Coordination of BMP-3b and cerberus is required for head formation of *Xenopus* embryos

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Abstract

Bone morphogenetic proteins (BMPs) and their antagonists are involved in the axial patterning of vertebrate embryos. We report that both BMP-3b and BMP-3 dorsalize *Xenopus* embryos, but act as dissimilar antagonists within the BMP family. BMP-3b injected into *Xenopus* embryos triggered secondary head formation in an autonomous manner, whereas BMP-3 induced aberrant tail formation. At the molecular level, BMP-3b antagonized nodal-like proteins and ventralizing BMPs, whereas BMP-3 antagonized only the latter. These differences are due to divergence of their pro-domains. Less BMP-3b than BMP-3 precursor is proteolytically processed in embryos. BMP-3b protein associated with a monomeric form of Xnr1, a nodal-like protein, whereas BMP-3 did not. These molecular features are consistent with their expression profiles during *Xenopus* development. XBMP-3b is expressed in the prechordal plate, while xBMP-3 is expressed in the notochord. Using antisense morpholino oligonucleotides, we found that the depletion of both xBMP-3b and cerberus, a head inducer, caused headless *Xenopus* embryos, whereas the depletion of both xBMP-3 and cerberus affected the size of the somite. These results revealed that xBMP-3b and cerberus are essential for head formation regulated by the Spemann organizer, and that xBMP-3b and perhaps xBMP-3 are involved in the axial patterning of *Xenopus* embryos.

Keywords: BMP-3b; GDF-10; BMP-3; Osteogenin; Cerberus; Axial patterning; Head formation; Self-differentiation; Spemann organizer

Introduction

Interactions between bone morphogenetic proteins (BMPs) and BMP binding proteins regulate the axial patterning of vertebrate embryos (Thomsen, 1997). Studies of *Xenopus* embryos have shown that BMP-mediated interaction is predominantly involved in dorsal–ventral patterning of the embryo. However, loss-of-function studies in mouse and zebrafish suggest that BMP-mediated interaction affects anterior–posterior, as well as dorsal–ventral patterning, since the depletion of BMP binding proteins generates headless embryos (Schulte-Merker et al., 1997; Bachiller et al., 2000). These findings raise the following issues about the embryonic functions of BMPs and of BMP binding proteins. First, BMP-mediated interaction is directly or indirectly related to head formation as a consequence of anterior patterning. Second, axial patterning by BMPs and by BMP binding proteins appears to involve more BMP-related molecules and/or other signaling pathways, such as the Wnt signaling pathway (Kiecker and Niehrs, 2001). Third, BMP-mediated patterning has been reconsidered from the perspectives of neural induction and patterning, since BMP binding proteins have been identified through searches for neural inducers (Harland, 2000; Wilson and Edlund, 2001). The present study examines head formation and axial patterning regulated by BMP-mediated interactions.

Head formation and axial patterning of amphibian embryos are regulated by a small group of cells called the...
Spemann organizer that forms in the dorsal equatorial region of the early gastrula (Harland and Gerhart, 1997). The organizer comprises at least a superficial and a deep layer of cells (Vodicka and Gerhart, 1995). The superficial layer becomes dorsal mesoderm and has typical organizing activities (Shih and Keller, 1992). In contrast, the deep layer becomes anterior–dorsal endoderm and mesoderm, and it might have head-organizing activity. However, the deep layer of cells has no organizing activity, or less than the superficial layer (Shih and Keller, 1992; Schneider and Mercola, 1999). During gastrulation, the organizer cells differentiate to dorsal mesoderm (that is, prechordal plate and notochord) and produce vertical and planar inducers to specify adjacent ectoderm and endoderm along the anterior–posterior and dorsal–ventral axes (Shih and Keller, 1992; Horb and Slack, 2001). In particular, prechordal plate mesoderm specifies anterior neuroectoderm and pharyngeal endoderm, which are progenitors for the organization of head structures, and the mesoderm therefore directs head formation (Ruiz i Altaba, 1992; Barlow, 2001). Shortly thereafter, specification of dorsal mesoderm along the anterior–posterior axis becomes critical for embryonic head formation and whole body patterning. However, the molecular mechanisms remain uncertain.

Many studies have identified secreted and transcriptional factors for dorsal mesoderm induction and specification. Among these factors, nodal and its signaling molecules, such as Smad2, are key to dorsal mesoderm formation (Whitman, 2001). A recent study using anti-phospho Smad2 antibody has revealed that nodal signaling is dynamically regulated in *Xenopus* embryos (Lee et al., 2001). Consistent with previous investigations of nodal (Agius et al., 2000; Gritsman et al., 2000), Smad2 is phosphorylated (that is, activated) in the dorsal mesoderm at the start of gastrulation. However, Smad2 phosphorylation is attenuated in the mesoderm during gastrulation, suggesting that the inhibition of nodal signaling is related to the specification of dorsal mesoderm along the anterior–posterior axis.

Cerberus, an antagonist of nodal, BMP-4, and Xwnt-8, is expressed in the anterior endomesoderm region of the Spemann organizer (Piccolo et al., 1999). Consistent with *Xenopus* cerberus, mouse cerberus-like protein is expressed in the anterior visceral endoderm, which is the head-organizing center of mouse embryos (Belo et al., 1997). The overexpression of cerberus and of cerberus-like protein in *Xenopus* embryos causes ectopic head formation and an enlarged head respectively, yet a loss of the *Cerberus* function in mice does not obviously perturb embryogenesis (Belo et al., 2000; Shawlot et al., 2000; Stanley et al., 2000). Thus, the embryonic functions of cerberus appear to be coordinated with other factors that specify dorsal mesoderm along the anterior–posterior axis.

BMP-3b (also called GDF-10) and BMP-3 (also called osteogenin) are structurally different from other members of the BMP family. BMP-3b was originally isolated from the rat femur, but its biological activity remains unclear (Takao et al., 1996). BMP-3 has been copurified with BMP-1 and with BMP-2 that can induce ectopic bone formation in adult animals. However, recombinant BMP-3 alone does not possess such activity, but instead is a BMP-2 antagonist (Luyten et al., 1989; Wozney, 1989; Daluiski et al., 2001). Although BMP-3b shares 81% amino acid sequence identity with BMP-3 in the ligand domain, their endogenous functions may not be identical. The distribution of BMP-3b mRNA in adult tissues differs from that of BMP-3 (Takao et al., 1996; Hino et al., 1996). Moreover, regulation of the BMP-3b gene in osteoblastic cells is opposite that of BMP-3. When osteogenesis is enhanced or suppressed in vitro, BMP-3b transcription correlates with osteogenic activity, while that of BMP-3 is inversely related (Hino et al., 1999). Their embryonic functions are also uncertain. Both BMP-3b (GDF-10) and BMP-3 are transcribed at an early stage (8.5 dpc) of mouse development, whereas targeted disruption of either BMP-3b (GDF-10) or BMP-3 results in no identifiable phenotype (Dudley and Robertson, 1997; Zhao et al., 1999; Daluiski et al., 2001). Although their expression in the mouse embryo does not overlap, BMP-3b and BMP-3 might complement each other.

To further understand the basis of these observations, we thoroughly examined the embryonic distribution and functions of BMP-3b and BMP-3 in *Xenopus* and directly compared them with those of other members of the BMP family. The present study shows the embryonic distribution of xBMP-3b and xBMP-3. *Xenopus* BMP-3b is expressed early in a subset of ectodermal cells and later in the prechordal plate, while xBMP-3 is expressed early in the ectoderm and mesoderm and later in the notochord. We then demonstrate that BMP-3b leads to secondary head formation, whereas BMP-3 does not. At the molecular level, BMP-3b interferes with mesoderm induction by ventralizing BMPs (BMP-2 and ADMP) and dorsal mesoderm inducers (Xnr1 and derrière). In contrast, BMP-3 interferes with the induction by only ventralizing BMPs. We examine these features of BMP-3b and BMP-3 by Western blotting and by immunoprecipitation. We also analyze the embryonic functions of these BMPs and cerberus using antisense morpholino oligonucleotides (MOs). Individual injections of MOs for the xBMPs and for cerberus alone in the organizer region have no identifiable phenotypic effect during *Xenopus* development. However, the simultaneous injection of xBMP-3b and cerberus MOs triggers gastrulation defects as well as headlessness in *Xenopus* embryos. Our results generally suggest that xBMP-3b together with cerberus is related to the regional specification of dorsal mesoderm and that regulation of xBMP-3b/3 activity mediates the anterior–posterior patterning of *Xenopus* embryos.
Materials and methods

Cloning Xenopus BMP-3b and BMP-3

Xenopus cDNAs were identified by PCR using total RNAs extracted from embryos and frontoparietal bone, and the following degenerate primers: BMP-3b, 5'-GCRCAN-GTNTCNACNSWCAT-3' and 5'-TGGGAYGARCCNMGNTNTG-3'; BMP-3, 5'-GCNTGYCARTYYCCNATG-CC-3' and 5'-GCRCANSWyTCNACNGTCTC-3'. The 5' and 3' ends of the cDNAs were cloned by using the RACE System (Gibco-BRL). Full-length cDNAs were isolated by PCR using Pyrobest DNA polymerase (Takara, Japan) and primers as follows: xBMP-3b, 5'-ATCCCAAGATGCAGA-GTAACCTTTA-3' and 5'-AATTGATGCTTTAACAATAATCCTTT-3'; BMP-3, 5'-AGTAGGCTTGTGAGATAGATC-3' and 5'-ACCTTTGTGTGCTTGCTGTICAG-3'. We verified 10 independent cDNAs by sequencing.

Embryological assays

Microinjection and explant cultures proceeded as described (Nishimatsu and Thomsen, 1998). We produced UV embryos by irradiating the vegetal hemisphere of dejellied eggs 25 min after fertilization with a 245-nm UV source for 90 s. This procedure provided embryos with a dorsocentral index grade of 1 or 2 (Kao and Elinson, 1988). In situ hybridization and β-gal staining proceeded as described (Tracy et al., 1998). Antisense and sense probes were synthesized in the presence of digoxigenin-UTP and colored with BM purple (Roche). After hybridization, embryos were postfixed and bleached in 1% hydrogen peroxide/5% formaldehyde/0.5 × SSC by gentle shaking under fluorescent light. For histological analysis, embryos were embedded in paraffin or in 0.5% gelatin/30% egg albumin/20% sucrose as described (Shamim et al., 1999), then sections of paraffin blocks were cut at 10 μm and mounted directly or counterstained with nuclear Fast Red (Merck). Sections of gel albumin were cut at 40 μm on a Vibratome and colored for microscopy by using AQUATEX (Merck). The following primers for RT-PCR were applied as described (Nishimatsu and Thomsen, 1998): xBMP-3b (Accession No. AB059564), 5'-CTCTCGCAGGTATTTAACATGATTTGCCGC-3' and 5'-GCTATGCTTTAACAATAATCCTTT-3'; BMP-3, 5'-AGTAGGCTTGAGATAGATC-3' and 5'-ACCTTTGTGTGCTTGCTTGGTICAG-3'. We verified 10 independent cDNAs by sequencing.

Constructions of chimeras and tagged proteins

Chimeras, Flag, and Myc-tagged proteins were constructed by PCR amplification and by cDNA fragment exchange. Template DNAs were subcloned into the pCS2+ plasmid. Table 1 lists the primers and fragments. DNA was digested with restriction enzymes and recombined, then all mutations were confirmed by DNA sequencing. Flag- and Myc-tagged proteins had the same biological activity as the wild types.

Biochemical analyses

Embryo extracts were prepared as follows. One nanogram of mRNA encoding Flag BMP-3b or BMP-3 was injected into animal hemispheres at the four-cell stage. The injected animal pole was isolated at stage 8.5 to remove the vegetal hemisphere containing a large amount of yolk proteins. Animal caps were harvested at stage 11 and lysed in Laemmli buffer. An amount of lysate equivalent to four caps was Western blotted. Conditioned media transfected with expression plasmids using FuGENE-6 (Roche) were collected 3 days posttransfection, and immunoprecipitated using FLAG Tagged Protein Immunoprecipitation (SIGMA) or Immunoprecipitation (Roche) Kits with anti-Myc monoclonal antibody (9E10; Santa Cruz Biotechnology). Conditioned medium concentrated to one-tenth of the original volume over CENTRIPREP (Millipore) adjusted with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) was incubated with 20 μl of M2-agarose beads or anti-Myc antibody plus protein G agarose at 4°C overnight. After three washes with lysis buffer, immunoprecipitates were resolved by SDS–PAGE in Laemmli buffer with or without 5% β-mercaptoethanol. An amount of the mixture equivalent to 10 ml (reducing conditions) or 20 ml (nonreducing conditions) of culture medium was Western blotted as follows.

Samples were collected on 16% SDS polyacrylamide gels and electroblotted onto PVDF membranes that were subsequently incubated with anti-Flag monoclonal antibody (M2; Sigma), or anti-Myc polyclonal antibodies (A14;
Table 1
DNA fragments and PCR primers used to construct chimera and tagged proteins

<table>
<thead>
<tr>
<th>Chimera</th>
<th>N-terminal region DNA fragment or PCR primer</th>
<th>C-terminal region DNA fragment or PCR primer</th>
</tr>
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<tbody>
<tr>
<td>BMP-3b/BMP-3 (99)</td>
<td>BMP-3b (HindIII)</td>
<td>5’-CGGAGGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCT-3’ (HindIII)</td>
</tr>
<tr>
<td>BMP-3b/BMP-3 (202)</td>
<td>5’-TTGAGGATATCCTGAGGCTTGGATCAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
<tr>
<td>BMP-3b/BMP-3 (287)</td>
<td>5’-GTAAGAGGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
<tr>
<td>BMP-3b/BMP-3 (285)</td>
<td>5’-CCACGTGCAAGCTGAGGCTTGGATCAGGCTGAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
<tr>
<td>BMP-3/xBMP-2</td>
<td>5’-CATGCAAGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
<tr>
<td>hBMP-2/BMP-3</td>
<td>5’-GAGAGGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
<tr>
<td>Myc-xNr1</td>
<td>5’-CTGCAAGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCTGAGGCT-3’ (EcoRV)</td>
<td>BMP-3 (EcoRV and KpnI)</td>
</tr>
</tbody>
</table>

Note: Primers used to amplify either N-terminal or C-terminal region of cDNA are listed. BMP-3b and BMP-3 cDNAs were from rat and BMP-2 was from Xenopus and human. Other PCR primers were derived from the pCS2 plasmid: 5’-CGGAGGCTACATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCT-3’ for the N-terminal region, 5’-GTATCCTATCCTTCTGAGGCTTGGATCAGGCTGAGGCTGAGGCTGAGGCT-3’ for the C-terminal region, respectively. Restriction sites designed in each primer are boxed. Underlines represent sequences encoding either Flag tag (BMP2/3b chimera, BMP-3b, and BMP-3), or Myc tag epitope.
Santa Cruz) for 1 h in 1% skim milk. Tagged proteins were detected by using HRP-conjugated secondary antibodies and chemiluminescence (ECL Plus; Amersham). The results shown were reproduced in three to six independent experiments.

**Morpholino antisense procedure**

Fluorescein-labeled morpholino antisense oligonucleotides (MOs) synthesized by GENE TOOLS LLC (Oregon) were dissolved in amphibian Ringer’s buffer (88 mM NaCl/1 mM KCl/15 mM Tris/HCl, pH 7.5) to a concentration of 1 mM (equivalent to 8.5 mg/ml) as stock solutions. The respective positive and negative controls were MOs for β-catenin and globin. The morpholino sequences were: xBMP-3 MO, 5'-AAGACATAACTAGAGAGAATTGC-3’; BMP-3 MO, 5’-GAGCCAGGGGGCCACACCTCT-GCCA-TAC-3’; cerberus MO, 5’-AGATCCTGAGTACATTTAGTAAACAT-3’.

We assayed in vitro interference by MOs using the TNT Coupled Reticulocyte Lysate System (Promega), according to the manufacturer’s instructions. For the in vivo assay, a total of 10 ng of MOs were injected into two dorsal vegetal cells of eight-cell embryos. Marker gene expression and the lineage of cells targeted with MOs were analyzed by double staining (Sive et al., 2000). After in situ hybridization, the first antibody was inactivated in 4% paraformaldehyde for 30 min, followed by 0.1 M glycine–HCl (pH 2.2) containing 0.1% Tween 20 for 15 min. Fluorescent MOs were traced and visualized by using an anti-fluorescein antibody (Roche) and BCIP (Gibco), respectively. For rescue experiments, a total of 100 pg of linearized plasmids (rBMP-3b/pCS2+ and/or mdCerberus/pCS2+) was co-injected with 5 ng each of xBMP-3b and cerberus MOs. We generated mdCerberus, which was resistant to the cerberus MO, by PCR using the primers, 5’-GGGAATTCATGCGTTGGAACGTGAGATAATCGAGGGATTAGAGA-TGTATC-3’ and 5’-CGTCTAGACTCAATGTTGCGAGGCGTGA-TGTATC-3’. Lower-cases represent ten mismatches introduced within the target sequences of cerberus MO. The biological activity of mdCerberus was the same as that of the wild type. All interference assays and rescue experiments were repeated at least twice.

**Results**

**Embryonic expression of Xenopus BMP-3b and BMP-3**

BMP-3b and BMP-3 constitute a unique subgroup within the BMP family, and their embryonic functions remain uncertain. To examine the roles of these proteins, we first identified Xenopus orthologues and investigated their expression during development. Xenopus BMP-3b protein is composed of 443 amino acids and has 53% identity with rat and human BMP-3b (Fig. 1). Similarly, xBMP-3 consists of 458 amino acids and shares 62% identity with mammalian orthologues. Although we performed PCR using genomic DNA as well as cDNAs from embryos and adult tissues, we could not isolate any more similar proteins from Xenopus.

Temporal expression profiles of xBMP-3b and xBMP-3 were demonstrated by RT-PCR (Fig. 2A). Transcripts of both xBMPs were detected soon after the onset of zygotic expression and at all later stages. xBMP-3 is also transcribed in the ovary and a small amount is inherited as maternal RNA, which is detectable by increasing the number of PCR cycles (data not shown).

The spatial expression of xBMP-3b and xBMP-3 was determined by whole-mount in situ hybridization. The expression of xBMP-3b was initially detected on the dorsal–lateral ectoderm before the neural plate became visible (Fig. 2B). The expression had extended to the entire neural plate by the neurula stage, and was enhanced in two bilaterally symmetrical stripes in the neural fold from the rostral to the caudal regions (Fig. 2C and D). In addition, xBMP-3b mRNA was detected in the prechordal plate and endoderm (Fig. 2E). At the tailbud stage, additional expression appeared at the rhombencephalon as well as at the dorsal side of the otic vesicles and remained beyond the tadpole stage (Fig. 2F). At the hatching stage, xBMP-3b was enriched from the dorsal mesenchyme to the olfactory pit and optic cup as well as posterior to the stomodeum (Fig. 2G–I). The expression of xBMP-3b was transient in the ventricular chamber (Fig. 2H and J). We also detected xBMP-3b in the dorsal midline of the spinal cord, and in bilateral stripes at the epaxial and hypaxial edges of the somite, where they would be restricted in the tail (Fig. 2K). At the swimming tadpole stage, xBMP-3b mRNA was detected on the ventral side of pharyngeal pouches 1, 2, and 3, and adjacent to the cement gland (Fig. 2L and M). The distribution of xBMP-3b mRNA is reminiscent of the chordal part of the chondrocranium as seen from the dorsal side (Fig. 2N and O). Cells expressing xBMP-3b accumulated around the notochord and neural tube where head mesoderm should appear (Coulx et al., 1993; Sadaghiani and Thiébaut, 1987, for Xenopus cranial development; see Kuratani et al., 1999 for review).

We initially detected xBMP-3 in the embryonic ectoderm and throughout the marginal zone from the late blastula (data not shown) through the gastrula stages (Fig. 2P and Q). Expression was restricted to the prechordal and chordal mesoderm by the neurula stages (Fig. 2R). At the tailbud stage, xBMP-3 appeared in a subset of cells in the cephalic neural crest (Fig. 2S). Expression of both extended anteriorly as development proceeded (Fig. 2T). At hatching, more xBMP-3 was expressed in the anterior neurocranium and cement gland (Fig. 2U–W). In the mid thorax, xBMP-3 was localized in the notochord (Fig. 2X). In short, Xenopus BMP-3a and BMP-3 are diversely expressed in embryos, but tend to mark the dorsal ectodermal and mesodermal cells.
BMP-3b and BMP-3 are BMP-2 antagonists

We investigated the effect of BMP-3b and BMP-3 overexpression in Xenopus embryos. Synthetic mRNAs encoding xBMP-3b or xBMP-3 (1 ng each) were injected into the ventral marginal zone of four cell embryos. The injection of xBMP-3b or BMP-3 prevented closure of the blastopore on the ventral side during gastrulation.

Fig. 1. Isolation of Xenopus BMP-3b and BMP-3 cDNAs. (A) Protein sequence comparison of Xenopus BMP-3b and BMP-3 with rat orthologues. Conserved amino acid residues in all four proteins are dotted and same amino acids within BMP-3b or BMP-3 are shaded. Dashes indicate spaces introduced to optimize alignment. Boxes represent cleavage sites; one is tetrabasic and the other is an alternative site determined by protein purification and N-terminal sequence analysis. GenBank Accession nos. for xBMP-3b and xBMP-3 are AB059564 and AB059563, respectively. (B) Phylogenetic relationships among complete precursors of human, zebrafish, and Xenopus BMP proteins. Dendrogram was generated by using the UPGMA method (GENETYX program, SDC Ltd, Japan). We used the underlined proteins (Figs. 5 and 6). Although ADMP is considered to be a BMP-3-like protein, it is more closely related to BMP-2 and BMP-4. XBMP-7 (Nishimatsu et al., 1992) has been renamed xBMP-7R, since a Xenopus orthologue of mammalian BMP-7 (OP-1) has been identified (Wang et al., 1997).

Fig. 2. Xenopus BMP-3b and BMP-3 are expressed in dorsal ectodermal and mesodermal tissues. (A) Temporal expression analyzed by RT-PCR. BMP-3b and BMP-3 are expressed at quite low levels. After whole-mount in situ hybridization, embryos were stained for 6 days to detect xBMP-3b (B–O) and overnight for xBMP-3 (P–X). (B) Dorsal view of stage 11 embryo. Anterior region faces top. BMP-3b expression appears on dorsolateral ectoderm before neural plate is visible. Expression extends to entire neural plate until stage 12.5 (C), and localizes upon four stripes at neural plate midline and edge. (D) Expression in anterior neural plate at stage 14. Solid line indicates level of section. (E) Sagittal section of embryo. Anterior faces left. BMP-3b expression is intense in ectoderm and faint in mesoderm and endoderm. Arrows highlight BMP-3b expression in prechordal plate. (F) Lateral view of stage 24 embryo. BMP-3b is expressed in hindbrain and dorsal side of otic vesicle and neural tube. (G) BMP-3b is expressed in lips of somites and transiently in the heart. (H) Close-up view of (G). (I) Dorsal view of (H). (J) Ventral view of (H). (K) Transverse section through trunk of embryo in (H). BMP-3b is expressed in both epaxial and hypaxial dermamyotome, and dorsal half of neural tube. (L) At stage 41, expression in dermamyotome is restricted to tail. (M) Close-up of head shown in (L). BMP-3b is expressed in dorsal side of otic vesicles, optic cup, nasal pit, stomodeum, and ventral visceral pouches. (N) Dorsal view of embryo shown in (M). (O) Transverse section of embryo shown in (L). BMP-3b is expressed in head mesoderm around notochord and neural tube. (P) Vegetal view of stage 10.5 embryo. Dorsal faces top. BMP-3 is expressed in ectoderm and mesoderm. (Q) Sagittal section of embryo shown in (P). Dorsal side is to right. (R) Sagittal section of stage 15 embryo. Anterior faces left. BMP-3 is expressed in prechordal plate and chordamesoderm. (S) BMP-3 expression appears around otic vesicle at stage 24. (T) At stage 30, BMP-3 is expressed in cranial neural crest derivatives and cement gland. (U) Close-up view of head shown in (T). (V) Dorsal view of (U). (W) Transverse section through head in (T). BMP-3 is localized in anterior neurocranium and dorsomeral wall of otic vesicle. (X) Transverse section through trunk. BMP-3 expression is restricted to notochord.
Table 2

Secondary head formation by *Xenopus* and rat BMP-3b

<table>
<thead>
<tr>
<th>Injected mRNA</th>
<th>Dose/embryo</th>
<th>n</th>
<th>Secondary head formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Xenopus</em> BMP-3b</td>
<td>1 ng</td>
<td>34</td>
<td>35.3</td>
</tr>
<tr>
<td></td>
<td>500 pg</td>
<td>34</td>
<td>17.6</td>
</tr>
<tr>
<td></td>
<td>250 pg</td>
<td>33</td>
<td>9.1</td>
</tr>
<tr>
<td>rat BMP-3b</td>
<td>1 ng</td>
<td>36</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>500 pg</td>
<td>37</td>
<td>43.2</td>
</tr>
<tr>
<td></td>
<td>250 pg</td>
<td>34</td>
<td>14.7</td>
</tr>
</tbody>
</table>

*Note.* n, number of scored embryos. *Xenopus* BMP-3b triggered less secondary head formation than rat BMP-3b. This may be because inefficient translation causes less production of xBMP-3b than of rBMP-3b protein (see Fig. 8A).

Fig. 3. BMP-3b and BMP-3 have apparently different dorsalizing activities in *Xenopus* embryos. (A–C) *Xenopus* embryos injected with BMP-3b or BMP-3 mRNA. Overexpression of BMP-3b led to ectopic head formation, whereas BMP-3 generated aberrant tail formation. Synthetic mRNAs (1 ng each) encoding xBMP-3b (A), xBMP-3 (B), or β-galactosidase (β-gal; C) were injected into marginal zone of two ventral blastomeres at the four cell stage. (D–G) Differentiation of ventral marginal zone explants (VMZs) injected with BMP-3b or BMP-3. Typical explants are shown in circles. (D) VMZs injected with rBMP-3b RNA generated head-like structures, consisting of a eye and cement gland and eye, and that they developed like dorsal marginal zone explants. These effects of BMP-3b in embryos and in the VMZ are comparable to those of cerberus, an organizer factor (Bouwmeester et al., 1996). In contrast, VMZ injected with BMP-3 mRNA elongated and expressed melanocytes, a process that is characteristic of dorsalization. The effect of BMP-3 was similar to that of the dominant negative forms of BMP-2, 4, and 7 (Nishimatsu and Thomsen, 1998). Molecular marker analyses of the VMZs in Fig. 3D–E also revealed that both BMP-3b and BMP-3 have dorsalizing activities, but that they act distinctly in *Xenopus* embryos (Fig. 3G). Ventral marginal zones injected with both BMP-3b and BMP-3 induced the expression of dorsal mesodermal and ectodermal markers, *cardiac actin* and NCAM, and VMZ injected with BMP-3b also expressed the anterior marker genes, *xAG1* (cement gland), *βB1-crystallin* (lens), and *rhodopsin* (retina).

We analyzed the expression of early marker genes in embryos by whole-mount in situ hybridization. The distribution of cells expressing BMP-3b or BMP-3 was traced by coinjecting the mRNA for β-galactosidase (β-gal). Fig. 3H shows that injected BMP-3b but not BMP-3 led to the ectopic expression of *Otx2*. Cells expressing BMP-3b that were stained light blue completely overlapped brown *Otx2* staining and thus appeared dark blue. Cells expressing BMP-3b as well as BMP-3 did not exhibit the brown staining of *Xwnt-8* expression, revealing that both BMP-3b and BMP-3 dorsalize ventral mesoderm. Moreover, we examined whether BMP-3b affects cerberus-mediated head induction. The results showed that BMP-3b did not elevate cerberus expression, indicating that BMP-3b acts independently of, or downstream from, cerberus during head formation.

**BMP-3b and head formation**

Explants of VMZ injected with BMP-3b developed head-like structures independently of signals from the Spemann organizer, which generates signals for induction, morphogenesis, and self-differentiation to coordinate embryonic development (Harland and Gerhart, 1997). The effect of BMP-3b in the VMZ indicates that BMP-3b is involved in organizer signaling. Several organizer factors as well as Vg1, an organizer inducer, can restore the dorsal axial structure of embryos ventralized by ultraviolet (UV) irradiation. We examined the ability of BMP-3b to perform this task and attempted to trace the cell fate by coexpressing BMP-3b with the lineage tracer β-gal.

Fig. 4A shows embryos exposed to UV of which the vegetal blastomere at the eight-cell stage was coinjected with BMP-3b and β-gal mRNAs. The expression of BMP-3b generated a head structure containing cyclopia and cement glands, but did not rescue the trunk–tail structure.
Fig. 4. BMP-3b triggers head formation in an intrinsic developmental pathway. To examine head formation by BMP-3b, we performed lineage tracing and rescue of embryos exposed to UV. (A) Injection of rBMP-3b mRNA (1 ng) rescued anterior structure of UV-ventralized embryos. Cells expressing BMP-3b were localized in head region (60%, n = 15). (B) Section of an embryo shown in (A). Cells were labeled in notochord (nt), heart (ht), optic cup, and adjacent to cement gland, suggesting that cells expressing BMP-3b differentiated into “head-forming cells” and organized head structures. (C) Cells expressing BVg1 (50 pg), an organizer inducer, were localized in endoderm, suggesting that they induced head-forming cells (50%, n = 14; Thomsen and Melton, 1993). (D) Injection of cerberus (1 ng) led to formation of smaller head than that with BMP-3b (60%, n = 25; Bouwmeester et al., 1996). (E) Control embryo.
Fig. 5. Distinct activities of BMP-3b and BMP-3 are caused by differences in their pro-domains. (A) Chimera analysis of BMP-3b and BMP-3 for localizing BMP-3b head-forming activity. Pro-domain of BMP-3b protein is required for head formation. Activity and score of head formation are tabulated on the right. Numbers represent positions of recombination. (B) Schematic representation of chimeras between either BMP-3b or BMP-3, and BMP-2. Pro-domains of BMP-3b and BMP-3 were fused to ligand domain of BMP-2, and ligand domain of BMP-3 was combined with BMP-2 pro-domain. Synthetic mRNAs encoding these chimeras were injected into marginal zones of two ventral blastomeres at the four cell stage. (C) Injecting BMP3b/2 chimera mRNA (1 ng) perturbed tail formation and induced cement gland (arrows) (96%, n = 25). (D) Overexpression of BMP3/2 mRNA (500 pg) ventralized embryos (100%, n = 25). (E) BMP2/3b chimera (50 pg) generated same phenotype as BMP-3 (100%, n = 24). (F) Animal cap assay of chimera functions. Caps injected with mRNAs (200 pg each) were isolated at stage 8.5, and harvested at stage 23 to score by RT-PCR. BMP-3b induced NCAM and xAG1, whereas BMP-3 induced these markers and HoxB9. BMP3b/2 and BMP2/3b chimeras induced NCAM and xAG1, whereas BMP3/2 induced globin, suggesting that pro-domain of BMP-3, but not of BMP-3b allows cleavage of ligand domain. Moreover, ligand domains of BMP-3 and BMP-3b have activity antagonistic to that of BMP-2. We also found that BMP-2 and its cleavage site mutant (cmBMP-2) induced HoxB9, a posterior neural marker. This observation may relate to a previous finding that HoxB9 is expressed weakly in lateral plate mesoderm as well as in the spinal chord (Wright et al., 1990; see also Fig. 9J).

Fig. 6. BMP-3b and BMP-3 differentially interact with TGF-β family members. Synthetic mRNAs for BMP-2 (A), ADMP (B), Xnr1 (C), derrière (D), and caALK4 receptor (E) were injected into animal caps and their mesoderm-inducing activities were challenged by increasing doses of BMP-3b or BMP-3. Combinations and doses (pg) of each mRNA sample are indicated above lanes. Animal caps were harvested at stage 12 for scoring by RT-PCR. Brachyury (Xbra) and NCAM served as pan-mesodermal and pan-neural markers, respectively. A 25-fold excess of BMP-3b (*) interfered with Xbra expression by all ligands, whereas BMP-3 reduced expression induced by BMP-2 and ADMP. Both of BMP-3b and BMP-3 alone induced NCAM expression in animal caps (A), indicating that they are neural inducers.
A

Xenopus embryo

<table>
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<tr>
<th>Control</th>
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<th>Flag-BMP-3</th>
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CHO cell

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WB: anti-Flag (mAb)

B

<table>
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<tr>
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<th>Myc-ADMP</th>
<th>Myc-Derrière</th>
<th>Myc-Xnr1</th>
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IP: anti-Myc (mAb) |

IP: anti-Flag (mAb) + WB: anti-Myc (pAb)

C

Reduced condition

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<th>+BMP-3</th>
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(kD)

D

Non-reduced condition

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(kD)

E

Interaction with members of the TGF-β family

<table>
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<td>ADMP</td>
<td>Derrière</td>
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<tr>
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<td>antagonistic</td>
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<td>heterodimer</td>
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Sections of the embryos revealed that cells expressing BMP-3b had differentiated into tissues that organize head structures, namely notochord, heart, optic cup, and cells adjacent to the cement gland (Fig. 4B). We also injected Bvg1 (50 pg), which leads to the production of active Vg1 that can fully restore the axial structure in embryos exposed to UV (Thomsen and Melton, 1993). Cells expressing Bvg1 were localized in the endoderm and induced head structures (Fig. 4C). Injected cerberus led to the formation of a smaller head and a large endodermal mass as reported (Bouwmeester et al., 1996). The shape of the embryos rescued with cerberus differed from those injected with BMP-3b (Fig. 4A and D). Cells expressing cerberus in embryos exposed to UV were located around the head region, but were more widely distributed than cells expressing BMP-3b. These observations indicated that BMP-3b triggers head formation through an intrinsic developmental pathway, whereas Vg1 and cerberus do so via inductive interaction. Since BMP-3b may not cause head formation by induction, we refer to the ability of BMP-3b as “head-forming.”

**Differences between BMP-3b and BMP-3**

We showed that BMP-3b acts differently from BMP-3. However, the BMP-3b and BMP-3 ligand domains share 81% amino acid identity. To identify the region of BMP-3b responsible for head-forming activity, we constructed chimeric proteins between BMP-3b and BMP-3 in which the transition was located within their conserved amino acid sequences. The results summarized in Fig. 5A indicate that the region of head-forming activity is located on the N-terminal pro-domain of BMP-3b. The chimera BMP-3b/BMP-3 (202), consisting of the N-terminal 202 amino acids from BMP-3b and the C terminus from BMP-3, retained head-forming activity. However, the BMP-3b/BMP-3 (99) chimera lost, while the antipodal chimera BMP-3/BMP-3b (99) possessed the activity. These results showed that the amino acid sequence from 99 to 202 is required for head formation by BMP-3b, and that the sequence is relatively conserved between rat and *Xenopus*, but divergent from that of BMP-3 (Fig. 1A).

This is rather unusual, because members of the TGF-β family usually express biological activities in the ligand domain, which binds to the receptor. The pro-domain of proteins in the TGF-β family simply modifies the activity of the ligand domain through control of its assembly, secretion, and turnover (Gray and Mason, 1990; Thomsen and Melton, 1993; Constam and Robertson, 1999). These observations implied that the pro-domain of BMP-3b modifies the activity of the ligand domain, which it shares with BMP-3.

To define the function of the pro-domains of BMP-3b and BMP-3, we constructed chimeric proteins consisting of the pro-domain of BMP-3b or BMP-3 combined with the ligand domain of BMP-2 and designated these constructs BMP3b/2 and BMP3/2, respectively (Fig. 5B). We then tested their ventralizing activities and compared them with the wild type and with cleavage site-mutated BMP-2 (Nishimatsu and Thomsen, 1998). The BMP3b/2 chimera perturbed the tail structure and induced cement glands (Fig. 5C). This phenotype is similar to that generated by the cleavage mutant BMP-2. In contrast, the BMP3/2 chimera ventralized embryos as effectively as wild type BMP-2 (Fig. 5D). These results imply that the BMP-3b and BMP-3 pro-domains respectively interfere and proceed with maturation of the ligand domain. We also examined this notion using the BMP-3b ligand domain chimera. Since the pro-domain of BMP-2 produces the cognate ligand domain in embryos (Thomsen and Melton, 1993), the ligand domain of BMP-3b was fused to the pro-domain of BMP-2 (BMP2/3b). Injection of the BMP2/3b chimera triggered a malformed tail, but not secondary head formation, which is similar to the action of BMP-3 (Fig. 5E and Fig. 3B). This finding also suggests that the ligand domain of BMP-3b has the same activity as that of BMP-3 and opposite activity to that of BMP-2. Finally, we confirmed these activities in animal caps (Fig. 5F). Caps injected with mRNAs (200 pg each) were harvested at stage 23, and examined by RT-PCR. In line with the phenotype of the embryos, both BMP3b/2 and BMP2/3b chimeras induced NCAM and xAG1 expression, whereas BMP3/2 induced globin. These results suggest that BMP-3b pro-domain interferes with the maturation of its ligand domain, while BMP-3 proceeds with the maturation of its ligand domain. Moreover, the ligand domains of both BMP-3b and BMP-3 have antagonistic activity to that of BMP-2.

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*Fig. 7. Divergence of proteolytic processing and heterodimer formation of BMP-3b and BMP-3.* (A) Less BMP-3b than BMP-3 precursor was cleaved in *Xenopus* embryos and in CHO cells. Extracts of embryos injected with Flag-tagged BMP mRNAs (left panel) and conditioned media of CHO cells transfected with the plasmid DNAs (right panel) were Western blotted. Controls were from wild type embryos and from CHO cells that had been transfected with vector DNA, respectively. All samples were resolved under reducing conditions. Molecular mass (kDa) of proteins (left), deduced structures, and Flag epitope (right) are shown. Two bands generated by N-glycosylation were grouped at about 25 and 16 kDa, respectively (lane: BMP-3 expressed in CHO cells). (B) Myc-tagged proteins of the TGF-β family were constructed and expressed in CHO cells to test interactions with BMP-3b and BMP-3. Although we transfected cells with equal amounts of each plasmid, Xnr1 precursor was not effectively processed and secreted from CHO cells. (C, D) Immunoprecipitation and Western blotting revealed that BMP-3b directly interacted with all TGF-β members, whereas BMP-3 interacted with all except Xnr1, in CHO cells. Coprocipitated BMP-3b and BMP-3 are shown at 75 and 25 kDa in (C). Apparent molecular weight of Xnr1 (*) under reducing and nonreducing conditions did not change, suggesting that BMP-3b forms noncovalent complexes with Xnr1. (E) Summary of molecular characteristics of BMP-3b and BMP-3 including results of Figs. 5 and 6. Both BMP-3b and BMP-3 oppose BMP-2 and ADMP. BMP-3b also blocks derrière and Xnr1. Types of BMP-3b antagonism are instructed by the pro-domain, which controls cleavage and assembly of the ligand domain. Incomplete processing of BMP-3b might reduce mesoderm-inducing activity of derrière and complex formation between BMP-3b and Xnr1 might result in altered Xnr1 function.
Head formation in the *Xenopus* embryo involves the inhibition of BMP-2/4, nodal, Xwnt-8, and ADMP activities (Glinka et al., 1997; Piccolo et al., 1999; Dosch and Niehrs, 2000). Since all of these factors except Xwnt-8 are members of the TGF-β family that induce mesoderm in the embryo, we used the animal cap assay to examine whether either of BMP-3b or BMP-3 interferes with mesoderm induction by these factors. We co-injected increasing doses of BMP-3b or BMP-3 mRNA and a mesoderm-inducing dose of BMP-2, ADMP, or Xnr1. We also used derrière and constitutively active ALK4 receptor (ca-ALK4) as controls. Derrière is a member of the TGF-β family that induces mesoderm in a nodal-like manner (Sun et al., 1999). Ca-ALK4 induces mesoderm independently of binding to its ligand (Hoodless et al., 1999). Fig. 6 shows that a 25-fold excess of BMP-3b mRNA interfered with the expression of Xbra, a pan-mesodermal marker, induced by all ligands. In contrast, an equivalent dose of BMP-3 repressed expression by BMP-2 and ADMP (i.e., ventralizing factors), but not by Xnr1 or derrière (that is, dorsal mesoderm inducers). Neither BMP-3b nor BMP-3 blocked expression by the ca-ALK4 receptor, implying that the interference results via ligand production and receptor binding (Fig. 6E). The expression profiles of Xbra were identical to those of another mesodermal marker, Xcad3 (data not shown). We also examined the expression of NCAM, a pan-neural marker, in animal caps injected with BMP-2 (Fig. 6A), because Xnr1 and derrière induce BMP-binding proteins like chordin and lead to NCAM expression. Both BMP-3b and BMP-3 increased NCAM expression while they suppressed Xbra. In short, these results suggest that BMP-3b antagonizes ventralizing factors and dorsal mesoderm inducers, whereas BMP-3 blocks ventralizing factors, but not dorsal mesoderm inducers. These antagonistic characteristics are consistent with their head formation and dorsalization activities.

**Divergent biosynthesis of BMP-3b and BMP-3**

Chimera analyses and interference assays in animal caps raise the possibility that BMP-3b and BMP-3 exert antagonistic actions through the proteolytic processing and assembly of precursors. We therefore used Western blotting to examine their biosynthesis in *Xenopus* embryos. We inserted the Flag epitope into the ligand domains, and then mRNAs encoding Flag-tagged BMP-3b and BMP-3 were injected into the embryo. At the gastrula stage, the embryos were lysed for Western blotting against anti-Flag antibodies (Fig. 7A, left panel). We detected BMP-3b as intense (75 kDa) and faint (25 kDa) bands that corresponded to precursor and cleaved ligand proteins, respectively. In contrast,

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**Fig. 8. Inhibition of both xBMP-3b and cerberus causes headless embryos.** (A) Specificity of morpholino antisense oligonucleotides (MO) of xBMP-3b, xBMP-3 and cerberus. Translation of mRNAs encoding *Xenopus* proteins is inhibited, but neither rat BMP-3b/3 nor mdCerberus are affected by MOs. Target sequences for cerberus MO are altered in mdCerberus mutant, but not the amino acid sequences. Messenger RNAs and proteins were prepared from plasmid DNAs as described in Materials and methods. (B) Schematic diagram of embryonic stage and injection point of MOs. (C–E) Independent injections of xBMP-3b, xBMP-3, and cerberus MOs (10 ng) did not perturb *Xenopus* embryos. (F, G) Simultaneous injection of xBMP-3b and cerberus MOs (5 ng each) led to headlessness (54%, n = 22), whereas that of xBMP-3 and cerberus MOs did not (0%, n = 21). (H) Injection of β-catenin MO (10 ng) caused headless embryos (92%, n = 25). (I–L) Histological sections of embryos injected with xBMP-3b and cerberus MOs (F), xBMP-3 and cerberus MOs (G), β-catenin MO (H), and wild type. sm, somite; nc, notochord; sc, spinal cord; bi, blood island.
BMP-3 was identified as a major band at 24 kDa, indicating that the BMP-3 precursor is readily cleaved in *Xenopus* embryos.

We next investigated the processing and secretion of BMP-3b and BMP-3 in CHO cells. Conditioned media of CHO cells were collected after 3 days of transfection with plasmids for Flag-BMP-3b or BMP-3. The media were purified by using beads with affinity for Flag-tagged proteins, and then Western blotted. Like the embryo extracts, the precursor of BMP-3b, but not of BMP-3, was detected in the medium (Fig. 7A, right panel). Cleaved mature proteins of BMP-3b and BMP-3 were identified at about 16 and 25 kDa. These findings indicated that BMP-3b precursor is secreted like BMP-3, but that the precursor is not completely cleaved. These results appear to be consistent with those of the chimera analyses showing that the BMP-3b pro-domain interferes with the maturation of its ligand domain. Regulated cleavage of the precursor may result in a different action of the ligand, since the responsiveness of cells may change during development.

We also tested whether BMP-3b and BMP-3 form heterodimers with members of the TGF-β family. To compare the findings with those of the interference assay in animal caps, we produced Myc-tagged BMP-2, ADMP, derrière, and Xnr1 in CHO cells (Fig. 7B). We then transfected the expression vectors for the Myc-tagged proteins with either Flag-BMP-3b or BMP-3 at a 1:1 ratio. Conditioned media were immunoprecipitated with the anti-Flag antibody then Western blotted against the anti-Myc antibody. Fig. 7C shows that BMP-3b coprecipitated with all TGF-β family members tested, whereas BMP-3 precipitated with all except Xnr1 under reducing conditions. A comparison with Fig. 7B shows that the interaction of BMP-3b or BMP-3 with derrière altered the proportion of derrière precursor and mature proteins, suggesting that cleavage of the derrière precursor could be promoted. However, BMP-3b precursor was not processed by such interactions (Fig. 7C, lower panel).

To investigate heterodimer formation, all samples were resolved under non-reducing conditions and Western blotted (Fig. 7D). Both BMP-3b and BMP-3 formed heterodimers with BMP-2, ADMP, and derrière. The BMP-3b heterodimeric structures included more precursor than those of BMP-3 and therefore contained larger molecular forms. We also reconfirmed that BMP-3b interacts with Xnr1. The molecular mass of the complex remained unchanged under non-reducing and reducing conditions, indicating that BMP-3b does not covalently bind Xnr1. These results are essentially consistent with those of the interference assay in animal caps (Fig. 7E). However, further analysis is required not only to assess whether the complex formation of BMP-3b and Xnr1 leads to impaired gastrulation movements. Lineage of cells targeted by MOs and expression of marker genes were monitored at gastrula, neurula and tadpole stages. (Left) Embryos injected with MOs for both xBMP-3b and cerberus. (Right) Control embryos injected with globin MO. Light blue staining represents cells targeted by MOs. Brown staining represents expression of markers as indicated on right corner. Dorsal side faces top in panels of gastrula and tadpole, and anterior side faces top in panels of neurula. Injection of MOs for xBMP-3b and cerberus did not affect dorsal lip formation and expression of organizer genes at beginning of gastrulation, but led to delayed blastopore closure and downregulation of Otx2 expression by neurula stage. Consequently, suppressing xBMP-3b and cerberus functions elevated expression of a ventral–posterior marker, Xcad1.
Involvement of xBMP-3b in head formation and axial patterning

We found that xBMP-3b is expressed in the prechordal plate and anterior neuroectoderm as well as in the dorsal–posterior neural plate (Fig. 2E). This observation indicated that xBMP-3b is expressed in conjunction with differentiation of the organizer cells and that it contributes to embryonic head formation. To test this notion, we performed loss-of-function analyses in Xenopus embryos using antisense morpholino oligonucleotides (Heasman et al., 2000). We designed morpholino oligonucleotides (MO) for xBMP-3b, xBMP-3, and cerberus as controls and confirmed the specificity of the MOs using an in vitro transcription and translation kit. Fig. 8A shows that the MOs inhibited translation of the Xenopus proteins, but not of the rat orthologues or of modified cerberus (mdCerberus), in which target sequences for cerberus MO were altered to those without the substitution of any amino acids. We next injected the MOs for each of xBMP-3b, xBMP-3, and cerberus into the dorsal vegetal cells and into points below the equatorial cleavage plate at the eight-cell stage (Fig. 8B). Injection of the MOs alone did not obviously perturb the Xenopus embryos, suggesting that their related molecules have some functional redundancies. (Fig. 8C–E). Although BMP-3b and cerberus are structurally divergent, BMP-3b and cerberus generate secondary head formation in Xenopus embryos. Moreover, both xBMP-3b and cerberus are expressed in relation to differentiation of the organizer cells. These observations suggest that these proteins coordinate formation of the Xenopus larval head.

We therefore co-injected either xBMP-3b or xBMP-3 MO together with cerberus MO into the dorsal marginal zone. Fig. 8F and G shows that xBMP-3b co-injected with cerberus MOs triggered gastrulation defects and headless embryos, whereas co-injected xBMP-3 and cerberus MOs did not cause such deformities. The effects of xBMP-3b and cerberus MOs were also compared with that of the positive control embryos injected with MOs for xBMP-3b and cerberus.
control, β-catenin MO, which blocks head formation (Fig. 8H; Heasman et al., 2000). Histological analysis of transverse sections from embryos injected with xBMP-3b and cerberus MOs showed that the embryos had a somite, notochord and an enlarged blood island, but lacked the neural tube (Fig. 8I). These observations suggest that mesodermal tissues are formed but ventralized and that neural tissues are disrupted by the simultaneous inhibition of xBMP-3b and cerberus. In contrast, sections of embryos injected with xBMP-3 and cerberus MOs had a relatively smaller somite than those of the wild type (Fig. 8J and L), whereas embryonic sections injected with β-catenin MO revealed an enlarged spinal cord and blood island (Fig. 8K).

We observed the expression of marker genes in embryos injected with fluorescent MOs for xBMP-3b and cerberus at the gastrula, neurula, and tadpole stages by whole-mount in situ hybridization. The lineage of cells targeted with MOs was traced and visualized by staining with anti-fluorescein antibody. At the beginning of the gastrula, normal dorsal lip was traced and visualized by staining with anti-situ hybridization. The lineage of cells targeted with MOs was restored, whereas the mdCerberus plasmid alone recovered the entire head and part of the tail structure (Table 3). We also injected embryos with both rBMP-3b and mdCerberus plasmids, or mdCerberus plasmids at a lower concentration. However, mdCerberus could not completely restore the tail structure (Fig. 10C, and data not shown). This finding might be related to divergent action between BMP-3b and cerberus, since the findings of our initial gain-of-function studies implied that BMP-3b acts intracellularly, whereas cerberus acts extracellularly. Altogether, our results show that xBMP-3b and cerberus cooperate to bring about head formation and axial patterning in Xenopus embryos.

### Discussion

The present study examined the embryonic function and distribution of BMP-3b and of BMP-3 in Xenopus embryos. Overall, our results revealed that BMP-3b and BMP-3 have dorsalizing activities, but that they function differently to control axial patterning of the embryo. We also showed that xBMP-3b in cooperation with cerberus is involved in Xenopus larval head formation. We discuss the mechanisms of head formation and axial patterning mediated by BMP-3b and BMP-3.

#### Head formation by BMP-3b

We demonstrated that BMP-3b triggers ectopic head formation via an intrinsic developmental pathway in Xenopus embryos. Our comparative study of BMP-3b and BMP-3 significantly clarified the molecular mechanism of head formation. Interference assays in animal caps indicated that BMP-3b antagonizes ventralizing BMPs (BMP-2 and ADMP) and dorsal mesoderm inducers (derrière and Xnr1), whereas BMP-3 antagonizes only ventralizing BMPs. The BMP-3b antagonism appears to occur at the time of intracellular processing and assembly. The precursor of BMP-3b is cleaved less than that of BMP-3, which might explain why BMP-3b can reduce mesoderm induction by derrière. BMP-3b also forms a noncovalent complex with Xnr1, and might diminish the mesoderm-inducing activity of Xnr1. In this regard, Xnr1 may interact with the head-forming region of BMP-3b determined by our chimera analysis. The interaction of BMP-3b and Xnr1 is unique within the TGF-β
family. These characteristics of BMP-3b could result in autonomous head formation.

We also showed that Xenopus BMP-3b indeed participates in embryonic head formation by coordinating with cerberus. During Xenopus development, xBMP-3b and cerberus are expressed in cells that differentiate from the organizer. Cerberus is first induced in the anterior endomesodermal cells of the organizer, then levels of xBMP-3b are elevated in the prechordal plate mesoderm. The simultaneous inhibition of xBMP-3b and cerberus in the organizer region delays gastrulation movements and results in headless embryos. These results agree with the findings of our gain-of-function studies and those of others (Piccolo et al., 1999). BMP-3b and cerberus trigger secondary head formation, and antagonize ventralizing BMPs as well as nodal-like proteins. Biochemical analyses revealed that BMP-3b interacts with these proteins at the level of intracellular space, whereas cerberus binds at the extracellular space. Based on these findings and the ideas of others (Lee et al., 2001; Bally-Cuif and Boncinelli, 1997; Beddington and Robertson, 1998), we interpret the mechanism of head formation by xBMP-3b and cerberus as follows. Initially, cerberus secreted from anterior endomesodermal cells in the organizer protects neighboring cells against nodal and ventralizing factors (such as BMP-2, ADMP, and Wnt proteins), although nodal is essential for induction of the mesodermal cells and for cerberus expression. As gastrulation proceeds, xBMP-3b accumulates in the anterior mesodermal cells and alters nodal function by forming a noncovalent complex. When cerberus and xBMP-3b successively block nodal and ventralizing factors from interacting with the anterior mesodermal cells, transcription factors (Otx2 etc) in the cells could proceed the expression of the downstream genes and complete self-differentiation as “head-organizing cells.”

This interpretation is supported by the results of an investigation by Joubin and Stern (1999). They argued that cells in the organizer are not committed when they are induced, but rather are maintained by interactions with neighboring cells during gastrulation. In short, cooperation between BMP-3b and cerberus may protect the ability of cells in the organizer to anteriorize and trigger head formation of the Xenopus embryo.

In addition, our explanation of BMP-3b and cerberus in embryos is consistent with the diverse functions of nodal, which is involved in the sequential endomesoderm specification, anterior–posterior and left–right patterning of early development (Whitman, 2001). These functions of nodal appear to be achieved through interaction with BMP-3b and cerberus. BMP-3b is secreted with a monomeric form of Xnr1, a nodal-like protein. Because the Xnr1 monomer is structurally similar to lefty and antivin, which are major regulators of left–right patterning (Meno et al., 1996; Thische and Thiese, 1999), the Xnr1 monomer and BMP-3b complexes could play a role in patterning.

**BMP-3b and BMP-3 as dorsalizing BMPs**

We demonstrated that both BMP-3b and BMP-3 antagonize ventralizing BMPs, such as BMP-2 and ADMP, and that they act as dorsalizing BMPs. These findings agree with those of Daluiski et al. (2001) who noted that BMP-3 antagonizes BMP-2 signaling and dorsalizes Xenopus embryos. Our biochemical analyses extend their observation by showing that BMP-3b and BMP-3 directly interact with ventralizing BMPs by forming heterodimers. The biological activity of such heterodimers remains unknown, but our interference assays in animal caps suggest that BMP-3b/3 heterodimers with BMP-2 do not have mesoderm-inducing activity. This is consistent with and closely related to our previous observation that mesoderm induction initiated by injecting BMP-2 mRNA is triggered by another heterodimer consisting of BMP-2 and endogenous BMP-7 in animal caps (Nishimatsu and Thomsen, 1998). BMP-2 coexpressed with either BMP-3b or BMP-3 might inhibit heterodimer formation between BMP-2 and BMP-7 in animal caps and thereby abolish mesoderm induction. Further analyses using recombinant proteins are required to define the dynamics of BMP heterodimers as well as homodimers.

Whether BMP-3 and BMP-3b bind to receptors and mediate signal transduction via Smad proteins (Massagué, 2000) remains unclear. Recombinant BMP-3 protein antagonizes BMP-2 signaling through activin receptors (ALK-4 and ActRII) in P19 cells (Daluiski et al., 2001). However, we showed that both BMP-3 and BMP-3b, unlike activin, do not induce mesoderm in animal caps (Fig. 6). Therefore, BMP-3 can bind to activin receptors, but the downstream signaling molecules might not be those used by activin. Alternatively, dorsalizing BMPs might also bind to a specific receptor in Xenopus embryos and directly mediate its signal. Members of the TGF-β family mediate signals through type I and type II transmembrane Ser/Thr kinase receptors and the Smad signaling pathway (Massagué, 2000). Our results and those of Daluiski et al. provide evidence of cross talk between dorsalizing and ventralizing BMPs at the extracellular level, which raises the important issue of how such cross talk triggers interaction with Smad proteins at the intracellular level.

**Axial patterning by BMP-3b and BMP-3**

The embryonic expression profiles and our results of functional analyses indicate that BMP-3b and BMP-3 are involved in the “dorsalization” of embryos and embryonic tissues. Xenopus BMP-3b is expressed in a small group of ectodermal cells that give rise to dorsal neurons and the neural crest. xBMP-3 is expressed in the entire ectoderm and mesoderm then localized in the dorsal-most mesoderm, namely the notochord. When overexpressed in animal caps, both BMP-3b and BMP-3 induced NCAM, suggesting that they dorsalize ectoderm (Sasai and De Robertis, 1997). Reduced function of xBMP-3b together with cerberus af-
fects dorsal–anterior patterning as a consequence of impaired gastrulation movements. Although we do not have direct evidence as to whether xBMP-3 is involved in the axial patterning of embryos, xBMP-3 must cooperate with other dorsaling factors and mediate the signal.

Our comparative studies of BMP-3b and BMP-3 raise a question regarding axial patterning by BMP proteins. Although Hawley et al. (1995) showed that inhibiting BMP-4 and -7 ligand production leads to dorsalization, inhibiting BMP-3b and -3 ligand production may not cause ventralization, but rather, anteriorization. Our chimera analyses and Western blotting showed that the BMP-3b pro-domain interferes with maturation of the cognate ligand domain, while the BMP-3 pro-domain proceeds with the maturation in *Xenopus* embryos. The difference between BMP-3b and BMP-3 precursors involves the ectopic expression of Otx2, an anterior marker, and secondary head formation. We also found that both BMP-3b and -3 ligand domains have dorsaling activities and antagonize ventralizing BMPs. These findings altogether indicate that processing of the BMP-3b/3 precursors affects anterior-posterior patterning, whereas ligand competition between BMP-3b/3 and ventralizing BMPs establishes dorsal–ventral patterning. A more through analysis of native BMP ligand production in embryos is required to substantiate whether biosynthesis and antagonistic interaction of BMP proteins are indeed involved in embryonic head formation and the axial patterning of *Xenopus* and other vertebrates. Furthermore, such BMP-mediated reactions and interactions for head formation and axial patterning should be regulated and affected by the Wnt and other signaling pathways (Kiecker and Niehrs, 2001; Pera et al., 2001; Tsuda et al., 2002). Communications with these signaling pathways also remain important topics for further investigation.

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