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学 位 論 文 題 目	**STUDIES ON BONE MORPHOGENETIC PROTEIN-BINDING PROTEINS IN THE EARLY DEVELOPMENT OF *XENOPUS LAEVIS***
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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, he 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, he 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.

論文の審査結果の要旨

初期発生は異なる細胞集団の相互作用によって進行する。その細胞間相互作用には細胞増殖因子がメディエーターとして重要な機能を担っていることが知られている。さらに、細胞増殖因子はそれらの活性を制御する結合タンパク質などによって厳密に制御されていることも明らかにされつつある。主にアフリカツメガエルなど両生類を用いた研究から、骨形成タンパク質(bone morphogenetic protein, BMP) は中胚葉の腹側化因子として、また外胚葉における神経分化の抑制因子として作用していることが明らかにされている。しかしながら、BMP 活性の制御機構の全貌については不明な点が多かった。

本申請者は形態形成に必須の胚領域（オーガナイザー）に発現するフォリスタチン (follistatin) が TGF-β スーパーファミリーに属する BMP と相互作用することを表面プラズモン共鳴解析装置を用いて明らかにしたほか、ツメガエル初期胚においてフォリスタチンは BMP 活性を阻害することを示した。さらに、BMP とのタンパク質相互作用をモニターすることによって胚抽出液より BMP 結合タンパク質を同定し、その機能解析を行った。これらの研究は初期発生における細胞増殖因子の活性制御機構をタンパク質間相互作用に注目し解析することによって、発生生物学に新しい研究アプローチをもたらしたものといえる。本研究によって得られた成果は以下の通りである。

（1）カイコ虫体を用いたタンパク質発現系によって BMP-2、BMP-4 のリガンドタンパク質および両者の受容体であるタイプ I BMP 受容体（BMPRIA）の細胞外ドメインを高発現させ、純化した。また、両タンパク質の結合カイネティクスを表面プラズモン共鳴解析装置 BIACORE を用いて解析した。

（2）フォリスタチンは BMP と同様、TGF-β スーパーファミリーに属するアクチビンの結合タンパク質として知られていたが、本研究によってフォリスタチンは BMP にも結合しうることを証明し、フォリスタチンの初期胚における過剰発現で起こる異所的な神経誘導はアクチビンではなく BMP を阻害した結果であることを示した。これにより、今まで混沌としていたフォリスタチンによる神経誘導作用の解釈が、BMP 活性の阻害によるものであると結論づけられた。

（3）初期胚中に存在する新規 BMP 結合タンパク質を探索するために、BMP を固定化した BIACORE センサーチップを用いて初期胚抽出液から BMP 結合活性をスクリーニングした。結合活性を有する画分を純化し、結合タンパク質のひとつが Ep45 と呼ばれる肝臓で産生され卵へ輸送されるセリンプロテアーゼインヒビター様構造をもつタンパク質であることを明らかにした。Ep45 は TGF-β ファミリーの中でも TGF-β やアクチビンには結合しないことから、BMP に特異的な結合タンパク質であることがわかる。さらに、Ep45 は BMP とフォリスタチンの結合を阻害すること、キモトリプシンによる BMP の N 末端側のプロセシングを阻害することなどを明らかにした。

学位論文として提出された研究結果について口頭発表させた後、審査委員が論文内容について試問した。さらに、申請者の関連研究分野の一般知識およびその背景となる基礎知識についても口頭試問により審査した。これらの試問に対する申請者の応答はいずれも的確であったことから、関連領域に十分な知識を有しているものと思われる。

また、提出された論文は英語で書かれており、英語の能力についても適正であると考えられた。さらに、論文の一部は国際誌である Proc. Natl. Acad. Sci., USA および J. Biol. Chem.に発表されている。これらの結果をもとに審査委員会は申請者の学力は学位取得に値するものと判断した。