J. Exp. Biomed. Sci. 12 (2006) 343-348

Functional Analysis of the BMP4 Antagonists During *Drosophila* Embryo and Wing Development

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Drosophila Sog and vertebrate Noggin play important roles during development. They function as antagonists against BMP4 signaling and induce neural ectoderm during embryogenesis. They are also engaged in appendage formation by inhibiting BMP4 signaling during late development. To understand further functions of Sog, Supersog, which is a more potent form of Sog, and Noggin BMP4 antagonists during development, I performed the molecular genetic analysis using *Drosophila* embryogenesis and wing formation as assay systems. In cellular blastoderm embryos, Sog inhibited Dpp signaling, *Drosophila* BMP4 signaling, whereas Supersog or Noggin did not block Dpp signaling. During wing formation, Sog inhibited Sax type I receptor of Dpp signaling whereas Noggin inhibited Tkv type I receptor of Dpp signaling. However, Supersog inhibited both Sax and Tkv type I receptors. These results suggest that functions of BMP4 antagonists are developmental stage dependent and indicate that each BMP4 antagonist inhibits BMP4 signaling by blocking different BMP4 receptors.

Key Words: BMP4 antagonist, *Drosophila*, Embryo, Wing, Development

INTRODUCTION

Bone morphogenic proteins (BMP) function as morphogens in vertebrates and invertebrate development and are required for various cell fate decisions during development (Massague and Chen, 2000; Balemans and Van Hul, 2002; Cadigan, 2002). In *Drosophila*, short gastrulation (Sog) inhibit Dpp signaling, which is a functional ortholog of vertebrate BMP4 signaling, as an extracellular antagonist (Francois et al., 1994; Biehs et al., 1996). During embryogenesis, *sog* mutants show the phenotype of decreased neural ectoderm whereas *dpp* mutants have the phenotype of increased neural ectoderm. Sog promotes neural ectoderm formation by blocking Dpp signaling (Biehs et al., 1996). In vertebrates, injection of *noggin* mRNA into frog embryos induces dorsal ectoderm to form neural tissue (Smith and Harland, 1992). Noggin blocks BMP4 signaling as an extracellular antagonist by binding to the BMP4 ligand and inhibiting BMP4 binding to its receptors (Zimmerman et al., 1996; McMahon et al., 1998).

During wing development in *Drosophila*, *sog* and *dpp* genes are involved in wing vein formation. The *dpp* gene is expressed in wing vein precursors and promotes wing vein formation whereas the sog gene is expressed in the intervein cells and suppresses wing vein formation. In the intervein region, Sog inhibits Dpp signaling to suppress vein formation (Yu et al, 1996). During the digit formation of chicken limb development, Noggin soaked beads between digits changes the identities of digits. BMP4 signaling activity between the digits determines the identity of digits, which is modulated by BMP4 antagonist Noggin (Dahn and Fallen, 2000).

Sog contains four copies of a cysteine repeat (CR) domain consisting of ~70 amino acids defined by 10 cysteins and a tryptophan (W) residue between the first two cysteines (Francois et al., 1994). The CR1, located after a transmembrane (TM) domain, is separated from CR2-CR4 by the intervening 565 amino acids (Fig. 1). During wing development Sog inhibits Gbb, which is another BMP4 ortholog in *Drosophila* (Wharton et al., 1991; Khalsa et al., 1998).

*Received: October 4, 2006
Accepted after revision: November 2, 2006
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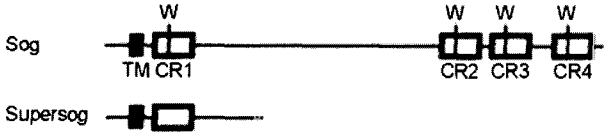

Fig. 1. Sog and Supersog protein structures. Sog contains 1,024 amino acids with four copies of a cysteine repeat (CR) domain consisting of ~70 amino acids defined by 10 cysteins and a tryptophan (W) residue between the first two cysteines. Supersog consists with CR1 and the part of intervening amino acids.

However, Supersog, a more potent form of Sog consisting with CR1 and the part of intervening amino acids (Fig. 1), inhibits both Dpp and Gbb and functions as the broad BMP signaling antagonist (Yu et al., 2000; Yu et al., 2004).

BMP ligands transduce a signal through the dimerization of type I and type II receptor kinases (Heldin et al., 1997). In *Drosophila*, type I receptors are Tkv and Sax while a type II receptor is Punt (Brummel et al, 1994; Nellen et al., 1994; Letsou et al., 1995). Dpp transduces a signal through the Tkv/Punt receptor dimmer whereas Gbb transduces a signal through the Sax/Punt receptor dimmer. Both Tkv/Punt and Sax/Punt receptor kinases activate downstream Smad kinase cascades and turn on target genes (Haerry et al., 1998).

In this report, I investigated developmental functions of Sog, Supersog, and Noggin BMP4 antagonists using *Drosophila* early embryogenesis and wing formation as assay systems.

MATERIALS AND METHODS

1. Fly stocks

Several independent transgenic lines for each pUAS construct were produced by the p-element mediated germline transformation. Double over-expressing pUAS lines were generated genetically. Bicoid-Gal4 driver (bcd>) is expressed in the anterior portion of early embryos up to the blastoderm stage (Janody et al., 2000). MS1096-Gal4 driver (ubi>) is ubiquitously expressed during wing development (Capdevila and Guerrero, 1994).

2. Generation of constructs

cDNAs encoding the full length of coding regions of *sog, noggin, and dpp* (Yu et al., 2000), *supersog* (Yu et al., 2004), and dominant negative (DN) form of *sax* and *tkv* (Haerry et al., 1998) were inserted into the pUAS expression vector

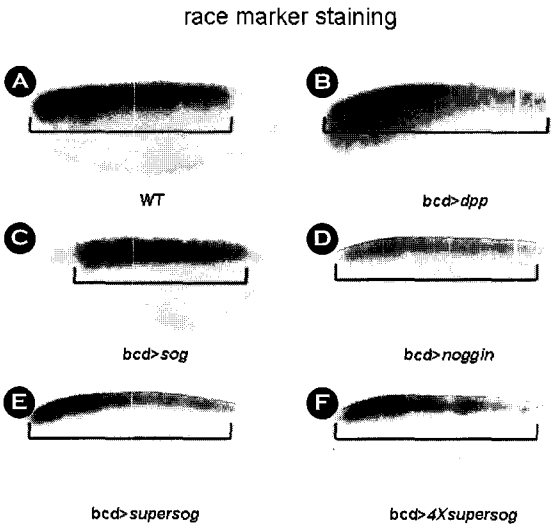

Fig. 2. *In situ* hybridization with the *race*, a target gene of Dpp signaling, probe in various cellular blastoderm embryos. **(A)** Wild-type embryo. **(B)** bcd>*dpp* embryo. **(C)** bcd>*sog* embryo. **(D)** bcd>*noggin* embryo. **(E)** bcd>*supersog* embryo. **(F)** bcd>*4Xsupersog* embryo.

(Brand and Perrimon, 1993) using appropriate restriction sites.

3. *In situ* hybridization to whole mount embryos

In situ hybridization to whole mount embryos was performed with digoxigenin-labeled probes and visualized as a blue alkaline phosphatase precipitate (O'Neill and Bier, 1994).

4. Mounting fly wings

Wings from adult flies were dissected in ethanol and mounted in the Canadian Balsam mounting medium.

RESULTS

1. Sog, not Supersog or Noggin, inhibited Dpp signaling in early embryogenesis

Using the UAS/Gal4 genetic binary system (Brand and Perrimon, 1993), I investigated expression of the *race* gene, which is a target gene of Dpp signaling (Tatei et al., 1995), in bcd>*sog*, bcd>*supersog*, bcd>*4Xsupersog*, bcd>*noggin*, and bcd>*dpp* cellular blastoderm embryos (Fig. 2). Wild-type embryo showed expression of the *race* marker gene in the dorsal part and up to 80% of an embryo from the anterior tip (Fig. 2A, bracket). In the bcd>*dpp* embryo, the anterior part of *race* expression was thickened, confirming

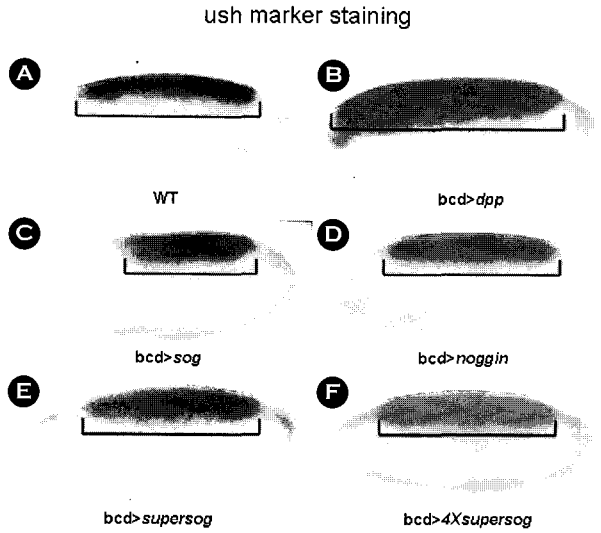

Fig. 3. *In situ* hybridization with the *ush*, a target gene of Dpp signaling, probe in various cellular blastoderm embryos. **(A)** Wild-type embryo. **(B)** bcd>*dpp* embryo. **(C)** bcd>*sog* embryo. **(D)** bcd>*noggin* embryo. **(E)** bcd>*supersog* embryo. **(F)** bcd>*4Xsupersog* embryo.

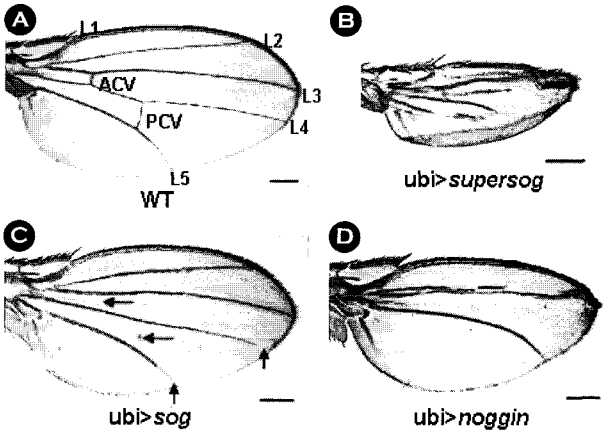

Fig. 4. Wing phenotypes of *sog*, *supersog*, and *noggin* BMP4 antagonist genes with the ubiquitously expressed MS1096-Gal4 driver (ubi>). **(A)** Wild-type wing. **(B)** ubi>*supersog* wing. **(C)** ubi>*sog* wing. **(D)** ubi>*noggin* wing. Bars are 0.2 mm.

that *race* is a target gene of Dpp signaling (Fig. 2B). In the bcd>*sog* embryo, the anterior part of *race* expression was missing compared to those from wild-type and bcd>*dpp* embryos (Fig. 2C compared to 2A & 2B), indicating that Sog blocks Dpp signaling in early embryos. However, in bcd>*supersog* and bcd>*noggin* embryos, *race* expression was up to 80% of the embryo from the anterior tip, which was similar with that of the wild-type embryo even though intensities of expression were weak (Fig. 2D & 2E compared to 2A). Moreover, bcd>*4Xsupersog* containing 4 copies of *supersog* trangenes also showed the similar expression pattern as bcd>*supersog* (Fig. 2F compared to 2E). These results indicate that Supersog and Noggin can not inhibit Dpp signaling in the blastoderm embryos.

I also investigated expression of the *ush* gene, which is another target gene of Dpp signaling (Rusch and Levine, 1997), in bcd>*sog*, bcd>*supersog*, bcd>*4Xsupersog* bcd>*noggin*, and bcd>*dpp* cellular blastoderm embryos (Fig. 3). The *ush* gene was expressed in the dorsal middle part of the wild-type embryo (Fig. 3A, bracket). The *ush* expression was thickened and expended to the anterior side in the bcd>*dpp* embryo, confirming that *ush* is a target gene of Dpp signaling (Fig. 3B). In the bcd>*sog* embryo, the lack of the anterior part of *ush* expression comparing with wild-type and bcd>*dpp* embryos was observed (Fig. 3C compared to 3A & 3B). It indicates that Sog blocks Dpp signaling in early embryos. In bcd>*supersog*, bcd>*4Xsupersog* bcd>*noggin* embryos, *ush* was expressed in the dorsal middle part like that of the wild-type embryo even though intensities were strong (Fig. 3D, 3E, & 3F compared with 3A). These results also indicate that Supersog and Noggin can not inhibit Dpp signaling in the blastoderm embryos.

2. Phenotypes of *sog*, *supersog*, and *noggin* genes were different in *Drosophila* wing

Drosophila wild-type wing has obvious five longitudinal veins, designated as L1-L5, anterior cross vein (ACV), and posterior cross vein (PCV) (Fig. 3A). When the *sog* gene was expressed with the MS1096-Gal4 driver (ubi>), ACV, PCV, and tips of L4 and L5 were missing (Fig. 4C, arrows). The phenotype of ubi>*supersog* wing was different with the phenotype of ubi>*sog* wing. The size of ubi>*supersog* wing was small and the veins were truncated or missing (Fig. 4B). The phenotype of ubi>*noggin* wing was also different with the phenotype of ubi>*sog* or ubi>*supersog* wing. L2 and L3 were fused, and L4 and CVs were missing (Fig. 4D). These results indicate that BMP4 antagonists may inhibit different BMP4 ligands and showed different phenotypes in the wing.

3. Sog, Supersog, and Noggin inhibited different type I receptors of BMP4 signaling

Dominant negative form of Sax (DN-sax) or Tkv (DN-tkv) receptors, which can bind ligands but can not transduce a signal into down-stream signaling components, were used

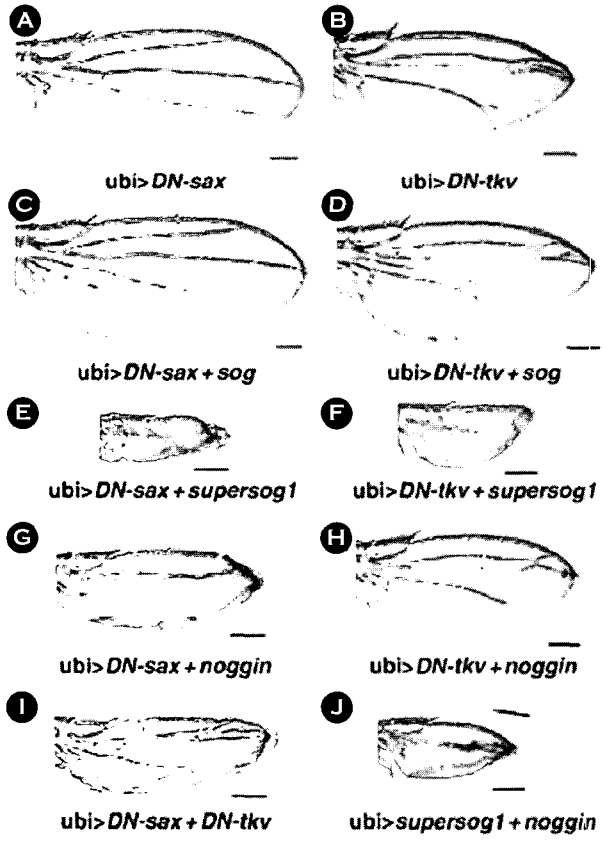

Fig. 5. Genetic interactions of *sog*, *supersog*, and *noggin* BMP4 antagonist genes with *sax* and *tkv* type I receptor genes of Dpp/ BMP4 signaling. **(A)** ubi>*Dominant Negative(DN)-sax* wing. **(B)** ubi>*DN-tkv* wing. **(C)** ubi>*DN-sax+sog* wing. **(D)** ubi>*DN-tkv+sog* wing. **(E)** ubi>*DN-sax+supersog* wing. **(F)** ubi>*DN-tkv+supersog* wing. **(G)** ubi>*DN-sax+noggin* wing. **(H)** ubi>*DN-tkv+noggin* wing. **(I)** ubi>*DN-sax+DN-tkv* wing. **(J)** ubi>*supersog+noggin* wing. Bars are 0.2 mm.

to investigate the genetic interactions with BMP4 antagonists. Ubi>*DN-sax* wing showed the thin, truncated L5, and missing PCV phenotype while Ubi>*DN-tkv* wing showed the fused L2/L3 and L4/L5 and missing ACV and PCV phenotype (Fig. 5A & 5B). The wing phenotypes of Ubi> *DN-sax+sog* and Ubi>*DN-tkv+sog* were indistinguishable with the phenotypes from Ubi>*DN-sax* and Ubi>*DN-tkv* wing respectively (Fig. 5C & 5D compared to Fig. 5A & 5B). However, Ubi>*DN-sax+supersog* and Ubi>*DN-tkv+ supersog* showed very small size wing and all fused vein phenotypes, which were very strong phenotypes compared with the phenotypes from Ubi>*DN-sax* and Ubi>*DN-tkv* wing (Fig. 5E & 5F compared to Fig. 5A & 5B). These results indicate that Supersog blocks both Sax and Tkv receptors. In addition, ubi>*DN-sax+noggin* and ubi>*DN-sax+DN-tkv* wing showed the similar phenotypes each other (Fig. 5G & 5I) while ubi>*DN-tkv+noggin* wing showed the very similar phenotype with ubi>*noggin* (Fig. 5H & 4D). The phenotype of ubi>*supersog+noggin* was also very similar to that of ubi>*DN-tkv+supersog* (Fig. 5J & 5F). These results indicate that Noggin inhibits the Tkv receptor of Dpp signaling.

DISCUSSION

Various BMP4 antagonists were found during vertebrate and invertebrate development. In vertebrate embryogenesis, Noggin, Chordin, Sog otholog in vertebrates, Follistain BMP4 antagonists are produced from the Spaman's organizer and block BMP4 signaling to form neural ectoderm (Piccolo et al., 1996; Zimmerman et al., 1996; Iemura et al., 1998). But, in *Drosophila* embryogenesis, only Sog blocks Dpp signaling to induce neural ectoderm (Biehs et al., 1996). It is interesting that, when either Supersog or Noggin was expressed in early *Drosophila* embryos, they did not suppress the Dpp signaling (Fig. 2 & 3). It suggests that Supersog or Noggin did not function and can not induce neural ectoderm in early *Drosophila* embryos. These results also suggest that, even though the overall mechanisms of neural ectoderm induction by Sog/Dpp in *Drosophila* and BMP4 antagonists/BMP4 ligands in vertebrates are evolutionary conserved (Holley et al., 1995; De Robertis and Sasai., 1996), the detailed mechanisms are different.

Sog, Supersog, Noggin showed different phenotypes in the fly wing (Fig. 4), confirming that each BMP4 antagonist inhibits different BMP4 ligand: Sog blocks Gbb, Noggin blocks Dpp, and Supersog blocks both Dpp and Gbb (Yu et al., 2000). It also indicates that only Sog is working both embryogenesis and wing formation as an BMP4 antagonist while functions of Supersog and Noggin are limited to late development.

Dpp functions through Tkv type I receptor while Gbb functions through Sax type I receptor during wing formation (Haerry et al., 1998). The results of genetic interactions between BMP4 antagonists and type I receptors turned out that Sog blocks Sax receptor, Noggin blocks Tkv receptor, and Supersog blocks both Sax and Tkv receptors (Fig. 5). These data collectively indicate that Sog blocks Gbb ligand - Sax receptor signaling, Noggin blocks Dpp ligand - Tkv receptor signaling, and Supersog blocks both Dpp ligand - Tkv receptor and Gbb ligand - Sax receptor signaling during

Drosophila wing development.

In this study using Drosophila early embryogenesis and wing formation as assay systems, I found that each BMP4 antagonist was developmental stage dependent and inhibited different BMP4 type I receptors. Because BMP4 antagonists play important roles during embryo development, organogenesis, and limb development in vertebrates, defects of BMP4 signaling induce embryonic lethality or malformations of organs. The results from this study will be applicable to investigate BMP signaling in vertebrates including human.

Acknowledgements

This work was supported by the grant No. RO1-2003-000-10762-0 from the basic research program of KOSEF.

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