STUDIES ON BONE MORPHOGENETIC PROTEIN-BINDING PROTEINS IN THE EARLY DEVELOPMENT OF *XENOPUS LAEVIS*

SHUN-ICHIRO IEMURA

DOCTOR OF SCIENCE

DEPARTMENT OF MOLECULAR BIOMECHANICS

SCHOOL OF LIFE SCIENCE

THE GRADUATE UNIVERSITY FOR ADVANCED STUDIES

1999

CONTENTS

ABBREVIATIONS

ActRI:	activin receptor type I
ALK:	activin receptor-like kinase
ALP:	alkaline phosphatase
BIA:	biomolecular interaction analysis
BmNPV:	*Bombyx mori* nuclear polyhedrosis virus
BMP:	bone morphogenetic protein
BMPRI:	BMP receptor type I
BSA:	bovine serum albumin
CA-ALK:	constitutively active ALK
cDNA:	complementary DNA
DAI:	dorsoanterior index
DAN:	differential screening-selected gene aberrative in neuroblastoma
DNA:	deoxyribonucleic acid
DTT:	dithiothreitol
ECD:	extracellular domain
ECM:	extracellular matrix
HBS:	HEPES buffered saline
HEPES:	*N*-(2-hydroxyethyl) piperazine-*N*'-2-ethanesulfonic acid
HRP:	horseradish peroxidase
mRNA:	messenger RNA
NTA:	nitrilo-tri-acetic acid
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
PMSF:	phenylmethylsulfonyl fluoride
PVDF:	polyvinylidene fluoride
RNA:	ribonucleic acid
RT-PCR:	reverse transcription-PCR

RU:	resonance unit
sBMPR:	soluble form of BMP type I receptor
SDS:	sodium dodecyl sulfate
SDS-PAGE:	SDS-polyacrylamide gel electrophoresis
SPR:	surface plasmon resonance
TBS:	Tris buffered saline
TFA:	trifluoroacetic acid
TGF-β:	transforming growth factor-β
Tris:	tris (hydroxymethyl) aminomethane

ABSTRACT

Almost all multicellular organisms arise from a single cell. After fertilization, it gives rise to diverse cell types, and then these differentiated cells are organized into tissues and organs of the body. In this embryonic development, a series of sequential embryonic inductions, especially mesoderm and neural induction, are essential processes for determining the individual basic body plan. Therefore, it is important to elucidate what signaling molecules are involved in the inductions, and how these molecules function in the cell-to-cell communication. To date, several signaling molecules acting in early developmental processes have been identified, and among them, bone morphogenetic protein (BMP), which is a member of the transforming growth factor-β (TGF-β) superfamily, is thought to play a key role in early events regulating morphogenesis. Recently, it has been demonstrated that BMP activity is antagonized by the factors (noggin and chordin) released by the Spemann's organizer, and that such an antagonism takes place by the direct binding between BMP and noggin, or chordin. These findings revealed that such a negative regulation of BMP activity by its binding proteins is essential for the pattern formation of early embryos.

In this thesis, I will focus on the regulatory mechanisms of BMP activity by BMP-binding proteins at the protein level. To analyze the interaction of BMP and its binding proteins, I introduced a surface plasmon resonance (SPR) biosensor (BIACORE). This sensor was developed for monitoring biomolecular interactions in real time, using non-invasive optical detection principle based on SPR, and can detect a change in mass concentration at the sensor surface as molecules bind or dissociate. To perform SPR analysis on BMP and its receptor, firstly, large-scale expression and purification of xBMP-4 and its soluble form of type I receptor (sBMPR) was performed using a silkworm expression system (chapter 2). Secondly, the interaction between BMP-4 and follistatin, which is also expressed in the Spemann's organizer region and suspected to bind directly to BMP-4, was analyzed (chapter 3). Lastly, the screening of the BMP-4-binding protein was performed, and the interaction between BMP-4 and

isolated proteins was analyzed (chapter 4). In addition, the interaction was confirmed by *in vivo* functional analysis using mRNA microinjection into early *Xenopus* embryos.

In chapter 2, to enable the analysis of protein-protein interactions, large scale preparation of BMP-4 and sBMPR was performed using a silkworm expression system. From the hemolymph recovered from infected larvae (approximately 2,000 larvae), about 1 mg of xBMP-4 and 20 mg of sBMPR were purified by liquid chromatography. This receptor was in monomer form in solution, and bound to BMP-4 but not to activin A or TGF-β1. The SPR studies showed that the association rate constant (k_a) of sBMPR for BMP-4 is 3.81×10^4 $M^{-1}s^{-1}$, and that the dissociation rate constant (k_d) is 3.69×10^{-4} s^{-1} (K_D=9.6 nM). This affinity was similar to that of the intact membrane-associated receptor expressed on COS cells. The biological activity of expressed BMP-4 was confirmed by alkaline phosphatase (ALP) activity in BMP responsive cell lines such as mouse osteoblastic cells MC3T3-E1 and bone marrow stromal cells ST2. The BMP-binding ability of expressed sBMPR protein to BMP was confirmed as an inhibition of BMP-induced ALP activity by the addition of sBMPR protein.

In chapter 3, functional analyses of follistatin in the development of *Xenopus* embryos were performed using the SPR sensor and mRNA microinjection method. Follistatin, originally known as an activin-binding protein, is localized to the Spemann's organizer of early *Xenopus* gastrula, as well as chordin and noggin. Until now, it is found that follistatin induces the secondary body axis when overexpressed in ventral blastomeres, and that it can induce neural tissue in ectoderm without affecting mesoderm. These observations indicate that follistatin might inhibit not only activin but also BMPs through direct binding.

To examine the antagonism between follistatin and BMPs (BMP-4 or BMP-7), mRNA microinjection assay was performed. BMP-4 and BMP-7 caused a ventralized embryo that lacked the anterior head structure and notochord when they were dorsally overexpressed by mRNA microinjection. These effects of BMPs were inhibited by coinjection with follistatin mRNA. On the contrary, the dorsalizing effect of follistatin

in ventral side was repressed when coinjected with BMP mRNAs. These findings reveal that follistatin and BMPs inhibit each other.

Next, the interactions between follistatin (FS-288) and BMPs were analyzed by the SPR sensor. While the affinity of FS-288 for BMPs is lower than that for activin A, the results clearly indicated that FS-288 binds to BMP-4, -7 homodimers, and BMP-4/7 heterodimer. In contrast to this, TGF-β1, other member of TGF-β superfamily, did not bind to FS-288. The kinetic parameters for the binding of FS-288 to BMP-4 were determined that the association rate constant (k_a) is 1.16×10^5 $M^{-1}s^{-1}$, and that the dissociation rate constant (k_d) is 2.7×10^{-3} s^{-1} (K_D=23 nM).

The inhibitory mechanism of follistatin for BMP-4 was analyzed by the biosensor, and compared with those of noggin and chordin. As previously reported, noggin and chordin bind to BMP-4 directly, and inhibit the interaction between BMP-4 and its receptor. In contrast, it was suggested that follistatin, BMP-4, and sBMPR form a trimeric complex, but follistatin does not interfere the binding of BMP-4 to sBMPR.

In chapter 4, novel BMP-4-binding proteins, which may regulate the multipotent BMP activity in development, were screened using the SPR sensor as a specific monitor. Two BMP-4-binding proteins were isolated from *Xenopus* embryo extracts by 3-step chromatography. Comparisons of N-terminal amino acid sequences established that they are Ep45 and lipovitellin 1. Lipovitellin 1 is an egg yolk protein that is processed from vitellogenin, while Ep45 is a member of the serine protease inhibitor (serpin) superfamily. Both Ep45 and vitellogenin are synthesized under estrogen control in the liver. Because it has been reported that vitellogenin binds to both activin and BMP, subsequent functional analyses were performed for Ep45.

The binding specificity of Ep45 and the kinetic parameters for the binding of Ep45 to BMP-4 were demonstrated by SPR studies. The results indicated that Ep45 can interact only with BMP-4 among TGF-β family ligands, activin A, TGF-β1, and BMP-4 so far tested. Moreover, the kinetic parameters for the binding of Ep45 to BMP-4 were calculated that the association rate constant (k_a) and the dissociation rate constant (k_d) are 1.06×10^4 $M^{-1}s^{-1}$ and 1.6×10^{-4} s^{-1}, respectively (K_D=15.1 nM).

Next, functional analyses of Ep45 in the early development of *Xenopus* embryos were performed using the SPR biosensor and mRNA microinjection. When Ep45 mRNA was microinjected into embryos, it had no effect on the development. Furthermore, analysis using the SPR sensor indicated that Ep45 does not inhibit the binding of BMP-4 to sBMPR. These results suggest that Ep45 is not a negative regulator of BMP-4. Subsequently, the influence of Ep45 on the interaction of BMP-4 with three organizer factors, which are noggin, chordin, and follistatin, was investigated. In microinjection assay, Ep45 had no effect on dorsalization induced by chordin or noggin, but inhibited dorsalization by follistatin in a dose-dependent manner. This result is further supported by the SPR biosensor. Namely, Ep45 inhibited the binding of BMP-4 to follistatin in a dose-dependent manner. Taking into consideration their affinities to BMP-4, it is assumed that Ep45 does not interfere with the high affinity binding of BMP to BMP-binding proteins such as noggin, or chordin. By contrast, because Ep45 binds to BMP-4 with higher affinity than follistatin, Ep45 is thought to be able to interfere the binding of follistatin to BMP-4. Taken together, it is likely that Ep45 is a specific inhibitor of follistatin against BMP.

In conclusion, this work demonstrates the differentially controlled mechanisms of regulation of BMP activity. Because the multipotent activities of polypeptide growth factors are essential for a variety of patterning events during not only early development, but also organogenesis, it is thought to be regulated through various mechanisms. Accordingly, the isolation of new molecules that bind to growth factors, and functional analyses of these growth factor-binding proteins would provide the key to understanding the mechanisms of development.

CHAPTER 1

GENERAL INTRODUCTION

The development of a multicellular organism begins with a single cell which is radially symmetric, and seemingly unpatterned. After fertilization, the egg gives rise to various kinds of cells, and then they are organized into each fated tissues and organs of the body. In the precise patterning of tissues and organs, it is thought that various inductive events are of critical importance. Especially, early embryonic inductions, particularly mesoderm and neural induction, are essential for embryogenesis.

The first experimental demonstration of embryonic induction was published by Hans Spemann and Hilde Mangold in 1924 (Spemann and Mangold, 1924). In this transplantation experiments, they demonstrated that when a tissue of gastrula blastopore lip of the donor newt is grafted to the ventral side of a host embryo, the transplant is not only maintained as blastopore lip, but it also redirects the fate of surrounding tissue. Namely, the grafted dorsal blastopore lip induces the host's ventral tissues to change their fates to form a neural tube and dorsal mesoderm tissue, resulting in formation of a secondary body axis (Fig. 1-1). Because of these observation, it is thought that during normal development, blastopore lip cells 1) self-differentiate into the chordamesoderm and other mesodermal structures, 2) induce the flanking mesoderm to axial mesoderm and lateral plate mesoderm, and 3) directly induce neural tissue from dorsal ectoderm (Gilbert, 1994). These are reasons why the dorsal blastopore lip of the amphibian embryo is called the "Spemann's organizer". The anatomical equivalent of the amphibian blastopore lip is found in the other vertebrates. In the mouse and the chick, it is called node, while in zebrafish, it is called embryonic shield. The essential role of organizers is also well conserved among vertebrates (Hemmati-Brivanlou and Melton, 1997; Lemaire and Kodjabachian, 1996; Sasai and De Robertis, 1997).

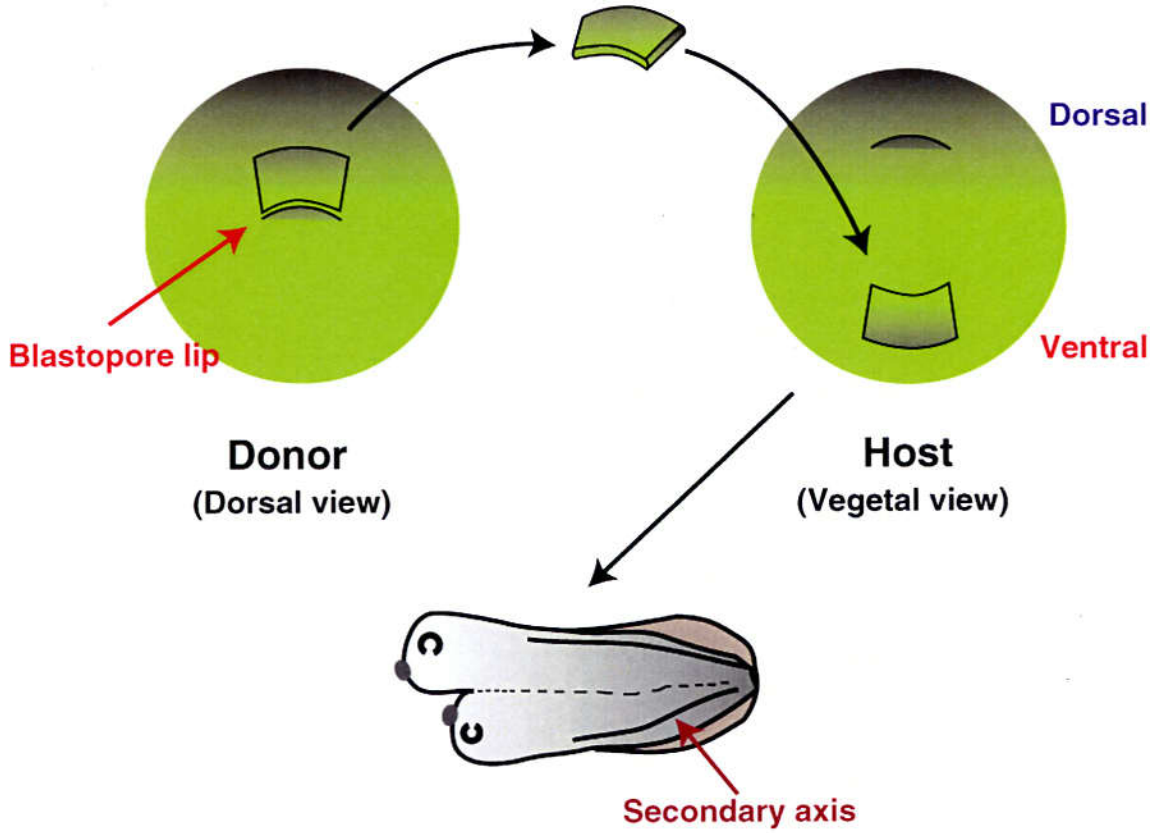

Figure 1-1. Spemann's organizer transplant experiments.

A graft of the dorsal region (blastopore lip) of a gastrula-stage donor embryo to the ventral region of a host embryo induces formation of a secondary body axis. The grafted donor blastopore lip differentiated into the new neural tube, notochord and somites.

Based on Spemann's findings and additional embryological experiments, an amphibian *Xenopus* model for dorsal-ventral patterning has been proposed (Harland, 1994; Kimelman et al., 1992; Slack, 1991). In this model, because of the dominant behavior of the organizer, dorsal tissues are thought to be positively patterned by an active signal released from the organizer (Fig. 1-2A). Namely, it indicates that ventral tissue is the ground (default) state, and that dorsal tissue is the dominant state. This model, however, is found to conflict with results of recent molecular studies (Graff, 1997; Thomsen, 1997). These studies suggest that ventral tissues, which is dominant state, are positively patterned by an active ventral signal, and that this signal is bone morphogenetic protein (BMP) (Fig. 1-2B).

BMPs, which are members of the transforming growth factor-β (TGF-β) superfamily, are dimeric secreted glycoproteins. These proteins were originally identified by their ability to induce ectopic bone formation in mammals (Urist, 1965). Thereafter, BMPs are found to be expressed in *Xenopus* embryos (Nishimatsu et al., 1992) and to possess a variety of embryological functions in vertebrates (Hogan, 1996). At present, these proteins are thought to play essential roles in dorsoventral patterning of embryos (Harland, 1994; Sive, 1993). For instance, in early *Xenopus* embryos, BMP family ligands inhibit dorsal mesoderm formation and induce ventral mesoderm formation (Graff et al., 1994; Hawley et al., 1995; Maeno et al., 1994; Sasai et al., 1995; Schmidt et al., 1995; Suzuki et al., 1994). Moreover, in the ectoderm, BMPs have been shown to inhibit neural cell differentiation and to promote epidermal differentiation (Hawley et al., 1995; Sasai et al., 1995; Wilson and Hemmati-Brivanlou, 1995). In addition to two germ layers, BMP ligand is also found to be involved in dorsoventral patterning of the endoderm (Sasai et al., 1996). Furthermore, when endogenous BMP signal is blocked, ventral mesoderm is converted to follow dorsal fates, and ventral ectoderm, epidermis, is converted to dorsal ectoderm, namely neural tissue (Graff et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Suzuki et al., 1994). These lines of evidence suggest that formation of ventral tissues in three germ layers requires an active BMP signal, and that inactivation of this signal

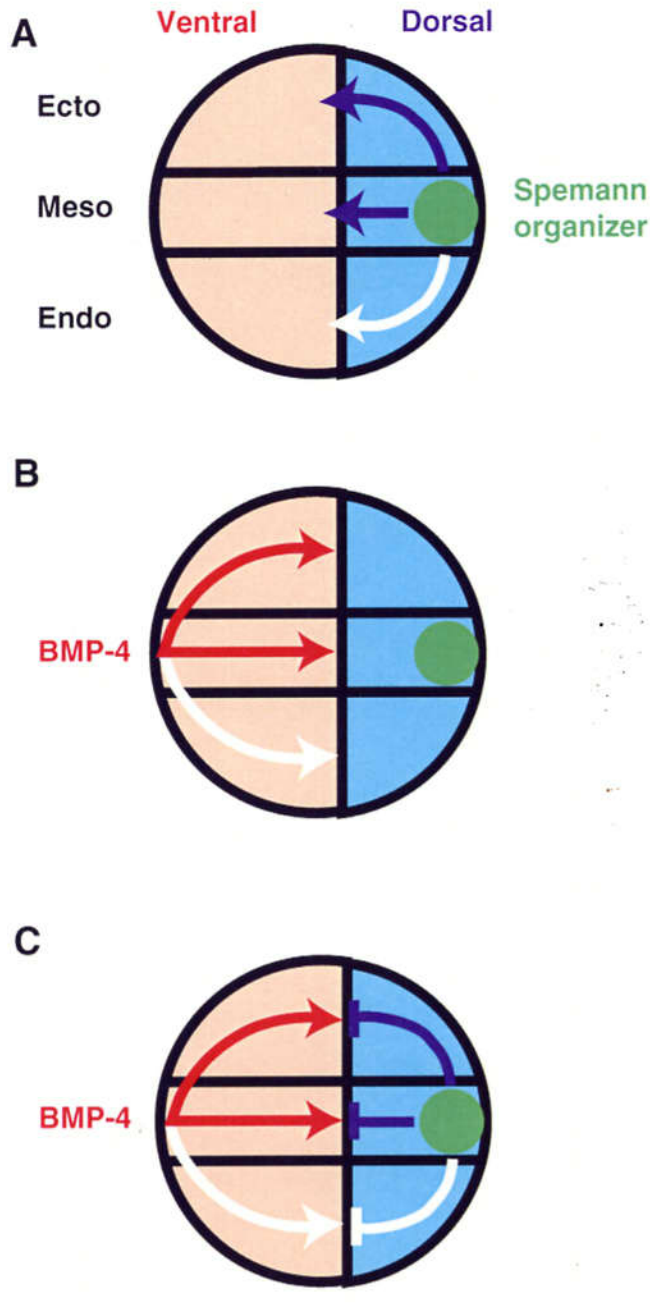

Figure 1-2. Models of early dorsal-ventral patterning.

(A) Previous embryological experiments suggest that the Spemann's organizer induces dorsal fates by sending an active inducing signal and that ventral fates are a ground state. (B) Molecular experiments suggest that active BMP signals induce ventral tissues and that blocking BMP signaling leads to formation of dorsal fates. (C) Recent studies reconcile the differences between these two views as the organizer signals function by blocking BMP signaling. The white lines indicate patterning effects in the endoderm that appear likely but there have yet to be definitively proven. Ect, ectoderm; Meso, mesoderm: Endo, endoderm. (Modified from Graff, 1997)

leads to formation of dorsal tissues including neural tissue (Fig. 1-2B).

The case of interest here is how the Spemann's organizer organizes the body axis. What role does the dorsal signal from the organizer play? The answer to this question has been revealed by several detailed studies. In these studies, it is demonstrated that BMP activity is negatively regulated by inhibitors of the BMPs, noggin, and chordin, which are expressed in the Spemann's organizer, and are thus called organizer factors (Graff, 1997; Thomsen, 1997). These have been shown to neuralize ectoderm directly (Sasai et al., 1994; Smith and Harland, 1992). Moreover, recent reports demonstrate that these organizer factors can bind to BMP-4 directly, and thus inhibit the anti-neural activity of BMP-4 (Piccolo et al., 1996; Zimmerman et al., 1996). These findings can account for the results of previous experiments, which was suggestive of active signal from the organizer. Namely, the organizer signals antagonize active ventral signals rather than actively facilitate dorsal fates (Fig. 1-2C).

In addition to these organizer factors, gremlin, cerberus, and DAN, which belong to the DAN family, have been found to block BMP signaling by direct binding to BMP-2 (Hsu et al., 1998). Gremlin is not expressed during gastrulation, although its activities are similar to those of noggin and chordin. Rather, it is implicated in neural crest development in later embryogenesis (Hsu et al., 1998). Cerberus has a potent head inducing activity that can lead to the formation of an ectopic head when its mRNA is microinjected into *Xenopus* embryos. It is expressed in the anterior endomesoderm of *Xenopus* gastrulae (Bouwmeester et al., 1996). Interestingly, cerberus can antagonize not only BMP activities but also those of *Xenopus* nodal-related (Xnr) -1, -2, and Wnt-8 by direct binding (Hsu et al., 1998; Piccolo et al., 1999). DAN is a putative zinc-finger transcription factor down-regulated in transformed cells (Ozaki and Sakiyama, 1993). Subsequently, DAN overexpression was shown to delay entry into S phase in normal cells, and to have tumor suppressor activity in transformed cells (Ozaki and Sakiyama, 1994). These results suggest that BMP activities are regulated throughout development by various BMP-antagonists with different affinities and binding specificities.

The main theme of this thesis is the analysis of regulatory mechanisms of BMP activity by BMP-binding proteins. To analyze the interaction in detail, I introduced a surface plasmon resonance biosensor (SPR biosensor). This sensor uses the optical phenomenon surface plasmon resonance to monitor biomolecular interactions (Fig. 1-3) (Löfås et al., 1991; Raether, 1977). One of the components (referred to as the *ligand*) in the interaction is immobilized on the surface of the sensor chip and the other (referred to as the *analyte*) flows over the surface in free solution. Detection depends on changes in the mass concentration of molecules at the biospecific interface, and does not require any labeling of interactants.

In chapter 2, large-scale expression and purification of active xBMP-4 and soluble form of BMP-2, and -4 receptor (sBMPR) to be available for analysis of protein-protein interactions was performed. And then, the interaction between BMP-4 and sBMPR was analyzed. In chapter 3, the functional analyses of follistatin was performed. Firstly, using mRNA microinjection, it was demonstrated that follistatin inhibits the BMP activities. Next, interactions between BMPs and follistatin were analyzed by the SPR sensor. Moreover, the mechanism by which follistatin inhibits BMP activity was explored. In chapter 4, using the SPR sensor as a specific monitor, BMP-binding proteins expected to regulate BMP activity in development were isolated, and analyzed the function. In the last chapter, chapter 5, I summarize the presented results, and give some suggestions for further studies.

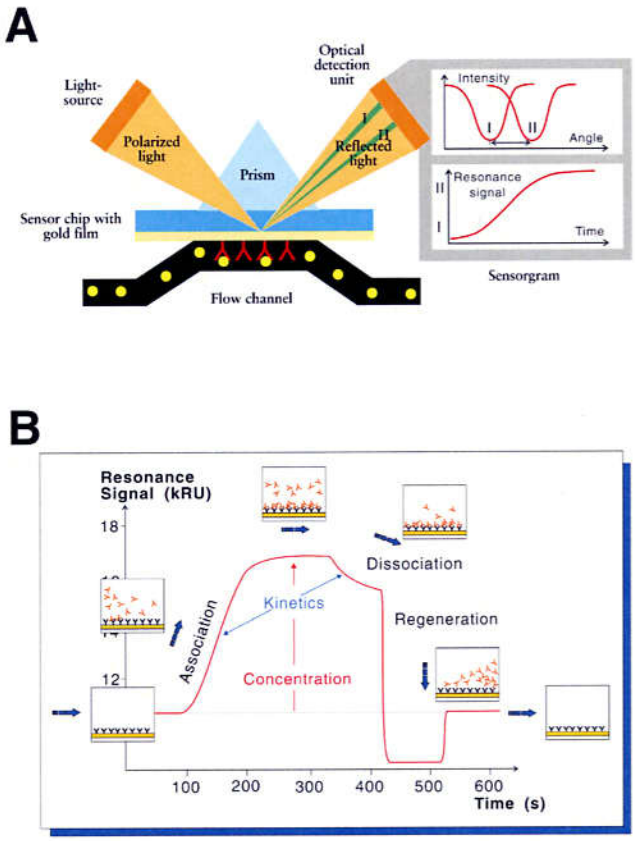

Figure 1-3. Real-time BIA (biomolecular interaction analysis).

BIA is a biosensor technology for monitoring interactions between two or more molecules. The detection principle relies on the optical phenomenon of surface plasmon resonance (SPR), which detects changes in the refractive index of the solution close to the surface of the sensor chip. (A) The SPR angle is sensitive to the mass concentration of molecules close to the sensor chip surface. As this concentration changes, the SPR angle shifts and produces a response. This response is expressed in resonance units (RU). (B) The continuous display of RU as a function of time, referred to as a sensorgram, thus provides a record of the progress of association and dissociation. During sample injection, analyte binds to the surface-attached ligand, resulting in an increase in signal. At the end of the injection, the sample is replaced by a continuous flow of buffer and the decrease in signal reflects dissociation of interactant from the surface-bound complex. A response of 1000 RU corresponds to a change in surface concentration of 1 ng/mm^2. (Adopted from BIA journal, 1995)

CHAPTER 2

EXPRESSION AND PURIFICATION OF BMP-4 AND SOLUBLE FORM OF BMP TYPE I RECEPTOR

SUMMARY

Here I describe large-scale expression and purification of xBMP-4 and sBMPR using a silkworm expression system. sBMPR was in monomer form in solution and bound to xBMP-4 but not to activin A or TGF-β1. SPR studies showed that kinetic parameters of sBMPR for BMP-4 consisted of a relatively rapid association rate constant (k_a=3.81±0.19×10^4 M^{-1}s^{-1}) and an extremely slow dissociation rate constant (k_d=3.69±0.26×10^{-4} s^{-1}). From these two kinetic parameters, affinity was determined to be similar to that of the intact membrane-associated receptor expressed on COS cells. sBMPR inhibited the alkaline phosphatase activity in BMP responsive cell lines such as mouse osteoblastic cell MC3T3-E1 and bone marrow stromal cell ST2. These data indicated that expressed proteins have biological activities, and that the extracellular domain of type I receptor for BMP-2 and BMP-4 is sufficient for high-affinity binding to its ligands and should prove useful in understanding the role of BMP-2/4 *in vivo*, because a suitable high-affinity anti-BMP antibody has yet to be developed.

INTRODUCTION

It was demonstrated that ectopic bone inducing activity exists in bone tissue, and this activity was called BMP (Urist, 1965). Thereafter, it was revealed that BMPs are members of the TGF-β superfamily (Wozney et al., 1988), and that their structures are conserved from flies to mammals (Kingsley, 1994). At present, it is found that BMPs play crucial roles not only in bone formation, but also in the development of vertebrates (Hogan, 1996).

BMP receptors are a family of transmembrane serine/threonine kinases (Massagué, 1996; ten Dijke et al., 1996). These receptors are divided into two distinct classes, type I, and type II receptors. In cases of TGF-β and activin, the first step of their signaling is the binding of ligands to their type II receptors, and then the type I receptors are recruited to ligand-bound type II receptors, forming probably a heterotetramer. By this heteromeric interaction, intrinsic kinase activity of type II receptor phosphorylates the intracellular domain of the type I receptor, and the signal is transduced (Yamashita et al., 1994). In contrast, both BMP-2 and BMP-4 are able to bind to their type I receptor without their type II receptor (Graff et al., 1994; Koenig et al., 1994; Suzuki et al., 1994; ten Dijke et al., 1994). Although BMP type II receptors alone bound to ligands weakly, the binding was facilitated by presence of type I receptor (Liu et al., 1995; Nohno et al., 1995; Rosenzweig et al., 1995). As well as the signaling of the other TGF-β family ligands, heteromerization of these receptors mediated by BMP ligands, and then phosphorylation of the glycine-serine rich cytoplasmic domain (GS domain or type I box) of type I receptors are necessary for initiating biological responses (Massagué, 1996; ten Dijke et al., 1996). The dominant-negative (dn) form of type I receptor, which lacks the serine/threonine kinase domain, enables BMP loss-of-function (Graff et al., 1994; Suzuki et al., 1994). Overexpressed dn type I receptor in ventral blastomeres of *Xenopus* embryos leads to formation of a secondary body axis that means the blocking of BMP signal. These findings indicate that the extracellular domain (ECD) of type I receptor is sufficient to mediate stable

binding to BMPs and subsequent formation of heteromeric complex with the endogenous type II receptor.

In this thesis, to study BMP-BMP-binding protein interactions, and effects of BMP-binding proteins on the interaction between BMP and BMP type I receptor using the SPR biosensor, firstly, *Xenopus* BMP-4 (xBMP-4) and a soluble form of the ECD of type I receptor fused to the *myc*-epitope sequence (sBMPR) were produced using a silkworm expression system. This system, in which ligand and its receptor are co-expressed as a complex, facilitates a high level of expression of the active form of these recombinant proteins that often become insolubilized in the *Escherichia coli* expression system (Maeda et al., 1985). Secondly, using the SPR biosensor, the interaction of sBMPR with xBMP-4 was analyzed, and binding specificity of sBMPR was examined. Lastly, biological activities of these recombinant proteins were confirmed using BMP-responsive cell lines such as MC3T3-E1 cells and ST2 cells.

EXPERIMENTAL PROCEDURES

Construction of sBMPR and *Xenopus* BMP-4 (xBMP-4) expression vectors

To express sBMPR (Fig. 2-1), a truncated mutant of cDNA that lacks both transmembrane and serine/threonine kinase domains (coding sequences for amino acids 1-152) was amplified from mTFR11 cDNA cloned in BlueScript SK(-) (Suzuki et al., 1994) by polymerase chain reaction (PCR), using a universal forward primer and a TFR11*myc* reverse primer. The TFR11*myc* primer, 3'-GAAACTACCGTCGTAGGCTCTCGTCTTCGACTAGAGGCTCCTCCTGGACATC, was designed so that one *myc* epitope sequence (EQKLISEEDL) would be added at the carboxyl terminus of the mutant protein (Kolodziej and Young, 1991). The PCR reaction was performed in a volume of 100 µl containing 10 µl of the reverse transcriptase reaction, 10 mM KCl, 20 mM Tris-HCl, pH 8.0, 10 mM $(NH_4)_2SO_4$, 6 mM $MgSO_4$, 0.1% Triton X-100, 1 unit of Pfu DNA polymerase (Stratagene), 40 ng of template DNA, and 50 pmole each of the forward and the reverse primers. After 30 cycles of denaturation (95 °C, 1 min), annealing (55 °C, 45 s), and extension (72 °C, 2 min), the PCR product was recovered from low melting temperature agarose gels, and subcloned into a unique *Nur*I site of baculovirus transfer vector pBm4 (Kobayashi et al., 1992). DNA sequencing was done using an automated DNA sequencer (373A, Applied Biosystems, Inc.). The full-length xBMP-4 cDNA was isolated from pUC19/xBMP-4 by digesting with restriction enzyme *Eco*RI (Nishimatsu et al., 1992). The fragment was blunted with T4 DNA polymerase and ligated into the *Nur*I site of the pBm4 vector.

Large-scale expression of recombinant proteins in silkworm larvae

The resulting transfer vectors, pBm4-sBMPR and pBm4-xBMP-4, were independently cotransfected with genomic DNA of wild type BmNPV into BmN4 insect cells (Nohsankoh, Japan), using a Lipofectin technique (Felgner et al., 1987) (Life Technologies, Inc.). Four days after cotransfection, the culture supernatant was

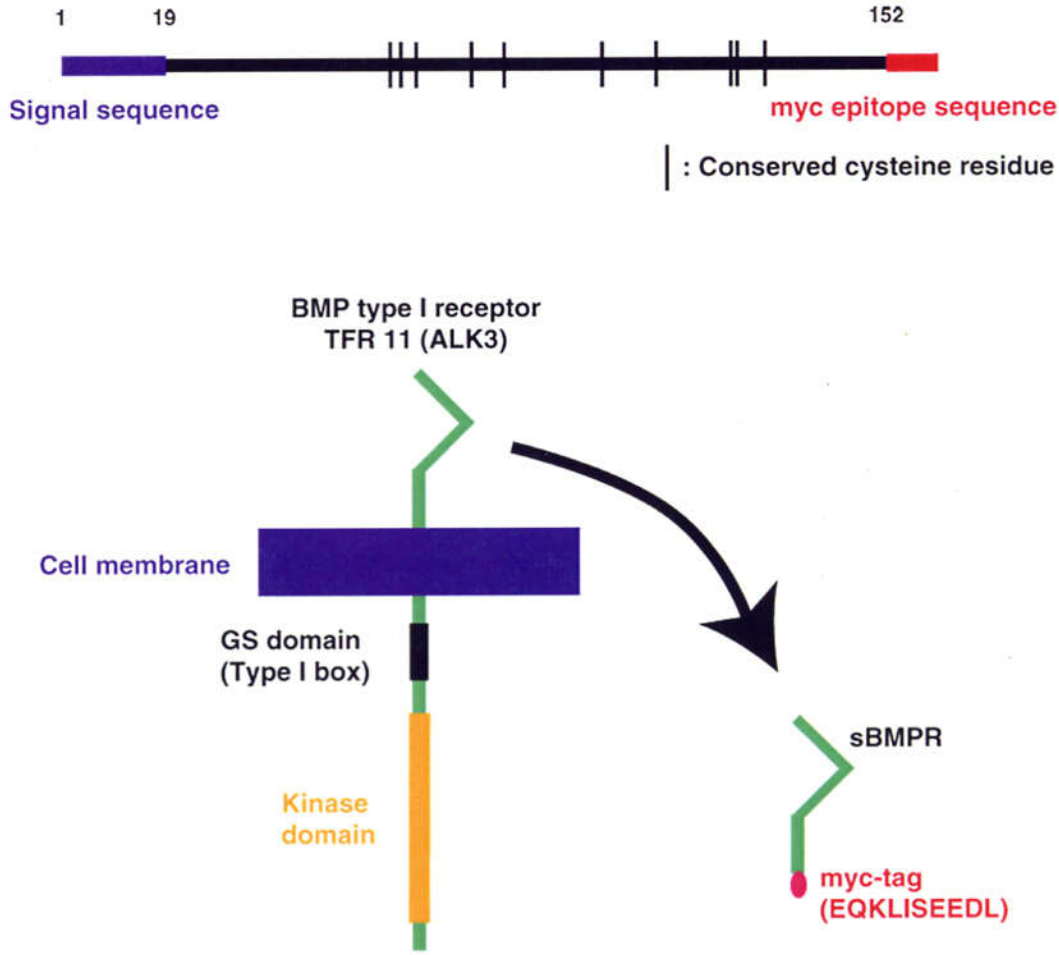

Figure 2-1. Structure of sBMPR.

sBMPR is the extracellular domain of mTFR 11 (ALK3). sBMPR cDNA codes for a 152-amino acid including a 19-amino acid signal sequence, and a 10-amino acid *myc* epitope sequence (EQKLISEEDL) at its carboxyl terminus. Conserved cysteine residues (10 residues) are indicated by black bars.

collected, and recombinant viruses, in which the polyhedrin gene was replaced with sBMPR or xBMP-4 cDNA, were cloned by the end-point dilution method on 96-well plate (Maeda et al., 1985). End-point dilution was performed three times to clone the viruses genetically. The recombination of viruses was confirmed by extracting the viral genomic cDNAs and by PCR with primers designed to anneal the insertions, sBMPR and xBMP-4 (data not shown). The recombinant viruses were propagated and stored at -80 °C for subsequent use. The fifth instar larvae of the silkworm (Kinshu X Showa) fed artificial diets (Nohsankoh, Japan) were obtained from Kanebo (Aichi, Japan). About 10^5 plaque-forming units of the recombinant viruses (50 µl) were coinjected subcutaneously into the larva. Four days after the infection, abdominal legs of the larva were pierced and the hemolymph recovered as described (Ishida et al., 1994) was stored at -80 °C.

Western blot analysis

Immunodetection of sBMPR and xBMP-4 was performed by Western blotting. After electrophoresis under reducing condition on a 15% SDS acrylamide gel, proteins were blotted onto polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford) using a semidry Western transfer apparatus (Pharmacia Biotech AB, Uppsala). The membrane was blocked with 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1.0% Tween 20 containing 5% nonfat dry milk for 1 hr at room temperature, and then reacted with either anti-*myc* monoclonal antibody, 9E10 (Kolodziej and Young, 1991) or anti BMP-4 polyclonal antibody, Ab383 (Ueno et al., 1992) overnight at 4 °C. The membrane then reacted with a horseradish peroxidase (HRP)-conjugate antibody (secondary antibody) for 1 hr room temperature. Western blot was developed with the chemiluminescent ECL kit (Amersham) as described by the manufacturer.

Purification of recombinant proteins

The hemolymph recovered from infected larvae was precipitated with 75% ammonium sulfate, resolved in two-volume of Tris-buffer (20 mM Tris-HCl, pH 7.4)

and applied to a phenyl-Sepharose column (Pharmacia Biotech AB) that was preequilibrated with the Tris-buffer containing 1 M ammonium sulfate. The column was washed with the preequilibrated buffer, and then eluted by stepwise decreasing concentration of ammonium sulfate (0.5 and 0 M). The fraction recovered from 0 M concentration elution, containing BMP-4, was pooled and dialyzed against Tris-buffer. The dialyzed sample was loaded on a heparin-Sepharose CL-6B column (Pharmacia Biotech AB), and then the column was washed with the Tris-buffer. sBMPR containing in the pass-through and this wash fraction was pooled. Moreover, the column was washed with Tris-buffer containing 4 M urea, and eluted by stepwise concentration of NaCl (0.25, 0.5, and 1 M). The 0.5 M-NaCl fraction, containing BMP-4, was further separated on the Resource S column (Pharmacia Biotech AB) in the same buffer, developed with a gradient from 0 to 1.0 M NaCl. The immunoreactive fraction was pooled, acidified to pH 3.0 with acetic acid, and applied to a μRPC C2/C18 column (Pharmacia Biotech AB, SMART system). The column was preequilibrated with 0.1% trifluoroacetic acid (TFA), and was eluted with a linear gradient from 0 to 80% acetonitrile containing 0.1% TFA. The main peak eluted at 60% acetonitrile was pooled and stored at 4 °C until use.

The initial two purification steps of sBMPR were the same as that of BMP-4 , i.e. 75% ammonium sulfate precipitation and phenyl-Sepharose column chromatography. Following these steps, the fraction, containing immunoreactivity of anti-*myc* antibody 9E10 analyzed by Western blot, was dialyzed against Tris-buffer and loaded on a Resource Q column (Pharmacia Biotech AB) preequilibrated with the same buffer. The column was eluted with a linear gradient from 0 to 0.5 M NaCl. The immunoreactive fraction in 0.2 M elution was further separated by gel filtration performed on Superdex 75 (Pharmacia Biotech AB) equilibrated in TBS (20 mM Tris-HCl, 0.1 M KCl, pH 7.4). sBMPR was eluted as the main peak of low molecular mass (<20 kDa).

Peptide growth factors and antibodies

Purified recombinant human activin produced in insect cells was a gift from Dr. Y. Eto (Ajinomoto, Kawasaki). Recombinant human TGF-β1 was purchased from King Jyohzo (Tokyo, Japan). The 9E10 cell line, which produces anti-*myc* antibody, was provided by Dr. A. Hemmati-Brivanlou (Rockefeller University, New York). The anti-*myc* monoclonal antibody was purified from the growth medium of 9E10, using a protein A affinity column.

Surface plasmon resonance studies

Binding experiments and kinetic analysis were performed using the BIACORE2000 (Biacore AB, Uppsala). The basic principles and its use have been documented (Johnsson et al., 1991; Karlsson et al., 1994). Purified BMP-4 described above, recombinant human activin A, and recombinant human TGF-β1 were prepared in immobilization buffer (10 mM acetate buffer, pH 4.5) at a concentration of 10 μg/ml and were immobilized on sensor chips (CM5, certified grade, Biacore AB) at a flow rate of 20 μl/min at 25 °C for 150 s by the amine coupling method (Johnsson et al., 1991). The immobilization levels for BMP-4, activin, and TGF-β1 were 1031, 1543, and 1012 RUs, respectively. For binding studies, sBMPR was injected over a range of concentrations between 10 nM and 12.5 μM at 25 °C for 60-150 s. Before the analysis, the flow rate of the analyte injection was optimized to minimize mass transport limitations (Karlsson et al., 1994). A flow rate of 30 μl/min was found to be sufficient to overcome the mass transport limitation (data not shown). The kinetic parameters, association rate constant (k_a) and dissociation rate constant (k_d), were determined from three independent experiments, using BIA evaluation software version 2.1 (Biacore AB). The buffer for sample dilution and running buffer for BIACORE2000 was HBS (10 mM HEPES, 150 mM NaCl, and 0.005% Tween 20, pH 7.4). Prior to data collection, several methods for surface regeneration after ligand binding were evaluated. Injection of 100 mM HCl (20 μl) efficiently removed the bound proteins and

preserved the binding capacity of the sensor chip surface. BMP-4 surfaces were stable for over 100 binding and regeneration cycles (data not shown).

Assessment of biological activity of xBMP-4 and sBMPR

Biological activities of xBMP-4 and sBMPR were examined using two different cell lines. MC3T3-E1 cells were provided by Dr. M. Kumegawa. ST2 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). These cells were inoculated at a density of 3×10^3 cells/well in 24-well plates and cultured with α-modified Minimum Essential Medium (Life Technologies, Inc., Grand Island, NY) containing 10% fetal bovine serum (Life Technologies, Inc.), and antibiotics (100 units/ml of penicillin-G and 100 μg of streptomycin). To examine the biological activity of sBMPR, alkaline phosphatase (ALP) activity was determined on day 6 of the culture, using an established technique (Suzuki et al., 1993). ST2 cells were grown in the presence of BMP-4 (100 ng/ml) and various concentrations of sBMPR. ALP activity was determined on day 3 of culture (Yamaguchi et al., 1996).

RESULTS

Expression and purification of xBMP-4 and sBMPR

Approximately 1,500-ml hemolymph was recovered from about 2,000 silkworm larvae coinfected with recombinant viruses, in which the polyhedrin gene was replaced with xBMP-4 or sBMPR. To confirm the synthesis of BMP-4 and sBMPR in the silkworm expression system, 0.5 μl of silkworm hemolymph was subjected to electrophoresis on a 15% SDS polyacrylamide gel. Under reducing conditions, BMP-4 was not clearly observed by Coomassie blue staining in infected hemolymph (Fig. 2-2, lane 3), whereas two specific bands (16 and 18 kDa) was detected by Western blot analysis using anti-BMP-2 and -4 antibody, Ab383 (Fig. 2-2, lane 6). This result indicates that BMP-4 is produced in silkworm larvae. In contrast to BMP-4, a defuse band of sBMPR with a relative molecular mass of ~20 kDa was observed by Coomassie blue staining in hemolymph recovered from larvae infected with recombinant viruses but not in non-infected or in wild virus-infected hemolymph (Fig. 2-2, lane 1-3). Western blot analysis showed that this band was recognized by the anti-*myc* monoclonal antibody, 9E10 (Fig. 2-2, lane 9), thereby suggesting the production of *myc*-epitope tagged sBMPR in larvae.

To purify BMP-4 and sBMPR from the hemolymph, several column chromatographies were performed (Fig. 2-3). Prior to these, the recovered hemolymph was precipitated with 75% ammonium sulfate. After the precipitate was dissolved in Tris-buffer, these proteins were purified using phenyl-Sepharose hydrophobic chromatography (Fig. 2-3Aa). sBMPR was eluted broadly (Fig. 2-3Ac), while BMP-4 was eluted at 0 M ammonium sulfate (Fig. 2-3Ab), and each pool was collected. To purify BMP-4, the fraction eluted at 0 M ammonium sulfate was further purified by heparin-Sepharose chromatography (Fig. 2-3B). In this step, BMP-4 was separated from sBMPR. Pass-through fraction containing sBMPR was pooled, while 0.25 M-NaCl eluted fraction including sBMPR was abolished because 4 M urea was found to inactivate sBMPR (data not shown). 0.5 M-NaCl fraction, containing BMP-4, next

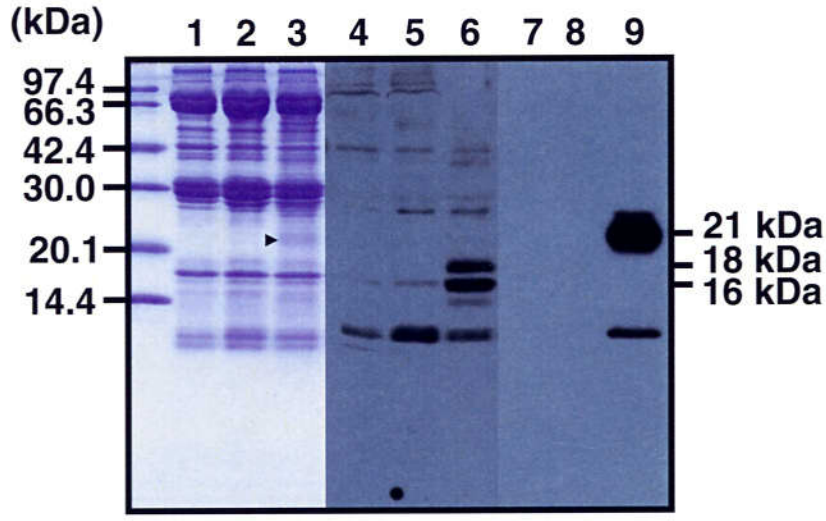

Figure 2-2. Production of sBMPR and BMP-4 in silkworm larvae.

The fifth instar larvae of silkworm were coinfected with xBMP-4- and sBMPR-recombinant viruses (lanes 3, 6, and 9), and the hemolymph was recovered. Under reducing conditions, 0.5 μl of the hemolymph was separated by 15% SDS-PAGE, and proteins were visualized by Coomassie blue staining (lanes 1-3). xBMP-4 and sBMPR were analyzed by Western blots with anti-BMP-4 antibody (Ab383) (lanes 4-6), and anti-*myc* antibody (9E10) (lanes 7-9), respectively. For control experiments, the hemolymph of noninfected (lanes 1, 4, and 7), and wild virus-infected (lanes 2, 5, and 8) larvae was also examined. An arrowhead indicates the band of sBMPR (lane 3).

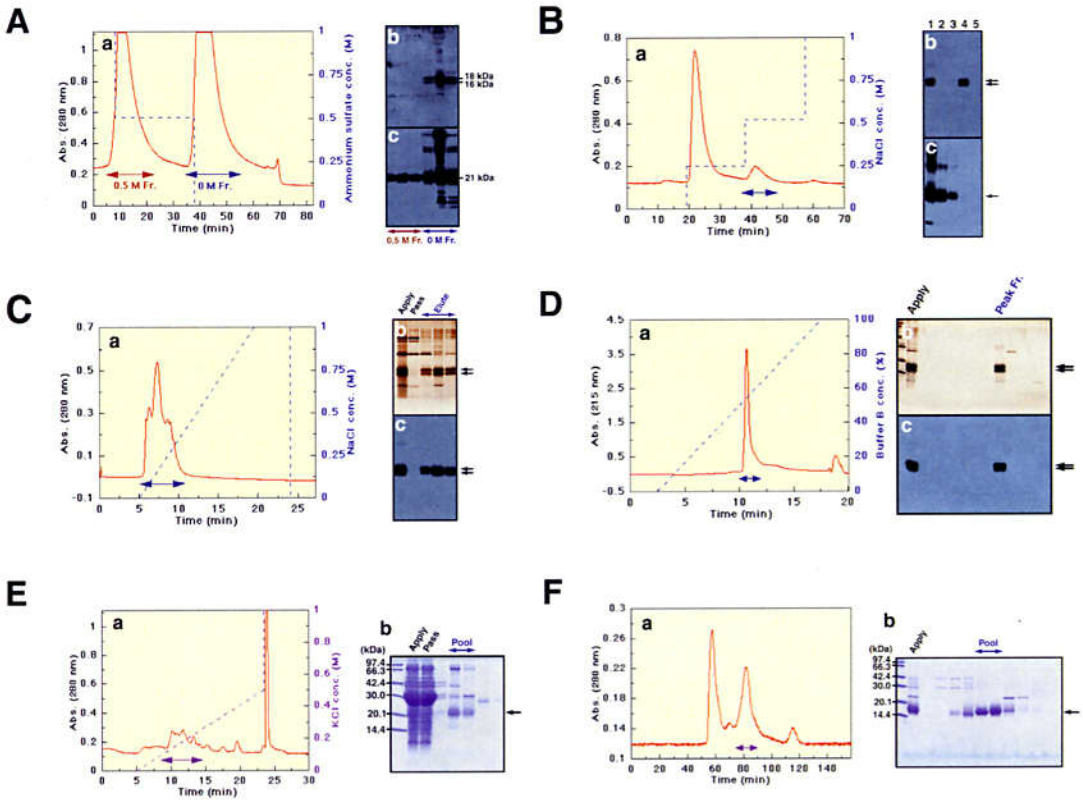

Figure 2-3. Sequential chromatographic purification of BMP-4 and sBMPR.

(A) (a) Phenyl-Sepharose chromatography. (B) (a) Heparin-Sepharose chromatography. (A) (B) BMP-4 (b) and sBMPR (c) were analyzed by Western blot analysis. The lane numbers are indicated that lane 1, apply sample; lane 2, pass-through fraction; lane 3, 0.25 M NaCl; lane 4, 0.5 M NaCl; lane 5, 1.0 M NaCl eluted fractions. (C) (a) Resource S chromatography. (D) (a) μRPC C2/C18 chromatography. (C) (D) BMP-4 was visualized by silver staining (b), and by Western blotting (c). (E) (a) Resource Q chromatography. (F) (a) Superdex 75 chromatography. (E) (F) sBMPR was analyzed by Coomassie blue staining (b). Black arrows indicate bands of BMP-4 and sBMPR. Each sample was subjected to electrophoresis on 15% SDS gel under reducing conditions. BMP-4 and sBMPR were recognized by Ab383 and 9E10, respectively.

separated by Mono S ion-exchange chromatography (Fig. 2-3C). Immunoreactive fractions were collected (Fig. 2-3Cc), and further purified by μRPC C2/C18 reverse-phase chromatography (Fig. 2-3D). The purity of BMP-4 was >98%, determined by silver staining of SDS-PAGE (Fig. 2-4A).

Next, to purify sBMPR, the fraction eluted from phenyl-Sepharose at 0.5 M NaCl (Fig. 2-3A), and the pass-through fraction from the heparin-Sepharose column were collected, and separated by Resource Q ion-exchange chromatography (Fig. 2-3Ea). In this step, most hemolymph proteins were removed (Fig. 2-3Eb). The eluted fractions containing sBMPR were further purified by Superdex 75 gel-filtration chromatography (Fig. 2-3Fa). The purity of sBMPR was >95%, determined by Coomassie blue staining (Fig. 2-4C). In these purifications, the amounts of xBMP-4 and sBMPR were 1 mg and 20 mg, respectively.

The binding specificity of sBMPR to TGF-β family ligands

The ability of sBMPR to bind known TGF-β family proteins was examined using the SPR biosensor (Fig. 2-5A). Purified xBMP-4, activin A, or TGF-β1 was immobilized on a sensor chip surface, and then sBMPR (5 μg/ml) was injected to flow over the sensor chips as an analyte. As sBMPR flowed over immobilized BMP-4, a rising slope of the RU was evident, and after the injection, the RU decreased slowly. However, this change in sensorgram did not occur when sBMPR was injected over either an activin A or a TGF-β1 immobilized sensor chip surface. This was identical to the control injection with mock-coupled sensor chip surface in the absence of proteins (Fig. 2-5A). To detect the *myc* epitope tag of sBMPR, injection of anti-*myc* monoclonal antibody, 9E10, followed the injection of sBMPR over the xBMP-4 surface. After binding of sBMPR to BMP-4 immobilized on the sensor chip, a second increase of 1,000 RU was observed by sequential injection of 9E10 (3 μg/ml). When 9E10 injected alone (blank surface) or injected with a 10-fold molar excess of *myc* epitope peptide (EQKLISEEDL), this second response during the injection of 9E10 was precluded (Fig. 2-5B), indicating that the second response was specific binding of 9E10 to the protein

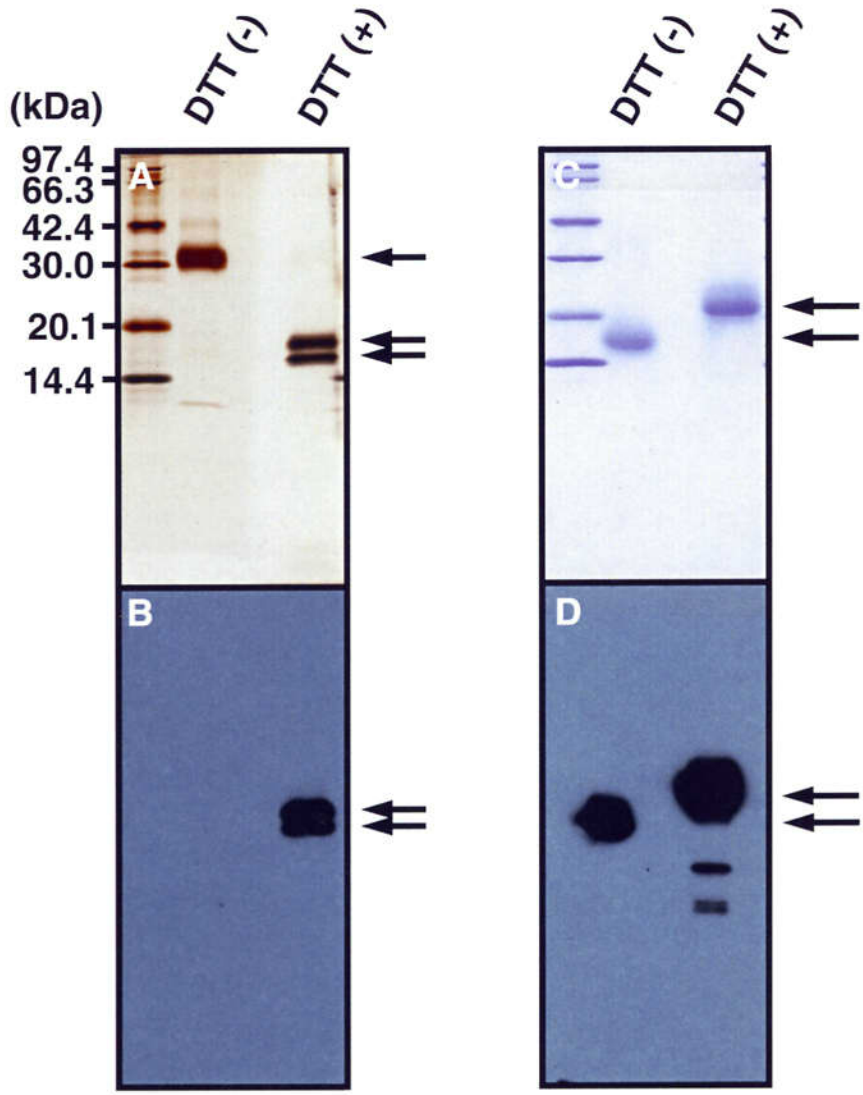

Figure 2-4. SDS-PAGE and Western blot analysis of BMP-4 and sBMPR.

Purified BMP-4 and sBMPR were analyzed by SDS-PAGE with silver (A), and Coomassie blue staining (C), respectively. Western blot analysis of BMP-4 (B) and sBMPR (D) was performed using Ab383 and 9E10, respectively. These analyses were performed under nonreducing (DTT (-)) and reducing (DTT (+)) conditions. Black arrows indicate bands of BMP-4 and sBMPR.

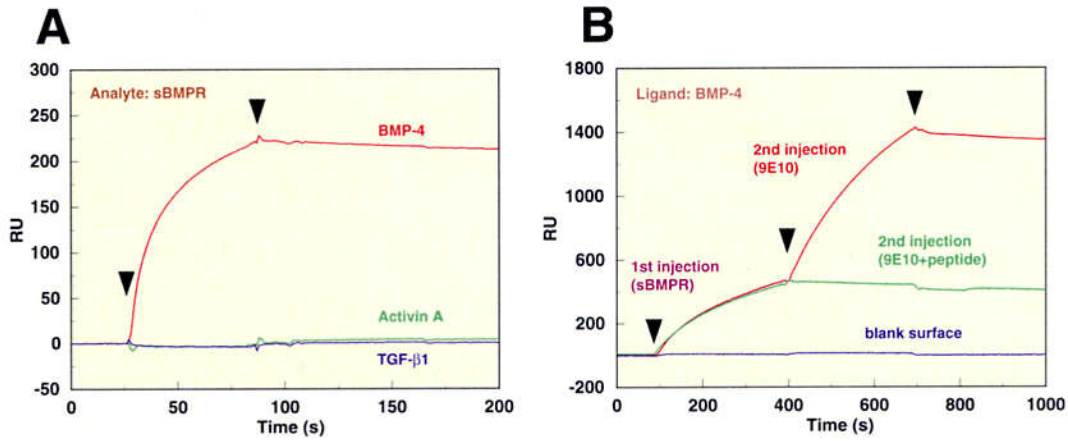

Figure 2-5. Interaction of sBMPR to immobilized xBMP-4.

(A) xBMP-4, activin A, and TGF-β1 were immobilized on the sensor chip surface. The purified sBMPR, at the concentration of 5 μg/ml, was injected over these surfaces at a flow rate of 10 μl/min for 60 s at 25 °C. For the blank runs, all samples were injected over mock-coupled sensor chip surfaces containing no protein simultaneously with each experimental run. All curves were corrected for background by subtracting the blank run, using BIAevaluation software version 2.1 (BIACORE AB). Arrowheads represent the initiation and termination of injections. (B) anti-*myc* antibody at a concentration of 3 μg/ml was injected after the injection of sBMPR over the xBMP-4 immobilized surface in the absence (9E10) or presence of a 10-fold molar excess of *myc* epitope peptide (9E10+peptide). For a control experiment, the first injection of sBMPR was replaced by running buffer (blank surface). Both proteins were injected at the flow rate of 10 μl/min for 150 s.

containing the *myc* epitope sequence, that is sBMPR. These results clearly suggested that the ECD of TFR11 was sufficient for binding to BMP-4 and did not bind either activin A or TGF-β1.

Kinetic analysis

To determine kinetic parameters, association rate constant and dissociation rate constant, increasing concentration of sBMPR was injected over the immobilized BMP-4 on the sensor chip (0.1-12.5 μM) (Fig. 2-6). Sensorgrams consisted of a rapid increase in RU during injections and an extremely slow decrease of dissociation phase (Fig. 2-6A); curves were analyzed using non-linear least squares methods. Dissociation phase (140-200 s) gave a good fit to a single exponential interaction model at all concentrations, and the dissociation rate constant, k_d, was calculated to be $3.69\pm0.26\times10^{-4}$ s^{-1} (Fig. 2-6C). However, association phase (80-130 s) was fitted to a single exponential interaction model only at a low concentration range (<3 μM), and the association rate constant, k_a, was estimated to be $3.81\pm0.19\times10^{4}$ $M^{-1}s^{-1}$ (Fig. 2-6B). The apparent equilibrium dissociation constant (K_D) determined from these two rate constants (k_d/k_a) was 9.6 nM.

sBMPR inhibits the biological activity of BMP-4

The biological activities of expressed sBMPR and BMP-4 were confirmed using culture cells. Firstly, ALP activity of MC3T3-E1 cells was measured to determine if sBMPR could compete with membrane-bound BMP receptors on cultured cells and could act as an antagonist of BMPs. MC3T3-E1 cells are well characterized as an osteoblastic cell line that differentiates into mature osteoblasts responding to BMPs and shows high ALP activity. The cells produce several family members of BMP and respond in an autocrine manner. RT-PCR analysis showed that this cell line expresses BMP-2 and BMP-4 of transcripts[1]. Fig. 2-7A shows the dose-dependent inhibition effect of sBMPR on ALP activity in MC3T3-E1 cells on day 6 of culture. Maxmum

[1] Natsume, T., Hatta, T., Kobayashi, Y., and Ueno, N.; unpublished observation.

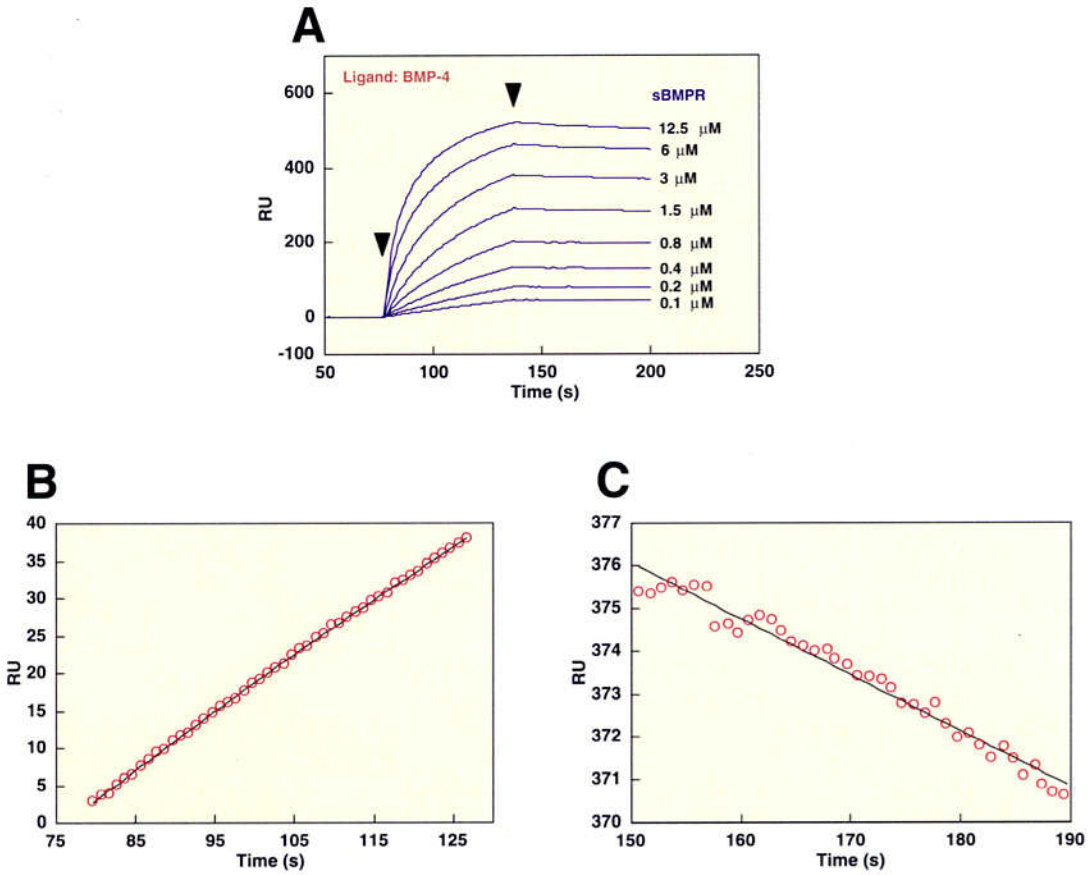

Figure 2-6. Kinetic analysis of sBMPR binding to BMP-4.

(A) To determine rate constants of the sBMPR for BMP-4, sBMPR was injected over the BMP-4 immobilized surface at different concentrations (0.1-12.5 μM) at a flow rate of 30 μl/min. Arrowheads represent the initiation and termination of injections. A single exponential binding model ($A+B \rightleftarrows AB$) was fitted to the binding curves shown in A. (B) (C) These data are representative examples of the curve fitting of the association and dissociation phases, respectively. The actual data points are shown as symbols, and the fitted curves are shown as solid lines.

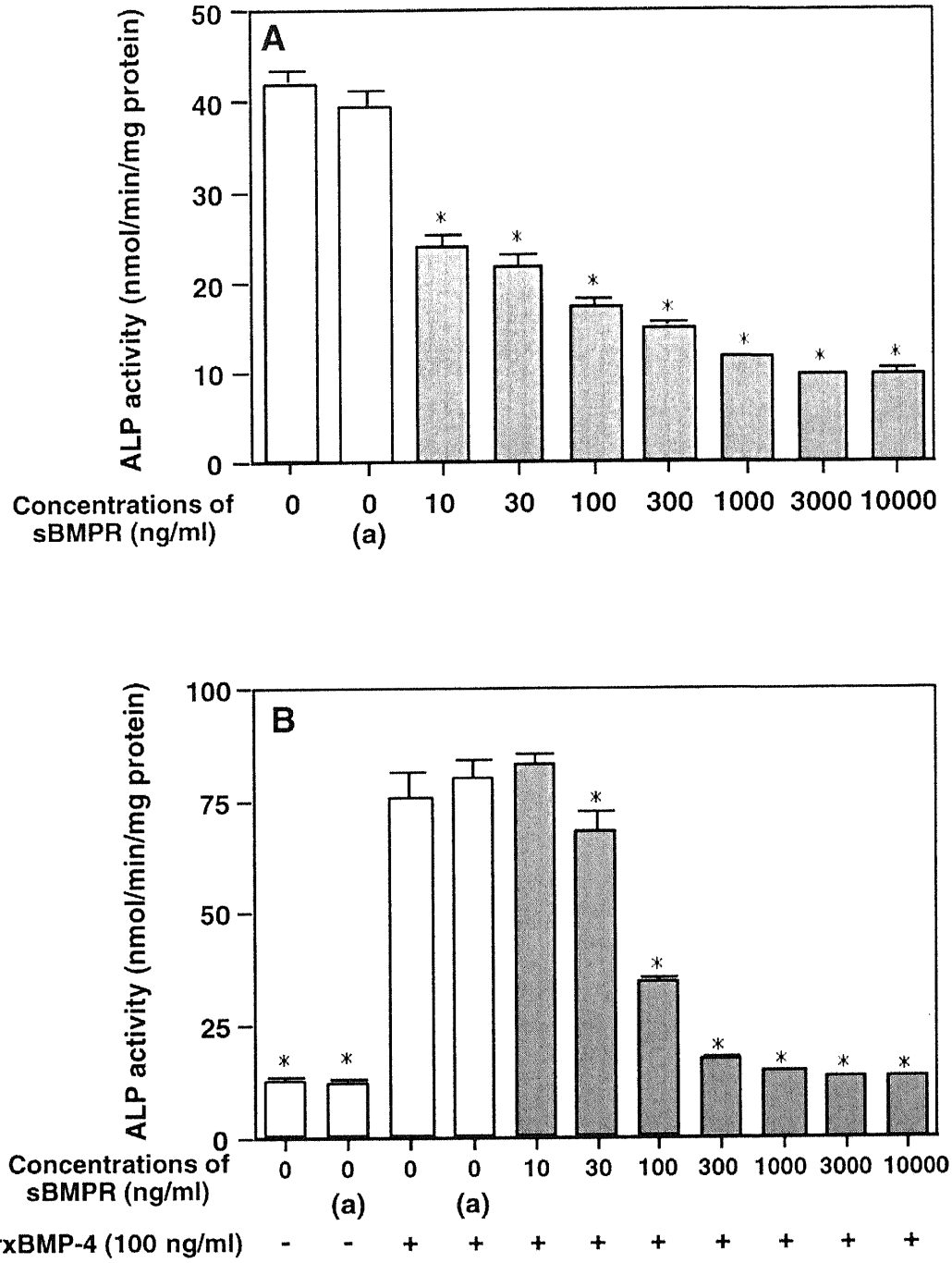

Figure 2-7. Biological activities of BMP-4 and sBMPR.

MC3T3-E1 and ST2 cells were inoculated at a cell density of 3×10^4 cells/well in 24-well plates and cultured. (A) The indicated concentrations of sBMPR were added to the culture medium of MC3T3-E1 cells on day 1 of the culture, and ALP activity was determined on day 6. (B) ST2 cells were grown in the absence (-) and presence (+) of xBMP-4 (100 ng/ml) with the indicated concentrations of sBMPR. After 3 days of treatment, ALP activity was determined. Data are expressed as the means (bars, S. E.) of three different wells. (a) For control culture, the buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) used for sample dilution was added.

inhibition was observed with a concentration of 3 μg/ml of sBMPR. Added sBMPR was considered to bind to BMPs secreted into the culture medium and to act as a antagonist by competing with wild membrane-bound receptors; the major type I receptor for BMP expressed on the cell was TFR11 (ALK3), which was cloned using a cDNA library of MC3T3-E1 cells (Suzuki et al., 1994). Next, I tested whether xBMP-4 can induce the differentiation of bone marrow stromal cells ST2, and sBMPR can block this differentiation. ST2 cells do not exhibit osteoblastic features, such as increase in ALP activity, in control culture. However, when the cells are cultured in the presence of purified xBMP-4 (100 ng/ml), the level of ALP activity increased 6-fold over that of control culture. This effect of BMP-4 on ALP activity was suppressed when sBMPR was added, dose-dependently (Fig. 2-7B). In the presence of 1 μg/ml of sBMPR, the ALP activity was reduced to basal level, and 50% inhibition was obtained at about 100 ng/ml of sBMPR (molar ration of added sBMPR to BMP-4 was ~2:1). These results suggest that both purified sBMPR and BMP-4 have intact biological activities, and that sBMPR can completely inhibit the activity of BMP-2 and BMP-4.

DISCUSSION

The silkworm expression system using a baculovirus vector is a powerful system for the large-scale expression of active proteins. In this study, to produce a large amount of BMP-4 and sBMPR efficiently, these proteins were coexpressed using this system. Although the expression level by coexpression was predicted to be decreased compared with independent expression because of limitation of productivity per silkworm, it was shown in the coexpression that sBMPR level was equal and BMP-4 level was rather increased as examined by Western blot analysis (data not shown). Because previous reports suggested that BMPs mediate apoptosis (Graham et al., 1994; Zou and Niswander, 1996), it is likely that overexpressed BMP-4 alone in silkworm leads to cell death of several types of cells, resulting in the decrease in total expression level of BMP-4. In contrast, because the effect of BMP-4 is assumed to be neutralized by coexpressed sBMPR, resulting in reduction of cell death, the total expression level of BMP-4 was probably increased. In addition to the high level of expression, expressed BMP-4 and sBMPR can be easily separated from each other by heparin-Sepharose chromatography (Fig. 2-3Bb, c) in keeping with their intact activities. This led to a milligram scale productions of xBMP-4 and sBMPR demonstrating that this cotransfection is very efficient.

In purification steps, loss of BMP-4 protein was observed by Western blot analysis (data not shown). Although the cause is not clear, its sticky nature (Reddi, 1994) may be involved in the loss of BMP-4 even under 4 M urea condition. Purified BMP-4 was detected by Western blot analysis using Ab383 antibody (Fig. 2-4B), which recognizes the monomer form of BMP-4 and BMP-2. Under reducing condition, BMP-4 was detected as 16- and 18-kDa proteins. These molecular masses were greater than that predicted by cDNA sequence (114 residues; 12.9 kDa). It is thought that these were generated by difference of glycosylation because xBMP-4 has two potential N-glycosylation sites (Nishimatsu et al., 1992).

In purification of sBMPR, its immunoreactivity was eluted from the phenyl-Sepharose column at both 0.5 and 0 M ammonium sulfate (Fig. 2-3Ac). Although it was thought to be attributed to conformational differences in protein folding of sBMPR, this is not likely because the SPR analysis indicated that both pools have similar BMP-binding activity (data not shown). As the 0 M-fraction contains BMP-4, while 0.5 M-fraction does not (Fig. 2-3Ab), it is likely that sBMPR in 0 M-fraction forms a complex with BMP-4 in hemolymph, and that sBMPR in 0.5 M-fraction is liberated from BMP-4. Together with this 0.5 M-fraction, sBMPR separated from BMP-4 by heparin-Sepharose chromatography (Fig. 2-3B) was further purified. In purification of sBMPR, I could not take advantage of the *myc*-tag; sBMPR was not retained on a 9E10-coupled affinity gel. Because sBMPR has only one *myc* sequence tagged, it may be too weak to retain sBMPR on the affinity column.

In SDS-PAGE analysis, the purified sBMPR migrated on the gel at a molecular mass of ~16 and ~22 kDa, under nonreducing and reducing conditions, respectively (Fig. 2-4C, D). Molecular masses of the observed bands in SDS-PAGE were greater than that predicted from cDNA sequence (129 residues; 1,430 Da). This was presumably the result of glycosylation on the two potential N-glycosylation sites of the molecule (Suzuki et al., 1994). A molecular mass of 16 kDa was also determined by matrix-assisted laser desorption mass spectrometry, indicating that sBMPR is monomeric (data not shown), despite the presence of 10 conserved cysteine residues of ECD. This was further supported by a global analysis of equilibrium sedimentation, using an analytical ultracentrifuge. Data from this yielded a single molecular mass of 16±0.5 kDa and indicated that sBMPR is a monomer in solution and does not aggregate further[2]. Recently, it has been demonstrated that all cysteines of ECD formed five intramolecular disulfide bridges and no unpaired cysteine residues[3]. Differences in molecular mass under reducing conditions in SDS-PAGE analysis would be the result of disruption of these intramolecular disulfide bonds.

---

[2] Natsume, T., Hatta, T., Ueno, N., and Kobayashi, Y, manuscript in preparation.

[3] Nishiuchi, Y., Natsume, T., Yamazaki, T., Hatta, T., Ueno, N., and Kobayashi, Y, manuscript in preparation.

The affinity determined by this method is similar to K_D values (0.5-3.5 nM) for intact type I receptors for BMPs binding to their ligand (Graff et al., 1994; Koenig et al., 1994; Suzuki et al., 1994). To give confidence in kinetic parameters of the sBMPR/BMP-4 interaction, reverse orientation experiment was also performed, i.e., sBMPR was immobilized on the sensor chip surface and BMP-4 was flowed as an analyte. However, the injected BMP-4 was absorbed in the micro flow channel of the instrument and hardly reached flow cells of the sensor chip surface at neutral pH range (data not shown), probably because of the sticky nature of BMP-4 (Reddi, 1994). Thus, using a sBMPR immobilized surface was not feasible for reproducible data collection and quantitative analysis.

In this chapter, I have succeeded in expression and purification of large-scale bioactive BMP-4 and sBMPR. sBMPR described here has facilitated structure-function analysis of the ECD of the type I receptor for BMP-2 and BMP-4. These data show that sBMPR retains high-affinity binding and forms a stable complex with BMP-4 because of an extremely slow dissociation rate constant. Because only the active form (mature form) of BMPs is capable of binding to its receptor (Miyazono et al., 1994), sBMPR might be useful to detect active forms of BMP-2 and BMP-4 as well as for use as a BMP antagonist. In addition, these proteins have been proven to be useful for studies of the interaction of BMP-4 with its binding proteins as described in following chapters.

CHAPTER 3

DIRECT BINDING OF FOLLISTATIN TO A COMPLEX OF BONE-MORPHOGENETIC PROTEIN AND ITS RECEPTOR

SUMMARY

In early development of *Xenopus* laevis, it is known that activities of polypeptide growth factors are negatively regulated by their binding proteins. In this study, follistatin, originally known as an activin binding protein was shown to inhibit all aspects of BMP activity in early *Xenopus* embryos. Furthermore, using the SPR biosensor, it was demonstrated that follistatin can directly interact with multiple BMPs at significantly high affinities. Interestingly, follistatin was found to be non-competitive with the BMP receptor for ligand binding and to form a trimeric complex with BMP and its receptor. The results suggest that follistatin is also likely to act as an organizer factor in early amphibian embryogenesis by inhibiting BMP activities by a different mechanism from that used by chordin and noggin.

INTRODUCTION

TGF-β family ligands are known to regulate a variety of cell differentiation processes that lead to morphogenesis in early vertebrate development (Kingsley, 1994). In *Xenopus laevis*, two classes of the TGF-β family ligands are believed to determine the dorsoventral pattern of the mesoderm in early gastrula embryos (Sive, 1993). The first class of ligands includes those that are related to activin and Vg1. This group has been shown to induce formation of the dorsal mesoderm, which gives rise to a variety of tissues including muscle and the notochord (Asashima et al., 1990; Smith et al., 1990; Thomsen and Melton, 1993). The second class includes the BMP family of ligands, which inhibit dorsal mesoderm formation and induce cells to take on ventral fates, such as that of blood cells, and is also involved in the dorsoventral specification of ectodermal cell fate by inducing the epidermis and inhibiting neural cell differentiation as mentioned in chapter 1. These biological activities of TGF-β ligands are known to be negatively regulated by their specific binding proteins (Graff, 1997; Thomsen, 1997). For example, follistatin is capable of binding activin extracellularly and this binding inhibits the activation of activin receptors (Nakamura et al., 1990). Therefore, follistatin has been considered to be an important component in the regulation of mesoderm induction (Fukui et al., 1993). Similarly, among BMPs, at least BMP-4 is known to be strictly regulated by its binding proteins, chordin and noggin. Interestingly, the expression of both chordin and noggin is restricted to the Spemann's organizer of early *Xenopus* gastrula (Sasai et al., 1994; Smith and Harland, 1992), suggesting that they may act as organizer factors inhibiting the anti-neural activity of BMP-4. These observations led to the notion that the inhibition of polypeptide growth factor activity by binding proteins is a key step in the control of early developmental events (Moon et al., 1997).

However, the specificity of ligand recognition by binding proteins is not fully understood. For example, recent studies on follistatin using *Xenopus* embryos demonstrate that the binding specificity of follistatin is a complicated issue. Follistatin

induces the secondary body axis when overexpressed in ventral blastomeres (Sasai et al., 1995) and in ectoderm it can induce neural tissue (Hemmati-Brivanlou et al., 1994). These observations indicate that follistatin might inhibit not only activin, but also BMPs through direct binding, because many of the phenotypes caused by the overexpression of follistatin in early *Xenopus* embryos are similar to those obtained by overexpression of dominant-negative BMP receptors.

Follistatin is a glycosylated monomeric protein originally discovered in ovarian follicular fluid as a suppressor of pituitary follicle-stimulating hormone (FSH) secretion (Ueno et al., 1987), and then identified as a activin-binding protein (Nakamura et al., 1990). Molecular cloning analyses of its cDNA and genomic DNA revealed that there are two types of alternatively spliced FS mRNAs in human (Shimasaki et al., 1988). These precursor FSs encode 317 and 344 amino acid residues, which include a common 29-amino acid signal peptide, and thus, the mature forms of FS would consist of 288 (FS-288) and 315 (FS-315) amino acid residues, respectively (Fig. 3-1). Mature FS-315 has an extra 27-amino acid residues at the carboxy-terminal (acidic region), while mature FS-288 is lacking in this region. The predicted primary structures of FS in human, pig, and rat are highly conserved (>98%). In *Xenopus* follistatin, precursor FSs encode 319 and 346 amino acid residues, which has an 84% homology with human and porcine FSs (Hemmati-Brivanlou et al., 1994; Tashiro et al., 1991) (unpublished data) (Fig. 3-1).

In this chapter, to solve the problem of the specificity of follistatin, firstly the effect of follistatin on all BMP activities in *Xenopus* embryos was tested using microinjection assay. Secondly, to confirm whether the effect of follistatin was due to the direct binding of follistatin to BMPs, the interaction of these proteins was examined using the SPR biosensor used in chapter 2. Finally, the inhibition mechanism by follistatin was further explored.

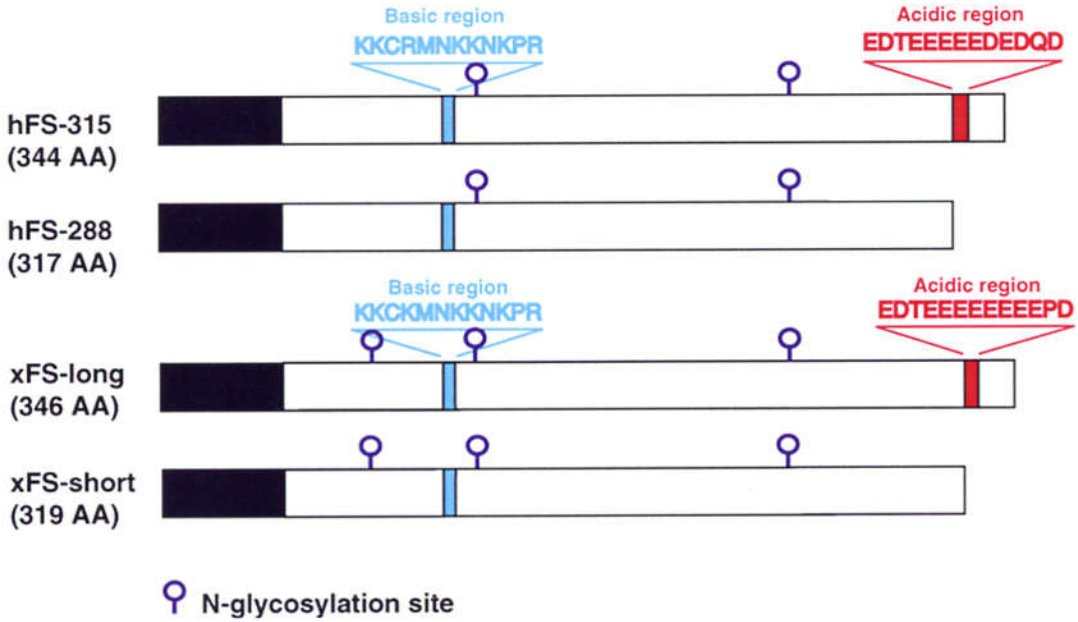

Figure 3-1. Structures of human and *Xenopus* follistatins.

Schematic representation of the human and *Xenopus* follistatins. Human or *Xenopus* follistatin cDNAs encoding a precursor of 344 and 317, or 346 and 319 amino acids, respectively, result from alternative splicing. Two structurally characteristic regions, acidic and basic regions, indicate red and blue boxes, respectively. Two potential sites of N-linked glycosylation in human, and three in *Xenopus* are present in the precursor proteins. Signal sequences are indicated by black boxes.

EXPERIMENTAL PROCEDURES

Embryo manipulations

Xenopus embryos were obtained by artificial fertilization and 2- or 4-cell stage embryos were microinjected with synthetic RNAs as previously described (Suzuki et al., 1997). For evaluation of mesodermal markers, dorsal or ventral marginal region, respectively, was excised when the injected embryo reached stage 10. For animal cap assay, presumptive ectoderm fragments were collected when the injected embryos reached stage 9.

Reverse transcription-polymerase chain reaction (RT-PCR)

For the detection of molecular marker expression, total RNA was isolated using TRIzol (GIBCOBRL) according to manufacturer's instructions and analyzed by RT-PCR. Primers were as follows: as a dorsal organizer marker goosecoid: upstream, 5'-ACTACTATGGACAGTTGCACG-3', downstream, 5'-TTCTGATTCCTCTGATGAAGATC-3'; a ventrolateral mesoderm marker Xvent1: upstream, 5'-TTCCCTTCAGCATGGTTCAAC-3', downstream, 5'-GCATCTCCTTGGCATATTTGG-3'; a definitive ventral mesoderm marker αT1globin: upstream, 5'-TTGCTGTCTCACACCATC-3', downstream, 5'-TCTGTACTTGGAGGTGAG-3'; an internal input control histone H4: upstream, 5'-ATAACATCCAGGGCATCACC-3', downstream, 5'-ACATCCATAGCGGTGACGGT-3'.

Recombinant proteins

Recombinant BMP-4 and sBMPR were obtained as described in chapter 2. Recombinant human follistatin (FS-288) was a gift from Dr. S. Shimasaki (Inouye et al., 1991), recombinant activin A was a gift from Dr. Y. Eto (Ajinomoto, Inc.), *Xenopus* BMP-4/7 heterodimer (BMP-4/7) and *Xenopus* BMP-7 (BMP-7) were gifts from Dr. Y. Fujisawa (Takeda Chemical Industries Ltd.), and *Xenopus* chordin was a gift from Dr.

S. Piccolo (Piccolo et al., 1996). Recombinant human TGF-β1 was purchased (King Jyouhzo). *Xenopus* noggin producing cell line, Chinese hamster ovary (CHO) B3 cell line, was a gift from Dr. R. Harland (Lamb et al., 1993). Noggin protein was purified from the conditioned medium by Mono S (Pharmacia Biotech AB) chromatography. The purity of the protein was >98%, determined by SDS-PAGE (data not shown).

Protein-protein interaction analysis by the SPR biosensor

Sample proteins were injected over the surfaces at a flow rate of 20 μl/min at 25 °C for 120 s. For the blank runs, all samples were injected over mock-coupled sensor chip surfaces containing no protein simultaneously with each experimental run. All curves were corrected for background by subtracting the blank run, using BIAevaluation software version 3.0 (BIACORE AB). BMPs, activin A, TGF-β1, and FS-288 were immobilized on the sensor chip surface (CM5, certified grade, BIACORE AB) by the amine coupling method. sBMPR that was biotinylated using sulfo-NHS-biotin (PIERCE) was immobilized on the sensor chip surface (SA5, research grade, BIACORE AB) which had been pre-immobilized with streptavidin. The sequential experiment was performed using the coinjection method (BIACORE AB). Arrowheads represent the initiation and termination of injections. Other conditions are described in chapter 2.

Chemical cross-linking and two-dimensional electrophoresis

FS-288 (450 ng) and BMP-4 (450 ng) were incubated for 1 hr at room temperature in 100 μl of HBS containing 0.02% Tween 20. Dithiobis (sulfosuccinimidylpropionate) (PIERCE) was added to a final concentration of 0.15 mM, incubated for 30 min at room temperature, and stopped by adding Tris-HCl (pH 8.0) to a final concentration of 50 mM. Diagonal SDS-PAGE analysis was performed as previously described (Ueno et al., 1992). Briefly, samples (10 μl) were electrophoresed in 0.2×7 cm strips of 1 mm-thick 12.5% polyacrylamide gel without reducing agents. Each gel strip was removed after the first electrophoresis and incubated in a solution containing 5% 2-mercaptoethanol for 1 hr at 37 °C. After

incubation, gel strip was placed onto a 15% SDS slab gel (7.5×9 cm) and then electrophoresed in the second dimension under reducing condition. Visualization of the proteins was performed by Western blotting as described below.

Western blotting analysis

Western blot analysis was described in chapter 2. Briefly, protein samples, resolved by two-dimensional electrophoresis, were electroblotted onto PVDF membranes (MILLIPORE). The membrane was blocked, and then reacted with the antibodies mixture of BMP-4 antibody (Ab97) and follistatin antiserum (Rb32) at 4 °C overnight (Inouye et al., 1991; Nishimatsu et al., 1993). The membrane was then reacted with a secondary antibody for 1 hr at room temperature. Western blot was developed with the chemiluminescent ECL plus kit (Amersham) as described by the manufacturer.

RESULTS

Inhibition of BMP-induced ventralization by follistatin

Using mRNA microinjection method, the interaction of BMPs with follistatin was analyzed. Firstly, the effect of follistatin on ventralizing activity of BMPs was investigated. The dorsal injection of 200 pg of BMP-2 (data not shown) or BMP-4 mRNA ventralized embryos to an average dorsoanterior index (DAI) of 0-1 (Fig. 3-2Ab) (Suzuki et al., 1994). In contrast, 500 pg of BMP-7 mRNA was required to cause a similar amount of ventralization (Fig. 3-2Ac), which is consistent with the previous observation that the ventralizing activity of BMP-7 mRNA is relatively weak compared with that of BMP-4 mRNA (Suzuki et al., 1997). To examine the effect of follistatin on BMPs, follistatin mRNA was coinjected with BMP mRNAs into the two dorsal blastomeres of 4-cell embryos. Coinjection of 100 pg follistatin mRNA with 200 pg of BMP-4 partially rescued the ventralized phenotype to DAI 4 (Fig. 3-2Ad). Complete rescue, however, was not achieved even at 300 pg of follistatin mRNA (data not shown). In contrast, only 10 pg follistatin mRNA was needed to significantly rescue the ventralized phenotype of embryos treated with 500 pg of BMP-7 mRNA (data not shown), and 50 pg effected almost complete rescue (Fig. 3-2Ae). These results suggest that follistatin can antagonize BMP-7 more efficiently than BMP-4. This antagonistic effect was further confirmed using molecular markers expressed in the dorsal marginal (equatorial) region using RT-PCR (Fig. 3-2B). Expression of the dorsal mesoderm marker, goosecoid, which is suppressed by BMP-4 and BMP-7 mRNA overexpression, was restored by the coinjection of follistatin mRNA. In particular, follistatin effectively returned the BMP-7-suppressed goosecoid expression to normal levels. Conversely, a ventral mesoderm marker, Xvent1, ectopically induced in the dorsal mesoderm by BMPs, was suppressed by follistatin to normal levels and a definitive ventral mesoderm marker, αT1globin, induced by BMP, was also down-regulated by the overexpression of follistatin (lane 2-5). Thus, by both criteria, i. e., morphology and molecular markers, the preferential inhibition of BMP-7 by follistatin in dorsal

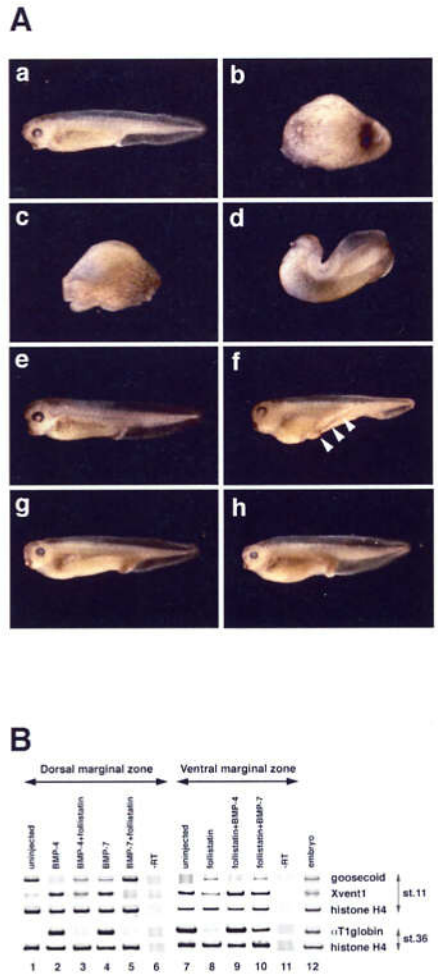

Figure 3-2. Inhibitory interaction between follistatin and BMPs.

(A) Phenotypes of *Xenopus* embryos dorsally co-injected with BMP mRNA and follistatin mRNA. BMP-4 mRNA (200 pg), either alone (b) or with 100 pg of follistatin mRNA (d), and BMP-7 mRNA (500 pg), either alone (c) or with 50 pg of follistatin mRNA (e), were injected into the equatorial region of the two dorsal blastomeres at the 4-cell stage as previously described (Jones et al., 1992). In contrast, equatorial regions of two ventral blastomeres at the 4-cell stage were injected with follistatin mRNA (60 pg), either alone (f) or with 500 pg of BMP-4 mRNA (g) or with 2 ng of BMP-7 mRNA (h). Embryos were evaluated and photos were taken at the tadpole stage. (a) uninjected embryo. (B) Expression of molecular markers in dorsal and ventral marginal explants. Embryos were either uninjected (lane 1, 6, 7, 11, 12) or injected with the mRNAs indicated on the top of each lane into the equatorial region of two dorsal (lane 2-5) or ventral blastomeres (lane 8-10) at the 4-cell stage. The amounts of mRNA for dorsal injection were 200 pg of BMP-4 (lane 2), 200 pg of BMP-4 plus 200 pg of follistatin (lane 3), 500 pg of BMP-7 (lane 4), and 500 pg of BMP-7 plus 50 pg of follistatin (lane 5). For ventral injection, 60 pg of follistatin mRNA, 500 pg of BMP-4 mRNA, and 2 ng of BMP-7 mRNA were used. Lane 12 shows the expression of markers in whole embryos and lane 6 and 11 show the control reactions with no reverse transcription step. Dorsal or ventral marginal zones were excised and explanted at the early gastrula stage and incubated until sibling embryos reached stages 11 and 36.

mesoderm was confirmed.

BMP can rescue dorsalization and neuralization induced by follistatin

It has been shown that follistatin induces the dorsal mesoderm and a secondary body axis in *Xenopus* when it is ventrally overexpressed (Sasai et al., 1995). These effects are indistinguishable from those caused by the ventral overexpression of a dominant-negative BMP receptor that inhibits both BMP-2 and BMP-4 (Graff et al., 1994; Suzuki et al., 1994). Therefore, it has been speculated that follistatin might inhibit the BMP signaling pathway (Sasai et al., 1995; Thomsen, 1997). To confirm this speculation, BMPs and follistatin were coinjected into the two ventral blastomeres of 4-cell embryos. Follistatin mRNA (60 pg) ventrally injected was sufficient to induce a secondary axis although it lacks anterior structures such as a brain and eyes (Fig. 3-2Af, arrowheads). To examine whether BMPs can rescue the secondary axis formation by follistatin, coinjection of follistatin mRNA (60 pg) with BMP-4 mRNA (50 pg) was performed, and partially suppressed the formation of secondary axis (data not shown). Furthermore, 500 pg of the mRNA almost completely suppressed it (Fig. 3-2Ag). In contrast, no significant inhibitory effect of BMP-7 mRNA was observed with 500 pg (data not shown), and 2 ng was required to suppress the secondary axis to a level similar to that of embryos rescued by 500 pg of BMP-4 mRNA (Fig. 3-2Ah). Analysis of molecular marker expression further demonstrated that follistatin-induced dorsal marker gene induction and ventral marker suppression were reciprocally returned to normal levels by the coinjection of BMP mRNAs (Fig. 3-2B), although the rescuing effect of BMP-7 (lane 10) appeared to be weak compared with that of BMP-4 (lane 9).

Follistatin interacts with BMP extracellularly

Next, to test whether follistatin antagonizes BMPs extracellularly or whether it acts on the BMP pathway intracellularly, constitutively active BMP type I receptors (substitution of Gln to Thr in the GS domain), which are ActRI (CA-ALK2) and

BMPRIA (CA-ALK3), were used (Akiyama et al., 1997; Suzuki et al., 1997). In theory, the extracellular interaction of follistatin with BMPs should not inhibit the ligand-independent ventralizing signal generated by the constitutively active receptors. As shown in Fig. 3-3, the dorsal overexpression of constitutively activated forms of both CA-ALK2 (Fig. 3-3Aa) and CA-ALK3 (data not shown) resulted in partially ventralized embryos without head formation as previously reported. As expected, ventralization by the constitutively active receptor overexpression was not inhibited by follistatin coexpression (Fig. 3-3Ab). Molecular marker expression also demonstrated that the effects of constitutively active receptors were not rescued by the coinjection of follistatin mRNA (Fig. 3-3B). These results suggest that the interaction between follistatin and BMPs occurs extracellularly.

Direct binding of follistatin to BMPs

To ascertain that follistatin binds BMPs directly, the SPR biosensor (BIACORE) was used. First, the ability of follistatin to bind known TGF-β family proteins was tested. Purified activin A, TGF-β1, or BMP-4 was immobilized on a BIACORE sensor chip, and then FS-288 (5 μg/ml) was injected to flow over the sensor chips as an analyte. During the injection of FS-288 over the activin A surface, a rising slope of resonance signal was evident, indicating binding, and after injection the resonance unit decreased slowly. However, this change in resonance signal was not detectable when the FS-288 was injected to flow over the TGF-β1-immobilized sensor chip surface. These results clearly suggest that FS-288 binds strongly to activin A but not to TGF-β1 (Fig. 3-4A). Interestingly, FS-288 showed significant binding to the BMP-4 homodimer although the level of response was relatively low compared with that obtained with activin A, even though the same amount of protein was fixed on the sensor chip. To test whether the interaction between BMP-4 and FS-288 is specific, I carried out a competition assay using BIACORE. As shown in Fig. 3-4B, soluble FS-288 competed with the binding of BMP-4 to FS-288 immobilized on the sensor chip in a dose-dependent manner. This result clearly demonstrates that the interaction between

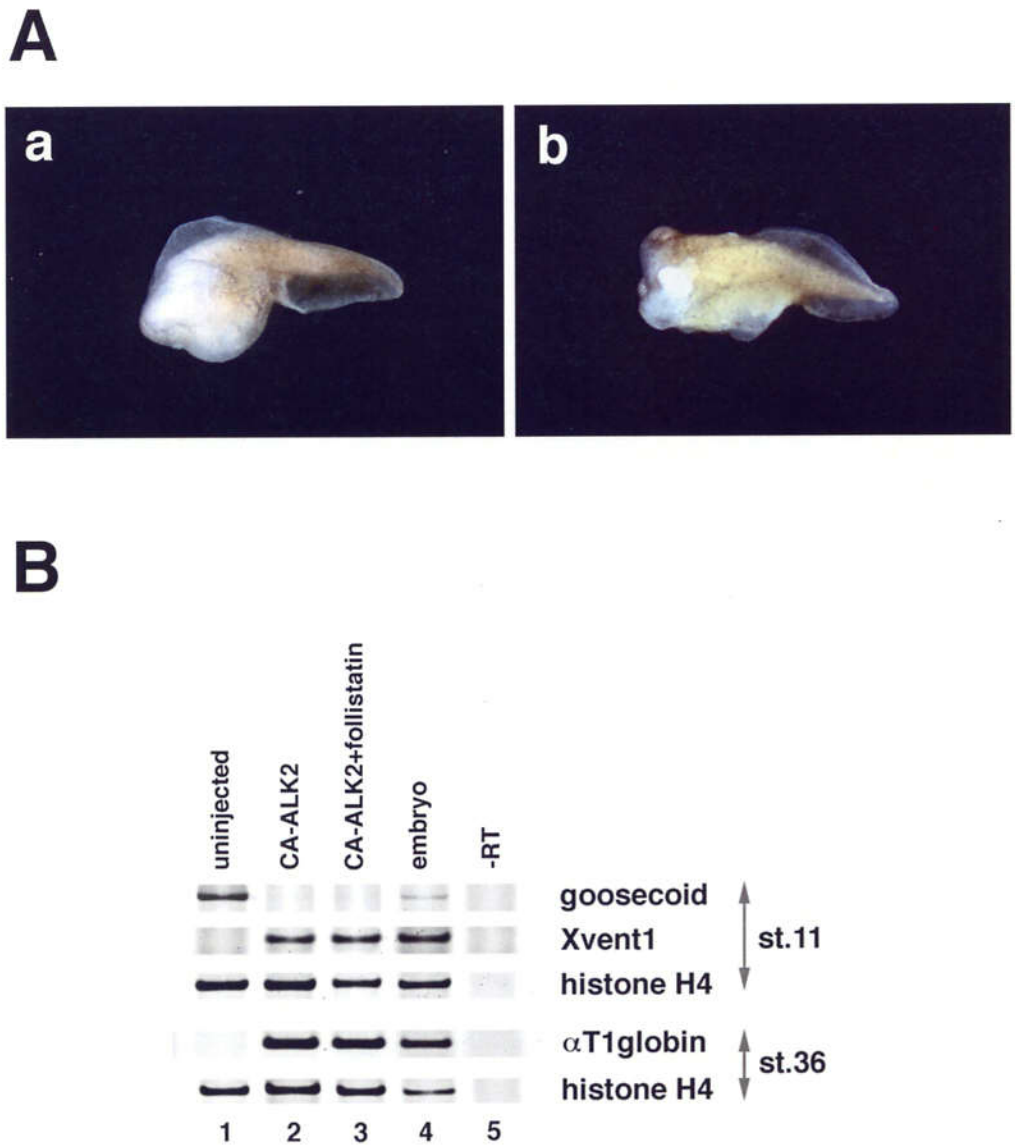

Figure 3-3. Failure of follistatin to block intracellular BMP signaling.

(A) Phenotype of ventralized embryos by the dorsal injection of mRNA for constitutively active BMP signaling receptor, with or without follistatin mRNA. 50 pg of mRNA for a constitutively activated form of *Xenopus* ActRI (CA-ALK2) was injected dorsally with (b) or without (a) 200 pg of follistatin mRNA as described in the legend for Fig.3-2. (B) Expression of molecular markers in dorsal marginal explants of embryos that were uninjected (lane 1), injected with 500 pg of mRNA for CA-ALK2 alone (lane 2), and co-injected with 500 pg of CA-ALK2 and 200 pg of follistatin (lane 3). Dorsal mRNA injection and RT-PCR analysis were performed as described in Fig 3-2. Co-injection of follistatin mRNA did not reduce the effect of CA-ALK2 overexpression for both dorsal (goosecoid) and ventral (Xvent1, αT1globin) markers. Similar results were obtained in the experiments using CA-ALK3 (data not shown).

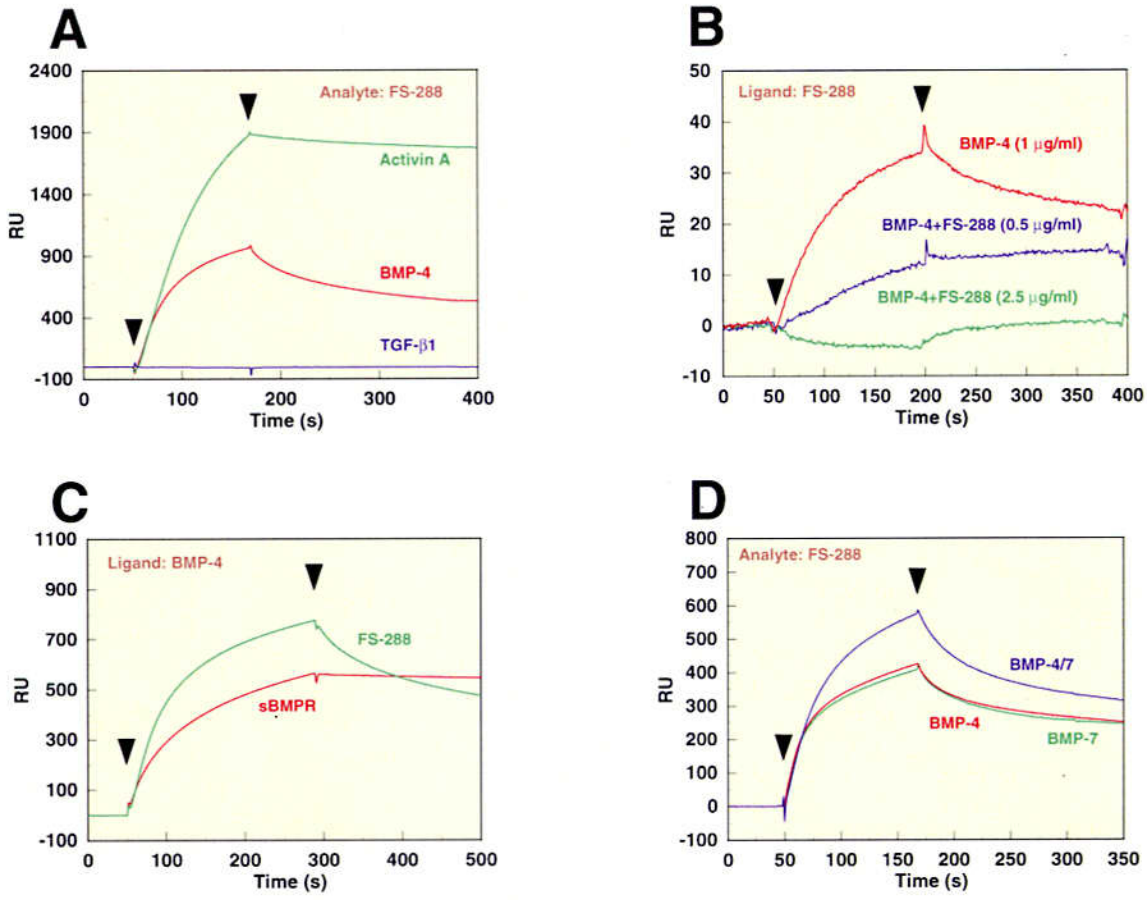

Figure 3-4. Direct binding of follistatin to immobilized BMPs.

(A) BMP-4, activin A, and TGF-β1 were immobilized on the sensor chip surface. The immobilization levels of BMP-4, activin A, and TGF-β1 were 2904, 2543, and 2681 RU, respectively. FS-288, at a concentration of 5 μg/ml, was injected over these surfaces. (B) FS-288 (807 RU) was immobilized on the sensor chip surface. BMP-4 (1 μg/ml) alone was injected over the surface (red line). BMP-4 (1 μg/ml) and FS-288 (0.5 or 2.5 μg/ml) were incubated for 30 min at room temperature. Each mixture was injected over the surface. Excess soluble FS-288 (2.5 μg/ml) almost completely abolished the binding activity on the FS-288 surface. (C) FS-288 and sBMPR, at the concentration of 5 μg/ml and 20 μg/ml, respectively, were injected over the surface of immobilized BMP-4, separately. (D) BMP-4, BMP-4/7, and BMP-7 were immobilized on the sensor chip surface. The immobilization levels of these ligands were 622, 768, and 697 RU, respectively. FS-288 at 5 μg/ml was injected over these surfaces.

follistatin and BMP is specific.

To further confirm this physical interaction by conventional biochemical way, chemical cross-linking analysis was performed. Follistatin and BMP-4 were mixed, chemically linked, and separated on two-dimensional electrophoresis. Each protein was visualized by Western blotting using specific antibodies for BMP and follistatin. As shown in Fig. 3-5B, BMP-4 bound to FS-288 migrated from 30 kDa to higher molecular weights (about 60, 90, 180, and 200 kDa<), while BMP-4 alone, without cross-linking (Fig. 3-5A) or with cross-linking (data not shown) did not shift significantly. This result supports that follistatin-BMP complex is indeed present in the reaction mixture.

Next, the kinetic parameters for the binding of FS-288 to BMP-4 were determined and compared to those for a soluble form of BMPRIA (sBMPR) that has been shown to bind BMP-4 homodimer independently of the BMP type II receptor (chapter 2). The association rate constant for FS-288 (k_a=1.16×10^5 M^{-1}s^{-1}) was faster than that for the soluble receptor binding to BMP-4 (k_a=3.81×10^4 M^{-1}s^{-1}). In contrast, following the injection, the dissociation of FS-288 was significantly faster than that of sBMPR (Fig. 3-4C). The dissociation rate constant of FS-288 was estimated from the resonance data (k_d=2.7×10^{-3} s^{-1}). From the association and dissociation rate constants, the affinity of FS-288 for BMP-4 was calculated to be 23 nM, which is lower than that for sBMPR (9.6 nM) (chapter 2).

Furthermore, the specificity of FS-288 for each of the BMPs was examined (Fig. 3-4D). FS-288 bound both BMP-2 (data not shown) and BMP-4 homodimers in an identical manner, as would be expected from the similarity of their primary sequences. It also bound BMP-7, whose primary structure is relatively distant from BMP-2 and -4, with almost the same binding profile as obtained with BMP-2 and -4. Interestingly, FS-288 showed a higher binding affinity for the BMP-4/7 heterodimer compared with other BMP homodimers.

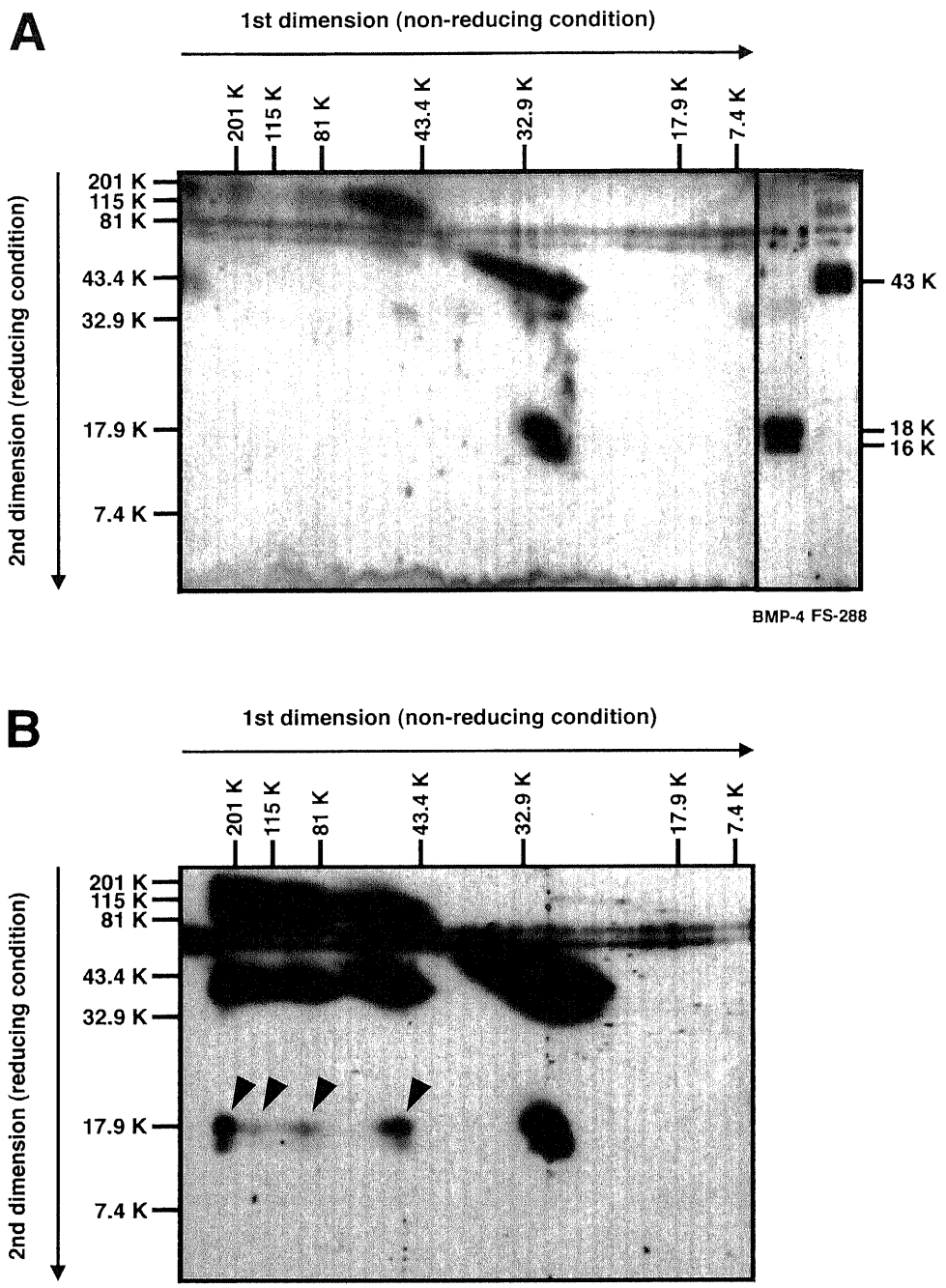

Figure 3-5. Diagonal SDS-PAGE analysis of FS-288-BMP-4 complex.

(A) For comparison of molecular mass, a mixture of FS-288 and BMP-4 was analyzed by two-dimensional electrophoresis. While FS-288 (32 kDa) tends to shift to higher molecular weight region (>60 kDa) on 1st dimension electrophoresis probably by self aggregation, a major band of BMP-4 shows the estimated molecular mass of dimeric form (30 kDa). FS-288 was detected as a 43 kDa protein, and BMP-4 was detected as 16 and 18 kDa proteins, which were generated due to difference of glycosylation, under reducing condition. (B) Cross-linked FS-288-BMP-4 complex was analyzed. Proteins were separated by two-dimensional electrophoresis, and subjected to Western blotting. At least, four shifted bands (arrowheads) of BMP-4, which were not seen in (A), were detected. Relative molecular mass is indicated horizontally for non-reducing, vertically for reducing condition.

The different inhibitory mechanisms of BMP-4 antagonists

Next, the mechanism by which follistatin inhibits BMP activity was examined. Chordin and noggin have been shown to interfere with BMP-4's ability to bind the BMP receptor and to inhibit BMP activity in a competitive manner (Piccolo et al., 1996; Zimmerman et al., 1996). In fact, as shown in Fig. 3-6, a mixture of BMP-4 and chordin (A), or noggin (B) showed no detectable binding capacity for sBMPR. Interestingly, FS-288 appears to bind BMP-4 in a non-competitive manner; a mixture of FS-288 and BMP-4 was still able to bind soluble BMP receptor, suggesting that they form trimeric complex (Fig. 3-6C). To confirm this possibility, BMP-4 and FS-288 were sequentially added to the receptor using the biosensor. After binding of BMP-4 to sBMPR immobilized on the sensor surface, a second increase of resonance signal was observed by sequential injection of FS-288 (Fig. 3-6D). This result suggests that the binding site of BMP-4 for its receptor may be distinct from that for follistatin, and that follistatin inhibits BMP-4 activities by a different mechanism from that used by chordin and noggin (Fig. 3-7).

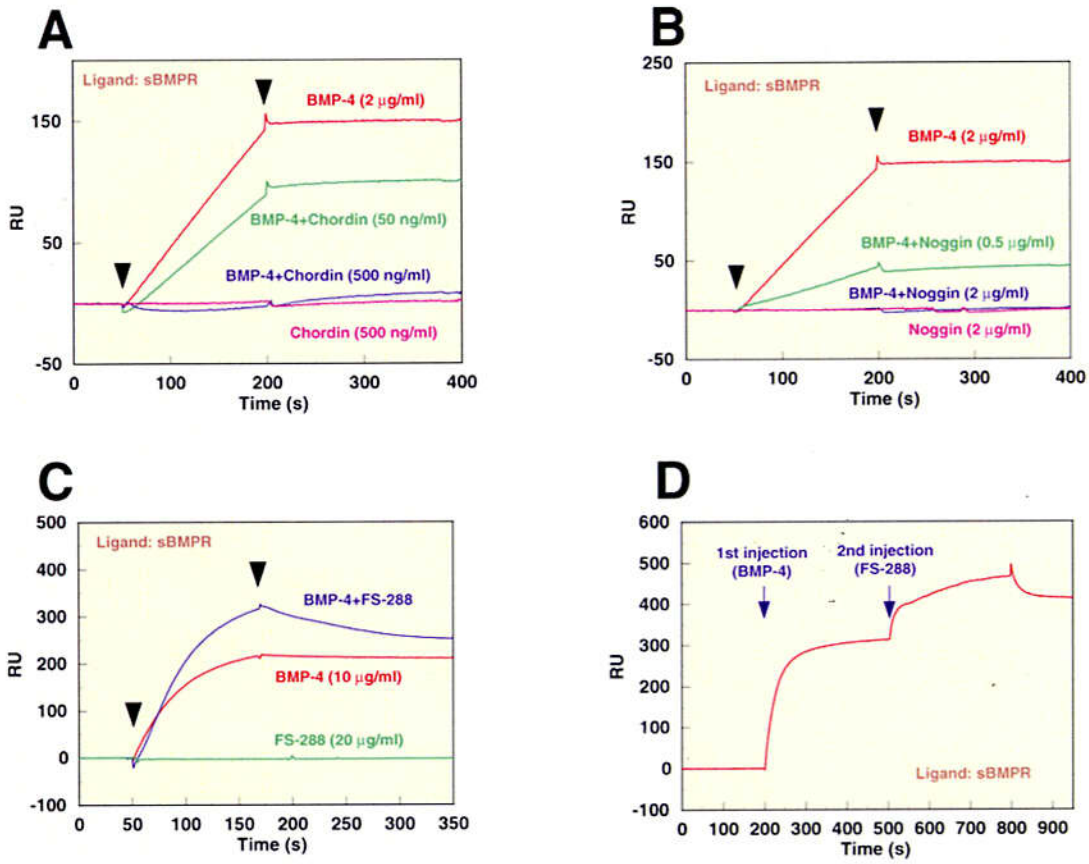

Figure 3-6. Different competitive mechanisms of three BMP-4 antagonists.

(A) Mixture of BMP-4 (2 μg/ml) with chordin (50 or 500 ng/ml) was incubated for 30 min, and injected over the surface immobilized sBMPR (the immobilization level was 1530 RU). (B) As described above, mixture of BMP-4 (2 μg/ml) with noggin (0.5 or 2 μg/ml) was incubated, and injected over the sBMPR (1530 RU) surface. (C) Mixture of BMP-4 (10 μg/ml) with follistatin (20 μg/ml) was incubated, and injected over the sBMPR (684 RU) surface. (D) FS-288 was injected after the injection of BMP-4 over the sBMPR-immobilized (684 RU) surface by using of the coinjection method. The concentrations of BMP-4 and FS-288 were 10 μg/ml and 5 μg/ml, respectively. Both proteins were injected at a flow rate of 10 μl/min for 300 s.

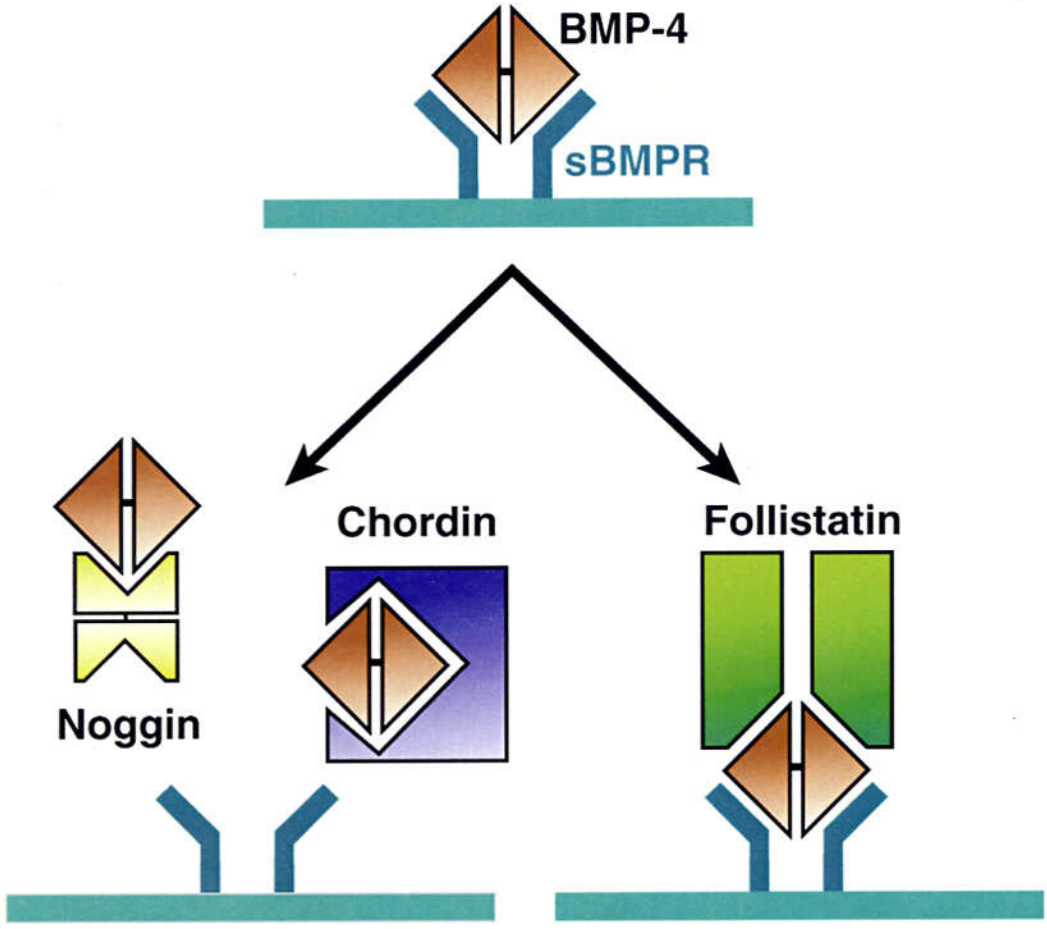

Figure 3-7. Possible inhibitory mechanisms of the BMP action by follistatin, chordin and noggin.

Chordin and noggin inhibit the binding of BMP-4 to its type I receptor (sBMPR). On the other hand, follistatin can bind to sBMPR through BMP-4, forming a trimeric complex.

DISCUSSION

One of the major roles of embryonic BMPs in *Xenopus* is to cause the ventralization of the mesoderm during gastrulation (Dale et al., 1992; Jones et al., 1992). All three BMP subtypes identified so far, namely BMP-2, -4, and -7 (Nishimatsu et al., 1992) cause a ventralized embryo that lacks the anterior head structure and notochord when they are dorsally overexpressed by mRNA microinjection. Therefore, BMP activity is easily examined by microinjecting mRNA, and the potency can be evaluated by observing the extent of ventralization (Suzuki et al., 1997).

One intriguing observation in above experiments (Fig. 3-2) showing the antagonism between BMPs and follistatin is related to the specificity of follistatin to BMP subtypes. In dorsal blastomeres, BMP-7 mRNA is inhibited by the overexpression of follistatin more efficiently than BMP-4 mRNA, suggesting that follistatin may have a higher affinity for BMP-7 than BMP-4. However, this is unlikely because, first, in ventral blastomeres and animal cap cells, BMP-4 mRNA appeared to be more efficient in blocking follistatin and, second, BMP subunits tend to form heterodimers (Hazama et al., 1995), thus it can not be assumed that follistatin blocked BMP-7 homodimers. Namely, it is very likely that exogenously introduced BMP mRNA contributed to the formation of heterodimers with related BMP subunits translated by endogenous mRNAs. Supporting this possibility, a cleavage mutant of BMP-7 inhibits the processing of, not only BMP-7, but also BMP-4, presumably by heterodimerization (Hawley et al., 1995). It is therefore possible that follistatin efficiently blocked BMP-2/7 or BMP-4/7 heterodimer whose ventral mesoderm-inducing activity is significantly higher than that of BMP homodimers (Suzuki et al., 1997). This suggestion is consistent with the observation that follistatin binds to BMP-4/7 heterodimer more efficiently than to homodimers. Alternatively, the amount of BMP-4/7 heterodimer generated by BMP-7 mRNA injection may be rather low in dorsal blastomeres because the amount of BMP-4 mRNA is limited, particularly on the dorsal side of the gastrula

embryo (Hemmati-Brivanlou and Thomsen, 1995). This may account for the observation that a smaller amount of follistatin mRNA could overcome the ventralizing activity and rescue the ventralized phenotype caused by BMP-7 mRNA injection, while higher doses were required to rescue the phenotype caused by BMP-4 mRNA injection. In the latter case, the injection of BMP-4 mRNA may lead to the efficient formation of heterodimer with BMP-7, whose transcript is uniformly distributed and abundant in early embryos (Suzuki et al., 1997). In ventral blastomeres, on the other hand, BMP-4 mRNA injection may result in the formation of BMP-4/7 heterodimer which efficiently cancels the antagonistic effect of follistatin, while BMP-7 mRNA injection generates mostly BMP-7 homodimers and only trace amounts of BMP-4/7 heterodimer, both of which are less effective at rescuing embryos dorsalized by follistatin.

Next, I confirmed that this interaction between BMP and follistatin occurs extracellularly (Fig. 3-3), because there remains a possibility that follistatin interacts with the BMP signaling pathway intracellularly, acting through a specific membrane receptor that has not yet been identified, although it is generally accepted that follistatin binds activin extracellularly. At least three type I receptors, which are ALK2, ALK3, and ALK6, have so far been shown to mediate BMP signaling. Using two constitutively activated BMP type I receptors (CA-ALK2 and CA-ALK3), it was revealed that follistatin can not inhibit the ligand-independent BMP signaling. Therefore, interaction between BMP and follistatin was thought to take place extracellularly.

In the SPR biosensor studies, since BMP-4 is immobilized on the sensor surface by the amine coupling method that may lead to random immobilization, I determined these values of rate constants from the sensorgrams at the low concentrations of FS-288 to obtain more reliable values (Schuck, 1997). Although the association and dissociation rate constants are considered to be semi-quantitative, these data are thought to be valuable in the biological context. To give confidence in these results obtained from the biosensor, firstly, immunoprecipitation assays were performed. However, I failed to show the direct interaction, which is most likely to be due to the

fast dissociation rate of BMP-follistatin complex. Namely, during the process of washing, BMP-follistatin complex is likely to be dissociated. This led me to carry out the chemical cross-linking. As expected, by using this method, the formation of the complex between BMP-4 and follistatin was confirmed.

In this chapter, I proposed the mechanism by which follistatin inhibits BMP activity. As another feasible inhibition mechanism, follistatin bound to BMP might be captured by extracellular matrix (ECM). It was previously shown that follistatin (FS-288) bound to activin was captured by heparan sulfate proteoglycans on the cell surface, and that cell-surface associated follistatin inhibits the activin-receptor binding (de Winter et al., 1996; Sugino et al., 1993). However, because the amount of FS-288 protein (high affinity for ECM) in embryos is not clarified, and a complex with BMP, follistatin, and ECM is not confirmed as yet, this possibility remains to be further investigated.

The present study demonstrated that follistatin can inhibit the effects of all three BMP subunits: BMP-2, -4, and -7. Follistatin was previously shown to inhibit the growth inhibitory effect of OP-1 (BMP-7 homodimer) on Mv1Lu cells (Yamashita et al., 1995) at high concentrations. This report is the first to reveal the negative effect of follistatin on BMP family proteins by direct binding. Although in a manner different from the other known organizer factors, noggin and chordin, the restricted expression of follistatin mRNA in the Spemann's organizer and its ability to block BMPs by binding to them support the idea that follistatin is also likely to act as an organizer factor in *Xenopus* embryogenesis. However, *in vivo* function remains controversial. I will discuss this issue in the next chapter.

CHAPTER 4

ISOLATION AND CHARACTERIZATION OF BONE MORPHOGENETIC PROTEIN-BINDING PROTEINS FROM THE EARLY *XENOPUS* EMBRYOS

SUMMARY

Using a SPR biosensor as a sensitive and specific monitor, I have isolated two distinct BMP-binding proteins, and identified them as lipovitellin 1 and Ep45, respectively. Lipovitellin 1 is an egg yolk protein that is processed from vitellogenin. Both vitellogenin and Ep45 are synthesized under estrogen control in the liver, secreted, and taken up by developing oocytes. In this chapter, I have shown that, of the TGF-β family members tested, Ep45 can bind only to BMP-4, while lipovitellin 1 can bind to both BMP-4 and activin A. Because of this difference in specificity, I have focused on and further studied Ep45. Kinetic parameters were determined by SPR studies, and showed that Ep45 associated rapidly with BMP-4 (k_a=1.06×10^4 M^{-1}s^{-1}) and dissociated slowly (k_d=1.6×10^{-4} s^{-1}). In *Xenopus* embryos microinjected with Ep45 mRNA, Ep45 blocked the ability of follistatin to inhibit BMP activity and to induce a secondary body axis in a dose-dependent manner, while it had no effect on the other BMP antagonists, chordin and noggin. These results support the possibility that Ep45 interacts with BMP to modulate its activities *in vivo*.

INTRODUCTION

BMPs are thought to play essential roles in the dorsoventral patterning of the embryo as mentioned in chapter 1. It is generally accepted that these important biological activities are negatively regulated by inhibitors of the BMPs (Graff, 1997; Thomsen, 1997). It has been demonstrated that so-called organizer factors, such as noggin, chordin, and follistatin, can bind to BMP-4 directly, and that negatively regulate BMP-4 activity (Piccolo et al., 1996; Zimmerman et al., 1996) (chapter 3). In addition to these organizer factors, gremlin, cerberus, and DAN, which belong to the DAN family, have been found to block BMP signaling by direct binding (chapter 1). In this way, BMP activities are likely to be regulated throughout development by various BMP-antagonists with different affinities and binding specificities.

In this chapter, I screened *Xenopus* embryo extracts to identify novel BMP-4-binding molecules that may regulate the multipotent BMP activity in development. To do the screening, the SPR biosensor was used. Because of its high sensitivity for monitoring real time protein-protein interactions (Lackmann et al., 1996), this technique replaced the conventional biological and immunochemical assays. By combined with liquid chromatography, two BMP-binding proteins are purified. They are identified as lipovitellin 1 and Ep45, using their N-terminal amino acid sequences and biochemical analyses. Lipovitellin 1, which is processed from vitellogenin, is a 120-kDa yolk platelet protein (Berridge and Lane, 1976; Gerber-Huber et al., 1987). The precursor protein, vitellogenin (210 kDa), is synthesized under estrogen control in the liver, transported to the ovary, and processed there to the yolk proteins lipovitellin and phosvitin (Berridge and Lane, 1976; Clemens, 1974). Ep45 (45 kDa) is also synthesized under estrogen control in the liver, and released into the blood stream (Holland et al., 1992; Holland and Wangh, 1987). The amino acid sequence indicates that Ep45 is a secreted glycoprotein, and a member of the serine protease inhibitor (serpin) superfamily. Moreover, Ep45 is a Ni^{2+} binding protein, and is implicated in Ni^{2+}-induced teratogenesis (Beck et al., 1992; Sunderman, 1993).

Because of previous report that vitellogenin binds to both activin and BMP[4], subsequent functional analyses were performed for Ep45. Firstly, the binding specificity of Ep45 was analyzed. Because Ep45 has no effect on the early *Xenopus* development and on the binding of BMP-4 to sBMPR in the SPR analysis, secondly, the effects of Ep45 on the binding of BMP-4 to organizer factors, noggin, chordin, and follistatin were analyzed. These results suggested that Ep45 blocked the binding of BMP-4 to follistatin specifically.

[4] Nakamura, T. personal communication.

EXPERIMENTAL PROCEDURES

Measurements and analysis by SPR biosensor

All measurements of binding activity in each purification step, binding experiments, and kinetic analysis were performed using the BIACORE2000 (Biacore AB). Samples were injected over the surfaces of sensor chips at a flow rate of 20 μl/min at 25 °C for 150 s. For the controls, all samples were simultaneously injected over mock-coupled sensor chip surfaces containing no protein with each experimental run. All curves were corrected for background by subtracting the blank run, using BIAevaluation software version 3.0 (Biacore AB). The BMP-binding activity in resonance unit (RU) was determined 30 s after sample injection. BMP-4, activin A, TGF-β1, and FS-288 were immobilized on the sensor chip surface (CM5, certified grade, Biacore AB) by the amine coupling method. Biotinylated sBMPR was immobilized on a sensor chip surface (SA5, research grade, Biacore AB) as described in chapter 3.

To determine kinetic parameters, 503 RU of BMP-4 was immobilized on the sensor chip surface (CM5) by the amine coupling method. The flow rate was 20 μl/min at 25 °C for an injection time of 150 s. The bound Ep45 was then allowed to dissociate for 120 s.

Recombinant proteins

xBMP-4 and sBMPR were purified as described in chapter 2. Human follistatin (FS-288), human activin A, *Xenopus* chordin, human TGF-β1, and *Xenopus* noggin were obtained as described in chapters 2 and 3.

Purification of BMP-binding proteins from *Xenopus* embryo

Extract (20 ml) from *Xenopus* embryos (approximately 8×10^3) at stage 8-10 was prepared as previously described (Ueno et al., 1992). It was dialyzed against 25 mM sodium acetate buffer, pH 5.5 at 4 °C, and then applied to a POROS SP/H cation ion-

exchange column (Perseptive Biosystems, Framingham). The column was eluted by a NaCl concentration gradient (0-0.5 M) and the BMP-binding activity of each fraction (10 ml) was monitored by the biosensor. The major peak (3.3 min) of BMP-binding activity was pooled, then separated on a Superose 12 gel filtration column (Pharmacia Biotech AB) preequilibrated with HBS. The three major protein peaks were pooled, acidified to pH 3.0 with 1.0 N acetic acid, and separated on a mRPC C2/C18 reverse phase column (Pharmacia Biotech AB) preequilibrated with 0.1% trifluoroacetic acid (TFA). The fractions containing the main peak of BMP-binding activity were pooled. For purification of Ep45 under neutral conditions, the Ep45-enriched fraction separated by Superose 12 was applied to NTA (nitrilo-tri-acetic acid)-agarose charged with Ni^{2+} (Qiagen, Valencia), preequlibrated with 20 mM Tris-HCl, pH 7.0, 5 mM $CaCl_2$ (Beck et al., 1992). Ep45 was eluted with an imidazole gradient from 0 to 0.2 M. The fractions of each chromatography step were tested for Ep45 by SDS-PAGE and using the biosensor. Protein concentration was determined using the BCA protein assay reagent kit (Pierce) according to the manufacturer's instructions, except for fractions from the µRPC C2/C18 column, for which it was determined by comparison with the absorbance (peak area) of a standard protein (BSA, Sigma, St. Louis).

Amino acid sequence analysis and identification of Ep45 and vitellogenin

N-terminal amino acid sequence analysis was performed on a Hewlett-Packard protein sequencer operated with the routine 3.1 sequencer program. Ep45 (30 pmole) and lipovitellin 1 (10 pmole) purified by the µRPC C2/C18 column were sequenced. *Xenopus* Ep45 and vitellogenin A2 were identified in a BLAST search of the SWISS-PROT data base (Altschul et al., 1990).

Embryo manipulations

Embryo manipulations were performed as described in chapter 3.

Assay of serine protease inhibitor activity

A reaction mixture of Ep45 (5 μg/ml), BMP-4 (2 μg/ml), and α-chymotrypsin (5 μg/ml; Sigma) in 0.1 M Tris-HCl, pH 8.5, 5 mM $CaCl_2$, 20 μg/ml BSA was incubated for 60 min at 37 °C (Beck et al., 1992). Samples were subjected to electrophoresis on a 15% gel and electroblotted onto PVDF membranes (Millipore, Bedford) as described in chapter 2. The membrane was blocked, incubated with the BMP-4 antibody (Ab97) at 4 °C overnight (Nishimatsu et al., 1993), and then reacted with a secondary antibody for 1 h at room temperature. Western blots were developed using the chemiluminescent ECL plus kit (Amersham, Buckinghamshire) according to the manufacturer's instructions.

RESULTS

Isolation of BMP-binding proteins from *Xenopus* embryos

Two BMP-binding proteins from *Xenopus* embryos were isolated using the BIAcore biosensor as a detection system. Both Ep45 and lipovitellin 1 were purified by 3-step column chromatography (Fig. 4-1). After the embryos extract was dialyzed against chromatography buffer, it was first purified by POROS SP/H cation ion-exchange chromatography (Fig. 4-1A). Although the column was eluted by a NaCl concentration gradient (0-0.5 M), the highest activity was eluted with concentrations above 0.5 M NaCl. These last peak fractions with BMP-binding activity were eluted at 3.3 min and were pooled (10 ml). The samples were next separated by Superose 12 gel-filtration chromatography (Fig. 4-1B). Three major protein peaks (20 min, Vo; 30 min, A; 35 min, B) were obtained and the fractions containing each peak were pooled. Each peak was further separated by μRPC C2/C18 reverse-phase chromatography. After this chromatography, the BMP-binding activity of the first peak (Vo) in Superose 12 was lost (data not shown) while both pools A and B gave single peaks with significant BMP-binding activities (Fig. 4-1C and D, asterisks). Each peak was collected and analyzed by SDS-PAGE (Fig. 4-2A, B). A 45-kDa protein from pool B and a 120-kDa protein from pool A were detected. This result also shows that the proteins were purified to near homogeneity. The isolated proteins yielded specific binding responses to BMP-4 of 2.0×10^6 and 2.2×10^6 RU/mg, respectively (Table 4-1). Approximately 5.7% (45-kDa protein) and 5.2% (120-kDa protein) of the total BMP-binding activity in the starting material (extract) were recovered as 6 and 5 μg pure protein, respectively.

Amino acid sequence analysis of the 45-kDa and 120-kDa proteins

The N-terminal amino acid sequences of the purified 45-kDa and 120-kDa proteins were analyzed using an automated liquid-phase sequencer, and 20 and 29 amino acid residues, respectively, were determined (Fig. 4-2C, D). A BLAST search

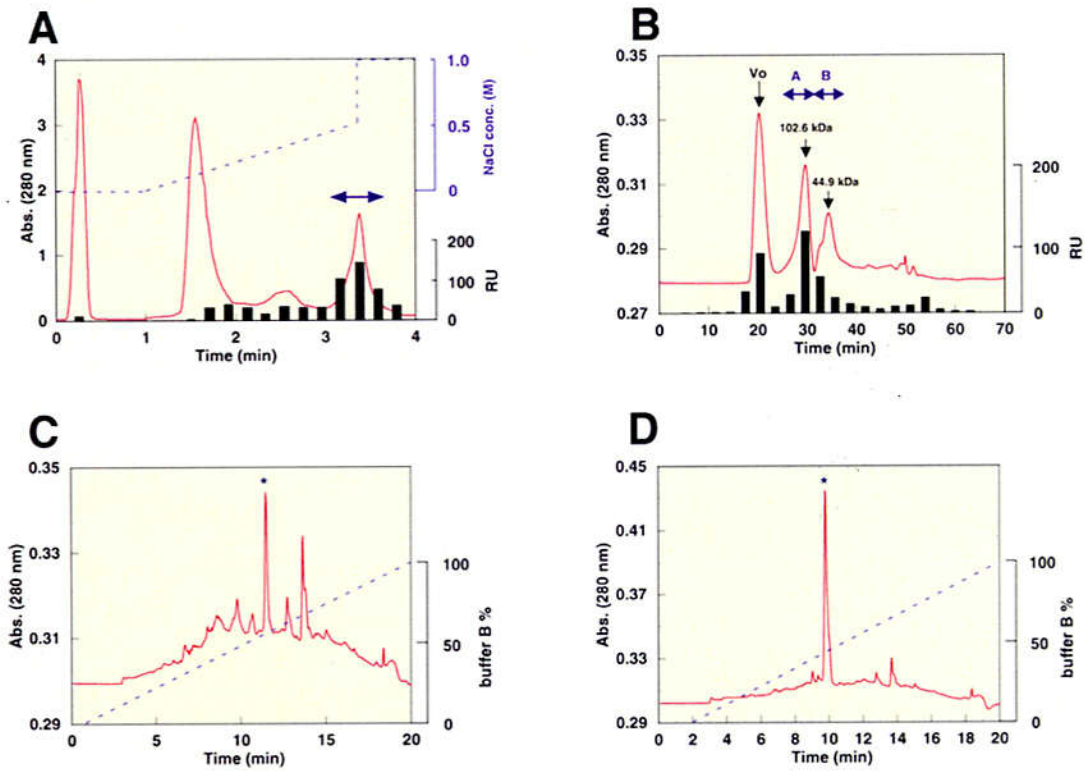

Figure 4-1. Sequential chromatographic purification of BMP-binding proteins.

(A) POROS SP/H chromatography. (B) Superose 12 chromatography. Vo: void volume. (C) (D) μRPC C2/C18 chromatography. Pools A and B from (B) were separated (C and D, respectively). Each BMP-binding activity was eluted in a sharp peak (asterisks). Buffer B was 80% CH_3CN in 0.1% TFA. Bar chart indicates the BMP-binding activity in each fraction as described in Experimental Procedures.

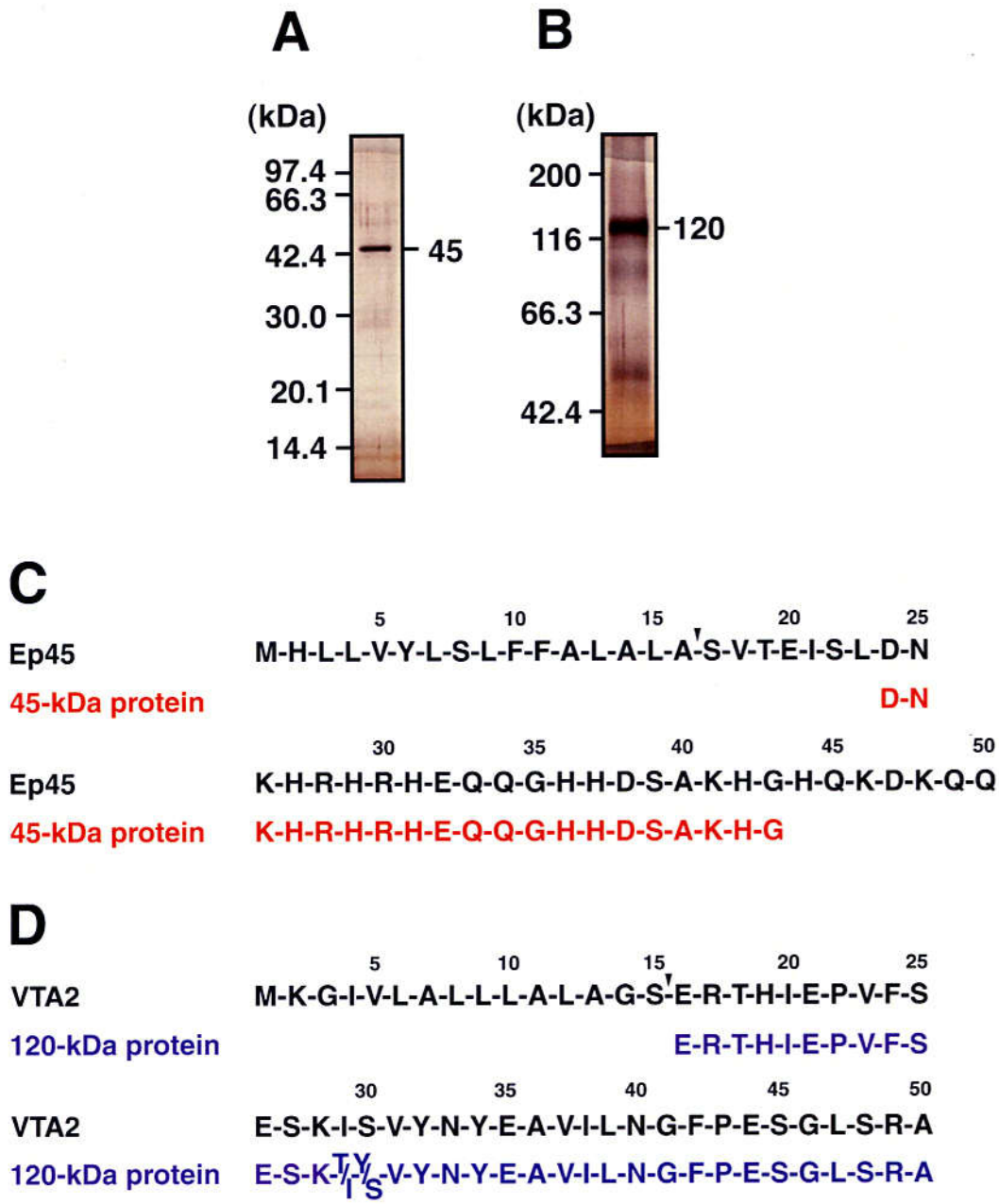

Figure 4-2. SDS-PAGE and amino acid sequence analyses of BMP-binding proteins.

(A) (B) Active fractions separated by reverse phase chromatography were analyzed by SDS-PAGE with silver staining. (A) Aliquots of the fraction containing the activity from Fig.4-1D were subjected to electrophoresis on 12.5% SDS gels under reducing conditions. (B) Aliquots of the fraction containing the activity from Fig.4-1C were subjected to electrophoresis on 7.5% SDS gels under reducing conditions. (C) The amino acid sequence of the 45-kDa protein corresponded exactly to the amino acid sequence of Ep45. The arrowhead after A-16 indicates the predicted position of cleavage of the signal peptide. The 45-kDa protein obtained lacked 7 amino acid residues compared with full-length, mature Ep45. (D) The amino acid sequence of the 120-kDa protein corresponded to the amino acid sequence of vitellogenin A2 precursor (VTA2).

Table 4-1. Purification of BMP-binding proteins

Purification step	Total protein [a] (mg)	Total response [b] (RU)	Specific response (RU/mg)	Yield (%)	Purification (-fold)
Extract	12	$2.1X10^5$	$1.8X10^4$	100	
POROS SP/H	1.2	$6.4X10^4$	$5.3X10^4$	30.5	2.9
Superose 12 (Pool A)	0.02	$1.3X10^4$	$6.5X10^5$	6.2	36.1
μRPC C2/C18	0.005	$1.1X10^4$	$2.2X10^6$	5.2	120
Superose 12 (Pool B)	0.06	$1.4X10^4$	$3.0X10^5$	6.7	16.7
μRPC C2/C18	0.006	$1.2X10^4$	$2.0X10^6$	5.7	110

a. Protein was measured as described in "Experimental Procedures."

b. Response was expressed as RU at 30 sec. after sample injection.

showed that the 45-kDa protein was Ep45, and that the 120-kDa protein was vitellogenin A2 precursor. Comparison of the amino acid sequence of purified 45-kDa protein with a previously reported sequence predicted from Ep45 cDNA (Holland et al., 1992) showed that the 45-kDa protein lacked only 7 N-terminal amino acid residues of full length, mature Ep45 (Fig. 4-2C). Despite the correspondence of the N-terminal amino acid sequence, the molecular weight of the 120-kDa protein was not consistent with that of vitellogenin A2 precursor (210 kDa). Given the molecular weight of this protein, it is most likely to be lipovitellin 1, which is a cleavage product of vitellogenin (Berridge and Lane, 1976).

The binding specificity of Ep45 and lipovitellin 1 to TGF-β family ligands

The ability of Ep45 and lipovitellin 1 to bind known TGF-β family proteins was tested using the BIAcore biosensor (Fig. 4-3). Purified activin A, TGF-β1, or BMP-4 was immobilized on a sensor chip surface, and then Ep45 (5 μg/ml) or lipovitellin 1 (3 μg/ml) was injected to flow over the sensor chips as an analyte. As lipovitellin 1 flowed over immobilized BMP-4, a rising slope of resonance signal was observed, indicating binding was evident, and after injection, the resonance signal decreased slowly. Moreover, this change in resonance signal was also detectable when lipovitellin 1 flowed over activin A immobilized on a sensor chip surface (Fig. 4-3B). However, no change in resonance signal was detectable on the TGF-β1-immobilized surface. In contrast to lipovitellin 1, Ep45 showed a detectable signal only when it was injected to flow over the BMP-4-immobilized sensor chip surface, but not over either activin A or TGF-β1 (Fig. 4-3A). Because Ep45 can interact only with BMP-4 among the TGF-β family ligands tested, and its role in development is poorly understood, I decided to focus on Ep45 and to exclude lipovitellin 1 from further consideration.

To determine kinetic parameters, Ep45 was injected at increasing concentrations and flowed over BMP-4 immobilized on a sensor chip (95-1,500 nM). The sensorgrams obtained were analyzed using the BIAevaluation 3.0 program. The association rate constant, k_a, was calculated to be 1.06×10^4 $M^{-1}s^{-1}$, and the dissociation

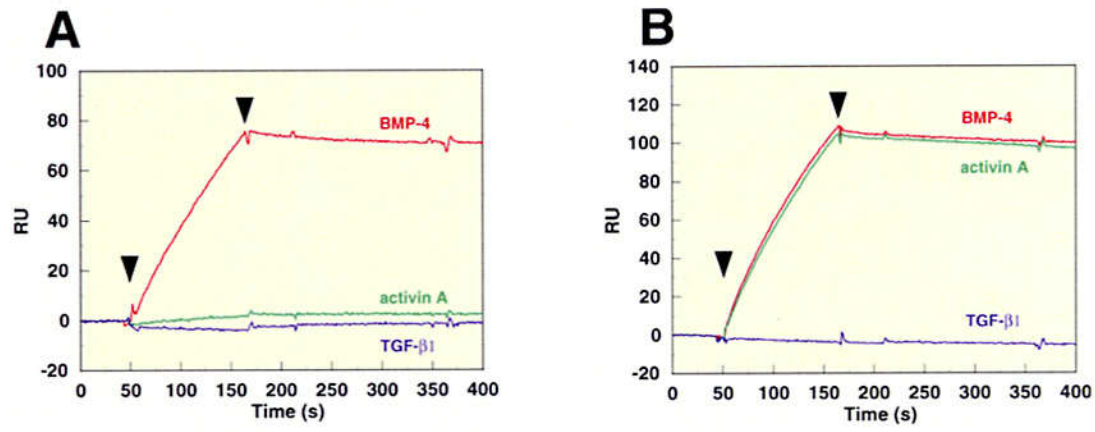

Figure 4-3. Binding specificity of Ep45 and lipovitellin 1.

BMP-4, activin A, and TGF-β1 were immobilized on the sensor chip surface. The amounts of immobilized BMP-4, activin A, and TGF-β1 were 2650, 2614, and 2409 RU, respectively. (A) Ep45 (5 μg/ml) was injected to flow over the sensor chips as an analyte. (B) lipovitellin 1 (3 μg/ml) was injected to flow over the surfaces. Arrowheads represent the initiation and termination of injections.

rate constant, k_d, was calculated to be 1.6×10^{-4} s^{-1}, which is slow. From these two rate constants (k_d/k_a), the apparent equilibrium dissociation constant (K_D) was calculated to be 15.1 nM.

Ep45 blocks the activity of follistatin

To clarify the biological function of Ep45 in binding to BMP-4 directly, Ep45 mRNA was microinjected into *Xenopus* embryos. I expected, based on previous studies (chapter 3) (Hsu et al., 1998; Piccolo et al., 1996; Zimmerman et al., 1996), that if Ep45 inhibits endogenous BMPs through direct binding, it might cause dorsalization of mesoderm and lead to secondary axis formation. Although 100-2,000 pg of Ep45 mRNA was injected into the two dorsal or ventral blastomeres of 4-cell embryos, it had no effect on the embryonic phenotype or on the expression of marker genes (data not shown). This result suggested that Ep45 does not affect BMP-BMP receptor interaction. To confirm this, Ep45 was tested whether it blocks the binding of BMP-4 to its receptor using the BIACORE biosensor. sBMPR was immobilized on a BIACORE sensor chip (422 RU), then BMP-4 (1 μg/ml) was injected to flow over the chip. As shown in Fig. 4-4, Ep45 did not influence the binding of BMP-4 to its type I receptor even at the higher concentrations, which is consistent with the fact that Ep45 did not induce dorsal mesoderm by inhibiting endogenous BMP-4 in embryos.

Next, I attempted to test whether Ep45 could influence the interaction between BMP-4 and the BMP-binding proteins, chordin, noggin, or follistatin. Chordin, noggin or follistatin mRNA was coinjected with Ep45 mRNA into the two ventral blastomeres of 4-cell embryos. These three factors independently induce dorsal mesoderm and a secondary body axis in embryos when they are ventrally overexpressed (Sasai et al., 1995; Sasai et al., 1994) (chapter 3) (Fig. 4-5Ab, e, h). Coinjection of Ep45 and chordin, or noggin did not inhibit the dorsalizing effect of these proteins (Fig. 4-5Ac and d, or f and g). In contrast, Ep45 did inhibit the dorsalizing signal of follistatin in a dose-dependent manner (Fig. 4-5Ai and j). To confirm that this result was due to the direct interference of Ep45 with the BMP-4-follistatin interaction, a competition assay

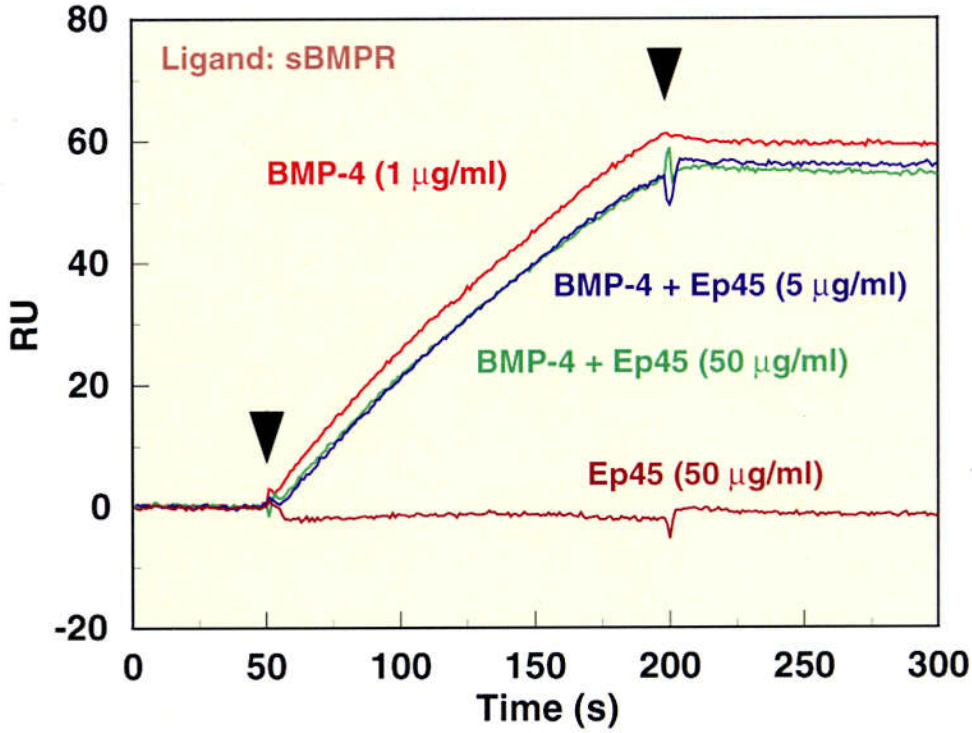

Figure 4-4. No effect of Ep45 on the binding of BMP-4 to sBMPR.

sBMPR (422 RU) was immobilized on the sensor chip surface. BMP-4 (1 μg/ml) and Ep45 (5 or 50 μg/ml) were incubated for 30 min at room temperature, then injected to flow over the surface. Arrowheads represent the initiation and termination of injections.

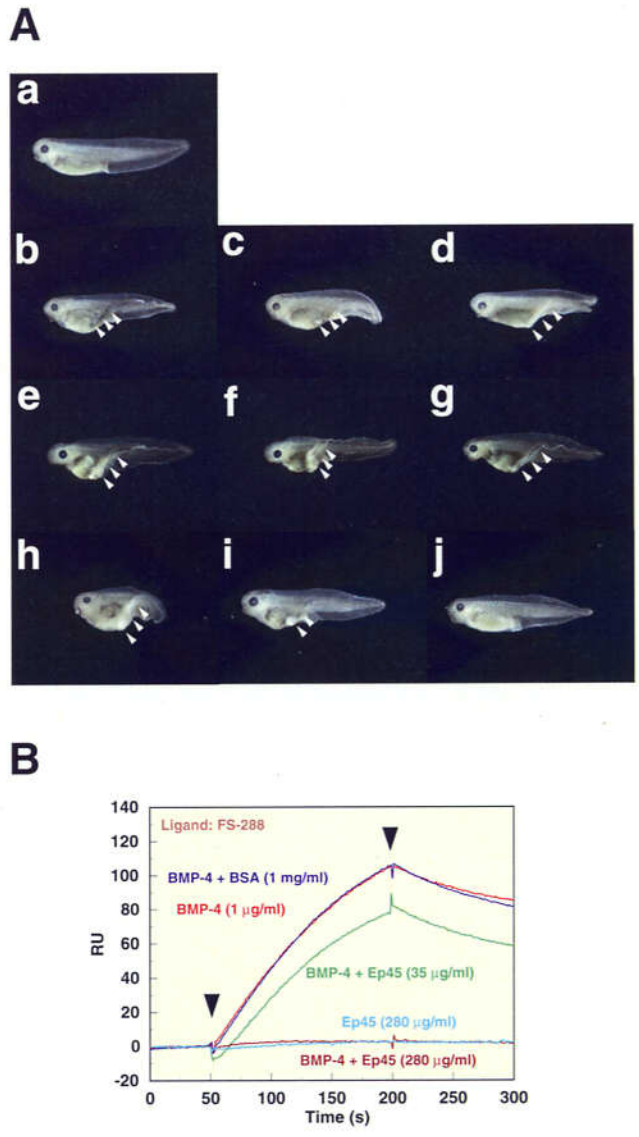

Figure 4-5. Inhibitory effect of Ep45 on the binding of BMP-4 to follistatin.

(A) Phenotypes of *Xenopus* embryos ventrally coinjected with Ep45 mRNA and chordin, noggin, or follistatin mRNA. 500 pg of chordin mRNA (b), 1 pg of noggin mRNA (e), or 50 pg of follistatin mRNA (h) was injected into the equatorial region of the two ventral blastomeres at the four-cell stage as previously described (Jones et al., 1992). 500 pg of chordin mRNA was coinjected with 1 ng (c), or 2 ng (d) of Ep45 mRNA. 1 pg of noggin mRNA was coinjected with 500 pg (f), or 2 ng (g) of Ep45 mRNA. In contrast, 50 pg follistatin mRNA was coinjected with 500 pg (i), or 2 ng (j) of Ep45 mRNA. (a) Uninjected embryo. Embryos were evaluated and photographed at the tadpole stage. Arrowheads indicate a secondary axis. (B) Dose-dependent inhibition of the binding of BMP-4 to follistatin by Ep45. FS-288 was immobilized on the sensor chip surface (1432 RU). BMP-4 (1 μg/ml) was injected to flow over the sensor chip as an analyte. After incubating for 30 min at room temperature, BMP-4 (1 μg/ml) was coinjected with 1 mg/ml of BSA, with 35 μg/ml of Ep45, or with 280 μg/ml of Ep45. 280 μg/ml of Ep45 alone was injected to over the surface. Arrowheads represent the initiation and termination of injections.

was carried out using the biosensor. As shown in Fig. 4-5B, Ep45 competed with the binding of BMP-4 to FS-288 immobilized on the sensor chip in a dose-dependent manner, suggesting that Ep45 inhibits BMP-4-follistatin interaction by direct binding to BMP-4.

DISCUSSION

It is broadly accepted that many proteins such as enzymes, polypeptide growth factors, and transcription factors are negatively regulated by their specific binding proteins (Perrimon and McMahon, 1999). This emphasizes the importance of screening for binding proteins for biologically active substances. BIACORE technology is useful and rapidly expanding in a variety of scientific fields, not only for kinetic analyses of protein-protein interactions but also for screening for novel binding molecules. Using this new screening technique, I have searched for and isolated two BMP-binding proteins, lipovitellin 1 and Ep45. The purification of these proteins was performed by using 3-step chromatography (Fig. 4-1 and Table 4-1). The relative amounts of Ep45 and lipovitellin 1 proteins were estimated to be 0.05% (6 μg) and 0.04% (5 μg) of total protein, respectively, and both proteins appeared to be abundant in the *Xenopus* embryo. In this experiment, I did not isolate the other known BMP-binding proteins such as noggin and chordin, which bind to BMP with high affinities, probably because the relative amounts of noggin, chordin, and the other binding proteins may be far lower than those of Ep45 and lipovitellin 1. If the side fractions of each chromatography step were further analyzed, it might be possible to purify such less-abundant proteins.

Lipovitellin 1 is known to be generated from vitellogenin in the egg yolk (Berridge and Lane, 1976). Its precursor, vitellogenin, binds both activin and BMP[5]. The SPR data also suggests that at least the lipovitellin 1 region of the vitellogenin protein binds to BMP and activin. Although the potential regulatory relationship between lipovitellin 1 (or vitellogenin) and BMP-4 is poorly understood in embryos, I decided to exclude it from further analyses because vitellogenin has already been shown to bind to both BMP and activin, and its binding specificity is lower than that of Ep45.

In this study, I did not find that Ep45 affected the binding of BMP-4 to its type I receptor (Fig. 4-4), despite its direct binding to BMP-4. Ep45 neither inhibited the

[5] Nakamura, T. personal communication.

binding of BMP to type I receptor as chordin or noggin does nor affected the BMP-BMP receptor binding profile as does follistatin (chapter 2). These results are consistent with the results obtained from *in vivo* experiments by microinjection of Ep45 mRNA, where ventrally overexpressed Ep45 mRNA did not change the fate of the ventral region of the embryo (data not shown). These findings suggest that Ep45 does not inhibit the function of BMP-4 in the *Xenopus* embryo. Rather, I found that in *Xenopus* Ep45 inhibited the antagonistic interaction of follistatin with BMP-4 but not of chordin or noggin. Subsequent BIACORE analysis further confirmed that Ep45 interfered with the direct interaction between BMP-4 and follistatin. One reason for this difference may be that follistatin, chordin, and noggin have different binding affinities. Follistatin binds to BMP-4 with lower affinity (K_D=23 nM) than chordin (K_D=0.3 nM) and noggin (K_D=19 pM) (chapter 3) (Piccolo et al., 1996; Zimmerman et al., 1996), while Ep45 binds to BMP-4 with higher affinity (K_D=15.1 nM) than follistatin. Although the kinetic data of chordin or noggin for BMP-4 was obtained by different methods, this tendency is thought to be correct because chordin or noggin can be coprecipitated with BMP-4, but follistatin can not (Piccolo et al., 1996; Zimmerman et al., 1996) (chapter 3). On the basis of these observations, it is assumed that Ep45 does not interfere with the high affinity binding of BMP to BMP-binding proteins. Since DAN-family member proteins bind to BMPs with high affinities and are coprecipitated with BMPs (Hsu et al., 1998; Piccolo et al., 1999), Ep45 probably has no effect on these interactions. These findings led me to the conclusion that Ep45 is a specific inhibitor of BMP-follistatin binding.

Now this specificity of Ep45 to inhibit follistatin activity against BMP raises a question about the *in vivo* role of follistatin in the early development of *Xenopus* embyos. Although the expression of Ep45 protein in the organizer region has so far not been ascertained, it has been shown that Ep45 mRNA expression is broad, spatially (Fig. 4-6) and temporally (data not shown), and that Ep45 protein appears to be abundant in embryos, as described in this chapter. Therefore, in the presence of Ep45, it is most likely that follistatin cannot act as an organizer factor by binding to BMP. Furthermore, this explains why dorsal overexpression of Ep45 did not perturb normal

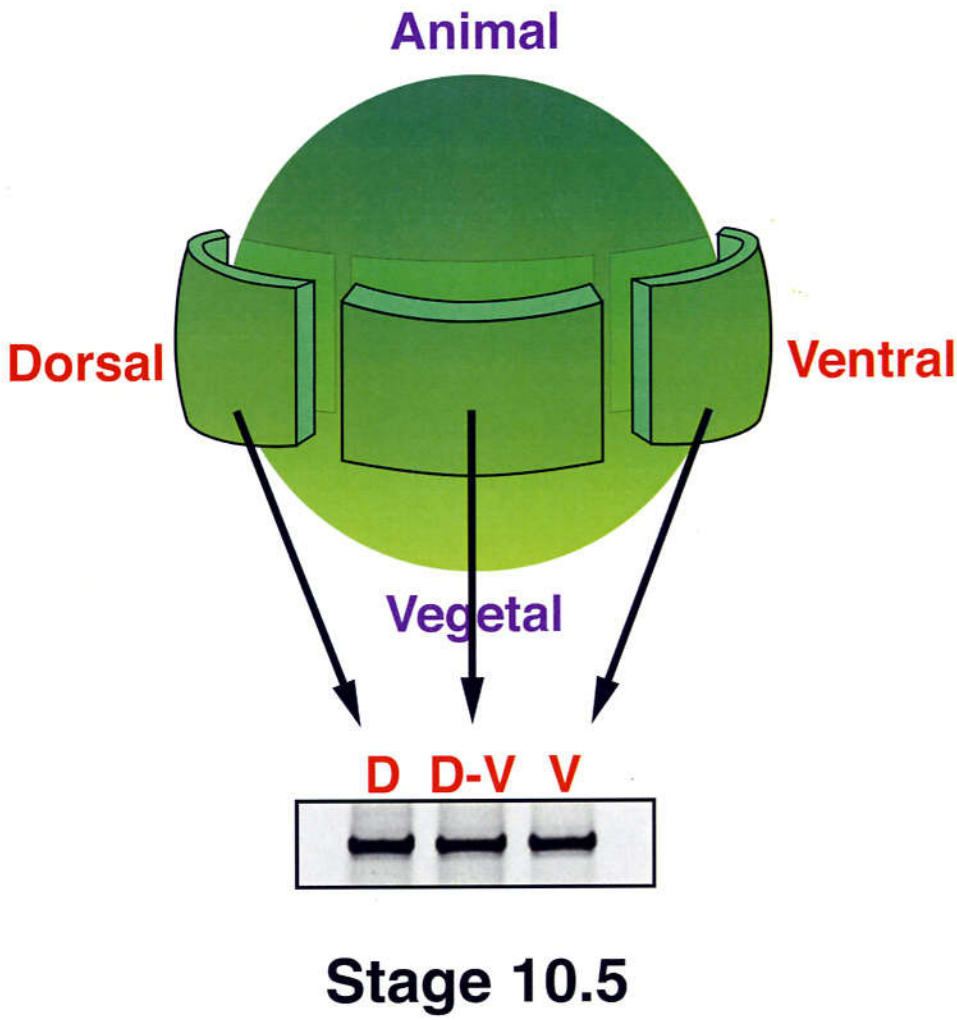

Figure 4-6. Expression of Ep45 mRNA

Ep45 mRNA was expressed broadly from dorsal to ventral marginal region at the early gastrula. Embryos at stage 10.5 were dissected into dorsal, dorsal-ventral, and ventral parts. RNA from each part was analyzed by RT-PCR.

neural development. It is presumed that neural induction occurred antagonizing BMP activity not by follistatin but by noggin and chordin that have redundant BMP-binding activities. In zebrafish, it has been shown that follistatin mRNA is expressed in anterior paraxial regions, but not in the organizer region, in spite of having the same dorsalizing properties as its *Xenopus* homologue (Bauer et al., 1998). In the mouse, also follistatin mRNA is not expressed in the node, the mouse equivalent of the Spemann's organizer, or the notochord (Albano et al., 1994; Feijen et al., 1994). Furthermore, follistatin knock out mice exhibit neither aberrant neural development nor defects in early dorsoventral patterning (Matzuk et al., 1995). Taken together, in different vertebrate species, it is assumed that follistatin probably functions as a BMP antagonist in later processes of development, but not in early patterning process.

Ep45 belongs to the serine protease inhibitor (serpin) superfamily (Holland et al., 1992). It may protect the BMP protein from some serine proteases by binding to BMP. Therefore, I tested whether Ep45 inhibits the activity of α-chymotrypsin (Fig. 4-7). BMP-4 was partially digested by α-chymotrypsin to yield lower molecular weight forms, as described in a previous study showing that a proteolytic cleavage with trypsin occurs at the N-terminus of BMP-2 (Koenig et al., 1994). These results indicate that both α-chymotrypsin and trypsin are capable of cleaving the N-terminus of BMP-4. Interestingly, the addition of Ep45 significantly blocked the limited proteolytic digestion of BMP-4 by α-chymotrypsin.

Both BMP-2 and -4 have a basic amino acid region in their N-terminus that binds heparin (Ruppert et al., 1996). These sites have been postulated to be important in the storage and stabilization of BMPs. Also, interactions of BMPs with ECM via heparin-binding sites are thought to be important during development for the establishment of morphogenetic gradients by limiting the free diffusion of BMPs. Ep45 may help regulate the ECM-binding behavior of BMPs. The role of the protease inhibitors and the relationship between BMPs and protease inhibitors in development remain to be further investigated.

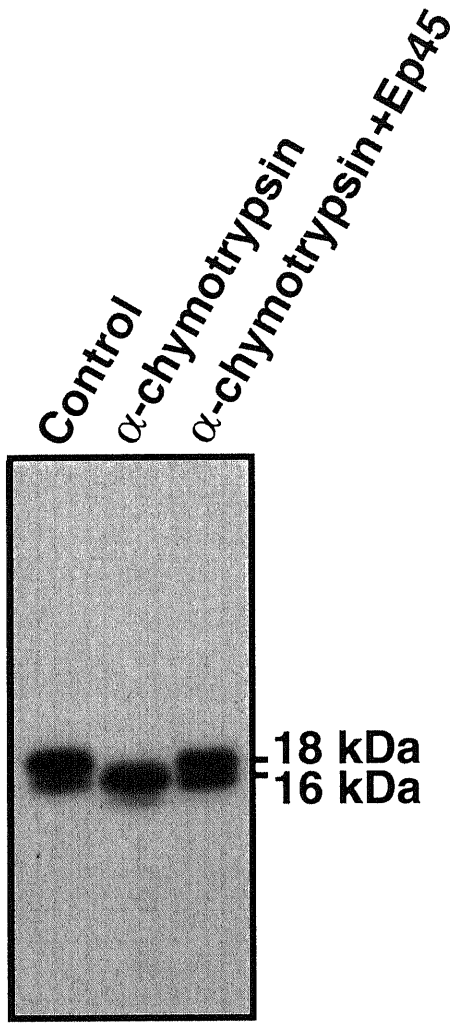

Figure 4-7. Inhibitory effect of Ep45 on bovine pancreatic α-chymotrypsin activity.
Activity of Ep45 as a serine protease inhibitor was tested by Western blot analysis as described under "Experimental Procedures."

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDIES

BMPs play a crucial role in development of vertebrates from early to late processes. Although signaling mechanisms of BMPs have been disclosed, it was not clear how BMPs activities are regulated. It is thought that such regulations occurs at several levels, e.g., of transcription, translation, protein maturation, and interaction with its receptor, and so on. In each case, biomolecular interactions are of basic importance (e.g. protein-protein, protein-nucleic acid, and protein-carbohydrate). In 1996, biochemical analyses demonstrated that noggin and chordin, which are so-called organizer factors, bind to BMP-4 directly to negatively regulate BMP's activities.

In this thesis, the BIACORE technology was proven to be valuable to analyze the protein-protein interactions. To promote studies at protein level, in chapter 2, large-scale expression and purification of xBMP-4, and sBMPR was performed using silkworm expression system. As a result of co-infection of recombinant viruses of xBMP-4 and sBMPR, an efficient milligram-scale purification was achieved. Using these purified proteins, in chapter 3, it was demonstrated that follistatin can bind to BMPs directly, and that forms a trimeric complex with BMP-4 and sBMPR to inhibit BMP signal, as in the no case with chordin or noggin. Lastly (chapter 4), Ep45, which is a newly identified BMP-binding protein, was isolated. This protein was found to have no effect on the binding of BMP to its receptor, but inhibit the binding of BMP to follistatin. Together with the expression pattern of Ep45 and the abundance of its protein, these results suggested that follistatin probably has a minimum role as an organizer factor, and may not be involved in early dorsoventral patterning (Fig. 5-1). Presumably, follistatin acts as a BMPs-antagonist at later stage of development.

In spite of these novel findings in this study, several problems remain to be clarified. One of them is how follistatin inhibits BMP activities after forming a trimeric complex with BMP and its type I receptor. Although this bound follistatin is thought to

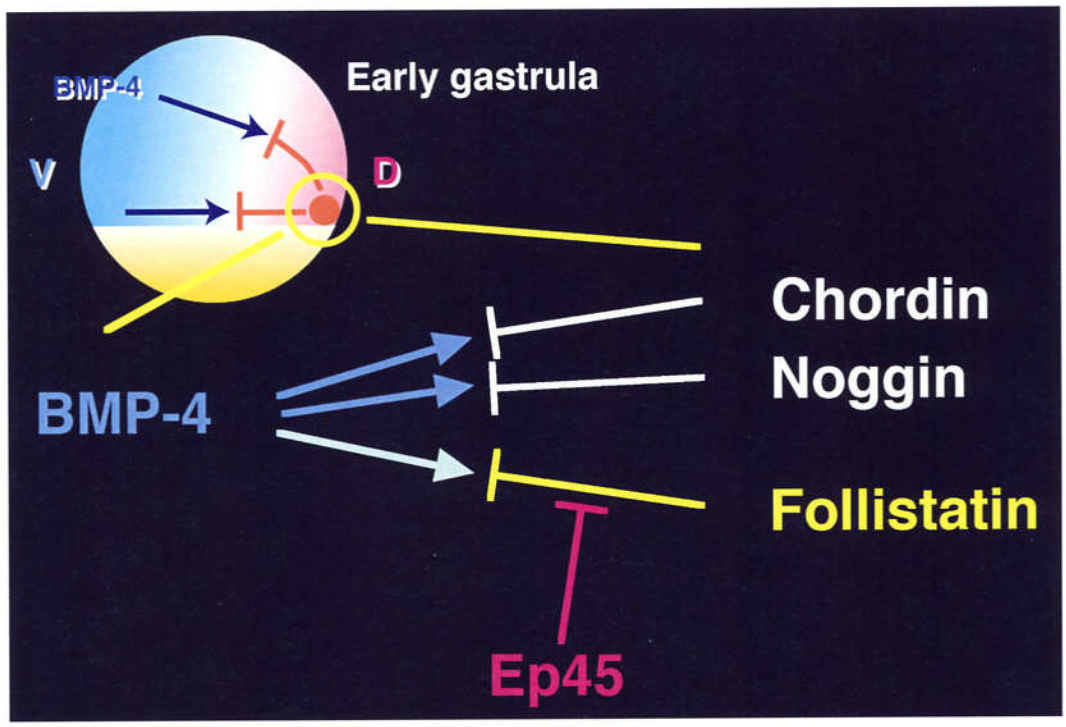

Figure 5-1. Summary

Follistatin can directly bind to BMP-4 and inhibit its activity as well as other organizer factors, chordin and noggin. However, the inhibitory mechanism of follistatin for BMP-4 is different from those of others. Ep45 inhibits the binding of follistatin to BMP-4 although Ep45 has no effect on the binding of chordin or noggin to BMP-4. In early gastrula *Xenopus* embryo, since Ep45 mRNA is expressed in dorsal marginal zone and Ep45 protein is abundant in the embryo, follistain is unlikely to act as an organizer factor by binding to BMP.

result in inhibiting the interaction of BMP type I receptor with type II receptor, experimental demonstrations using type II receptor have yet to be done. Moreover, as described in chapter 3, further investigation is needed to confirm whether a complex with BMP and follistatin is captured by ECM. As regarding Ep45, to assess whether follistatin functions as an organizer factor, it is important to show localization of Ep45 protein at all stage in development. Furthermore, the intriguing issue of the function of Ep45 as a serine protease inhibitor in early development remains to be investigated.

Here I described the several novel findings regarding the regulation of BMP activity. However, the elucidation of the negative regulatory mechanisms by binding proteins has just been started. For further understanding, factors involved in the regulation have to be isolated. Although several known factors that directly bind to BMPs may be used repeatedly to regulate BMP activities at all developmental stages, more unknown factors may exist, because it is thought that multipotent BMP activities are regulated by a variety of mechanisms at various biological phases. In-depth analyses of the interaction of isolated factors with BMPs will lead to a better understanding of early developmental events regulated by BMPs.

ACKNOWLEDGMENT

I wish to express my gratitude to all those whose support and encouragement made this dissertation. First of all, I wish to thank my research advisor, Professor Naoto Ueno for his exact guidance and constant encouragement throughout this study. And I wish to thank Drs. Yoshitaka Nagahama, Ken-ichiro Morohashi, and Hiroyuki Takeda for their reviews and appropriate comments. I also wish to thank Dr. Tohru Natsume for leading me to the new world of SPR biosensor and for helpful discussions. Furthermore, I would like to thank Drs. Shuichiro Tomita for providing baculovirus and helpful discussion, Yuzuru Eto for activin A protein, Ali Hemmati-Brivanlou for the 9E10 cell line, Shunichi Shimasaki for human follistatin (FS-288) protein and anti-follistatin antiserum (Rb32), Yukio Fujisawa for *Xenopus* BMP-4/7 heterodimer and BMP-7 homodimer proteins, Stefano Piccolo for *Xenopus* chordin protein, Richard Harland for the CHO B3 cell line which produces *Xenopus* noggin protein, and Ken Cho for beneficial comments and discussions. I also wish to express special thanks to Mr. Takamasa S. Yamamoto and Ms. Chiyo Takagi for their excellent techniques of embryonic experiments, beneficial discussions, and appropriate advices. Many thanks are also extended to all the members of Division of Morphogenesis, National Institute for Basic Biology.

This work described in this thesis was carried out at the Research and Development Center, Nippon Meat Packers, Inc. (chapter 2), and the Department of Developmental Biology, National Institute for Basic Biology (NIBB) (chapter 3 and 4).

REFERENCES

Akiyama, S., Katagiri, T., Namiki, M., Yamaji, N., Yamamoto, N., Miyama, K., Shibuya, H., Ueno, N., Wozney, J. M., and Suda, T. (1997). Constitutively active BMP type I receptors transduce BMP-2 signals without the ligand in C2C12 myoblasts. Exp. Cell Res. *235*, 362-369.

Albano, R. M., Arkell, R., Beddington, R. S., and Smith, J. C. (1994). Expression of inhibin subunits and follistatin during postimplantation mouse development: decidual expression of activin and expression of follistatin in primitive streak, somites and hindbrain. Development *120*, 803-813.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Asashima, M., Nakano, H., Shimada, K., Kinoshita, K., Ishii, K., Shibai, H., and Ueno, N. (1990). Mesoderm induction in early amphibian embryos by activin A (erythroid differentiation factor). Roux's Arch. Dev. Biol. *198*, 330-335.

Bauer, H., Meier, A., Hild, M., Stachel, S., Economides, A., Hazelett, D., Harland, R. M., and Hammerschmidt, M. (1998). Follistatin and noggin are excluded from the zebrafish organizer. Dev. Biol. *204*, 488-507.

Beck, B. L., Henjum, D. C., Antonijczuk, K., Zaharia, O., Korza, G., Ozols, J., Hopfer, S. M., Barber, A. M., and Sunderman, F., Jr. (1992). pNiXa, a Ni(2+)-binding protein in Xenopus oocytes and embryos, shows identity to Ep45, an estrogen-regulated hepatic serpin. Res. Commun. Chem. Pathol. Pharmacol. *77*, 3-16.

Berridge, M. V., and Lane, C. D. (1976). Translation of Xenopus liver messenger RNA in Xenopus oocytes: vitellogenin synthesis and conversion to yolk platelet proteins. Cell *8*, 283-297.

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

Clemens, M. J. (1974). The regulation of egg yolk protein synthesis by steroid hormones. Prog. Biophys. Mol. Biol. *28*, 69-108.

Dale, L., Howes, G., Price, B. M., and Smith, J. C. (1992). Bone morphogenetic protein 4: a ventralizing factor in early Xenopus development. Development *115*, 573-585.

de Winter, J. P., ten Dijke, P., de Vries, C. J., van Achterberg, T. A., Sugino, H., de Waele, P., Huylebroeck, D., Verschueren, K., and van den Eijnden-van Raaij, A. J. (1996). Follistatins neutralize activin bioactivity by inhibition of activin binding to its type II receptors. Mol. Cell. Endocrinol. *116*, 105-114.

Feijen, A., Goumans, M. J., and van den Eijnden-van Raaij, A. J. (1994). Expression of activin subunits, activin receptors and follistatin in postimplantation mouse embryos suggests specific developmental functions for different activins. Development *120*, 3621-3637.

Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc. Natl. Acad. Sci. U S A *84*, 7413-7417.

Fukui, A., Nakamura, T., Sugino, K., Takio, K., Uchiyama, H., Asashima, M., and Sugino, H. (1993). Isolation and characterization of Xenopus follistatin and activins. Dev. Biol. *159*, 131-139.

Gerber-Huber, S., Nardelli, D., Haefliger, J. A., Cooper, D. N., Givel, F., Germond, J. E., Engel, J., Green, N. M., and Wahli, W. (1987). Precursor-product relationship between vitellogenin and the yolk proteins as derived from the complete sequence of a Xenopus vitellogenin gene. Nucleic Acids Res. *15*, 4737-4760.

Gilbert, S. F. (1994). Developmental Biology (Massachusetts: Sinauer Associates, Inc.), pp. 586-622.

Graff, J. M. (1997). Embryonic patterning: to BMP or not to BMP, that is the question. Cell *89*, 171-174.

Graff, J. M., Thies, R. S., Song, J. J., Celeste, A. J., and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm- inducing signals override dorsal signals in vivo. Cell *79*, 169-179.

Graham, A., Francis-West, P., Brickell, P., and Lumsden, A. (1994). The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature *372*,

684-686.

Harland, R. M. (1994). The transforming growth factor beta family and induction of the vertebrate mesoderm: bone morphogenetic proteins are ventral inducers. Proc. Natl. Acad. Sci. U S A *91*, 10243-10246.

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

Hazama, M., Aono, A., Ueno, N., and Fujisawa, Y. (1995). Efficient expression of a heterodimer of bone morphogenetic protein subunits using a baculovirus expression system. Biochem. Biophys. Res. Commun. *209*, 859-866.

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. (1997). Vertebrate embryonic cells will become nerve cells unless told otherwise. Cell *88*, 13-17.

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.

Hogan, B. L. M. (1996). Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes Dev. *10*, 1580-1594.

Holland, L. J., Suksang, C., Wall, A. A., Roberts, L. R., Moser, D. R., and Bhattacharya, A. (1992). A major estrogen-regulated protein secreted from the liver of Xenopus laevis is a member of the serpin superfamily. Nucleotide sequence of cDNA and hormonal induction of mRNA. J. Biol. Chem. *267*, 7053-7059.

Holland, L. J., and Wangh, L. J. (1987). Estrogen induction of a 45 kDa secreted protein coordinately with vitellogenin in Xenopus liver. Mol. Cell. Endocrinol. *49*, 63-73.

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.

Inouye, S., Guo, Y., DePaolo, L., Shimonaka, M., Ling, N., and Shimasaki, S. (1991). Recombinant expression of human follistatin with 315 and 288 amino acids: chemical and biological comparison with native porcine follistatin. Endocrinology *129*, 815-822.

Ishida, N., Tsujimoto, M., Kanaya, T., Shimamura, A., Tsuruoka, N., Kodama, S., Katayama, T., Oikawa, S., Matsui, M., Nakanishi, T., and et, a. (1994). Expression and characterization of human bone morphogenetic protein-2 in silkworm larvae infected with recombinant Bombyx mori nuclear polyhedrosis virus. J. Biochem. *115*, 279-285.

Johnsson, B., Löfås, S., and Lindquist, G. (1991). Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal. Biochem. *198*, 268-277.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V., and Hogan, B. L. (1992). DVR-4 (bone morphogenetic protein-4) as a posterior-ventralizing factor in Xenopus mesoderm induction. Development *115*, 639-647.

Karlsson, R., Roos, H., Färgestam, L., and Persson, B. (1994). Methods (Orlando) *6*, 99-110.

Kimelman, D., Christian, J. L., and Moon, R. T. (1992). Synergistic principles of development: overlapping patterning systems in Xenopus mesoderm induction. Development *116*, 1-9.

Kingsley, D. M. (1994). The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes Dev. *8*, 133-146.

Kobayashi, J., Imanishi, S., Inoue, H., Ohsuye, K., Yamaichi, K., Tsuruoka, N., and Tanaka, S. (1992). High level expression of a frog alpha-amidating enzyme, AE-II, in cultured cells and silkworm larvae using a Bombyx mori nuclear polyhedrosis virus expression vector. Cytotechnology *8*, 103-108.

Koenig, B. B., Cook, J. S., Wolsing, D. H., Ting, J., Tiesman, J. P., Correa, P. E., Olson, C. A., Pecquet, A. L., Ventura, F., Grant, R. A., Chen, G.-X., Wrana, J. L., Massagué, J., and Rosenbaum, J. S. (1994). Characterization and cloning of a receptor for BMP-2 and BMP-4 from NIH 3T3 cells. Mol. Cell. Biol. *14*, 5961-5974.

Kolodziej, P. A., and Young, R. A. (1991). Epitope tagging and protein surveillance. Methods Enzymol. *194*, 508-519.

Lackmann, M., Bucci, T., Mann, R. J., Kravets, L. A., Viney, E., Smith, F., Moritz, R. L., Carter, W., Simpson, R. J., Nicola, N. A., Mackwell, K., Nice, E. C., Wilks, A. F., and Boyd, A. W. (1996). Purification of a ligand for the EPH-like receptor HEK using a biosensor-based affinity detection approach. Proc. Natl. Acad. Sci. U S A *93*, 2523-2527.

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. Science *262*, 713-718.

Lemaire, P., and Kodjabachian, L. (1996). The vertebrate organizer: structure and molecules. Trends Genet. *12*, 525-531.

Liu, F., Ventura, F., Doody, J., and Massagué, J. (1995). Human type II receptor for bone morphogenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. Mol. Cell. Biol. *15*, 3479-3486.

Löfås, S., Malmqvist, M., Ronnberg, I., Stenberg, E., Liedberg, B., and Lundstrom, I. (1991). Sensors Actuators *B5*, 79-84.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y., and Furusawa, M. (1985). Production of human alpha-interferon in silkworm using a baculovirus vector. Nature *315*, 592-594.

Maeno, M., Ong, R. C., Xue, Y., Nishimatsu, S., Ueno, N., and Kung, H. F. (1994). Regulation of primary erythropoiesis in the ventral mesoderm of Xenopus gastrula embryo: evidence for the expression of a stimulatory factor(s) in animal pole tissue. Dev. Biol. *161*, 522-529.

Massagué, J. (1996). TGFbeta signaling: receptors, transducers, and Mad proteins. Cell *85*, 947-950.

Matzuk, M. M., Lu, N., Vogel, H., Sellheyer, K., Roop, D. R., and Bradley, A. (1995). Multiple defects and perinatal death in mice deficient in follistatin. Nature *374*, 360-363.

Miyazono, K., ten Dijke, P., Yamashita, H., and Heldin, C. H. (1994). Signal transduction via serine/threonine kinase receptors. Semin. Cell Biol. *5*, 389-398.

Moon, R. T., Brown, J. D., Yang-Snyder, J. A., and Miller, J. R. (1997). Structurally related receptors and antagonists compete for secreted Wnt ligands. Cell *88*, 725-728.

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., and Sugino, H. (1990). Activin-binding protein from rat ovary is follistatin. Science *247*, 836-838.

Nishimatsu, S., Suzuki, A., Shoda, A., Murakami, K., and Ueno, N. (1992). Genes for bone morphogenetic proteins are differentially transcribed in early amphibian embryos. Biochem. Biophys. Res. Commun. *186*, 1487-1495.

Nishimatsu, S., Takebayashi, K., Suzuki, A., Murakami, K., and Ueno, N. (1993). Immunodetection of Xenopus bone morphogenetic protein-4 in early embryos. Growth Factors *8*, 173-176.

Nohno, T., Ishikawa, T., Saito, T., Hosokawa, K., Noji, S., Wolsing, D. H., and Rosenbaum, J. S. (1995). Identification of a human type II receptor for bone morphogenetic protein-4 that forms differential heteromeric complexes with bone morphogenetic protein type I receptors. J. Biol. Chem. *270*, 22522-22526.

Ozaki, T., and Sakiyama, S. (1993). Molecular cloning and characterization of a cDNA showing negative regulation in v-src-transformed 3Y1 rat fibroblasts. Proc. Natl. Acad. Sci. U S A *90*, 2593-2597.

Ozaki, T., and Sakiyama, S. (1994). Tumor-suppressive activity of N03 gene product in v-src-transformed rat 3Y1 fibroblasts. Cancer Res. *54*, 646-648.

Perrimon, N., and McMahon, A. P. (1999). Negative feedback mechanisms and their roles during pattern formation. Cell *97*, 13-16.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature *397*, 707-710.

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.

Raether, H. (1977). , Volume 9, G. Hass, M. H. Francombe and R. W. Hoffman, eds. (New York: Academic Press).

Reddi, A. H. (1994). Bone and cartilage differentiation. Curr. Opin. Genet. Dev. *4*, 737-744.

Rosenzweig, B. L., Imamura, T., Okadome, T., Cox, G. N., Yamashita, H., ten Dijke, P., Heldin, C. H., and Miyazono, K. (1995). Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc. Natl. Acad. Sci. U S A *92*, 7632-7636.

Ruppert, R., Hoffmann, E., and Sebald, W. (1996). Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. Eur. J. Biochem. *237*, 295-302.

Sasai, Y., and De Robertis, E. M. (1997). Ectodermal patterning in vertebrate embryos. Dev. Biol. *182*, 5-20.

Sasai, Y., Lu, B., Piccolo, S., and De Robertis, E. M. (1996). Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. EMBO J. *15*, 4547-4555.

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.

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.

Schmidt, J. E., Suzuki, A., Ueno, N., and Kimelman, D. (1995). Localized BMP-4 mediates dorsal/ventral patterning in the early Xenopus embryo. Dev. Biol. *169*, 37-50.

Schuck, P. (1997). Use of surface plasmon resonance to probe the equilibrium and dynamic aspects of interactions between biological macromolecules. Annu. Rev. Biophys. Biomol. Struct. *26*, 541-566.

Shimasaki, S., Koga, M., Esch, F., Cooksey, K., Mercado, M., Koba, A., Ueno, N., Ying, S. Y., Ling, N., and Guillemin, R. (1988). Primary structure of the human follistatin precursor and its genomic organization. Proc. Natl. Acad. Sci. U S A *85*, 4218-4222.

Sive, H. L. (1993). The frog prince-ss: a molecular formula for dorsoventral patterning in Xenopus. Genes Dev. *7*, 1-12.

Slack, J. M. W. (1991). From Egg to Embryo. Regional Specification in Early Development., 2nd Edition (Cambridge: Cambridge University Press).

Smith, J. C., Price, B. M., Van Nimmen, K., and Huylebroeck, D. (1990). Identification of a potent Xenopus mesoderm-inducing factor as a homologue of activin A. Nature *345*, 729-731.

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.

Spemann, H., and Mangold, H. (1924). Induction of embryonic primordia by implantation of organizers from a different species. In Foundations of Experimental Embryology, B. H. Willier and J. M. Oppenheimer, eds. (New York: Haufer), pp. 144-184.

Sugino, K., Kurosawa, N., Nakamura, T., Takio, K., Shimasaki, S., Ling, N., Titani, K., and Sugino, H. (1993). Molecular heterogeneity of follistatin, an activin-binding protein. Higher affinity of the carboxyl-terminal truncated forms for heparan sulfate proteoglycans on the ovarian granulosa cell. J. Biol. Chem. *268*, 15579-15587.

Sunderman, F., Jr. (1993). Search for molecular mechanisms in the genotoxicity of nickel. Scand. J. Work. Environ. Health *1*, 75-80.

Suzuki, A., Kaneko, E., Maeda, J., and Ueno, N. (1997). Mesoderm induction by BMP-4 and -7 heterodimers. Biochem. Biophys. Res. Commun. *232*, 153-156.

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

Suzuki, A., Nishimatsu, S., Shoda, A., Takebayashi, K., Murakami, K., and Ueno, N. (1993). Biochemical properties of amphibian bone morphogenetic protein-4 expressed in CHO cells. Biochem. J. *291*, 413-417.

Suzuki, A., Thies, R. S., Yamaji, N., Song, J. J., Wozney, J. M., Murakami, K., and Ueno, N. (1994). A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. Proc. Natl. Acad. Sci. U S A *91*, 10255-10259.

Tashiro, K., Yamada, R., Asano, M., Hashimoto, M., Muramatsu, M., and Shiokawa, K. (1991). Expression of mRNA for activin-binding protein (follistatin) during early embryonic development of Xenopus laevis. Biochem. Biophys. Res. Commun. *174*, 1022-1027.

ten Dijke, P., Miyazono, K., and Heldin, C. H. (1996). Signaling via hetero-oligomeric complexes of type I and type II serine/threonine kinase receptors. Curr. Opin. Cell. Biol. *8*, 139-145.

ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C. H., and Miyazono, K. (1994). Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. J. Biol. Chem. *269*, 16985-16988.

Thomsen, G. H. (1997). Antagonism within and around the organizer: BMP inhibitors in vertebrate body patterning. Trends Genet. *13*, 209-211.

Thomsen, G. H., and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. Cell *74*, 433-441.

Ueno, N., Ling, N., Ying, S. Y., Esch, F., Shimasaki, S., and Guillemin, R. (1987). Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle- stimulating hormone. Proc. Natl. Acad. Sci. U S A *84*, 8282-8286.

Ueno, N., Shoda, A., Takebayashi, K., Suzuki, A., Nishimatsu, S., Kikuchi, T., Wakimasu, M., Fujino, M., and Murakami, K. (1992). Identification of bone morphogenetic protein-2 in early Xenopus laevis embryos. Growth Factors *7*, 233-240.

Urist, M. R. (1965). Bone: formation by autoinduction. Science *150*, 893-899.

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

Wozney, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988). Novel regulators of bone formation: molecular clones and activities. Science *242*, 1528-1534.

Yamaguchi, A., Ishizuya, T., Kintou, N., Wada, Y., Katagiri, T., Wozney, J. M., Rosen, V., and Yoshiki, S. (1996). Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem. Biophys. Res. Commun. *220*, 366-371.

Yamashita, H., ten Dijke, P., Franzen, P., Miyazono, K., and Heldin, C. H. (1994). Formation of hetero-oligomeric complexes of type I and type II receptors for transforming growth factor-beta. J. Biol. Chem. *269*, 20172-20178.

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.

Zou, H., and Niswander, L. (1996). Requirement for BMP signaling in interdigital apoptosis and scale formation. Science *272*, 738-741.