

The Spemann Organizer Signal noggin Binds and Inactivates Bone Morphogenetic Protein 4

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Summary

Signals released by the Spemann organizer of the amphibian gastrula can directly induce neural tissue from ectoderm and can dorsalize ventral mesoderm to form muscle. The secreted polypeptide noggin mimics these activities and is expressed at the appropriate time and place to participate in the organizer signal. Neural induction and mesoderm dorsalization are antagonized by bone morphogenetic proteins (BMPs), which induce epidermis and ventral mesoderm instead. Here we report that noggin protein binds BMP4 with high affinity and can abolish BMP4 activity by blocking binding to cognate cell-surface receptors. These data suggest that noggin secreted by the organizer patterns the embryo by interrupting BMP signaling.

Introduction

In *Xenopus*, the neural plate and the bulk of the somitic mesoderm are induced during gastrulation. Spemann and others demonstrated that a signaling center at the dorsal lip of the gastrula blastopore could direct neighboring tissues to assume neural or dorsal mesodermal rather than epidermal and ventral mesodermal fates (reviewed in Hamburger, 1988). More recent work suggests that this Spemann organizer signaling center secretes a cocktail of polypeptides that induce both neural and dorsal mesodermal fates (Kessler and Melton, 1994; Slack, 1994).

The first molecule shown to have the properties expected of a Spemann organizer signal, noggin, was identified in an expression screen for activities that induce dorsal structures in *Xenopus* embryos (Smith and Harland, 1992). *noggin* is expressed in the organizer at the gastrula stage and encodes a novel ~32 kDa glycoprotein that is secreted as a homodimer. Soluble noggin protein mimics organizer activities in the induction of a muscle marker in explants of gastrula ventral marginal zone that would otherwise be fated to form ventral mesoderm (Smith et al., 1993). noggin also induces anterior neural markers in animal cap ectoderm that would otherwise become epidermis (Lamb et al., 1993). noggin's mechanism in Spemann organizer signaling remained elusive, since no cell-surface receptor transducing the noggin signal has been identified.

Organizer signals such as noggin may be antagonized by members of the bone morphogenetic protein (BMP) class of the transforming growth factor β (TGF β) gene superfamily. These dimeric secreted glycoproteins were

first identified by their ability to induce ectopic bone development in mammals; BMPs have since been shown to act as mesoderm ventralizers and epidermal inducers in *Xenopus* assays, at the expense of the dorsal mesoderm and neural tissue induced by the Spemann organizer (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Re'em-Kalma et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Wilson and Hemmati-Brivanlou, 1995). Conversely, disruption of BMP signaling mimics organizer activity: injection of mRNAs encoding dominant negative BMP4 receptors or unprocessable BMP precursors induces neural tissue and dorsalizes mesoderm (Graff et al., 1994; Maéno et al., 1994; Suzuki et al., 1994; Hawley et al., 1995; reviewed in Harland, 1994).

While noggin could be exerting its effects on BMP signaling via an independent signal transduction pathway, it is suggestive that another TGF β , the potent mesoderm inducer activin, can be inactivated by high affinity binding to a secreted protein, follistatin (Nakamura et al., 1990; Schneyer et al., 1994). By analogy, noggin protein could act by binding BMP4 directly, before it has reached its target, thus preventing it from transmitting its signal through its receptors. This model is supported by epistasis experiments in *Drosophila*, where no noggin equivalent has been identified. Injection of *Xenopus* noggin RNA into fly embryos can block the effects of the fly BMP2/4 homolog decapentaplegic (*dpp*), but noggin has no effect on embryos coinjected with an activated mutant *dpp* receptor, suggesting that its antagonizing activity is exerted upstream of the *dpp* receptor (Holley et al., this issue of *Cell*).

The opposing activities of noggin and BMP4 in the developing *Xenopus* embryo led us to examine whether noggin antagonized BMP4's effects in other systems, whether there was a direct physical interaction between the two proteins, and whether this mechanism could account for noggin's ability to participate in Spemann organizer signaling.

Results

noggin Blocks BMP4 Activity in a Dose-Dependent Fashion

The antagonism between the biological activities of noggin and BMP4 in the *Xenopus* embryo could be complex and indirect; we therefore examined this interaction in a direct, quantitative bioassay for BMP protein activity. The murine bone marrow stromal cell line W-20-17 responds to treatment with BMP4 protein by inducing alkaline phosphatase activity (Thies et al., 1992). Preincubation of BMP4 with 16 nM noggin protein for 1 hr abolishes its activity over a range of BMP4 doses from 0.1 pM to 6.66 nM (Figure 1). noggin alone has a small but reproducible inhibitory effect in the absence of added BMP4, suggesting that it may antagonize activities present in serum or secreted by the W-20-17 cells.

At lower concentrations, noggin inhibits the activity of a molar equivalent of BMP4. At 420 pM BMP4, preincubation with 160 pM noggin (38% of 420 pM) reduces

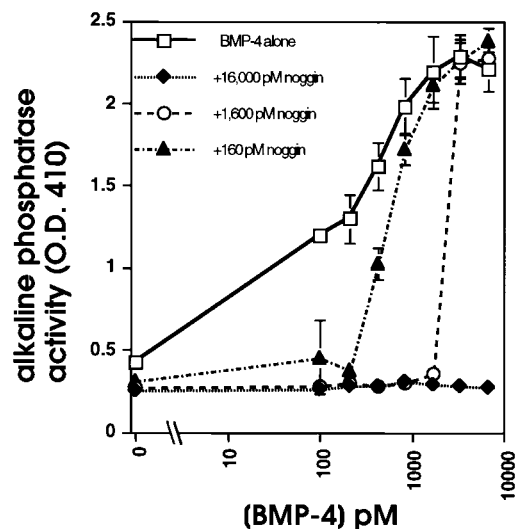


Figure 1. noggin Blocks BMP4 Activity in a Bone Marrow Stromal Cell Line Assay in a Dose-Dependent Fashion

Induction of alkaline phosphatase activity was assayed in W-20-17 murine bone marrow stromal cells. Triplicate serial dilutions of BMP4 at 0, 100, 210, 420, 830, 1,660, 3,330, and 6,660 pM were preincubated alone (squares) or with purified human noggin at 160 pM (triangles), 1,600 pM (circles), or 16,000 pM (diamonds) for 1 hr prior to addition to cells. Alkaline phosphatase activity was assayed 24 hr later. At each point, noggin blocks a molar equivalent of BMP4 activity.

specific alkaline phosphatase activity by 39%; at 830 pM BMP4, 160 pM noggin (19%) inhibits 16% of the activity. This linear relationship between noggin dose and inhibition of BMP4 activity is consistent with a direct interaction between the two proteins. Moreover, the persistence of the linear dose-dependence at very low concentrations suggests that the affinity of noggin for its effector (whether BMP4 or another molecule) in this system may be quite high. For example, if the K_d of noggin for a "receptor" (not present in large excess) were 10 nM, at 160 pM we might expect that only a small fraction of the noggin present would be effective. Instead, 160 pM noggin quantitatively inhibits BMP4 activity, consistent with an affinity several orders of magnitude higher.

noggin Protein Associates with BMP4

A simple model explaining noggin's ability to inhibit BMP4 signaling in both *Xenopus* and mammalian cell culture is one in which noggin protein binds BMP4 directly and prevents activation of its signal transduction pathway. To test this hypothesis, we first analyzed BMP4's ability to associate directly with noggin bound to sepharose beads

To facilitate identification and quantitation of BMP4 protein, we constructed a myc-tagged version of BMP4, expressed it in COS cells, and confirmed that the supernatants had biological activity. *Xenopus* noggin protein was purified from transfected COS supernatant and, to make it easier to manipulate, covalently bound to CNBr-activated sepharose beads. As a control for nonspecific binding, another set of sepharose beads was subjected to an identical coupling protocol in the absence of noggin protein. BMP4-myc COS supernatants were then

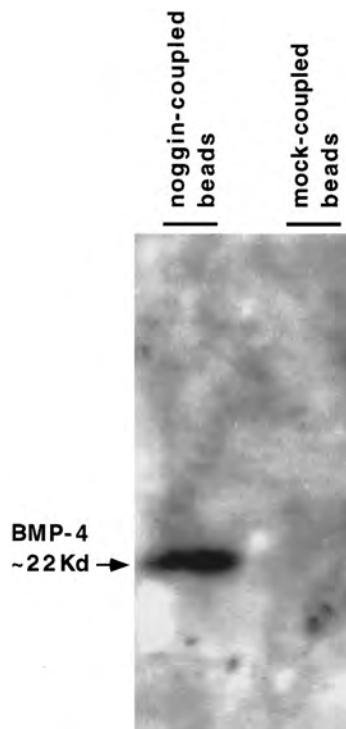


Figure 2. BMP4 Protein Associates With Immobilized noggin

COS supernatant containing myc-tagged BMP4 was incubated with a *Xenopus* gastrula extract (as nonspecific competitor) plus either noggin-coupled sepharose beads or control mock-coupled beads, washed, and then Western blotted with anti-myc antibody. BMP4-myc immunoreactivity specifically associated with noggin-coupled beads.

incubated with both sets of beads in the presence of 1 mg/ml of a *Xenopus* gastrula extract as nonspecific competitor (Figure 2). Beads were washed and analyzed by Western blot for myc immunoreactivity. BMP4-myc was observed to associate with noggin-coated beads but not mock-coupled beads. Use of either gastrula extract or bovine serum albumin (BSA) at 1 mg/ml (data not shown) as nonspecific competitor did not interfere with binding, suggesting that the interaction of noggin and BMP4 is sufficiently specific to occur in the complex biochemical environment of the gastrula, but is not dependent on the presence of auxiliary proteins.

noggin Binds BMP4 with Very High Affinity

Many growth factors, including the BMPs, have very high affinities for their receptors ($K_d \approx 10^{-9} - 10^{-10}$ M; Koenig et al., 1994; Iwasaki et al., 1995). For noggin to block BMP signaling effectively in our model, it must either bind at least as tightly to or be present in large excess over its target cytokine. To quantitate the affinity of noggin for BMPs, we incubated a range of concentrations of purified human BMP4 and radioiodinated BMP4 tracer with a constant concentration of purified human noggin that had been fused to an immunoglobulin Fc domain (hnogFc). noggin-BMP complexes were then isolated by allowing hnogFc to bind directly to protein G-sepharose beads, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the 125 I-BMP4

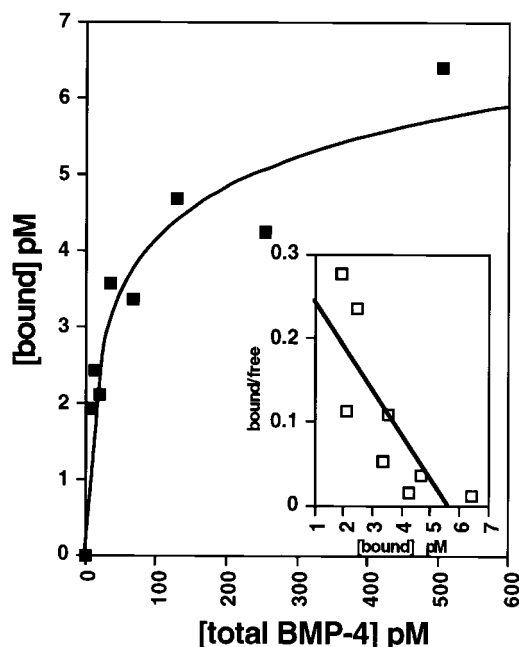


Figure 3. noggin Binds BMP4 with High Affinity

Purified human BMP4 at 0 to 500 pM, with trace 125 I-BMP4, was incubated with Fc-tagged human noggin for 1 hr at 4°C, precipitated with protein G-sepharose, and quantitated by SDS-PAGE and phosphorimager. Scatchard analysis (inset) yields an apparent K_d of 19 pM.

tracer was quantitated. Nonspecific binding was determined by two methods: binding in the absence of hnogFc and binding in the presence of 20 nM (~5000-fold excess) unlabeled BMP4; both methods gave approximately equal background levels.

Following subtraction of nonspecific binding, coprecipitating BMP4 counts were normalized with respect to the specific activity of 125 I-BMP4 at each total BMP4 concentration to determine the absolute concentrations of bound and free ligands (Figure 3). Scatchard analysis of these data produces an apparent K_d of 19 pM. The other known TGF β -binding protein, follistatin, has a comparable affinity for its target, activin (1.3–200 pM; Nakamura et al., 1990; Schneyer et al., 1994). This strikingly high affinity suggests that noggin could effectively compete with cell-surface receptors for binding to BMP ligands.

Specificity of noggin-BMP Interactions

While BMP4 has been the most-studied of the BMPs in *Xenopus* embryogenesis, related activities, such as BMP2 and BMP7/OP-1, have been identified (Plessow et al., 1991; Nishimatsu et al., 1992); other TGF β family members, such as activin and Vg-1, appear to play very different roles (Kessler and Melton, 1994; Slack, 1994). To test whether noggin interacted specifically with BMP4 or whether it could bind a range of TGF β family members, we incubated trace 125 I-BMP4 with Fc-tagged human noggin and a range of concentrations of unlabeled TGF β s. BMP4, BMP2 (92% amino acid homology to BMP4), BMP7 (58%), and the more distantly related

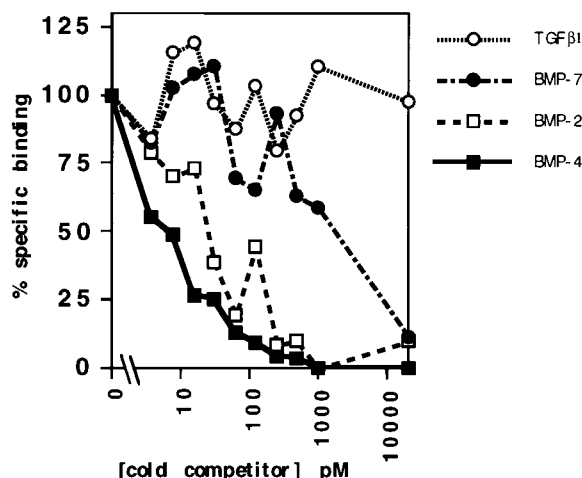


Figure 4. noggin Preferentially Binds BMP2 and -4

Fc-tagged human noggin was incubated with 125 I-BMP4 alone (=100% specific binding) or with serial dilutions (4, 8, 16, 31, 62, 125, 250, 500, 1,000, 20,000 pM) of purified human BMP4 (closed squares), BMP2 (open squares), BMP7 (closed circles), or porcine TGF β 1 (open circles) and then precipitated and quantitated as in Figure 2. BMP2 and -4 competed effectively with 125 I-BMP4 for binding to human noggin. At higher concentrations, BMP7 was also able to displace 125 I-BMP4. TGF β 1 did not compete for binding.

porcine TGF β 1 (35%) were included as competitors, and the incubation was followed by precipitation and quantitation as in the previous experiment. Following subtraction of nonspecific binding, bound counts at each concentration of unlabeled ligand were expressed as a percentage of the specific 125 I-BMP4 counts bound to noggin in the absence of cold competitor (=100% specific binding).

BMP4 and BMP2 effectively competed with 125 I-BMP4 for binding to noggin, while BMP7 bound less tightly and TGF β not at all (Figure 4). BMP2 and -4 are very closely related, so it is not surprising that both bind noggin. The observation that low picomolar concentrations of unlabeled BMP4 inhibit >50% of the 125 I-BMP4 binding detected is consistent with the very high affinity suggested by Scatchard analysis. BMP7, despite its reduced affinity relative to BMP4, is coexpressed with noggin in the organizer and might still experience some binding *in vivo*. A concentrated COS cell supernatant of myc-tagged activin also did not compete at nanomolar concentrations, but the data are not strictly comparable since the activin was not purified (data not shown). Purified TGF β 1 does not compete with BMP4 for binding, demonstrating that noggin can discriminate among related proteins.

noggin Prevents BMP4 from Binding Its Receptors

Having determined that noggin binds BMP4 very tightly, we wished to describe the mechanism by which it blocked BMP4 activity. We used an affinity-labeling assay to distinguish between the following alternatives: first, the noggin-BMP4 complex binds BMP4 receptors normally (but fails to initiate normal signaling), or second, the noggin-BMP4 complex fails to bind BMP4 receptors.

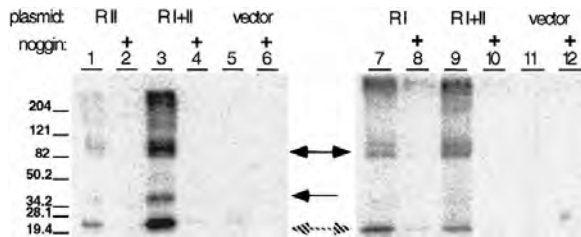


Figure 5. Preincubation with noggin Prevents BMP4 from Binding Its Receptors

2.5 nM 125 I-BMP4 was preincubated with or without 50 nM purified human noggin, then allowed to bind to COS7 cells that had been transfected with pCMV5-BMPRII-His (lanes 1 and 2), pCMV5-BMPRI-HA (lanes 7 and 8), both (lanes 3, 4, 9, and 10), or pCMV5 alone (vector) (lanes 5, 6, 11, and 12), and chemically cross-linked with BS³. Lanes 1–6 were precipitated using the His tag on the type II BMP receptor; lanes 7–12 with the HA tag on the type I BMP receptor. 125 I-BMP4 alone affinity-labeled either receptor type (lanes 1 and 7, hatched arrows); binding was enhanced by cotransfection of both receptors (lanes 3 and 9). Receptor-transfected cells were also enriched for binding of 125 I-BMP4 monomer (closed arrows) and crosslinked dimer (single-headed arrow). No binding was detected in the absence of transfected receptors (vector alone, lanes 5, 6, 11, and 12). The presence of 20-fold excess noggin in the preincubation step abolished affinity labeling of receptor complexes (lanes 2, 4, 8, and 10) as well as coprecipitation of 125 I-BMP4 monomer and dimer.

We transfected COS cells with expression constructs containing either or both of the type I and II BMP receptors bearing influenza virus haemagglutinin (HA) epitope and histidine (his) tags, respectively (Liu et al., 1995). The use of different tags enabled us to assay binding to the two receptor types independently. 125 I-BMP4 was preincubated for 1 hr with or without a 100-fold molar excess of purified human noggin protein, then allowed to bind to transfected COS monolayers, washed, and chemically crosslinked. Receptor- 125 I-BMP4 complexes were precipitated from cell extracts using either of the tags on the receptors, and visualized by SDS-PAGE and autoradiography (Figure 5). noggin protein abolished 125 I-BMP4 labeling of both type I and type II receptors, indicating that BMP4 complexed with noggin is not capable of binding its receptors.

An Antibody That Blocks noggin's Biological Activity Also Blocks BMP4 Binding

The ability to bind to BMP proteins, prevent them from interacting with their receptors, and block their activities in a bioassay strongly suggests that noggin exerts its inductive activities in the *Xenopus* embryo via a direct BMP-binding mechanism. We tested this hypothesis by asking whether reagents that block noggin's biological activity in a *Xenopus* explant assay could also interfere with the formation of a noggin-BMP complex.

A panel of anti-*Xenopus* noggin monoclonal antibodies was generated and screened for the ability to inhibit dorsalization of ventral marginal zones by noggin protein (Smith et al., 1993). One antibody, MAb1A4, was shown to be effective in blocking induction of muscle actin by *Xenopus* noggin; several others, including MABs2C3

and 5B1, did not significantly affect noggin activity (Figure 6A). Antibodies alone had no effect on explants. MAB1A4's effects were specific for *Xenopus* noggin, since induction of muscle actin by human noggin protein or by injection of mRNAs encoding two alternative dorsalizing agents—chordin and a dominant negative BMP receptor—was not inhibited by the presence of this antibody (Figure 6D).

We then compared the blocking antibody, MAB1A4, with the nonblocking antibodies 2C3 and 5B1 for their ability to immunoprecipitate a noggin-BMP4 complex. A 50-fold molar excess of each antibody was preincubated with noggin protein for 1 hr prior to the addition of 125 I-BMP4. All three antibodies precipitated approximately equal amounts of noggin protein as measured by western blot; however, the blocking antibody 1A4 coprecipitated significantly less 125 I-BMP4 (Figure 6B). Quantitation of noggin and BMP4 in a separate experiment indicates that the blocking antibody precipitates 15-fold less BMP4 per unit of noggin than do the other antibodies (Figure 6C). The most likely explanation is that the antibody that blocks noggin's biological activity in the ventral marginal zone assay does so by binding to and occluding the face of noggin that interacts with BMPs. Antibodies that do not block activity presumably bind elsewhere on noggin, and can therefore coimmunoprecipitate the complex. The small amount of BMP4 coprecipitated by the blocking antibody may result from immune complexes formed in which only one subunit of the noggin dimer is bound by antibody, leaving the other subunit free to bind BMP4.

We have also reproduced in *Xenopus* the results of Holley et al. (1996), by showing that injected noggin RNA fails to dorsalize embryos or neuralize animal caps coinjected with a constitutively active mutant BMP receptor RNA (Hoodless et al., 1996) (L. Z., D. Hsu, and R. M. H, data not shown). These results complement the biochemical data, which predict that an activated receptor would be epistatic to activities outside the cell.

Together with our antibody analysis showing that noggin's biological activity correlates with its ability to bind BMPs, these observations strongly suggest that noggin acts via a direct BMP-binding mechanism *in vivo*.

Discussion

We propose a model in which secreted noggin mediates aspects of Spemann organizer function by blocking intercellular BMP signaling. *In vivo*, noggin expressed in the dorsal tissues of developing embryos may combat ventralizing signals, and help to unmask expression of neural or dorsal mesodermal markers. In explant assay systems, noggin may inactivate a reservoir of BMP protein trapped on the cell-surface and in extracellular matrix. Dissociated cells from animal cap explants can be neuralized by noggin treatment, but they also "autoneuralize" if dispersed for 4 hr, consistent with a slow loss of a signal from the cell surface. Treatment with BMP4 suppresses autoneuralization and restores the epidermal phenotype characteristic of intact caps (Wilson and Hemmati-Brivanlou, 1995). The large dose of exogenous noggin protein required to neuralize animal caps (Lamb

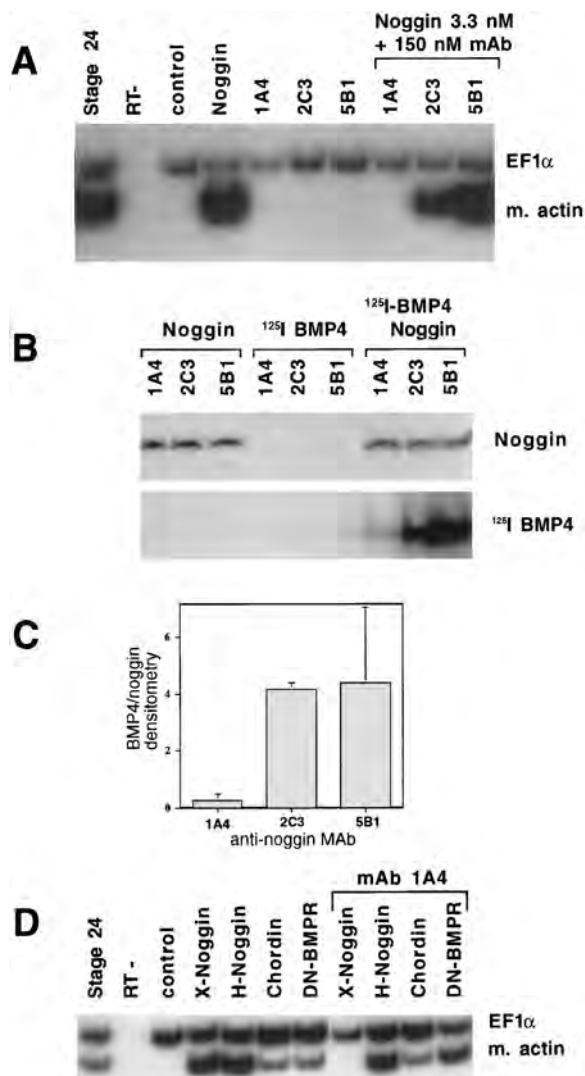


Figure 6. Antibody Inhibition of noggin Activity and noggin-BMP4 Complex Formation

(A) Antibody inhibition: ventral marginal zones of stage 10.5 *Xenopus* embryos were dissected and incubated in control medium or medium containing 150 nM monoclonal anti-noggin antibody 1A4, 2C3, or 5B1 with or without 3.3 nM *Xenopus* noggin protein; RNA was then harvested at stage 24 and subjected to reverse transcription-PCR for the dorsal marker muscle actin and EF1α. Anti-noggin antibody 1A4 blocked noggin's ability to induce muscle actin, while antibodies 2C3 and 5B1 did not. Antibodies alone had no effect in this assay.

(B) Coimmunoprecipitation using anti-noggin monoclonal antibodies: MAb's 1A4, 2C3, and 5B1 were incubated with noggin alone, ¹²⁵I-BMP4 alone, or noggin plus ¹²⁵I-BMP4. All three antibodies precipitate approximately the same amount of noggin protein as detected by Western blot, but MAb 1A4 fails to coprecipitate significant ¹²⁵I-BMP4.

(C) Quantitation of coimmunoprecipitation: ¹²⁵I-BMP4 signal from each immunoprecipitation was quantitated on a phosphorimager, then normalized to the noggin Western blot signal for the same precipitation to give the amount of ¹²⁵I-BMP4 precipitated per unit noggin. MAbs 2C3 and 5B1 coprecipitate 5- to 20-fold more ¹²⁵I-BMP4 than does MAb 1A4.

(D) Blocking MAb1A4 is specific for *Xenopus* noggin: ventral marginal zones were treated essentially as in (A), except that explants were also treated with purified human noggin protein (H-noggin) or were dissected from embryos that had been injected with 0.5 ng

et al., 1993) is also consistent with the proposed mechanism, since the noggin must diffuse into intercellular spaces to block the BMP signals. While the data presented here do not formally disprove the existence of an independent noggin signal transduction system, they do provide a rationale for most of noggin's observed effects. Efforts to describe a cell-surface noggin receptor, including binding assays, biochemical approaches, and expression screens, have not uncovered evidence for a more conventional noggin-binding component (L. B. Z., R. M. H., A. Economides, N. Stahl, unpublished data).

While noggin binds BMP2 and -4 with very high affinity, other TGFβ family members with which noggin may interact continue to be identified in the developing *Xenopus* embryo. Several of these have ventralizing activities similar to BMP4's, yet are expressed in the dorsal regions from which BMP4 is excluded. Repression of ventralizers in these regions during gastrulation is potentially more critical to the embryo than elimination of diffusing BMP4 activities. Our data suggest that BMP7 has a lower affinity for noggin, but at higher concentrations can compete away BMP4 binding. BMP7 is also capable of forming a highly active heterodimer with BMP4 (3–10 times more active than either BMP4 or -7 homodimers (Aono et al., 1995) that could also be subject to regulation by noggin. Another BMP-related molecule, anti-dorsalizing morphogenetic protein (ADMP), is expressed specifically in the organizer, yet ventralizes embryos when injected dorsally, in its normal site of expression (Moos et al., 1995). While not closely related to BMP2 and -4, ADMP is a candidate target for inactivation by noggin by virtue of its apparently contradictory organizer-specific expression and robust ventralizing activity. The involvement of diverse homo- and heterodimeric BMP activities, counteracted by noggin and potentially other organizer-secreted factors with distinct specificities, such as chordin and follistatin (Hemmati-Brivanlou et al., 1994; Sasai et al., 1994), suggests that dorsoventral axis formation may be regulated with considerable redundancy and specificity.

Detection and quantitation of endogenous noggin and BMP proteins in the embryo remains problematic. The very high affinity of noggin for BMP2 and -4 suggests these molecules are likely to interact where coexpressed. Effects where expressing cells are separated, or on other BMPs and TGFβ family members, are more difficult to predict. Recent evidence has suggested that the *Drosophila* BMP2/-4 homolog decapentaplegic may act as a morphogen during wing formation, eliciting distinct cellular responses as it diffuses away from its source (Nellen et al., 1996). noggin that is overexpressed in RNA-injected *Xenopus* embryos can also diffuse (J. D. J. and R. M. H., unpublished data), but diffusion may only occur when low affinity binding sites in the extracellular matrix are saturated by excess noggin. noggin does bind heparin, which suggests such an association with extracellular matrix (L. B. Z. and R. M. H.,

chordin mRNA or 2 ng of a dominant-negative BMP receptor mRNA (DN-BMPR) at the two cell stage. MAb 1A4 specifically blocked the dorsalizing effects of *Xenopus* noggin but not human noggin, chordin, or DN-BMPR.

unpublished data). In any case, noggin is likely to affect BMP activity gradients by acting as a sink for diffusible BMPs.

BMP activity may be subject to quenching by noggin in several embryonic tissues. noggin displays a complex pattern of expression later in embryogenesis, notably in developing skeleton and central nervous system (L. B. Z., R. M. H., L. Brunet, S. Takada, J. A. McMahon, and A. P. McMahon, unpublished data). BMPs are widely coexpressed with noggin in the developing skeleton. In the spinal cord, noggin is expressed in the roofplate (Smith and Harland, 1992, and unpublished data). BMP signaling has been implicated in the differentiation of dorsal cell types in the chick neural tube, with BMP4 and -7 expressed in the dorsal neural tube and the adjacent epidermal ectoderm, respectively (Liem et al., 1995). noggin could also modulate a later role for BMP2 in instructing neural crest cells to differentiate into neurons (Shah et al., 1996). In the mediolateral patterning of somites, an unidentified signal from the neural tube has been shown to antagonize BMP4 secreted by the lateral plate (Pourquie et al., 1996). BMP7 has also been shown to induce dendritic growth in rat sympathetic neurons (Lein et al., 1995); noggin, which has a complex pattern of expression in the developing brain (Valenzuela et al., 1995), could be involved in modulation of neurite arborization. In addition to its normal role in animal development, noggin may also prove a useful pharmacological reagent with which to manipulate BMP signaling in a variety of systems.

Here we describe a mechanism by which noggin may participate in Spemann organizer function. Do the rest of the candidate secreted organizer molecules act similarly, or on different pathways? Other treatments that induce neural tissue in *Xenopus* include expression of dominant negative activin and BMP receptors, both of which block the BMP signaling pathway, and injection of mRNAs encoding chordin or the other known TGF β -binding protein, follistatin (Hemmati-Brivanlou et al., 1994). Chordin appears to be the homolog of the *Drosophila* secreted factor short gastrulation, which antagonizes decapentaplegic (Holley et al., 1995; Sasai et al., 1995). While neither follistatin nor chordin bear significant sequence similarity to noggin or each other, both may have the potential to act by binding BMPs. It is an attractive possibility that many of the Spemann organizer's long-studied instructive properties may result from its ability to prevent other instructions, specifically ventralizing BMP-like signals, from reaching their targets.

Experimental Procedures

Design and Purification of Proteins

Purified recombinant human BMP2, -4, and -7 were the kind gift of Genetics Institute, Cambridge, Massachusetts. myc-tagged BMP4 protein was generated by using polymerase chain reaction (PCR) to introduce a half myc-tag containing an EcoRV site upstream of the first cysteine of the mature N-terminal domain of BMP4 (Nishimatsu et al., 1992) and, to facilitate cloning, an XhoI site at the 3' end. This fragment was then used to replace the mature domain of a myc-tagged dorsalin construct, dsl 501 (Basler et al., 1993; gift of T. Jessell, Columbia University), by cloning into the EcoRV site in the middle of the myc tag and the XhoI site in the downstream polylinker of pMT21. The dorsalin pro-region of this chimera is then

cleaved during maturation, resulting in a myc-tagged BMP4 molecule of the expected size (Aono et al., 1995). PCR primers: 5': BMP4myc(S): GCTG ATA TCC GAG GAG GAC CTG AAA CAT TGC CGG AGG CAT TC; 3': B4MC-D: CG CTC GAG TCA ACG GCA CCC ACA CCC (gift of Paul Wilson). This construct, pMT21BMP4myc(s), was then transfected into COS cells using lipofectamine (GIBCO-BRL, Gaithersburg, Maryland), and the supernatants were quantitated by Western blot with reference to known amounts of myc-tagged noggin as well as by biological activity in *Xenopus* explant assays (Dale et al., 1992). To construct Fc-tagged noggin, an SrfI-NotI human IgG1 fragment (Davis et al., 1994; Economides et al., 1995) was fused to human noggin using an oligonucleotide encoding the following peptide bridge sequence to incorporate the SrfI site: (noggin)... SECKCSC (end)- VAAGPGG (bridge)- EPKSCDKTHTCPP CPAP (hinge of human IgG1); this construct has specific activity indistinguishable from wild-type frog or human noggin proteins in *Xenopus* explant assays (data not shown). Fc-tagged human noggin was purified from COS supernatants and untagged human noggin was purified from baculovirus-infected insect cells as previously described (Lamb et al., 1993), and were the gift of Regeneron Pharmaceuticals Inc., Tarrytown, New York. myc-tagged activin-conditioned medium was made by transfection of COS cells with the plasmid pKB590 (a gift of K. Basler and T. Jessell, Columbia University). TGF β 1 purified from porcine platelets was purchased from R&D Systems, Minneapolis, Minnesota.

Sephacrose-noggin Preparation and BMP4myc Binding Assay

Xenopus noggin was purified from COS supernatants as previously described (Lamb et al., 1993) and coupled to CNBr-activated sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. Mock-coupled beads were exposed to an identical coupling and blocking protocol in the absence of noggin protein. Ten microliters of noggin- or mock-coupled beads were then incubated with 250 microliters of BMP4myc COS supernatant (~ 0.2 μ g/ml BMP4) and 1 μ g/ml gastrula extract in 1 ml of binding buffer (20 mM HEPES [pH 7.6], 10 mM MgCl₂, 300 mM NaCl, 0.1 mM PMSF, 5 μ g/ml leupeptin, and 0.1% CHAPS) for 2 hr at 4°C. Beads were then washed three times in 1 ml of binding buffer, boiled in sample buffer, and then subjected to Western analysis with 9E10 anti-myc monoclonal antibody.

Affinity Labeling and Precipitation of BMP Receptors

Radiolabeling of BMP4 was performed using Iodo-Beads (Pierce), with slight modifications. In brief, one iodobead was washed in phosphate-buffered saline (PBS), then incubated 5' at room temperature with 5 μ l (500 μ Ci) ¹²⁵I-Nal in 445 μ l of PBS. Two micrograms of BMP4 was brought to 50 μ l in 10 mM acetic acid, mixed with the reacting iodobead, and incubated for 5' at room temperature and 5' on ice. The iodobead was then removed, carrier protein (BSA) was added to 1 μ g/ml, and the solution was desalted over Pierce GF-5 columns according to the manufacturer's instructions.

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (DME 10%), transfected with lipofectamine and pCMV5-BMPRII-His, pCMV5-BMPRI-HA, or pCMV5 (kind gifts of Joan Massagué, Columbia University) and subjected to binding 36 to 48 hr later.

Whole cell binding and affinity labeling were performed using established procedures (Massagué, 1987), except that a protease inhibitor cocktail (0.1 mM PMSF, 0.5 μ g/ml leupeptin, pepstatin, chymostatin, aprotinin, and antipain) was added during the preincubation and binding procedures to decrease nonspecific binding (Koenig et al., 1994). In brief, ~ 2.5 nM ¹²⁵I-BMP4 was preincubated for 1 hr with or without 50 nM purified human noggin in binding buffer (128 mM NaCl, 5 mM KCl, 5 mM MgSO₄, 1.2 mM CaCl₂, 50 mM HEPES [pH 7.8], 2 mg/ml BSA) prior to binding to cell monolayers in 6-well plates, 0.67 ml per well for 3.5 hr at 4°C. The binding solution was then aspirated and monolayers were rinsed 3 times in ice-cold binding buffer and twice with crosslinking buffer (binding buffer without BSA or protease inhibitors). Crosslinking was performed using 750 mM bis(sulfosuccinimidyl) suberate (BS³) (Pierce) for 15' at 4°C. Cells were lysed for 20 min at 4°C in 50 mM Tris (pH 7.4), 150 mM NaCl, 0.75% Triton X-100 plus protease inhibitors. Extracts were clarified by centrifugation, and ¹²⁵I-BMP4-receptor complexes

were purified using the HA epitope and His tags on the receptors as previously described (Carcamo et al., 1995) and visualized by SDS-PAGE and phosphorimager.

BMP Activity Assays

W-20-17 cells (Thies et al., 1992)—a gift of Genetics Institute, Cambridge, Massachusetts—were plated in 96-well plates at 10^4 cells per well. Twenty-four hours later, serial dilutions of BMP4 in DME 10% were preincubated for 1 hr at room temperature with or without purified noggin protein, then applied to the cells. Each ligand concentration was assayed in triplicate. After an additional 24 hr incubation, cells were washed once with PBS and lysed by freeze-thawing twice in 50 μ l of water per well. Cell lysates were then assayed for alkaline phosphatase activity in 50 mM glycine, 0.05% Triton X-100, 4 mM $MgCl_2$, and 5 mM *p*-nitrophenol phosphate [pH 10.3], for 20 min at 37°C. The reaction was quenched by the addition of NaOH, and the absorbance at 410 nm was read on a Dynatech MR400 plate reader.

Coprecipitation Assays

For both quantitation of BMP4 affinity for noggin and for competition with other TGF β family members, ~ 5 pM ^{125}I -BMP4 was incubated with serial dilutions of purified unlabeled BMP2, -4, -7, or TGF β 1 in 0.5 ml binding buffer (128 mM NaCl, 5 mM KCl, 5 mM $MgSO_4$, 1.2 mM $CaCl_2$, 50 mM HEPES [pH 7.8], 2 mg/ml BSA) and 20 pM Fc-tagged human noggin (hnogFc) for 1 hr at 4°C, rotating. Protein G sepharose beads were then added, allowed to bind the Fc domain of hnogFc for another 30 min, washed four times with binding buffer, and boiled in loading buffer. Half of each sample was subjected to SDS-PAGE and coprecipitating ^{125}I -BMP4 was quantitated on a phosphorimager. Nonspecific binding was determined by two methods: binding in the absence of hnogFc, and binding in the presence of 20 nM (~ 5000 -fold excess) unlabeled BMP4.

Coprecipitation with a panel of purified anti-noggin monoclonal antibodies was performed essentially as above, with antibodies added at 50-fold molar excess over noggin (150 nM of each antibody, 3.3 nM *Xenopus* noggin, and ^{125}I -BMP4), except that 200 mM NaCl was added to the wash buffer. For quantitative analysis, duplicate samples were immunoprecipitated, blotted, and quantitated by Western blot/ECL and densitometry for noggin and by phosphorimager for ^{125}I -BMP4.

Ventral Marginal Zone Assay

Ventral marginal zone assays were performed essentially as described (Smith et al., 1993), with 3.3 nM *Xenopus* or human noggin and 150 nM antibody where indicated, in modified Danilchik's medium (53 mM NaCl, 5 mM Na_2CO_3 , 4.5 mM potassium gluconate, 32 mM sodium gluconate, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mg/ml BSA, and $1 \times$ antibiotic/antimycotic solution [Sigma #A9903]). Blocking effects were observed at a 10-fold molar excess of antibody (data not shown). Chordin (0.5 ng) and dominant negative BMP receptor transcripts (2 ng) were injected at the two-cell stage. Explants were harvested at stage 24 and analyzed for muscle actin and EF1 α expression by reverse transcription-PCR (Rupp and Weintraub, 1991; Wilson and Melton, 1994).

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