Development 126, 3347-3357 (1999)
Printed in Great Britain © The Company of Biologists Limited 1999
DEV3918

Xenopus GDF6, a new antagonist of noggin and a partner of BMPs

Chenbei Chang and Ali Hemmati-Brivanlou*

Department of Molecular Embryology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA
*Author for correspondence (e-mail: brvnlou@rockvax.rockefeller.edu)

Accepted 21 May; published on WWW 5 July 1999

SUMMARY

In *Xenopus*, ectodermal cell fates are determined by antagonistic interaction between the BMP subfamily of TGF-β ligands and the organizer-specific secreted factors (e.g. noggin, chordin and follistatin). Inhibition of BMP function by these factors can convert cells from an epidermal to a neural cell fate. In this study, we report that GDF6, a new member of the *Xenopus* TGF-β family, can function in antagonistic interaction with neural inducers. GDF6 induces epidermis and inhibits neural tissue in dissociated cells, and this activity is blocked by the presence of noggin. We demonstrate that GDF6 binds directly to the neural inducer noggin. Furthermore, we find that GDF6 and BMP2 can form heterodimers and the process seems to require cotranslation of the proteins in the same cells. In normal embryos, GDF6 and BMP2 are coexpressed in several places, including the edge of the neural plate at early neurula stages, suggesting that GDF6 may synergize with BMPs to regulate patterning of the ectoderm. Our data show for the first time that noggin can bind directly to and inhibit another TGF-β family member: GDF6. In addition, BMP and GDF6 heterodimers may play an important role in vivo to regulate cell fate determination and patterning.

Key words: GDF6, BMP, neural inducer, epidermal inducer, noggin

INTRODUCTION

In *Xenopus*, neural tissue is induced from dorsal ectoderm during gastrula stages. Factors secreted from the Spemann organizer act on the overlying ectoderm to induce formation of the neural plate, which is then further patterned along its anterior-posterior and medial-lateral axes (for reviews, see Wilson and Hemmati-Brivanlou, 1997; Chang and Hemmati-Brivanlou, 1998). Organizer-specific secreted factors, such as noggin, follistatin, chordin, cerberus and Xnr-3, have recently been identified which seem to mediate the patterning of ectoderm toward the neural fate (Smith and Harland, 1992; Lamb et al., 1993; Hemmati-Brivanlou et al., 1994; Sasai et al., 1994, 1995; Smith et al., 1995; Bouwmeester et al., 1996; Hansen et al., 1997). Injection of RNA encoding these proteins in *Xenopus* embryos diverts ectodermal explants (animal caps) from an epidermal to a neural fate. Ectodermal cells also adopt a neural fate when dissociated for an extended period of time in calcium- and magnesium-free buffer in the absence of any exogenous factors (Godsave and Slack, 1989; Grunz and Tacke, 1989). These results have led to the current model that neural is the default fate for ectodermal cells (Hemmati-Brivanlou and Melton, 1994). In normal embryos, endogenous signals present in the ectoderm block neural differentiation and inhibition of these negative signals by the organizer-specific secreted factors is required for the neural tissue to form.

Using a cell dissociation assay, it was found that Bone Morphogenetic Proteins (BMPs), belonging to the transforming growth factor-β (TGF-β) family, can mediate inhibition of neural and induction of epidermal fate (Wilson and Hemmati-Brivanlou, 1995). BMP4 and BMP2, as well as BMP7 to a lesser extent, can repress neural-specific marker expression and induce epidermal genes in dissociated animal cap cells (Suzuki et al., 1997a). In intact ectodermal explants, endogenous BMP signaling takes place, which maintains ectoderm in an epidermal fate. Various treatments that disrupt BMP signaling in animal caps, including the introduction of truncated BMP receptors or dominant negative BMP ligands, lead to direct conversion of epidermal cells to neural cells (Hawley et al., 1995; Sasai et al., 1995; Suzuki et al., 1995, 1997a; Xu et al., 1995).

Recently, it has been shown that organizer-specific secreted factors induce neural tissue by antagonizing BMP activity, in most cases through direct binding to BMP proteins (perhaps with the exception of Xnr-3, whose mechanism on neural induction is yet unknown). Both noggin and chordin interact with BMP4 and BMP2 with high affinity, while noggin also binds to BMP7 but with a much lower affinity (Piccolo et al., 1996; Zimmerman et al., 1996). Cerberus binds to BMP2 and inhibits BMP function (Hsu et al., 1998). Binding of these neural inducers to BMPs prevents the association of BMPs with their cognate receptors, thus blocking the BMP signal transduction pathway at the ligand level. The antagonistic interaction of organizer-secreted factors with BMPs seems to be sufficient to induce neural tissue. Other non-organizer factors, such as Gremlin and DAN, which are related to Cerberus, also interact antagonistically with BMP2 and they too induce neural tissue in animal caps (Hsu et al., 1998).

These results suggest that binding to BMPs to prevent their signaling downstream may be the only function of these factors in neural induction.

Though many of the neural inducers bind to BMPs, they seem to differ in their binding affinities for particular BMP ligands. Some of these factors also interact with ligands other than BMPs in the TGF-β family. Cerberus, for example, also antagonizes the function of Xnr2, a *Xenopus* nodal related factor (Hsu et al., 1998). The different specificity of BMP antagonists suggests that they have overlapping and distinct functions in vivo. Follistatin, which was originally identified as an activin inhibitor, has recently been reported to bind to BMPs directly (Fainsod et al., 1997; Iemura et al., 1998). Though several studies indicate that follistatin may preferentially inhibit BMP7 function (Yamashita et al., 1995; Liem et al., 1997; Iemura et al., 1998), binding assays show, surprisingly, that follistatin may bind to BMP2, BMP4 and BMP7 with equal affinities (Iemura et al., 1998). In addition, unlike noggin and chordin, follistatin-BMP4 complexes are still able to bind to BMP receptors (Iemura et al., 1998). Follistatin-BMP7 complexes have not been tested in receptor-binding assay and there is no functional assay for the consequence of the BMP4 binding. It is therefore not known whether follistatin selectively inhibits BMP7 activity after binding to ligands. Further experiments are required to correlate follistatin's BMP-binding ability and its BMP-antagonizing activity.

In *Xenopus* embryos, in addition to antagonistic interaction of BMPs with neural inducers, another level of regulation seems to exist during neural induction to reduce the level of selective BMP genes in the dorsal ectoderm. BMP2 and BMP7 RNAs are expressed in the entire presumptive ectoderm at gastrula stages, a time when neural induction occurs. BMP4 transcripts, on the contrary, are excluded at this stage from the dorsal side, including the dorsal ectoderm which is fated to become the future neural plate (Hemmati-Brivanlou and Thomsen, 1995). Thus, neural induction seems to involve suppression of the BMP inhibitors at the transcription level. To see if neural inducers can downregulate transcription of BMP4 or other related factors, we performed a TGF-β family-specific differential screen experiment. Using this method, we cloned the *Xenopus* GDF6 (Growth and Differentiation Factor 6), which is most homologous to the mouse GDF6 gene and can be repressed at the transcriptional level in dissected animal cap explants by the neural inducers noggin and follistatin at gastrula stages. We show that GDF6, like the BMPs, induces epidermal genes and inhibits neural markers in dissociated cells, and this activity is blocked by the presence of noggin. Interestingly we find that GDF6 binds directly to noggin with an affinity similar to that of BMP2. In addition, we demonstrate that GDF6 can form heterodimers with BMP2 and the two genes are coexpressed in several places in normal embryos. Our results suggest that the GDF subfamily of TGF-β ligands can engage in antagonistic interactions with neural inducers; and GDFs may synergize with BMPs through heterodimer formation to regulate endogenous neural induction and patterning.

MATERIALS AND METHODS

Cloning of GDF6 by differential screening of TGF-β family ligands and plasmid construction

Total RNA from midgastrula stage embryos was amplified by RT-PCR with primers hybridizing to the conserved domain of all TGF-β members. Sequences of the two degenerate TGF-β primers are TGF1: 5′-GCGGAATTCGGNTGGVANRANTGGRT-3′ and TGF2: 5′-GCGAAGCTTYTBNGTNGGNRCRCARCA-3′ (R=A/G, Y=C/T), encoding peptides GW(D/Q/N/K)(D/N)W(I/V) and (K/E/Q)TP(A/V)-CC, respectively. PCR fragments were cut with *Eco*RI and *Hin*dIII and inserted into the pBluescript vector. Duplicate filters of the transformed colonies were screened with labeled TGF-β PCR probes from two different sources. One source was PCR products from ectodermal explants injected with 0.2 ng noggin or 2 ng follistatin RNA, the other was from uninjected animal caps. RNAs from both sources were extracted when control embryos reached mid-late gastrula (stages 11-12). Colonies that hybridized only to the second probe but not to the first one were TGF-β members that were downregulated by noggin or follistatin at gastrula stages. Several of such clones encoded a 180 bp fragment that was most homologous to the mouse *GDF6* gene. We used this fragment as a probe to screen a *Xenopus* stage 28 head library (Hemmati-Brivanlou et al., 1991). Among 10^6 plaques that we screened, 9 positive clones with 1.8 kb insert were obtained, all of them had the same restriction digestion pattern. Sequences of these clones confirmed that they all encoded the *Xenopus GDF6* gene. The gene was subsequently cloned into the pSP64T vector by PCR cloning method. Two primers used for PCR are GDF6-N: 5′-GCGAGATCTACCATGGATACATACAGGAGC-3′ and GDF6-C: 5′-GCGAGATCTCTATTACCTACATCCACAGGA-CTCTA-3′. The PCR products were cut with *Bgl*II and inserted into the *Bgl*II site of the pSP64T vector. For G6myc construct, two additional PCR primers are used: GDF6-myc(R) 5′-GCGGAATA-TTAGCTTCTGTTCTCTTTTGCCATGCCTGCTGTT-3′ and GDF6-myc(F): 5′-AAGCTGATATCTGAAGAAGACTTGCATGGAAGG-AAATCCAGGCTG-3′. The PCR products with the primer set of GDF6-N/GDF6-myc(R) were cut with *Ssp*I and ligated to the *Eco*RV digested PCR products using primers GDF6-C and GDF6-myc(F). The resulting fragment was cut with *Bgl*II and inserted into the pSP64T vector.

Embryos, RNA preparation, microinjection and ectodermal explants

Pigmented and albino *Xenopus* embryos were obtained and staged as described before (Hemmati-Brivanlou and Harland, 1989). RNA used for microinjection was synthesized with SP6 RNA polymerase on linearized DNA templates as described previously (Chang et al., 1997). p64T-GDF6 and p64T-G6myc were linearized with *Eco*RI enzyme, while p64T-BMP2 was linearized with *Xba*I. The template for activin and AVg1 RNA synthesis were made as reported before (Kessler and Melton, 1995). RNA was injected into the animal pole of 2-cell-stage embryos and animal caps were explanted at blastula stage (stage 9). Total RNA was extracted from the animal caps at the indicated stages and RT-PCR was performed to assay for specific marker expression, as previously described (Chang et al., 1997).

RT-PCR

RT-PCR was performed as previously described (Chang et al., 1997). Primers used for analysis of GDF6 expression were GDF6-U: 5′-GAATACGGATCTGCTTTGCAGG-3′ and GDF6-D: 5′-GGAC-TCAGTTTGAACAGGTGCC-3′, and 25 cycles were used in the PCR reaction. Other primer sequences were described previously (Chang et al., 1997).

Whole-mount in situ hybridization

In situ hybridization was performed on albino embryos with the digoxigenin-labeled probe as described before (Harland, 1991). GDF6 probe was synthesized with T7 RNA polymerase on *Not*I linearized pBS-GDF6 template.

Oocyte injection and protein analysis

Xenopus stage 5-6 oocytes (Smith et al., 1991b) were isolated

manually in MBS buffer (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM Hepes, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$). Each oocyte was injected with 30 to 40 ng of RNA encoding GDF6, G6myc, BMP2, BMP7, activin and Vg1. The injected oocytes were incubated in MBS buffer containing 0.5 mg/ml BSA for 2-3 days before the supernatant was collected and analyzed by SDS-PAGE (i.e. by electrophoresis). To label protein samples with [^{35}S]methionine and cysteine, radiolabeled amino acid mix was added into the oocyte culture medium at 1:40 dilution (New England Nucleotide). For the dissociated animal cap assay, the oocyte-conditioned medium was added at a 10- to 20-fold dilution in Ca^{2+} and Mg^{2+}-free buffer. For protein immunoprecipitation assay, 30-200 μl of oocyte supernatant was incubated with 5 μl anti-myc antibody. Protein binding was performed at room temperature in TBST (20 mM Tris, 100 mM NaCl, 0.1% Tween-20, pH7.6) containing 0.5 mg/ml BSA for 1 hour. The protein G-coupled agarose beads were present in the binding solution or added after 1 hour and the binding was continued for another hour. The beads were then washed four times with TBST before they were dissolved in protein loading buffer and analyzed by SDS-PAGE. For quantitative protein-binding competition analysis, the amount of proteins in conditioned oocyte medium was quantified by Bio-Rad DC protein assay kit, using the manufacturer's protocol that is based on Lowry method (Lowry et al., 1951). In addition, part of the secreted proteins were labeled and independently checked by SDS-PAGE.

RESULTS

Cloning of GDF6 by differential screening of TGF-β ligands

To identify TGF-β ligands that may be transcriptionally regulated by neural inducers at an early developmental stage, we employed a modified differential screening strategy based on the reverse transcription-polymerase chain reaction (RT-PCR) method. This strategy allows us to identify TGF-β ligands that are either upregulated or downregulated at the onset of neural induction. In this screening protocol, cDNAs from *Xenopus* late gastrula embryos are amplified with primers hybridizing to the conserved domain of all TGF-β members. Duplicate filters containing these PCR clones are screened with two different TGF-β probes, both of which are PCR products made from cDNA of animal caps explanted at blastula stages and incubated to late gastrula stages. One probe is from uninjected animal caps, while the other is from animal caps neuralized by noggin or follistatin RNA injection. Colonies that hybridize to only one of the probes represent differentially regulated TGF-β ligands. With this scheme, we did not uncover any TGF-β members that are upregulated by neural inducers, but we did isolate several clones that seem to be downregulated by the neural inducers follistatin or noggin. Sequence analysis of these clones revealed that most of them encoded a *Xenopus* homolog of the GDF6 (Growth and Differentiation Factor 6) gene. A full-length GDF6 cDNA was obtained by screening a *Xenopus* stage 28 head library. As shown in Fig. 1A, like all other TGF-β members, GDF6 has a signal peptide at its N terminus (underlined) and a consensus protein processing sequence RXXR (boxed). The mature peptide of *Xenopus* GDF6 shows high homology to that of other GDF ligands, especially to the product of the mouse *GDF6* gene (Storm et al., 1994), with more than 90% identity at the amino acid level (Fig. 1B). Outside the GDF subfamily, GDF6 is most closely related to the BMPs, with 55% and 53% amino acid identity to *Xenopus* BMP2 and BMP4, respectively (Fig. 1C).

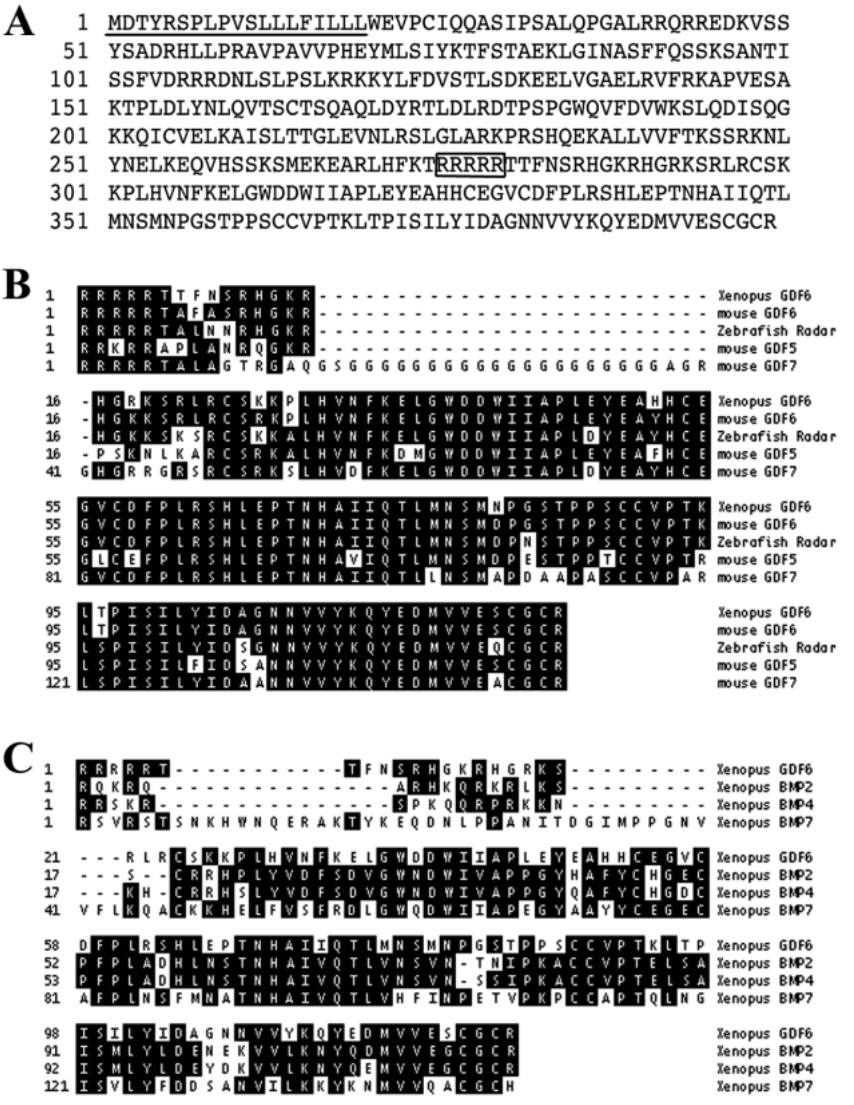

Fig. 1. *Xenopus* GDF6 is a close homolog of GDFs and BMPs. (A) Sequence of the *Xenopus* GDF6 protein. The signal peptide is underlined and the proteolytic processing site is boxed. (B) GDF6 is highly conserved between species. Alignment of *Xenopus* GDF6 mature region with that of GDF6, GDF5 and GDF7 from other species. *Xenopus* GDF6 is more than 90% identical to mouse GDF6. (C) GDF6 is homologous to BMPs. Alignment of the mature region of *Xenopus* GDF6 with that of *Xenopus* BMPs. GDF6 is about 50% identical to BMP2, BMP4 and BMP7 in their mature regions, while it is less homologous to activin and other TGF-βs. GenBank accession number for *Xenopus* GDF6 is AF155125

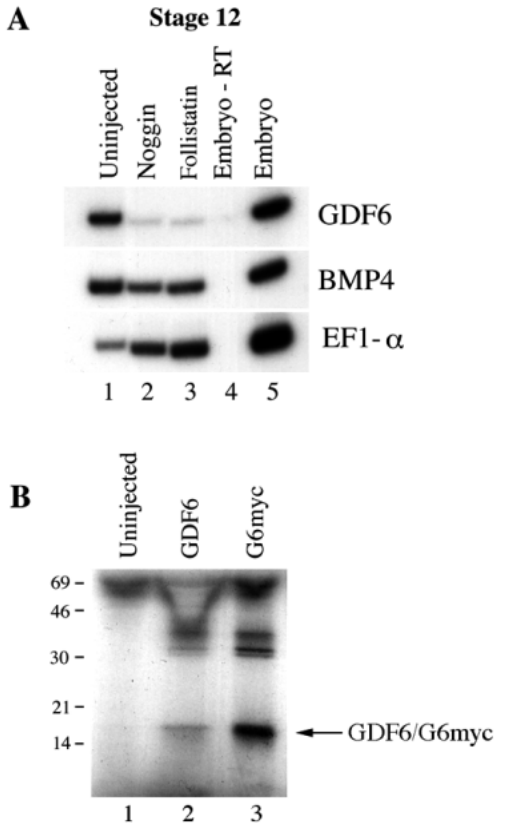

Fig. 2. GDF6 is downregulated by neural inducers at gastrula stages at the RNA level and is efficiently secreted from oocytes. (A) GDF6, but not BMP4, transcripts are significantly downregulated by neural inducers noggin and follistatin at late gastrula stages (stage 11-12). Embryos were injected at the 2-cell stage with 0.2 ng noggin or 2 ng follistatin RNA (lanes 2 and 3, respectively). Animal caps were dissected at blastula stage and incubated to late gastrula stage before they were processed for RT-PCR assay. Lane 1 is from uninjected control embryos, while lanes 4 and 5 are from whole embryos with (lane 5) or without (lane 4) reverse transcriptase during RT reaction. (B) GDF6 is secreted from *Xenopus* oocytes. Oocytes were injected with RNAs encoding GDF6 (lane 2) or a myc-tagged GDF6 (G6myc, lane 3). The oocyte culturing medium was collected 2 days later and analyzed on 10% SDS-PAGE. While uninjected oocytes did not secret any proteins at a significant level (lane 1), the supernatant from oocytes injected with GDF6 and G6myc contains two bands, corresponding to the processed mature and the pro-region of the protein.

Since GDF6 was isolated by differential screening, we wanted to see if the gene could be regulated at the RNA level by neural inducers at gastrula stages. We compared GDF6 expression in animal caps injected with noggin or follistatin RNA with that in uninjected control caps at late gastrula stage (stage 12, Nieuwkoop and Faber, 1967). As shown in Fig. 2A, GDF6 transcription can be detected in uninjected control explants at stage 12 (lane 1). This expression, however, is suppressed by the presence of the neural inducers noggin or follistatin (compare lanes 2 and 3 with lane 1 in Fig. 2A), confirming the results from the differential screening strategy. In contrast, BMP4 transcription, which is inhibited in the dorsal region in normal embryos at this stage, is less significantly downregulated by the neural inducers (Fig. 2A). The result suggests that a different mechanism may exist to repress BMP4 transcription at the dorsal side of gastrula embryos in vivo.

To test whether GDF6 is a secreted factor, we injected GDF6 RNA into *Xenopus* oocytes and incubated them in medium containing [^{35}S]methionine and cysteine labeling mix. The supernatant was collected 2 days later and resolved on the denaturing SDS protein gel. Two prominent bands about 32 kDa and 14 kDa in size were detected that correspond to the processed pro-region and the mature region of the GDF6 protein respectively (lane 2, Fig. 2B). Uninjected oocytes did not secrete any detectable proteins (lane 1 in Fig. 2B). When a myc-tag was added to the N terminus of the mature GDF6, the resulting protein (G6myc) was also secreted and processed (lane 3 in Fig. 2B). Thus GDF6 and G6myc are secreted efficiently from oocytes and the addition of the myc-tag to the N terminus of the mature GDF6 did not affect the secretion of GDF6.

Expression of GDF6 during development

To see if GDF6 is expressed at an appropriate time to play a role in the regulation of neural induction and/or patterning, we assayed GDF6 expression at different developmental stages by RT-PCR. Fig. 3A shows that GDF6 mRNA can first be detected weakly after the midblastula transition when zygotic transcription begins and is maintained at low levels until midgastrula stages, at which time the transcription of GDF6 increases and remains relatively constant until at least tailbud stages. To see if the early expression of GDF6 is localized preferentially in specific regions at gastrula stages, we analyzed GDF6 transcription in dissected early gastrula (stage 10+) embryos. Fig. 3B shows that GDF6 expression is restricted to the animal caps of early gastrula embryos and cannot be detected above background level in the rest of the embryos (compare lane 1 with lanes 2 to 4). The detection of Vg1 in the vegetal region, chordin in the dorsal and Xvent1 in the ventral marginal zones attest to the accuracy of microsurgery in these experiments (Fig. 3B, Weeks and Melton, 1987; Sasai et al., 1994; Gawantka et al., 1995; Onichtchouk et al., 1996). As GDF6 expression can be downregulated in isolated animal caps by several neural inducers at gastrula stages, we wanted to test whether restricted GDF6 expression in the embryonic ectoderm is differentially distributed. We therefore dissected dorsal and ventral animal caps at stage 10 and assayed for the region-specific markers Zic3 and Vent1 (Gawantka et al., 1995; Nakata et al., 1997). As previously reported, we detected Zic3 signal in the dorsal ectoderm and Vent1 in the ventral ectoderm (Fig. 3C), confirming the accuracy of our dissection. GDF6, like Vent1, is downregulated in the dorsal explant at this early gastrula stage. In contrast, BMP4 RNA, which is excluded from the dorsal lip, is present at equal levels at stage 10 (Fig. 3C, Hemmati-Brivanlou and Thomsen, 1995). This in vivo expression pattern is consistent with our results obtained from animal cap assays (Fig. 2A) and demonstrates that GDF6 and BMP4 may be subject to different transcriptional regulation at early gastrula stages.

To study in more detail the distribution of GDF6 transcripts in embryos at different stages of development, we performed whole-mount in situ hybridization (Harland, 1991). At early gastrula stages, GDF6 is weakly expressed in the animal region

(not shown). Expression is enhanced at the border of the dorsal and ventral ectoderm from mid-gastrula onward, seen as two stripes reminiscent of the early neural-crest-specific Slug

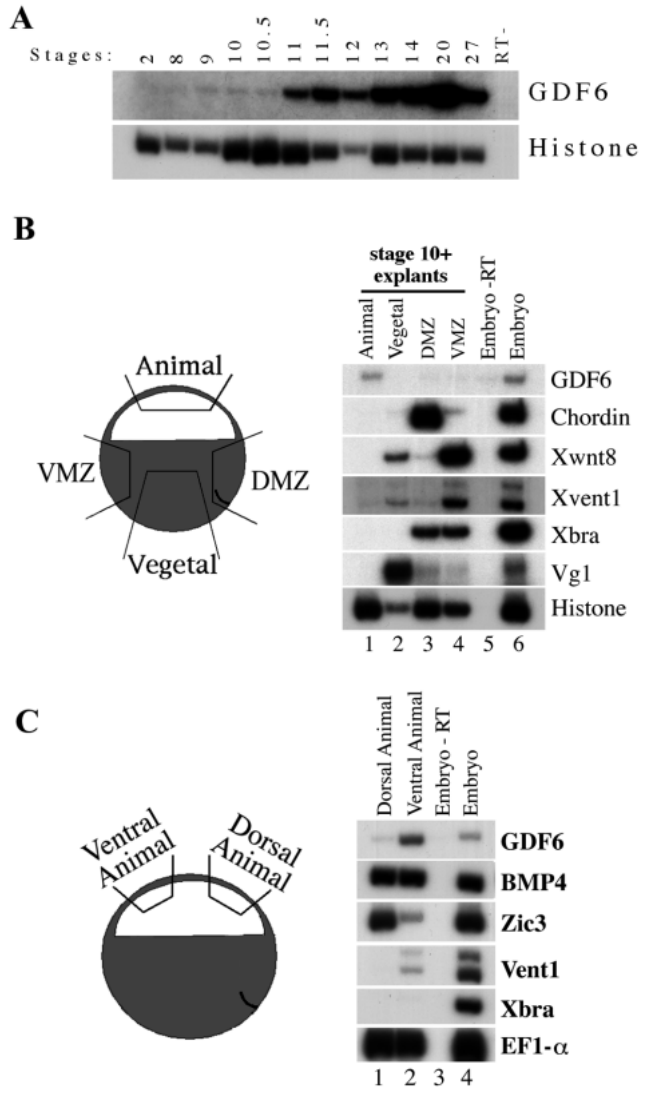

Fig. 3. Early zygotic expression of GDF6 is restricted to the animal cap. **(**A) GDF6 is expressed weakly after the midblastula transition. The expression increases significantly around the mid-gastrula stages and maintains its level until at least tailbud stages. (B) GDF6 expression is restricted to the animal caps of early gastrula embryos. Early gastrula stage (stage 10+) embryos were dissected into animal and vegetal (lanes 1 and 2), dorsal marginal zone and ventral marginal zone (DMZ and VMZ respectively, lanes 3 and 4) regions. The dissection scheme is shown at the left. RT-PCR method was used to assay for region-specific markers (Chordin is a dorsal and Xvent1 is a ventral marker, Xwnt8 is expressed ventral-laterally, Vg1 is a marker for vegetal pole and Xbra, *Xenopus* brachyury, is a pan-mesoderm marker) as well as GDF6 expression. GDF6 is expressed only in the animal caps at this early gastrula stage. (C) GDF6 transcripts are downregulated in the dorsal ectoderm at early gastrula stages. Stage 10 embryos were dissected into dorsal and ventral animal caps (scheme shown at the left). RT-PCR was used to assay for dorsal animal (lane 1) and ventral animal pole (lane 2) markers. Zic3 is a dorsal-specific marker, while Vent1 is a ventral marker. Xbra is a pan-mesodermal marker. Unlike BMP4, which is uniform at this stage in animal caps, GDF6 is downregulated in the dorsal animal region (compare lane 1 with lane 2).

expression domain (Fig. 4A; Nieto et al., 1994). At neural plate stages, GDF6 is detected symmetrically at the edges of the neural plate as well as in two stripes at the anterior of the neural plate (Fig. 4B,C). The expression at the border of the neural plate persists in neurula embryos (Fig. 4D-F), and is present as a salt-and-pepper pattern rather than a uniform staining of the whole neural fold region (e.g. Fig. 4E, inset). This dotted expression of GDF6 remains after neural tube closure and occupies the dorsal midline region of the neural tube (Fig. 4H). At tailbud stages, GDF6 is expressed at the dorsal hindbrain and the dorsal midline of the spinal cord (Fig. 4I-K). Staining of GDF6 in a subset of cells in the neural folds and the dorsal neural tube during embryonic development likely demarcates the premigratory neural crest cells in these regions. Consistently, GDF6 expression is excluded from the most anterior border of the neural plate, a region where no neural crest cells are observed (Fig. 4D,F). The rostral border of GDF6 in the neural plate is marked by two patches of expression at approximately the level of the diencephalon (Fig. 4D,F). These two patches come together as the neural tube closes and later develop into a continuous band at a position corresponding to the olfactory placode at tailbud stages (Fig. 4D,F,G,H,L). In addition, GDF6 staining is observed just outside the anterior neural folds in the eye buds at late neurula stages (Fig. 4D,F). In tailbud embryos, this expression is localized to the dorsal retina of the developing eyes (Fig. 4G,I,L), which is coincidental with the expression domain of BMP4 in the eyes at these stages (Hemmati-Brivanlou and Thomsen, 1995). Unlike BMPs, no GDF6 expression outside of the ectoderm is observed at the stages that we examined, which indicates that GDF6 may only participate in ectodermal cell fate determination. Though the embryonic expression of the mouse GDF6 is not reported at this time, the expression pattern of *Xenopus* GDF6 is very similar to that of Radar, a Zebrafish GDF6 homolog (Rissi et al., 1995), suggesting that they may have conserved function during embryogenesis.

GDF6 can function as a neural inhibitor and an epidermal inducer

GDF6 is downregulated by neural inducers at gastrula stages, suggesting that inhibition of this gene may be relevant to neural induction. To determine whether overexpression of GDF6 can block neuralization of animal caps, we performed a cell dissociation assay in the presence or absence of GDF6 protein. Animal caps were dissected at blastula stage and dissociated in calcium (Ca^{2+})- and magnesium (Mg^{2+})-free buffer for 4 hours, either with or without soluble GDF6 protein obtained from oocyte supernatant. The cells were then reaggregated in the presence of Ca^{2+} and Mg^{2+} and incubated until sibling control embryos reached mid-neurula stages, at which point they were processed for expression of tissue-specific markers by RT-PCR. As shown in Fig. 5A, when animal caps were dissociated, rather than left intact, their identity was shifted from an epidermal to a neural fate. Neural markers NCAM (neural cell adhesion molecule, Kintner and Melton, 1987) and OtxA (Lamb et al., 1993), as well as cement gland marker XAG-1 (Hemmati-Brivanlou et al., 1990), were turned on, while the epidermal keratin gene (Jonas et al., 1985) was switched off by dissociation (compare lane 2 with lane 1). Conditioned medium from uninjected oocytes had no effect on this gene expression pattern (lane 3 in Fig. 5A). When

supernatant collected from GDF6-injected oocytes was included during dissociation, however, epidermal marker expression was restored and neural genes were repressed (compare lane 5 with lane 2 in Fig. 5A). The change of cell fate by GDF6 occurred in the absence of mesoderm induction, as the mesodermal marker Brachyury (Xbra) was not expressed (Fig. 5A, Smith et al., 1991a). Consistent with previous reports, BMP2-conditioned oocyte medium also had neural inhibition and epidermal induction activity (lane 4, Suzuki et al., 1997a). In addition, when we normalized the amount of BMP2 and GDF6 in the oocyte-conditioned medium (see Materials and Methods) and tested the ability of the two proteins to induce epidermal markers in dissociated cells, we found that GDF6 and BMP2 induced epidermis at a similar range of dilution (not shown). Our data suggest that GDF6 acts, like the BMPs, as a neural inhibitor and an epidermal inducer in *Xenopus* ectodermal explants.

In embryos, the epidermal induction activity of BMPs can be antagonized by neural inducers derived from the organizer. To see if the epidermal inducing activity of GDF6 can also be blocked by neural inducers, we tested the effect of noggin on GDF6 in the cell dissociation assay. As shown in Fig. 5B, while GDF6 protein can induce epidermal keratin and repress neural genes such as NRP-1 (Richter et al., 1990) and OtxA, its activity is blocked by noggin protein when they are coincubated during cell dissociation (compare lanes 3 and 4 in Fig. 5B). This result indicates that noggin can inhibit epidermal induction by GDF6.

Noggin binds directly to GDF6

The BMP family of neural inhibitors (BMP2, BMP4 and BMP7) can associate directly with the neural inducers noggin and chordin (Piccolo et al., 1996; Zimmerman et al., 1996). Binding of BMPs to these neural inducers prevents the association of BMP ligands to their receptors; the resulting blockage of BMP signaling in cells alters their fate from an epidermal to a neural fate (Piccolo et al., 1996; Zimmerman et al., 1996). Since the epidermal inducing activity of GDF6 can be blocked by noggin, we wanted to address whether GDF6 could also bind directly to noggin and if so whether the binding, like that of BMPs, was specific. We performed a competition experiment (Fig. 6A). An unlabeled noggin-Fc protein (Ng-Fc, Zimmerman et al., 1996) was used to precipitate [^{35}S]methionine- and cysteine-labeled GDF6 or BMP2. In the absence of Ng-Fc, a very low background of GDF6 binding to protein G beads was observed (lane 3 in Fig. 6A). The binding signal was strongly enhanced in the presence of Ng-Fc (compare lane 5 with lane 3), suggesting the association of GDF6 with Ng-Fc. Similarly BMP2 was only precipitated in the presence of Ng-Fc (compare lane 6 with lane 4 in Fig. 6A). Addition of unlabeled GDF6 (lanes 7 and 8) or BMP4 (lanes 9 and 10) competed the binding of noggin-Fc to both radiolabeled BMP2 and GDF6. To see if other TGF-β ligands can also compete with GDF6 to bind to noggin, we performed the experiment with activin β_B as well as secreted Vg1 protein (AVg1, Kessler and Melton, 1995). As shown in Fig. 6B, while BMP2-conditioned oocyte medium can compete with GDF6 for noggin, neither activin nor Vg1 attenuates the GDF6 signal, indicating that these two proteins do not bind to noggin and cannot compete with GDF6 (lanes 5-8 in Fig. 6B). The competition experiment suggests that binding of GDF6 to noggin is specific and that GDF6 and BMPs may recognize the same site on noggin protein.

Although several members of the BMP family bind to noggin, they do so with different affinities. BMP2 and BMP4 bind to noggin with a higher affinity than does BMP7 (Zimmerman et al., 1996). In order to determine whether GDF6 binds to noggin with an affinity similar to one of the BMP ligands, we performed a dose-dependent binding competition experiment (Fig. 6C). Binding of ^{35}S-labeled GDF6 to Noggin-Fc was challenged with different amounts

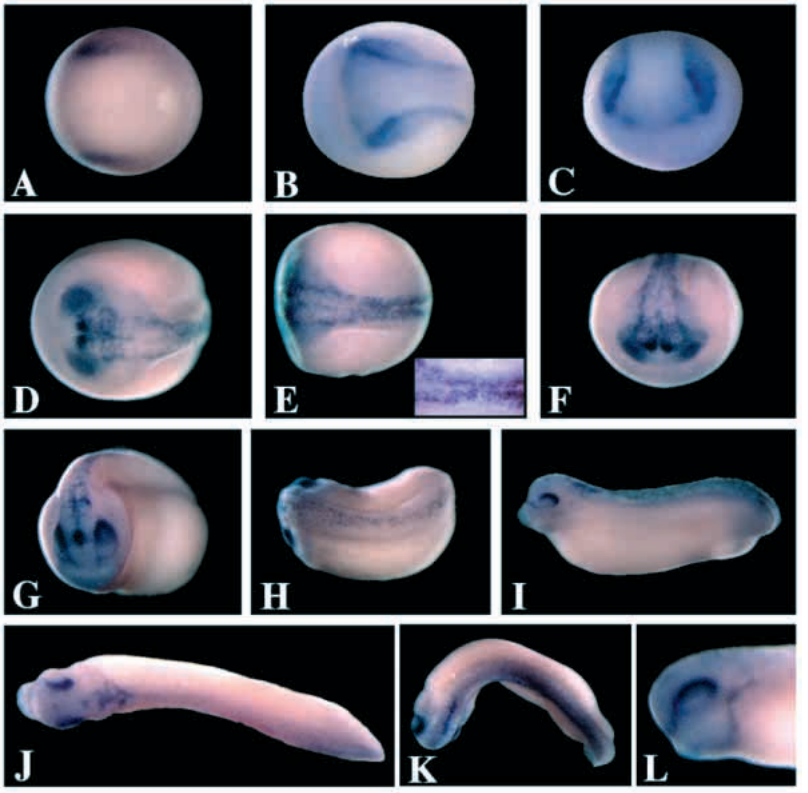

Fig. 4. Spatial expression of GDF6 during early *Xenopus* development. (A) GDF6 is expressed at the border of the dorsal and ventral ectoderm around stages 11 to 12. (B,C) At neurula stages, GDF6 is expressed at the edges of the neural plate, as well as in two patches in the anterior neural plate. (D-F) At late neurula stages 18-19, GDF6 is distributed in a salt-and-pepper pattern in the neural folds (inset of panel E), possibly representing the premigratory neural crest cells. In addition, GDF6 is expressed in olfactory placode and in the eye field. (G,H) After the neural tube closure, GDF6 occupies the dorsal midline of the neural tube, again in a dotted fashion. The expression in the olfactory placode persists, and the staining in the eye field becomes localized to the dorsal part. (I-L) At tailbud stages, GDF6 is seen at the dorsal hindbrain and the dorsal midline of the spinal cord. It is also expressed in the dorsal retina of the developing eyes. Embryos are viewed from dorsal side in A, B, D, E, H, J and K, with anterior end to the left. In C, F and G, the embryos are viewed head on from anterior end. Embryos in I and J are viewed from lateral side with anterior end to the left

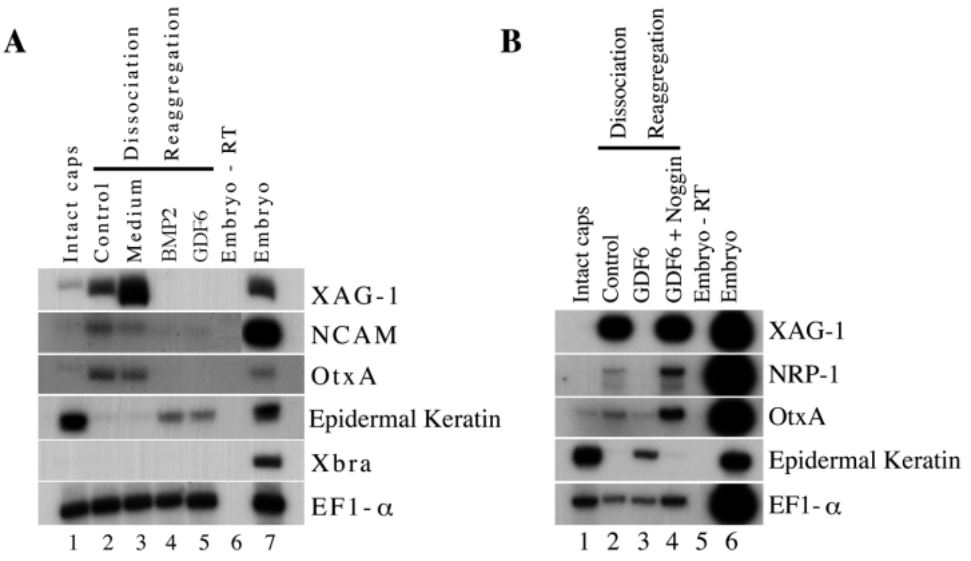

Fig. 5. GDF6 inhibits neural tissue and induces epidermis in dissociated animal caps, and its activity is blocked by noggin. (A) Animal caps from stage 9 embryos were dissociated in Ca^{2+}- and Mg^{2+}-free medium for 4 hours before reaggregated and cultured to neurula stages. Lane 1 is from the intact caps, which expresses the epidermal marker epidermal keratin and does not express neural genes such as NCAM and OtxA. Lanes 2-5 are animal caps dissociated for 4 hours. Lanes 3-5 contain conditioned oocyte medium collected from uninjected control oocytes (lane 3), BMP2-injected (lane 4) and GDF6-injected (lane 5) oocytes. BMP2 and GDF6 convert the cells from a neural to an epidermal fate. Lanes 6 and 7 are whole embryo control, with (lane 7) or without (lane 6) reverse transcriptase during the RT reaction. (B) Noggin blocks epidermal inducing activity of GDF6. Lane 1 is from intact animal caps, while lanes 2 to 4 are from cells dissociated for 4 hours. GDF6-conditioned medium is present in samples in lanes 3 and 4, and noggin protein is present in samples on lane 4 during cell dissociation.

of unlabeled GDF6, BMP2 or BMP7, which were collected from oocyte-conditioned medium (see Materials and Methods). As shown in Fig. 6C, binding of the radiolabeled GDF6 to noggin is effectively competed away by the presence of 3- to 5-fold of unlabeled GDF6 or BMP2 (lanes 2 to 9, representative of three independent experiments). In contrast, unlabeled BMP7 cannot compete with GDF6 for noggin binding even at 10-fold excess (lanes 10 to 13, Fig. 6C). This result indicates that GDF6 and BMP2 bind to noggin with similar affinities, while BMP7 binds to noggin with a lower affinity.

Heterodimer formation between GDF6 and BMP2

Different members of the BMP subfamily of TGF-β ligands can form heterodimers. In the case of BMP4 and BMP7, the heterodimer is more potent as a mesoderm inducer (Suzuki et al., 1997b). To test whether BMP and GDF6 can also dimerize, we coinjected the RNAs encoding BMP2 and a myc-tagged GDF6 (G6myc) into *Xenopus* oocytes and collected the conditioned supernatant 2 days later to analyze the interaction between BMP2 and GDF6 by coimmunoprecipitation. An anti-myc antibody precipitates G6myc, but not BMP2, from conditioned oocyte medium (lanes 1 and 2 in Fig. 7). When the RNAs encoding the two genes were coinjected into the oocytes, BMP2 is coprecipitated by the anti-myc antibody along with G6myc (lane 3 of Fig. 7). The coprecipitation of BMP2 and G6myc seems to require coexpression of the two genes in the same cells, as BMP2 cannot be precipitated with G6myc when oocyte-conditioned medium containing BMP2 and G6myc are mixed together (not shown). A reverse experiment using a flag-tagged BMP2 (B2flag) and an anti-flag antibody produced the same result. GDF6, which could not be precipitated by the antibody, was coprecipitated with B2flag when the two genes were coexpressed in the oocytes (data not shown). These results show that BMP2 and GDF6 can interact with each other to form a stable complex when coexpressed in the same cells.

DISCUSSION

In this study, we report the characterization of GDF6 as a novel epidermal inducer and neural inhibitor in *Xenopus* embryos. Several other GDFs have been implicated in regulation of developmental processes, such as GDF5 in joint formation (Storm and Kingsley, 1996), GDF9 in ovarian folliculogenesis (Dong et al., 1996) and, more recently, GDF7 in a subset of dorsal sensory interneuron specification (Lee et al., 1998). Our results, however, show for the first time that a GDF family ligand can also be involved in neural development at an early step. Like BMP family of neural inhibitors, GDF6 is a direct target for noggin. Binding of GDF6 to noggin blocks the epidermal inducing activity of GDF6. In addition, we show that GDF6 can heterodimerize with BMP2, raising the interesting possibility that heterodimer formation between members of TGF-β family ligands may play an important role in vivo during embryogenesis.

Regulation of TGF-β ligands at the RNA level by organizer-specific factors

Organizer-secreted factors may regulate BMP/GDF activity in the dorsal ectoderm by several means. They may (i) trigger a direct repression of BMP/GDF transcription, (ii) regulate the stability of their RNA and (iii) antagonize the function of existing proteins. In the case of BMP4, its transcripts are excluded from the organizer of early gastrula embryos, then also from the future neural plate at midgastrula stages. The expression pattern indicates that downregulation of this gene may be important for neural induction. In our study, we find that none of several organizer-secreted neural inducers, such as noggin, chordin and follistatin, repress BMP4 transcription significantly in animal caps at gastrula stages. These results suggest that other factors are involved in downregulation of BMP4 transcripts. Goosecoid, an organizer-specific transcription factor, has been shown to reduce the size of the BMP4 expression domain in the marginal zone of gastrula

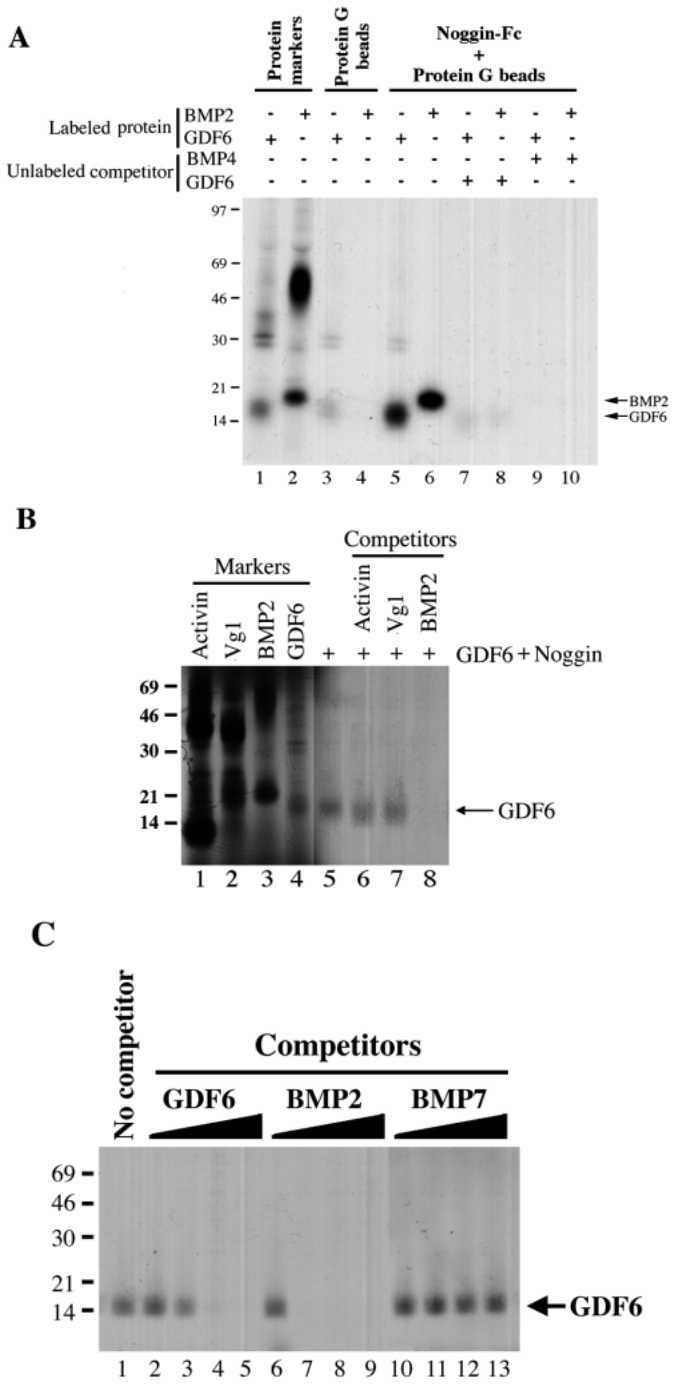

Fig. 6. (A) GDF6 binds specifically to noggin. Lanes 1 and 2 are markers for labeled GDF6 and BMP2, respectively. Lanes 3 and 4 are the two proteins incubated only with the protein G beads in the absence of noggin-Fc. Lanes 5 to 10 are the two proteins coincubated with noggin-Fc and protein G beads. Lanes 7 and 8 include unlabeled GDF6, while lanes 9 and 10 include BMP4 as specific competitor for noggin binding. Odd-numbered lanes contain labeled GDF6 protein, and even-numbered lanes contain labeled BMP2 protein. Binding of GDF6 or BMP2 to noggin can be competed away by unlabeled BMP4 or GDF6 ligands. (B) BMP2, but not activin or Vg1, can compete with GDF6 for noggin binding. Lanes 1 to 4 are markers for secreted TGF-β ligands activin, Vg1, BMP2 and GDF6. Lanes 5-8 contain noggin-Fc with labeled GDF6, and lanes 6-8 include binding competitors activin, Vg1 and BMP2, respectively. (C) GDF6 binds to noggin with an affinity closer to BMP2 than to BMP7. Lane 1 is binding of ^{35}S-labeled GDF6 to noggin-Fc in the absence of any competitors, while the rest of the lanes are samples containing 1:1 (lanes 2, 6 and 10), 3:1 (lanes 3, 7 and 11), 5:1 (lanes 4, 8 and 12) and 10:1 (lanes 5, 9 and 13) ratio of unlabeled GDF6 (lanes 2-5), BMP2 (lanes 6-9) or BMP7 (lanes 10-13) to labeled GDF6. Binding of ^{35}S-labeled GDF6 to noggin is competed away by the presence of 3- to 5-fold of unlabeled GDF6 or BMP2, while BMP7 cannot compete for GDF6 binding even at 10-fold excess.

embryos (Fainsod et al., 1994). It is not known, however, whether Goosecoid also represses BMP4 transcription in animal caps. As Goosecoid does not induce neural tissue in ectodermal explants, it will be important to determine the effect of goosecoid on BMP4 expression in animal caps. Recently a winged helix transcription factor, BF-2, has been identified that strongly represses BMP4 transcription, this activity seems to directly correlate with its ability to induce neural markers in animal caps (Mariani and Harland, 1998). Since BF-2 is not expressed at early gastrula stages, other factors belonging to this family of transcription factors may play a role in early regulation of BMP4 expression. In contrast to BMP4, GDF6 expression is suppressed by noggin, chordin and follistatin at gastrula stages in animal caps. In early gastrula embryos, GDF6 is expressed at a lower level in the dorsal than in the ventral ectoderm, supporting the notion that organizer-secreted factors may downregulate GDF6 expression. The exact mechanism for GDF6 transcriptional regulation is unknown. As these neural inducers function to block BMP and GDF signaling (see below), one mechanism might be through positive feedback regulation by GDF and/or BMP members of TGF-β ligands. GDF6 expression, however, does not seem to be upregulated by BMP4. In isolated animal caps, GDF6 transcripts diminish by neurula stages. Overexpression of BMP4 in these caps is not able to enhance GDF6 transcription (data not shown). In normal neurula stage embryos, GDF6 is expressed mainly at the border of the neural plate and the epidermis, a region where intermediate BMP concentration is proposed to exist (Wilson et al., 1997; Marchant et al., 1998). These results suggest that a balance between neural inducers and BMPs may be important to maintain GDF6 transcription. The difference in regulation of BMP4 and GDF6 expression by these organizer-secreted factors may reflect different pathways that the embryos employ to downregulate genes with epidermalizing activity from the future neural plate.

Antagonistic interaction between GDF6 and noggin

The repression of GDF6 transcription by several neural inducers suggests that GDF6 may play a role in neural induction and/or patterning. Consistent with this idea, GDF6 protein can inhibit neuralization and revert cells from a neural to an epidermal fate in dissociated animal caps. Furthermore the epidermal inducing activity of GDF6 is inhibited by noggin at the protein level, indicating that the neural inducer can antagonize GDF6 function in regulation of ectodermal cell fate determination. Biochemical analysis presented in this study revealed that one mechanism for this antagonistic interaction is the binding of noggin directly to GDF6 and the binding can be competed away by unlabeled BMP4, BMP2 or GDF6 ligands, but not by the related factors activin or Vg1. The

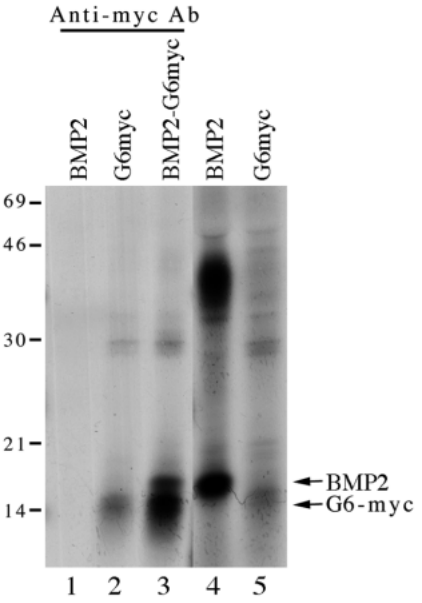

Fig. 7. Heterodimer formation between GDF6 and BMP2. Conditioned oocyte supernatant was precipitated with anti-myc antibody. The antibody precipitates G6myc, but not BMP2 protein (compare lanes 2 and 1). However when the two genes are coexpressed in the oocytes, BMP2 can be coimmunoprecipitated with G6myc, suggesting heterodimer formation between the two proteins (lane 3). Lanes 4 and 5 are markers for BMP2 and G6myc, respectively.

results suggest that the binding of GDF6 to noggin is specific. Among the BMP family of ligands, BMP7 has been shown to bind to noggin at a significantly lower affinity than BMP2 or BMP4 (Zimmerman et al., 1996). As both BMP7 and GDF6 are about 50% identical to BMP2/4 at the protein level, they may assume a similar low affinity to noggin. Surprisingly, however, by quantitative competition experiment, BMP7 is shown unable to compete with GDF6 to bind to noggin even at 10-fold excess while both GDF6 and BMP2 compete the binding at 3- to 5-fold range. The result suggests that GDF6 binds to noggin at an affinity closer to BMP2/4 than to BMP7, and that there may be a difference in how different BMP/GDF ligands are involved in epidermal induction and neural inhibition in vivo. As GDF6 is expressed over the animal region in early gastrula embryos, the antagonistic interaction between noggin and GDF6 may be important to determine the neural cell fate in dorsal ectoderm. In agreement with the notion, blocking the signal pathway with a dominant negative GDF6 ligand leads to expansion of cement gland and neural tissue in vivo, while injection of wild-type GDF6 RNA into early *Xenopus* embryos results in mild ventralization phenotype (data not shown).

The interaction of GDF6 and noggin may not be limited to neural inhibition and epidermal induction. During neurula stages, GDF6 is expressed in a salt-and-pepper fashion at the border of the neural plate, which seems to contribute subsequently to the prospective neural crest (Fig. 4). At these stages, noggin is expressed in the notochord, which underlines the ventral midline of the neural plate. The exclusive expression pattern of these two secreted factors near the perspective dorsal and ventral neural tube suggests that antagonistic interaction between noggin and GDF6 may also play an important role in patterning neural tissue. It has been suggested that, in *Xenopus*, antagonistic interaction between other BMPs and neural inducers is involved in the determination of cell fates along the border of the neural plate and the epidermis, such as cement gland and neural crest (Morgan and Sargent, 1997; Wilson et al., 1997; Marchant et al., 1998). Similarly the expression pattern of GDF6 suggests that it may participate in regulation of neural crest formation. This function of GDF6 is currently under investigation.

GDF6 as a potential partner of BMPs

Both GDF6 and BMPs have the ability to induce epidermis and inhibit neural tissue, and their activities can be blocked by a dominant negative GDF6 mutant (data not shown). The data suggest that GDF6 and BMPs may function in a similar way. There are several steps at which GDF6 and BMP signals may converge in the process of ectodermal cell fate determination, e.g. at the level of receptor binding or downstream signal transducers. Interestingly, in addition to possibly sharing a similar pathway, we find that BMP2 and GDF6 can directly associate with each other to form heterodimers when they are coexpressed in oocytes. Dimer formation between GDF6 and BMP2 seems to require coexpression of the two genes in the same cells, as a mixture of oocyte-conditioned medium containing GDF6 and BMP2 proteins, respectively, does not lead to dimer formation. BMP4, a close homolog of BMP2, may also interact with GDF6; the dominant negative GDF6 mutant blocks mesodermal and epidermal induction by BMP4 when they are coinjected into the embryos (data not shown). In normal embryos, heterodimer formation may occur in the region where GDF6 and BMP2/BMP4 expression overlaps, such as the ventral ectoderm of gastrula embryos and the border of the neural plate of neurula embryos. GDF6 and BMP4 are also coexpressed in the dorsal retina of the developing eyes. Heterodimers between GDF6 and BMPs may thus play a role in these regions to regulate ectodermal cell fate determination. It is unclear at this time whether GDF6/BMP dimers, if they form in vivo, have different function(s) than either of the homodimers alone. In the case of the BMPs, BMP4/BMP7 heterodimers are much more effective at mesoderm induction in animal caps than a mixture of the two homodimers (Suzuki et al., 1997b). It is possible that GDF6/BMP heterodimers are also more potent than homodimers in determination of specific cell fates of ectodermal cells.

In summary, we have cloned the *Xenopus GDF6* gene and showed that it may be involved in ectodermal cell fate determination through antagonistic interaction with specific neural inducers. As *Xenopus* GDF6 is highly homologous to GDF6 in other vertebrates, with about 90% amino acid identity in their mature region, it is likely that GDF6 may also be involved in antagonistic interaction with neural inducers in these species to regulate the ectodermal cell fate specification. Consistent with this idea, embryonic expression of the Zebrafish *GDF6* homolog, *Radar*, is very similar to that of the *Xenopus* gene (Rissi et al., 1995), suggesting a conserved function of GDF6 across species.

We would like to thank Aris Economides (Regeneron Inc., NY) for providing noggin-Fc protein and for technical advice on protein

binding, Atsushi Suzuki for providing flag-tagged BMP2 construct. We thank Drs. Curtis Altman, Bart Eggen, Dan Weinstein, Georgio Lagna and Atsushi Suzuki for critical reading of the manuscript. The work is supported by NIH grant HD 32105-01 to A. H.-B., who is a McKnight, Klingenstein and Merck scholar.

REFERENCES

Bouwmeester, T., Kim, S.-H., Sasai, Y., Lu, B. and DeRobertis, E. M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. *Nature* **382,** 595-601.

Chang, C., Wilson, P. A., Mathews, L. S. and Hemmati-Brivanlou, A. (1997). A Xenopus type I activin receptor mediates mesodermal but not neural specification during embryogenesis. *Development* **124**, 827-837.

Chang, C. and Hemmati-Brivanlou, A. (1998). Cell fate determination in embryonic ectoderm. *J. Neurobiol.* **36,** 128-151.

Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N. and Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**, 531-535.

Fainsod, A., Deissler, K., Yelin, R., Marom, K., Epstein, M., Pillemer, G., Steinbeisser, H. and Blum, M. (1997). The dorsalizing and neural inducing gene follistatin is an antagonist of BMP-4. *Mech. Dev.* **63**, 39-50.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J.* **13,** 5015-5025.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *EMBO J* **14**, 6268-6279.

Godsave, S. F. and Slack, J. M. W. (1989). Clonal analysis of mesoderm induction in Xenopus laevis. *Dev. Biol.* **134**, 486-490.

Grunz, H. and Tacke, L. (1989). Neural differentiation of Xenopus laevis ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Differ. Dev.* **28**, 211-218.

Hansen, C. S., Marion, C. D., Steele, K., George, S. and Smith, W.C. (1997). Direct neural induction and selective inhibition of mesoderm and epidermis inducers by Xnr3. *Development* **124**, 483-492.

Harland, R. M. (1991). In situ hybridization: an improved wholemount method for Xenopus embryos. *Methods Cell Biology* **36**, 675-685.

Hawley, S. H. B, Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W. and Cho, K. W. Y. (1995). Disruption of BMP signals in embryonic Xenopus ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

Hemmati-Brivanlou, A. and Harland, R. M. (1989). Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Hemmati-Brivanlou, A.,de la Torre, J. R., Holt, C. and Harland, R. M. (1991). Cephalic expression and molecular characterization of *Xenopus* En-2. *Development* **111**, 715-724.

Hemmati-Brivanlou, A., Kelly, O. G. and Melton, D. A. (1994). Follistatin, an antagonist of activin, is expressed in the Spemann organizer and displays direct neuralizing activity. *Cell* **77**, 283-295.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

Hemmati-Brivanlou, A. and Thomsen, G. H. (1995). Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. *Dev. Genet.* **17**, 78-89.

Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M. and Harland, R. M. (1998). The Xenopus dorsalizing factor Gremlin identifies a novel family of secreted proteins that antagonize BMP activities. *Mol. Cell* **1,** 673-683.

Iemura, S., Yamamoto, T. S., Takagi, C., Uchiyama, H., Natsume, T., Shimasaki, S., Sugino, H. and Ueno, N. (1998). Direct binding of follistatin to a complex of bone-morphogenetic protein and its receptor inhibits ventral and epidermal cell fates in early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **95,** 9337-9342.

Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **82**, 5413-5417.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-2164.

Kintner, C. R. and Melton, D. A. (1987). Expression of *Xenopus* N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Lamb, T. M., Knecht, A. K., Smith, W. C., Stachel, S. E., Economides, A. N., Stahl, N., Yancopolous, G. D. and Harland, R. M. (1993). Neural induction by the secreted polypeptide noggin. *Sci.* **262**, 713-718.

Lee, K. J., Mendelsohn, M. and Jessell, T. M. (1998) Neuronal patterning by BMPs: a requirement for GDF7 in the generation of a discrete class of commissural interneurons in the mouse spinal cord. *Genes & Dev.* **12,** 3394-3407.

Liem Jr., K. F., Tremml, G. and Jessell, T. M. (1997). A role for the roof plate and its resident TGFβ-related proteins in neuronal patterning in the dorsal spinal cord. *Cell* **91,** 127-138.

Lowry, O.H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193,** 265-275.

Marchant, L., Linker, C., Ruiz, P., Guerrero, N. and Mayor, R. (1998). The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev. Biol.* **198,** 319-329.

Mariani, F. V. and Harland, R. M. (1998). XBF-2 is a transcriptional repressor that converts ectoderm into neural tissue. *Development* **125,** 5019-5031.

Morgan, R. and Sargent, M. G. (1997). The role in neural patterning of translation initiation factor eIF4AII; induction of neural fold genes. *Development* **124**, 2751-2760.

Nakata, K., Nagai, T., Aruga, J. and Mikoshiba, K. (1997). Xenopus Zic3, a primary regulator both in neural and neural crest development. *Proc. Natl. Acad. Sci. USA* **94**, 11980-11985.

Nieto, M. A., Sargent, M. G., Wilkinson, D. G. and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Sci.* **264**, 835-839.

Nieuwkoop, P. D. and Faber, J. (1967). Normal table of Xenopus laevis (Daudin). North-Holland, Amsterdam.

Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1996). The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling dorsoventral patterning of *Xenopus* mesoderm. *Development* **122**, 3045-3053.

Piccolo, S., Sasai, Y., Lu, B. and De Robertis, E. M. (1996). Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell* **86**, 589-598.

Richter, K., Good, P. J. and Dawid, I. B. (1990). A developmentally regulated, nervous system-specific gene in Xenopus encodes a putative RNA-binding protein. *New Biol.* **2**, 556-565.

Rissi, M., Wittbrodt, J., Delot, E., Naegeli, M. and Rosa, F. M. (1995). Zebrafish Radar: a new member of the TGF-β superfamily defines dorsal regions of the neural plate and the embryonic retina. *Mech. Dev.* **49,** 223-234.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.

Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and Bmp-4 antagonistic patterning signals in Xenopus. *Nature* **376**, 333-336.

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991a). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Smith, L. D., Xu, W. and Varnold, R. L. (1991b). Oogenesis and oocyte isolation. *Methods in Cell Biology* **36**, 45-60.

Smith, W. C. and Harland, R. M. (1992). Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell* **70**, 829-840.

Smith, W. C., McKendry, R., Ribisi Jr., S. and Harland, R. M. (1995). A nodal-related gene defines a physical and functional domain within the Spemann organizer. *Cell* **82**, 37-46.

Storm, E. E., Huynh, T. V., Copeland, N. G., Jenkins, N. A., Kingsley, D. M. and Lee, S.-J. (1994). Limb alterations in brachypodism mice due to mutations in a new member of the TGFβ-superfamily. *Nature* **368,** 639-643.

Storm, E. E. and Kingsley, D. M. (1996). Joint patterning defects caused by single and double mutations in members of the bone morphogenetic protein (BMP) family. *Development* **122**, 3969-3979.

Suzuki, A., Shioda, N. and Ueno, N. (1995). Bone morphogenetic protein acts as a ventral mesoderm modifier in early Xenopus embryos. *Dev. Growth Differ.* **37**, 581-588.

Suzuki, A., Kaneko, E., Ueno, N. and Hemmati-Brivanlou, A. (1997a). Regulation of epidermal induction by BMP2 and BMP7 signaling. *Dev. Biol.* **189**, 112-122.

Suzuki, A., Kaneko, E., Maeda, J. and Ueno, N. (1997b). Mesoderm

induction by BMP-4 and -7 heterodimers. *Biochem. Biophys. Res. Commun.* **232**, 153-156.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861-867.

Whitman, M. (1998). Smads and early developmental signaling by the TGFβ superfamily. *Genes Dev.* **12,** 2445-2462.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Wilson, P. A. and Hemmati-Brivanlou, A. (1997). Vertebrate neural induction: inducers, inhibitors, and a new synthesis. *Neuron* **18**, 699-710.

Wilson, P. A., Lagna, G., Suzuki, A. and Hemmati-Brivanlou, A. (1997). Concentration-dependent patterning of the *Xenopus* ectoderm by BMP4 and its signal transducer Smad1. *Development* **124**, 3177-3184.

Xu, R. H., Kim, J., Taira, M., Zhan, S., Sredni, D. and Kung, H. F. (1995). A dominant-negative bone morphogenetic protein 4 receptor causes neuralization in Xenopus ectoderm. *Biochem. Biophys. Res. Commun.* **212**, 212-219.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C.-H. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130,** 217-226.

Zimmerman, L. B., De Jesus-Escobar, J. M. and Harland, R. M. (1996). The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell* **86**, 599-606.