, which were predicted to play important roles in binding to the receptor complexes, and examined their capabilities of binding to TGF-β and BMP receptor complexes as well as of inhibiting TGF-β and BMP signals. We found that two amino acid residues within the basic surface groove (Lys-312 and Lys-316) of Smad7 are required for the specific inhibition of TGF-β signals, while two amino acids within L3 loop (Lys-401 and Arg-409) are essential for the inhibition of both TGF-β and BMP signals. Moreover, the mutation of Lys-312 or Lys-316 abolished the binding ability to TGF-β receptor complexes and inhibitory activity of Smad2 phosphorylation, whereas that of Lys-401 or Arg-409 abolished the binding ability to both TGF-β and BMP receptor complexes and the inhibitory activity of phosphorylation of Smad2 and 5. These results suggest that the Smad7 MH2 domain plays important roles in the specific inhibition of TGF-β superfamily signals via specific binding to receptor complexes.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Single amino acid mutations in the MH2 domain of Smad7 were made by PCR-based methods as described previously (26) with some modification. PCR products were carried out with Pfu polymerase (Invitrogen). The obtained fragments were digested by BamHI and XbaI and introduced into pCDEF3 (27) or pCS2 (28). All constructs were verified by sequencing. Other constructs that were used have been described previously (6, 10, 24).

Cell Culture—COS-7, 293T, and R-mutant mink lung epithelial cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

Transfection, Immunoprecipitation, and Immunoblotting—COS-7 and 293T cells were transiently transfected using FuGENE 6 (Roche Applied Science). Immunoprecipitation and immunoblotting were performed as described previously (29) using anti-hemagglutinin (HA) 12CA5 (Roche Applied Science) for immunoprecipitation, anti-HA 3F10 (Roche Applied Science), anti-FLAG M2 (Sigma), anti-phospho-Smad1/5 antibody (Cell Signaling Technology), and anti-phospho-Smad2 antibody (Cell Technology) for immunoblotting.

Luciferase Assay—R-mutant mink lung epithelial cells were transiently transfected with an appropriate combination of reporter constructs, expression plasmids, and pcDNA3. Total amounts of transfected DNAs were the same in each experiment. Luciferase activities were normalized using cotransfected sea pansy luciferase activity under the control of thymidine kinase promoter.

Affinity Cross-linking and Immunoprecipitation—Iodination of TGF-β3 and BMP-6, affinity cross-linking, and subsequent immunoprecipitation were performed as described previously (6). Briefly, recombinant TGF-β3 and BMP 6 were iodinated using the chloramine-T method, and cross-linking was performed with disuccinimidyl suberate (Pierce). Cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody followed by SDS-PAGE. Cross-linked receptor complexes were visualized using a Fuji BAS 1800 Bio-Image Analyzer (Fuji Photo Film).

Embryo Manipulation and Microinjection, Reverse Transcriptase-PCR in Xenopus Embryos and Explants—Xenopus embryo manipulation and microinjections were performed as described previously (26). RNA extracts from animal caps, dorsal marginal zone (DMZ), and ventral marginal zone (VMZ), and reverse transcriptase-PCR were carried out as described previously (30). Primer sequences are available upon request.

RESULTS AND DISCUSSION

A Basic Surface Composed of Five Basic Amino Acids in the MH2 Domain of Smad7—Analyses using a deletion mutant of Smad7, which contained only its MH2 domain, showed that the Smad7 MH2 domain was capable of interacting with TGF-β type I receptors (TβR-I) and was essential for inhibition of TGF-β and BMP signaling (24). A highly positively charged groove surrounding the L3 loop in the MH2 domain of Smad2 has been identified as a region that interacts with the L45 loop in TβR-I containing acidic amino acid residues (31, 32). Because the Smad7 MH2 domain is highly homologous to that of Smad2, we inspected three-dimensional structures of the Smad7 MH2 domain based on the previously reported three-dimensional structure of the Smad2 MH2 domain (32).

As shown in Fig. 1A, the Smad7 MH2 domain has a highly positively charged groove (blue area) that is also found in the Smad2 MH2 domain suggesting that Smad7 interacts with type I receptors through this basic surface. The basic surface of Smad7 is composed of five amino acid residues, Lys-306, Lys-312, Lys-316, Lys-401, and Arg-409 (Fig. 1B). A multiple sequence alignment showed that corresponding amino acids in Smad2, 4, and 6, with the exception of Lys-312, are also basic amino acids (Fig. 1C). Lys-401 and Arg-409 are located in a region corresponding to the L3 loop that is important for specific R-Smad interaction with type I receptors (32).
Differential Roles of the Basic Amino Acids in the Basic Surface in the Inhibition of TGF-β Superfamily Signals—To elucidate the basic amino acids in the basic surface (including the L3 loop) that contribute to the inhibition of TGF-β superfamily signals by Smad7, we introduced mutations in the five basic amino acids to convert them to glutamic acid (K306E, K312E, K316E, K401E, and R409E) and investigated the biological functions of the mutants using transcriptional assays. We used two different promoter-reporter constructs, p3TP-Lux and 3GC2-Lux, which preferentially respond to TGF-β/activin and BMP signals, respectively. As shown in Fig. 2A, the wild type and K306E mutant of Smad7 potently inhibited the transcriptional activation of p3TP-Lux induced by TGF-β3 (1 ng/ml) in the presence of transfected TβR-I. In contrast, p3TP activation by TGF-β3 was not inhibited by the K312E, K316E, K401E, or R409E mutant of Smad7. Similar results were ob-

TABLE I
Dorsalizing activity of Smad7 variants in Xenopus whole embryos

mRNAs encoding Smad7 variants were injected in the equatorial region of two ventral blastomeres (100 pg/embryo). The total number of injected embryos surviving to the tadpole stage (N) and the percentage of these embryos showing either a secondary axis, or hyperdorsalization with no apparent secondary axis, is indicated. Data are pooled from multiple experiments. Ectopic expression of Smad7 wild type, K306E, K312E, and K316E mutants resulted in hyperdorsalization of most embryos, whereas more embryos exhibited secondary axes when Smad7 K401E and K409E mutants were overexpressed. When 100 pg of β-globin RNA was injected, embryos developed normally.
The wild type and K306E mutant of Smad7 repressed the transcriptional activation of 3G2C-Lux by 200 ng/ml of BMP7 (Fig. 2B). In contrast, K401E and R409E mutants exhibited weaker inhibition. Interestingly, the K312E and K316E mutants potently inhibited BMP signals. Similar results were obtained using Id1-Lux, which contains the promoter region of Id1 and responds to BMP signals (data not shown). These results suggest that Lys-401 and Arg-409, but not Lys-312 and Lys-316, in the Smad7 MH2 domain are essential for inhibition of BMP signals.

**Differential Roles of the Four Basic Amino Acids in Regulation of Endogenous Activin/Nodal and BMP Signals in Early Xenopus Embryos**—Next, we studied the biological functions of the Smad7 mutants by examining their effects on endogenous activin/Nodal and BMP signals in Xenopus early embryos. During Xenopus early embryogenesis, XSmad7 (also called Smad7 in Xenopus), an ortholog of Smad7, was shown to inhibit both activin and BMP signals (33–35). In addition, the overexpression of XSmad7 in dorsal equatorial regions induced spina bifida, which might be caused by a gastrulation defect (23). During Xenopus early embryogenesis, activin/Nodal signals induce the expression of various direct target genes, such as goosecoid (gsc), in the DMZ of stage 10.5 embryos, whereas gsc is not expressed in the VMZ (Fig. 3A and Refs. 36 and 37). We therefore studied the effects of Smad7 variants on gsc expression. Expression of the gsc gene in the DMZ was partially inhibited by the ectopic expression of wild type and K306E mutant Smad7 in the DMZ (Fig. 3A). In contrast, the expression of gsc was hardly blocked by ectopic expression of the K312E, K316E, K401E, or R409E mutants suggesting that Lys-312, Lys-316, Lys-401, and Arg-409 are necessary for inhibition of endogenous activin/Nodal signals in Xenopus embryos.

Endogenous BMP signals have been shown to regulate ventral mesoderm fates and inhibit neural differentiation during Xenopus embryogenesis (38). In agreement with previous reports (33–35), an ectopic expression of the wild type Smad7 in the ventral equatorial region induced secondary axis formation and hyperdorsalization, which exhibits as a short trunk because of the lack of notochord (Fig. 3B). The frequency of the dorsalizing phenotype induced by Smad7 was 76% (Table I). Ectopic expression of the K306E, K312E, and K316E mutants caused a similar frequency of the phenotypes. In contrast, expression of the K401E and R409E mutants caused different frequencies of the phenotypes, i.e., more secondary axis formation than hyperdorsalization (Fig. 3B and Table I).

To further study the ability of the Smad7 mutants to inhibit endogenous BMP signals, we examined a neural-specific marker in ectodermal explants (animal caps). In agreement with previous reports (33–35), ectopic expression of Smad7 induced expression of N-CAM, a pan-neural marker (Fig. 3C). The K306E, K312E, and K316E mutants induced significant expression of N-CAM in animal caps, whereas the K401E and R409E mutants caused only a weak induction of expression. The induction of N-CAM expression by Smad7 variants is a direct consequence of mesoderm induction. This reflects their neuralizing activities, as expression of the mesodermal marker muscle actin was not induced by any of the variants.

These results obtained in Xenopus embryos further support the results obtained by transcription assays in cultured mammalian cells. Four amino acids (Lys-312, Lys-316, Lys-401, and Arg-409) were essential for inhibition of activin/Nodal signals, whereas only two amino acids (Lys-401 and Arg-409) were required for the inhibition of BMP signals (Figs. 2 and 3). The other two basic residues (Lys-312 and Lys-316) play less important roles in inhibition of BMP signaling in Xenopus embryos.
Roles of the Basic Amino Acids in the Interaction with Type I Receptors—We investigated whether the Smad7 mutants physically interact with receptor complexes containing the constitutively active forms of TβR-I/activin receptor-like kinase-5 (referred to as TβR-I (TD)) and TβR-II or those containing BMPR-IB/activin receptor-like kinase-6 (referred to as BMPR-IB (QD)) and BMPR-II. In the following experiments, constructively active type I receptors showed results essentially similar to those obtained with the wild type receptors in the presence of type II receptors; however, a more potent interaction with Smad7 was obtained with the constructively active type I receptors than with the wild type receptors (data not shown). We thus used the constructively active type I receptors in the following experiments. COS-7 cells were cotransfected with TgR-I (TD), TgR-II, and Smad7 variants. Immunoprecipitation of the Smad7 constructs followed by the immunoblotting of TgR-I (TD) and TgR-II revealed that the wild type and K306E mutant of Smad7 strongly bound TgR-I and TgR-II (Fig. 4A, lanes 4 and 5, respectively, and Supplemental Fig. 1A). In contrast, the K312E, K316E, K401E, and R409E mutants of Smad7 showed a relatively weak interaction with TgR-I (Fig. 4A, lanes 6, 7, 8, and 9, respectively, and Supplemental Fig. 1A). However, immunoprecipitation of the Smad7 variants followed by the immunoblotting of BMPR-IB (QD) and BMPR-II revealed that only the K401E and R409E mutants of Smad7 exhibited reduced interaction with the receptor complexes (Fig. 4B, lanes 8 and 9, respectively, and Supplemental Fig. 1B).

We further studied the physical interaction of the Smad7 mutants with receptor complexes using cross-linking of radiiodinated ligands and receptor complexes followed by immunoprecipitation of the Smad7-receptor complexes. COS-7 cells were cotransfected with Smad7 and constructively active type I and II receptors, affinity cross-linked using 125I-labeled ligands, and subjected to immunoprecipitation of the Smad7 variants. As shown in Fig. 4, C and D, the TgR-II-TgR-I complex was not efficiently immunoprecipitated by the K312E, K316E, K401E, or R409E mutants, whereas the BMPR-II-BMPR-IB complex was efficiently immunoprecipitated by the K312E and K316E mutants, similar to the wild type and the K306E mutant of Smad7. These results are consistent with those obtained by functional assays (Figs. 2 and 3) suggesting that Lys-401 and Arg-409 are necessary for inhibition of both TGF-β and BMP signals by Smad7 through physical interaction with corresponding receptor complexes, whereas Lys-312 and Lys-316 are necessary for its inhibition of BMP signals.

Repression of R-Smad Phosphorylation by Smad7 Variants Correlates with Their Inhibition of Transcription Induced by TGF-β/BMP Signals—I-Smads have been reported to regulate TGF-β/BMP signaling through various mechanisms such as competition with R-Smads for receptor activation, ubiquitin-dependent degradation of receptors by Smurfs, prevention of complex formation between R-Smads and Co-Smads, and transcriptional repression in the nucleus. To further elucidate the mechanisms by which Smad7 variants differentially inhibit the TGF-β/BMP signals, the abilities of the Smad7 variants to inhibit phosphorylation of Smad2 and 5 were examined in transfected COS-7 cells (Fig. 5A and B, respectively). Consistent with the results obtained with transcriptional activation assays (Fig. 2), the wild type and K306E mutant of Smad7 strongly inhibited phosphorylation of Smad2 (Fig. 5A, lanes 4 and 5, respectively). In contrast, the K312E, K316E, K401E, and R409E mutants of Smad7 showed relatively weak inhibition (lanes 6, 7, 8, and 9, respectively). However, only the K401E and R409E mutants of Smad7 exhibited reduced inhibition of Smad5 phosphorylation (Fig. 5B, lanes 8 and 9, respectively). These results suggest that loss of abilities of Smad7 variants to repress TGF-β/BMP signals might occur mainly at the receptor level through competition with R-Smads for receptor activation. In addition, we found that the wild type and K306E mutant of Smad7 enhanced Smurf1-mediated ubiquitination of TgR-I (TD), whereas the K312E, K316E, K401E, or R409E mutant did not (Supplemental Fig. 2) suggesting that the loss of abilities of Smad7 variants to inhibit TGF-β signals might occur through ubiquitin-dependent degradation of type I receptors.

Analysis of the three-dimensional structure of MH2 domain of Smad2 revealed that it contains a highly positively charged groove next to the L3 loop, which might be important for receptor binding (32). Cytoplasmic regions of type I receptors for the TGF-β superfamily contain an L45 loop, which determines the specificity of interactions with R-Smads. The L45 loop is located near the GS region, which is phosphorylated upon ligand binding and becomes very acidic. Thus, in addition to the interaction between the L45 loop of the type I receptors and the L3 loop of R-Smads, the interaction between the phosphorylated GS region of the type I receptors and the basic surface of R-Smads might provide binding affinity.

The MH2 domain of Smad7 is well conserved with those of Smad2, 4, and 6. As shown in Fig. 1C, Lys-312 and Lys-316 in Smad7 are located in the region that corresponds to the basic surface groove next to the L3 loop, whereas Lys-401 and Arg-409 are located in the L3 loop. Our present findings revealed that Lys-401 and Arg-409 are essential for interaction with both TgR-I and BMPR-I suggesting that the L3 loop of Smad7...
might be the major binding site to the type I receptors. Lys-312 and Lys-316 are also necessary for an interaction with TβR-I, indicating that Smad7 also interacts with TβR-I through the basic surface groove in the MH2 domain. In contrast, the K312E and K316E mutants were able to bind BMP receptor complexes suggesting that Smad7 interacts with BMPR-I mainly through the L3 loop and does not require the other part of the basic surface of the MH2 domain. Thus, the present study suggests that the MH2 domain of Smad7 interacts with type I receptors for TGF-β and BMP in different ways, and this may play an important role in specific inhibition of TGF-β superfamily signals.

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