Isolation and Characterization of Bone Morphogenetic Protein-binding Proteins from the Early Xenopus Embryo*

(Received for publication, December 1, 1998, and in revised form, May 19, 1999)

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Bone morphogenetic proteins (BMPs),1 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 (1). BMPs are now known to be expressed in vertebrate embryos (2) and to possess a variety of embryological functions (3). In early Xenopus embryos, the BMP family of ligands inhibit dorsal mesoderm formation and induce ventral mesoderm formation (4). Furthermore, in ectoderm, BMPs have been shown to inhibit neural cell differentiation and to promote epidermal differentiation (5). In this way, BMPs are thought to play essential roles in the dorsoventral patterning of the embryo (6, 7).

Using a surface plasmon resonance biosensor as a sensitive and specific monitor, we have isolated two distinct bone morphogenetic protein (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 paper, we have shown that of the TGF-β family members tested, Ep45 can bind only to BMP-4, whereas lipovitellin 1 can bind to both BMP-4 and activin A. Because of this difference in specificity, we have focused on and further studied Ep45. Kinetic parameters were determined by surface plasmon resonance studies and showed that Ep45 associated rapidly with BMP-4 ($k_\text{a} = 1.06 \times 10^4 \text{M}^{-1} \text{s}^{-1}$) and dissociated slowly ($k_\text{d} = 1.6 \times 10^{-4} \text{s}^{-1}$). 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, whereas it had no effect on other BMP antagonists, chordin and noggin. These results support the possibility that Ep45 interacts with BMP to modulate its activities in vivo.

Bone morphogenetic proteins (BMPs),1 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 (1). BMPs are now known to be expressed in vertebrate embryos (2) and to possess a variety of embryological functions (3). In early Xenopus embryos, the BMP family of ligands inhibit dorsal mesoderm formation and induce ventral mesoderm formation (4). Furthermore, in ectoderm, BMPs have been shown to inhibit neural cell differentiation and to promote epidermal differentiation (5). In this way, BMPs are thought to play essential roles in the dorsoventral patterning of the embryo (6, 7).

It is generally accepted that these important biological activities are negatively regulated by inhibitors of the BMPs (8, 9), noggin, follistatin, and chordin, which are expressed in the Spemann’s organizer and are thus called organizer factors. All of these have been shown to be neutralized ectoderm directly (10–12). Recent reports demonstrate that these organizer factors can bind to BMP-4 directly and thus inhibit the anti-neural activity of BMP-4 (13–15). 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 and BMP-4 (16, 17). Gremlin is not expressed during gastrulation, although its activities are similar to those of noggin, chordin, and follistatin. Rather, it is implicated in neural crest development in later embryogenesis (16). 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 normally expressed in the anterior endomesoderm of Xenopus gastrulae (18). These results suggest that BMP activities are regulated throughout development by various BMP antagonists with different affinities and binding specificities.

Here, we screened Xenopus embryo extract to find novel BMP-4-binding molecules that may regulate the multipotent BMP activity in development. To do the screening, we used a surface plasmon resonance biosensor. Because of its high sensitivity for monitoring real time protein-protein interactions (19), this technique replaced the conventional biological and immunochemical assays. We purified two BMP-binding proteins and identified them as lipovitellin 1 and Ep45, respectively, using their N-terminal amino acid sequences and biochemical analyses. Lipovitellin 1, which is processed from vitellogenin, is a 120-kDa yolk platelet protein (20, 21). 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 (20, 22). Ep45 (45 kDa) is also synthesized under estrogen control in the liver and released into the blood stream (23, 24). 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 Ni²⁺−binding protein and is implicated in Ni²⁺−induced teratogenesis (25, 26). In this report, we performed functional analyses of Ep45 in the interaction between BMP and its antagonist follistatin and found that Ep45 blocked the binding of BMP to follistatin.

EXPERIMENTAL PROCEDURES

Measurements and Analysis by Surface Plasmon Resonance Biosensor—All measurements of binding activity in each purification step, binding experiments, and kinetic analysis were performed using the BIACORE2000™ (Biacore AB, Uppsala, Sweden). The basic principles behind the technique and its successful use have been previously documented (27). 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 treated in the same manner as the experimental samples. The injection and running buffer was 10 mM sodium acetate, pH 5.0, containing 150 mM NaCl and no protein. Detection was made at a wavelength of 320 nm.

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§ The abbreviations used are: BMP, bone morphogenetic protein; FS-288, follistatin-288; TGF-β, transforming growth factor-β; sBMPR, soluble form of mouse BMP type IA receptor; RU, resonance unit; PAGE, polyacrylamide gel electrophoresis.

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* This work is supported by “Research for the Future” program of the Japan Society for the Promotion of Science Grant JPSP-RFTF96L000406. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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binding activity was eluted in a sharp peak (*asterisks*) by the amine coupling method. The surface (1050 RU) as described above. Ep45 was immobilized on a sensor chip surface (CM5, certified grade, Biacore AB) by the amine coupling method (28). sBMPR that was biotinylated using sulfo-NHS-biotin (Pierce) was immobilized on a sensor chip surface (SA5, research grade, Biacore AB) that had been immobilized previously with streptavidin (15).

To examine whether the binding of Ep45 to BMP-4 is dependent on the conformation of BMP-4, BMP-4 was reduced with 1% 2-mercaptoethanol at 37 °C for 1 h. The sample was diluted with CF3COOH 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 units (RU) was determined 30 s after sample injection. The running and sample dilution buffer was HBS (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, and 0.005% Tween 20, pH 7.4). BMP-4, activin A, TGF-β1, and FS-288 were immobilized on the sensor chip surface (CMS, certified grade, Biacore AB) by the amine coupling method (28). sBMPR which was biotinylated using sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC) was immobilized on the sensor chip surface (CM5, research grade, Biacore AB) that had been immobilized previously with streptavidin (15).

To determine kinetic parameters, 503 RU of BMP-4 was immobilized on the sensor chip surface (CMS) 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—Recombinant Xenopus BMP-4 and the extracellular domain of the mouse BMP type I receptor (sBMPR) were obtained using a silkworm expression system (29). Recombinant human follistatin (FS-288) was a gift from Dr. Y. Eto (Ajinomoto, Tokyo), and activin A was a gift from Dr. S. Shimasaki (30), recombinant human TGF-β1 was a gift from Dr. S. Piccolo (14). Recombinant human TGF-β1 was purchased (King Jyouhzo, Hyogo, Japan).

**Chemical Cross-linking and Two-dimensional Electrophoresis**—

**Embryo Manipulations—**Xenopus embryos were obtained by artificial fertilization and 2- or 4-cell stage embryos were microinjected with synthetic RNAs as described previously (33). To evaluate mesodermal markers, the dorsal or ventral marginal region was excised when injected embryos reached stage 10. For the animal cap assay, presumptive ectoderm fragments were collected when the injected embryos reached stage 8.5.

**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 pmol) and vitellogenin 1 (10 pmol) 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 (32).

**Embryo Purification of BMP-binding Proteins from Xenopus Embryo—**Extract (20 ml) from Xenopus embryos (approximately 8,000) at stage 8–10 was prepared as described previously (31). It was dialyzed against 25 mM sodium acetate buffer, pH 5.5, at 4 °C and then applied to a POROS SPH cation ion exchange column (Perseptive Biosystems, Framingham, MA). 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 and then separated on a Superose 12 gel filtration column (Amersham Pharmacia Biotech) pre-equilibrated with HBS. The three major protein peaks were pooled, acidified to pH 3.0 with 1.0 n acetic acid, and separated on a μRPC C2/C18 reverse phase column (Amersham Pharmacia Biotech) pre-equilibrated with 0.1% CF3COOH (trifluoroacetic acid). 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 nitrotriacetic acid-agarose charged with Ni2+ (Qiagen), pre-equilibrated with 20 mM Tris-HCl, pH 7.0, 5 mM CaCl2 (25). 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 by 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 (bovine serum albumin; Sigma).
Chemical cross-linking and two-dimensional electrophoresis were performed as described previously (15). Briefly, BMP-4 (75 nm) and Ep45 (750 nm) were incubated for 1 h at room temperature in 100 μl of HBS. Dithiobis (sulfosuccinimidylpropionate) (Pierce) was added to a final concentration of 0.5 μm and incubated for 30 min. After Tris-HCl (pH 8.0) was added to a final concentration of 50 mM to quench the reaction, the sample was subjected to diagonal SDS-PAGE analysis. BMP-4 was detected by Western blotting using BMP-4 antibody (Ab697).

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 6.5, 5 m NaCl, 20 mg/ml bovine serum albumin was incubated for 80 min at 37°C (25). Samples were subjected to electrophoresis on a 15% gel and electroblotted onto polyvinylidene difluoride membranes (Millipore, Bedford). The membrane was blocked with 20 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20 containing 5% nonfat dry milk, incubated with the BMP-4 antibody (Ab697) at 4°C overnight (34), and then reacted with a horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Western blots were developed using the chemiluminescent ECL plus kit (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

RESULTS

Isolation of BMP-binding Proteins from Xenopus Embryos—We isolated two BMP-binding proteins from Xenopus embryos using the BIAcore biosensor as a detection system. Both Ep45 and lipovitellin 1 were purified by three-step column chromatography (Fig. 1). After the embryo extract was dialyzed against chromatography buffer, it was first purified by POROS SP/H cation ion exchange chromatography (Fig. 1A). Although the column was eluted by a NaCl concentration gradient (0–0.5 M), the highest activity was eluted at concentrations above 0.5 M NaCl. The 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. 1B). Three major protein peaks (20 min, peak Vo; 30 min, peak A; 35 min, peak B) were obtained, and the fractions containing each peak were pooled. Each peak was further separated by μRPC C2/C18 reverse phase chromatography (Fig. 1C). We isolated two BMP-binding proteins from Xenopus embryos using the BIAcore biosensor as a detection system. Both Ep45 and lipovitellin 1 were purified by three-step column chromatography (Fig. 1A). After the embryo extract was dialyzed against chromatography buffer, it was first purified by POROS SP/H cation ion exchange chromatography (Fig. 1A). Although the column was eluted by a NaCl concentration gradient (0–0.5 M), the highest activity was eluted at concentrations above 0.5 M NaCl. The 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. 1B). Three major protein peaks (20 min, peak Vo; 30 min, peak A; 35 min, peak 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), whereas both pools A and B gave single peaks with significant BMP binding activities (Fig. 1, C and D, asterisks). Each peak was collected and analyzed by SDS-PAGE (Fig. 2, A and 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 × 10⁶ and 2.2 × 10⁶ RU/mg, respectively (Table I). 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 of pure protein, respectively.

Amino Acid Sequence Analysis of the 45- and 120-kDa Proteins—The N-terminal amino acid sequences of the purified 45- and 120-kDa proteins were analyzed using an automated liquid phase sequencer, and 20 and 29 amino acid residues, respectively, were determined (Fig. 2, C and D). A BLAST search 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 (24) showed that the 45-kDa protein lacked only seven N-terminal amino acid residues of full-length, mature Ep45 (Fig. 2C). Despite the correspondence of the N-terminal amino acid sequence, the molecular mass of the 120-kDa protein was not consistent with that of vitellogenin A2 precursor (210 kDa). Given the molecular mass of this protein, it is most likely to be lipovitellin 1, which is a cleavage product of vitellogenin (20).

The Binding Specificity of Ep45 and Lipovitellin 1 to TGF-β Family Ligands—The ability of Ep45 and lipovitellin 1 to bind

| Purification step | Total protein | Total response | Specific response | Yield | Purification |
|------------------|--------------|----------------|------------------|-------|--------------|
| Extract          | 12           | 2.1 × 10⁵      | 1.8 × 10⁴        | 100   | 2.9          |
| POROS SP/H       | 1.2          | 6.4 × 10⁴      | 5.3 × 10⁴        | 30.5  | 2.9          |
| Superose 12 (pool A) | 0.02      | 1.3 × 10⁴      | 6.5 × 10³        | 6.2   | 36.1         |
| μRPC C2/C18      | 0.005        | 1.1 × 10⁴      | 2.2 × 10⁴        | 5.2   | 120          |
| Superose 12 (pool B) | 0.06      | 1.4 × 10⁴      | 3.0 × 10³        | 6.7   | 16.7         |
| μRPC C2/C18      | 0.006        | 1.2 × 10⁴      | 2.0 × 10⁶        | 5.7   | 110          |

* Protein was measured as described under “Experimental Procedures.”

* Response was expressed as RU at 30 s after sample injection.
Isolation and Characterization of BMP-binding Proteins

known TGF-β family proteins was tested using the BIAcore biosensor (Fig. 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 (Fig. 3B). Moreover, this change in resonance signal was also detectable when lipovitellin 1 flowed over activin A immobilized on a sensor chip surface. 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. 3A). Because Ep45 can interact only with BMP-4 among the TGF-β family ligands we tested and its role in development is poorly understood, we decided to focus on Ep45 and to exclude lipovitellin 1 from further consideration.

To confirm this physical interaction between Ep45 and BMP-4, we chemically cross-linked the Ep45-BMP-4 complex and performed two-dimensional electrophoresis (Fig. 4). Separated BMP-4 was visualized by Western blotting using specific antibody for BMP-4. As shown in Fig. 4A, BMP-4 bound to Ep45 migrated from 30 kDa to 120 kDa, whereas BMP-4 alone with cross-linking (Fig. 4B) did not shift significantly. These results suggest that BMP-4 binds to Ep45 directly in the reaction mixture.

Next, to examine whether this binding of Ep45 to BMP-4 is dependent on the conformation of BMP-4, the interaction of Ep45 with reduced BMP-4 was determined using the surface plasmon resonance biosensor. When reduced monomer BMP-4 was injected over the Ep45 immobilized sensor chip, the response was not detected (data not shown). This result suggests that at least tertiary structure of BMP-4 was required to interact with Ep45.

To determine kinetic parameters, we injected Ep45 at increasing concentrations and allowed it to flow 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 (kₐ) was calculated to be 1.06 × 10⁴ M⁻¹ s⁻¹, and the dissociation rate constant (k₅) was calculated to be 1.6 × 10⁻⁴ s⁻¹, which is slow. From these two rate constants (kₐ / k₅), 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, we micro-injected Ep45 mRNA into Xenopus embryos. We expected, based on previous studies (13–16), 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, we tested whether Ep45 blocks the binding of BMP-4 to its receptor using the BIACORE biosensor. sBMPR was immobilized on a BIACORE sensor chip (900 RU), and then BMP-4 (1 μg/ml) was injected to flow over the chip. As shown in Fig. 5, 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, we attempted to test whether Ep45 could influence the interaction between BMP-4 and the BMP-binding proteins, chordin, noggin, and follistatin. Chordin, noggin, or follistatin mRNA was coinjected with Ep45 mRNA into the two ventral blastomeres of 4-cell embryos (Fig. 6A). Chordin, noggin, and follistatin independently induce dorsal mesoderm and a secondary body axis in embryos when they are ventrally overexpressed (12, 15, 35) (Fig. 6B). Injection of Ep45 and chordin or noggin did not inhibit the dorsalizing effect of these proteins (Fig. 6A, panels c and d or panels f and g). In contrast, Ep45 did inhibit the dorsalizing signal of follistatin in a dose-dependent manner (Fig. 6A, panels i and j). To confirm that this result was due to the direct interference of Ep45 with the BMP-4-follistatin interaction, we carried out a competition assay using the biosensor. As shown in Fig. 6B, 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 (36). 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, we have searched for and isolated two BMP-binding proteins, lipovitellin 1 and Ep45. 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 our experiment, we 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 (20). Its precursor, vitellogenin, binds both activin and BMP.2 Our results also suggest 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, we 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, we did not find that Ep45 affected the binding of BMP-4 to its type I receptor (Fig. 5), despite its direct binding to BMP-4. Ep45 neither inhibited the binding of BMP to type I receptor as chordin does nor affected the BMP-BMP receptor binding profile as does follistatin (15). These results are consistent with the results we 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, we found that in Xenopus Ep45 inhibited the antagonistic interaction of follistatin with BMP-4 but not of chordin or noggin. Our 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) (13–15), whereas 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 and noggin for BMP-4 was obtained by different methods, this tendency is thought to be correct because chordin and noggin have different binding affinities. Therefore, in the presence of Ep45, it is most likely that follistatin cannot act as an organizer factor by binding binding to BMP. Furthermore, this explains why dorsal overexpression of Ep45 did not perturb normal 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, despite having the same dorsalizing properties as its Xenopus homologue (37). In the mouse, follistatin mRNA is also not expressed in the node, the mouse equivalent of the Spemann's organizer, or the notochord (38, 39). Furthermore, follistatin knock-out mice exhibit neither aberrant neural development nor defects in early dorsoventral patterning (40). 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 earlier patterning process.

Ep45 belongs to the serine protease inhibitor (serpin) superfamily (24). It may protect the BMP protein from some serine proteases by binding to BMP. In fact, we tested whether Ep45 inhibits the activity of $\alpha$-chymotrypsin (Fig. 7). BMP-4 was partially digested by $\alpha$-chymotrypsin to yield lower molecular mass forms, as described in a previous study showing that a proteolytic cleavage with trypsin occurs at the N terminus of BMP-2 (41). The addition of Ep45 significantly blocked the limited proteolytic digestion of BMP-4. In addition, this experiment together with the previous study demonstrates that both $\alpha$-chymotrypsin and trypsin are capable of cleaving the N terminus of BMP-4.

Both BMP-2 and -4 have a basic amino acid region in their N terminus that binds heparin (42). These sites have been postulated to be important in the storage and stabilization of BMPs (43, 44). Also, interactions of BMPs with the extracellular matrix 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 extracellular matrix-binding behavior of BMPs. The role of the protease inhibitors and their relationship between BMPs and protease inhibitors in development remain to be investigated.

Acknowledgments—We thank Drs. Y. Eto, Ajinomoto Inc. for the activin A, S. Piccolo and E. De Robertis for the chordin-containing medium, Y. Sasai for the Xenopus chordin plasmid, S. Shimasaki for the human FS-288 protein, A. Hemmati-Brivanlou for the Xenopus follistatin plasmid, and R. Harland for the Xenopus noggin plasmid. We also thank Dr. K. Cho for beneficial comments and discussions.

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