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Cellular interpretation of multiple TGF-β signals: intracellular antagonism between activin/BVg1 and BMP-2/4 signaling mediated by Smads

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SUMMARY

During early embryogenesis of *Xenopus*, dorsoventral polarity of the mesoderm is established by dorsalizing and ventralizing agents, which are presumably mediated by the activity of an activin/BVg1-like protein and Bone Morphogenetic Proteins (BMP), respectively. Interestingly, these two TGF-β subfamilies are found in overlapping regions during mesoderm patterning. This raises the question of how the presumptive mesodermal cells recognize the multiple TGF-β signals and differentially interpret this information to assign a particular cell fate. In this study, we have exploited the well characterized model of *Xenopus* mesoderm induction to determine the intracellular interactions between BMP-2/4 and activin/BVg1 signaling cascades. Using a constitutively active BMP-2/4 receptor that transduces BMP-2/4 signals in a ligand-independent fashion, we demonstrate that signals provided by activin/BVg1 and BMP modulate each other's activity and that this crosstalk occurs through intracellular mechanisms. In assays using BMP-2/4 and activin/BVg1-specific reporters, we determined that the specificity of BMP-2/4 and activin/BVg1 signaling is mediated by Smad1 and Smad2, respectively. These Smads should be considered as the mediators of the intracellular antagonism between BMP-2/4 and activin/BVg1 signaling possibly through sequestration of a limited pool of Smad4. Consistent with such a mechanism, Smad4 interacts functionally with both Smad1 and -2 to potentiate their signaling activities, and a dominant negative variant of Smad4 can inhibit both activin/BVg1 and BMP-2/4 mediated signaling Finally, we demonstrate that an activin/BVg1-dependent transcriptional complex contains both Smad2 and Smad4 and thereby provides a physical basis for the functional involvement of both Smads in TGF-β-dependent transcriptional regulation. Thus, Smad4 plays a central role in synergistically activating activin/BVg1 and BMP-dependent transcription and functions as an intracellular sensor for TGF-β-related signals.

Key words: activin, BMP, mesoderm, TGF-β, Vg1, Smad, *Xenopus*

INTRODUCTION

TGF-β signal transduction requires the interaction between two distinct types (types I and II) of transmembrane serine/threonine kinase receptors (Wrana et al., 1994; reviewed in Massagué, 1996; Derynck and Feng, 1997). For activin and TGF-β1, binding of the ligand to a type II receptor results in interaction with a type I receptor and leads to transphosphorylation of the type I receptor by the type II receptor kinase. Phosphorylation results in activation of the type I receptor and subsequent signaling by the heteromeric receptor complex. This paradigm implicates the type II receptor as a high affinity receptor responsible for ligand binding and the type I receptor with a low affinity for ligand as an activator or effector of signal transduction. However, it has been noted that BMP receptors behave in a slightly different manner, whereby the high affinity is determined by the heteromeric combination of type I and II receptors (Letsou et al., 1995; Rosenzweig et al., 1995).

Recently the Smads, which are related to *Drosophila* Mad, have been identified as important components of TGF-β-related signal transduction pathways in a variety of species (Raftery et al., 1995; Sekelsky et al., 1995; Newfeld et al., 1996; Graff et al., 1996; Hoodless et al., 1996; Thomsen, 1996; Liu et al., 1996; Savage et al., 1996; Zhang et al., 1996; Yingling et al., 1996). *Drosophila* Mad and its homolog in vertebrates (Smad1) are essential for signaling in Dpp and BMP-2/4 pathways and can elicit biological responses characteristic of BMP-2/4 (Hoodless et al., 1996; Graff et al., 1996; Thomsen, 1996; Liu et al., 1996; Newfeld et al., 1996). While Smad1 mediates BMP-2/4 signaling, Smad2 and Smad3

transduce activin and TGF-β signaling, respectively (Graff et al., 1996; Baker and Harland, 1996; Eppert et al., 1996, Zhang et al., 1996). It is thought that initiation of a particular TGF-β-related pathway results in phosphorylation of a specific Smad protein and subsequent translocation from the cytoplasm to the nucleus (Hoodless et al., 1996, Macias-Silva et al., 1996, reviewed in Derynck and Zhang, 1996). Once inside the nucleus, Smads may function as transcriptional activators (Liu et al., 1996). Accordingly, *Xenopus* Smad2 has been shown to be part of a transcriptional complex containing a winged helix protein that interacts with the activin-responsive Mix 2 promoter (Chen et al., 1996) and Mad interacts directly with a defined promoter element in the vestigial gene (Kim et al., 1997).

TGF-β signaling has been implicated in numerous processes during vertebrate embryonic development (Wall and Hogan, 1994; Smith, 1995; Hogan, 1996). One of the most intensely studied areas of the TGF-β action during development is the induction and patterning of amphibian mesoderm when multiple TGF-β signaling molecules are present at that time (reviewed by Smith, 1995). In particular, the TGF-β-related molecules, activin, Vg1, BMP, and nodal-related factors may mimic or be directly involved in patterning the mesoderm (Green and Smith, 1990; Dale et al., 1993; Thomsen and Melton, 1993; Jones et al., 1992; Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1995; Smith et al., 1995). Activin and BVg1, which corresponds to a processed form of Vg1 cleaved from a hybrid precursor with the pre-pro region of BMP-4 and mature Vg1 (Dale et al., 1993; Thomsen and Melton, 1993) are both able to induce dorsal types of mesoderm from naive ectoderm (animal cap tissue) and when ectopically expressed in the ventral region of a developing *Xenopus* embryo (Green and Smith, 1990; Dale et al., 1993; Thomsen and Melton, 1993). BMP-2 and-4, conversely, are ventralizing factors and can block the dorsalizing effects of activin in naive ectoderm (Jones et al., 1992; Dale et al., 1992). Furthermore, elimination of BMP-2/4 signals in the ventral region of the embryo by expressing a dominant negative BMP-2/4 receptor or ligand allows dorsal signals to induce a secondary axis (Graff et al., 1994; Suzuki et al., 1994; Hawley et al., 1995; Schmidt et al., 1995). Similarly, eliminating BMP-2/4 signals through antisense RNA allows the expression domain of dorsal-specific molecules to be extended into the ventral region of the embryo (Steinbeisser et al., 1995). Thus, removing BMP-2/4 signals unmasks a dorsalizing activity that is present throughout the underlying endoderm (Watabe et al., 1995). These results emphasize the importance of antagonistic signals in mediating mesodermal patterning during *Xenopus* development. Thus, cells within the equatorial region of the *Xenopus* embryo (presumptive mesoderm) are likely to receive and integrate both dorsal (e.g. activin/BVg1-like) and ventral (e.g. BMP-2/4) signals, and interpret these differential signals, and accordingly modulate gene expression to assign a certain fate.

While the antagonism between activin/BVg1 and BMP signals is essential for proper patterning of the mesoderm, the mechanism underlying this phenomenon is not well understood. Two types of mechanisms are conceivable, depending on their extracellular versus intracellular nature. In the extracellular medium, BMPs may interact with activin receptors and thereby interfere with activin receptor activation. Consistent with this notion is the fact that BMP-2/4, BMP-7 and *Drosophila* Dpp bind not only to BMP-2/4 receptors (Childs et al., 1993; Brummel et al., 1994), but also to receptors that were thought to be specific for activin (ten Dijke et al., 1994; Yamashita et al., 1995, Rosenzweig et al., 1995). Another extracellular mechanism could involve sequestration of BMP-2/4 by interacting proteins. Accordingly, chordin and noggin proteins have been shown to interact directly with BMPs, which prevents them from interacting with their cognate receptors and thus allows underlying activin/BVg1-like signals in the dorsal region of the embryo to induce dorsal mesoderm (Piccolo et al., 1996; Zimmerman et al., 1996). An intracellular mechanism could involve signaling mediators that are differentially activated following stimulation by activin/BVg1 and BMP-2/4 and activation of their respective receptor. At present there are no data that substantiate such an intracellular mechanism of convergence and differential interpretation.

To address this question and to better understand TGF-β signaling, we evaluated whether the antagonism occurs through intracellular means. Our rationale was to generate a constitutively active BMP-2/4 type I receptor (CABR) that can transduce BMP-2/4 like signals in the absence of ligand. This would allow us to examine a possible intracellular signaling antagonism between activin/BVg1 and BMP-2/4 without any extracellular influence. Our data suggest that the antagonism between the signals from BMP-2/4 and activin/BVg1 occurs intracellularly. Furthermore, the intracellular convergence of TGF-β-related signals is mediated by Smad proteins, and Smad4 appears to be a focal point for sensing the levels of different TGF-β signals. Finally, we demonstrate that the interaction between Smad2 and -4 and their association with the promoter directly regulates a transcriptional response of an activin/BVg1 target gene. These results link components of a TGF-β-related signaling cascade from the activated receptor at the cell surface to gene expression in the nucleus.

MATERIALS AND METHODS

Embryo manipulations

Xenopus embryos were obtained by in vitro fertilization of eggs with testes homogenates. Embryos were dejellied in 2% cysteine and staged according to Nieuwkoop and Faber (1994). For animal cap assays, embryos were injected animally at either the 2 or 4 cell stage with 2-4 nl of solution. Animal caps were dissected at stage 8-8.5 and cultured in 1× Barth's solution. Dorsal and ventral regions of the embryos were assigned based upon pigmentation differences as described in Cho et al. (1991). Amounts of RNA and DNA injected are indicated in figure legends.

Receptor binding assay

Recombinant human activin was iodinated using the CGU method according to Howard and Knauer (1986). Animal cap explants were dissected from stage 8 embryos which were uninjected or injected with ΔE-CABR mRNA (up to 1.6 ng). The explants were dissociated by incubating in calcium/magnesium-free *Xenopus* Ringer solution for 30 minutes. The dissociated cells were washed with binding buffer (phosphate-buffered saline containing 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, and 1 mg/ml bovine serum albumin) three times and incubated on ice in the same buffer with ^{125}I-activin in the absence or presence of increasing amounts of unlabeled activin or human recombinant TGF-β_1 for 3 hours. Cells were washed with the binding buffer three times, and the bound ^{125}I-activin was measured using a gamma counter.

Xvent2-Luc reporter plasmid

A 1.8 kb *Pst*I-*Eco*RI digested fragment of the Xvent2 genomic subclone (Xvent2g1-2) was PCR amplified using the following two oligonucleotides (upstream primer; 5′TAATACGACTCTCACTAT-AGGC-3′, downstream primer; 5′-GGGAAGCTTCTGTATTAGTC-CTTGTG-3′), and subcloned into *Bam*HI and *Hin*dIII digested pOLuc vector (de Wet et al., 1987). The resulting construct (Xvent2-Luc) contains approximately 250 bp of Xvent2 promoter sequences.

Expression plasmids for constitutively active BMP receptor (CABR) and extracellular-deleted (ΔE) CABR

A cDNA clone encoding a *Xenopus* BMP Type I receptor (ALK3) was obtained from N. Ueno (Hokkaido University, Japan; Suzuki et al., 1994). To create a constitutively activated BMP receptor (CABR), the codon encoding amino acid 228 (relative to translation start) was mutated from glutamine to aspartic acid. The mutation was introduced by an overlapping, two-step PCR strategy described by Wieser et al. (1995) using the following primers: SP6, Q-D no. 1 (sense, GTACTATAGCAAAGGACATCCAAATGGTTCGAC), Q-D no.2 (antisense, GTCGAACCATTTGGATGTCCTTTGCTATAGTAC), and an pSP64 polylinker primer (GAATTCGAGCTCGCCGGG).

To prepare the CABR lacking the extracellular ligand binding domain, the cDNA encoding the CABR was used as a template in another overlapping, two-step PCR strategy. In this case, two PCRs were carried out encompassing the first 40 amino acids of the receptor (reaction no. 1, using the SP6 and ΔE no. 1 [GAGGTGACCACTCT-CACACGTGTAGTTGGCCTG] primers), and amino acids 140 to the carboxy terminus (reaction 2, using the pSP64 polylinker primer and ΔE no. 2 [TACACGTGTGAGAGTGGTCACCTCAAGGAGCCT] primers). Primers were designed such that the 3′ end of reaction no. 1 overlapped with the 5′ end of reaction no. 2. When aliquots from the two reactions were mixed and subjected to PCR using the 5′ (sense) primer from reaction no. 1 and the 3′ (anti-sense) primer from reaction no. 2, a product deleting 60% of the extracellular domain was created (ΔE-CABR). This was subcloned into a pSP64 based vector (Krieg and Melton, 1984) and used for RNA synthesis. Like the CABR, the ΔE-CABR is able to provide specific BMP signals. However, 5- to 10-fold more ΔE-CABR mRNA is required to obtain equivalent phenotypes when compared to the wild-type CABR (data not shown).

Construction of dominant negative Smad expression plasmids

Dominant negative mutants of *Xenopus* Smad1 and -2 were constructed using pSP64TEN-DOT1 and pSP64TEN-DOT2 (a gift from D. A. Melton), encoding *Xenopus* Smad-1 and 2, respectively. C-terminal truncation of 39 amino acids for Smad1 and -2 were obtained by PCR amplification using SP6 as a upstream primer and the downstream primers (5′GGGAATTCCTAACCCCATCCCTTGACAA3′ for Smad-1Dc, and 5′GGGAATTCCTAACCCCAGCCTTTTACAA3′ for Smad-2Dc), followed by *Eco*RI digestion and subcloning into pSP64TEN. Details of the creation of dominant negative Smad4 can be found in Zhang et al. (1996).

Reverse transcriptase-PCR

RNA for RT-PCR was isolated by the method of Chomzynski and Sacchi (1987). RT-PCR was carried out according to the method of Blitz and Cho (1995). Primers for PCR are listed in Blitz and Cho (1995), and Hawley et al. (1995) except for Xvent1, which can be found in Gawantka et al. (1995).

Luciferase assays

Luciferase assays were carried out according to the method of Watabe et al. (1995). Briefly, embryos were injected animally into each blastomere at the two cell stage with 4 nl of a RNA/reporter gene cocktail. Amounts are indicated in the figures or their legends. Animal caps were isolated at stage 8-8.5, cultured for 3 hours in 1× Barth's and ten animal caps for each sample were harvested for luciferase assay. Because absolute levels of reporter gene activity were influenced by the batch of eggs, each experiment was carried out using eggs laid by a single female. Each assay was repeated at least twice with a representative experiment being shown.

Electrophoretic mobility shift assay and western blot analysis

Soluble protein extracts were prepared by homogenizing animal caps in extraction buffer (50 mM HEPES, pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml leupeptin), and centrifuging to remove debris and yolk. The supernatant was collected, protein concentration determined and aliquots frozen at –80°C. For gel shifts, sense and antisense oligonucleotides corresponding to the A/VRE (Watabe et al., 1995) were annealed and end-labeled with [γ-^{32}P]ATP. Probe containing 40×10^3 c.p.m. was mixed with equal amounts of extract in binding buffer (20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 0.05 mM EDTA, 8% glycerol, 1 mM DTT, 5 μg/ml poly dI-dC) for 30 minutes on ice. Binding reactions were separated on a 5% non-denaturing polyacrylamide gel. For western blot analysis, gel shift was performed using A/VRE oligos as described above with minor modifications. Unlabeled A/VRE oligo was used for the gel shift assay, and the amount of protein extracts used for the assay was 5 times higher than the normal reaction volume. The complex was electrotransferred to nitrocellulose membrane overnight. The membrane was incubated with either anti-Flag (Kodak) or anti-myc (Oncoscience) antibodies (1:1000 dilution), and subjected to staining using ECL kit according to the manufacture's instructions (Amersham).

RESULTS

Generation of a constitutively active BMP receptor

Previous results in *Xenopus* demonstrated that BMP-4 was able to block the dorsal mesoderm-inducing activity of activin (Jones et al., 1992; Dale et al., 1992). In a similar manner, we have confirmed and extended these observations by demonstrating that BMP-4 interferes with the mesoderm-inducing activity of not only activin but also of BVg1 (data not shown). To investigate whether the activin/BVg1 and BMP-2/4 antagonism occurs intracellularly, we have generated a constitutively active BMP-2/4 type I receptor (CABR) that can transduce BMP signaling in a ligand-independent fashion. Using a PCR strategy (see Materials and Methods), the codon for Glu228 immediately downstream from the conserved GS domain was changed into an Asp codon. This alteration in protein sequence has been demonstrated to constitutively activate a type I TGF-β receptor (Wieser et al., 1995) as well as the Thick veins type I Dpp receptor of *Drosophila* (Nellen et al., 1996; Hoodless et al., 1996). To eliminate any possible influence of extracellular ligands on this activated BMP receptor, we eliminated 60% of the extracellular region, thereby creating the CABR variant named ΔE-CABR.

These activated receptors were tested by several independent assays for their ability to provide BMP-specific signals. mRNAs encoding these receptors were able to block the formation of dorsoanterior tissues (e.g. eyes, cement glands) in a dose-dependent manner when injected dorsally (data not shown). Additionally, these mRNAs were able to revert the secondary axis phenotype caused by injection of dominant-negative BMP type I receptor mRNA or mRNAs encoding

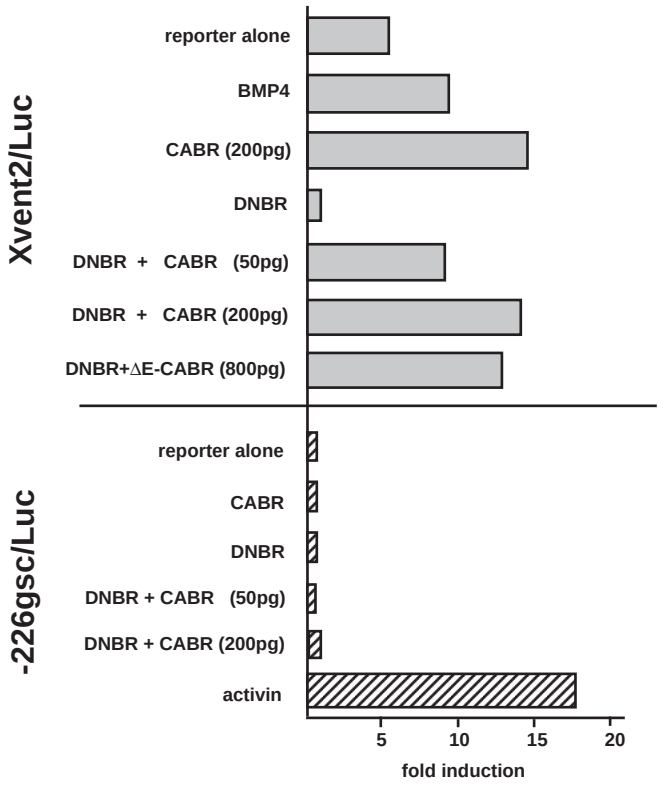

Fig. 1. Specificity of constitutively active BMP receptors. Luciferase assay showing activation of Xvent2 and goosecoid (gsc) luciferase (Luc) reporter constructs. Embryos are injected in the animal pole at the two-cell stage with either a gsc or Xvent2-luciferase reporter gene construct together with the indicated mRNAs. At blastula stage, animal caps were dissected manually, cultured for 3 hours, and subjected to luciferase assays. Overexpression of BMP4 and CABR induced the Xvent2 promoter. Expression of DN-BR reduces 'basal' Xvent-Luc reporter gene activity, presumably by inhibiting BMP2 and BMP7. When the Xvent2-Luc reporter was coinjected with DN-BR together with increasing concentrations of CABR or ΔE-CABR, the reporter gene activity increased in a dose-dependent manner. The CABR does not affect –226gsc-Luc activity.

cleavage mutant BMP-4 and BMP-7 (data not shown) (Graff et al., 1994; Suzuki et al., 1994; Hawley et al., 1995). Finally, we used a luciferase reporter plasmid (Xvent2-Luc) in which luciferase expression is controlled by a 250 bp promoter fragment of Xvent2, a gene which responds specifically to BMP signaling (Onichtchouk et al., 1996). As shown in Fig. 1, overexpression of BMP-4 and CABR stimulated luciferase expression from the Xvent2 promoter but not from the –226gsc-Luc plasmid, in which luciferase activity is under the control of an activin/BVg1-specific response element (Watabe et al., 1995). The level of luciferase induction from the Xvent2 promoter (5-fold induction) by these proteins is apparently muted due to the presence of BMP-2 and -7 in the ectoderm of early gastrula embryos (Nishimatsu et al., 1992; Suzuki et al., 1993). The endogenous BMPs in the animal caps provide an endogenous stimulation of the expression of Xvent2-Luc, since we can reduce the 'basal' Xvent2-Luc reporter activity by coexpressing a dominant-negative BMP receptor (DN-BR). When the basal BMP activity is eliminated by expression of dominant-negative BMP type I receptor, expression of CABR and ΔE-CABR still strongly induces luciferase expression from the Xvent2-promoter, further illustrating the independence of the activated receptor for ligand. Taken together, our results indicate that both the CABR and ΔE-CABR transduce BMP-2/4-specific signals in a ligand-independent fashion.

The antagonism in signaling by TGF-β-related molecules occurs intracellularly

The properties and activity of CABR and ΔE-CABR allowed us to test whether the dorsal and ventral mesoderm-inducing signals originating from stimulation by activin and BMP-2/4 are differentiated and integrated intracellularly. Since these constitutively active receptors circumvent the requirement for ligand and eliminate possible interactions with other TGF-β-related molecules, we rationalized that if CABR and ΔE-CABR were able to override the dorsalizing effects of activin/BVg1, then the two signaling pathways must converge intracellularly.

We first used the animal cap assay in which the mesoderm-inducing effects of activin/BVg1 and BMP-2/4 have been well documented. As mentioned, exogenous application of BMP-2/4 has been shown to block the dorsalizing effects of activin (Jones et al., 1992; Dale et al., 1992). Thus, we tested whether CABR could block the dorsalizing effects of activin. Treatment of animal caps with activin results in their elongation, which is indicative of the formation of dorsal tissues including notochord and muscle. Both the CABR and ΔE-CABR reduced, in a dose-dependent manner, the degree of extension induced by activin (Fig. 2A, and data not shown). This phenotypic effect was accompanied by a decrease in dorsal mesoderm-specific markers and an increase in ventral markers (Fig. 2B,C).

BVg1 has, similarly to activin, the capacity to induce dorsal mesoderm in animal caps and to activate dorsal mesoderm-specific markers (e.g. goosecoid, muscle actin, orthodenticle). While specific receptors for BVg1 have not been isolated, we assume that, similar to other TGF-β-related factors, BVg1 transduces a signal through a heteromeric receptor system. Consistent with this model, a cytoplasmically truncated activin receptor is able to block BVg1 signals in a dominant negative manner (Kessler and Melton, 1995). We, therefore, tested whether expression of CABR was also able to block the dorsalizing effect of BVg1, as is the case for activin. Our results show that CABR expression not only blocked the activin-induced but also the BVg1-induced dorsal mesoderm induction (Fig. 2D). Since the activated BMP-2/4 receptors signal independently from ligands, we conclude that the activin/BVg1 and BMP-2/4 signals converge and antagonize intracellularly.

While ΔE-CABR is incapable of binding to extracellular ligand, it is formally possible that ectopic expression of ΔE-CABR interferes with or downregulates endogenous activin receptors and in this way promotes its inhibitory activity. We therefore evaluated the ability of activin to interact with cell surface receptors in dissociated animal cap cells isolated from uninjected and ΔE-CABR expressing embryos. This assay measured the ability of the cell surface receptor to bind ^{125}I-activin and the competition of unlabeled activin with the radiolabeled ligand. As shown in Fig. 3, expression of ΔE-CABR (up to 1.6 ng mRNA) did not affect the interactions between ^{125}I-activin and its receptor. This indicates that ΔE-CABR expression does not spuriously disturb receptor-ligand interactions. Further, since the amount of bound ^{125}I-activin

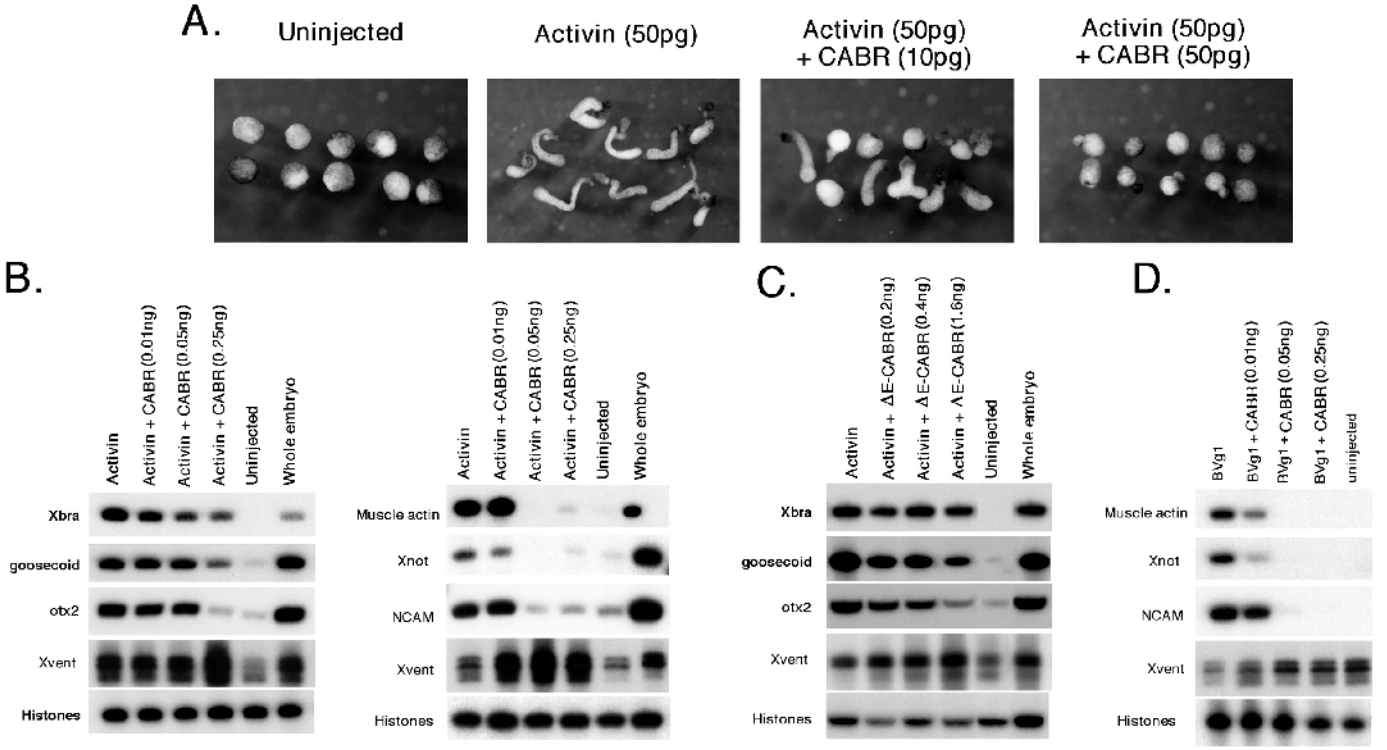

Fig. 2. BMP signaling can antagonize dorsal signals intracellularly. (A) Effect of CABR on activin signaling. Activin mRNA alone was able to induce dorsal types of mesoderm as indicated by the elongation of the animal caps. When intracellular BMP signaling was provided by the coinjection of mRNA encoding the CABR, the elongation was reduced in a dose dependent manner. (B) The reduction of elongation was directly associated with the loss of dorsal-specific markers such as goosecoid, otx2, muscle actin, Xnot and NCAM. The increase in the ventral marker, Xvent (Onichtchouk et al., 1996), indicates that the CABR mRNA induces ventral mesoderm at the expense of dorsal mesoderm. The decrease in Xbra expression was not consistently reproducible but may reflect differences in induction of Xbra by ventral versus dorsal signaling molecules. Left panel shows marker expression of animal caps isolated at stage 8 and cultured to stage 11.5 equivalent; right panel, animal caps cultured to stage 22 equivalent. (C) Similar intracellular antagonism is observed when using ΔE-CABR, a variant of CABR which lacks 60% of its extracellular domain. (D) Intracellular BMP signaling can also antagonize the dorsalizing effects of BVg1. A similar dose-dependent decrease of dorsal-specific markers is accomplished when increasing amounts of CABR mRNA are coinjected. The expression of gsc, otx2, N-CAM and Xnot in uninjected samples is higher than normal in this particular set of experiments, and not usually observed.

was not reduced, the presence of ΔE-CABR does not cause downregulation of activin receptors. These data are consistent with an intracellular convergence of activin/BVg1 and BMP-2/4 signals.

Activin/BVg1 and BMP-2/4 signals converge on a cis-element within the goosecoid promoter

We next evaluated the molecular mechanism of intracellular convergence and integration of activin/BVg1 and BMP signaling by assessing the transcriptional regulation of known target genes that are responsive to activin or BMP-2/4. As previously shown (Jones et al., 1992; Dale et al., 1992) and illustrated in Fig. 2, the expression of specific genes can be regulated by the antagonizing signals of activin and BMP-2/4. However, an examination of the endogenous expression levels of these responsive genes is not sufficiently informative since it does not allow us to discriminate between two possible modes of intracellular convergence at the transcription level. One possible mechanism would be that the transcriptional regulation could be determined by separate activin/BVg1 and BMP-2/4 *cis*-response elements within the promoter of a given gene that are differentially activated or repressed depending on the incoming activin/BVg1 or BMP signals. In this case, there is not necessarily a direct competition between transcriptional modulators. Alternatively, the convergence of the signals may occur by direct competition or interaction of mediators (cytoplasmic or nuclear) that signal through a single responsive promoter element.

To distinguish between these two possibilities, we used a reporter assay in which luciferase expression is controlled by a previously identified *cis*-acting DNA element that responds specifically to activin/BVg1 and not to BMP-2/4, i.e. the activin/BVg1 response element or A/VRE (also previously known as the DE) (Watabe et al., 1995). This response element was identified as a 226 bp segment in the activin/BVg1-responsive goosecoid (*gsc*) gene. If the differential interpretation of activin/BVg1 and BMP signals is mediated through distinct promoter/enhancer elements, expression of CABR will not affect the stimulation of transcription from the A/VRE element by activin/BVg1 signaling. However, if the antagonizing signals converge prior to the direct activation of a target gene or compete for the same response element, the induction of the A/VRE activity by activin or BVg1 will be affected by the CABRs. The luciferase reporter plasmid containing the 226 bp gsc promoter region with its A/VRE was coinjected with mRNA encoding activin or BVg1 in the presence or absence

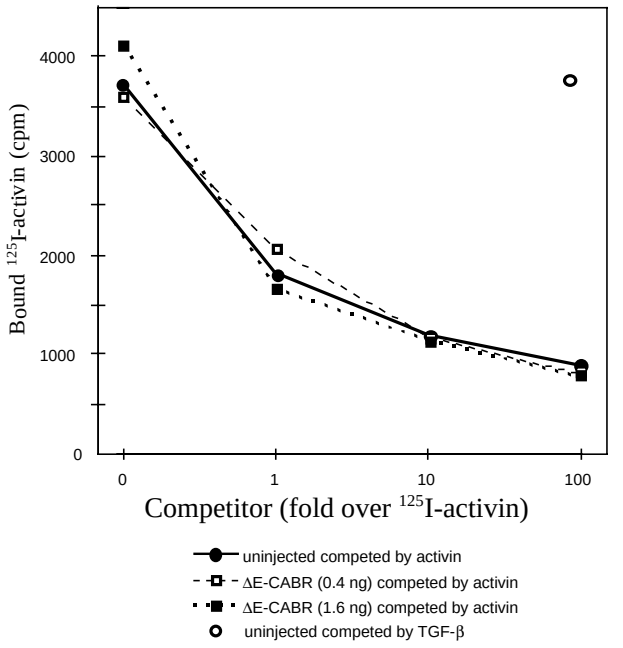

Fig. 3. ΔE-CABR does not interfere with activin ligand/receptor interaction. Dissociated animal cap cells were incubated with ^{125}I-activin for 3 hours on ice in the presence of increasing concentrations of unlabeled activin. The amount of bound ^{125}I-activin was measured. A 100-fold molar excess of unlabeled activin was able to block approximately 80% of the ^{125}I-activin from receptor interactions, while TGF-β did not interfere with the interaction. The competition curves of uninjected cells and ΔE-CABR-expressing cells (0.4 ng and 1.6 ng per embryo) are identical, indicating that ΔE-CABR does not interfere with activin ligand/receptor interactions or downregulate activin receptors.

of the CABR mRNA. Consistent with our previous observations (Watabe et al., 1995), luciferase expression from the gsc promoter was induced about 30-fold by BVg1. However, when BMP-2/4 signaling was provided by coinjection of CABR mRNA, the induction was repressed up to 10-fold in a dose dependent manner (Fig. 4A). This result suggests that the induction of transcription of a gene by activin/BVg1 signaling can be directly antagonized by converging signals from the BMP-receptor pathway.

The 226 bp region of the gsc promoter used in this reporter assay (Fig. 4A) contains two separable elements that synergize to recapitulate endogenous *gsc* expression. One of these is the A/VRE element which responds only to activin/BVg1 signaling, whereas the other one, the proximal element, responds to wnt signaling (Watabe et al., 1995). To eliminate any possible influence from the proximal element, we tested luciferase expression driven by a multimer of the A/VRE element (DE(6X)gscLuc) which is sufficient to respond specifically to activin or BVg1 signals and not to wnt (Watabe et al., 1995). Fig. 4B,C shows that the induction of this multimer reporter construct by either BVg1 or activin was repressed by the presence of the ΔE-CABR in a manner similar to the entire 226 bp region of the gsc promoter. Furthermore, Fig. 4D shows that BMP-2/4 signaling, assessed by transcription from the Xvent2 promoter (Onichtchouk et al., 1996; Fig. 1) can occur in the presence of high concentrations of activin (80 pg mRNA), as long as strong BMP signals are transduced. The results suggest that the repression of transcription of DE(6X)gscLuc at high concentrations of ΔE-CABR (Fig. 4C) is not simply due to inhibition of activin signaling, but is compensated by corresponding increases in BMP signaling.

Taken together, our results show that the activin/BVg1 and BMP-2/4 intracellular signals converge at the A/VRE site or at a level of signaling mediators that is upstream from A/VRE binding. Furthermore, repression of the gsc promoter activity by BMP-2/4 signaling is accompanied by transcriptional induction of the BMP-specific response element found in the Xvent2 promoter.

The intracellular antagonism is bidirectional

All of our results outlined thus far have demonstrated the ability of BMP-2/4 signals to antagonize activin/BVg1 signaling, suggesting that BMP-induced ventralizing signals unidirectionally inhibit these dorsalizing signals. However, increasing amounts of activin can overcome the antagonizing effects of BMP-2/4 signals provided by ΔE-CABR on transcription from the A/VRE reporter element (Fig. 4E). Thus, the antagonism between activin and BMP-2/4 signaling is not simply a block of activin/BVg1 signaling by BMP-2/4 receptor signals. In contrast, the interference is bidirectional, which suggests that there exists an intracellular cross-talk between the signaling cascades induced by members of the TGF-β superfamily, which can be observed at the level of transcription from the A/VRE element.

The bidirectional nature of activin/BVg1 and BMP-2/4 signaling can account for the observations of secondary axis formation by both loss-of-function of BMP and gain-of-function of activin or BVg1 signaling. During normal development, the ventral region of the embryo is specified by active BMP-2/4 signals. Thus, removal of BMP signals could unmask underlying activin/BVg1-like signals and allow formation of a secondary axis. In contrast, ectopic overexpression of dorsalizing factors such as activin and BVg1 in the ventral region of a *Xenopus* embryo (Thomsen et al., 1990; Dale et al., 1993) can override and antagonize BMP-2/4 signals and induce a secondary axis.

A specific A/VRE binding protein is influenced by the antagonism between BMP and activin/BVg1 signals

The antagonistic effects of activin/BVg1 and BMP-2/4 on the gsc promoter prompted us to examine the role of DNA binding proteins that may influence transcription from this DNA segment. Using an A/VRE probe, we identified a specific A/VRE binding complex (ABC) that is induced within 20 minutes after animal caps are treated with activin (Fig. 5A). Since the accumulation of ABC was activin-dependent, we determined the effect of intracellular BMP signaling on ABC complex formation. To test this possibility, embryos were first injected with mRNA encoding ΔE-CABR at the two cell stage. Animal caps were isolated at blastula stage, treated with soluble activin for 60 minutes, and extracts prepared for gel-shift analysis. Whereas activin alone induced the formation of ABC, the presence of BMP-2/4 receptor signals from the ΔE-CABR reduced ABC formation (Fig. 5B). These results indicate that the antagonism between activin/BVg1 and BMP-2/4 signaling that affects transcriptional activity at the A/VRE

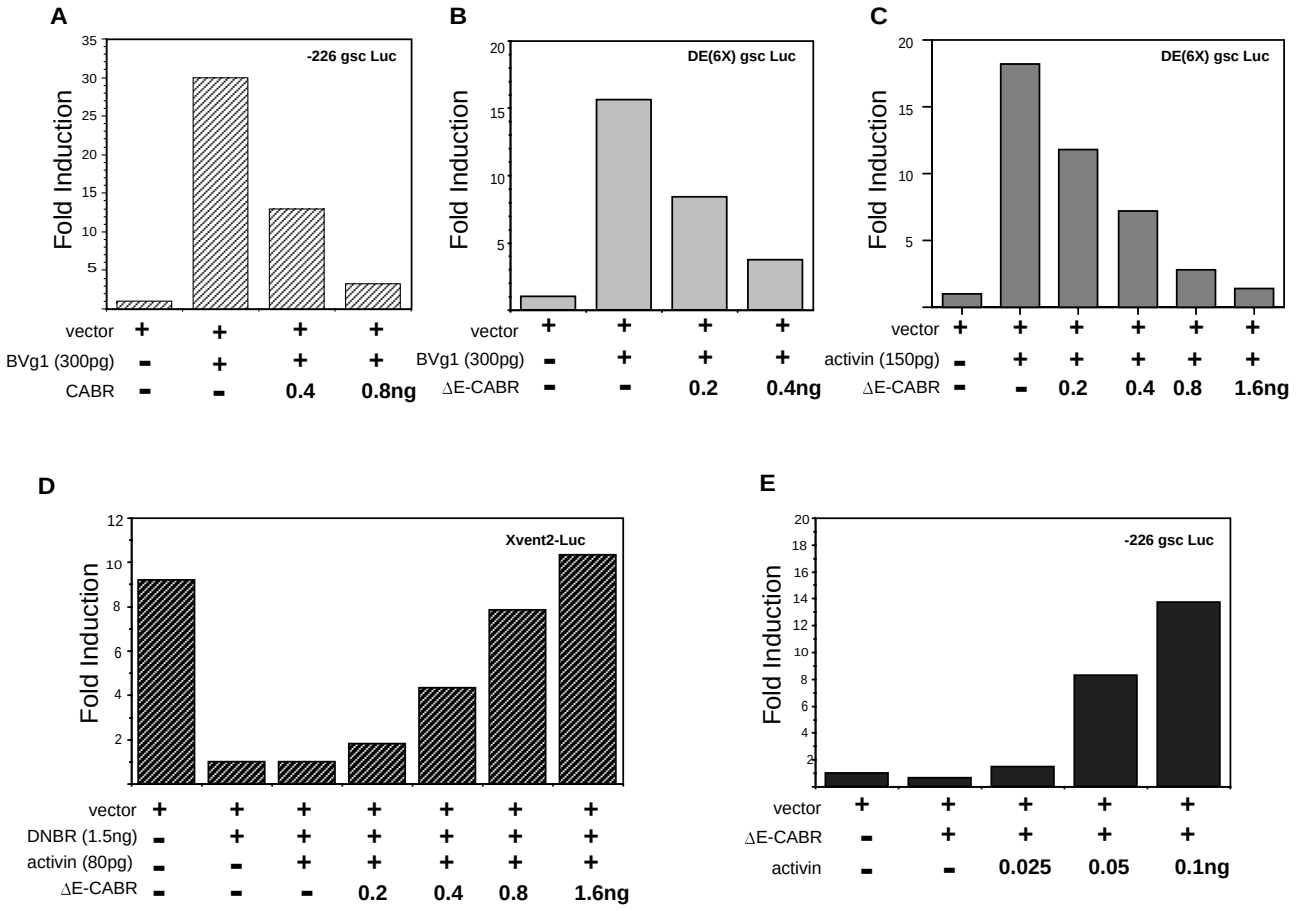

Fig. 4. Induction of the goosecoid (gsc) promoter is regulated by the intracellular antagonism between dorsal (activin/BVg1) and ventral (BMP) signals. (A) A luciferase reporter construct containing 226 bp (–226) of the gsc promoter (Watabe et al., 1995) was injected with BVg1 alone or coinjected with increasing amounts of CABR. As previously demonstrated (Watabe et al., 1995), BVg1 alone induced the reporter construct. The induction was suppressed in a dose-dependent manner with increasing amounts of CABR mRNA. (B,C) The induction by either BVg1 (300 pg) or activin (150 pg) of a hexamer of the distal element (DE6X, also known as the activin/Vg1 response element, A/VRE) are similarly suppressed by intracellular BMP signals provided by the ΔE-CABR. While the DE(6X)gsc/Luc construct responds to activin/Vg1 signals, the induction level is usually somewhat lower than that of –226gsc/Luc. (D) A control experiment to show that BMP signaling can occur in the presence of high amounts (80 pg mRNA) of activin. Induction of the BMP-specific response element, Xvent2, occurs in a dose-dependent manner. (E) The action BMP is not unidirectional as increasing amounts of activin are able to override the suppressive effects of intracellular BMP signals. For all experiments 40 pg of –226 gsc Luc or 20pg of DE(6X) gsc Luc was injected. Each experiments were repeated at least twice.

site (Fig. 4) may be due to differences in formation of the ABC complex that interacts directly with the promoter element.

Synergistic effects of Smads in both activin/BVg1 and BMP signaling

Smads have previously been shown to mediate intracellular signals provided by TGF-β-related proteins (Derynck and Zhang, 1996), and ectopic expression of Smad1 and Smad2 are sufficient to mimic BMP-2/4 and activin/BVg1 signaling, respectively (Graff et al., 1996; Thomsen, 1996; Baker and Harland, 1996). The role of Smads as mediators in the intracellular TGF-β-related signaling cascades raises the possibility that they may play a role in the observed intracellular convergence and antagonism between activin/BVg1 and BMP-2/4 signaling. Using the animal cap assay, we tested whether ectopic expression of Smad1 or Smad2 is sufficient to specifically activate transcription of the luciferase gene from the A/VRE or Xvent2 promoter elements. These experiments would demonstrate not only the specificity of Smad-mediated signaling but, more importantly, that they act through distinct *cis*-acting transcriptional elements for activin/BVg1 (A/VRE) and BMP-2/4 (Xvent2) signals. mRNAs encoding Smads1-4 were coinjected with the A/VRE-Luc or Xvent2-Luc reporter plasmids into developing embryos. Animal caps were isolated at blastula stage, cultured for an additional 90 minutes, and luciferase reporter activity was measured. As shown in Fig. 6, Smad2 and 1, when injected alone, weakly but specifically induced the expression of the A/VRE- and Xvent2-luciferase reporters, respectively. Since Smad4/DPC-4 has been shown to interact and synergize with Smad1, -2 and -3 (Zhang et al., 1996, 1997, Lagna et al., 1996), the weak induction of luciferase expression could be due to a synergy with endogenous Smad4. To test if Smad4 could synergize with the other Smads in similarly activating transcription from the A/VRE and Xvent2 promoter elements, we coinjected Smad4 with the other Smads and the reporter genes. A strong synergistic acti-

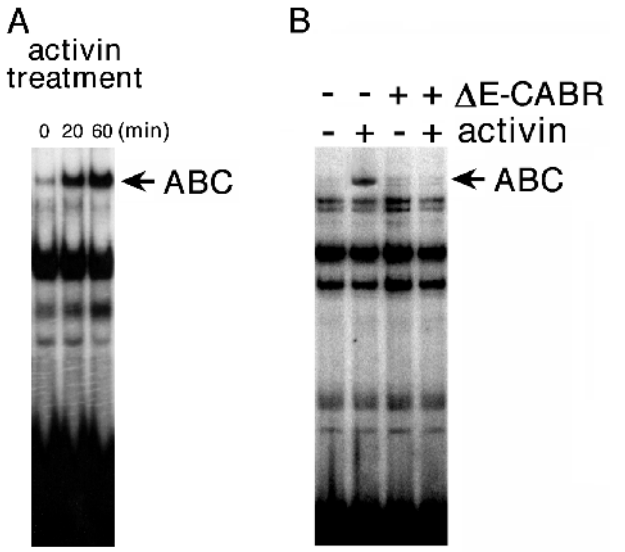

Fig. 5. Antagonism between activin/BVg1 and BMP-2/4 signals affects binding of transcription factors to the goosecoid A/VRE: Electrophoretic mobility shift assays with animal cap extracts. (A) Animal caps excised at stage 8 were treated with soluble activin (200 ng/ml) for the indicated times. A specific A/VRE binding complex (ABC) is induced within 20 minutes of activin treatment above a basal level of activity (zero minutes). (B) Similarly, animal caps isolated from uninjected or ΔE-CABR injected embryos were treated with or without 200 ng/ml activin for 60 minutes. In the absence of ΔE-CABR, induction of ABC is detected. However, in the presence of ΔE-CABR, this induction is blocked, suggesting that BMP signaling transduced by the ΔE-CABR eliminates all induction of ABC by activin. We note that the intensity of ABC is variable and batch dependent.

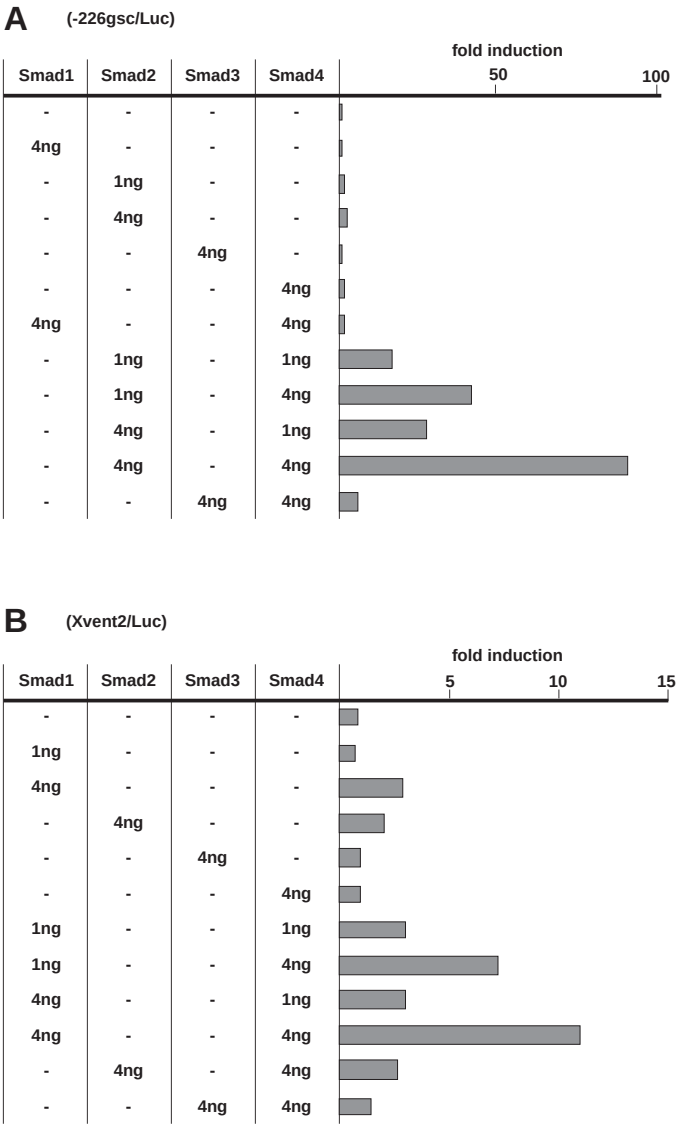

Fig. 6. Synergistic effects of Smads in activin/BVg1 and BMP signaling. Smad mRNAs were microinjected into embryos together with either –226gsc-Luc (A) or Xvent2-Luc reporter gene (B). Animal caps isolated at the blastula stage were cultured in saline for 3 hours, and assayed for luciferase activity. On their own, ectopic expression of Smads had little or no effect on reporter genes. However, co-expression of Smad2 and Smad4 synergistically induces the expression of –226gsc-Luc reporter gene activity 50-100 fold over uninjected animal caps, while co-expression of Smad1 and Smad4 synergistically induces the Xvent2-Luc reporter gene up to 15 fold. Smad3 and Smad4 did not activate reporter genes by themselves or in combination with each other demonstrating the specificity of action of Smad proteins.

vation (as much as 90-fold induction) of transcription from the A/VRE element was observed when Smad2 and -4 were coexpressed (Fig. 6A), whereas Smad4 synergized with Smad1 to induce transcription of the Xvent2-Luc reporter gene (Fig. 6B). Similarly, we found synergistic induction of endogenous Xvent2 and gsc in animal caps after coexpression of Smad1 or -2 together with Smad4 (data not shown). These results illustrate the direct synergistic activation of activin/BVg1 and BMP-2/4-specific *cis*-regulatory promoter elements by Smads and support the notion that Smad4 can potentiate the activity of Smad1 and Smad2 in mediating BMP and activin/BVg1 signaling, respectively.

Smads are effectors in the intracellular antagonism

Smad4 synergizes with Smad1, -2 and -3 and is thought to be required for signaling by these Smads and the corresponding TGF-β receptor complexes (Lagna et al., 1996; Zhang et al., 1996, 1997; the present study; see Fig. 9). Furthermore, Smad4 interacts physically with these Smads and this interaction is thought to be essential for biological activity (Lagna et al., 1996, Wu et al., 1997). Therefore, the convergence and antagonism between the activin/BVg1 and BMP signaling pathways might occur as a result of competition of Smad1 and -2 for Smad4 as a common mediator. In such a scenario, we would predict that the availability of the Smad4 may be limiting such that the relative strength between two TGF-β-related signals would be determined by the level and nature of initial receptor activation. For example, activation of a large number of activin receptors would lead to sequestration of the available Smad4 to mediate activin signaling and concomitantly mute the effects of BMP-2/4 receptor signaling, which also requires Smad4.

To evaluate this possibility, we generated dominant-negative variants of Smad1, Smad2 and Smad4 (DN-Smad1, DN-Smad2, and DN-Smad4 respectively) by deleting a short carboxy-terminal segment of Smad1 and Smad2. A similar

deletion of Smad3 was shown to behave as a dominant-negative mutant in TGF-β signaling, whereas DN-Smad1 was unable to inhibit this response (Zhang et al., 1996). Furthermore, overexpression of DN-Smad1 does not affect the synergistic activity of Smad3 and -4 (Zhang and Derynck, unpublished), further illustrating the specificity of these dominant negative versions. We tested if expression of DN-Smad1 or -2 mRNA, injected separately, could block both activin/BVg1 and BMP-2/4 signaling pathways. Synthetic mRNA encoding either DN-Smad1 or DN-Smad2 was microinjected into embryos together with the –226gsc-Luc reporter plasmid. Animal caps were isolated at blastula stage, treated with activin, and the luciferase activity was measured. We found that both DN-Smad1 and DN-Smad2 inhibited the induction of the reporter gene by activin (Fig. 7A). Similar experiments were performed to examine the effects of DN-Smad1 and DN-Smad2 on BMP-2/4 signaling, but with a minor modification; embryos were coinjected with mRNAs encoding both a DN-BR and a constitutively active ΔE-CABR to induce BMP-2/4 signaling in the absence of endogenous ligand. As shown in Fig. 7B, both DN-Smad1 and DN-Smad2 inhibited the activation of the Xvent2-Luc reporter, indicating that BMP-2/4 signaling mediated by ΔE-CABR had been blocked.

A model in which Smad4 is a common effector in activin/BVg and BMP-2/4 signaling and is the limiting factor in TGF-β signaling was further tested by evaluating the effects of overexpression of a dominant negative or wild type Smad4. This model would predict that a dominant negative variant of Smad4 would inhibit both activin/BVg1 and BMP-2/4-mediated signaling. mRNA encoding DN-Smad4 (Zhang et al., 1996) was microinjected into embryos together with the –226gsc-Luc reporter plasmid. Animal caps were isolated at blastula stage, treated with activin, and luciferase activity measured. We found that DN-Smad4 inhibited the induction of the reporter gene by activin (Fig. 7A). Similar experiments were performed to examine the effects of DN-Smad4 on BMP-2/4 signaling. As shown in Fig. 7B, DN-Smad4 inhibited the activation of the Xvent2-Luc reporter, indicating that BMP-2/4 signaling mediated by ΔE-CABR had been blocked. Since Smad1 and -2 mediate distinct TGF-β signals (Graff et al., 1996), and interact functionally and physically with Smad4 (Lagna et al., 1996; Zhang et al., 1996, 1997; Wu et al., 1997), the observed inhibition is likely to reflect the involvement of Smad4 as a common effector of both signaling pathways.

If the antagonism between activin/BVg1 and BMP signaling pathways occurs as a result of Smad4 sequestration and Smad4 is indeed the limiting factor in TGF-β signaling pathways, cells should be able to overcome the antagonism in the presence of excess amounts of Smad4. Consistent with this hypothesis, Fig. 7C shows that the antagonistic effect of BMP signaling by CABR on activin signaling was specifically eliminated in the presence of excess Smad4, but not by excess Smad1 or Smad2. These results support our interpretation that crosstalk and antagonism between activin/BVg1 and BMP-2/4 signaling occurs as a result of intersecting signaling pathways, in which Smad4, may be the focal point of convergence.

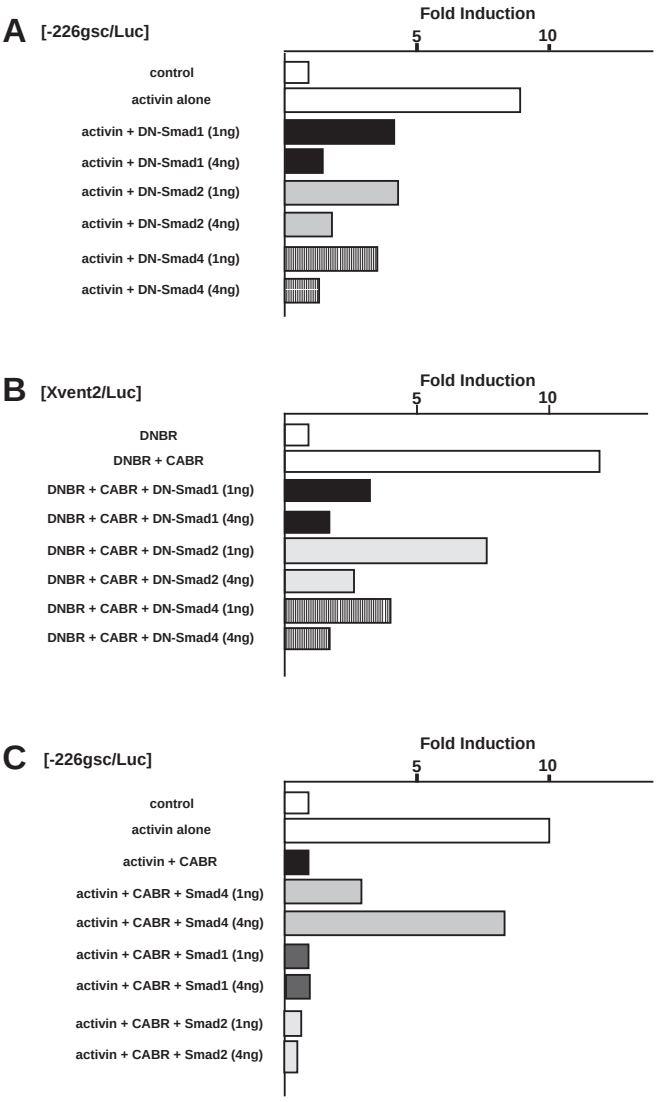

Fig. 7. Smads mediate the intracellular antagonism through a limited pool of Smad4. (A) mRNAs encoding DN-Smad1, DN-Smad2 and DN-Smad4 were microinjected into embryos at the two cell stage together with –226gsc-Luc. Animal caps were isolated at blastula stage, challenged with activin for 3 hours, and subjected to luciferase assays. (B) For Xvent2-Luc activity, the reporter gene was injected together with mixtures of mRNAs containing DN-BR (1 ng), CABR (400 pg), and DN-Smads. DN-Smad1, DN-Smad2 and DN-Smad4 were able to inhibit both activin and BMP signaling in a dose-dependent manner demonstrating convergence of TGF-β-related signaling through the Smads. (C) The antagonistic effects of activin and BMP-2/4 signals can be overcome by the presence of excess Smad4 (1-4 ng), but not by Smad1 and Smad2, indicating that Smad4 is the limiting factor in mediating multiple TGF-β-related signals.

Smads participate in the formation of an activin-induced binding complex (ABC)

Smads are thought to function as regulators of transcription in the nucleus since activation of TGF-β-related receptors results in Smad phosphorylation and translocation to the nucleus (Graff et al., 1996; Hoodless et al., 1996; Baker and Harland, 1996, Macias-Silva et al., 1996). Furthermore, the C-terminal segments of Smads have been shown to regulate transcription of a reporter gene (Liu et al., 1996, Wu et al., 1997) and Smad2 has been shown to form a complex with a winged-helix transcription factor that interacts with the Mix2 promoter (Chen et

al., 1996). To further investigate the role of Smads in transcriptional regulation, we tested whether Smad2 and -4, which mediate activin signaling, play a role in the formation of the ABC at the A/VRE element in response to activin. Cell extracts were prepared from embryos expressing FLAG epitope-tagged Smad2 and myc epitope-tagged Smad4, and used for gel-retardation analysis using ^{32}P-labeled A/VRE oligonucleotide as an interaction probe. We detected increased amounts of ABC formation in cellular extracts isolated from embryos coexpressing Smad2 and Smad4, while coexpression of Smad1 and Smad4 had no effect on ABC formation (Fig. 8A). Importantly, ABC appears to be composed of both Smad2 and Smad4. First, both anti-myc and anti-Flag antibodies were able to supershift the ABC complex (Fig. 8B). Second, both anti-myc and anti-Flag western blot analyses revealed the presence of Smad2-Flag and Smad4-myc in the ABC complex (Fig. 8C). These findings demonstrate that Smads interact and participate in establishing a transcriptional complex, and suggests a mechanistic basis for the central role of Smad4 in mediating signaling by TGF-β-related factors. Considering the synergistic effects of Smad2 and Smad4 on A/VRE-Luc transcriptional activation (Fig. 8A) together with their interactions with the transcription complex at the A/VRE site, we conclude that both Smad2 and Smad4 are central mediators of activin/BVg1 signaling.

DISCUSSION

How growth factor-mediated signaling leads to changes in gene expression and ultimately to changes in cell fate or function is a fundamental question in biology. For the TGF-β superfamily, results over the past few years have revealed that TGF-β-related ligands are received at the cell surface by heteromeric complexes of two receptor types and that the Smad family of proteins are part of the intracellular signaling cascade that transduces the TGF-β-initiated signals (Derynck and Zhang, 1996, Massagué, 1996). These findings demonstrate how TGF-β-related molecules transduce signals but do not address how crosstalk between multiple TGF-β-mediated signals is regulated and integrated. This question is particularly important during *Xenopus* mesoderm formation where multiple TGF-β-related factors have overlapping spatiotemporal activities, and where it remains to be determined how cells receiving such a variety of signals from a single family of factors interpret the information to direct their differentiation along a defined lineage.

Signaling by multiple TGF-β-related factors could, in principle, be regulated at several levels to ensure proper assignment of cell fate and function. First, the level of available ligand may be regulated. This could occur by controlling not only the expression but also the maturation or processing of TGF-β-related molecules, as has been suggested in the case of Vg1 (Thomsen and Melton, 1993; Dale et al., 1993). Alternatively, the availability of TGF-β ligands may be extracellularly regulated by sequestration, which has been demonstrated by the interactions of chordin and noggin with BMPs (Piccolo et al., 1996; Zimmerman et al., 1996). Yet another possibility is that ligand stimulation may be regulated at the level of receptor binding, whereby different ligands may compete for binding to the same receptors, and in this way block each other's ability to activate the receptors. BMPs and activin can bind to the same receptor complexes and could physically interfere with each other's ligand/receptor interactions (Childs et al., 1993; Brummel et al., 1994; ten Dijke et al., 1994; Yamashita et al., 1995, Rosenzweig et al., 1995). These models deal only with extracellular modes of regulating TGF-β signaling and contrast with possible intracellular mechanisms. Here, we provide evidence for an intracellular mechanism of regulation and convergence of the different TGF-β signaling pathways.

Dorsalizing activin/BVg1 and ventralizing BMP signals converge intracellularly

Previous studies have demonstrated that activin-mediated

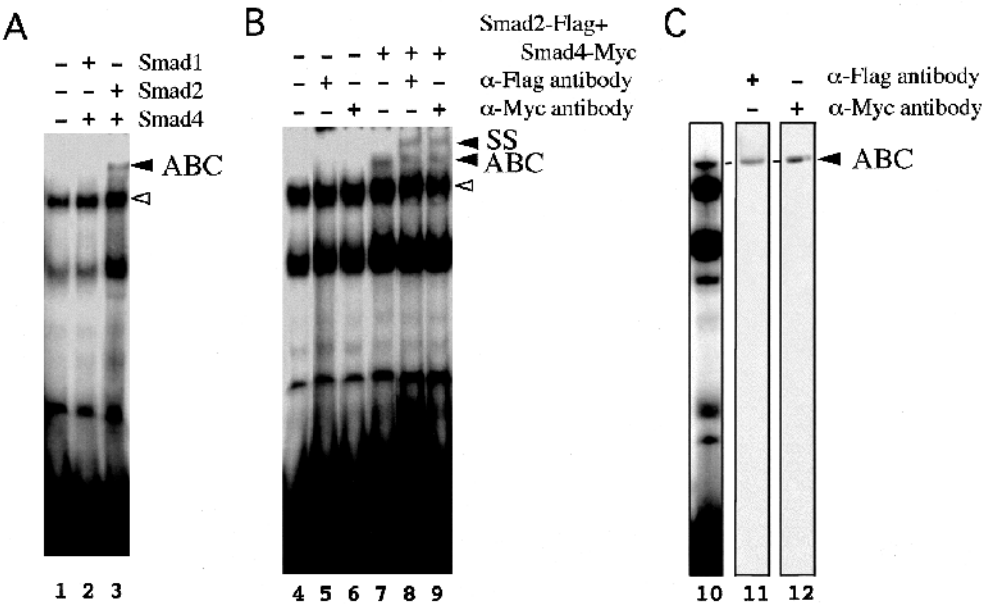

Fig. 8. Smad2 and Smad4 participate in the formation of an activin-induced binding complex (ABC). Synthetic mRNAs encoding flag epitope-tagged Smad1 and Smad2 were microinjected together with myc epitope-tagged Smad4 into 2-cell stage embryos. Extracts were prepared from animal caps and incubated with a ^{32}P-labeled gsc A/VRE probe. Increased amounts of ABC formation was detected in cellular extracts isolated from cells coexpressing Smad2 and Smad4 (A). ABC (B; lane 7) was supershifted by both anti-Flag (lane 8) and anti-myc antibodies (lane 9). Western blot analysis of gel shift (C; lane 10) also revealed the presence of both Smad2 (lane 11) and Smad4 (lane 12) in ABC, suggesting that both Smad2 and Smad4 are part of the ABC. Formation of ABC was specific for Smad2 and -4 since other combinations did not induce ABC formation and were not found in a supershifted complex. The open arrow indicates a second, independent A/VRE binding complex. We note that the intensity of ABC is variable and batch dependent.

signals could be blocked by the presence of BMP-4 ligand (Dale et al., 1992; Jones et al., 1992) and that signals from these two pathways antagonize each other (Fainsod et al., 1994; Steinbeisser et al., 1995). However, these observations did not provide a mechanistic basis of this antagonism and did not allow a discrimination between extracellular or intracellular mechanisms. In the current study, we have used an activated variant of a type I BMP-2/4 receptor to show that activin/BVg1-mediated signals can be attenuated by BMP-2/4 signaling, independent of BMP ligand. This antagonism regulates the expression of the activin/BVg1 target gene, *gsc*, and can be modulated by increasing amounts of activin/BVg1 signaling or BMP-2/4 signaling by ΔE-CABR. Our results demonstrate a crosstalk mechanism between intracellular signals induced by activated activin/BVg1 and BMP-2/4 receptors, and suggest a mechanism of differential regulation during mesodermal patterning when multiple TGF-β signaling pathways are active.

During mesoderm formation, different members of the TGF-β superfamily are present including BMPs, nodal-related factors and activin/BVg1-like activity. While the mRNAs for these factors may not be present at the same time, the activities of these factors overlap in gastrulating embryos. For instance, the induction of a secondary axis after removing BMP-2/4 signals from the ventral region of the embryos may be caused by unmasking maternal activin/Vg1-like activity. In accordance with this, observations by Jones et al. (1996) suggest that BMP-4 is likely to inhibit activin/BVg1 signaling during gastrulation. While ectopic expression of BMP-4 mRNA failed to inhibit induction of *gsc* expression in embryos, the maintenance of expression was reduced. This mode of BMP-4 activity is consistent with its expression pattern in embryos, which peaks during gastrulation. The role of BMP-2 and BMP-7 during gastrulation has not been established, although it is known that they are maternally provided (Nishimatu et al., 1992).

We note that the inhibitory effect of BMP4 mRNA expression on *gsc* expression is different from that of CABR. When BMP-2/4 signaling was provided in a ligand-independent manner by CABR, the induction of activin and BVg1 was blocked at the onset of gastrulation. The observed differences in *gsc* activation between BMP-4 mRNA injection and CABR expression may be due to the different onset of BMP-2/4 signaling resulting from these manipulations. BMP-4 translated from injected mRNA requires maturation and processing and subsequent activation, which may not occur until gastrulation, the time when Jones et al. (1996) demonstrated BMP-4 activity. In contrast, CABR does not require any post-translational events and, thus, may be capable of transducing BMP-2/4 signals as soon as the receptors are expressed, i.e. before gastrulation.

Interaction between Smads

Smad proteins have been shown to directly mediate specific TGF-β-related signaling in a range of species from *Drosophila* and *C. elegans* to mammalian cells (Graff et al., 1996; Liu et al., 1996; Eppert et al., 1996; Thomsen, 1996; Baker and Harland, 1996; Raftery et al., 1995; Savage, 1996; reviewed in Derynck and Zhang, 1996). Current evidence suggests that Smad1 mediates BMP-2/4 signaling whereas Smad2 and -3 mediate activin and TGF-β responses. Our results support this notion and demonstrate a role for Smad4 in which it synergizes with Smad1 and -2 to induce transcription from activin/BVg1- and BMP-2/4-responsive reporter genes, respectively. These data are consistent with the observed synergy of Smad4 with Smad3 in mediating TGF-β-like responses (Zhang et al., 1996) and with Smad1 or Smad2 in mediating BMP-2/4 and activin-like responses, respectively (Zhang et al., 1997). Furthermore, while this work was in progress, Lagna et al. (1996) also reported that expression of endogenous *gsc* and *Xvent1* genes required functional Smad4, and that Smad2 and -4 can synergize to induce expression from the PAI-1 promoter. Our data now provide additional evidence that Smad4 synergizes with both Smad1 and 2, and show that this synergy occurs at the level of transcription from activin/BVg1- and BMP-2/4-responsive promoters. Results with the gsc A/VRE reporter suggest that the antagonism between BMP-2/4 and activin/BVg1 signaling is likely due to competition for a single transcription factor (i.e. Smad 4). The observation that Smad4 can physically interact with Smad1 and -2 is consistent with this notion (Lagna et al., 1996). Taken together, these results suggest that Smad4 may be a focal point for Smad action and function as a regulator of the intracellular interplay between different TGF-β superfamily signals. While the specificity of signaling may be mediated by Smad1, -2 and 3, the relative signaling strengths may be regulated by a limited pool of Smad4.

The ability of Smad4 to physically associate with the other Smads in heteromeric complexes (Lagna et al., 1996; Wu et al., 1997; Shi et al., 1997) and the requirement of the heteromeric complex formation for Smad activity suggest a possible mechanism for the observed intracellular antagonism between activin/BVg1 and BMP-2/4 signaling (see Fig 9). Based on our current knowledge, the activated activin/BVg1 and BMP-2/4 receptor complexes may directly induce phosphorylation of associated Smad2 and 1, respectively, which in turn can form a complex with Smad4 and undergo nuclear translocation. Therefore, we propose that activation of two TGF-β-related signaling pathways will result in a competition for Smad4, and that the outcome of this competition will determine the relative strengths and antagonism of the signals, which lead to the promotion of a particular differentiation pathway and cell fate. In support of this notion, we have shown that dominant-negative Smad4 mutants can inhibit both activin/BVg1 and BMP signaling. Furthermore, we demonstrated that Smad4 is indeed a limiting factor in TGF-β signaling pathways since an excess of Smad4 can override each of these antagonistic effects. The additional observations that increased Smad4 expression results in the formation of both ventral and dorsal mesoderm (Lagna et al., 1996; Zhang et al., 1997), and that a dominant negative Smad4 can inhibit the activity of Smad1 and Smad2 on mesoderm induction (Lagna et al., 1996) and prevents nuclear translocation of the other Smads (Zhang et al., 1997) are all consistent with this notion. Finally, we provide direct evidence that both Smad2 and -4 associate to form the ABC transcriptional protein complex that binds to the activin/BVg1-responsive A/VRE promoter element (Fig. 8).

In vivo relevance of competition for Smad4

What is the in vivo significance of our overexpression and dominant-negative studies? Since activin and BMP signaling

cascades were manipulated by either providing exogenous activin or BMP signaling or by inhibition of their signaling by dominant-negative Smads, one may argue that the signaling pathways may be artificially saturated. Therefore, a signaling component utilized by both activin and BMP pathways would become limiting, and this may not occur in vivo. A definitive answer to this question will require further analysis of the relative abundance of the Smad proteins in vivo and the relative levels of free Smad4 and Smad1/Smad4 and Smad2/Smad4 complexes. However, our evidence suggests that the experimental conditions in animal caps may resemble the in vivo situation where TGF-β signaling may occur at saturating levels. First, we have previously shown that the reporter gene containing a multimerized A/VRE is activated approximately 25-35 fold in activin-treated animal caps when compared to untreated control animal caps (ectodermal tissue), and this appears to be the maximum level of induction that can be achieved using varying concentrations of activin (Watabe et al., 1995). When the same reporter gene was microinjected into various regions of embryos to detect endogenous levels of activin/BVg1 activity, we found that the reporter gene was activated to a similar extent (35 fold) in mesoderm/endoderm regions, where activin/BVg1 activity is thought to reside, when compared to the activity detected in ectoderm (Watabe et al., 1995). The similarity in the maximum attainable activity of A/VRE-containing reporter genes between activin-treated animal caps and embryonic mesoderm in situ suggest that activin signaling in the embryos operates at a maximal level. Second, expression of CABR was able to activate the Xvent2 promoter to a level of only two-fold higher than the endogenous BMP activity level present in animal caps (see Fig 1), implying that BMP signaling also reaches near saturation conditions in animal caps. Third, the finding that overexpression of Smad2 and -4 can hyperinduce the reporter gene over 90-fold, i.e. much higher than the levels achieved in the experiments above, suggests that Smad -4 may be a limiting factor in activin/BVg1 signaling in vivo. Taken together, these results suggest that near saturation conditions of TGF-β-related signaling indeed occur in vivo, and that the cellular interpretation of relative TGF-β signaling activity may be mediated by the competition for Smad4.

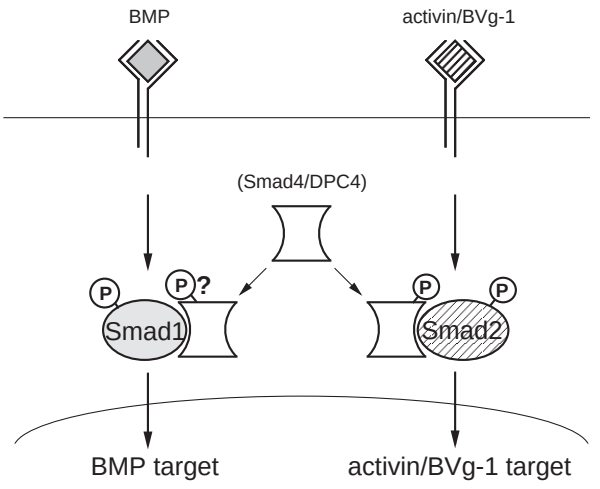

Fig. 9. Model for intracellular cross-talk between activin and BMP signaling. BMP signaling is mediated by both Smad1 and Smad4 proteins, while activin/BVg1 signaling is mediated by both Smad2 and Smad4. Smad1, 2, and 3, appear to mediate the specificity of the signal, and Smad4 may regulate signal strength. If both activin/BVg1 and BMP signaling cascades are activated in the same cell, the relative strength of the signals may be reflected in the competition for Smad4.

Smads as transcriptional regulators of TGF-β signaling

Smads are now clearly recognized as mediators of intracellular signaling by TGF-β-related factors, and their possible role as nuclear transcription regulators receives increasing experimental support. Smad1 fused to a DNA binding domain can behave as transcriptional activator in transfected mammalian cells (Liu et al., 1996) and Smad3 can act similarly in yeast (Wu et al., 1997). Furthermore, Smad2 can bind to the DNA binding FAST-1 protein that directly interacts with the Mix2 promoter (Chen et al., 1996) and Mad binds directly to a promoter sequence in the vestigial gene (Kim et al., 1997). We have extended these observations by demonstrating that not only Smad2 but also Smad4 participate in the formation of a transcriptional complex on a direct target gene, gsc, which is induced by activin. Considering the functional similarity with the activin-dependent interaction of Smad2 with the Mix2 promoter and its interaction with the DNA-binding FAST-1, it is likely that the heteromeric Smad2/4 complex following nuclear translocation also has to interact with a DNA-binding transcription factor to function as transcriptional regulator of the gsc gene. In the case of the Mix2 promoter, FAST-1 is the protein partner that confers DNA-binding (Chen et al., 1996). However, the A/VRE sequence in the gsc promoter differs from the activin-responsive element in the Mix2 promoter, which might suggest the involvement of a different DNA-binding protein. Furthermore, our initial characterization of a gsc A/VRE DNA-binding protein indicates that it is distinct from FAST-1 and binds specifically to the gsc A/VRE and not to the Mix2 activin-responsive element (T. W. and K. C. unpublished observations). It remains to be determined if and what other factors are involved in forming the ABC complex on the gsc promoter. However, the differences in the activin-responsive sequences and transcription factors that are involved in transcription of the *gsc* and *Mix2* genes suggest that both common (i.e. Smads) and unique transcription factors may mediate activin signals. Further characterization of A/VRE binding proteins and their interactions with Smads should facilitate our understanding of the signaling by activin and other TGF-β-related factors.

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