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Endogenous patterns of TGFβ superfamily signaling during early *Xenopus* development

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SUMMARY

Transforming growth factor β (TGFβ) superfamily signaling has been implicated in patterning of the early *Xenopus* embryo. Upon ligand stimulation, TGFβ receptors phosphorylate Smad proteins at carboxy-terminal SS(V/M)S consensus motifs. Smads 1/5/8, activated by bone morphogenetic protein (BMP) signaling, induce ventral mesoderm whereas Smad2, activated by activin-like ligands, induces dorsal mesoderm. Although ectopic expression studies are consistent with roles for TGFβ signals in early *Xenopus* embryogenesis, when and where BMP and activin-like signaling pathways are active endogenously has not been directly examined.

In this study, we investigate the temporal and spatial activation of TGFβ superfamily signaling in early *Xenopus* development by using antibodies specific for the type I receptor-phosphorylated forms of Smad1/5/8 and Smad2. We find that Smad1/5/8 and two distinct isoforms of Smad2, full-length Smad2 and Smad2Δexon3, are phosphorylated in early embryos. Both Smad1/5/8 and Smad2/Smad2Δexon3 are activated after, but not before, the mid-blastula transition (MBT). Endogenous activation of Smad2/Smad2Δexon3 requires zygotic transcription, while Smad1/5/8 activation at MBT appears to involve transcription-independent regulation. We also find that the competence of embryonic cells to respond to TGFβ superfamily ligands is temporally regulated and may be a determinant of early patterning.

Levels of phospho-Smad1/5/8 and of phospho-Smad2/Smad2Δexon3 are asymmetrically distributed across both the animal-vegetal and dorsoventral axes. The timing of the development of these asymmetries differs for phospho-Smad1/5/8 and for phospho-Smad2/Smad2Δexon3, and the spatial distribution of phosphorylation of each Smad changes dramatically as gastrulation begins. We discuss the implications of our results for endogenous functions of BMP and activin-like signals as candidate morphogens regulating primary germ layer formation and dorsoventral patterning of the early *Xenopus* embryo.

Key words: Smad, TGFβ, Phosphorylation, Mesoderm induction, Signal transduction, *Xenopus laevis*

INTRODUCTION

Mesoderm formation in the early *Xenopus laevis* embryo involves integration of patterning signals across both animal-vegetal and dorsoventral axes. Mesoderm is initially specified at the equatorial marginal zone of the pregastrula embryo (reviewed in Harland and Gerhart, 1997; Heasman, 1997). Over the past 10 years, many studies have focused on the identification of signals that can induce and/or pattern early mesoderm. While the formation and patterning of mesoderm clearly involves multiple interacting signal pathways (Harland and Gerhart, 1997), Transforming Growth Factor β (TGFβ) superfamily signals have emerged as central components of the inductive process (reviewed in Heasman, 1997). Addition of activin, a TGFβ superfamily ligand, to prospective ectodermal cells can induce a broad range of mesodermal markers and tissues (Green et al., 1992). Conversely, inhibition of TGFβ superfamily signaling in intact embryos by expression of a dominant inhibitory type II activin receptor, which broadly inhibits TGFβ superfamily signaling (Hemmati-Brivanlou and Melton, 1992; Kessler and Melton, 1995; Schulte-Merker et al., 1994), inhibits mesoderm induction and dorsal axis formation (Hemmati-Brivanlou and Melton, 1992).

Two functionally distinct TGFβ signaling pathways are critical in the induction and patterning of mesoderm: (1) activin-like signals, which induce primarily dorsal mesoderm and (2) bone morphogenetic protein (BMP) signals, which ventralize mesoderm. Activin and B-Vg1, a processed and active form of Vg1 (Dale et al., 1993; Thomsen and Melton, 1993), can both induce dorsal mesoderm and endoderm in animal cap explants, and can also induce a secondary dorsal axis when ectopically expressed on the ventral side of the embryo (Dale et al., 1993; Kessler and Melton, 1995; Thomsen and Melton, 1993; Thomsen et al., 1990). Activin-like ligands, Xnr1, Xnr2, Xnr4 and derrière, can dorsalize marginal zone explants (Jones et al., 1995; Joseph and Melton, 1997; Sun et

al., 1999). Expression of dominant inhibitors of these activin-like signals results in significant disruption of mesodermal or endodermal specification (Dyson and Gurdon, 1997; Joseph and Melton, 1998; Osada and Wright, 1999; Sun et al., 1999), indicating that each of these ligands is important in early patterning. BMP ligands can induce prospective ectoderm to form ventral mesoderm, ventralize dorsal mesoderm and suppress neuralization of prospective epidermis (Clement et al., 1995; Dale et al., 1992; Jones et al., 1992; Suzuki et al., 1997b; Wilson and Hemmati-Brivanlou, 1995). Elimination of BMP signaling in the ventral side of the embryo by overexpression of dominant negative BMP receptors or ligands induces a secondary axis and neuralizes animal caps (Frisch and Wright, 1998; Graff et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Suzuki et al., 1994), suggesting that BMP signaling is required to specify ventral pattern in both the ectoderm and mesoderm. These results emphasize the importance of antagonistic TGFβ superfamily pathways in mesoderm formation and in patterning of the *Xenopus* embryo.

Smad proteins are intracellular transducers of TGFβ signals (reviewed in Massagué, 1998), and distinct Smads transduce different subsets of TGFβ superfamily ligands. Overexpression experiments have demonstrated that Smad1, Smad5 and Smad8 specifically transduce BMP signals (Chen et al., 1997a; Graff et al., 1996; Suzuki et al., 1997a; Thomsen, 1996; Yamamoto et al., 1997), whereas Smad2 and Smad3 mediate activin-like and TGFβ signals (Baker and Harland, 1996; Eppert et al., 1996; Graff et al., 1996; Macías-Silva et al., 1996). In response to ligand stimulation, Smad proteins are phosphorylated on the last two serine residues within carboxyl SS(V/M)S motifs; these modifications are essential for transduction of TGFβ signals (Abdollah et al., 1997; Hoodless et al., 1996; Kretzschmar et al., 1997; Liu et al., 1997; Macías-Silva et al., 1996, 1998; Souchelnytski et al., 1997). Receptor-phosphorylated Smads associate with Smad4, which functions as a shared partner for activin and BMP signaling; then Smad complexes translocate from the cytoplasm to the nucleus to activate transcription (reviewed in Massagué, 1998). Smad2 acts in combination with the site-specific DNA binding protein FAST-1 to regulate dorsal and pan-mesodermal gene expression (Chen et al., 1996, 1997b; Watanabe and Whitman, 1999; Yeo et al., 1999), but how Smads 1, 5 and 8 regulate specific transcriptional changes in the induction of ventral mesoderm is not known. Inhibition of Smad2 signaling in early embryos by expression of dominant negative mutants disrupts dorsoanterior pattern (Hoodless et al., 1999), further supporting the hypothesis that Smad signaling is important in the early patterning of mesoderm.

While overexpression studies have indicated that TGFβ signaling pathways are important in *Xenopus laevis* embryonic patterning, understanding how they are important for patterning requires direct knowledge of when and where these pathways are actually active endogenously (reviewed in Whitman, 1998). Since zygotic transcriptional responses do not begin until after the mid-blastula transition (MBT) (Newport and Kirschner, 1982), the study of mesodermal transcription has shed little light on the extent to which patterning signals act before MBT. Ligand-stimulated C-terminal phosphorylation is required for the signaling activity of the receptor-regulated Smads, and therefore provides a direct means with which to assess the activity of endogenous TGFβ superfamily signaling pathways. We have developed antibodies to detect the activation-specific phosphorylation of the receptor-regulated Smads.

In this study, we have examined TGFβ superfamily signaling in early embryos using antibody preparations that specifically recognize C-terminally phosphorylated, activated Smads, and that distinguish phosphoSmad2 from phosphoSmad1/5/8. Smad activation is not detectable before the MBT, and cellular responsiveness to ligands, as well as expression of the ligands themselves, is an important component of endogenous regulation of TGFβ signaling. While Smad2 activation at MBT is dependent on zygotic transcription, Smad1/5/8 phosphorylation is not, indicating that a transcription-independent timer regulates the onset of BMP pathway activity at MBT. We also examine the spatial distribution of Smad phosphorylation in early embryos, and find a temporally complex pattern of asymmetric Smad activation across the dorsoventral and animal-vegetal axes between MBT and gastrulation. The examination of endogenous TGFβ signaling complements earlier work on ectopic manipulation of TGFβ signaling to elucidate how this superfamily of factors may function as morphogens in the early embryo.

MATERIALS AND METHODS

Antibodies

Anti-phosphoSmad antisera were raised by immunization of rabbits with the peptide KKK-NPISSpVSp (Smad1) or KKK-SSpMSp (Smad2) containing two C-terminal phosphoserine residues coupled to keyhole limpet hemocyanin (KLH), mixed with Freund's adjuvant (Persson et al., 1998). Crude sera were precipitated with 50% ammonium sulfate, redissolved and dialyzed into phosphate-buffered saline (PBS). They were then loaded onto phosphoSmad peptide columns and eluted with 100 mM glycine, pH 2.5, and 100 mM triethylamine, pH 11, as described (Harlow and Lane, 1988). Both high and low pH eluates contained specific anti-phosphoSmad activity; pH 11 eluates were used for western blots described here.

Anti-Smad1/5 (N18) antibody from Santa Cruz Biotechnology was used at a dilution of 1/1000 in Tris-buffered saline plus Tween 20 (TBST: 10 mM Tris, pH 8, 150 mM NaCl, 0.2% Tween 20). Anti-Smad2 antibody from Transduction Laboratories was used at a dilution of 1/250 in 5% milk in TBST. Anti-Flag antibody (SIGMA) was used for western analysis at a dilution of 1/10,000 in TBST. For immunoprecipitation, 1 μl anti-Flag antibody preparation (3 μg/μl) was added to cleared lysates (see below). Anti-actin antibody (SIGMA) was used at a dilution of 1/500 in TBST. Secondary antibodies were anti-goat IgG-HRP antibodies (Santa Cruz) (1/10,000 dilution), goat anti-rabbit-HRP antibodies (Boehringer Mannheim) (1/5,000 dilution), and donkey anti-mouse-HRP $F(ab')_2$ fragments (Jackson ImmunoResearch Laboratories, Inc.) (1/10,000 dilution). Each secondary HRP-conjugated antibody was diluted in 2% milk in TBST.

Western blot analysis

For anti-phosphoSmad2 and anti-Smad2 analysis, embryos were homogenized (20 μl per embryo) in modified RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8, 1% NP40, 0.5% deoxycholate, 2 mM EDTA, 20 μg/ml aprotinin, 40 μg/ml leupeptin, 4 μg/ml pepstatin, 0.75 mM PMSF, 25 mM β-glycerophosphate, 1 mM Na_3Vo_4, 100 mM NaF). Lysates were centrifuged at 14,000 ***g*** for 10 minutes in a tabletop centrifuge at 4°C, suspended in twice their volume of 2× Laemmli buffer but not boiled. They were separated by 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to

nitrocellulose (Protran). For anti-phosphoSmad2 and anti-actin immunoblotting, membranes were blocked overnight at room temperature (RT) with 5% polyvinyl-pyrrolidone (PVP) (Calbiochem) in TBST. Blots were rinsed in TBST, and primary antibody was added for 3 hours at RT. For anti-Smad2 immunoblotting, membranes were blocked for 1 hour at RT with 5% milk in TBST, incubated overnight with primary antibody, and then washed. Secondary antibody was added for 1 hour at RT, followed by washing and development with chemiluminescent reagents (ECL, Amersham).

For western blot analysis of anti-phosphSmad1 and Smad1, embryos were homogenized (10 μl per embryo) in lysis buffer (20 mM Tris, pH 8, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 2 mM EDTA, 1% NP40, 20 μg/ml aprotinin, 40 μg/ml leupeptin, 4 μg/ml pepstatin, 0.75 mM PMSF, 1 mM Na_3Vo_4). Lysates were prepared for electrophoresis/western blotting as above. Membranes were blocked at room temperature for 1 hour either with 1% PVP for anti-phosphoSmad1, or with 2% milk in TBST for anti-Smad1. For anti-actin western blotting, membranes were blocked for 1 hour with 1% PVP. After several washes in TBST, membranes were incubated overnight with the antibodies. Detection was then done with HRP-labeled secondary antibodies and ECL as above.

Lysis solutions containing ionic detergents (e.g. modified RIPA buffer) extracted highly abundant yolk proteins, limiting the amount of sample that could be loaded and thereby preventing detection of a strong anti-phosphoSmad1 signal. We have confirmed that lysate buffers with or without ionic detergents quantitatively extract both total Smads and phospho-Smads (not shown). Some experiments were done with modified RIPA buffer to confirm that anti-phosphoSmad1 and anti-Smad1 antibodies give the same patterns in western blot analysis.

Immunoprecipitations

Embryos were lysed in modified RIPA buffer and centrifuged (as above). Immunoprecipitations were performed in a total volume of 200 μl with at least 10 embryo equivalents per lane. Immunoprecipitations were carried out for 3-4 hours with rocking at 4°C, washed once with PBS/0.5 M NaCl, once with PBS/1% NP40 and once with PBS. For lambda protein phosphatase (λ-PPtase) treatment, beads were resuspended in λ-PPtase reaction buffer with 2 mM $MnCl_2$ for 4 hours at 30°C with or without λ-PPase and then washed. All immunoprecipitates were boiled in 2× Laemmli buffer, separated by 7.5% SDS-PAGE, and transferred to nitrocellulose. Membranes were blocked for 1 hour with 5% milk or 1% PVP and incubated overnight with primary anti-Flag antibody. Thereafter immunoblotting was carried out as above. Signals were not obscured by crossreacting IgG bands.

Embryo manipulation

Embryos were collected from *Xenopus* females and artificially fertilized as previously described (Watanabe and Whitman, 1999). Staging of embryos was according to Nieuwkoop and Faber (1967). Embryos were dejellied before first cleavage in 3% cysteine and injected at the two-cell stage with 5-10 nl RNAs and/or 50 pg α-amanitin (Boehringer Mannheim) (Newport and Kirschner, 1982). α-amanitin-injected embryos did not undergo gastrulation movements and died at stage 10.5/11 (Sible et al., 1997; Stack and Newport, 1997).

Dissections were done in 0.7× MMR. Embryos or explants were washed in 0.1× MMR or dH_2O before freezing at –80°C for storage. Cytoskeletal actin serves as a control for equivalent cellular volume (Tannahill and Melton, 1989) for all experiments. Since cell size varies greatly across the animal-vegetal axis, we standardized animal, marginal and vegetal tissues for cell volume by comparing titrations of explant lysates by western blot analysis for cytoskeletal actin (data not shown). Results presented here were produced after this standardization. For dorsoventral dissections, dorsal regions of embryos were marked with Nile Blue (Peng, 1991) from 4- to 16-cell stages to allow accurate identification of the dorsal side for dissection before gastrulation. From stage 10, the dorsal blastopore lip served as an indicator of the dorsal side. Dissection has previously been demonstrated to activate the FGF signaling pathway or its downstream effector MAP kinase in early *Xenopus* embryos (Christen and Slack, 1999; LaBonne and Whitman, 1997). To control for dissection artifacts, lysates from whole embryos were compared to an equivalent volume of dorsal plus ventral halves for dorsoventral bisections; whole embryos serve as a similar control for animal/ marginal/vegetal dissections.

In vitro transcription

Capped mRNAs were synthesized in vitro using the SP6 mMessage mMACHINE kit (Ambion).

Reverse transcription-polymerase chain reaction

For RT-PCR, total RNA was extracted from whole embryos or dissected explants by the proteinase K/phenol method and PCR reactions were performed as previously reported (LaBonne and Whitman, 1994; Watanabe and Whitman, 1999). 25 cycles were used for PCR amplification of *Xbra* and *gsc* (Hemmati-Brivanlou et al., 1994a) genes and 22 cycles for amplification of *ODC* and *XSox17β* (Hudson et al., 1997).

Cloning of Smad2Δexon3

cDNA from stage-8 embryos was prepared as above. PCR was performed with primers to the *Xenopus* Smad2 sequence that span exon3: primer 2-2A up 5′-GCA AAA CAT GTC GTC CAT CT-3′ and primer 2-4 down 5′-TAT TCA CAA TTT TCG ATT GC-3′ (27 cycles, annealing temperature 50°C). Primer binding sites are indicated in Fig. 3A. RT-PCR products were digested with *Afl*III (a site upstream of the initiating ATG that was contained in primer 2-2Aup) and blunted with Klenow fragment, and with *Bsg*I (a site 3′ to exon3 but within the MH1 domain of *Xenopus* Smad2). Digested products were separately gel-purified and each was cloned into pCS2-6myc-tagged *Xenopus* Smad2 deleted of its amino terminus with *Cla*I (blunted with Klenow) and *Bsg*I. Restriction digestion indicated that constructs containing these RT-PCR products were distinct sizes. Smaller clones that underwent automated sequencing of their N termini demonstrated loss of exon3, nucleotides 237-326. Sequencing also demonstrated one nucleotide change from the previously published *Xenopus* Smad2 sequence (base pair 92, A to G) that changes one amino acid (residue 31, D to G) (Graff et al., 1996).

Immunohistochemistry

For anti-phosphoSmad1 and anti-phosphoSmad2 analysis, embryos were fixed in MEMFA (0.1 M MOPS, pH 7.4, 2 mM EGTA, pH 8, 1 mM $MgSO_4$, 3.7% formaldehyde) for 2 hours, stored in Dent's fixative (20% DMSO in methanol) overnight at –20°C and then rehydrataed in PBS. Fixed embryos embedded in 2% low-melt agarose (Nusieve GTG agarose, FMC Bioproducts) were bisected (with respect to the dorsoventral axis where indicated) with a disposable microscalpel (Feather Safety Razor Company) under PBS. Pigment was bleached by a 1 hour treatment in fresh H_2O_2 (2% or 10%) (Sigma), 5% formamide, 0.5× SSC under bright light at room temperature with gentle agitation. Despite bleaching, the animal pole region showed significant, non-specific, non-nuclear staining. After washing in PBT (PBS, 2 mg/ml BSA, 0.1% Triton X-100), embryos were blocked for 2 hours with PBT plus 10% normal goat serum (Jackson ImmunoResearch Laboratories) dialyzed against PBS to remove sodium azide. Primary antibody incubation (anti-phosphoSmad1 at dilution 1:20, anti-phosphoSmad2 at dilution 1:25) carried out overnight at 4°C was followed by washes with PBS for 2 hours. To confirm that the signal reflected specific detection of phosphoSmad epitopes, phosphoSmad peptides were added to primary antibody incubations at a final concentration of 1 or 5 μg/ml. Secondary antibody incubation with HRP-conjugated goat anti-rabbit

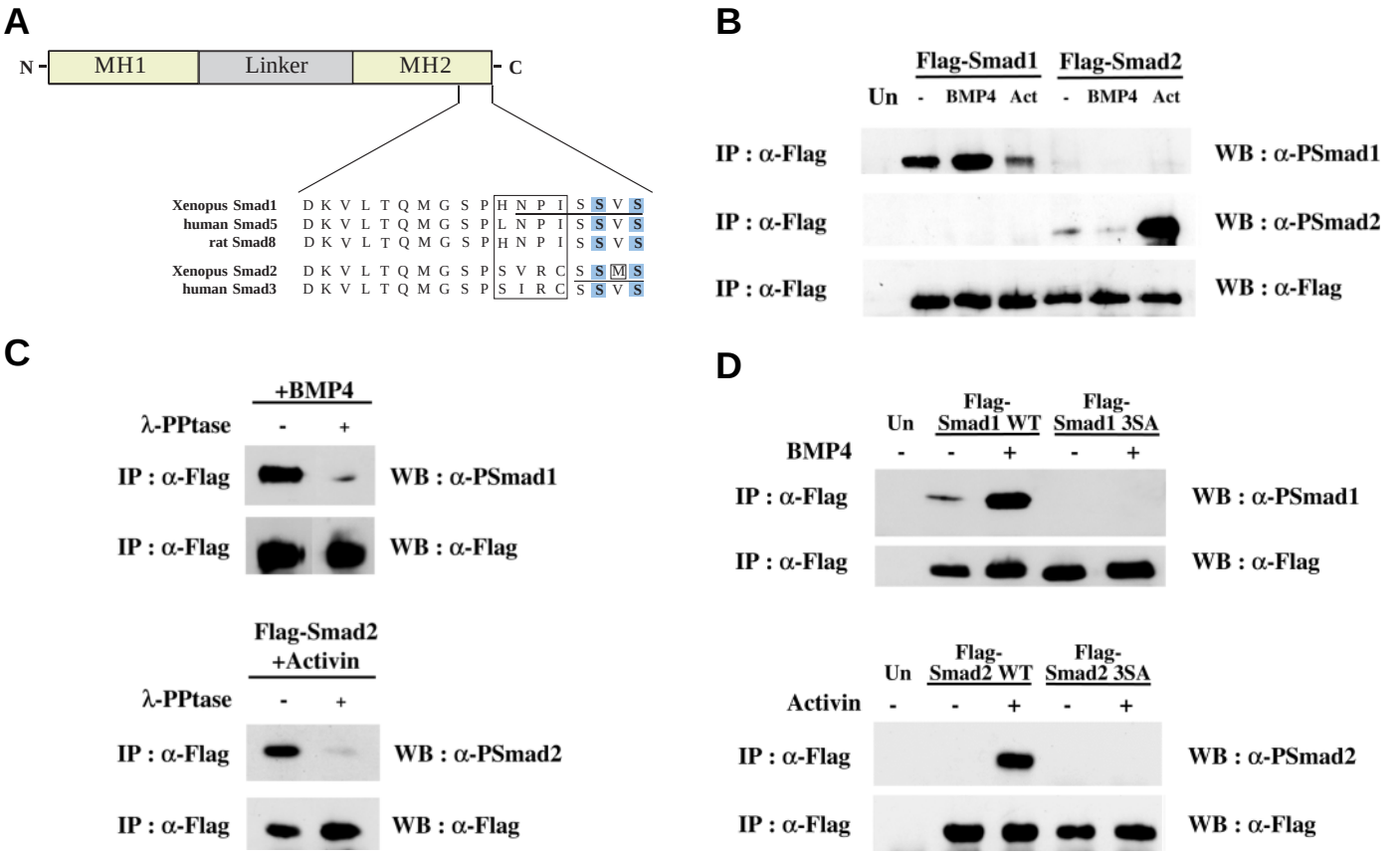


Fig. 1. Anti-phosphoSmad antibodies specifically detect activation of overexpressed Smad1 and Smad2 in immunoprecipitation/western blot analyses. (A) Diagram of antigen sites. Schematic illustration of the highly conserved amino (MH1) and carboxyl (MH2) terminal domains of Smad proteins. The underlined amino acid sequences correspond to the sequences of the synthesized phosphopeptides against which anti-phosphoSmad antibodies were raised. The location of serine residues phosphorylated by TGFβ type I receptors are highlighted. Residues that are not conserved are boxed. (B) Anti-phosphoSmad antibodies distinguish activated Smad1 from activated Smad2. Two-cell-stage *Xenopus* embryos were injected at the animal pole with mRNAs encoding either Flag-Smad1 or Flag-Smad2 (100 pg/embryo), alone (–) or together with BMP4 or activin (Act) mRNAs (100 pg/embryo). Control embryos were not injected (Un). At stage 10, embryos were lysed and immunoprecipitated with anti-Flag antibodies for immunoblotting with either anti-phosphoSmad1 or anti-phosphoSmad2 antibodies. Anti-Flag western blot analysis confirmed that equivalent amounts of Flag-Smad proteins were immunoprecipitated. (IP, immunoprecipation; WB, western blotting.) (C) Lambda protein phosphatase (λ-PPtase) treatment eliminates recognition of activated Smad proteins by anti-phosphoSmad antibodies. Two-cell-stage *Xenopus* embryos were injected with mRNAs encoding either Flag-Smad1 with BMP4 or Flag-Smad2 with activin. At stage 10, embryos were lysed and immunoprecipitated with anti-Flag antibodies. Mock-treated and λ-PPtase-treated half-precipitates were analyzed by anti-phosphoSmad western blotting. (D) Anti-phosphoSmad antibodies detect activating phosphorylations of Smad proteins. Two-cell-stage embryos were injected at the animal pole with mRNAs encoding wild-type (WT) or mutant (3SA) forms of Flag-Smad1 or Flag-Smad2, either alone or in combination with BMP4 or activin mRNAs. Embryos were harvested at stage 10, and levels of phosphorylation of overexpressed Flag-Smad proteins were determined by anti-phosphoSmad western blot analysis.

antibody (dilution 1:250) was carried out for 1 hour at room temperature and followed by washes as above. Staining was developed with ImmunoPure Metal-enhanced DAB Substrate Kit (Pierce), as per the manufacturer's instructions for 5-10 minutes. Images were captured immediately with NIH Image software on a Kodak DCS420 camera attached to a Zeiss Axiophot microscope.

RESULTS

Characterization of activation-specific anti-phosphoSmad1 and anti-phosphoSmad2 antibodies

We obtained and purified antibodies against peptides that correspond to the phosphorylated carboxyl-terminal sequences of Smad1/5/8 and Smad2 to examine temporal and spatial patterns of endogenous TGFβ signaling during *Xenopus* embryogenesis (Fig. 1A; see Materials and Methods). Smad1/5/8 phosphorylation is induced by BMP signaling but not by the activin signaling pathway whereas the converse is true for Smad2 phosphorylation (Baker and Harland, 1996; Hoodless et al., 1996; Kretzschmar et al., 1997; Macías-Silva et al., 1996, 1998). To distinguish between these highly conserved but functionally very different TGFβ signaling pathways, our antibodies must be able to differentiate between activated Smad1/5/8 and activated Smad2.

Anti-phosphoSmad antibodies specifically detect activation of overexpressed Smad1 and Smad2 in immunoprecipitation/western blot analysis

To characterize the specificity of anti-phosphoSmad purified

antibodies, Flag-tagged human Smad proteins were expressed in *Xenopus* embryos, either alone or together with BMP or activin, for immunoprecipitation/western blot analysis. Anti-Flag immunoprecipitates were split for analysis with anti-phosphoSmad1, anti-phosphoSmad2 and anti-Flag antibodies. When Flag-Smad1 is overexpressed, anti-phosphoSmad1 antibodies reveal a basal signal that is strongly increased in response to BMP4; neither the endogenous signal nor the BMP4-induced phosphoSmad1 signal is detected by anti-phosphoSmad2 antibodies (Fig. 1B). When Flag-Smad2 is overexpressed, activin stimulation leads to a strong signal that is detected by anti-phosphoSmad2 antibodies, but not by anti-phosphoSmad1 antibodies (Fig. 1B). Each of the anti-phosphoSmad antibodies definitively distinguishes between overexpressed Smad1 and Smad2 and appears to recognize receptor-activated Smad proteins.

Two approaches were used to confirm that signals observed with anti-phosphoSmad antibodies reflected C-terminal Smad phosphorylation: (1) elimination of signal by enzymatic dephosphorylation and (2) mutation of phosphorylation sites in Smads. Lysates from embryos overexpressing Flag-Smad1 with BMP or Flag-Smad2 with activin were precipitated with anti-Flag antibody, and then treated or mock-treated with lambda protein phosphatase. Lambda protein phosphatase treatment eliminates signal recognition by each of the anti-phosphoSmad antibodies (Fig. 1C), but has no effect on total tagged protein levels, indicating that antibody recognition is dependent on phosphorylation of the Smad proteins. To confirm that anti-phosphoSmad antibodies specifically recognized phosphorylation at the C-terminal SSXS motif regulated by upstream receptors, *Xenopus* embryos were injected with mRNA encoding mutants of Smad1 (Flag-Smad1 3SA) or Smad2 (Flag-Smad2 3SA) in which the three C-terminal serine residues were substituted by alanine residues (Kretzschmar et al., 1997; Macías-Silva et al., 1996). Immunoprecipitation/western blot analysis demonstrates that alanine mutation of the three serine residues in the SS(V/M)S motif of Smad1 or Smad2 abolishes BMP4- or activin-induced phosphoSmad signals (Fig. 1D), indicating that each of the anti-phosphoSmad antibodies specifically detects Smads phosphorylated at their receptor-regulated C-terminal sites.

Anti-phosphoSmad1 and anti-phosphoSmad2 antibodies detect endogenous activated Smads in direct western blot analysis

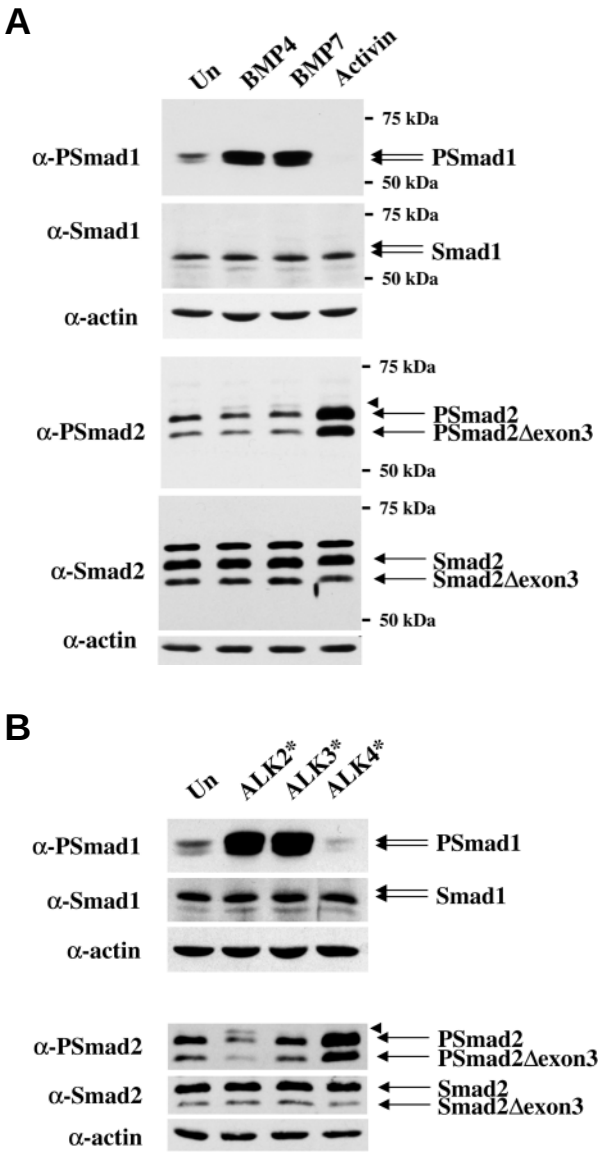
We next examined the ability of our anti-phosphoSmad antibodies to specifically detect endogenous Smad proteins activated by expression of TGFβ superfamily ligands or activated receptors. *Xenopus* embryos were injected with mRNAs encoding either activin or BMP ligands or constitutively active TGFβ type I receptors (Activin receptor-Like Kinase *) ALK2*, ALK3* or ALK4*. ALK2*, ALK3* and ALK6* have been shown to activate the BMP signaling pathway (Armes and Smith, 1997; Macías-Silva et al., 1998), leading to Smad1 phosphorylation (Chen et al., 1997a; Hoodless et al., 1996; Kretzschmar et al., 1997). In contrast, constitutively active ALK4*, which mimics activin induction of a secondary axis when overexpressed ventrally in *Xenopus* embryos (Armes and Smith, 1997), induces Smad2 phosphorylation (Hoodless et al., 1999). Immunoprecipitation of lysates with Smad1/5/8-specific or Smad2/3-specific antibodies confirmed the identity of endogenous Smads (data not shown). Anti-phosphoSmad1 analysis demonstrated that phosphorylation of endogenous Smad1 was increased in embryos overexpressing BMP ligands (Fig. 2A) or activated BMP type I receptors (ALK2* and ALK3*) (Fig. 2B), while total Smad1 protein levels were unaffected. Expression of activin (Fig. 2A) or ALK4* (Fig. 2B) induced phosphorylation of endogenous Smad2 but did not affect Smad2 protein levels. Activin/Alk4* overexpression inhibited endogenous Smad1 phosphorylation (Fig. 2A,B), probably reflecting the induction of BMP antagonists by activin-like signaling (Sasai et al., 1994; Smith and Harland, 1992). These observations demonstrate that anti-phosphoSmad antibodies differentiate between Smad1 and Smad2 activated by different TGFβ superfamily ligands and type I receptors.

Finally, we validated our anti-phosphoSmad antibodies by demonstrating that the inhibition of endogenous BMP or activin-like signaling pathways eliminates signal detection by anti-phosphoSmad antibodies. Overexpression of a dominant inhibitory BMP type I receptor (tBR) (Graff et al., 1994; Suzuki et al., 1994) eliminated endogenous Smad1 phosphorylation while Smad2 phosphorylation was unaffected (Fig. 2C). In contrast, expression of an activin dominant inhibitory type II receptor (Δ1XAR1), which inhibits both BMP and activin-like signaling in embryos (Hemmati-Brivanlou and Melton, 1992; Kessler and Melton, 1995; Schulte-Merker et al., 1994), reduced endogenous phosphorylation of both Smad1 and Smad2. These results demonstrate that our anti-phosphoSmad antibody preparations are sufficiently sensitive and specific to assay endogenous levels of TGFβ superfamily signals in the early *Xenopus* embryo.

Identification of proteins detected by anti-phosphoSmad and anti-Smad antibodies

Western blot analysis with anti-phosphoSmad1 antibodies identifies a doublet in embryonic lysates. This doublet may reflect post-transcriptional modification of Smad1 transcript or protein, or reactivity with a closely related Smad. Both bands react with anti-Smad1 as well as anti-phosphoSmad1 antibodies; reactivity of these bands with anti-Smad1 antibody is blocked by a specific blocking peptide, and the upper band comigrates exactly with overexpressed, full-length *Xenopus* Smad1 (not shown). Smad1, Smad5 and Smad8 share an identical C-terminal sequence, very high overall sequence homology and very similar molecular masses (Chen et al., 1997a; Graff et al., 1996; Nishimura et al., 1998). These proteins are not distinguishable immunologically, but as they are regulated by the same type I receptors, C-terminal phosphorylation of any of them should reflect activation of a BMP signaling pathway. Our anti-phosphoSmad1 antibodies react with ectopically expressed murine Smad5 in a BMP-regulated manner (data not shown). Smad5 and Smad8 have not been identified in *Xenopus*, and for simplicity we will refer to the endogenous BMP-regulated Smad signal seen in embryos as ‘Smad1’. Endogenous Smad5 or Smad8 may also contribute to this signal, but whether they do or do not, this anti-phosphoSmad1 signal appears to reflect activation of a BMP signaling pathway.

The endogenous signal detected by both the anti-



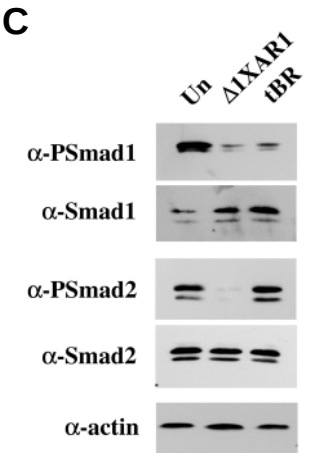


Fig. 2. Anti-phosphoSmad1 and anti-phosphoSmad2 antibodies detect endogenous activated Smads in direct western blot analyses. (A) Differential activation of endogenous Smad1 and Smad2 by TGFβ agonists. Lysates of uninjected embryos (Un) or embryos injected at the animal pole at the two-cell stage with mRNAs encoding BMP4, BMP7 or activin ligands (100 pg/embryo), were analyzed by western blotting with anti-phosphoSmad and anti-Smad antibodies. Cytoskeletal actin served as a loading control. *Xenopus* Smad proteins ran between 55 and 60 kDa. Arrows indicate 'Smad1' (Smad1/5/8) and Smad2/Smad2Δexon3. Additional bands detected by anti-Smad1 not indicated by an arrow are not inhibited by coincubation of the primary antibody with a specific blocking peptide, and therefore appear to be non-specific (not shown). Refer to text and Fig. 3 for additional explanation concerning the identification of Smad proteins. (B) Differential activation of endogenous Smad1 and Smad2 by activated TGFβ type I receptors. Two-cell-stage embryos were injected in the marginal zone with mRNAs encoding the dominant activated forms of TGFβ type I receptors (Activin receptor-Like Kinases *) ALK2* (200 pg/embryo), ALK3* (200 pg/embryo) and ALK4* (100 pg/embryo) for comparison to uninjected embryos (Un), and analysed as in A. (C) Expression of dominant negative TGFβ receptors specifically eliminates phosphoSmad signals. mRNA encoding either a dominant negative BMP type I receptor (tBR; 1 ng/embryo) or a dominant inhibitory activin type II receptor (Δ1XAR1; 1 ng/embryo), which has been shown to inhibit all TGFβ superfamily signaling pathways, was injected into animal and vegetal poles of two-cell-stage embryos for comparison with uninjected controls (Un). Embryos were lysed at stage 10 and analyzed by western blot analysis.

phosphoSmad2 and anti-Smad2 antibodies runs consistently as a well-resolved doublet. Anti-phosphoSmad2 analysis demonstrates that both bands show increased intensity with activin overexpression (Fig. 2A), suggesting that each is phosphorylated in response to ligand stimulation. We hypothesized that these two proteins could be Smad2, Smad3 or Smad2Δexon3 (Fig. 3A). Smad2 and Smad3 are separate genes that have 92% sequence identity; both are regulated by phosphorylation at their C termini by TGFβ/activin type I receptors, which phosphorylate Smad2 at the C-terminal SSMS motif and Smad3 at SSVS (Abdollah et al., 1997; Liu et al., 1997; Macías-Silva et al., 1996; Souchelnytski et al., 1997). Smad2 and Smad2Δexon3 are derived from alternate splicing of the same transcript; Smad2Δexon3 transcripts are present in human cell lines at low levels (Takenoshita et al., 1998; Yagi et al., 1999), but have not been reported in *Xenopus* embryos. Smad2 and Smad2Δexon3 differ functionally in that Smad2Δexon3 can bind DNA and Smad2 cannot. Exon 3 encodes an insert in the MH1 domain of Smad2 that prevents this region from binding DNA; deletion of this exon in Smad2Δexon3 results in a Smad2 isoform that is active in DNA binding (Dennler et al., 1998). The presence or absence of this DNA binding activity in Smad2 has been reported to substantially alter transcriptional regulation in Smad containing complexes (Labbé et al., 1998; Yagi et al., 1999).

Smad2 has been cloned from *Xenopus* embryos (Graff et al., 1996), but Smad3 and Smad2Δexon3 have not. To begin to identify which activin signal-mediating Smad proteins are present during early *Xenopus* development, we compared the migration of overexpressed *Xenopus* Smad2 or human Smad3 to that of the endogenous Smad bands. Anti-Smad2 antibody recognizes overexpressed Smad2 and Smad3; *Xenopus* Smad2 migrates at the same size as the larger endogenous band while human Smad3 is smaller than either endogenous band (Fig. 3B). Anti-phosphoSmad2 immunoblotting demonstrates that the endogenous bands and overexpressed *Xenopus* Smad2 are phosphorylated (Fig. 3B). We do not detect phosphorylation of overexpressed Smad3, presumably because the phosphorylated epitope differs from Smad2 by the same amino acid that distinguishes Smad2 from Smad1

(Fig. 1). These results suggest that the larger endogenous protein is Smad2. The smaller endogenous protein detected by anti-phosphoSmad2 and anti-Smad2 antibodies is smaller than overexpressed full-length Smad2, but larger than Smad3, suggesting that it may be the Smad2 splice variant Smad2Δexon3.

To determine whether Smad2Δexon3 is present during early *Xenopus* development, we performed RT-PCR on cDNA from stage-8 embryos, targeting the amino-terminal region of Smad2 that spans exon3. Two bands containing Smad2-derived sequences were amplified, corresponding in size to the amplification products predicted for the Smad2 sequence with exon3 (459 bp) and without exon3 (369 bp) (Fig. 3A,C). Cloning and sequence analysis indicated that the larger band encodes the N-terminal region of Smad2, while the smaller band encodes an N-terminal region of Smad2 lacking exon3. This *Xenopus* Smad2Δexon3 construct has the same splice junction as human Smad2Δexon3 (Takenoshita et al., 1998), indicating that these exon-intron boundaries within Smad2 are conserved between mammals and amphibians.

To compare the apparent size of our newly cloned *Xenopus* Smad2Δexon3 to the endogenous Smad2-like bands, we replaced the N-terminal region of full-length Smad2 with the PCR-derived N-terminal regions of Smad2 and Smad2Δexon3 and expressed these constructs in embryos. Western blot analysis demonstrates that the larger endogenous band comigrates with full-length Smad2, while the smaller endogenous band comigrates with ectopic Smad2Δexon3 (Fig. 3B). From unfertilized eggs through at least to stage 12, transcripts of Smad2 and Smad2Δexon3 are present and are translated, while Smad3 protein cannot be detected. These observations indicate that a DNA binding form of Smad2, like the previously described non-DNA binding form of Smad2, is a major component of the activin-like signaling pathway in the early embryo.

Temporal regulation of activation of TGFβ signaling pathways in early *Xenopus laevis* development

We next investigated the timing of endogenous Smad activation during early embryogenesis. In *Xenopus* embryos, zygotic transcription begins at the mid-blastula transition (MBT; stage 8) (Newport and Kirschner, 1982); before this stage intercellular signaling pathways must be derived strictly from maternal components. RNAs encoding BMP ligands and the activin-like ligand Vg1 are expressed maternally, suggesting that both of these TGFβ superfamily signaling pathways might be active before MBT (Clement et al., 1995; Dale et al., 1992; Nishimatsu et al., 1992; Weeks and Melton, 1987). To test this, *Xenopus* unfertilized eggs or embryos from stages 2 to 20 were lysed and analyzed by western blotting using anti-Smad1 and anti-phosphoSmad1 antibodies. As demonstrated in Fig. 4A, Smad1 phosphorylation was not detected in unfertilized eggs or embryos until stage 8. Smad1 protein was found to be expressed maternally at low levels, and we observed an increase in the level of total Smad1 protein at MBT. Smad1 phosphorylation was not seen before MBT even when Smad1 was expressed ectopically to high levels (data not shown), indicating that the absence of endogenous phosphoSmad1 before MBT is not attributable to low levels of total Smad1. The level of Smad1 phosphorylation gradually increased from stage 9 to stage 11 and remained nearly constant until at least stage 20 (Fig. 4C). Our results suggest that, despite maternal expression of BMP ligands, BMP receptors and Smad1, Smad1 signaling is zygotically regulated.

To investigate activin-like signaling during early developmental patterning, we examined Smad2 phosphorylation from cleavage to gastrula stages. Phosphorylated Smad2/Smad2Δexon3 is detected in unfertilized eggs at a low level and in post-MBT embryos, but not in pre-MBT embryos. Through these early pre-MBT stages Smad2 and Smad2Δexon3 protein levels increased gradually (Fig. 4B). Phosphorylation of Smad2/Smad2Δexon3 was not detected before MBT (stage 8) and declined after stage 10.5 to low levels by stage 12, even though Smad2 and Smad2Δexon3 proteins were present at nearly constant levels through blastula and gastrula stages (Fig. 4D). Our results indicate that signaling through Smad2/Smad2Δexon3 in the early embryo is a zygotic process that is activated over a brief period of time.

Smad2, but not Smad1, requires zygotic transcription for endogenous activation

The appearance of phosphorylation of Smad1 and Smad2 at MBT suggests that they may require zygotic transcription for endogenous activation. To examine this, we tested the effect of the RNA polymerase II inhibitor α-amanitin (Newport and Kirschner, 1982) on endogenous phosphorylation of Smad1 or Smad2. Smad2/Smad2Δexon3 activation is inhibited by α-amanitin while Smad1 activation is not (Fig. 5A). Total levels of Smad proteins are unaffected by α-amanitin treatment (data not shown). To confirm that α-amanitin injection completely inhibits zygotic transcription, inhibition of expression of the immediate early zygotic gene *gsc* was demonstrated (Fig. 5B). Our results indicate that Smad2/Smad2Δexon3, but not Smad1, requires zygotic transcription for endogenous regulation, and suggest that although both Smad1 and Smad2/Smad2Δexon3 are activated after MBT, their mechanisms of endogenous regulation are distinct.

A novel timing mechanism regulates cellular responsiveness to TGFβ superfamily ligands at MBT

We next examined the timing of responsiveness of Smad1/Smad2 phosphorylation to ligand stimulation. Lysates from pre-MBT (stage 6-7) or post-MBT (stage 10) embryos injected with BMP4, activin or B-Vg1 (the processed and activated form of Vg1) (Dale et al., 1993; Thomsen and Melton, 1993) were analyzed. We also examined the effect of inhibiting zygotic transcription on ligand-induced Smad1 or Smad2 phosphorylation.

Endogenous Smad1 phosphorylation is not detected before MBT, and even ectopic expression of BMP ligands does not stimulate Smad1 phosphorylation prior to MBT (Fig. 6A). Either a critical component of the BMP/Smad1 signal transduction pathway is missing, or a mechanism for inhibiting this pathway is active during cleavage stages. α-amanitin injection does not prevent the appearance of Smad1 phosphorylation in response to BMP at MBT (Fig. 6A), indicating that Smad1 activation by exogenous, as well as endogenous BMP stimuli, is regulated by a timing mechanism that is independent of zygotic transcription.

While endogenous Smad2/Smad2Δexon3 phosphorylation

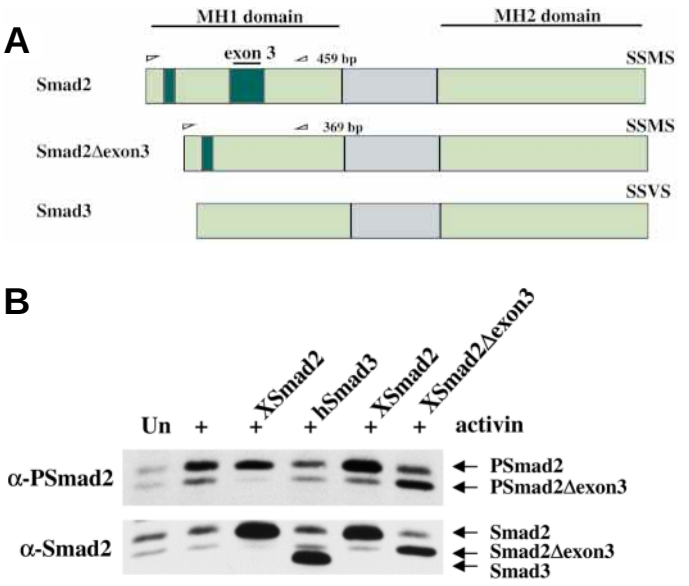


Fig. 3. Identification of proteins detected by anti-phosphoSmad2 and anti-Smad2 antibodies. (A) Diagram of activin/TGFβ-inducible Smads. Schematic comparison of Smad2, Smad2Δexon3 and Smad3 proteins, and receptor-phosphorylation sites (blue). Highly conserved MH1 and MH2 domains are separated by linker regions (grey). PCR primers that span exon3 of *Xenopus* Smad2 genes are indicated (arrwoheads), and expected sizes of the PCR products are noted. (B) Overexpression of Smads allows identification of endogenous bands. Two-cell-stage embryos were injected with activin (100 pg/embryo) alone or with *Xenopus* Smad2, human Smad3 or the full-length Smad2 or Smad2Δexon3 clones (100 pg/embryo) derived from *Xenopus* Smad2 RT-PCR products (see Fig. 3C and text), for comparison with uninjected controls (Un). Embryos were harvested at stage 10 for western blot analysis. (C) Smad2 and the splice variant Smad2Δexon3 are expressed during early *Xenopus* development. RNA from stage-8 embryos was (+RT) or was not (–RT) reverse transcribed for PCR with primers to *Xenopus* Smad2 that span exon 3 (see Fig. 3A and Materials and Methods). PCR products were separated on a 1× TAE/3% agarose gel that was incubated in ethidium bromide for UV visualization.

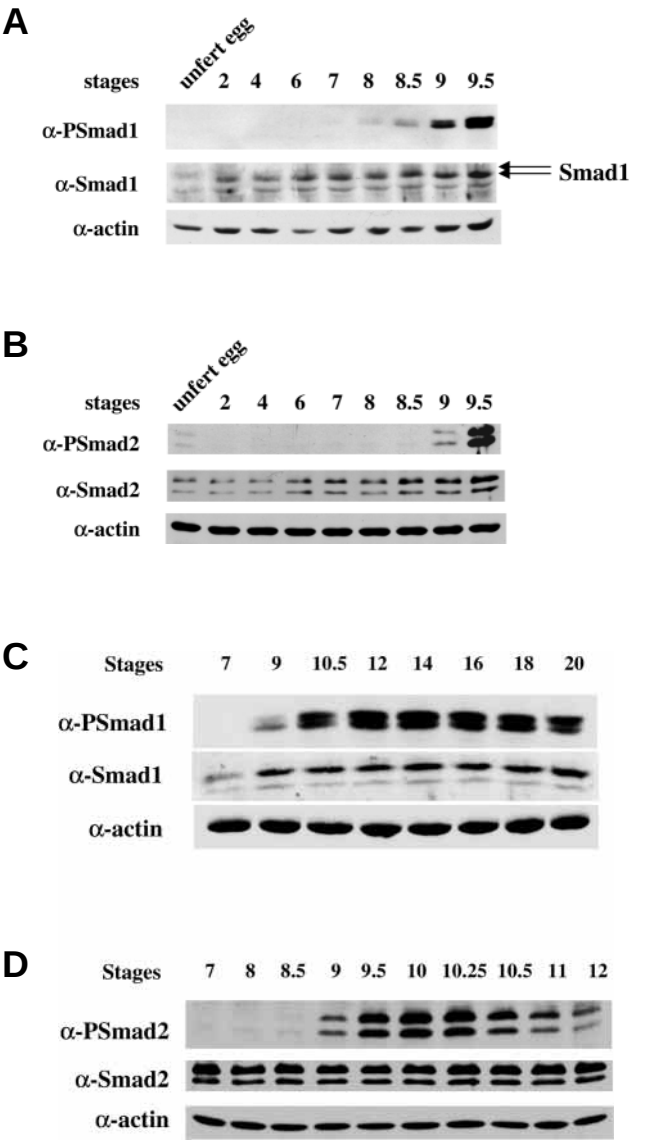


Fig. 4. Temporal activation of TGFβ signaling pathways in early *Xenopus* development. Protein extracts from embryos harvested at the indicated stages were subjected to western blot analysis with either anti-phosphoSmad1 and anti-Smad1 antibodies or anti-phosphoSmad2 and anti-Smad2 antibodies. Anti-phosphoSmad1 and anti-phosphoSmad2 western blots were overexposed to allow maximum detection of signal. Additional bands detected by anti-Smad1 not indicated by an arrow are not inhibited by coincubation of the primary antibody with a specific blocking peptide, and therefore appear to be non-specific (not shown). (A) Temporal activation of BMP signaling pathways from unfertilized eggs to stage 9.5. (B) Temporal activation of activin-like signaling pathways from unfertilized eggs to stage 9.5. (C) Temporal activation of BMP signaling pathways from blastula to neurula stages. (D) Temporal activation of activin-like signaling pathways during blastula and gastrula stages.

was not detected until after MBT, overexpression of activin RNA induced phosphorylation of Smad2 and Smad2Δexon3 before MBT (Fig. 6B). The Smad2 signaling pathway is therefore competent for activation from at least cleavage stage 6.5. Ectopic expression of B-Vg1 (BMP2-Vg1; Thomsen and Melton, 1993), however, induced phosphorylation of Smad2 and Smad2Δexon3 only after the MBT (Fig. 6C), indicating that Smad2 activation by B-Vg1 requires signaling components distinct from those required by activin. As for BMP responsiveness, responsiveness to B-Vg1 is not prevented by inhibition of zygotic transcription (Fig. 6C). Overexpression of A-Vg1 (activin-Vg1; Kessler and Melton, 1995) produced the same results, indicating that the ligands themselves, and not their proregions and cleavage sites, are correlated with pathway activation. These observations indicate that pre-MBT embryos lack components of the signaling pathway necessary for responsiveness to the active form of Vg1, and that regulation of these components is post-transcriptional.

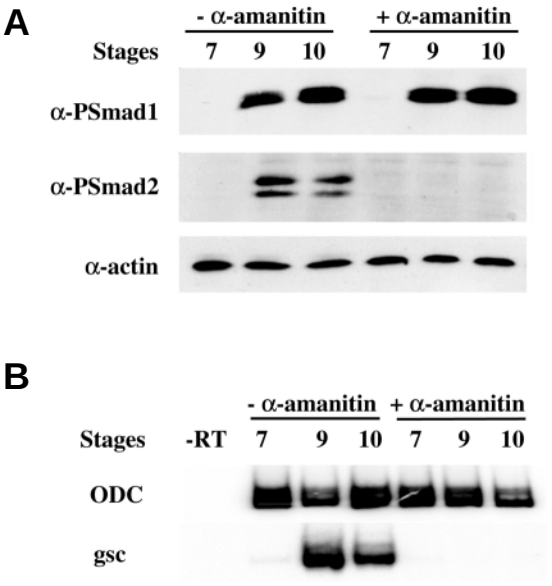


Fig. 5. Activation of endogenous Smad2/Smad2Δexon3, but not of Smad1, requires zygotic transcription. (A) α-amanitin inhibits phosphorylation of Smad2 but not phosphorylation of Smad1. Two-cell-stage embryos were injected into animal and vegetal poles with 50 pg of α-amanitin. When sibling embryos reached the indicated stages, control embryos (– α-amanitin) and α-amanitin-injected embryos (+ α-amanitin) were harvested and lysed for western blot analysis with anti-phosphoSmad1 and anti-phosphoSmad2 antibodies. (B) α-amanitin inhibits zygotic transcription in the *Xenopus* embryo. RT-PCR analysis was performed on mRNA extracted from uninjected embryos (– α-amanitin) or α-amanitin-injected embryos (+ α-amanitin) harvested as before, to detect zygotic expression of the immediate early mesodermal gene *goosecoid (gsc)*. Maternally expressed *ODC* was assessed as a loading control. –RT,whole embryo RNA mock-transcribed without reverse transcriptase.

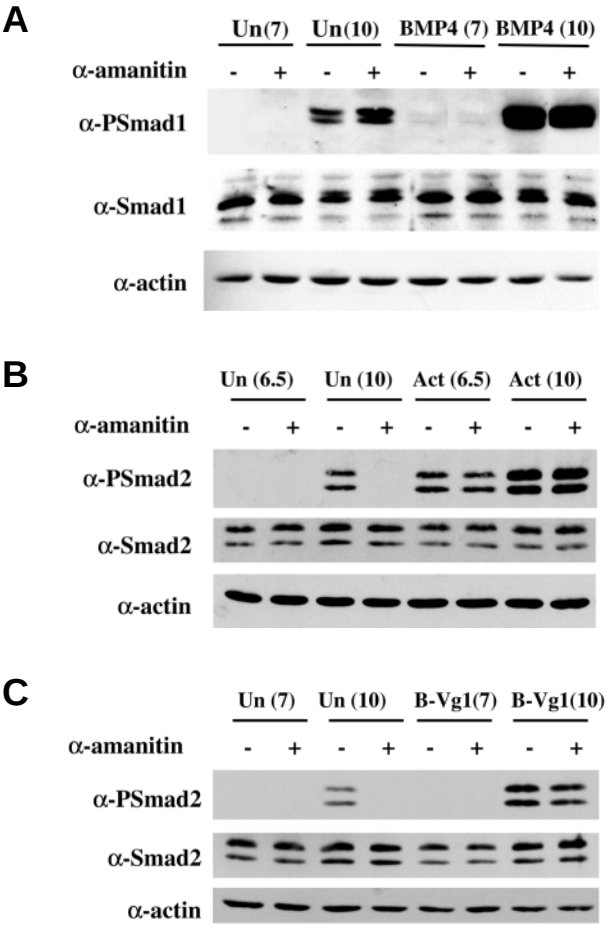


Fig. 6. Overexpression of Smad ligand-activators identifies a novel developmental timing mechanism. (A) BMP ligand activates Smad1 after MBT without requirement for zygotic transcription. Two-cell-stage embryos were injected at the animal and vegetal poles with 50 pg of α-amanitin alone or in combination with BMP4 mRNA (1.5 ng/embryo). Embryos were harvested when uninjected siblings (Un) reached stages 7 and 10. Smad1 activation was monitored by western blot analysis using anti-phosphoSmad1 antibodies. (B) Activin ligand activates Smad2/Smad2Δexon3 before and after MBT without requirement for zygotic transcription. Two-cell-stage embryos were injected vegetally with 50 pg of α-amanitin alone or in combination with activin (Act) mRNA (100 pg/embryo) injected marginally. Embryos were harvested when uninjected siblings (Un) reached stages 6.5 and 10. Activation of Smad2/Smad2Δexon3 was monitored by western blot analysis using anti-phosphoSmad2 antibodies. (C) B-Vg1 ligand activates Smad2/Smad2Δexon3 after MBT without requirement for zygotic transcription. Embryos were injected as above with α-amanitin alone or in combination with B-Vg1 mRNA (100 pg/embryo). Embryos were harvested when uninjected siblings (Un) reached stages 7 and 10. western blot analysis was carried out as for activin (see above).

Spatial localization of TGFβ signaling pathways in early *Xenopus laevis* development

Smad activation across the animal-vegetal axis

To examine the localization of Smad activation across the animal-vegetal axis, we dissected animal, marginal and vegetal regions from early gastrula embryos and assayed them for Smad phosphorylation. To confirm that lysates were enriched for the indicated tissues, dissected regions were examined by RT-PCR for the pan-mesodermal gene *Xbra* (Smith et al., 1991) and the endodermally enriched gene *Xsox17β* (Hudson et al., 1997). At stage 10+, marginal and vegetal explants, respectively enriched in *Xbra* and *Xsox17β* genes, are found to contain comparable amounts of phosphoSmad1. These levels are higher than those found in animal cap explants, even though all explants contain the same amount of total Smad1 protein (Fig. 7A,D). To examine the timing of Smad1 activation more precisely, we dissected embryos from stages 9.5 to 10.5 and compared the levels of Smad1 activation over time in animal caps, marginal zones and vegetal bottoms. Although marginal and vegetal explants contain comparable levels of Smad1 from stage 9.5 to stage 10.5, the level of phosphoSmad1 in animal caps is low at stage 9.5 and increases through stage 10.5 (Fig. 7B). These results suggest that BMP functions in each of the primary germ layers but does not distinguish among them.

Activin-like signaling has been shown to be necessary and sufficient for induction of mesoderm and endoderm in the embryo (Hemmati-Brivanlou and Melton, 1992; Henry et al., 1996; Thomsen et al., 1990; Yasuo and Lemaire, 1999). At stage 10, activated Smad2/Smad2Δexon3 is distributed

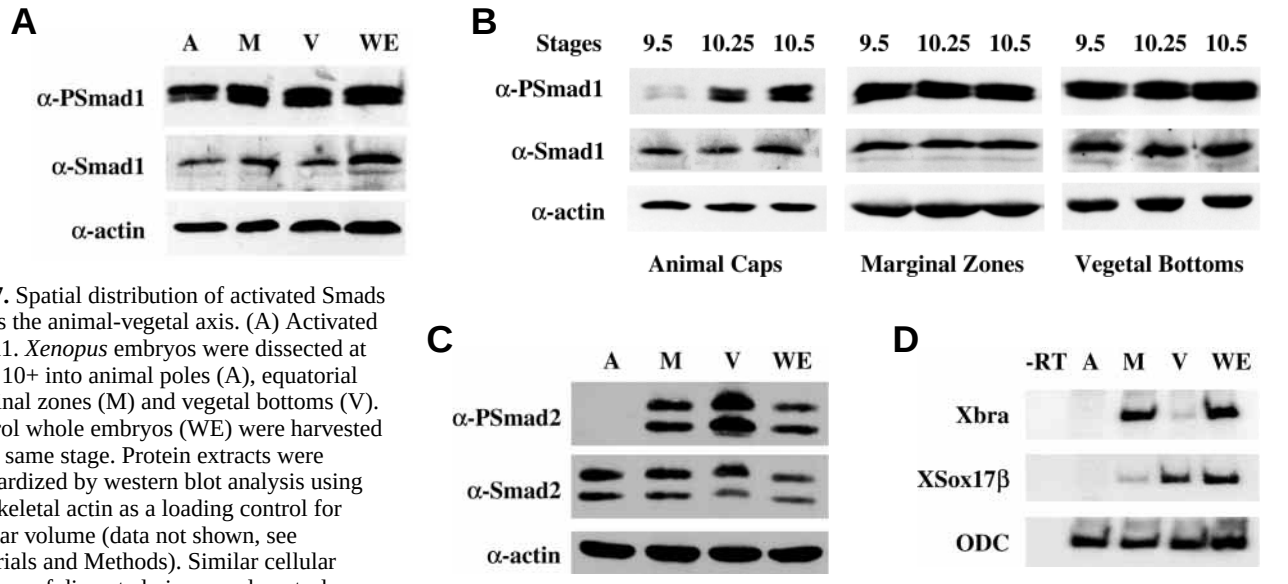


Fig. 7. Spatial distribution of activated Smads across the animal-vegetal axis. (A) Activated Smad1. *Xenopus* embryos were dissected at stage 10+ into animal poles (A), equatorial marginal zones (M) and vegetal bottoms (V). Control whole embryos (WE) were harvested at the same stage. Protein extracts were standardized by western blot analysis using cytoskeletal actin as a loading control for cellular volume (data not shown, see Materials and Methods). Similar cellular volumes of dissected pieces and control whole embryos were loaded and analyzed by western blotting. (B) Time course of Smad1 activation in explants. Animal poles (caps), marginal zones and vegetal bottoms were dissected and harvested at stages 9.5, 10+ and 10.5 as previously described. For each type of explant, Smad1 activation over time was monitored by western blot analysis. (C) Activated Smad2/Smad2Δexon3. *Xenopus* embryos were dissected at stage 10 into animal caps (A), marginal zones (M), and vegetal bottoms (V). Whole embryos (WE) were collected at the same stage. Cytoskeletal actin was used to standardize dissected pieces and whole embryos with respect to cell volume (data not shown; see Materials and Methods). (D) RT-PCR analysis of animal poles (A), equatorial marginal zones (M) and vegetal bottoms (V). cDNA standardized as above was analyzed by RT-PCR for the pan-mesodermal marker *Xbra* and the endodermally enriched gene *Xsox17β. ODC* RNA was assessed as a loading control. −RT, whole embryo RNA mock-transcribed without reverse transcriptase.

unevenly across the animal-vegetal axis. Phosphorylated Smad2/Smad2Δexon3 is most abundant vegetally, moderate marginally and undetectable in the animal pole (Fig. 7C,D). With respect to the cytoskeletal actin control, animal and marginal regions contain similar amounts of Smad2/Smad2Δexon3, while vegetal tissues contain less. Despite apparent under-representation in the vegetal bottom, phosphorylation of Smad2/Smad2Δexon3 is highest in this region. These observations are consistent with the idea that high levels of an activin-like signal are involved in the specification of both endoderm and mesoderm.

Smad activation across the dorsoventral axis

To examine activation of BMP signaling across the dorsoventral axis, we dissected *Xenopus* embryos at stages 9, 9.5 and 10.25 into ventral and dorsal halves. Accuracy of dissections was confirmed by RT-PCR for the expression of genes specifically expressed in ventral or dorsal mesoderm or both (data not shown). At stage 9, Smad1 is activated both in ventral and dorsal halves of embryos. By stage 9.5, however, activated Smad1 is enriched in the ventral halves. At stage 10.25, most of the activated Smad1 is localized to ventral halves, while the total amount of Smad1 remains constant across the dorsoventral axis (Fig. 8A). We therefore conclude that BMP signaling is not localized dorsoventrally at the onset of transcription at MBT, but only becomes so with the onset of gastrulation, suggesting that BMPs are effectors rather than initiators of ventral patterning.

Smad2 signaling across the dorsoventral axis was also examined at late blastula to early gastrula stages (Fig. 8B). At stage 9, Smad2/Smad2Δexon3 phosphorylation is predominantly dorsal; by stage 9.5, Smad2/Smad2Δexon3 phosphorylation has increased ventrally but remains enriched dorsally; by stages 10 and 10.25, Smad2/Smad2Δexon3 is phosphorylated at similar levels in dorsal and ventral halves. At each stage, Smad2 and Smad2Δexon3 are similarly expressed in dorsal and ventral halves. In contrast to Smad1 phosphorylation, which is symmetrically distributed in the late blastula and becomes asymmetric as gastrulation begins, Smad2/Smad2Δexon3 phosphorylation is asymmetrically distributed well before gastrulation and becomes symmetrically distributed as gastrulation proceeds (Fig. 8).

Immunohistochemical detection of activated Smads in the early gastrula embryo

To visualize activation of BMP and activin-like signaling pathways at higher spatial resolution, we examined early gastrula-stage embryos by immunohistochemical analysis. Both anti-phosphoSmad1 and anti-phosphoSmad2 antibodies detect specific signals that are non-uniformly distributed in the embryo and that show subcellular localization to nuclei, as predicted (reviewed in Massagué, 1998). Signals detected by both anti-phosphoSmad antibodies are specific since addition of phosphoSmad peptides eliminates immunoreactivity (data not shown).

At stage 10+, endogenous activated Smad1 is restricted to the ventral side of the embryo in ectoderm, mesoderm and endoderm (Fig. 9B), as previously shown by western blot analysis (Fig. 7A,B). Inhibition of endogenous BMP signaling by overexpresssion of noggin (Smith and Harland, 1992) abolishes endogenous phosphorylation of Smad1 (Fig. 9B), while ectopic activation of the BMP signaling pathway

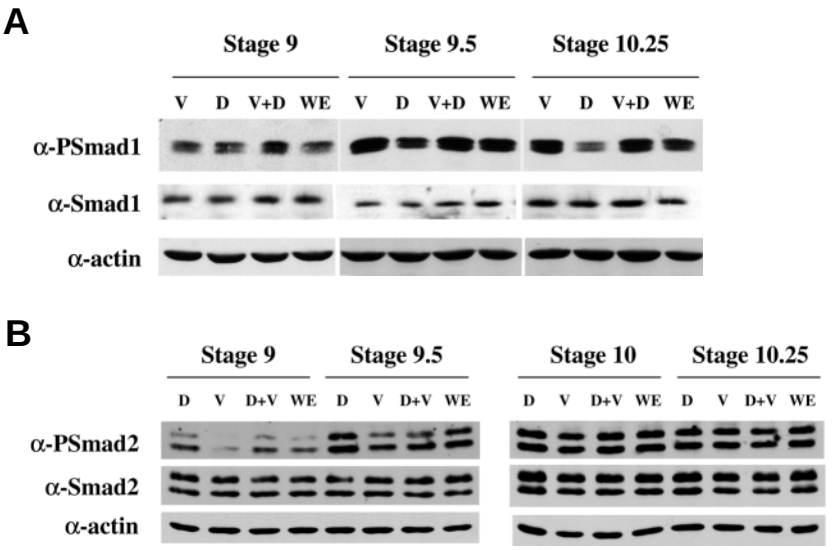


Fig. 8. Spatial distribution of activated Smads across the dorsoventral axis. (A) Activated Smad1. Embryos were dissected at stages 9, 9.5 and 10.25 into ventral (V) and dorsal (D) halves. Smad1 activation in ventral and dorsal halves was monitored by western blot analysis with anti-phosphoSmad1 antibodies. Levels of cytoskeletal actin and total Smad1 served as loading controls. Mean values for Smad1 activation in ventral and dorsal tissues together (V+D) did not exceed Smad1 activation in whole embryo (WE) samples, indicating that dissection does not result in ectopic activation of Smad1 phosphorylation (see Materials and Methods). (B) Activated Smad2/Smad2Δexon3. *Xenopus* embryos were bisected at stages 9, 9.5, 10 and 10.25 into dorsal (D) and ventral (V) halves. For each time point, the same volume of lysate was loaded, as indicated by the cytoskeletal actin control. Whole embryos (WE) and dorsal plus ventral halves (D+V) served as controls for dissection artifacts (see Materials and Methods).

enhances both the distribution and strength of the phosphoSmad1 signal. Overexpression of BMP4 at high concentration in the marginal zone results in even distribution of signal across the dorsoventral axis (Fig. 9C).

Endogenous activated Smad2/Smad2Δexon3 is detected in the dorsal and ventral marginal zones and in the endoderm, but not in the animal cap, of the stage 10+ gastrula (Fig. 9D), as predicted by western blot analysis of dissected regions (Fig. 7C). Inhibition of activin-like signaling by overexpression of the dominant negative activin type II receptor eliminates detection of signal in the marginal zone and vegetal bottom (Fig. 9E). Overexpression of activin RNA, which strongly induces Smad2 activation (Fig. 2A), expands immunoreactivity to the animal cap region and intensifies it (Fig. 9F). In both uninjected embryos and even activin-injected embryos, immunostaining of the endoderm is not evenly distributed and appears weaker than marginal zone staining. This differs from the distribution of Smad2 phosphorylation seen in western blot analysis of dissected embryos, and may reflect poor probe penetration of the yolky vegetal endoderm, as has often been observed for RNA in situ analysis. Despite this limitation, the distribution of Smad phosphorylation observed using immunohistochemistry is generally consistent with that observed by western blot, indicating that immunohistochemistry will be useful for the analysis of more complex spatial patterns of Smad activation in later development.

DISCUSSION

Examination of the endogenous state of TGFβ signaling pathways is necessary to understand where, when and how these signals act to pattern the developing embryo. Antibodies specific for phosphorylated, active Smads are powerful new tools for this purpose. Our antibodies reliably recognize the activated forms of the signal transducing Smads, and distinguish transducers of BMP and activin-like signaling pathways. Both of these antibodies recognize multiple Smad isoforms on western blot analysis, however, reflecting additional complexities in endogenous Smad signaling. The observation that Smad2Δexon3 represents a major portion of the total Smad protein regulated by activin-like signals is particularly significant in that it, unlike Smad2, contains intrinsic DNA binding activity (Dennler et al., 1998; Takenoshita et al., 1998). In experiments with the mouse goosecoid promoter, Labbé et al. (1998) have shown that the replacement of Smad2 with Smad3 in a complex with mouse FAST results in inhibition, rather than activation, of transcription. Because Smad2Δexon3 shares with Smad3 the property of DNA binding (Yagi et al., 1999), Smad2Δexon3 may differ significantly from Smad2 in its effects on transcriptional regulation. The presence of two coregulated, but functionally distinct, Smad2 isoforms may be significant to the regulation of responsiveness to activin-like signals in the early embryo.

Timing of endogenous Smad activation

The timing of endogenous TGFβ superfamily signals signals in the pregastrula embryo has not previously been directly investigated. Using heterochronic Nieuwkoop recombinants of animal-vegetal explants, Jones and Woodland (1987) found that vegetal cells are competent to induce mesoderm from stage 6/6.5, significantly before the MBT. RNAs encoding BMPs 2, 4 and 7, as well as activin D and Vg1, an activin-like ligand, are present maternally (Clement et al., 1995; Dale et al., 1992; Nishimatsu et al., 1992; Oda et al., 1995; Weeks and Melton, 1987), suggesting a role for these signaling molecules in inductive signaling before the onset of zygotic transcription at MBT. More recent work, however, has demonstrated the importance of zygotic expression of activin-like ligands in meso-endodermal specification (Zhang et al., 1998). The maternal transcription factor VegT activates the zygotic transcription of several activin-like ligands at MBT (Clements et al., 1999), and ectopic expression of these ligands can rescue mesoderm induction following depletion of maternal VegT (Kofron et al., 1999). Taken together, these results strongly indicate that meso-endodermal induction is largely dependent on zygotic regulation of activin-like ligands by maternal VegT. We have sought to clarify these apparently contradictory observations regarding the onset of mesoderm-inducing signals by direct examination of the activation state of TGFβ signaling pathways both before and after MBT.

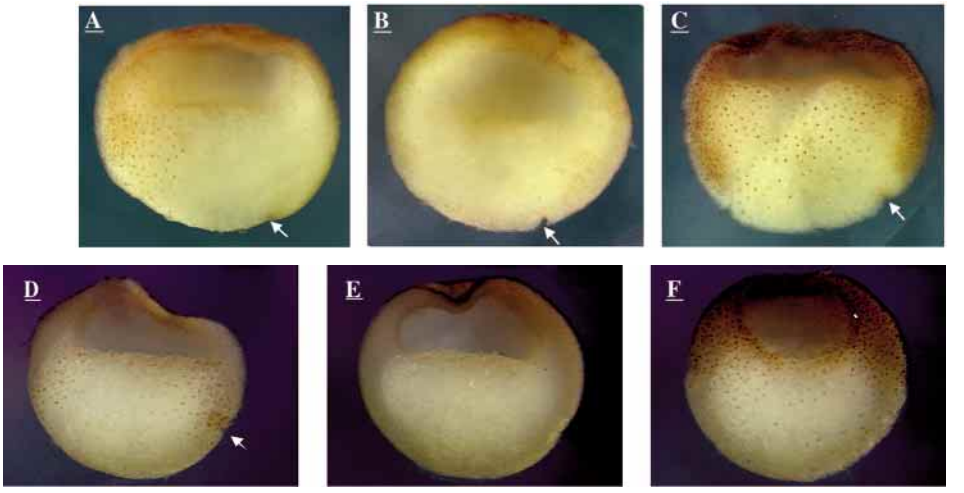


Fig. 9. Immunostaining of activated Smads across the dorsoventral axis. Stage 10+ embryos were fixed and bisected for immunohistochemistry. Dorsoventral orientation was determined by the dorsal blastopore lip, which is indicated with an arrow in (A-D). Embryo diameter is approximately 1 mm. Immunostaining with anti-phosphoSmad1 antibody was carried out with uninjected embryos (A) or embryos injected marginally with noggin (100 pg/embryo) (B) or BMP4 (500 pg/embryo) (C). Anti-phosphoSmad2 immunostaining was performed with uninjected embryos (D) or embryos injected marginally with Δ1XAR1 (1 ng/embryo) (E) or activin (50 pg/embryo) (F).

PhosphoSmad specific antibodies detect both activated Smad1 and activated Smad2 after MBT but not before. While these data do not absolutely rule out the presence of TGFβ signaling before MBT, we can state that pre-MBT Smad phosphorylation is below the limits of detection of this assay, and well below the levels of Smad activation associated with the induction of mesoderm. We find that Smad2/Smad2Δexon3 activation is dependent on transcription at MBT, consistent with recent work showing that the maternal meso-endodermal determinant VegT regulates zygotic transcription of activin-like ligands (Clements et al., 1999).

Even less is known about the termination of TGFβ superfamily signals during early development than is known about their onset. Endogenous phosphorylation of Smad1 and Smad2/Smad2Δexon3 appears roughly simultaneously after MBT, but shows very different patterns of maintenance as development progresses. After activation at MBT, Smad1 phosphorylation remains high throughout gastrulation and post-gastrula development. In contrast, Smad2/Smad2Δexon3 phosphorylation begins to fall at stage 10.5, relatively early in gastrulation, and is quite low by the end of gastrulation at stage 12. Smad2/Smad2Δexon3 phosphorylation can be stimulated ectopically by activin through stage 12 (data not shown), indicating that components of the activin signaling pathway remain present and functional through the end of gastrulation. Both Smad1 and Smad2 have been shown to be degraded through ubiquitin-mediated pathways (Lo and Massagué, 1999; Zhu et al., 1999); in the case of Smad2, this degradation is ligand-regulated. While total Smad1 or Smad2/Smad2Δexon3 levels do not fall significantly in the post-gastrula embryo, specific turnover of phospho-Smad2/Smad2Δexon3 in the absence of ongoing ligand stimulation might account for the overall reduction in Smad2 phosphorylation observed as gastrulation progresses. Duration of signaling may be as important as dose in the action of inducers, and therefore attenuation of Smad2 phosphorylation as gastrulation progresses may be an important step in patterning by activin-like signals.

While transcriptional regulation at MBT is clearly a control point for activin-like signals, our data demonstrate that ligand transcription is not the only mechanism regulating TGFβ superfamily activity in the early embryo. Regulation of responsiveness of embryonic cells to ligands, by a process independent of zygotic transcription but temporally linked to MBT, is also a critical determinant of endogenous signaling. Neither endogenous, maternal BMP RNA nor ectopically expressed BMPs can activate BMP signaling in the pre-MBT embryo, but both can do so after MBT. Similarly, ectopic expression of B-Vg1 can activate Smad2 only after MBT. Smad2 is phosphorylated in response to exogenous activin in pre-MBT embryos, indicating that all the components required for at least one Smad signaling pathway are present and active before MBT, but additional components appear to be necessary for BMP and B-Vg1 signaling. Ligand-specific receptors or receptor-Smad coupling proteins (e.g. SARA; Tsukazaki et al., 1998)) are the most obvious candidates for missing signaling components, but there may also be additional, as yet uncharacterized, regulatory steps. Transcription is not necessary for the transition that regulates the signaling components necessary for either BMP or B-Vg1 responsiveness. Whether the appearance of responsiveness at MBT reflects translation of new regulators or transducers, or reflects some time-dependent post-translational modification, the transcription-independent appearance of responsiveness to TGFβ superfamily ligands defines an important new regulatory transition that coincides with the MBT.

Spatial pattern of Smad activity in early embryos

Activin-like signals have been postulated to function as morphogens in the early embryo (Gurdon et al., 1994), and distinct doses of activin have been shown to be able to induce markers or tissues graded across the dorsoventral or animal-vegetal axes (Green et al., 1992; Henry et al., 1996; Hudson et al., 1997). The extent to which activin-like activity actually is graded across either or both of these axes has not been investigated. Across the animal-vegetal axis, Smad2/Smad2Δexon3 is phosphorylated most extensively in the vegetal bottom, moderately in the marginal zone, and undetectably in the animal cap. This pattern of Smad2/Smad2Δexon3 phosphorylation is consistent with the observed distribution of expression of RNAs encoding the activin-like ligands Vg1, Xnr1, Xnr2, Xnr4 and derrière (Clements et al., 1999; Jones et al., 1995; Joseph and Melton, 1997; Lustig et al., 1996a; Sun et al., 1999; Weeks and Melton, 1987), as well as with the distribution of the proposed meso-endodermal determinant VegT (Horb and Thomsen, 1997; Lustig et al.,

1996b; Stennard and Gurdon, 1996; Zhang and King, 1996). It is also consistent with the theory that the highest levels of activin-like signaling are associated with the induction of endoderm (Hemmati-Brivanlou and Melton, 1994b).

Across the dorsoventral axis, the spatial distribution of Smad2/Smad2Δexon3 phosphorylation changes as gastrulation begins. Phosphorylated Smad2/Smad2Δexon3 is enriched in the dorsal half of the embryo at stage 9.5; by stage 10.25, phospho-Smad2/Smad2Δexon3 is equally distributed between dorsal and ventral halves. This indicates that, while differential Smad2 activation indeed may have a role in distinguishing dorsal from ventral meso-endoderm, this distinction may be based on the differential timing of Smad2 activation in dorsal versus ventral meso-endoderm, rather than on a static spatial gradient of Smad2 activation across the dorsoventral axis.

The distribution of phospho-Smad1, like that of phospho-Smad2/Smad2Δexon3, changes as gastrulation begins, but the pattern of change is quite different for the two Smads. Prior to gastrulation, Smad1 phosphorylation is symmetric across the dorsoventral axis, and becomes asymmetric as gastrulation commences. In contrast, phospho-Smad2/Smad2Δexon3 is predominantly on the prospective dorsal side at stage 9, and symmetric across the dorsoventral axis by stage 10.25. Activin-like signals can induce a variety of inhibitors of BMP function (Sasai et al., 1994; Smith and Harland, 1992), and dorsal enrichment of Smad2 signaling in the late blastula may help to establish the subsequent suppression of BMP signals dorsally. The similarity in phosphoSmad1 levels in prospective endoderm, mesoderm and ectoderm at gastrulation indicates that while ectopic BMP expression can induce ventral mesodermal markers (Clement et al., 1995; Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995), and in some cases endodermal markers (M. Watanabe and M. W., unpublished results), endogenous BMP signaling is not likely to be a determinant of germ layer specification. The major role of the BMP/phosphoSmad1 signal seems likely to be the suppression of dorsal/neural specification in all three germ layers; the asymmetric distribution of Smad1 phosphorylation that mediates this patterning is established only at the onset of gastrulation.

Integration of signals in the establishment and patterning of germ layers

Smads are clearly not the only signal transducers involved in early embryonic patterning, and a full picture of this process will require an understanding both of the endogenous states of multiple signaling molecules and of how their effects are integrated. A fibroblast growth factor (FGF)/MAP kinase pathway is required ubiquitously in the pregastrula embryo for cellular competence to respond to inducing signals, and also is subsequently involved in maintaining mesodermal gene expression (Cornell and Kimelman, 1994; Kessler and Melton, 1995; Kimelman and Kirschner, 1987; Kroll and Amaya, 1996; LaBonne and Whitman, 1994; Schulte-Merker et al., 1994; Slack et al., 1987). The Wnt/β-catenin signaling pathway specifies the dorsal character of both mesoderm and endoderm (Wylie et al., 1996; Zorn et al., 1999). This specification may involve multiple mechanisms, including: (1) the synergistic action of β-catenin with TGFβ superfamily signals at promoters for dorsospecific genes (Crease et al., 1998; Watabe et al., 1995), (2) the dorsal activation of transcription of BMP antagonists (Sasai et al., 1994; Smith and Harland, 1992) and (3) additional, as yet uncharacterized, mechanisms. The endogenous activity of these multiple signal transduction pathways, and the crossregulatory interactions between them, are important pieces in the puzzle of how early embryonic pattern is established. PhosphoSmad antibodies provide a new tool for investigating not only where and when TGFβ signals are active, but also how they are regulated by interaction with other signaling pathways in the early embryo. For the study of the highly localized patterning events of postgastrula embryogenesis, application of these antibodies to immunohistochemistry should provide a high-resolution picture of the endogenous pattern of TGFβ superfamily signals.

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