Autoregulation of Xvent-2B; Direct Interaction and Functional Cooperation of Xvent-2 and Smad1*

Received for publication, September 5, 2001, and in revised form, October 31, 2001
Published, JBC Papers in Press, November 9, 2001, DOI 10.1074/jbc.M108524200

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Members of the Xvent-2 homeodomain transcription factor family are immediate response genes of BMP-4 signaling. The bone morphogenetic protein response element (BRE) of Xvent-2B was previously identified and characterized with respect to Smad1 and Smad4 binding sites. In this study, we further report on the transcriptional regulation of Xvent-2B. We provide evidence that Xvent-2B (Xvent-2) maintains its own expression through autoregulation. This activity was demonstrated for the endogenous gene by reverse transcriptase-PCR analysis and was found to be insensitive to cycloheximide. Localized by DNase I footprinting were several Xvent-2 binding sites within the proximal upstream region including the BRE. In the early Xenopus embryo, the BRE was shown to be sufficient to drive expression of a green fluorescent protein reporter in a similar pattern compared with the endogenous gene. Furthermore, Xvent-2B was able to activate the BRE in luciferase reporter assays, and in co-injection experiments Xvent-2B and Smad1 were found to synergistically activate the BRE. Moreover, glutathione S-transferase pull-down experiments demonstrated that Xvent-2B directly and specifically interacts with Smad1. This association was mediated by the MH1 domain of Smad1 and required the C-terminal domain of Xvent-2. The failure of an Xvent-2 mutant lacking the C terminus to stimulate the BRE underlines the significance of the C-terminal domain in the described autoregulatory loop.

Bone morphogenetic proteins (BMPs),1 as well as other members of the TGF-β superfamily of extracellular signaling molecules, are essential regulators of critical cellular events including cellular differentiation and apoptosis in early embryonic development (1, 2). BMP-4 has been shown to act as a morphogen in the patterning of the dorsal-ventral axis of the amphibian embryo, with high concentrations specifying the most ventral tissues (such as blood) and lower concentrations specifying muscle (3). Within the ectoderm, BMP-4 suppresses neural differentiation and promotes formation of epidermis (4). The Xvent-2 (Xvent-2B, Vox, Xbr-1, and Xom) and Xvent-1 (Xvent-1B and PV.1) homeodomain transcription factors have been identified as both targets and pivotal mediators of BMP function in Xenopus and Zebrafish (5–11). Consistent with the Xvents as downstream effectors of BMP, these genes are expressed in the ventral and lateral marginal zones and are excluded from the dorsal-most regions of early Xenopus embryos. Ectopic expression of the Xvents in the dorsal blastomeres mimics the BMP-induced ventralization of Xenopus embryos and results in the activation of BMP-induced genes. Moreover, these proteins can rescue the dorsalizing effects of the dominant negative BMP receptor.

The intracellular transducers of TGF-β signaling are the Smad family of proteins (reviewed in Refs. 12 and 13). Ligand binding to TGF-β receptors stimulates the transient association and subsequent activation of receptor-specific Smads by phosphorylation of C-terminal serine residues. Smad1, Smad5, and Smad8 are substrates for BMP receptors, whereas Smad2 and Smad3 are mediators of TGF-β and activin-like signaling (14–20). Phosphorylation by the type I receptor triggers heterodimerization of receptor-specific Smads with Smad4 and translocation to the nucleus, where the Smad complex directly participates in the regulation of target genes.

The Xvent-2B and Xvent-2 promoters, like other immediate response genes of TGF-β members, contain Smad binding elements that have been shown to confer BMP-4 inducibility (21, 22). We have previously localized a BMP-4 responsive region between –275 and –152 of the 5′-flanking region of Xvent-2B. This region was shown to contain two Smad1 binding sites and an essential CAGAC motif that serves as a Smad4 recognition element. The upstream Smad1 binding sites were not required for stimulation by BMP-4; however, these sites were shown to be required for full transcriptional activation. Although it has been shown that the Smads are sequence-specific transcriptional modulators, specificity of action requires the cooperation of the Smads with other transcription factors (reviewed in Refs. 23–25). These include examples of factors that synergistically cooperate with the Smads via binding to adjacent elements and/or direct interaction with the Smads.

The zinc finger protein OAZ has been identified recently as a Smad1 cofactor involved in the BMP-induced transcriptional activation of the Xvent-2 gene (22). OAZ is ubiquitously expressed in the early Xenopus embryo and thus is a good candidate for the Smad1/Smad4 cofactor required for the initial Xvent-2 activation. However, by early neurula stages expression of OAZ becomes restricted to the anterior neural plate, a region where expression of the Xvent-2 gene family is excluded (5, 9). This suggests the existence of additional factors that are required to maintain and restrict the Xvent-2 expression.

Because Xvent-2 has been shown to act not only as a repressor but also as an activator (26), we investigated in this study whether Xvent-2 can fulfill this role through an autoregulatory mechanism. We provide evidence that Xvent-2B can maintain its own transcriptional regulation. Furthermore, we demon-
strate that activation by Xvent-2B and Smad1 occurs in an additive fashion, and Xvent-2B can associate directly and specifically with Smad1.

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—His-Xvent-2 was generated by subcloning the Xvent-2 cDNA into pRSET vectors (Invitrogen) for expression in bacteria. GST fusion proteins were created by subcloning Xenopus Smad1, Xenopus Smad2, human Smad3, human Smad4, and Xvent-2 cDNAs into the pGex4T3 vector (Amersham Biosciences). The Smad1 deletion mutants were prepared using the following primers and cloned in the pCS2 vector as described: Smad1 linker/MH2 domains (aa 1–467) up-5’-CCGGGATCCATGAGTTCTTCCGACCCAT-3’ and down-5’-CCGGGATCCATAGATGTTGGCTATGGCTTC-3’ and Smad1 L domain (aa 132–209) up-5’-CCGGGATCCATGAGTTCCCATGAGACCGA-3’ (BanHI) and down-5’-CCGGGATCCATGAGTTCTTCCGACCCAT-3’. Smad1 promoter reporter constructs and Xvent-2B promoter were described previously (6, 21). The Xvent-2B (~275–1528) promoter pGL3 construct was linearized with HindIII, and the overhang was filled in to create a blunt fragment. The fragment was then excised by cleavage with KpnI and cloned into the KpnI/BglII sites of the reporter plasmid pGL3-Control.

**Microinjection and Luciferase Assays**—Xenopus embryos were obtained by in vitro fertilization, dejellied in 2% cysteine hydrochloride in 0.1× MSB (10 mM HEPES pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO3, 0.82 mM MgSO4, 0.41 mM CaCl2, 0.66 mM KNO3) and staged according to Nieuwkoop and Faber (27). Embryos were injected in the 4-cell stage into both dorsal blastomeres (total 10 nl, 20 pg/blastomere). MBSH containing 4% Ficoll. Deletion mutants were injected at the 8-cell stage. All experiments were performed with at least two different batches from at least two different females except for experiments in which Smad1 linker/MH2 domains were injected. In those experiments, 8-cell stage embryos were injected in one blastomere. The ability of Xvent-2B to activate Smad1 was measured by the dual luciferase assay system (Promega) according to the manufacturer’s protocol. The puromycin selection (275/275 fused to the minimal promoter (from pGL3 vector) at 5°C annealing temperature), Xvent-2 (upstream primer, 5’-CATCATCATTCCCTGGG-3’; downstream primer, 5’-TA- GAGGTTGATGAATGCTG-3’; 50 °C annealing temperature), or Xvent-2B (upstream primer, 5’-GGAGCTTAAATCCTCAAC-3’; downstream primer, 5’-GAGCGGATGACGCTG-3’; 50 °C annealing temperature).

**Preparation of Embryonic Extracts**—Embryos were injected dorsally at the 4-cell stage with Xvent-2 (400 pg) or Xvent-2B (400 pg) RNA. At stage 7.5 embryos were treated with 25 μg/ml cycloheximide (Sigma) until control embryos reached stage 10.5. Total RNA was isolated using RNeasy mini columns (Qiagen; RNeasy protocol for isolation of total RNA from animal tissues). DNase digestion as performed by the addition of 1.5 μl of RNase-free Dnase I (Roche) and 5 μl of 25 mM MgCl2 to 50 μl of total RNA. The reaction was incubated for 20 min at 37 °C followed by inactivation of Dnase by heating for 10 min at 75 °C. cDNA synthesis was performed under the following conditions: 1X RT reaction buffer (Amersham Biosciences), 10 ng of oligo(dT)12–18, 10 μl of random primer, 0.2 mM dNTPs, 26.8 units of RNAGuard™ RNase inhibitor (Amersham Biosciences), 10 units of Maloney murine leukemia virus reverse transcriptase (Amersham Biosciences), and 600 ng total RNA. cDNA synthesis products were ethanol precipitated and resuspended in 4 μl of water. cDNA fragments have been used: Histone H4 (upstream primer, 5’-CGGGAATAATTCGAGGAGC-3’; downstream primer, 5’- ATCATGAGTGGTCG-3’; 56 °C annealing temperature), Xvent-2 (upstream primer, 5’-CATCATCATTCCCTGGG-3’; downstream primer, 5’-TAGAGGTTGATGAATGCTG-3’; 50 °C annealing temperature), or Xvent-2B (upstream primer, 5’-GGAGCTTAAATCCTCAAC-3’; downstream primer, 5’-GAGCGGATGACGCTG-3’; 50 °C annealing temperature).

**RNase I Footprinting**—The desired promoter fragments were excised from pGL3 vector (~275–1528 fused to the minimal promoter (~32– +34) and 3’-labeled on one strand by a fill-in reaction with [α-32P]dUTP and Klenow DNA polymerase. Binding reactions were carried out on ice for 30 min at 30 °C using binding buffer (30 mM Tris-HCl pH 7.5, 30 mM KCl, 1 mM MgCl2, 1 mM dithiothreitol, and 13.2% glycerol) containing 1 μg of poly(dI-dC) and 1 ng of the gel-purified probe. After 5 min of pre-incubation the protein was added and incubated for 30 min. The concentration of MgCl2 was subsequently raised to 5 mM for Dnase I footprinting, and 0.065 units (free DNA) or 0.195 units (DNA + protein) of Dnase I were added at room temperature for 45 s. The DNase I digestion was stopped by addition of an equal volume of sample buffer (66% deionized formamide, 20 mM EDTA, and 660 mM sucrose). Sequencing reactions were performed according to the method of Maxam and Gilbert (30). After pre-electrophoresis for 2 h at 70 watts, samples were analyzed by 7% denaturing PAGE at 60 watts in 1x Tris borate EDTA.

**RESULTS**

**Positive Autoregulation of Xvent-2**—The ability of Xvent-2B to activate itself was studied in Xenopus embryos injected in both ventral blastomeres at the 4-cell stage with mRNA encoding the closely related Xvent-2. The Xvent-2 and Xvent-2B genes show identical expression patterns and appear to be functionally redundant. These genes probably arose either by the known tetraploidization within Xenopus laevis or by a separate gene duplication event. Although the amino acid sequences between Xvent-2 and Xvent-2B are highly conserved (83% overall sequence identity, 98% within the homeobox), the cDNA sequences are sufficiently divergent to allow the design of primers that can distinguish between the endogenous Xvent-2B and the injected Xvent-2B mRNA. Overexpression of Xvent-2 resulted in the activation of Xvent-2B and vice versa; Xvent-2B stimulated the transcription of Xvent-2 (Fig. 1, compare lanes 1 and 2). To test whether this activation required the de novo synthesis of downstream factors of Xvents, embryos were incubated in cycloheximide just before the onset of zygotic transcription at midblastula transition. This procedure allows for the translation of previously injected RNAs but prevents translation of zygotic transcripts. In both instances, the activation was insensitive to cycloheximide.
treatment, suggesting that the activatory effect is direct (Fig. 1, lanes 3 and 4). Because both proteins bind to identical target sequences and the proximal promoter sequences of both genes are highly conserved showing 95% nucleotide identity within the −240/+1 upstream region (6, 22), it is reasonable to assume that both Xvent-2 and Xvent-2B are subject to autoregulation. This conclusion is consistent with our previous observation that Xvent-2B, but not the more distantly related Xvent-1B, can activate the 5′-flanking region of Xvent-2B spanning from −275 to +34 (6).

**BRE-GFP-driven Expression in Xenopus**—As the previously characterized BRE (21) by sequence inspection contains putative Xvent-2B binding elements, we investigated the ability of this element to direct correct spatial and temporal expression in *Xenopus* embryos using a transgenic approach (29). The BRE (−275/−152) together with the minimal promoter were cloned in front of the GFP reporter, and the linearized construct was co-injected with isolated sperm nuclei into unfertilized eggs. GFP expression was found to be restricted to the ventral marginal zone of the early gastrula embryo (Fig. 2, B–D). As gastrulation proceeded, expression expanded dorsally but was excluded from the dorsal lip and later from the dorsal midline. Comparison of GFP expression with a whole mount in situ hybridization of endogenous Xvent-2B transcripts (Fig. 2A) demonstrates that the identified BRE is capable of driving correct spatial expression of the reporter gene in the early *Xenopus* embryo.

**Xvent-2 Binds Specifically to the Xvent-2B Promoter**—Initial gel shift experiments had shown that both Xvent-2 and Xvent-2B proteins bind to the Xvent-2B promoter (data not shown). To further demonstrate that Xvent-2 is directly involved in an autoregulatory circuit, DNase I footprinting experiments were performed with 32P-labeled DNA fragments of the Xvent-2B promoter. As shown in Fig. 3, increasing concentrations of bacterially expressed Xvent-2 resulted in a concentration-dependent protection of distinct regions within the Xvent-2B promoter spanning from −275 to −50, relative to the transcription start site. Three of the identified Xvent-2 binding sites are located within the delineated BMP response element of the Xvent-2B promoter. All protected regions contain at the core a 5′-TAAT-3′ homeodomain consensus binding site (31) that was also shown to be a preferred Xvent-2 binding motif (26, 32).

**Synergistic Activation of the BRE by Smad1 and Xvent-2**—To determine whether the BRE is sufficient to confer activation by Xvent-2B, promoter reporter assays were performed. Shown in Fig. 4 dorsal co-injection of Xvent-2B mRNA, with the luciferase reporter under the control of the BRE in transgenic *Xenopus* embryos at stage 10 (B), 11.5 (C), and 13 (D). GFP expression was visualized by fluorescence in living embryos. Note that in both the integrated (diffuse staining) as well as unintegrated DNA (punctuate staining) are not expressed within the dorsal lip or dorsal midline.
us to evaluate whether these two factors could physically interact using GST pull-down assays. RNA encoding Myc-tagged Xvent-2 was injected into both blastomeres of 2-cell stage embryos, and total cell lysates were prepared at stage 11 and incubated with GST or GST-Smad fusion proteins. Xvent-2B from these lysates specifically bound to GST-Smad1 but not to GST, GST-Smad4, or GST-Smad3 (Fig. 5).

To determine whether this interaction was direct, analogous pull-down experiments using GST-Xvent-2 and in vitro-translated Smad proteins were performed. In this assay, Smad1 bound strongly to GST-Xvent-2 but not to the GST control (Fig. 6, lanes 7 and 4). A slight interaction with Smad3 was observed in this assay; however, binding of Xvent-2 with Smad4 was not detected (Fig. 6, lanes 8 and 9).

Deletion mutants of Smad1 were created and used in the pull-down assay to map the Xvent-2 binding domain. In contrast to the MH2/linker mutant or the linker domain alone, a strong interaction was observed with the MH1/linker construct and the MH1 domain alone (Fig. 7, compare lanes 13 and 15 with lanes 12 and 14). Taken together, these results demonstrate that the Smad1 MH1 domain is required and sufficient to facilitate Xvent-2 binding.

To delineate the domains within Xvent-2 responsible for Smad1 binding, mutants were created in which the N- or C-terminal regions flanking the homeodomain were removed. GST-Smad1 strongly bound the mutant lacking the N-terminal 184 amino acids, whereas the removal of the C-terminal domain completely abolished binding (Fig. 8A, compare lanes 8 and 9). Thus, Xvent-2 domains that are critical for Smad1 are located outside of the homeodomain within the C terminus.

Further support for the significance of the C-terminal domain of Xvent-2 in the autoregulatory loop was gained from co-injections of the ~275/−152 promoter/luciferase construct with Xvent-2, Xvent-2ΔC, Xvent-2ΔN, and Xvent-1 RNA (Fig. 8B).
The Smad family of transcriptional factors interacts in a sequence-specific manner with regulatory elements within target genes. Because the DNA binding affinity of Smads is low, Smads are thought to cooperate with additional factors to increase specificity and selectivity (33). Smads have been shown to associate and synergize with a variety of transcription factors including ubiquitously expressed and tissue-specific transcription factors as well as those that receive regulatory signals from other signaling pathways (reviewed in Refs. 23, 24). A limited number of Smad binding partners are able to discriminate among members and form complexes with pathway-restricted Smads of the same class. The first such factor was the winged-helix/forkhead DNA-binding factor FAST-1, which in response to TGF-β and activin signaling binds to Smad4 via direct interaction with Smad2/3 and regulates transcription of the Mix2 and goosecoid genes (34–36).

The present study identifies Xvent-2 as a Smad1-specific coactivator. In contrast to the growing list of partners for Smad2/3/4, only a few such partners have been reported for Smad1 (23–24). OAZ (Olf-1/EBF-associated zinc finger) is the only known coactivator that is specific for vertebrate Smad1 (22). This multi-zinc finger protein was shown to form a complex with Smad1 via the MH2 domain in response to BMP signaling and activate a BRE within the Xvent-2 promoter. Whereas overexpression of a DNA-binding OAZ mutant that is not competent in Smad1 binding reduced expression of Xvent-2, the expression patterns of OAZ and Xvent-2 in Xenopus suggest that additional factors are involved in Xvent-2 transcriptional activation.

We have demonstrated through RT-PCR experiments in the presence of cycloheximide that Xvent-2 directly participates in its own maintenance through an autoregulatory mechanism. Consistently, DNase I footprinting analysis identified several Xvent-2 binding sites within the Xvent-2 BRE. Moreover, the BRE/minimal promoter was shown to drive correct spatial expression of the GFP reporter in transgenic Xenopus embryos at early developmental stages. This element was also sufficient to confer transcriptional activation by Xvent-2 in luciferase reporter assays. However, reporter co-injection assays using various deletion mutants within the BRE were unable to distinguish functionally between the various Xvent-2 binding sites. This element was also sufficient to confer transcriptional activation by Xvent-2B in luciferase reporter assays. However, reporter co-injection assays using various deletion mutants within the BRE were unable to distinguish functionally between the various Xvent-2 binding sites. The finding that Xvent-2 gene activation in the presence of cycloheximide depends on the previous synthesis of Xvent-2 protein is significant because it demonstrates that the activation is not occurring simply by the relief of repression mediated by dorsal genes (37). This mechanism was postulated because Xvent-2 and, for example, goosecoid have been shown to mutually repress their expression (5, 38).

Evidence that the Xvent-2B autoregulation can be modulated by Smad1 was obtained by Xvent-2B BRE luciferase reporter assays. Co-injection of Smad1 and Xvent-2B afforded higher levels of reporter activation than were obtained with either factor alone. This synergistic activation was also observed on a reporter mutated in the Smad4 binding site that was unresponsive to BMP-4 and Smad1/Smad4 co-injections. This is somewhat unexpected because overexpression of BMP-4 results in the activation of endogenous Xvent-2B that should be able to activate the promoter in the same manner as ectopically expressed Xvent-2B. However, sufficient levels of the endogenous Xvent-2 protein may not have been reached at the time the reporter assays were performed.

Neither Xvent-1 nor Xvent-2ΔC was able to stimulate the reporter activity as was observed with Xvent-2 and also with Xvent-2ΔN. These results are consistent with the notion that the C-terminal domain of Xvent-2 is required as a transcriptional activation domain that interacts with the MH1 domain of Smad1.

DISCUSSION

The Smad family of transcriptional factors interacts in a sequence-specific manner with regulatory elements within target genes. Because the DNA binding affinity of Smads is low, Smads are thought to cooperate with additional factors to increase specificity and selectivity (33). Smads have been shown to associate and synergize with a variety of transcription factors including ubiquitously expressed and tissue-specific transcription factors as well as those that receive regulatory signals from other signaling pathways (reviewed in Refs. 23, 24). A limited number of Smad binding partners are able to discriminate among members and form complexes with pathway-restricted Smads of the same class. The first such factor was the winged-helix/forkhead DNA-binding factor FAST-1, which in response to TGF-β and activin signaling binds to Smad4 via direct interaction with Smad2/3 and regulates transcription of the Mix2 and goosecoid genes (34–36).

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Smads have been shown to associate with several homeodomain transcription factors. Milk and Mixter, members of the paired-like homeodomain transcription family, mediate activation of the Xenopus goosecoid promoter by recruiting Smad2 (39). Association of Smad1 with the Hoxc-8 and Hoxa-9 repressors dislodges the homeodomain factors from the osteopontin promoter, thereby activating gene expression by relieving transcriptional repression (40–42). The same mechanism may also be utilized by the Smads to relieve the repression afforded by the Zn-finger/homeodomain protein, SIP-1 (43).

Fro, a series of GST pull-down assays we have demonstrated a direct interaction of Smad1 with Xvent-2 and delineated the respective interaction domains. The Xvent-2 interaction domain of Smad1 was mapped to the MH1 domain. In the case of Xvent-2, removal of the N-terminal amino acids relative to the homeodomain did not alter binding to Smad1; however, deletion of the C-terminal 85 amino acids completely abolished binding. These results demonstrate that regions essential for interaction of Xvent-2 with Smad1 are located outside of the homeodomain within the C terminus. The functional significance of the C-terminal domain was further illustrated by coinjections of the corresponding mutant with promoter/reporter constructs, which led to a loss of reporter gene activation. A Smad1 interaction domain of Hoxc-8 was also mapped to the MH1 domain; however, in contrast to Xvent-2, the homeodomain was shown to be sufficient for Smad1 binding (40). SIP-1 also employs a different mode of binding, with residues outside of the homeodomain responsible for the association to the MH2 domain within the Smads (43).

Members of the homeodomain transcription family have been shown to be context-specific transcription activators and repressors, with dual functionality achieved by association with specific cofactors. For example, NK-4 (tinman) activates the MH2 domain within the Smads (43).

The Xvent-2 family is known to be a direct target of BMP signaling, and represses target genes by recruiting the p300 coactivator with specific cofactors. For example, NK-4 (tinman) activates the transcriptional activity of the BMP-4 gene.

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J. Biol. Chem. 2002, 277:2097-2103.
doi: 10.1074/jbc.M108524200 originally published online November 9, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108524200

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