Selective Inhibitory Effects of Smad6 on Bone Morphogenetic Protein Type I Receptors

The inhibitory Smads, Smad6 and Smad7, play pivotal roles in negative regulation of transforming growth factor-β (TGF-β) family signaling as feedback molecules as well as mediators of cross-talk with other signaling pathways. Whereas Smad7 acts as a ubiquitous inhibitor of Smad signaling, Smad6 has been shown to effectively inhibit bone morphogenetic protein (BMP) signaling but only weakly TGF-β/activin signaling. In the present study, we have found that Smad6 inhibits signaling from the activin receptor-like kinase (ALK)-3/6 subgroup in preference to that from the ALK-1/2 subgroup of BMP type I receptors. The difference is attributable to the interaction of Smad6 with these BMP type I receptors. The amino acid residues responsible for Smad6 sensitivity of ALK-3 were identified as Arg-238, Phe-264, Thr-265, and Ala-269, which map to the N-terminal lobe of the ALK-3 kinase domain. Although Smad6 regulates BMP signaling through multiple mechanisms, our findings suggest that interaction with type I receptors is a critical step in the function of Smad6.

Bone morphogenetic proteins (BMPs) are a group of multifunctional cytokines, originally identified as proteins that induce bone and cartilage formation in ectopic tissues (1, 2). More than 10 proteins have been identified as members of the BMP family, some of which have also been termed OP (osteogenic protein), GDF (growth-differentiation factor), and CDMP (cartilage-derived morphogenetic protein). The BMP family can be divided into several subgroups based on structure, including the BMP-2/4 group (BMP-2 and BMP-4), OP-1 group (BMP-5, BMP-6, BMP-7/OP-1, and BMP-8/OP-2), GDF-5 group (GDF-5/CDMP-1, GDF-6/CDMP-2/BMP-13, and GDF-7/BMP-12), and BMP-9/10 group (BMP-9/GDF-2 and BMP-10). Ligands of the BMP family have distinct profiles of spatiotemporal expression, exhibit divergent biological activities, and have important in vivo functions during embryonic development, including tissue patterning and organogenesis, as well as tissue remodeling. Aberrant transduction of BMP signaling has been implicated in several diseases, including primary pulmonary hypertension (3), juvenile polyposis syndrome (4, 5), brachydactyly type A2 (6), and fibrodysplasia ossificans progressiva (7).

BMPs transmit signals through induction of heterotetrameric complexes consisting of two type II receptors and two type I receptors on the cell surface (8). Type II receptor kinase is constitutively active and activates type I receptor kinase through phosphorylation of the GS (Gly-Ser) domain of the type I receptor. Activated type I receptor kinase in turn phosphorylates receptor-regulated Smads (R-Smads) including Smad1, Smad5, and Smad8 (referred to as BMP-specific R-Smads). Phosphorylated BMP-specific R-Smads then form heteromeric complexes with Smad4, a common Smad, and translocate into the nucleus, where they regulate the transcription of various target genes, including Id1, Smad6, and Smad7 (9–11). Smad6 and Smad7, which are induced by BMPs, in turn inhibit the BMP signaling pathways and thus form a negative feedback loop (12, 13). Smad6 and Smad7 are thus classified as inhibitory Smads (I-Smads). Although Smad7 inhibits both TGF-β/activin and BMP signaling, Smad6 efficiently inhibits BMP signaling but only weakly inhibits TGF-β/activin signaling (14–17).

Three receptor serine/threonine kinases have been identified as type II receptors for BMPs, i.e. BMP type II receptor (BMPR-II), activin type II receptor (ActR-II), and activin type IIB receptor (ActR-IIB). When BMPR-II is expressed, it is preferentially utilized for signal transduction (18). Similarly, four different BMP type I receptors have been identified, including activin receptor-like kinase (ALK)-3 (also termed BMP receptor type 1A), ALK-6 (BMP receptor type IB), ALK-2 (ActR-I), and ALK-1. ALK-3 and ALK-6 are structurally quite similar and constitute a subgroup of type I receptors, whereas ALK-1 and ALK-2 in turn are structurally similar and form another subgroup. ALK-2 and ALK-3 are widely expressed in various tissues, whereas expression of ALK-1 and ALK-6 is restricted to certain types of cells and tissues. Utilization of type I receptors differs depending on BMP ligands; BMP-2 utilizes ALK-3 and ALK-6 (19, 20), whereas BMP-6 binds principally to ALK-2 (19). GDF-5 preferentially interacts with ALK-6 (20). BMP-9 is a ligand for
ALK-1 and ALK-2 (21–23). Type I receptors exhibit some preference in phosphorylation of downstream Smad proteins (24), which may determine the specificity of intracellular signaling. The variation in biological activities of BMPs is thus attributable to differences in affinity for their receptors. However, the signal transduction properties of the individual type I receptors are not yet clearly understood.

In the present study, we found that Smad6 inhibited ALK-3 in preference to ALK-2. Smad6 interacted efficiently with ALK-3 but only weakly with ALK-2, whereas Smad7 interacted with both ALK-3 and ALK-2. We identified the amino acid residues responsible for the Smad6 sensitivity of ALK-3, which are located in the N-terminal lobe of the ALK-3 kinase domain. Smad6 also efficiently suppressed ALK-6 signaling while minimally affecting ALK-1 signaling. We conclude that Smad6 principally inhibits the ALK-3/6 subgroup of BMP type I receptors through physical interaction, although multiple modes of action of Smad6 have been reported. Differences in sensitivity to negative feedback regulators appear to affect the signal transduction properties of each receptor, including the duration of receptor activation.

EXPERIMENTAL PROCEDURES

Cell Cultures and DNA Transfection—Mouse myoblast C2C12 cells and COS7 cells were obtained from the American Type Culture Collection (Manassas, VA). Mouse MC3T3-E1 osteoblastic cells were obtained from Dr. H. Kodama (Ohu University). Cells were grown in a 5% CO₂-humidified atmosphere at 37 °C. C2C12 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 20% fetal bovine serum (FBS) supplemented with 100 units/ml penicillin G and 100 µg/ml streptomycin (Invitrogen). COS7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS supplemented with 100 units/ml penicillin G and 100 µg/ml streptomycin. MC3T3-E1 cells were maintained in α-minimal essential medium (Sigma) containing 10% FBS, 100 units/ml penicillin G, 100 µg/ml streptomycin, and 2 mM L-glutamine (Invitrogen). Cells were transfected using FuGENE6 transfection reagent (Roche Diagnostics) according to the manufacturer’s recommendations.

DNA Constructs and Chemicals—The constructions of constitutively active forms of BMP type I receptors, wild-type BMP2R-II, Smad1, Smad5, Smad6, Smad6N, Smad6C, and Smad7 were described previously (14, 25, 26). ALK-2 and ALK-3 chimeras or point mutants were generated by a PCR-based approach using ALK-2-OD or ALK-3-OD as a template.

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All of the PCR products were sequenced. Recombinant human BMP-2 and BMP-6 were kindly provided by Astellas Pharma, Inc., and Dr. T. K. Sampath (Creative BioMolecules, Inc.), respectively.

Luciferase Assay—The transcriptional activation induced by BMPs was measured using BRE-Luc, luciferase reporter constructs containing BMP-responsive elements (27). C2C12 cells or MC3T3-E1 cells were seeded in duplicate in 12-well plates (Nunc Corporation) and were then transiently transfected with an appropriate combination of promoter-reporter constructs, expression plasmids, and pcDNA3. Twenty-four hours after transfection, the cells were stimulated with BMP-2 or BMP-6 and cultured for another 24 h. Cell lysates were then prepared, and luciferase activities in the lysates were measured by the Dual-Luciferase reporter system (Promega) using a luminometer (MicroLumat Plus, Berthold). When the cells were stimulated with constitutively active type I receptors, the cell lysates were prepared 24 h after transfection. Values were normalized using Renilla luciferase activity under control of cytomegalovirus promoter. In some experiments, levels of expression of recombinant proteins in lysates were determined by immunoblotting.

Immunoprecipitation and Immunoblotting—Cells were washed with phosphate-buffered saline and harvested with Nonidet P-40 lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. The lysates were then centrifuged. The supernatants were measured for protein concentrations and subjected to immunoprecipitation or immunoblotting as described previously (28). The antibodies used were as follows: anti-FLAG antibody (M2, Sigma), anti-hemagglutinin antibody (3F10, Roche Diagnostics), anti-α-tubulin antibody (DM1A, Sigma), and anti-phospho-Smad1/5/8 antibody (Cell Signaling). Anti-Smad5 antibody was prepared by immunizing a rabbit with mouse Smad5 (amino acid residues 217–262) expressed as a fusion protein with glutathione S-transferase.

Osteoblastic Differentiation—Cells were cultured in 2.5% FBS-containing medium for 4 h prior to stimulation. Osteoblastic differentiation of C2C12 cells and MC3T3-E1 cells was induced by 100 ng/ml recombinant human BMP-2 or BMP-6 in 2.5% FBS-containing medium (29).

Quantitative Real-time Reverse Transcription-PCR—Total RNA was extracted from C2C12 cells or MC3T3-E1 cells using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using SuperScript™ III reverse transcriptase (Invitrogen). Real-time PCR was performed using Power SYBR Green PCR master mix.

Figure 1. Smad6 differentially affects ALK-2 and ALK-3 signaling. A, effects of I-Smads on the transactivation of BRE-Luc induced by constitutively active ALK-2 or ALK-3 in C2C12 cells. Results are given as percentages of control. Error bars represent S.D. The three panels show levels of protein expression of transiently transfected ALK-2/3-OD (top panel), Smad6 and Smad7 (middle panel), and endogenous tubulin as a loading control (bottom panel). B, effects of I-Smads on the phosphorylation of Smad5 in transiently transfected C2C12 cells stimulated with ALK-2/3-OD or ALK-3-3QD. Phosphorylation of Smad5 was visualized by immunoblotting using anti-phospho-Smad1/5/8 antibody (top panel). The lower three panels show levels of protein expression of transiently transfected I-Smads and Smad5, ALK-2/3-OD, and endogenous tubulin as a loading control. E, effects of I-Smads on the transactivation of BRE-Luc induced by BMP-2 (25 ng/ml) or BMP-6 (40 ng/ml). Cells were stimulated for 24 h prior to harvest. Results are given as percentages of control. Error bars represent S.D. D, effects of BMP-2 and BMP-6 on osteoblastic differentiation of MC3T3-E1 cells. Expression of alkaline phosphatase (ALP) as well as Id1 was examined by quantitative real-time PCR analyses after cells were treated with BMP-2 or BMP-6 (100 ng/ml) for 1 or 48 h in the presence of 2.5% FBS. Error bars represent S.D. E, duration of Smad1/5 phosphorylation after stimulation of MC3T3-E1 cells with BMP-2 or BMP-6 (100 ng/ml). Cells were harvested at various periods of time after stimulation, and phosphorylation of Smad1/5 was determined by immunoblotting (top panel). The lower two panels show levels of protein expression of Smad5 and tubulin. Expression of tubulin was determined as a loading control. F, duration of Smad1/5 phosphorylation in control or Smad6-knocked-down cells after stimulation with BMP-2 or BMP-6 (100 ng/ml) in MC3T3-E1 cells. Phosphorylation of Smad1/5 was determined by immunoblotting (top panel). The lower two panels show levels of expression of Smad5 and of tubulin protein as a loading control. HA, hemagglutinin. NTC, negative control.
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Smad6 Inhibits ALK-3 Signaling in Preference to ALK-2 Signaling—Smad6 has been reported to efficiently inhibit BMP signaling but only weakly inhibit TGF-β/activin signaling (14–17). However, effects of Smad6 on each BMP type I receptor have not been well explored.

We first examined the inhibitory effects of Smad6 and Smad7 on BMP type I receptors ALK-2 and ALK-3 using their constitutively active mutants. The luciferase reporter assay was conducted in C2C12 mouse myoblast cells using a BMP-responsive reporter BRE-Luc (Fig. 1A). Smad7 equally inhibited the signaling induced by these ALKs. In contrast, Smad6 effectively inhibited ALK-3 signaling but only weakly inhibited ALK-2 signaling. Similar results were obtained when MC3T3-E1 mouse osteoblastic cells were used (data not shown). We next examined the effects of Smad6 and Smad7 on the phosphorylation of BMP-specific Smads by each ALK using exogenous Smad1 and Smad5 (Fig. 1B and supplemental Fig. 1). Consistent with the results shown in Fig. 1A, Smad7 effectively inhibited ALK-2- and ALK-3-mediated phosphorylation of Smad1 and 5, whereas Smad6 inhibited Smad1/5 phosphorylation by ALK-3 but only weakly inhibited that by ALK-2. These findings suggest that Smad6 differentially inhibits phosphorylation of BMP-specific R-Smads by each BMP type I receptor.

Effect of Smad6 on BMP-6-induced ALK-2 Activation and BMP-2-induced ALK-3 Activation—In C2C12 and MC3T3-E1 cells, BMP-6 mainly utilizes ALK-2, whereas BMP-2 utilizes ALK-3 for signaling (19), because ALK-6 is not expressed in these cells (19, 30). We thus examined the effects of I-Smads on BMP-6- and BMP-2-induced signaling in C2C12 cells (Fig. 1C). Smad7 inhibited signaling by these two BMP ligands to similar extents. In contrast, Smad6 more effectively inhibited BMP-2 signaling than BMP-6 signaling. These findings are consistent with our results that Smad6 preferentially inhibits ALK-3 signaling.

BMP-6-induced ALK-2 Activation Results in More Sustained Signaling than Does BMP-2-induced ALK-3 Activation—We next compared induction of osteoblastic differentiation by BMP-2 or BMP-6 (Fig. 1D). MC3T3-E1 cells were stimulated with 100 ng/ml BMP-2 or BMP-6 and harvested 0, 1, and 48 h after stimulation. The expression of target genes was then analyzed by quantitative real-time PCR. At 1 h after stimulation, BMP-2 and BMP-6 exhibited similar effects on the expression of Id1, a direct target gene of BMP signaling. Interestingly, a striking difference was observed in the induction of alkaline phosphatase, an osteogenic marker, at 48 h after stimulation. BMP-6 was more efficient in inducing alkaline phosphatase than BMP-2 in MC3T3-E1 cells. Similar results were obtained when C2C12 cells were used (data not shown). To elucidate the differences in potency in inducing alkaline phosphatase, we examined the phosphorylation of Smad1/5 by BMP-2 and BMP-6 in MC3T3-E1 cells (Fig. 1E). BMP-2 and BMP-6 induced similar levels of Smad1/5 phosphorylation at 1 h after stimulation, consistent with the results on Id1 induction. The phosphorylation of Smad1/5 induced by BMP-6 was, however, sustained up to 48 h after stimulation, whereas that by BMP-2 declined earlier. Similar results were obtained in C2C12 cells (data not shown). These findings suggest that BMP-6-induced ALK-2 activation results in more sustained signaling than does BMP-2-induced ALK-3 activation.

Endogenous Smads Less Effectively Inhibits BMP-6-induced ALK-2 Signaling than BMP-2-induced ALK-3 Signaling—We then examined whether the lower sensitivity of ALK-2 to Smad6 may account for the more sustained signaling induced by ALK-2. We knocked down Smad6 by transfecting siRNA duplex into MC3T3-E1 cells, because knockdown efficiency was higher in MC3T3-E1 cells than in C2C12 cells (data not shown). Smad6 mRNA expression was decreased to 40% of the control level (data not shown). We then examined the effect of Smad6 knockdown on BMP-induced phosphorylation of Smad1/5. Silencing of Smad6 resulted in more sustained phosphorylation of Smad1/5 by BMP-2 and BMP-6, with a more prominent effect in the case of BMP-2 (Fig. 1F). These findings suggest that BMP-6-induced ALK-2 activation causes more sustained phosphorylation of Smad1/5 due to the lower sensitivity of ALK-2 to Smad6.

The Differential Inhibitory Effects of Smads Can Be Attributed to Differences in Interaction with BMP Type I Receptors—to explore the mechanism of preferential inhibition of ALK-3 by Smad6, we examined the interaction of Smad6 and Smad7 with BMP type I receptors in transfected COS7 cells (Fig. 2A). I-Smads were immunoprecipitated, and the co-precipitated receptors were detected by immunoblotting. ALK-3 strongly interacted with Smad6 and Smad7, whereas ALK-2 only weakly interacted with Smad6 compared with Smad7. These findings suggest that the differential inhibitory effects of Smad6 can be attributed to differences in interaction with BMP type I receptors.

The IL-45 Loop Does Not Play a Major Role in Determining the Differential Sensitivity of BMP Type I Receptors to Smads—to determine the region responsible for differential sensitivity to Smad6, we divided the cytoplasmic domains of ALK-2 and ALK-3 into five regions based on sequence alignment between the two proteins (supplemental Fig. 2) and constructed five constitutively active ALK-2/ALK-3 chimeric receptors (Fig. 2B). These mutant proteins exhibited similar levels of signaling activity in luciferase reporter assay when they were expressed at the same levels in cells (data not shown). Region 1 (Arg-147–Gln-207 in ALK-2 and Tyr-176–Gln-233 in ALK-3) contains

RNA Interference and Oligonucleotides—Small interfering RNAs were introduced into MC3T3-E1 cells using HiPerFect transfection reagent (Qiagen) according to the manufacturer’s instructions with 25 nm of siRNA and 12 µl of transfection reagent per well of collagen-I-coated, 6-well plates (Sumitomo Bakelite Co.). HP GenomeWide siRNA (Qiagen, catalog number SI00177149) was used to knock down Smad6 (sense, 5'-GAUUCUACAUUGUCUUACA-3'; antisense, 5'-UUGUAAGACAUGUAAGAAC-3'). AllStars negative control siRNA (Qiagen, catalog number 1027280) was used (sequence not available) as a negative control.
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Identification of Amino Acid Residues in ALK-3 Responsible for Smad6 Sensitivity—To identify the amino acid residues responsible for the sensitivity to Smad6 in region 2 of ALK-3, we compared the amino acid sequences of region 2 in ALK-2 and ALK-3 (Fig. 3A). Region 2 consists of 36 amino acid residues, 21 of which are identical. Three clusters of divergent sequence were found in region 2 (Fig. 3A, underlined residues). We constructed three ALK-2 mutants in which these residues were replaced by the corresponding residues of ALK-3 (mutants ALK-2-m1, ALK-2-m2, and ALK-2-m3). Each of these mutants exhibited slightly increased sensitivity to Smad6 compared with ALK-2 (supplemental Fig. 3B, open bars). We next constructed six point mutants of ALK-2 in which each divergent amino acid residue was replaced by the corresponding residue in ALK-3 (Fig. 3A, asterisks). Four of the six mutants exhibited increased sensitivity to Smad6 compared with ALK-2 (supplemental Fig. 3B, open bars). We then examined various combinations of point mutations and finally found that a combination of four amino acid residues in ALK-3, Arg-238, Phe-264, Thr-265, and Ala-269, was sufficient to confer ALK-2 with sensitivity to Smad6 (Fig. 3A, boxed). An ALK-2 mutant with replacement at the corresponding positions by these four residues (E212R/S238F/S239T/K243A) exhibited a high degree of sensitivity to Smad6 (Fig. 3B). On the other hand, a reciprocal ALK-3 mutant (R238E/F264S/T265S/A269K) lost sensitivity to Smad6, although it still exhibited sensitivity to Smad7. Consistent results were obtained when we examined the phosphorylation of BMP-specific R-Smads by these receptors (Fig. 3C) and binding of I-Smads to these receptors (Fig. 3D). These findings suggest that Smad6 and Smad7 may interact with different sites on ALK-3. We further found that the C-terminal region of Smad6 (Smad6C, residues 315–496 containing the MH2 domain) interacted with ALK-3 through the critical four residues (Fig. 3E). We

the GS domain, where type I receptors are phosphorylated by type II receptors. Region 2 (Ile-208–Lys-243 in ALK-2 and Ile-234–Ala-269 in ALK-3) is thought to constitute the β structure with a conserved phosphate-binding loop and ATP binding site. Region 3 (Ser-244–Glu-287 in ALK-2 and Ser-270–Glu-313 in ALK-3) includes the αC helix, which is important for kinase activity, and the L45 loop, which is responsible for the specificity of interaction with R-Smads (31–33). The amino acid sequences of the L45 loop differ significantly between ALK-2 and ALK-3 (supplemental Fig. 2). Region 4 (Met-288–Pro-330 in ALK-2 and Asn-314–Pro-356 in ALK-3) contains the E6 loop, which has been implicated in the homo-oligomerization of type I receptors (34). Region 5 (Ala-331–Cys-509 in ALK-2 and Ala-357–Ile-532 in ALK-3) constitutes the C-terminal lobe of receptor kinase and contains the catalytic segment as well as the activation segment.

We then examined the inhibitory effects of Smad6 and Smad7 on signaling from five ALK-2/ALK-3 chimera receptors (Fig. 2C). Smad7 efficiently inhibited the signaling induced by all ALK-2/ALK-3 chimeras. Smad6 effectively inhibited neither signaling by ALK-2 nor that by the chimera receptors containing region 2 of ALK-2 (Fig. 2C, gray bars) but strongly inhibited signaling by ALK-3 and the chimera receptors containing region 2 of ALK-3 (Fig. 2C, open bars). These findings indicate that region 2 of ALK-3 is responsible for the sensitivity to Smad6. Notably, the L45 loop, which is located in region 3, does not appear to play a major role in determining the differential sensitivity to Smad6.

Identification of Amino Acid Residues in ALK-3 Responsible for Smad6 Sensitivity—To identify the amino acid residues responsible for the sensitivity to Smad6 in region 2 of ALK-3, we compared the amino acid sequences of region 2 in ALK-2 and ALK-3 (Fig. 3A). Region 2 consists of 36 amino acid residues, 21 of which are identical. Three clusters of divergent sequence were found in region 2 (Fig. 3A, underlined residues). We constructed three ALK-2 mutants in which these residues were replaced by the corresponding residues of ALK-3 (mutants ALK-2-m1, ALK-2-m2, and ALK-2-m3). Each of these mutants exhibited slightly increased sensitivity to Smad6 compared with ALK-2 (supplemental Fig. 3B, open bars). We next constructed six point mutants of ALK-2 in which each divergent amino acid residue was replaced by the corresponding residue in ALK-3 (Fig. 3A, asterisks). Four of the six mutants exhibited increased sensitivity to Smad6 compared with ALK-2 (supplemental Fig. 3B, open bars). We then examined various combinations of point mutations and finally found that a combination of four amino acid residues in ALK-3, Arg-238, Phe-264, Thr-265, and Ala-269, was sufficient to confer ALK-2 with sensitivity to Smad6 (Fig. 3A, boxed). An ALK-2 mutant with replacement at the corresponding positions by these four residues (E212R/S238F/S239T/K243A) exhibited a high degree of sensitivity to Smad6 (Fig. 3B). On the other hand, a reciprocal ALK-3 mutant (R238E/F264S/T265S/A269K) lost sensitivity to Smad6, although it still exhibited sensitivity to Smad7. Consistent results were obtained when we examined the phosphorylation of BMP-specific R-Smads by these receptors (Fig. 3C) and binding of I-Smads to these receptors (Fig. 3D). These findings suggest that Smad6 and Smad7 may interact with different sites on ALK-3. We further found that the C-terminal region of Smad6 (Smad6C, residues 315–496 containing the MH2 domain) interacted with ALK-3 through the critical four residues (Fig. 3E). We
**FIGURE 3. Identification of the amino acid residues in ALK-3 responsible for Smad6 sensitivity.**

A. Alignment of amino acid sequences of region 2 in ALK-2 and ALK-3. Divergent residues are shown in red. Three clusters of divergent sequences are underlined and denoted as m1, m2, and m3. Point mutants constructed for residues in ALK-2 are shown with asterisks. Residues responsible for differences in Smad6 sensitivity are boxed.

B. Effects of I-Smads on ALK-2, ALK-3, and their mutants were examined in luciferase reporter assay using BRE-Luc in C2C12 cells. Results are given as percentages of control. Error bars represent S.D. The three panels show the expression of each protein as indicated.

C. Effects of I-Smads on phosphorylation of Smad5 in transfected C2C12 cells stimulated with ALK-2, ALK-3, or their mutants. Phosphorylation of exogenous Smad5 was visualized by immunoblotting using anti-phospho-Smad1/5/8 (top panel). The lower three panels show the expression of each protein as indicated.

D. Physical interaction of I-Smads with ALK-2, ALK-3, and their mutants. COS7 cells were transfected with the indicated plasmids. I-Smads were immunoprecipitated (IP), and co-precipitated receptors were visualized by immunoblotting (top panel). Precipitated I-Smads (middle panel) and expression of receptors (bottom panel) are also shown. The relative intensities of I-Smad binding to ALKs and their mutants were quantified from the top panel by Image J. E. Physical interaction of Smad6N and Smad6C with ALK2, ALK-3, and their mutants was examined as in D. 2mt denotes ALK-2 (E212R/S238F/S239T/K243A); 3mt, ALK-3 (R238E/F264S/T265S/A269K). HA, hemagglutinin.
conclude that these four residues (R238/F264/T265/A269) in ALK-3 are responsible for interaction with the MH2 domain of Smad6 and confer Smad6 sensitivity to ALK-3.

**Smad6-sensitive Residues in ALK-3 Map to the N-terminal Lobe of the Kinase Domain**—We aligned amino acid sequences of region 2 in ALK-2 and ALK-3 of human, mouse, Xenopus, and zebrafish (z) origin are shown. Residues responsible for Smad6 sensitivity are boxed. β, three-dimensional structural model of the ALK-3 kinase domain based on the three-dimensional structure of ALK-5 (Protein Data Bank code 1B6C). The left panel shows a frontal view, and the right panel, an overhead view of the ALK-3 three-dimensional structure. Arg-238, Phe-264–Thr-265, and Ala-269 are conserved beyond species. We next mapped the four residues responsible for Smad6 sensitivity on a structural model of the ALK-3 kinase domain, which was constructed based on the ALK-5 kinase structure (Protein Data Bank code 1B6C) (Fig. 4, yellow). They are located adjacent to the L45 loop (Fig. 4B, green) but apart from the GS loop (Fig. 4B, cyan). They are not clustered and are instead scattered along the phospho-binding loop and the αC helix in the N-terminal lobe of the kinase domain. We then examined whether Smad7 affects the interaction of Smad6 with ALK-3, because Smad6 and Smad7 appear to interact with ALK-3 through different sites. As shown in Fig. 4C, increasing amounts of Smad7 competed with Smad6 for association with ALK-3, indicating that Smad7 interacts with ALK-3 in the vicinity of the Smad6 binding site.

**Smad6 Sensitivities of Other ALKs**—Seven type I receptors for the TGF-β family have been identified in mammals, among which ALK-3 and ALK-6 are structurally closely related and constitute a subgroup, whereas ALK-2 and ALK-1 constitute another subgroup. We compared the sequence of region 2 of ALK-3 with the corresponding regions in ALK-1, ALK-2, and ALK-6 (Fig. 5A). The residues responsible for Smad6 sensitivity are well conserved in ALK-6 but divergent in ALK-1 and ALK-2. We then examined the Smad6 sensitivity of ALK-1 and ALK-6 using luciferase reporter assay (Fig. 5B). All BMP type I receptors were efficiently inhibited by Smad6. In contrast, ALK-6 was sensitive to Smad6 (Fig. 5B, open bar), whereas ALK-1 was only weakly inhibited by Smad6 (Fig. 5B, gray bar). Our findings thus indicate that the two structurally distinct type I receptor groups transmitting BMP signals have distinct Smad6 sensitivity and might have distinct signaling properties.

![Image](Image69x539 to 159x628)

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![Image](Image116x355 to 257x391)

![Image](Image116x393 to 257x428)

![Image](Image116x430 to 257x465)

![Image](Image217x26 to 245x38)

![Image](Image218x28 to 222x22)

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**FIGURE 4. Residues of ALK-3 responsible for Smad6 sensitivity.** A, conservation of the amino acid residues responsible for Smad6 sensitivity of ALK-2 and 3 beyond species. Alignments of amino acid sequences of region 2 in ALK-2 and ALK-3 of human (h), mouse (m), Xenopus (x), and zebrafish (z) origin are shown. Residues responsible for Smad6 sensitivity are boxed. β, three-dimensional structural model of the ALK-3 kinase domain based on the three-dimensional structure of ALK-5 (Protein Data Bank code 1B6C). The left panel shows a frontal view, and the right panel, an overhead view of the ALK-3 three-dimensional structure. Arg-238, Phe-264–Thr-265, and Ala-269 are conserved beyond species. We next mapped the four residues responsible for Smad6 sensitivity on a structural model of the ALK-3 kinase domain, which was constructed based on the ALK-5 kinase structure (Protein Data Bank code 1B6C) (Fig. 4B, yellow). They are located adjacent to the L45 loop (Fig. 4B, green) but apart from the GS loop (Fig. 4B, cyan). They are not clustered and are instead scattered along the phospho-binding loop and the αC helix in the N-terminal lobe of the kinase domain. We then examined whether Smad7 affects the interaction of Smad6 with ALK-3, because Smad6 and Smad7 appear to interact with ALK-3 through different sites. As shown in Fig. 4C, increasing amounts of Smad7 competed with Smad6 for association with ALK-3, indicating that Smad7 interacts with ALK-3 in the vicinity of the Smad6 binding site.

**FIGURE 5. Smad6 differentially affects ALK-1/2 and ALK-3/6 signaling.** A, comparison of amino acid sequences of region 2 in ALK-1, -2, -3, and -6. Amino acid residues affecting Smad6 sensitivity are boxed. β, effects of I-Smads on luciferase reporter assay using BRE-Luc in C2C12 cells stimulated with each ALK receptor. Results are given as percentages of control. Error bars represent S.D. The three panels show levels of protein expression of transiently transfected ALKs, Smad6 and Smad7, and endogenous tubulin as a loading control. HA, hemagglutinin.

A269), which are responsible for the differences in Smad6 sensitivity, are conserved beyond species. We next mapped the four residues responsible for Smad6 sensitivity on a structural model of the ALK-3 kinase domain, which was constructed based on the ALK-5 kinase structure (Protein Data Bank code 1B6C) (Fig. 4B, yellow). They are located adjacent to the L45 loop (Fig. 4B, green) but apart from the GS loop (Fig. 4B, cyan). They are not clustered and are instead scattered along the phospho-binding loop and the αC helix in the N-terminal lobe of the kinase domain. We then examined whether Smad7 affects the interaction of Smad6 with ALK-3, because Smad6 and Smad7 appear to interact with ALK-3 through different sites. As shown in Fig. 4C, increasing amounts of Smad7 competed with Smad6 for association with ALK-3, indicating that Smad7 interacts with ALK-3 in the vicinity of the Smad6 binding site.

**Smad6 Sensitivities of Other ALKs**—Seven type I receptors for the TGF-β family have been identified in mammals, among which ALK-3 and ALK-6 are structurally closely related and constitute a subgroup, whereas ALK-2 and ALK-1 constitute another subgroup. We compared the sequence of region 2 of ALK-3 with the corresponding regions in ALK-1, ALK-2, and ALK-6 (Fig. 5A). The residues responsible for Smad6 sensitivity are well conserved in ALK-6 but divergent in ALK-1 and ALK-2. We then examined the Smad6 sensitivity of ALK-1 and ALK-6 using luciferase reporter assay (Fig. 5B). All BMP type I receptors were efficiently inhibited by Smad6. In contrast, ALK-6 was sensitive to Smad6 (Fig. 5B, open bar), whereas ALK-1 was only weakly inhibited by Smad6 (Fig. 5B, gray bar). Our findings thus indicate that the two structurally distinct type I receptor groups transmitting BMP signals have distinct Smad6 sensitivity and might have distinct signaling properties.
Inhibitory Effects of Smad6 on BMP Type I Receptors

**DISCUSSION**

Smad6 and Smad7 were originally identified as inhibitory proteins for TGF-β family signaling (12, 35, 36). Subsequently, it was determined that Smad6 effectively inhibits BMP signaling but only weakly inhibits that by TGF-β and activin (14–17), whereas Smad7 ubiquitously inhibits TGF-β family signaling. In the present study, we found that Smad6 efficiently inhibits signaling from the ALK-3/6 subgroup but only minimally inhibits that from the ALK-1/2 subgroup of BMP type I receptors. Smad6 thus appears to be an inhibitory Smad with narrow specificity, and ALK-3 and ALK-6 appear to be more tightly regulated BMP type I receptors.

Several mechanisms have been proposed for the inhibitory effect of Smad6 on BMP signaling. Smad6 stably binds to BMP type I receptors and inhibits phosphorylation of BMP-specific R-Smads, resulting in inhibition of BMP signaling (12). Furthermore, Smad6 recruits the E3 ubiquitin ligase Smurf1 (Smad ubiquitin regulatory factor 1) to the signaling receptor complex and enhances the down-regulation of type I receptors through proteasome-dependent degradation (37). In addition to these receptor-targeting mechanisms, alternative modes of action of Smad6 have also been reported. Smad6 has been shown to form a complex with Smad1 and interfere with complex formation between Smad1 and Smad4 (15). Smad6 has also been shown to enhance the degradation of BMP-specific R-Smads through recruitment of Smurf1 to a complex with activated BMP-specific R-Smads (37). Smad6 has also been shown to interact with transcriptional factors (38) and histone deacetylases 1 and 3 (39) to inhibit the transcription of specific genes. Interaction of Smad6 with transcriptional co-repressor CtBP (40) has also been reported.

As we have clearly demonstrated in the present study, the inhibitory effect of Smad6 was more potent on ALK-3/ALK-6-mediated signaling than on ALK-1/ALK-2-mediated signaling. We therefore conclude that the receptor-targeting mechanisms of Smad6 play principal roles in the inhibition of BMP signaling, although other mechanisms may play roles in this as well.

Although it has been established that I-Smads interact with type I receptors, the mode of interaction between I-Smads and type I receptors has remained unclear. The L45 loop of the N-lobe in the type I receptor protrudes from the kinase domain (34). The amino acid sequences of the L45 loop are conserved within each type I receptor subgroup but diverge between different subgroups. Because the L45 loop is a determinant of the specific activation of R-Smad isoforms (31–33), it is considered a binding site for R-Smads. Smad7 has been shown to compete with R-Smads in binding to type I receptors (35). It appears likely that I-Smads also interact with type I receptors through the region near the L45 loop. Itoh et al. (41), however, have previously reported that the introduction of a mutation in the L45 loop of ALK-5 to abolish interaction with Smad2 did not affect its interaction with Smad6 and Smad7, suggesting that R-Smads and I-Smads interact with ALK-5 through different sites. In the present study, we found that the L45 loop is not a principal determinant of the interaction of ALK-3 with Smad6. Smad6 interacted with the N-terminal lobe of the kinase domain of ALK-3 and competed with Smad7 in binding to ALK-3. These findings suggest that the R-Smads, Smad6, and Smad7 all interact with the N-terminal lobe of the kinase domain, although their binding sites differ. We have also found that the residues responsible for Smad6 binding are located surrounding the phosphate binding loop of the ALK-3 kinase domain. Smad6 may thus affect the ATP binding properties of ALK-3 upon interaction with it, although this remains to be clearly demonstrated.

Of the seven type I receptors for the TGF-β family, four receptors, i.e. ALK-1, ALK-2, ALK-3, and ALK-6, signal through phosphorylation of BMP-specific R-Smads. Although they have overlapping characteristics, each has unique activities in vivo. The germ line mutations of each of them have been implicated in distinct diseases; haploinsufficiency of the ALK1 gene leads to hereditary hemorrhagic telangiectasia type 2 (42). Missense mutations of the ALK2 gene, which may cause hyperactivity of ALK-2, have recently been identified in patients with fibrodysplasia ossificans progressiva (7). Mutations of the ALK3 gene have been found in some patients with juvenile polyposis (4). Missense mutations of the ALK6 gene cause brachydactyly type A2, a malformation of the hands (6). To ensure that each of them can play its unique role, their activities are finely regulated through differences in specificities for various ligands as well as differences in the pattern of spatiotemporal expression (43). Additionally, signaling specificities, including preference in phosphorylation of BMP-specific R-Smads, ability to transduce non-Smad signaling, and sensitivity to negative regulators, are also important in playing their unique roles.

In the present study, we have found that Smad6 preferentially inhibits the ALK-3/ALK-6 subgroup of BMP type I receptors. Targeted disruption of the gene coding for Smad6 has been reported to result in multiple cardiovascular abnormalities (44). Roles of Smad6 in atrioventricular cushion formation have been revealed by hyperplasia of the cardiac valves as well as outflow tract septation defects in knock-out mice, which reflect overgrowth of mesenchymal cells in the region of the atrioventricular canal. Recently, using the Tie1-Cre or Tie2-Cre system, mice in which Alk3 is specifically deleted in the endothelium/endocardium have been generated (45, 46). These mice also exhibited defects in atrioventricular cushion formation. Further analysis indicated that ALK-3 is required for proper proliferation of endocardial/mesenchymal cells through down-regulation of nuclear p21 as well as up-regulation of cyclin D1 (46). The cardiovascular phenotypes observed in the Smad6 knock-out mice are thus explained well by overactivity of ALK-3 in atrioventricular mesenchymal cells.

Both Smad6 and Smad7 are transcriptionally induced by various ligands of the TGF-β family and serve as negative feedback molecules (9, 11, 36). They are also induced by various ligands other than members of the TGF-β family, including epidermal growth factor (9), interferon-γ (47), and tumor necrosis factor-α (48), suggesting that signaling by the TGF-β family is considerably affected by cellular context. As found in the present study, Smad7 is a ubiquitous inhibitor of Smad signaling, whereas Smad6 is a pathway-specific inhibitor. Interestingly, these two I-Smads are differentially regulated in various cells by various stimuli, i.e. the magnitude as well as the duration of
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