Members of the transforming growth factor-β (TGF-β) family transmit signals from membrane to nucleus via intracellular proteins known as Smads. A subclass of Smad proteins has recently been identified that antagonize, rather than transduce, TGF-β family signals. Smad7, for example, binds to and inhibits signaling downstream of TGF-β receptors. Here we report that the C-terminal MAD homology domain of murine Smad7 (mSmad7) is sufficient for both of these activities. In addition, we show that mSmad7 interacts with activated bone morphogenetic protein (BMP) type I receptors (BMPR-1s), inhibits BMPR-I-mediated Smad phosphorylation, and phenocopies the effect of known BMP antagonists when overexpressed in ventral cells of Xenopus embryos. Xenopus Smad7 (XSmad7, previously termed Smad8) and mSmad7 are nearly identical within their bioactive C-domain, but have quite distinct N-domains. We found that XSmad7, similar to mSmad7, interacted with BMP and TGF-β type I receptors and inhibited receptor-mediated phosphorylation of downstream signal-transducing Smads. However, XSmad7 is a less efficient inhibitor of TβR-I-mediated responses in mammalian cells than is mSmad7. Furthermore, overexpression of XSmad7 in Xenopus embryos produces patterning defects that are not observed following overexpression of mSmad7, suggesting that mSmad7 and XSmad7 may preferentially target distinct signaling pathways. Our results are consistent with the possibility that the C-domain of antagonistic Smads is an effector domain whereas the N-domain may confer specificity for distinct signaling pathways.

Members of the transforming growth factor-β (TGF-β) family, such as TGF-β8, bone morphogenetic proteins (BMPs), and activins, are multifunctional cell-cell signaling molecules that play important roles in embryonic pattern formation and in regulation of cell proliferation in adults (1, 2). Individual family members transmit signals via distinct combination(s) of type I and type II transmembrane serine/threonine kinase receptors, both of which are required for signaling (3–5). Ligand binding stimulates formation of a heteromeric receptor complex, following which the type I receptor is phosphorylated by the constitutively active type II receptor kinase (6). The activated type I receptor then propagates the signal through transient interaction with, and phosphorylation of, pathway-restricted Smads (3–5). Whereas Smad2 and Smad3 act in the TGF-β and activin pathways (7–10), Smad1, Smad5, and MADH6/Smad8 act downstream of BMPs (11–15). All of these Smads have conserved amino (N)- and carboxy (C)-terminal domains that are also termed Mad homology (MH) 1 and MH2 domains, respectively. Receptor-mediated phosphorylation, which occurs on two serine residues in a Ser-Ser-Xaa-Ser motif at the end of the C-domain (16, 17), relieves the inhibitory activity of the N-domain and induces hetero-oligomerization with Smad4, a common mediator of TGF-β, activin, and BMP signaling (18, 19). This hetero-oligomeric Smad complex then translocates to the nucleus and interacts with DNA, either directly or in association with unrelated DNA binding proteins, to modulate transcription of TGF-β family target genes (20–22).

Recently, a distinct subgroup of distantly related Smads, including mammalian Smad6 (23) and Smad7 (24, 25), Xenopus Smad6 (XSmad6) (26) and Smad8 (now called XSmad7) (27) and Drosophila Dad (28), have been identified as intracellular antagonists of TGF-β family signaling. Smad6 and Smad7 form stable associations with activated TGF-β type I receptor (TβR-I), thereby preventing pathway restricted Smads from binding to, and being phosphorylated by, these receptors. Smad6 has also been shown to bind to and inhibit signaling downstream of BMP receptors (23) and to prevent formation of an active Smad4/Smad1 signaling complex by directly competing with Smad4 for binding to Smad1 (29). In Xenopus embryos, ectopically expressed murine Smad7 (mSmad7), XSmad7, or XSmad6 can partially antagonize activin-like signaling although both are much more potent inhibitors of BMPs (25–27, 29). Inhibitory Smads, like signal-transducing Smads, contain conserved N- and C-domains, but the function of each domain is unknown.

In the current studies, we have investigated the physical interaction of mSmad7 with BMP receptors and the functional consequences thereof. mSmad7 interacts with activated BMP type I receptors and can inhibit BMP signal transduction in mammalian cells and in Xenopus embryos. Thus Smad7 may act as a general TGF-β family inhibitor. We show that the C-domain of mSmad7 is sufficient for receptor interaction, and for inhibition of downstream signaling. The biochemical and functional properties of XSmad7 overlap extensively with those...
of mSmad7, but distinct functional differences between the two related Smads are observed. Our results are consistent with the possibility that the C-domain of antagonistic Smads is the effector domain whereas the N-domain may confer specificity for distinct signaling pathways.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—COS cells and Mv1Lu mink lung epithelial cells were obtained from American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle's medium (Life Technologies, Inc.) with 10% fetal bovine serum and antibiotics.

**Expression Constructs**—Expression constructs for TβR-I, TβR-II, BMPR-II, BMPR-IA, BMPR-IB, ActR-I, Smad1, Smad2, and Smad5 have been described (10). Expression constructs for Flag-tagged mSmad7 C-domain (7C; amino acids 204–426), Flag-tagged mSmad7 C-domain with C-tail deletion (7C3; amino acids 204–407), and Flag-tagged XSmad7 (F-XSmad7) were made by using polymerase chain reaction technique and subcloning in pcDNA3-Flag.

**Transient Transfections, Immunoprecipitations, and Immunoblots**—Transient transfections using the DEAE-dextran protocol, metabolic labeling of cells, immunoprecipitation, immunoblotting, and SDS-polyacrylamide gel electrophoresis were performed as described previously (10). Anti-Flag antibodies were purchased from Eastman Kodak Co. (New Haven, CT).

**Iodination of Ligands and Affinity Cross-linking**—TGF-β1 and BMP-7 were iodinated using the chloramine-T method according to Frolik et al. (30). Cross-linking was performed as described previously (10). Complexes of Smads and affinity-labeled receptors were immunoprecipitated with antiserum directed against Flag-tag in Smads. To determine expression levels of receptors, aliquots of cell lysates were immunoprecipitated with antisera against type I receptors. Expression of Smads was determined by Western blotting on aliquots of cell lysates.

**Transcriptional Response Assay**—Mv1Lu cells were transfected with p3TPLux, as described (10). Luciferase activity was measured as described (10) and normalized for transfection efficiency using the β-galactosidase reporter gene in pCMV5 vector (10). Results shown are representative of at least three independent experiments.

**Xenopus Embryo Culture and Manipulation**—Xenopus eggs were obtained, and embryos were microinjected and cultured as described (31). RNA was synthesized by in vitro transcription (31) of pcS2+XSmad7 and pcS2+mSmad7 (27). The latter construct was generated by subcloning the coding region of F-mSmad7 into pcS2+ (32). Embryonic stages are according to Nieuwkoop and Faber (33).

**RESULTS**

**Association of mSmad7 with BMP Receptors**—We investigated whether mSmad7 can associate with BMP receptors, in addition to binding to TGF-β receptors as previously shown (24, 25). COS cells transfected with cDNAs encoding F-mSmad7 in combinations with BMPR-III (wild type or kinase-deficient mutant) and BMPR-IA, BMPR-IB, or ActR-I (wild type or kinase-deficient) were affinity labeled with [32P]I-BMP-7, and F-Smad7 was immunoprecipitated from cell lysates with Flag antiserum. We found that Smad7 interacted with BMPR-IA, BMPR-IB (Fig. 1A), and ActR-I (data not shown). No interaction was observed between mSmad7 and a heteromeric complex of kinase-deficient type I receptor. Thus, interaction of mSmad7 with the BMP type I receptor requires that this receptor be phosphorylated by the BMP type II receptor. Phosphorylation may induce a conformational change that is required for mSmad7 type I receptor interaction.

**mSmad7 Inhibits BMP Receptor-induced Smad Phosphorylation**—In light of our finding that mSmad7 can associate with an active BMP receptor complex, we examined the effect of mSmad7 on BMPR-IB- and ActR-I-mediated phosphorylation of Smad1 or Smad5 (Fig. 1, B–E). COS cells were transfected with cDNAs encoding BMP receptors and Smad1 or Smad5 in the absence or presence of mSmad7. The phosphorylation level of Smad1 or Smad5 was examined by [32P]orthophosphate labeling and immunoprecipitation from cell lysates using Smad antisera. mSmad7 potently inhibited BMPR-IB-mediated phosphorylation of Smad1 and Smad5 in a dose-dependent manner (Fig. 1, B–E). In addition, mSmad7 inhibited ActR-I-mediated phosphorylation of Smad1 (Fig. 1, B and C).

**The MIH2 Domain of mSmad7 Is Sufficient for Receptor Interaction and Inhibitory Function**—Deletion mutant forms of mSmad7 were tested for their ability to interact with the TGF-β receptor complex, and to inhibit TGF-β signaling. We found that the isolated C-domain of mSmad7 interacted with the TGF-β receptor complex as efficiently as did wild-type mSmad7 (Fig. 2A), and was able to inhibit TβR-I-mediated phosphorylation of Smad2 (Fig. 2B). In some of the experiments, the inhibitory activity with mSmad7C was less than wild-type mSmad7. In contrast, as previously shown by Ha- yashi et al. (24), a small deletion of C-terminal residues of mSmad7 was found to abrogate receptor binding (data not shown).

Deletion mutant forms of mSmad7 were also tested for their ability to inhibit TGF-β-induced transcriptional activation of the plasminogen activator inhibitor-1 promoter, as assayed using the p3TPLux reporter construct. Consistent with the ability of the C-domain of mSmad7 to inhibit TβR-I-mediated phosphorylation of Smad2, this domain was sufficient to inhibit TGF-β-induced transcriptional activation, although it was less efficient at doing so than was wild-type mSmad7 (Fig. 2C). Deletion of the last 19 amino acid residues completely abrogated the ability of the mSmad7 C-domain to inhibit TGF-β-induced transcriptional activation of the 3TP promoter (data not shown).

**mSmad7 Is More Potent than XSmad7 in Inhibiting TGF-β-induced Responses**—XSmad7 shares 96% amino acid identity with mSmad7 within the C-domain (27). Given that this domain is sufficient for receptor binding, and for inhibition of several TGF-β-induced responses, one would predict that these proteins would behave identically with regard to receptor binding and inhibition of downstream signaling. Consistent with this possibility, XSmad7 interacted with the activated TβR-I, BMPR-IA, BMPR-IB, and ActR-I (data not shown). In addition, XSmad7 inhibited BMPRI-mediated phosphorylation of Smad1 and Smad5 in a dose-dependent fashion that was indistinguishable from that of mSmad7 (data not shown). However, whereas mSmad7 efficiently inhibited TβR-I-mediated phosphorylation of Smad2, XSmad7 was less effective at doing so (Fig. 3A). Interestingly, this correlated with the more pronounced effect of mSmad7 versus XSmad7 in inhibiting TGF-β-induced transcriptional activation of the p3TPLux reporter gene (Fig. 3B). Of note, mSmad7 is more effective than is XSmad7 at inhibiting activin signaling in a Xenopus mesoderm induction assay (27).

**Partial Secondary Axis Formation by mSmad7 and XSmad7**—To determine whether mSmad7 can inhibit transduction of BMP signals in vivo, we analyzed patterning defects caused by overexpression of mSmad7 in Xenopus embryos. Overexpression of known BMP antagonists, such as dominant negative BMP receptors or ligands, on the ventral side of Xenopus embryos can induce the formation of a partial secondary axis (reviewed in Ref. 34). Injection of 200 or 400 pg of RNA encoding mSmad7 near the ventral midline of four-cell embryos led to formation of a secondary dorsal axis in 90% (n = 100) or 96% (n = 79) of embryos, respectively (Fig. 4B). Secondary axis formation was observed in 94% (n = 98) of embryos injected with 200 pg of RNA encoding the isolated C-domain of mS-
FIG. 1. mSmad7 interacts with BMP receptor complexes and inhibits BMP-mediated Smad phosphorylation. A, association of N-terminal Flag-tagged mSmad7 (F-mSmad7) with BMPR-IA and BMPBR-IB in complex with BMPR-II. COS cells were transfected with various
mSmad7 C-domain is sufficient for binding to TGF-β receptor complex and inhibition of TGF-β-induced responses. A, association of wild-type F-mSmad7 (WT) and F-mSmad7C (7C) with TGF-β receptor complex. COS cells were transfected with F-mSmad7 (WT or 7C) in combination with TßR-I and TßR-II. The receptors were covalently affinity labeled with 125I-TGF-β1, and cell lysates were subjected to immunoprecipitation with Flag antiserum. B, mSmad7C inhibits TßR-I-mediated phosphorylation of Smad2. COS cells were transfected with F-Smad2 alone or together with F-mSmad7C or wild-type F-mSmad7 in the presence of BMPR-IB and BMPR-II; cells were labeled with [32P]orthophosphate and incubated in the absence or presence of BMP-7, and subjected to immunoprecipitation with Flag antisera. Expression of F-Smad2, F-mSmad7, and F-mSmad7C was determined by immunoblotting with Flag on aliquots of cell lysates. C, transfection of mSmad7C blocks TGF-β-induced p3TPLux transcriptional response but less efficient than wild-type mSmad7.

FIG. 2. mSmad7 C-domain is sufficient for binding to TGF-β receptor complex and inhibition of TGF-β-induced responses. A, association of wild-type F-mSmad7 (WT) and F-mSmad7C (7C) with TGF-β receptor complex. COS cells were transfected with F-mSmad7 (WT or 7C) in combination with TßR-I and TßR-II. The receptors were covalently affinity labeled with 125I-TGF-β1, and cell lysates were subjected to immunoprecipitation with Flag antiserum. B, mSmad7C inhibits TßR-I-mediated phosphorylation of Smad2. COS cells were transfected with F-Smad2 alone or together with F-mSmad7C or wild-type F-mSmad7 in the presence of BMPR-IB and BMPR-II; cells were labeled with [32P]orthophosphate and incubated in the absence or presence of BMP-7, and subjected to immunoprecipitation with Flag antisera. Expression of F-Smad2, F-mSmad7, and F-mSmad7C was determined by immunoblotting with Flag on aliquots of cell lysates. C, transfection of mSmad7C blocks TGF-β-induced p3TPLux transcriptional response but less efficient than wild-type mSmad7.
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**Fig. 3.** mSmad7 inhibits TGF-β-mediated signaling responses more effectively than XSmad7. (A) XSmad7 inhibits TβR-I-mediated phosphorylation of Smad2 less efficiently than mSmad7. Cells were transfected with F-Smad2 alone or together with F-mSmad7 or F-XSmad7 in the presence of TβR-I and TβR-II; cells were then incubated in the absence or presence of TGF-β. The level of F-Smad2 phosphorylation was determined by [32P]orthophosphate labeling and immunoprecipitation of cell lysates with Flag antiserum. Expression of F-Smads was analyzed by Western blotting with Flag antiserum on aliquots of cell lysates. B, transfection of mSmad7 in Mv1Lu cells blocks the TGF-β-mediated signaling response more effectively than XSmad7.

**Fig. 4.** Overexpression of mSmad7 in Xenopus embryos induces formation of a partial secondary axis and eye defects. Photomicrographs of control tadpole (A) and sibling tadpole (B) made to misexpress mSmad7 in ventral cells. Note induction of partial secondary dorsal axis (arrowheads). C and D, control (C) and mSmad7-RNA (D) injected sibling showing immunoreactive muscle in primary and secondary (arrowheads) axes; E, eye defects (arrowhead) and spina bifida (arrow) in an embryo made to overexpress XSmad7 in dorsal cells; F, fusion of eyes (arrowhead) in an embryo made to overexpress mSmad7 in dorsal cells.

**DISCUSSION**

Members of the TGF-β family play critical regulatory roles throughout vertebrate embryogenesis. They have been implicated as key players in mesoderm induction, in specification of ventral versus dorsal fates during early cleavage through gastrula stages, and in patterning of virtually all organs and tissues at later stages of development (reviewed in Refs. 35 and 36). The multifunctional nature of this family of ligands clearly implicates the need for tight control of their biological activity. Smads play a key role in controlling TGF-β signaling at the intracellular level. Pathway-restricted and common-mediator Smads, which become activated and form heteromeric com-
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plexes upon receptor activation, function to transduce TGF-β family signals. Pathway-restricted Smads may also negatively modulate these signal transduction cascades in some contexts. Specifically, Smad2 and Smad1 have been shown to compete with each other for sequestration of a limited pool of the common mediator, Smad4, thereby acting as intracellular antagonists of the BMP and activin signaling pathways, respectively (37). A distinct subfamily of Smads, which function to directly inhibit TGF-β family signaling by preventing formation of an active signal-transducing Smad complex, has recently been identified. In the present study, we have further characterized specific signaling pathways that are antagonized by murine and Xenopus Smad7 and have examined potential molecular mechanisms underlying their inhibitory activities.

Smad7 has previously been shown to inhibit signal transduction downstream of TGF-β and activin (24, 25). Our results demonstrate that mSmad7 is also a potent intracellular antagonist of BMPs, thereby implicating it as a broad spectrum inhibitor of TGF-β family signaling. Exposure of cells to TGF-β (25) strongly up-regulates mRNA expression of Smad7, consistent with the possibility that Smad7 participates in a negative feedback loop to regulate TGF-β responsiveness. Interestingly, we find that activin and BMP-7 are also potent inducers of expression of Smad7. Smad7 may therefore act as a general feedback inhibitor that decreases the ability of the cell to respond to all TGF-β family ligands following exposure to any one ligand.

The molecular mechanism by which members of the inhibitory Smad subfamily function is controversial. Mammalian Smad6 and Smad7 have both been shown to inhibit BMP and/or TGF-β signaling by competing with pathway-restricted Smads for association with the intracellular domain of TGF-β family type I receptors (23–25). A distinct mechanism of action for Smad6 has recently been proposed in which Smad6 competes with Smad4 for binding to Smad1 (29), rather than competing with Smad1 for receptor binding. The Smad6-Smad1 complex is apparently inactive, and prevents formation of active Smad1-Smad4 complexes. In the current studies, our data suggest that Smad7 inhibits BMP signaling by blocking access of Smad1 or Smad5 to the receptor. Specifically, Smad7 stably associates with BMP-7-induced heteromeric complexes of BMPR-II and BMPR-IA, BMPR-IB, or ActR-I and prevents receptor-mediated phosphorylation of the BMP pathway-specific Smads, Smad1 and Smad5. Our finding that interaction of Smad7 with BMP type I receptors is dependent on activation of these receptors by the BMP type II receptor kinase supports the physiological relevance of this interaction.

Our structure-function analyses demonstrate that the C-domain of mSmad7 is sufficient for interaction with activated type I receptors and for inhibitory function in mammalian cells and in Xenopus embryos. The C-domain of signal transducing Smads has also been shown to be sufficient for receptor interaction (38, 39). A short (19 amino acid residue) C-terminal truncation of this domain abrogates receptor interaction and inhibitory activity of mSmad7, suggesting that an intact C-domain is required for function. Hata et al. (29) report that the isolated C-domain of Smad6 is a more potent inhibitor of BMP signaling than is wild-type Smad6. In contrast, we find that the isolated C-domain of mSmad7 is less or similarly active at inhibiting TGF-β-induced responses in cultured cells than wild-type mSmad7.

Whereas the N-domain of signal-transducing Smads provides an inhibitory function, which can be relieved by receptor-mediated phosphorylation of the C-domain (40), antagonistic Smads are not substrates for TGF-β family receptors, and the function of the N-domain is less clear. Deletion of the N-domain from Smad6 relieves the ligand dependence of signal inhibition (29), suggesting that this domain plays a conserved role in silencing all subclasses of Smad proteins in the absence of ligand. Our results suggest that the N-domain of antagonistic Smads may also function to target specific signaling pathways for inhibition. This possibility is supported by our observation that mSmad7 and XSmad7, which share 96% amino acid identity in their C-domains but are less similar (51% identity) in their N-domains, differ in their ability to inhibit TGF-β signaling in cultured cells (present studies) and in their ability to block activin signaling in vivo (assays (27)). Furthermore, overexpression of XSmad7 in Xenopus embryos produces patterning defects that are not observed following overexpression of mSmad7, suggesting that mSmad7 and XSmad7 may preferentially target distinct signaling pathways. To rule out the possibility that these differences are due to species variability awaits the identification of the complete spectrum of inhibitory Smads in mammals and in Xenopus.

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