Xenopus SMOC-1 Inhibits Bone Morphogenetic Protein Signaling Downstream of Receptor Binding and Is Essential for Postgastrulation Development in Xenopus

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The bone morphogenetic protein (BMP) family of signaling molecules and their antagonists are involved in patterning of the body axis and numerous aspects of organogenesis. Classical biochemical purification and protein sequencing of highly purified fractions containing potent bone forming activity from bovine cartilage identified several BMPs together with a number of other proteins. One such protein was SMOC-2 (secreted modular calcium-binding protein-2), classified as belonging to the BM-40 family of modular extracellular proteins. Data regarding the biological function of SMOC-2 and closely related SMOC-1 remain limited, and their expression or function during embryological development is unknown. We therefore isolated the Xenopus ortholog of human SMOC-1 (XSMOC-1) and explored its function in Xenopus embryos. In gain-of-function assays, XSMOC-1 acted similarly to a BMP antagonist. However, in contrast to known extracellular ligand-binding BMP antagonists, such as noggin, SMOC antagonizes BMP activity in the presence of a constitutively active BMP receptor, indicating a mechanism of action downstream of the receptor. We provide several lines of evidence to suggest that SMOC acts downstream of the BMP receptor via MAPK-mediated phosphorylation of the Smad linker region. Loss-of-function studies, using antisense morpholino oligonucleotides, revealed XSMOC-1 to be essential for postgastrulation development. The catastrophic developmental failure observed following XSMOC knockdown resembles that observed following simultaneous depletion of three ligand-binding BMP antagonists described in prior studies. These findings provide a direct link between the extracellular matrix-associated protein SMOC and a signaling pathway of general importance in anatomic patterning and cell or tissue fate specification.

Patterning of the body axis, axial and appendicular skeleton, and various other structures requires many interacting signals expressed in complex spatial and temporal patterns. Among these signals are the bone morphogenetic proteins (BMPs) and their antagonists (for a review, see Ref. 1). Several proteins in the BMP subgroup of the transforming growth factor superfamily were identified by classical biochemical purification and protein sequencing of fractions containing potent bone forming activity from bovine cartilage (2). These fractions also contained proteins unrelated to the BMPs structurally, such as the Wnt antagonist Fzrb (3). Another protein, which could not be dissociated from osteoinductive activity following extensive purification, was identified as SMOC-2 (secreted modular calcium-binding protein-2). SMOC-2 and the closely related SMOC-1 have been classified as belonging to the BM-40 family of modular extracellular proteins (4, 5), because they contain a follistatin-like domain and a C-terminal extracellular calcium-binding domain (4, 5). They also contain two thyroglobulin-like domains and a novel domain without known homologs. The extracellular calcium-binding domain has been shown to bind calcium (5), but data regarding the biological function of SMOC1/2 remain limited. Furthermore, there are currently no published data on SMOC-1/2 function during embryological development. Both proteins are expressed in a wide variety of adult mouse tissues and are secreted by established cell lines of epithelial and mesenchymal origin. Immunofluorescence analyses have shown SMOC-1/2 to be associated with basement membrane structures (4, 5), and human vascular endothelial cells infected with adenovirus expressing SMOC-2 show SMOC-2 to be localized predominantly to the cell periphery (6). These data are consistent with a putative role of SMOC-2 as a regulator of extracellular matrix interactions and/or growth factor signaling. The related BM-40 family member SPARC (secreted protein acidic and rich in cysteine) binds to platelet-derived growth factor (7) and vascular endothelial growth factor (8) and indirectly influences the effects of basic fibroblast growth factor (9) and transforming growth factor β (10). In cell culture, SMOC-2 has been shown to potentiate cellular responses to basic fibroblast growth factor and vascular endothelial growth factor (6) and the mitogenic effects of epidermal growth factor and platelet-derived growth factor (11).

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.
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4 M. Moos and F. P. Luyten, unpublished observations.
The biochemical studies described above, together with the co-purification of SMOC with BMPs (2), suggested the possibility of a functional role during embryonic development. *Xenopus* provides a powerful system in which to examine gene function by both gain and loss of function. We therefore isolated the *Xenopus* ortholog of human SMOC-1 (XSMOC-1) and explored its function in *Xenopus* embryos. In gain-of-function assays, XSMOC-1 acted as a BMP antagonist, and loss-of-function studies revealed XSMOC-1 to be essential for postgastrulation development. In contrast to BMP antagonists described to date, several lines of evidence suggest that XSMOC-1 acts intracellularly, via the mitogen-activated protein kinase (MAPK) signaling pathway, rather than by extracellular binding to the ligands themselves.

**EXPERIMENTAL PROCEDURES**

**Isolation of *Xenopus* SMOC-1**—Initial cDNA sequences encoding *Xenopus* SMOC were obtained following 5'- and 3'-SMART™-RACE (Clontech) amplification using mRNA from stage 59 limbs and degenerate primers designed to sequences conserved between human SMOC1/2 located at the boundary of the follistatin-like and thyrogbolin-like domain 1 (5'-CCACACAYTGGGRYRTCTTTGCA-3') and the extracellular calcium-binding domain (5'-TGGARGCVCTCWCCACHGACATGGT-3'). Full-length *Xenopus* SMOC-1 (accession number EU287947) was obtained by reverse transcription (RT)-PCR using stage 59 limb cDNA and the primers 5'-CCTCCTATACAAGTCTCAGCCCTGA-3' and 5'-CTTCTTCTG GCCGGCTCTCCTA-3'. PCR products were cloned into pCR®-TOPO (Invitrogen) and confirmed by sequencing. XSMOC-1 was subsequently subcloned into pCS2 and pdNA3.

**Plasmids and Probes**—Zebrafish SMOC-2, obtained from the Zebrafish International Resource Center (clone ID CB488) as full-length expressed sequence tag in pSPORT1, was subcloned into pCS2 (provided by David Turner). BMP2, activin, and linker mutant Smad1 (LM-Smad1) were kind gifts from Gerald Thomsen, Sergei Sokol, and Joan Massagué, respectively. Noggin was isolated from stage 10.5 *Xenopus* cDNA by RT-PCR and confirmed by sequencing in both directions. Constitutively active chicken BMPR1B was kindly provided by Lee Marc's modified Ringer's solution, 1 mg/ml bovine serum albumin, 50 µg/ml gentamicin and 50 µl/gentamicin until noninjected siblings reached stage 17.

Perturbations of axial patterning were quantified by dorso-anterior index (17). Dark field images of embryos were photographed with low angle oblique illumination and a Zeiss Stemi-6 dissecting microscope.

**Immunoblotting**—XSMOC-1 (300 pg) was injected equatorially into each blastomere of *Xenopus* embryos at the four-cell stage, and animal caps, isolated at stage 9, were incubated in 0.7 X Marc's modified Ringer's solution, 1 mg/ml bovine serum albumin. 50 µg/ml gentamicin until sibling embryos reached stage 17. Animal caps were extracted on ice in 20 mM Tris, pH 7.5, 5 mM EDTA, 2 mM EGTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 3 mM benzamidine, 5 µM pepstatin A, 10 µM leupeptin, and 0.5% Nonidet P-40 in a volume of 10 µl/cap. Supernatants (10 µg/lane) were analyzed by SDS-PAGE using Novex 10% NuPAGE gels and the MES buffer system. Immunoblot analysis was performed using the mini-PROTEAN II system (Bio-Rad) and Immobilon™-P polyvinylidene difluoride membranes (Millipore). The diphtophos form of extracellular signal-regulated kinase (dp-ERK) was detected using the rabbit phospho-p44/p42 MAPK primary antibody (Cell Signaling), goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce), and SuperSignal® West Femto Maximum Sensitivity Substrate (Pierce).

**RT-PCR**—Separate pools of embryos or explants from at least two different fertilizations were prepared and analyzed for each condition reported. Total RNA was prepared with Trizol™ Invitrogen and treated with DNA-free™ DNase removal reagent (Ambion). RT was done using Taqman® RT reagents (Applied Biosystems) as described by the manufacturer, using 1 µg of total RNA/reaction; 2% of the cDNA obtained was used in each PCR. Amplification was performed in 10-µl reactions containing 40 mM Tricine-KOH, pH 8.7, 15 mM KOAc, 3.5 mM Mg(OAc)2, 0.375% bovine albumin, 2.5% Ficoll 400, 5 mM DMSO red, 200 µM dNTPs, 0.5 µM each primer, and 0.2 units of Advantage® 2 polymerase (Clontech). Each cycle comprised 94°C for 0 s, 55°C for 0 s, and 72°C for 40 s; a 1-min denaturation at 94°C preceded cycling, and a 2-min extension at 72°C was included after the final cycle. An Idaho Technologies air thermal cycler was used in all experiments, allowing momentary (setting of “0 s”) dwell times at the annealing and denaturation temperatures to increase amplification specificity. Optimal cycle numbers and annealing temperatures were
BMP Antagonist

determined for each primer set. PCR products were separated on 2% agarose gels in TAE buffer, stained with SYBR Green 1™ (Molecular Probes, Inc., Eugene, OR), and scanned using an Amersham Biosciences Fluorimeter. PCR analysis was performed at least twice for each cDNA to confirm that the amplifications were reproducible. The Xenopus primers for histone H4, Brachyury, cardiac actin, engrailed, keratin, Krox-20, N-CAM, N-tubulin, and Otx2 are given in Xenbase (available on the World Wide Web); those for Myf-5, Pax6, and XAG-1 are given by the De Robertis laboratory (available on the World Wide Web); those for XVent-1 are from Ref. 48; and those for NRP-1 are 5′-GAG-TCGCCAGAGACCGATGGA-3′ and 5′-CATGGCAATCATCCACCTTCCCA-3′.

Hybridization in Situ—cRNA probes were produced using MEGAscript T3, T7, or SP6 in vitro transcription kits (Ambion), incorporating digoxigenin. For whole mount hybridization in situ on Xenopus embryos, procedures outlined by Harland were followed (18), with modifications as described (16). For colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin and (16). For colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin and (16). For colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin and (16). For colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin and (16). For colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin.

RESULTS

Isolation and Characterization of Xenopus SMOC—Although mammals have two forms of SMOC, extensive attempts to isolate more than one form from Xenopus were unsuccessful, and searches of Xenopus (laevis and tropicalis) expressed sequence tag data bases and the Joint Genome Institute Xenopus tropicalis genomic data base (version 4.1) revealed only a single form. The Xenopus SMOC open reading frame is 74 and 50% identical to human SMOC-1 and SMOC-2, respectively. Therefore, the gene product is most likely the Xenopus ortholog of human SMOC-1. XSMOC-1 is composed of 463 amino acids, compared with 434 in human SMOC-1. The difference is due largely to an additional 19 amino acids at the C-terminal end and an additional 9 amino acids within a domain that lacks homology to other proteins (termed the nonhomologous domain). The domain structure of XSMOC-1 and mammalian SMOC1/2 is conserved (see Fig. S1). XSMOC-1 has a 25-amino acid leader sequence, followed by a predicted signal peptidase cleavage site between amino acids 25 and 26 (CFG-R). The identities between human SMOC-1 and XSMOC-1 within the conserved domains of the mature protein are as follows: follistatin-like domain, 72%; thyroglobulin-like domain 1, 93%; nonhomologous domain, 42%; thyroglobulin-like domain 2, 79%; calcium-binding domain, 88%.

XSMOC-1 first became detectable by RT-PCR at stage 12.5, corresponding to late gastrulation/early neurulation, and remained at consistent levels throughout neurula and tail bud stages (Fig. 1A). Hybridization in situ in whole embryos showed XSMOC-1 to be expressed initially at stage 12.5 at the anterior of the embryo with a dorso-ventral distribution (Fig. 1B). At stage 14, XSMOC-1 was localized within the anterior-ventral region and also lateral to the developing neural plate (Fig. 1C). This staining pattern continued throughout neurulation (Fig. 1, C–E). At later stages (stages 20–25), XSMOC-1 was localized dorsal to the cement gland, the ventral region of the developing eye (Fig. 1F), and the developing pronephros (Fig. 1, F–J). Expression was also observed in the mesencephalon and rhombencephalon (Fig. 1, J and J) with prolonged color development. By stage 30, XSMOC-1 was also observed in the pharyngeal arches (not shown). Transverse sections of overstained embryos at stage 25 confirmed the ventral eye expression domain (Fig. 1K) and revealed XSMOC-1 to be localized to the lateral regions of the midbrain (not shown) and hindbrain (Fig. 1L). In the trunk, expression was observed throughout the pronephros and in subepithelial neural crest cells migrating laterally to the somites (Fig. 1M).

Gain of Function of XSMOC-1 Produces a Phenotype and Molecular Marker Pattern Consistent with Action as a BMP Antagonist—Bilateral injection of mRNA (300 pg) encoding XSMOC-1 (Figs. 2 and 3) or zebrafish SMOC-2 (not shown) at the two-cell stage produced exaggerated dorsal/anterior structures, most prominently enlarged heads and cement
The phenotype was apparent at stage 17 (Fig. 2B). Whole mount hybridization in situ analysis of Sox2 expression demonstrated that relative to controls (Fig. 2C), the neural plate was expanded in the dorsalized embryos (Fig. 2D). Transverse sections taken through the anterior region of overstained embryos showed that, unlike controls (Fig. 2E), Sox2 expression occupied the majority of the tissue dorsal to the archenteron roof in XSMOC-1-overexpressing embryos (Fig. 2F). By stage 26, the dorsalization was more apparent (Fig. 3B), and histological analysis of sagittal sections revealed grossly hypertrophied columnar epithelium in the cement gland (Fig. 3D). Transverse sections through stage 33 XSMOC-1-overexpressing embryos showed enlargement of the neural tube and disorganized somites (Fig. 3F). Animal cap explants from embryos injected with XSMOC-1 mRNA expressed anterior neuroectodermal (Otx2 and XAG-1) and panneural (NCAM and NRP-1) markers but not the posterior neural marker Krox-20 (Fig. 4A). In addition, the epithelial marker keratin was down-regulated (Fig. 4A), supporting conversion from epithelial to neural cell fate. The biological effects of XSMOC-1 overexpression in these assays were consistent with that of a BMP antagonist (20, 21).

To examine whether XSMOC-1 action is cell-autonomous or is effective away from its point of origin, we assayed conjugated animal caps by whole mount hybridization in situ. Animal caps from wild-type embryos injected with control or XSMOC-1 mRNAs were conjugated to noninjected albino animal caps and analyzed by hybridization in situ for the anterior neuroectodermal marker Otx2 when sibling embryos reached stage 17. Otx2 was not detectable in the controls (Fig. 4B) but was readily detectable in the albino noninjected caps conjugated to XSMOC-1-expressing wild-type
caps (Fig. 4C), indicating that XSMOC-1 can act at a distance from its cellular origin.

Loss of Function of XSMOC-1 Arrests Development at Neurulation—Injection of Xenopus embryos with morpholino antisense oligonucleotides has been used widely and effectively to study the effects of blocking the synthesis of selected proteins (22). An antisense morpholino to XSMOC-1 (XSMOC-1 MO) located at positions 11002 to 11005 was designed to examine the effect of down-regulation of XSMOC-1 during early Xenopus development. Initial studies were conducted on embryos injected unilaterally with 6 ng of XSMOC MO at the two-cell stage (Fig. 5). At stage 17, mild abnormalities were observed in the developing neural axis (Fig. 5B). By stage 32, compared with controls (Fig. 5C), anterior defects (mild ventralization) were apparent (Fig. 5, D and E), and eye and other anterior structures were absent or severely dysmorphic on the injected side; corresponding structures on the noninjected side were also affected but less severely (Fig. 5, D and E). At stage 38, these differences were more obvious (Fig. 5G). Whole mount hybridization in situ studies of stage 32 embryos for Otx2 (Fig. 5, H and I) and Tbx2 (Fig. 5, J and K) revealed aberrant expression of these markers in the eye field on the XSMOC-1 MO-injected side (Fig. 5, I and K, right). Otx2 expression was diminished in the developing eye field on the noninjected side and was completely absent on the MO-injected side (Fig. 5I). Expression of Tbx2 on the noninjected side was similar to that of controls (Fig. 5, J and K, left), but expression in the eye field was diminished (Fig. 5K, left). On the MO-injected side, Tbx2 expression was absent from the eye region and branchial arches but was present in the cranial ganglia, otic vesicle, and frontonasal process (Fig. 5K, right).

Bilateral injections of 6 ng of XSMOC MO at the two-cell stage resulted in complete developmental arrest at the end of gastrulation (Fig. 6). Development appeared normal until late gastrulation (Fig. 6, D and E), and RT-PCR analyses revealed normal expression of the markers Brachyury, Goosecoid, and Myf-5 at stage 10.5 (Fig. 6G) and of cardiac actin, Otx2, and XAG at stage 12 (Fig. 6H). Developmental arrest immediately

**FIGURE 2.** Xenopus embryos overexpressing XSMOC-1 exhibit a dorsalized phenotype. A–D, dorsal views of stage 17 embryos injected bilaterally at the two-cell stage with 300 pg of GFP (A and C) or XSMOC-1 (B and D) mRNAs. XSMOC-1-injected embryos have exaggerated anterior and diminished posterior structures (B) with laterally expanded expression of the neural plate marker Sox2 (D). The arrows indicate the position of the neural tube. E and F, transverse sections taken through the anterior regions of overstained embryos C and D (white bars) show Sox2 expression throughout the dorsal tissues in XSMOC-1-injected embryos (F). The phenotypes shown for XSMOC-1 overexpression are typical for this stage and were observed in 95% of the embryos in three separate experiments (n = 96).

**FIGURE 3.** Dorsalization is pronounced in tadpoles overexpressing XSMOC-1. A–F, stage 26 Xenopus embryos injected bilaterally at the two-cell stage with 300 pg of GFP (A, C, and E) or XSMOC-1 mRNAs (B, D, and F). XSMOC-1-injected embryos were dorsalized, with exaggerated dorsal/anterior structures, particularly cement glands. The XSMOC-1 overexpression phenotypes shown in B were typical for this stage and were observed in 95% of the embryos in five independent experiments (n = 218). C and D, histological 3-mm plastic sections (modified Von Gieson stain) showing hypertrophic cement gland cells in XSMOC-1-overexpressing embryos (D). E and F, 7-mm paraffin sections (Feulgen, light green, orange G method) showing enlargement of the neural tube and disorganized somites in XSMOC-1-overexpressing embryos (F).
prior to neurulation appeared to be very abrupt and nearly total (Fig. 6F); the postgastrulation markers En-2, Pax6, and N-tubulin were expressed only weakly (Fig. 6I). Hybridization in situ analyses at stage 11–11.5 demonstrated some disturbance of the normal expression patterns of the organizer and presumptive notochord marker XNot and the myogenic marker Myf5 in XSMOC-1 MO-injected embryos (Fig. 7, A–D). At stage 12.5, XNot expression in the presumptive notochord of XSMOC-1 MO-injected embryos was abnormal, and the neural plate marker XSox2 was disturbed severely (Fig. 7, E–H). At stage 15, convergent extension associated with neurulation failed to occur in XSMOC-1 MO-injected embryos, and the XSox2 expression pattern was disrupted further (Fig. 7, I and J). Histological analysis of these embryos revealed the absence of the archenteron and any recognizable dorsal structures (Fig. 7L). These findings suggest that the effects of XSMOC-1 loss of function are specific to one or more processes occurring near the end of gastrulation and are not due to disruption of a more global process necessary for cell viability.

The specificity of the XSMOC-1 morpholino effect on Xenopus embryos was confirmed as follows. Co-injection of XSMOC-1 MO (12 ng) with zebrafish SMC2 mRNA (600 pg), which cannot hybridize to XSMOC-1 MO, produced partial to full rescue of bilaterally injected embryos (see Fig. S2). Injection of a second nonoverlapping XSMOC-1 antisense MO located at positions −39 to −63 (XSMOC-1 MO2) produced the same phenotype as XSMOC-1 MO (65% of embryos arrested prior to neurulation in three separate experiments; n = 96) in bilaterally injected embryos, at a dose of 30 ng/blasto-mere at the two-cell stage (not shown).

XSMOC-1 Blocks the Effects of BMP2 but Not Activin and Acts Downstream of the BMPR1B Receptor—Since overexpression of XSMOC-1 in Xenopus embryos produced a phenotype similar to that observed for BMP antagonists, we analyzed the effect of XSMOC-1 on BMP2 and activin activity. Both are members of the transforming growth factor β superfamily but signal via different serine-threonine kinase receptors. Overexpression of BMP2 in Xenopus embryos produced a strongly ventralized phenotype (23) (Fig. 8A) that could be rescued partially or completely by co-expression of XSMOC-1 (Fig. 8A). RT-PCR analysis demonstrated that BMP2-mediated induction of the ventral marker XVent was blocked completely by co-expression of XSMOC-1 in animal cap explants (Fig. 8B). In contrast, induction of Brachyury by activin was not inhibited by XSMOC-1 (Fig. 8C). Inhibition of BMP2 activity by XSMOC-1 was also demonstrated in mammalian cell culture (Fig. 8D).

To investigate whether XSMOC-1 acts by direct binding to ligand, we studied its effect in the presence of the constitutively active chicken BMP receptor 1B (caBMPR1B). Overexpression of caBMPR1B has been shown to promote signaling of the BMP2/4/7 family in the absence of bound ligand (25), and, consistent with this expectation, animal cap explants from Xenopus embryos injected with caBMPR1B mRNA expressed the ventralized phenotype (23) (Fig. 8E). As expected, the BMP antagonist noggin, which acts extracellularly by direct ligand binding, did not reverse this effect (Fig. 8E). However, expression of XVent-1 in caps from embryos injected with both XSMOC-1 and caBMPR1B mRNA was expressed only weakly (Fig. 8E), indicating that XSMOC-1 does not inhibit BMP signaling via direct binding to BMPs. It also suggests that XSMOC-1 acts downstream of the BMP receptor.

BMP receptors signal through C-terminal phosphorylation of Smad (for a review, see Ref. 26). This can be inhibited by activation of the MAPK/ERK pathway, which results in Smad phosphorylation within the linker region, effectively blocking C-terminal phosphorylation (27–29). To evaluate the possibility that XSMOC-1 acts via this mechanism, we studied the effect of XSMOC-1 in the presence of LM-Smad1. LM-Smad1 has four serine-to-alanine substitutions at conserved PXSP
and NRP-1) markers demonstrated that in the presence of U0126, there was a marked reduction in XSMOC-1 activity (Fig. 9D).

**DISCUSSION**

Previous studies on mammalian SMOC in adult tissues identified two closely related genes, SMOC-1 and -2, which have been characterized as extracellular calcium-binding proteins (4, 5) with angiogenic and growth factor-potentiating activities (6). Unlike mammals, the *Xenopus* genome appears to contain only one SMOC gene, the ortholog of mammalian SMOC-1. The domain structure of XSMOC-1 and mammalian SMOC1/2 is conserved, and there is a high degree of identity within each of the domains, with the exception of the region exhibiting no homology to other proteins (see Fig. S1). We observed XSMOC-1 to be a zygotic transcript initially expressed at the anterior of the embryo at the end of gastrulation and onset of neurulation (Fig. 1). In neurula embryos, XSMOC-1 was expressed lateral to the developing neural plate (Fig. 1C) and at the early tail bud stage was present in the early pronephric anlage (Fig. 1F). In addition to the pronephric expression, later tail bud embryos expressed XSMOC-1 in the ventral region of the developing eye (Fig. 1, H and K), the lateral aspects of the midbrain and hindbrain (Fig. 1, I, J, and L), and trunk neural crest cells passing laterally to the somites (Fig. 1M). To examine SMOC function during embryological development, we used various assays in the *Xenopus* model system.

Overexpression of XSMOC-1 in *Xenopus* embryos produced a dorsalized phenotype and pattern of marker induction suggestive of a BMP antagonist (19, 20). Similar to the BMP antagonists noggin and chordin, XSMOC-1 induced anterior (Otx2, NrP-1, and XAG) but not posterior (Krox 20) neural markers (Fig. 3). Co-expression experiments in *Xenopus* revealed that XSMOC-1 was able to inhibit the activity of BMP2, which signals through Smad1, -5, or -8 (31), but not activin, which signals through Smad2 or -3 (Fig. 8). Inhibition of BMP2 signaling by XSMOC-1 was also demonstrated in mouse 3T3 fibroblasts (Fig. 8D). Unlike noggin and chordin, which are first expressed in the Spemann organizer near the onset of gastrulation, XSMOC-1 was not expressed until the end of gastrulation (stage 12.5) and at the pole opposite to the organizer (Fig. 1B). This pattern is consistent with a developmental role for XSMOC-1 in processes initiated following the onset of gastrulation. At later stages (20–26), XSMOC-1 expression in the developing pronephros (Fig. 1, F–J and M) and the ventral region of the developing eye (Fig. 1, H and K) suggests a possible role in the organogenesis of these structures. Potential targets

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**FIGURE 5. Unilateral injection of XSMOC-1 antisense morpholino (MO) produces mild ventralization and anophthalmia on the injected side.** XSMOC-1 MO (6 ng) was injected into a single blastomere at the two-cell stage. At stage 17 (A and B), mild abnormalities were observed in the developing neural axis of XSMOC-1 MO-injected embryos (B). By stage 32 (C–E), MO-injected embryos were mildly ventralized (D and E) compared with controls (C). In addition, eyes were absent on the injected side (F); this was more apparent by stage 38 (G). Eye development appeared normal on the noninjected side (F). The XSMOC-1 MO phenotypes shown in D and E were typical for this stage and were observed in 90% of the embryos in five independent experiments (n = 164). H–K, whole mount hybridization in situ analyses of Otx2 (H and I) and Tbx2 (J and K) in stage 32 control (G and J) and XSMOC-1 MO-injected (I and K) embryos. The injected sides are displayed on the right. The arrows indicate the location of the eye fields.
for the BMP antagonist activity of XSMOC-1 would be BMP7 in the pronephros (31, 32) and BMP4, BMP7, and GDF6 in the developing eye (33–35).

Of the many BMP antagonists described to date, including noggin, chordin, follistatin, cerberus, dan, and gremlin (for a review, see Ref. 1), most act by direct interaction with BMP ligands to prevent receptor binding or activation. To test whether XSMOC-1 was acting by a similar mechanism, we used a constitutively active type I BMP serine/threonine kinase receptor (caBMPR1B), which activates BMP2/4/7 signaling even in the absence of ligand (25, 36). In the presence of caBMPR1B, noggin did not induce the expression of anterior neural markers in animal cap assays (Fig. 8E), consistent with expectations. If XSMOC-1 were acting by a similar mechanism, it would also be expected to be ineffective in the presence of the constitutively active receptor. This was not the case; XSMOC-1 continued to induce expression of anterior neural markers when co-expressed with caBMPR1B (Fig. 8E). We conclude, therefore, that the mechanism by which extracellular XSMOC-1 acts as a BMP antagonist appears not to be primarily via direct binding to BMPs but at a point downstream of the receptor.

Activated BMP receptor serine/threonine kinases phosphorylate intracellular Smads (R-Smads) at C-terminal serine residues, resulting in their translocation to the nucleus to form transcriptional complexes (for a review, see Ref. 26). An alternative mechanism for interfering with BMP signaling is via activation of the MAPK pathway upon ligand (e.g. epidermal growth factor, fibroblast growth factor, or insulin-like growth factor) binding to tyrosine kinases (27–29). The resulting intracellular phosphorylation of the MAPK, ERK, produces dp-ERK. This, in turn, phosphorylates Smad1, -5, and -8 on serine residues at four conserved PxSP sites within the linker region (27, 28). As a consequence of linker-phosphorylated Smad being bound by the ubiquitin ligase Smurf1, resulting in polyubiquitination and proteasome-dependent degradation in addition to inhibition of Smad nuclear translocation (29). This sequence of events leads to an inhibition of BMP signal transduction. It has been shown that a mutant form of Smad1 (LM-Smad1), which cannot be phosphorylated within the linker region, is unable to inhibit BMP activity (27). When LM-Smad1 was overexpressed in Xenopus embryos, XSMOC-1 activity was lost (Fig. 9A), indicating that...
XSMOC-1 elicits its effect on BMP signaling by inducing linker phosphorylation of Smad1, -5, or -8. If this is correct, then one might expect there to be an elevation in dp-ERK levels in response to X-SMOC1 overexpression. This was the case; immunoblot analysis of animal cap explants overexpressing XSMOC-1 demonstrated a dramatic increase in the level of dp-ERK (Fig. 9B). Further support for XSMOC-1 acting via the MAPK pathway came from studies using the MEK inhibitor U0126 (30). In the presence of U0126, XSMOC-1 activity, as measured by its ability to induce neural markers, was markedly reduced. Exactly how extracellular XSMOC-1 produces an elevation in intracellular dp-ERK will be the emphasis of further study.

The modular structure of XSMOC-1 may provide for its interaction at the cell surface, possibly via some kind of direct or indirect interaction with one or more receptor tyrosine kinase(s). Potential interactions involving the N-terminal SMOC follistatin-like domain are unlikely, since a deletion construct lacking this domain was found to have activity comparable with that of wild type XSMOC-1.5 Further characterization of the modules required for XSMOC-1 activity is ongoing.

Loss of function experiments using antisense morpholino oligonucleotides indicated that the expression of XSMOC-1 is

5 J. T. Thomas and M. Moos, unpublished results.
essential for development to proceed through neurulation and subsequent dorsal patterning. In the absence of XSMOC-1, gastrulation and neural induction appeared normal, but embryological development arrested just prior to neurulation (Fig. 6, A, B, and C), indicating disruption of developmental fields secondary to abnormal morphogenetic movements, are consistent with this possibility. Neurulation is defined as a set of morphogenetic or convergent extension movements that result in formation of the neural tube, future brain, and spinal cord (for a review, see Ref. 41). This process requires the coordinated reorganization of cell-cell and cell-matrix interactions. One influencing a set of events distinct in both space and time from those modulated by XSMOC-1.

The severity of the phenotype we observed in bilaterally injected morphant embryos prompted us to consider the possibility that loss of XSMOC-1 function was somehow affecting a vital "housekeeping" function. We believe this to be unlikely. First, XSMOC-1 MO-unilaterally injected embryos showed specific defects in anterior structures (Fig. 5), a result that would not be expected if a gene required by every cell was disrupted. Second, posterior structures were normal on both sides of unilaterally injected embryos. Third, the conjugated cap experiment (Fig. 4, B and C) demonstrated XSMOC-1 to be non-cell-autonomous, whereas housekeeping proteins are cell-autonomous. These observations are incompatible with a ubiquitous nonspecific housekeeping function for XSMOC-1.

It has been well documented that inhibition of BMP signaling is paramount for neural induction (for a review, see Ref. 1). During gastrulation, the Spemann organizer and closely neighboring tissue secrete a number of proteins that bind BMPs and antagonize BMP signaling in adjacent cells, allowing them to follow their default neural pathway (1, 38–40). Considerable functional redundancy exists between these BMP inhibitors; depletion of any one or even two does not result in loss of the neural plate (37). Simultaneous depletion of three (chordin, noggin, and follistatin) is required before complete failure of dorsal patterning is achieved (37). The apparent absolute requirement for XSMOC-1 for neurulation and subsequent development, suggested by our loss-of-function data (Fig. 6), is thus somewhat surprising. It is unusual that a protein with such an important function during development has no redundancy. Analyses of different genomes reveals that the nonredundancy of SMOC in Xenopus may be limited to amphibians, since there are two SMOC genes in zebrafish, chickens, mice, and humans.

In normal Xenopus embryos, many molecular markers associated with neural induction are present before XSMOC-1 expression can be detected. The expression of these early neural markers during gastrulation was not affected by XSMOC-1 depletion (Figs. 6G and 7, B and D), suggesting that the effect of XSMOC-1 is not on primary neural induction but on subsequent neurulation events needed for specification of the nervous system. In agreement with this hypothesis, the markers N-tubulin (neuronal differentiation), Engrailed (midbrain/hindbrain boundary), and Pax6 (anterior neural tissue), which are expressed postgastrulation, were markedly reduced (Fig. 6I). In addition, the neural plate marker Sox2 was expressed aberrantly (Fig. 7, F and J). The sudden and dramatic developmental arrest immediately following apparently normal gastrulation suggests that XSMOC-1 may have an essential function in one or more of the mechanical events necessary for neurulation. The abnormalities in spatial distribution of Myf5 and Xnot apparent in bilaterally injected stage 12 morphant embryos (Fig. 7, B and D), indicating disruption of developmental fields secondary to abnormal morphogenetic movements, are consistent with this possibility. Neurulation is defined as a set of morphogenetic or convergent extension movements that result in formation of the neural tube, future brain, and spinal cord (for a review, see Ref. 41). This process requires the coordinated reorganization of cell-cell and cell-matrix interactions. One
group of molecules involved in these types of interactions is the integrin family of transmembrane proteins. Integrins have a structural role, linking the extracellular matrix to the intracellular actin cytoskeleton, but also activate signaling pathways involved in these types of interactions is the integrin family of transmembrane proteins. Integrins have a structural role, linking the extracellular matrix to the intracellular actin cytoskeleton, but also activate signaling pathways involving cell migration, differentiation, growth, and survival (for a review, see Ref. 42). Interestingly, an abrupt arrest in cellular events.

In addition, our loss-of-function data demonstrate that a mechanism for establishing sharp developmental field boundaries interact with cell surfaces via the extracellular domains of integrins, and both the BM-40 family member SPARC (46) and mammalian SMOC-2 (11) have been shown to be involved in activation of the cytoplasmic integrin effector, integrin-linked kinase. There is also substantial evidence that integrins are involved in MAPK signaling (42, 47). Consequently, possible interactions between XSMOC-1, integrins, and/or receptor-tyrosine kinases warrant further investigation (Fig. 10).

In summary, we present evidence that the BM-40 family member XSMOC-1 acts as an antagonist of BMPs that signal through Smad1, -5, or -8 by activation of an intracellular MAPK cascade culminating with phosphorylation of the Smad linker region. The BMP antagonist activity of XSMOC-1, coupled with its localization within basement membranes, could allow for discrete localization of BMP inhibitory activity and provide a mechanism for establishing sharp developmental field boundaries. In addition, our loss-of-function data demonstrate that XSMOC-1 is essential to neurulation and subsequent developmental events.

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