DEVELOPMENTAL BIOLOGY **200,** 260–268 (1998)

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Smad7 Inhibits Mesoderm Formation and Promotes Neural Cell Fate in *Xenopus* Embryos

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We report the isolation and characterization of a new inhibitory Smad in *Xenopus,* which we have designated as *Xenopus* Smad7. Smad7 is present at fairly constant levels throughout early development and at blastula stages enriched in the ventral side of the animal hemisphere. The induction of mesoderm by TGF-β-like signals is mediated by receptor ALK-4 and we show that Smad7 blocks signaling of ALK-4 in a graded fashion: lower levels of Smad7 block activation of dorsal mesoderm genes and higher levels block all mesoderm genes expression. Smad7 is able to directly activate neural markers in explants in the absence of mesoderm or endoderm. This neural-inducing activity of Smad7 may be due to inhibition of BMP-4 signaling because Smad7 can also block BMP-4-mediated mesoderm induction. Thus, Smad7 acts as a potent inhibitor of mesoderm formation and also activates the default neural induction pathway. © 1998 Academic Press

Key Words: **Smads; germ layer; cell fate specification; TGF-β; ectoderm.**

INTRODUCTION

Understanding the processes that lead to formation of the germ layers in an embryo is key to understanding how the spatial organization of an animal emerges from a fertilized egg. During vertebrate embryogenesis, inductive processes involving cell interactions are known to play a key role in specification of cell fates and the formation of germ layers (reviewed in Jessell and Melton, 1992). In *Xenopus,* the marginal zone cells of the animal hemisphere adopt a mesodermal fate in response to inductive signals that originate from the underlying endoderm. Ectoderm arises from animal pole cells that do not become mesoderm. Dorsal ectoderm forms the nervous system in response to signals from the dorsal marginal zone (DMZ), the site of the Spemann's organizer, while ventral ectoderm does not receive these signals and forms epidermis (reviewed in Heasman, 1997).

This cascade of signaling relies in large part on secreted molecules of the TGF-β family (reviewed in Kessler and Melton, 1994). The TGF-β family members signal through heteromeric receptor complexes: the ligand binds to the type II receptors followed by the recruitment of a type I receptor into a complex which then initiates the signaling cascade (Wrana *et al.,* 1994). The type I receptors, known as ALKs (activin receptor-like kinases) activate downstream target proteins known as Smads which subsequently translocate to the nucleus and cooperate with other proteins to activate transcription (Chen *et al.,* 1997a). Although the exact identities of the signals utilized in the embryo remain unclear in many cases (Smith, 1995), isolation and characterization of receptors that mediate the signals of the TGF-β-like ligands and Smad proteins provide new tools to study the mechanisms that govern embryonic induction. In *Xenopus,* at the blastula stage, the induction of mesoderm and the formation of the Spemann's organizer appear to be mediated by the receptor ALK-4 and its downstream target, Smad2 (Bhushan and Vale, manuscript submitted; Baker and Harland, 1996; Chang *et al.,* 1997). During gastrula stages, the receptor ALK-3 and Smad1 mediate BMP-4 signals to actively maintain ventral fates in the animal hemisphere and to attenuate dorsalizing signals emanating from the organizer (Graff *et al.,* 1994). Such analyses are beginning to unravel how an early developing embryo controls spatial patterns of transcription factors through intercellular signaling.

A number of recent studies have suggested that the establishment of dorsal–ventral pattern of gene expression in the gastrula-stage *Xenopus* embryo involves the interaction of stimulatory and inhibitory signaling (reviewed in Wilson and Hemmati-Brivanlou, 1997). Noggin and chordin

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are dorsalizing factors secreted by the Spemann's organizer that inhibit BMP-4-mediated ventral signals by directly binding to and inactivating BMP-4 (Piccolo *et al.,* 1996; Zimmerman *et al.,* 1996). Thus active signaling by BMP-4 leads to the formation of ventral mesoderm in the marginal zone and epidermis in the ectoderm; inhibition of BMP-4 signaling promotes dorsal mesoderm and neural fate. These studies indicate that a common set of activating and inhibitory signaling molecules are involved in patterning the animal hemisphere in both mesoderm and ectoderm. The decision of animal hemisphere cells to form either mesoderm or ectoderm however lies with the responding tissue and the molecular basis of this competence remains unknown.

We report the isolation and characterization in *Xenopus* of a new member of the Smad family, the homologue of the human Smad7, that reveals another inhibitory signaling pathway involved in early patterning events. Smad7 inhibits its signaling mediated by ALK-4, and blocks the transcriptional activation of mesodermal genes in the marginal zone. Smad7 is able to directly induce neural tissue in animal pole explants in the absence of mesoderm and endoderm. The neural-inducing activity of Smad7 may be due to inhibition of BMP-4 signaling because Smad7 can block BMP-4-mediated mesoderm induction. The ability of Smad7 to block mesoderm induction while promoting ectodermal fate suggests that Smad7 may play a role in the decision of animal hemisphere cells to form either mesoderm or ectoderm.

MATERIALS AND METHODS

Isolation of Xenopus Smad7

Degenerate primers directed at the highly conserved C-terminal MH2 domain of human and rat Smad7 clones were used in the amplification in the polymerase chain reaction (PCR) from stage 11 embryo cDNA. The 360-bp fragment we obtained was subsequently used to screen a stage 17 phage library (gift of C. Kintner). The 14 positive clones analyzed revealed almost identical sequences. The complete nucleotide sequence of the longest clone containing the full-length *Xenopus* Smad7 has been deposited in GenBank under Accession No. AF045742.

Animals and Reagents

Embryos were obtained from *Xenopus laevis* adult frogs (NASCO and *Xenopus* 1) by hormone-induced egg-laying and *in vitro* fertilization using standard protocols and staged according to Nieuwkoop and Faber. For phenotypic analysis, embryos were injected in two blastomeres of four-cell embryos. The dorsal side of the four-cell embryos was identified by pigment and cell size difference between dorsal and ventral blastomeres. For *in situ* hybridization analysis, albino embryos were injected in both blastomeres at the two-cell stage without any dorsal/ventral bias.

Animal Pole Explant Assay

Two-cell-stage embryos were injected with RNAs spread in both blastomeres and allowed to develop until animal pole explants were cut at blastula stage (stage 9). Animal caps were dissected in 0.5× MMR containing penicillin and streptomycin. Animal caps were harvested for RNA using acid guanidinium–phenol–chloroform method (TRI Reagent). Expression of specific RNA transcripts was analyzed using an RT–PCR protocol as described (Wilson and Melton, 1994). Smad7 primers: Forward, 5′-cgatcggtgctcgtcc-3′; reverse, 5′-actccaccgcctgca-3′.

Plasmid Constructs and in Vitro Transcription

All constructs used for *in vitro* transcription and injection were inserted into a CS2+ vector (Turner and Weintraub, 1994). Most of the 5′ and 3′ untranslated sequences of all the cDNAs used were removed before inserting into the vector. RNA was synthesized in vitro using SP6 polymerase.

In Situ Hybridization and Probe Synthesis

The whole mount *in situ* hybridization protocol developed by Harland (1991) was used with minor modifications. Digoxigenin-labeled RNA probes were generated by *in vitro* transcription. Hybridization was detected with an alkaline-phosphatase-coupled anti-digoxigenin antibody and visualized using either NBT/BCIP or BM purple as a substrate (Boehringer-Mannheim). Stained embryos were fixed overnight in MEMFA, washed in ethanol, and photographed directly.

Histology

Embryos were washed in 70% ethanol before dehydrated through an ethanol series and embedded in paraffin using standard procedures. Tissues were sectioned at 10 μm on a rotary microtome and mounted on gelatin-subbed slides. Sections were then stained with eosin.

RESULTS

Xenopus Smad7 Is Enriched in the Ventral Side of the Animal Hemisphere at Blastula Stages

The *Xenopus* Smad cDNA we isolated encodes a predicted 382-residue polypeptide with structural features of the Smad family, such as the two conserved domains known as MH1 and MH2 (for mad homology). Sequence comparison with the known Smad family members revealed that the clone we isolated is distantly related to the pathway-restricted Smads (Smad1, Smad2, Smad3, Smad5, Smad8) and closely related to the human inhibitory Smad, Smad7 (Chen *et al.,* 1997b; Graff *et al.,* 1996; Hayashi *et al.,* 1997; Suzuki *et al.,* 1997a). The *Xenopus* clone as well as the human Smad7 cDNA lacks the carboxy-terminal SSXS motif that serves as a receptor phosphorylation site in receptor-regulated Smads (Kretzschmar *et al.,* 1997; Macias *et al.,* 1996). The human Smad7 is predicted to be 426 amino acid long and contains a few stretches of amino acids in the N-terminal MH1 domain not found in the *Xenopus*

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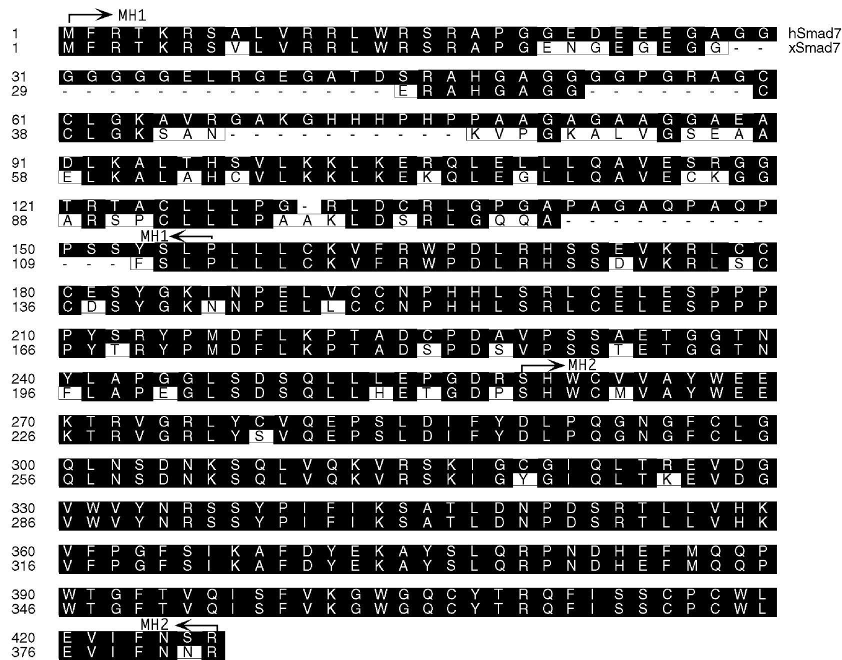

FIG. 1. Structure of *Xenopus* Smad7. Alignment of the predicted amino acid sequences of human Smad7 and *Xenopus* Smad7. Identical residues are shaded in dark. Mad homology domains, MH1 and MH2 are indicated by arrows.

clone (Fig. 1) (Topper *et al.,* 1997). Other than the absence of these loops, the *Xenopus* clone is virtually identical to the human Smad7 and thus we refer to it as *Xenopus* Smad7 (Fig. 1). We also found that the human Smad7 and *Xenopus* Smad7 behaved in a similar fashion in functional assays described later (data not shown). Smad7 is expressed maternally and zygotically transcribed at fairly constant levels during early development (Fig. 2, left). To further explore the distribution of Smad7 transcripts, blastula-stage embryos were dissected into animal and equatorial/vegetal regions and cultured until the mid-gastrula stage (stage 11). Smad7 was found to be localized predominantly in the animal pole region (Fig. 2, right). To determine the dorsal/ventral localization of Smad7, early-gastrula-stage embryos were dissected into dorsal and ventral halves and analyzed at mid-gastrula stage. Smad7 was found to be enriched in the ventral half of the embryo, though low levels of Smad7 could be detected in the dorsal hemisphere (Fig. 2, right). *In situ* hybridization techniques could not reliably detected Smad7 expression in blastula-stage embryos.

FIG. 2. Localization of Smad7 during development. (Left) RNA isolated from embryos at various stages as indicated in the figure was analyzed by RT–PCR for the expression of Smad7 transcripts. (Right) Blastula-stage embryos dissected into different regions and cultured until equivalent of early gastrula stage. AP, animal pole; M/V, marginal zone and vegetal region; D, dorsal half; V, ventral half; E, whole embryo; −RT, whole embryo extracted RNA in the absence of reverse transcriptase in the RT–PCR reaction.

Smad7 Blocks ALK-4 Signaling

Since specification of embryonic cell fate is known to involve TGF-β family signaling, we assessed the biological function of Smad7 during early development of *Xenopus* embryos. TGF-β family members such as activin can mimic the vegetal signals in the embryo and signal through ALK-4 and Smad2 to induce animal pole explants to form mesoderm (Bhushan and Vale, manuscript submitted; Baker and Harland, 1996; Chang *et al.,* 1997) . Cell culture studies suggest that the human Smad7 functions as an inhibitor of TGF-β signaling. Smad7 has been shown to associate with the type I TGF-β receptor, ALK-5 and to prevent signaling by presumably blocking Smad2/3 activation (Hayashi *et al.,* 1997). We tested whether Smad7 would inhibit ALK-4 signaling by coinjecting Smad7 RNA with constitutively active ALK-4 (CA-ALK-4) RNA into the animal pole of two-cell stage embryos. Two batches of animal pole explants from these embryos were cultured and RNA was extracted from the explants at the equivalent of mid-

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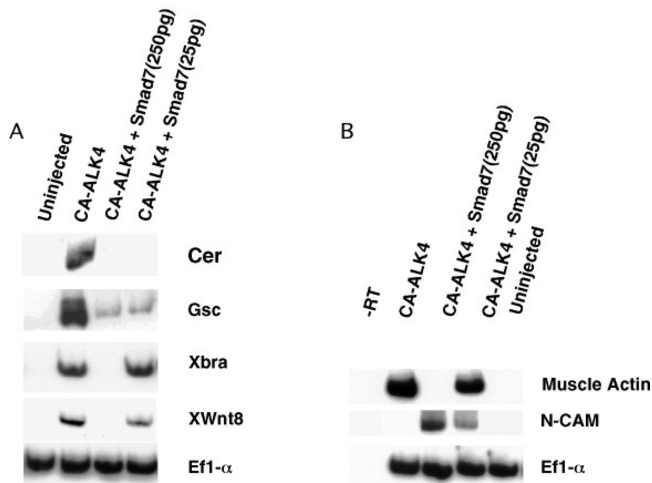

FIG. 3. Smad7 blocks ALK-4-mediated mesoderm induction in explant assays. (A) Animal pole explants from embryos injected with 500 pg of CA-ALK-4 alone and coinjected with Smad7 (amounts as indicated) were analyzed by RT–PCR at stage 11 for the expression of mesoderm-specific markers expressed at gastrula stages. EF1-α transcripts used as an internal standard. Cer, cerberus; Gsc, goosecoid; Xbra, brachyury. −RT lane not shown for this experiment. (B) Animal pole explants from embryos injected with 500 pg of ALK-4 alone and coinjected with Smad7 (amounts as indicated) were analyzed by RT–PCR at stage 17 for the expression of mesoderm and ectoderm differentiated markers. NCAM, neural-specific marker; muscle actin, somitic mesoderm marker; −RT, whole embryo extracted RNA in the absence of reverse transcriptase in the RT–PCR reaction.

gastrula-stage embryo (stage 11) and at late neurula stage (stage 17). At gastrula stages the transcriptional activation of a variety of mesoderm-specific genes can be analyzed in the animal pole explants that reflect the induction and pattern of mesoderm in the embryo. We have utilized four such markers that broadly represent spatially distinct mesoderm at gastrula stages: *Cerberus* (Cer), a secreted factor expressed in the endomesodermal cells, *goosecoid* (gsc), a homeobox gene expressed in the Spemann's organizer, *Xenopus brachyury* (Xbra), a gene present throughout the marginal zone, and *Wnt-8,* a member of the Wnt family expressed in the ventral mesoderm (Bouwmeester *et al.,* 1996; Cho *et al.,* 1991; Christian *et al.,* 1991; Smith *et al.,* 1991). Injection of 500 pg of CA-ALK-4 RNA alone activated of all four markers, while uninjected control explants did not express any of the mesoderm markers (Fig. 3A). Coinjection of 25 pg of Smad7 RNA along with 500 pg of CA-ALK-4 RNA significantly inhibited transcriptional activation of cer and gsc but did not affect induction of Xbra or Wnt-8. A 10-fold increase in the injected amount of Smad7 RNA (250 pg) however was sufficient to inhibit the expression of Xbra and Wnt-8 in addition to cer and gsc.

RNA extracted from explants at the equivalent of neurula stage embryos was analyzed for markers of differentiated tissue that derived from both mesoderm and ectoderm (Fig. 3B). Explants injected with CA-ALK-4 RNA induced muscle actin expression, a marker for somitic mesoderm. Coinjection of Smad7 RNA at low levels did not inhibit the expression of muscle actin, although higher levels of Smad7 blocked muscle actin expression completely. In this respect, Smad7 inhibition of muscle actin expression follows a similar profile as Xbra and Xwnt-8 analyzed at the gastrula stages. Thus, the analysis of gastrula- and neurula-stage mesoderm markers showed that Smad7 inhibited the formation of a range of mesoderm cell types by blocking signaling mediated by ALK-4. The analysis of the ectodermal marker, NCAM, a general neural marker (Kintner and Melton, 1987), however, yielded a surprising result. Explants coinjected with 250 pg of Smad7 and CA-ALK-4 RNA did not express muscle actin as just described but expressed NCAM. This experiment pointed to an intriguing result: inhibition of mesoderm markers by Smad7 coincided with induction of neural fate.

Smad7 Promotes Neural Fate

To directly study the role of Smad7 in the induction of ectodermal fates, we injected various levels of Smad7 RNA in the animal pole of two-cell stage embryos. Explants were dissected from blastula-stage embryos and RNA was extracted from the explants when sibling embryos reached the equivalent of late neurula stage (stage 17). Explants from embryos injected with Smad7 expressed high levels of the neural-specific marker, NCAM (Fig. 4A). Injection of as little as 25 pg of Smad7 RNA resulted in expression of NCAM. We also analyzed these explants for the expression of muscle actin and endodermin (edd), a panendodermal marker (Sasai *et al.,* 1996). No muscle actin or edd expression was detected in Smad7-injected embryos. To confirm that no mesoderm was induced, explants injected with various levels of Smad7 RNA were analyzed at mid-gastrula stage for the expression of panmesoderm marker, Xbra. No Xbra expression was detected in these explants (Fig. 4A). The induction of neural fate by Smad7 thus appeared to be direct, i.e., occurred in the absence of mesoderm or endoderm induction. Analysis of markers for other ectodermal fates such as the cement gland marker XAG (Sive and Bradley, 1996) revealed that explants from Smad7 embryos also expressed high levels of XAG. The epidermal fate marker, epidermal keratin, is normally expressed in uninjected caps and usually inhibited upon neural induction (Wilson *et al.,* 1997). In Smad7-injected explants, epidermal keratin expression was significantly reduced.

Several lines of evidence suggest that neural specification is under inhibitory control and that neural-inducing signals function by antagonizing BMP-4 in the ectoderm allowing neural tissue to form as a default state (reviewed in (Wilson and Hemmati-Brivanlou, 1997). We tested whether Smad7 could block BMP-4 signaling. BMP-4 has been shown to induce a variety of early response genes in ectodermal explants including Xwnt-8, Xbra, Vox (Xvent-

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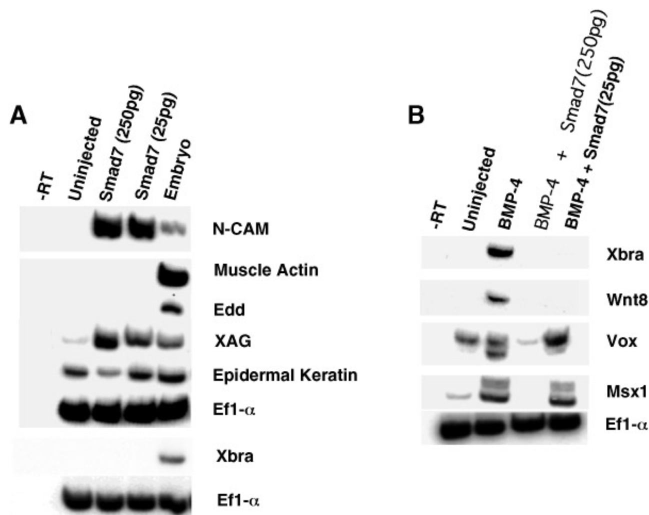

FIG. 4. Smad7 activates the default neural pathway. (A) Explants from embryos injected with Smad7 (amounts indicated) alone and analyzed for the expression of differentiated markers for all germ layers. EF1-α transcripts used as an internal standard. NCAM, neural-specific marker; edd, endodermin—panendoderm marker; XAG, cement gland marker; muscle actin, somitic mesoderm marker; epidermal keratin, epidermis marker; Xbra, *Xenopus* brachyury, pan-mesoderm marker. (B) Smad7 blocks BMP-4-mediated induction of mesoderm. Embryos were injected with 500 pg of BMP-4 RNA alone or coinjected with 500 pg of BMP-4 RNA and Smad7 RNA (amounts indicated). Explants at blastula stage were cultured until gastrula stage and analyzed for the expression of mesoderm markers by RT–PCR. −RT, whole embryo-extracted RNA in the absence of reverse transcriptase in the RT–PCR reaction.

2), and Msx1 (Onichtchouk *et al.,* 1996; Schmidt *et al.,* 1996; Suzuki *et al.,* 1997b). Embryos at the two-cell stage were injected with 500 pg of BMP-4 RNA alone or coinjected along with different amounts of Smad7 RNA. Explants were dissected at late-blastula stage and analyzed at mid-gastrula equivalent stage for the induction of these early response markers to BMP-4. Analysis of RNA extracted from mid-gastrula-stage explants (Fig. 4B) showed that BMP-4 can mediate induction of the ventral mesoderm marker, Xwnt-8, and the panmesodermal marker, Xbra. Vox and Msx1 are normally expressed in the nonneural ectoderm in addition to ventral mesoderm and uninjected explants express both these markers, though BMP-4 is capable of significantly increasing their levels of expression (Fig. 4B). Coinjection of low doses of Smad7 with BMP-4 completely blocks the induction of both Xbra and Xwnt8, although Vox and Msx1 are unaffected. At higher doses of injected Smad7, however, Vox and Msx1 expression in explants is also inhibited to levels lower than uninduced explants. These experiments show that Smad7 could promote neural fate in ectodermal explants by blocking BMP-4 signaling.

Smad7 Acts Downstream of the Receptor Activation

To ascertain where Smad7 acted in the signaling cascade, we also determined whether Smad7 was capable of inhibiting pathway-specific Smad involved in ALK-4- and BMP-4-mediated signaling. Explants injected with Smad2 and Smad1, in a ligand-independent fashion, activate dorsal and ventral markers, respectively (Graff *et al.,* 1996). Explants injected with 1 ng of Smad2 RNA expressed dorsal mesoderm marker, muscle actin, while explants injected with 1 ng Smad1 RNA expressed ventral mesoderm marker, globin (Fig. 5). Coexpression of 250 pg of Smad7 RNA had no effect on either Smad activity because muscle actin and globin induction was unaffected. It is important to note that this level of Smad7 was sufficient to inhibit all target genes activated by CA-ALK-4 and BMP-4. However, in explants coinjected with higher levels of Smad7 (1 ng), muscle actin and globin were not detected, suggesting that relatively high levels of Smad7 are required to block Smad2 and Smad1 activities.

Smad7 Blocks Mesoderm Formation in the Embryo

We tested whether Smad7 also blocked mesoderm signals in the marginal zone in the embryo. Albino embryos were injected with Smad7 RNA in the marginal zone of both blastomeres of two-cell-stage embryos. At early gastrula stage, embryos were analyzed by *in situ* hybridization for the induction of a range of mesoderm-specific genes. Two representative markers are shown here: goosecoid, which is expressed in the dorsal lip, and Vox (Xvent-2), a gene expressed in ventral mesoderm outside the dorsal lip region

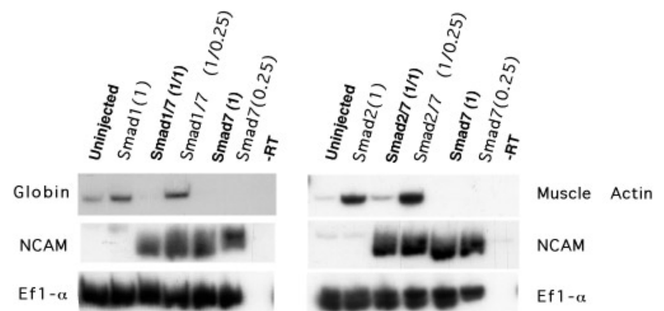

FIG. 5. Smad7 has similar effects on Smad1 and Smad2 activities. (Left) Animal pole explants from embryos injected with 1 ng of Smad1 alone and coinjected with Smad7 (amounts as indicated in ng) were analyzed by RT–PCR at stage 17 for the expression of globin, a ventral mesoderm marker. (Right) Animal pole explants from embryos injected with 1 ng of Smad1 alone and coinjected with Smad7 (amounts as indicated in ng) were analyzed by RT–PCR at stage 17 for the expression of muscle actin, somitic mesoderm marker. NCAM, neural-specific marker used as a positive control for Smad7; −RT, whole embryo-extracted RNA in the absence of reverse transcriptase in the RT–PCR reaction.

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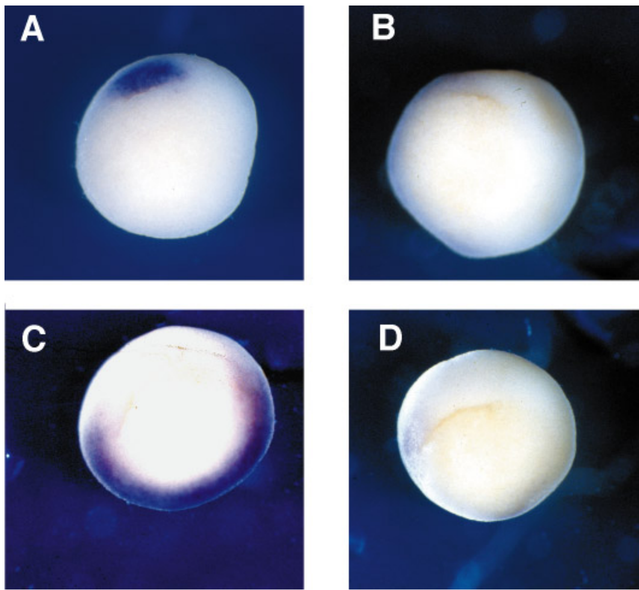

(Figs. 6A and 6C). In Smad7-injected embryos, expression of either goosecoid and Vox was not detected (Figs. 6B and 6D). Other markers such as Xbra and Xwnt-8 were also blocked by Smad7 (data not shown). The injection of human Smad7 in *Xenopus* embryos was also reported to block Xbra expression, suggesting that *Xenopus* and human Smad7 behave in a similar fashion (Nakao *et al.,* 1997).

Since Smad7 blocked gene expression in the mesoderm and the formation of mesoderm is crucial for the establishment of the body plan, we analyzed the phenotype of embryos that were injected with Smad7. Embryos injected with Smad7 RNA in the marginal zone of both blastomeres of two-cell-stage embryos did not initiate gastrulation, consistent with the inability of these embryos to form any mesoderm (data not shown). We targeted the expression of Smad7 by injecting either dorsal or ventral blastomeres of four-cell-stage embryos. Embryos injected in the dorsal blastomeres showed severely truncated anterior axes and

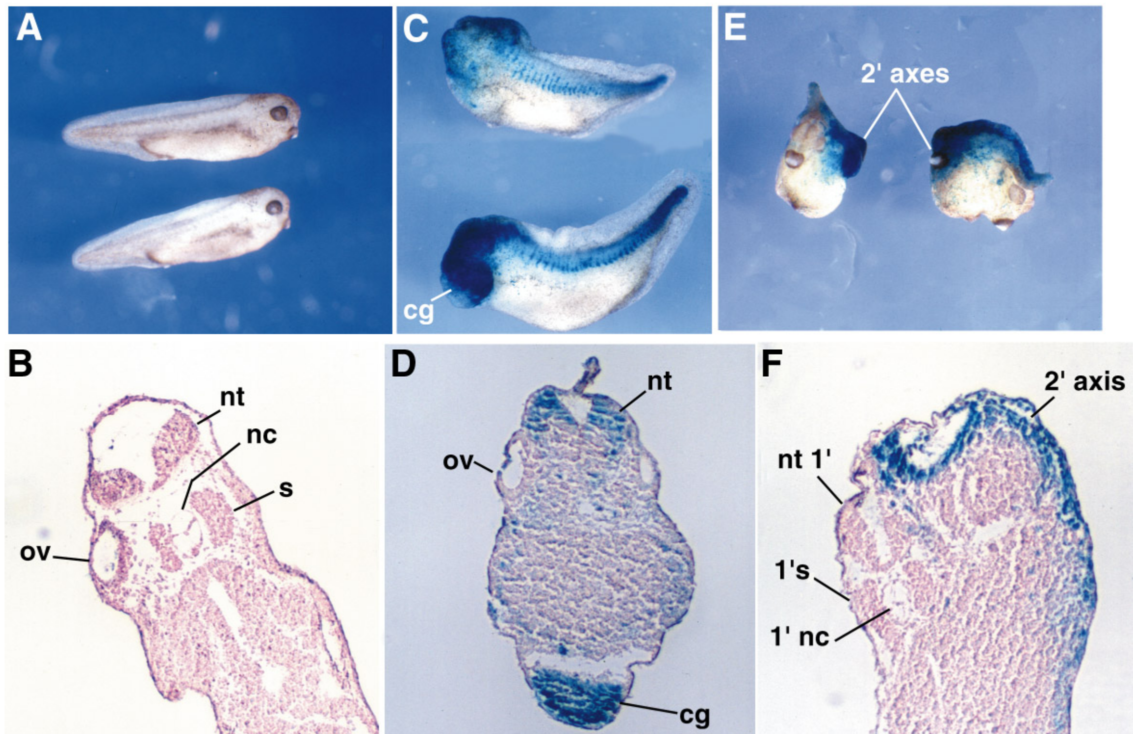

FIG. 6. (Top) Smad7 blocks activation of mesoderm markers in the embryo. Albino embryos were injected with 25 pg of Smad7 RNA in the marginal zone of both blastomeres at the two-cell stage. The embryos were fixed at gastrula stage 10. One-half and whole mount *in situ* hybridization was performed using Gsc or Vox antisense probes. (A) Uninjected embryo—Gsc is expressed in the dorsal lip. (B) Embryo injected with Smad7—Gsc is no longer detected ($n = 22$). (C) Uninjected embryo—Vox is expressed in the ventral mesoderm and excluded from the dorsal lip. (D) Embryo injected with Smad7—Vox is no longer detected ($n = 16$).

FIG. 7. (Bottom) Smad7 blocks mesoderm formation and affects body axis in the embryo. Embryos injected with 25 pg of Smad7 RNA. (A) Uninjected embryos. (B) Histological section of uninjected embryos. (C) Embryos injected in a dorsal blastomeres at four-cell stage ($n = 37$, 21). (D) Histological section of dorsally injected Smad7 embryos—note the lack of dorsal mesodermal tissue, an enlarged cement gland, and the presence of a neural tube. (E) Embryos injected with Smad7 RNA in ventral blastomeres at four-cell stage ($n = 28$)—note incomplete secondary axes. (F) Histological section of embryos injected with Smad7 ventrally—note that the secondary axis does not contain any notochord or somitic mesoderm. cg, cement gland; nt, neural tube; s, somites; ov, otic vessicle; nt, notochord.

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most lacked head structures, although these embryos had prominent cement glands (Fig. 7C). The β-gal lineage tracer patterns in the dorsal axes suggested that somites either were not formed at all or were disordered. Histological sections confirmed that these embryos lacked dorsal mesoderm structures such as notochord and somites (Fig. 7D). Interestingly, these embryos did appear to have, at least by histological criteria, a well-formed neural tube. Embryos injected in ventral blastomeres displayed incomplete secondary axes, consistent with the ability of Smad7 to block ventral mesoderm formation by BMP-4 (Fig. 7E). Histological sections showed that the secondary axes were devoid of any histologically defined mesodermal tissue and appeared disorganized (Fig. 7F).

DISCUSSION

Signals from the vegetal region of the *Xenopus* embryo induce overlying marginal zone cells to form mesoderm. The mesoderm-inducing signals are mimicked by TGF-β members, activin, Vg1 and nodal-related molecules and are likely to be mediated by the serine kinase receptor, ALK-4, and its downstream mediator, Smad2. In this study, we have investigated the role of a new member of the Smad family, *Xenopus* Smad7, in the induction of mesoderm. Smad7 is expressed at fairly constant levels throughout early development. At blastula and gastrula stages Smad7 transcripts are enriched in the ventral part of the animal hemisphere, although low levels of Smad7 transcript are detected in the dorsal side of the animal hemisphere. We demonstrate that Smad7 blocks the induction of mesoderm mediated by ALK-4. We also found the inhibitory activity of Smad7 in blocking transcriptional activation of mesoderm markers to be graded: lower levels of Smad7 blocked expression of dorsal mesoderm markers, while higher levels blocked all mesoderm. Since activation of dorsal mesoderm genes requires the highest concentration of inducer (Green and Smith, 1990), lower levels of Smad7 would presumably lead to partial inhibition of ALK-4 activity and that would be sufficient to block activation of the genes that require the highest activity. This suggests that Smad7 may play a negative regulatory role in the establishment of the pattern of mesodermal gene expression. The pattern of Smad7 RNA expression, i.e., higher in the ventral hemisphere and lower dorsally, is consistent with this proposed role. There is growing evidence of regulatory processes that underlie spatial organization of gene transcription in the embryo that utilizes negative control. The syncytial early *Drosophila* embryo sets up spatial transcription patterns that depend on both positive and negative transcription control (reviewed in Davidson, 1993). In *Xenopus,* activin has been shown to activate as well as inhibit gene transcription in animal explants and dissociated cells in a concentration-dependent manner (Green and Smith, 1990; Gurdon *et al.,* 1994), suggesting that the signal transduction mechanism involves some sort of negative regulatory control. Observations in the whole embryo suggest that a more cascade-like series of patterning involving negative feedback signaling may operate in the embryo, since even under experimental conditions, various induced tissue such as notochord, somites, lateral mesoderm, and ectoderm remain in proportion (Cooke, 1989). In addition, theoretical modeling that includes an inhibitory component is better at simulating the regulatory behavior of pattern formation in the embryo (Meinhardt, 1977). Whether Smad7 may play a role in a negative regulatory signaling cascade to control gene expression patterns in the embryo remains to be fully explored.

The human Smad7 was shown to function in cell culture studies as an intracellular antagonist of TGF-β signaling (Hayashi *et al.,* 1997; Nakao *et al.,* 1997). We have shown here that Smad7 blocks signaling mediated by the activin receptor ALK-4 as well as inhibit BMP-4 signaling. These results reveal that Smad7 could act as a general inhibitor of the TGF-β superfamily. Smad7 can efficiently block signaling from activated type I receptors, ALK-4 and ALK-5, and associates stably with these activated type I receptors (Hayashi *et al.,* 1997; Nakao *et al.,* 1997). This suggests that the Smad7 may interfere with the activation of the pathway-specific Smads. Our results show that Smad7, at levels more than sufficient to block the activity of activated receptors, does not block pathway-specific Smads, Smad1 and Smad2, activities. Unlike the related inhibitory Smad6 (Hata *et al.,* 1998), no direct physical interactions between Smad7 and pathway-specific Smads have been observed (unpublished data). Smad7 and Smad6 were however reported to form heterodimers (Topper *et al.,* 1997). It is possible that the block of pathway-specific Smads at high levels of Smad7 expression may be mediated through Smad6. Further biochemical analysis could help in the clarification of the specificity and the site of action of the inhibitory Smads.

The direct neural-inducing activity of Smad7 in ectodermal explants most likely originates in its ability to block BMP-4 signaling. The Spemann's organizer produces a host of secreted factors that have direct neural-inducing activity in animal pole explants. Two such secreted factors from the dorsal mesoderm, follistatin and cerberus, have activity similar to Smad7 (Bouwmeester *et al.,* 1996; Hemmati-Brivanlou *et al.,* 1994). Both follistatin and cerberus inhibit mesoderm induction and induce neural tissue in explants. Follistatin-blocking mesoderm induction in explants is consistent with its ability to inhibit activin; however, its neural-inducing activity may arise from follistatin inhibiting other members of the TGF-β family such as BMP-4. Unlike Smad7, however, follistatin does not prevent mesoderm formation in embryos (Schulte *et al.,* 1994). Cerberus suppresses dorsal and ventral mesoderm gene expression and promotes the formation of cement gland and neural structures. However, unlike Smad7, cerberus does activate the panendodermal marker, edd, in animal pole explants. It may, however, be worth pursuing the relationship of cerberus with Smad7, since it is possible that Smad7 could

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function downstream of cerberus and mediate some of its activities.

Noggin and chordin are two other factors expressed in the Spemann's organizer that have been shown to directly bind BMP-4 and this activity is sufficient to initiate the pathway for neural development (Piccolo *et al.,* 1996; Zimmerman *et al.,* 1996). Smad7, like noggin and chordin, blocks BMP-4-mediated activation of ventral mesoderm and thus initiates neural development in animal pole explants. However, three activities of Smad7 distinguish it from noggin and chordin. First, Smad7 is a potent inhibitor of all mesoderm, while noggin and chordin dorsalize ventral mesoderm. Second, noggin and chordin induce explants to activate edd expression and form endoderm in addition to neural tissue (Sasai *et al.,* 1996) while Smad7-expressing explants do not activate edd. Finally, blocking BMP-4 signaling in explants by injecting dominant negative BMP-4 receptor or noggin induces neural fate at the expense of other ectodermal cell types such as for cement gland and epidermis (Wilson *et al.,* 1997). Smad7, by contrast, induces high levels of cement gland marker and does not completely suppress the epidermal marker. The expression of epidermal keratin we observe, however, could be simply due to Smad7 RNA not distributed throughout the animal pole, thus allowing expression of this very abundant marker. These differences nevertheless indicate that despite the ability of Smad7 to directly induce neural tissue in explants, its role in the embryo may be quite different from the secreted BMP-4 antagonists such as noggin and chordin.

Our results lead us to speculate that the role of Smad7 in the embryo may more likely lie in the establishment of spatial limits for the expression of mesodermal genes. Since *in vitro* assays for mesoderm induction are based on the ability of animal pole explants to form mesoderm, animal pole cells are clearly capable of forming mesoderm; yet in the embryos, only cells in the marginal zone of the animal pole form mesoderm. Several mechanisms have been proposed that would limit the extent of induction such as the rate of diffusion, concentration, or the stability of the ligands (Gurdon, 1987; Weeks and Melton, 1987). On the other hand, ectodermal explants lose the ability to respond to mesoderm inducers at gastrula stages and thus temporal competence could also limit the extent of mesoderm formation (Slack, 1993). Whether Smad7 plays a role in establishing the germ layer domains in the early vertebrate embryo by limiting the spatial extent of induction remains to be determined. Nevertheless, the isolation and characterization of Smad7 as a potent inhibitor of mesoderm induction *in vitro* and *in vivo* suggests that Smad7 may negatively regulate embryonic induction in the embryo.

ACKNOWLEDGMENTS

We thank Drs. Gisele Deblandre and Chris Kintner for critical reading of the manuscript and for invaluable discussions. We are grateful to Dr. Chris Kintner for allowing A.B. to use instrumentation in his laboratory. We thank Drs. E. DeRobertis, J. Smith, N. Papalopulu, I. Dawid, P. Lemaire, and K. Cho for sharing reagents. This work was supported by NIH Grant HD 13527 and the Foundation for Medical Research. Inc. W.V. is a Foundation for Medical Research Senior Investigator.

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Received for publication February 10, 1998
Revised May 27, 1998
Accepted May 27, 1998

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