

HtrA1 serine protease inhibits signaling mediated by Tgf β family proteins

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Summary

HtrA1, a member of the mammalian HtrA serine protease family, has a highly conserved protease domain followed by a PDZ domain. Because HtrA1 is a secretory protein and has another functional domain with homology to follistatin, we examined whether HtrA1 functions as an antagonist of Tgf β family proteins. During embryo development, mouse *HtrA1* was expressed in specific areas where signaling by Tgf β family proteins plays important regulatory roles. The GST-pulldown assay showed that HtrA1 binds to a broad range of Tgf β family proteins, including Bmp4, Gdf5, Tgf β s and activin. HtrA1 inhibited signaling by Bmp4, Bmp2, and Tgf β 1 in C2C12 cells, presumably by

preventing receptor activation. Experiments using a series of deletion mutants indicated that the binding activity of HtrA1 required the protease domain and a small linker region preceding it, and that inhibition of Tgf β signaling is dependent on the proteolytic activity of HtrA1. Misexpression of *HtrA1* near the developing chick eye led to suppression of eye development that was indistinguishable from the effects of noggin. Taken together, these data indicate that HtrA1 protease is a novel inhibitor of Tgf β family members.

Key words: HtrA1, Tgf β , Bmp, Serine protease, Noggin, Follistatin

Introduction

HtrA (DegP) was initially identified in *E. coli* as a heat shock protein that was attributed to tolerance of elevated temperatures (for a review, see Clausen et al., 2002). It was soon found that *HtrA* exists ubiquitously in microbes, plants and animals. The HtrA family shares a highly conserved trypsin-like serine protease (SP) domain and one or two C-terminal PDZ domains. In the N-terminal region, each HtrA protein has domains that most likely modulate physiological function, for example, limiting the protein to specific cellular compartments.

Human *HtrA2* (*omi*) is the most characterized member of mammalian *HtrA* genes. HtrA2 induces apoptosis in a caspase-independent manner through its protease activity and in a caspase-dependent manner via its ability to activate caspases (Suzuki et al., 2001). The crystal structure of HtrA2 has recently been elucidated (Li et al., 2002). In the crystals, three monomers of HtrA2 are assembled into a pyramid-shaped structure. The trimer structure is essential for the proteolytic activity of HtrA2.

Human *HtrA1* (*L56* or *RSPP11*) was originally isolated as a gene whose expression was downregulated in a human fibroblast cell line after transformation with SV40 (Zumbrunn and Trueb, 1996). Although HtrA1 and HtrA2 share homologous SP domains and PDZ domains in the C-terminal regions, HtrA1 contains a signal sequence for secretion, as well as an insulin-like growth factor binding protein (IGFBP) domain and a Kazal-type serine protease inhibitor (KI) domain

in the N-terminal region (see Fig. 5 for HtrA1 structure). Downregulation of human *HtrA1* has been repeatedly observed in ovarian cancers (Shridhar et al., 2002) and melanomas, in close correlation with the malignant progression and metastasis of these tumors (Baldi et al., 2002). Overexpression of *HtrA1* in highly invasive melanomas was shown to suppress proliferation and migration of tumor cells (Baldi et al., 2002). *HtrA1* is thus considered to be a tumor suppressor gene in certain cancers.

In a study to profile changes in gene expression in osteoarthritic cartilage, Hu et al. observed that human *HtrA1* expression was several fold higher in articular chondrocytes of osteoarthritic patients (Hu et al., 1998). They noticed a high degree of homology between the N-terminal region of HtrA1 and Mac25, a presumed member of IGFBPs. Although Mac25 was initially identified as an IGFBP, the affinities of Mac25 to IGFs are orders of magnitude lower than those of authentic IGF binding proteins, such as IGFBP3 (Oh et al., 1996). Indeed, Kato et al. noted that Mac25 was more closely related to follistatin, an activin-binding protein, and showed that Mac25 actually bound to activin (Kato et al., 2000). It was therefore proposed that HtrA1 regulates biological processes by modulating growth-factor systems other than IGF, such as the system mediated by the transforming growth factor β (Tgf β) family to which activin belongs. In an attempt to examine this possibility, we investigated the properties of mouse HtrA1 with respect to Tgf β signaling.

The Tgf β family consists of more than 30 proteins in mammals, including Tgf β s, Bmps, growth/differentiation factors (Gdfs) and activin (for a review, see Massagué and Chen, 2000). These proteins are expressed in distinct and complex spatio-temporal patterns during embryogenesis and in adult tissues, playing pivotal roles in the development and homeostasis of these tissues. Although Tgf β signaling can be regulated at various steps, it is largely regulated extracellularly through receptor activation. A group of antagonists have been identified that bind to Bmp subfamily proteins in the extracellular space, thereby interfering the receptor activation. Noggin and chordin are antagonists of several Bmps, whereas follistatin is an antagonist of activin. New members, like Dan family proteins, are continuously being added to the list of Bmp antagonists (Baemans and Van Hul, 2002). However, there are only a few proteins known to be antagonists of the Tgf β subfamily.

We report that the expression pattern of mouse *HtrA1* shows a striking correlation with the sites where signaling of the Tgf β family regulates development. We also present evidence that HtrA1 binds to a broad range of Tgf β proteins and inhibits their signaling in vitro as well as in vivo. Surprisingly, the binding and inhibitory activities of HtrA1 on Tgf β proteins depend on the integrity of the HtrA1 serine protease domain.

Materials and methods

Plasmids and other reagents

A 604-bp fragment of mouse *HtrA1* was amplified using a set of PCR primers (MS-8F, 5'-CGCTTATCAAGATTGACCAACC-3'; and MS-8R, 5'-TTGATGACATCGCTGACG-3') that were designed from an EST (AA028600) sequence. This fragment was used as a probe to isolate full-length 2-kb cDNA of mouse *HtrA1* (GenBank Accession Number AF172994) from an adult mouse brain cDNA library. pCDNA3 vector (Invitrogen) was used to express full-length HtrA1 and its mutant forms in mammalian cells. Deletion mutants of HtrA1 were constructed using appropriate restriction enzyme fragments. To produce the S328A mutant, the serine at amino acid (aa) position 328 was substituted with alanine by PCR mutagenesis. Human *follistatin* cDNA (kind gift of N. Ueno, National Institute for Basic Biology, Okazaki) was tagged with a human myc epitope sequence at the 3' coding end and cloned into pCDNA3. Other DNAs obtained were: luciferase reporter plasmids, pGL3-Id985 WT and pGL3ti-(SBE)₄ from T. Katagiri, Showa University, Tokyo and W. Kruij, Biological Center, Kercklaan, respectively; mouse *Smad* cDNA from K. Miyazono, University of Tokyo; *Xenopus* noggin expression vector from Y. Takahashi, RIKEN, Kobe; mouse *Bmp4* cDNA from D. Constam, Swiss Institute for Experimental Cancer Research; and a constitutively active Bmp type I receptor (*caBMPRI-IB*) cDNA in pEF-BOS from T. Katagiri, Showa University, Tokyo. Human recombinant Bmp2 protein was provided by Yamanouchi Pharmaceuticals, Tokyo.

In situ hybridization of mRNA

Whole embryos and tissues were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) overnight at 4°C. Whole-mount and section in situ hybridization were performed as previously described (Sasakli and Hogan, 1994; Morimoto et al., 1996). Non-overlapping fragments of *HtrA1* cDNA, a *BalI-BamHI* fragment (nucleotide 644-1191) and a *BamHI-HincII* fragment (nucleotide 1191-1750), cloned in BluescriptKSII(+) (Stratagene) were used as templates to synthesize digoxigenin-labeled RNA probes using a DIG-RNA labeling kit (Boehringer).

Antibodies, immunostaining and western blot analysis

N- and C-terminal HtrA1 fragments (aa 28 to 143 and aa 385 to 480)

produced in *E. coli* were injected into rabbits to raise antibodies, and antiserum was affinity purified. Purified polyclonal antibodies against the N-terminal fragment were used for immunohistochemistry. This antibody did not cross react with mouse HtrA3. Immunohistochemical staining was carried out using the tyramide signal amplification-avidin-biotin-complex method as previously described (Toda et al., 1999). Adult bones were decalcified in 10% EDTA (pH 7.4), and embedded in paraffin wax. For western blot analysis, protein samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Membranes were probed with the appropriate primary antibodies and then with alkaline phosphatase-labeled secondary antibodies, followed by the BCIP/NBT color reaction.

GST pull-down assay

The cDNA fragments encoding the mature peptides of mouse Tgf β 1, Tgf β 2, Bmp4, Gdf5 and activin were cloned into the pGEX-4T-1 vector (Stratagene) in-frame using *BamHI* and *EcoRI* sites. All GST fusion proteins were expressed in insoluble fractions. They were solubilized in 8 M urea containing buffer and then renatured, as previously described (Groppe et al., 1998). The renatured proteins were dialyzed against the GST pull-down buffer [20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 0.05% NP-40, 10% glycerol]. HtrA1, its mutant forms and myc-tagged follistatin were produced in sf9 cells using a baculovirus system. Each sf9 culture supernatant was mixed with one of the GST fusion proteins and incubated overnight at 4°C in 400 μ l of 20 mM Tris-HCl (pH 8.0), 1.5 mM CaCl₂, 3 mM MgCl₂, 5% glycerol, 0.1% NP-40, 0.3-1.0 M NaCl, 5% fetal bovine serum (FBS) to minimize non-specific binding. Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) (20 μ l) were added to the mixture and incubated for 1 hour at 4°C. Beads were recovered by centrifugation and washed five times in 500 μ l GST pull-down buffer. Proteins bound to the beads were separated by SDS-PAGE and analyzed by western blotting, as described above. Pull-down assays in the presence of 1.0 M NaCl gave the most specific and reproducible results.

Solid phase binding assay

ELISA plate (Nunc) wells were coated with 50 μ l of either 10 μ g/ml of GST or GST-Bmp4 in 20 mM Tris-HCl (pH 7.5) and 500 mM NaCl at 4°C overnight. The wells were washed twice with a wash buffer [0.05% Tween 20 in TBS (20 mM Tris-buffered saline, pH 7.5)] and non-specific binding sites were blocked with 0.1% skimmed milk in wash buffer. Various amounts of myc-tagged HtrA1 protein in Dulbecco's Modified Essential Medium (DMEM) containing 10% FBS were added to the wells and incubated at 4°C overnight. The wells were then washed twice with wash buffer, incubated with anti-myc antibody, washed five times with wash buffer, and then incubated with alkaline phosphatase-conjugated anti-mouse IgG antibody. The bound phosphatase was assayed using *p*-nitrophenyl phosphate. The reaction was stopped with 100 mM EDTA, and absorbance at 405 nm was measured. The difference between the absorbance value for GST-Bmp4 and that for GST alone was considered to represent specific binding and was used to determine the K_d value. The concentration of HtrA1 in the medium was quantitated by western blotting with anti-HtrA1 antibody using bacterially produced HtrA1 as the standard.

Cell culture, transfection and luciferase assay

Mouse C2C12 myoblasts were cultured in DMEM supplemented with 15% FBS in 24-well plates. Four hours before transfection, growth medium was replaced with fresh medium containing 2% FBS. Transfection of cells was carried out using the calcium phosphate method with 125 ng of a β -galactosidase expression vector as an internal control and other plasmid DNAs, as described in the figure legends. Cell extracts were prepared 24 hours after transfection and assayed for luciferase activity using the Picagene kit (Toyo Ink Co. Ltd, Tokyo). Luciferase activity was corrected for transfection

efficiency using β -galactosidase activity. Transfection of C2C12 cells with the *Bmp4* or *caBMPR-IB* expression vector stimulated the pGL3-Id985WT reporter several fold, as described previously (Pearce et al., 1999; Katagiri et al., 2002). *Smad1* and/or *Smad4* expression further stimulated the pGL3-Id985WT or pGL3ti-(SBE)₄ reporters in the presence of *Bmp4* or *Tgfβ1*, respectively, although *Smad* expression alone did not activate those reporters.

Misexpression of *HtrA1* in chick embryo

HtrA1 cDNA was inserted into the pCAGGS vector in two orientations to express sense and antisense strands. Cultured 293T cells were co-transfected with an EGFP expression vector and a pCAGGS vector, containing sense or antisense cDNA, using Lipofectamine 2000 (Invitrogen). The pEF-Bos-noggin vector was used to express noggin. Cells were harvested 48 hours after transfection and suspended in a small amount of culture medium. Cell suspension (less than 0.1 μ l) was injected into the extraocular mesenchyme.

The pCAGGS-*HtrA1* vector and the EGFP vector were electroporated into the optic vesicles, as described previously (Koshiba-Takeuchi et al., 2000). After incubation for 24-27 hours, embryos were subjected to whole-mount in situ hybridization using a 1.4-kb *cVax* probe.

Results

Expression of *HtrA1*

HtrA1 mRNA was not detectable at 9.5 dpc or in earlier embryos by both northern analysis and RT-PCR (data not shown). It was first detected at 10.5 dpc and levels increased at 14.5 dpc, remaining then at the same level until born.

In order to precisely locate *HtrA1* expression, we carried out in situ hybridization and immunostaining of embryos and adult tissues. Two non-overlapping probes were used for in situ hybridization and both probes gave identical results. For immunostaining, we used polyclonal antibodies raised against the N-terminal region of *HtrA1* (aa 28-143). The results of immunostaining were consistent with those of in situ hybridization in various tissues (see Fig. 2D,E), ensuring the specificity of this antibody. In the 12.5 dpc embryo, *HtrA1* mRNA was expressed in a pattern closely associated with skeletal elements of the vertebral column and limbs (Fig. 1A). In the sagittal section of the 12.5 dpc embryo, expression of *HtrA1* was also found in the brain, the heart, the lungs, the gonads and the skin of the thoraco-abdominal region (Fig. 1B). Expression of *HtrA1* became enhanced at 14.5 dpc (Fig. 1C). In the skeletal tissues, *HtrA1* was expressed in rudiments of tendons and ligaments along the vertebrae, as well as in mesenchymal cells surrounding precartilaginous condensations (Fig. 1B,C,D). In the brain, *HtrA1* was expressed in specific regions of the neuroepithelium in the forebrain and hindbrain (Fig. 1B), in close association with development of the choroid plexus. This was clearly seen in the hindbrain of the 14.5 dpc embryo (Fig. 1E), where expression of *HtrA1* was detected in the neuroepithelium adjacent to the forming choroid plexus. The mature choroid plexus, however, did not express *HtrA1* (Fig. 1E). It has been reported that several members of the *Bmp* subfamily are expressed in the developing brain with patterns characteristic to each member. Interestingly, choroid plexus development sites are those regions where all *Bmps* are co-expressed (Furuta et al., 1997).

In the heart of 11.5 dpc embryos, *HtrA1* was expressed in the atrioventricular (AV) endocardial cushion and the outflow

tract (Fig. 1F). At 12.5 dpc, *HtrA1* was expressed in the endocardial cushion (Fig. 1B,G), and at 14.5 dpc it was strongly expressed in the outflow tracts, including valves (Fig. 1C). Development of AV cushions and outflow tracts depends on signaling by *Tgfβ* proteins. *Tgfβ* induces epithelial-mesenchymal transition (EMT) of endothelial cells in these regions, endowing them with mesenchymal properties (Nakajima et al., 2000). Indeed, *Tgfβ2* knockout mice show defects in the cardiac septa and valves due to disturbed EMT (Sanford et al., 1997). Another well-known example of EMT is seen in the regression of the Müllerian duct in male gonads (Roberts et al., 2002). A diverse member of the *Tgfβ* family, anti-Müllerian hormone (AMH), induces apoptosis and EMT in Müllerian duct cells, initiating regression. *HtrA1* was expressed in Müllerian duct cells and the surrounding mesenchyme in both male and female gonads (Fig. 1B,H). *HtrA1* mRNA was detected in the mesenchymal cells beneath the lung epithelium (Fig. 1B,C,I), where epithelial-mesenchymal interactions mediated by *Tgfβ* family members play important roles in development and branching of respiratory tracts (Warburton et al., 2000). In the skin of a 12.5 dpc embryo, both epidermis and dermis expressed *HtrA1* (Fig. 1B). *HtrA1* mRNA was also detected in the epithelial component of developing whiskers (Fig. 1J) at 14.5 dpc. At later stages, *HtrA1* protein was localized in the basal layer of epidermis and in the invading epidermal cells that formed the whisker rudiments (Fig. 1K). Noggin, a potent inhibitor of *Bmps*, is expressed in the dermal papilla and regulates hair follicle formation (Botchkarev et al., 1999). At 9 days after birth when whiskers are nearly fully developed, *HtrA1* protein was detected in the outer root sheet (Fig. 1L). *Tgfβ* signaling has been suggested to regulate catagen induction during hair follicle regression by promoting apoptosis in the outer root sheet (Foitzik et al., 2000).

A striking correlation between the expression patterns of *HtrA1* and the functioning sites of various *Tgfβ* proteins, together with the presence of a domain similar to follistatin in *HtrA1* protein, suggests that *HtrA1* plays a role in *Tgfβ*-mediated signaling.

HtrA1 expression in developing skeletal elements

Because skeletal tissues are the most intensively studied organs in relation to *Tgfβ* signaling during morphogenesis, and because expression of *HtrA1* was reported to increase in articular cartilage cells of human arthritic patients (Hu et al., 1998), we conducted a detailed examination of *HtrA1* expression during development of bones and related tissues. As described above for 12.5 and 14.5 dpc embryos, *HtrA1* was expressed in undifferentiated mesenchymal cells surrounding precartilaginous condensations but was not detected in the condensation cores (Fig. 1B,C,D). However, within the precartilaginous condensations, joint interzones, which later form joint cavities, expressed *HtrA1* (Fig. 2A,B). Rudiments of tendons and ligaments that develop from mesenchyme in the vicinity of the precartilaginous condensations expressed *HtrA1* (Fig. 1D; Fig. 2B,C). In the process of endochondral ossification, matured chondrocytes become hypertrophic, degenerate and are then replaced by calcified bones. Although precartilaginous condensations and chondrocytes did not express *HtrA1*, ossification centers were found to strongly express *HtrA1*. The primary ossification centers expressing *HtrA1* mRNA as well

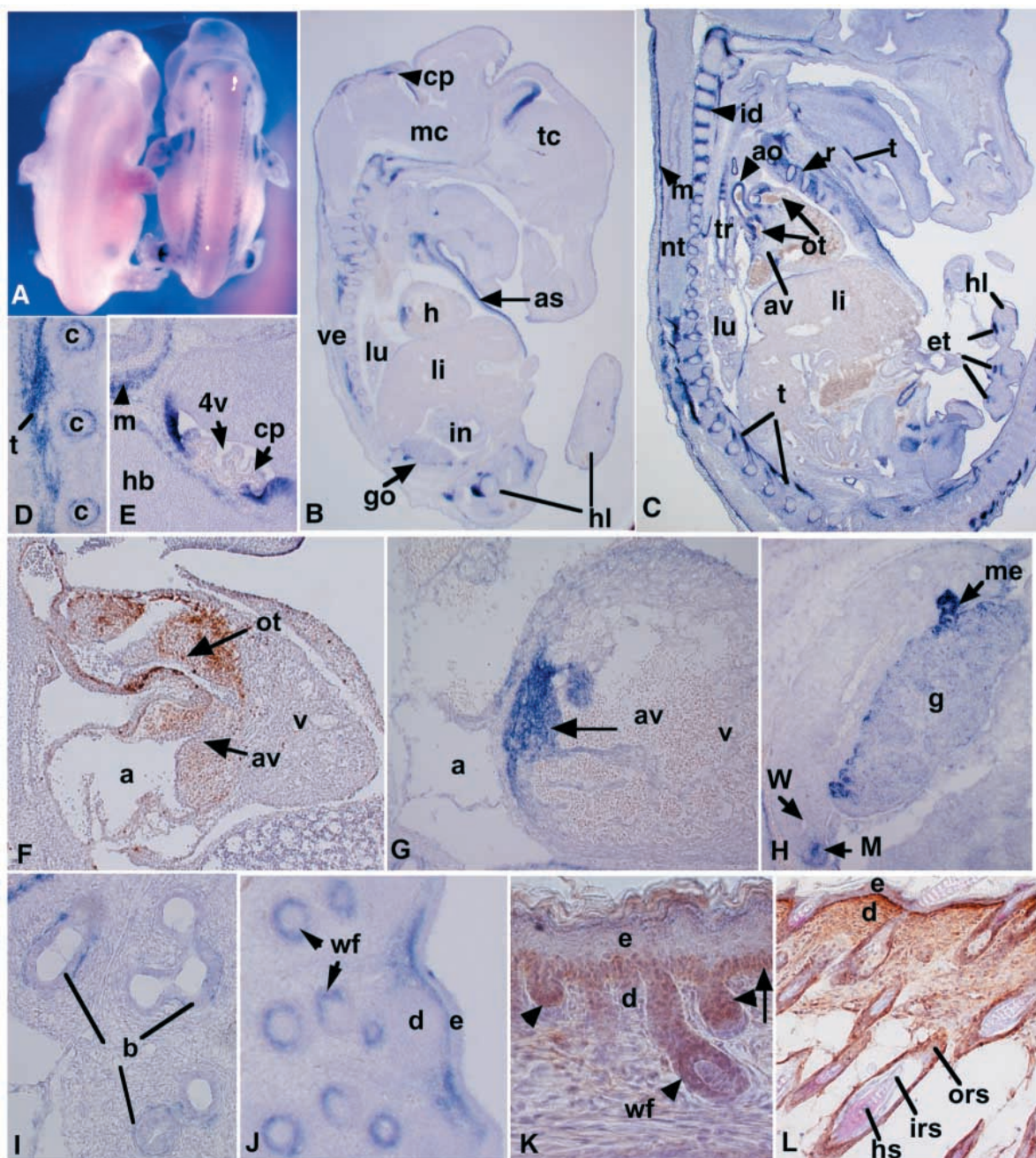


Fig. 1. Expression pattern of *HtrA1*. (A) Whole-mount in situ hybridization of 12.5 dpc embryo. Embryos were hybridized with a sense probe (left), or with an antisense probe (right). (B) In situ hybridization of a sagittal section of 12.5 dpc mouse embryo. *HtrA1* expression (blue signals) is seen in the vertebral column, hind limb, brain, gonad, heart, lung and abdominal skin. (C) In situ hybridization of a sagittal section of 14.5 dpc embryo. In addition to the sites mentioned in B, *HtrA1* expression is seen in the intervertebral disc, tracheal wall, cardiac outflow tracts and meninges. In the hind limb, *HtrA1* is expressed in the primordia of the extensor tendons. (D) Magnified view of B showing the vertebral column and ribs. (E) Magnified view of C showing the brain area near the fourth ventricle. Note that the meninges also expressed *HtrA1*. (F) Immunostaining of a transverse section through the heart at 11.5 dpc. *HtrA1* protein (brown) is detected in the AV cushion and outflow tract. (G) Magnified view of B showing the heart. *HtrA1* is expressed in the AV endocardial cushion. (H) Magnified view of B showing the gonad. The Wolffian duct does not express *HtrA1* but the mesonephric tubules do. (I) Magnified view of B showing the lung. (J) In situ hybridization of a section of 14.5 dpc maxillary skin. (K,L) Immunostaining of the skin. The eyelid of an 18.5 dpc embryo (K) and the skin from the back of a 9-day old pup (L) are shown. *HtrA1* protein is detected in the basal layer of epidermis (arrow), in the epithelial component of the developing whisker (arrowhead). *HtrA1* protein is also detected in the outer root sheets of whiskers of the 9-day pup. a, cardiac atria; ao, aorta; as, abdominal skin; av, atrioventricular cushion; b, bronchicles; c, condensing cartilage; cp, choroid plexus; d, dermis; e, epidermis; et, primordium of extensor tendon; g, gonad primordium; h, heart; hb, hind brain; hl, hind limb; hs, hair shaft; id, intervertebral disc; in, intestine; irs, inner root sheath; li, liver; lu, lung; m, meninges; mc, metacephalon; me, mesonephric tubules; nt, neural tube; ot, outflow tract; ors, outer root sheath; r, rib; t, tendon/ligament; tc, telencephalon; tr, trachea; v, cardiac ventricle; ve, vertebral column; W, Wolffian duct; wf, whisker follicle; 4v, 4th ventricle.

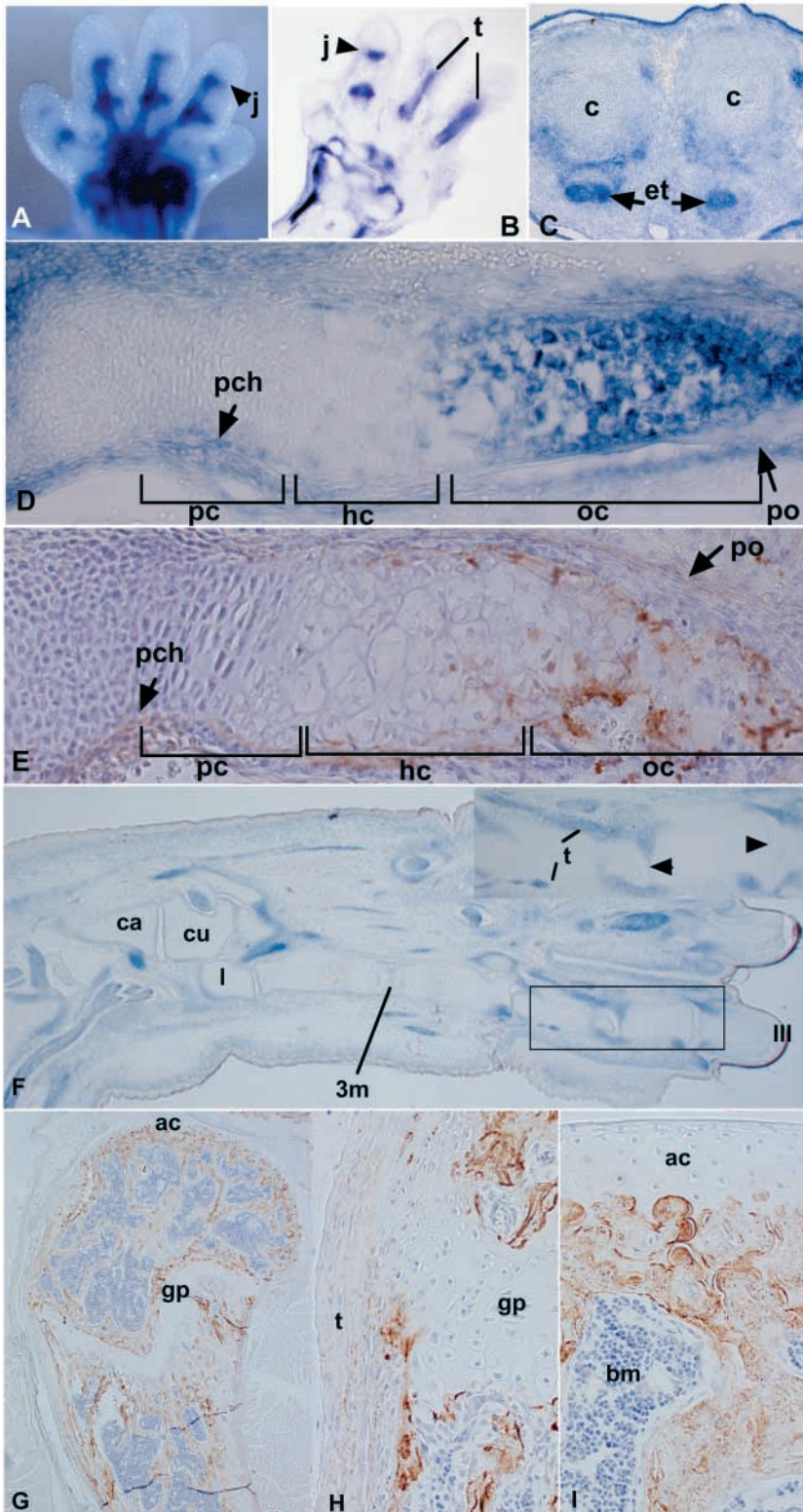


Fig. 2. *HtrA1* expression during the development of skeletal elements. (A) Whole-mount in situ hybridization of the 13.5 dpc forelimb. (B) Section of the 14.5 dpc limb. (C) In situ hybridization of a transverse section of the 14.5 dpc limb. (D) In situ hybridization of a 14.5 dpc rib section. (E) Immunostaining of a 15.5 dpc rib section. The localization of HtrA1 protein is in good agreement with that of mRNA shown in D. (F) In situ hybridization of the 17.5 dpc hind limb. Inset shows a magnified view of the boxed area. Low expression can be seen in a thin layer of the articular surface (arrowhead in inset). (G,H,I) Immunostaining of adult tibia. HtrA1 is detected in the bone matrix of the diaphysis and epiphysis. ac, articular cartilage; bm, bone marrow; c, cartilage nodule; ca, calcaneus; cu, cuboid; et, extensor tendon primordium; gp, growth plate; hc, hypertrophic cartilage; j, joint developing region; l, lateral cuneiform; oc, ossification center; pc, proliferating cartilage; pch, perichondrium; po, periosteum; t, tendon; III, digit III; 3m, 3rd metatarsal.

were detected only in the thin layer of articular surfaces (Fig. 2F). In adult bones, HtrA1 protein seemed to be deposited in the bone matrix (Fig. 2G,H,I), where Tgfb β 1 is known to accumulate at high concentrations (Kresse and Schönher, 2001). Cells in the tendons continued producing HtrA1 protein (Fig. 2H). No significant HtrA1 protein was detectable in the articular cartilage (Fig. 2G,I) or in the chondrocytes of the growth plate (Fig. 2G,H). However, when mice were challenged with an anti-collagen antibody cocktail in order to induce experimental arthritis, articular chondrocytes, especially those in the deep layer, started producing high levels of HtrA1 (A.T., unpublished).

The observed spatio-temporal expression patterns of *HtrA1* strongly suggest that HtrA1 regulates signaling of Tgfb β proteins.

Binding of HtrA1 to Tgfb β family proteins

In order to confirm the involvement of HtrA1 in Tgfb β signaling, we examined the binding activity of HtrA1 to Tgfb β family proteins. The mature forms of mouse Tgfb β 1, Tgfb β 2, Bmp4, Gdf5 or activin were expressed as GST-fusion proteins in *E. coli*. HtrA1 protein was expressed in a baculovirus expression system. First, we tested the binding of follistatin to these Tgfb β proteins. Follistatin bound most strongly to activin, weakly to Bmp4, but not to any other proteins (Fig. 3A,a). This binding specificity of follistatin was consistent with previous reports (Iemura et al., 1998), thus confirming the validity of our

binding assay. The GST pull-down assay revealed that HtrA1 protein bound to all Tgfb β family proteins tested (Fig. 3A,b).

The affinities of HtrA1 and follistatin for the Tgfb β family members were roughly compared by estimating the recovery of HtrA1 and follistatin in the GST pull-down assay. GST pull-down with GST-Bmp4 resulted in recovery of more than 3%

as the protein in the ribs of 14.5 and 15.5 dpc embryos are shown in Fig. 2, parts D and E, respectively. This figure also shows the rib perichondrium and periosteum expressing *HtrA1* (Fig. 2D,E). In the hind limb of a 17.5 dpc embryo, significant *HtrA1* expression persisted in tendons and ligaments, but expression in the forming joints was reduced and weak signals

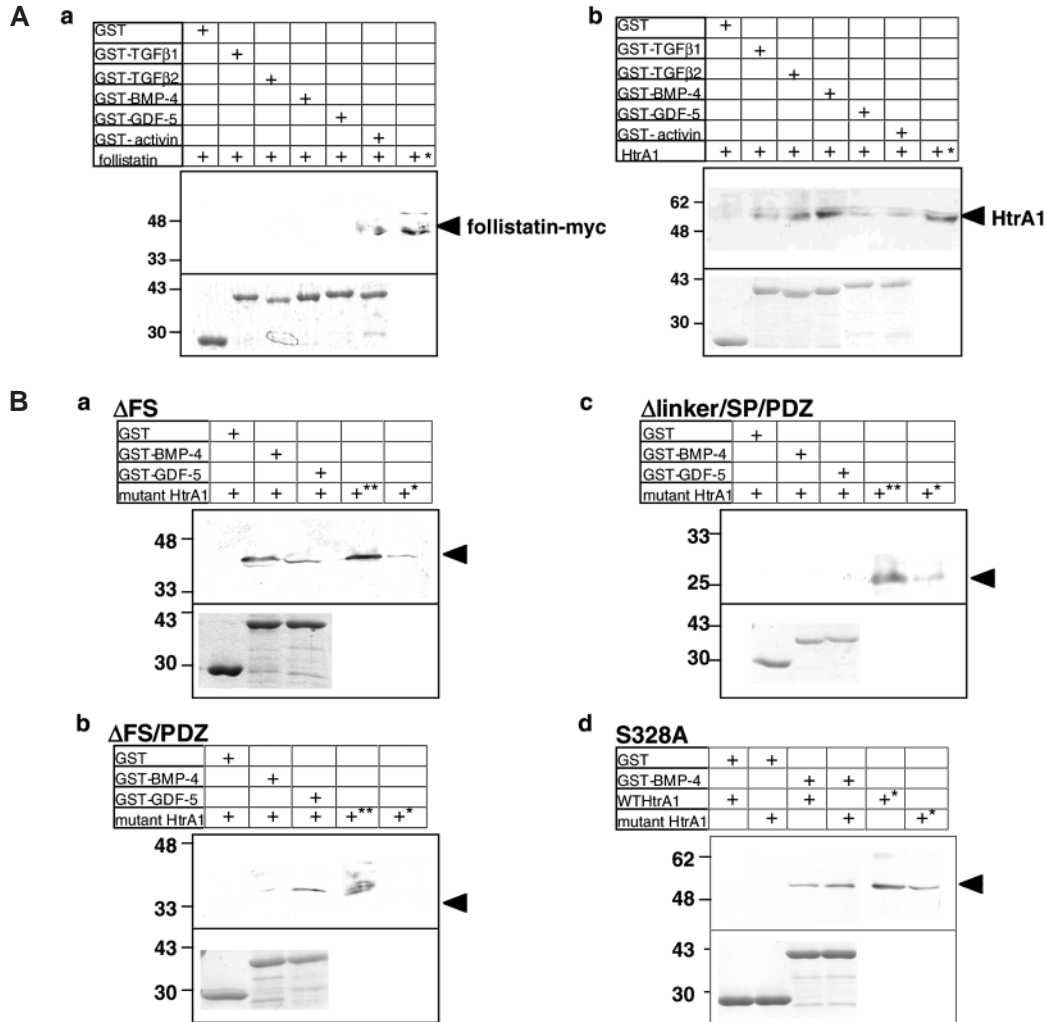


Fig. 3. Binding of HtrA1 to various Tgfβ family proteins. (A) Binding of HtrA1 and follistatin to Tgfβ family proteins. GST pull-down assay was carried out using GST-Tgfβ fusion proteins and either HtrA1 (b) or myc-tagged follistatin (a) in the presence 1.0 M NaCl. Bound HtrA1 or follistatin was detected by western blotting. The righthand lane in each panel (asterisk) was loaded with 3% input HtrA1 or follistatin. Lower panels show the recovered GST-Tgfβ fusion proteins stained with Coomassie blue. GST alone did not bind to HtrA1 (left-hand lanes). (B) Binding of mutant HtrA1 proteins to Bmp4 or Gdf5. Results of a GST pull-down assay with ΔFS (a), ΔFS/PDZ (b), Δlinker/SP/PDZ (c) and S328A (d) are shown. One or two lanes on the right were loaded with aliquots of input HtrA1 mutants; 10% (**) or 1% (*) of input in a and c, 20% (**) or 2% (*) in b, and 15% (*) of input in d. Arrowheads indicate positive positions of HtrA1 mutant proteins. (C) Solid phase binding assay of HtrA1. ELISA plate wells coated with GST or GST-Bmp4 were incubated with 100 μl of a solution containing myc-tagged HtrA1 protein (0.01-0.3 μg/ml). The binding of HtrA1 to GST (white triangle) or GST-Bmp4 (black square) was quantitated by colorimetric assay using anti-myc antibody.

(compare lanes 4 and 7 in Fig. 3A,b) but less than 10% (data not shown) of input HtrA1. This recovery was comparable to that of follistatin with GST-activin (Fig. 3A,a). This indicates that binding of HtrA1 to Bmp4 is significant. HtrA1 affinity for Tgfβ proteins decreased as follows:

Bmp4>Tgfβ2>Tgfβ1>activin>Gdf5 (Fig. 3A,b). HtrA1 did not show any significant affinity for epidermal growth factor (Egf) and basic fibroblast growth factor (bFgf) in this GST pull-down assay, supporting the specific binding of HtrA1 to Tgfβ proteins (data not shown).

To measure the binding affinity quantitatively, we developed a solid phase binding assay for HtrA1 (Fig. 3C). K_d of HtrA1 for Bmp4 was estimated to be 1.3 nM. This affinity was comparable with the reported affinities of follistatin (FS-315) for activin ($K_d=430$ pM) (Hashimoto et al., 2000), or of follistatin (FS-288) for Bmp4 ($K_d=23$ nM) (Iemura et al., 1998).

Inhibition of Bmp4 and Tgf β 1 signaling

We next examined whether HtrA1 was able to inhibit signaling mediated by Tgf β proteins. We assayed the effects of HtrA1 on Bmp2, Bmp4 and Tgf β 1 signaling by transcriptional assay in mouse C2C12 myoblast cells (Katagiri et al., 1994). For Bmp signaling we used a reporter plasmid, pGL3-Id985WT, in which a luciferase gene was driven by a Bmp-responsive promoter element derived from murine *Id* gene (Katagiri et al., 2002). Transfection of C2C12 cells with the *Bmp4* expression vector stimulated the *Id* promoter several fold, similar to as described by Pearce et al. (Pearce et al., 1999), and co-transfection of *Smad1* and *Smad4* expression vectors further stimulated the *Id* promoter up to 11-fold (Fig. 4A). Addition of the *HtrA1* expression vector inhibited Bmp4 signaling in a dose-dependent manner (Fig. 4A). The inhibitory activity of HtrA1 on Bmp4 signaling appeared to be weaker than that of noggin (Fig. 4A). The same promoter was similarly activated by addition of purified human recombinant Bmp2 protein to the culture medium. HtrA1 inhibited Bmp2 signaling to a similar degree as it did Bmp4 signaling (data not shown).

The effects of HtrA1 on Tgf β 1 signaling were examined using the pGL3ti-(SBE)₄ reporter. This reporter contains four tandem Smad-binding elements derived from the *Jun* promoter upstream of the luciferase gene (Jonk et al., 1998). Transfection of C2C12 cells with the *Smad4* expression vector and the addition of the recombinant human Tgf β 1 protein at a concentration of 10 ng/ml activated this reporter about 20-fold (Fig. 4B). Co-transfection of the HtrA1 expression vector inhibited Tgf β 1 signaling in a dose-dependent manner. Noggin is a specific antagonist of Bmps and does not bind to Tgf β 1 nor inhibit its signaling. Co-transfection of a noggin expression vector did not inhibit, but rather activated Tgf β 1 signaling.

The constitutively active Bmp4 receptor, caBMPR-IB, stimulates the *Id* promoter in the absence of ligands (Akiyama et al., 1997; Katagiri et al., 2002). HtrA1 did not inhibit the signal that originated from caBMPR-IB (Fig. 4D). This result excludes the possibility that HtrA1 inhibits intracellular signaling pathways.

Overexpression of *HtrA2*, a diverse member of the mammalian HtrA family, was shown to induce apoptosis (Li et al., 2002). We assayed cell death by propidium iodide staining after transfection of C2C12 with the *HtrA1* expression vector. The number of dead cells was not increased by *HtrA1* overexpression (data not shown). This excluded the possibility that *HtrA1* overexpression induces cell death in C2C12.

Domains necessary for binding and inhibition

The domains of HtrA1 protein involved in the binding to and the inhibition of signaling by Tgf β proteins were determined by producing a series of HtrA1 mutant proteins. A schematic presentation of the mutants is shown in Fig. 5. Because wild-type (WT) HtrA1 protein binds to Bmp4 and Gdf5 with the highest and the lowest affinities, respectively, we investigated

the binding of mutants to these two ligands. Inhibition on Bmp4 signaling was examined using the *Bmp4/Smad1/Smad4* co-transfection assay. Unexpectedly, we found that the protease domain and the linker region (aa 155-202) that precedes it, but not the follistatin-like domain, were essential for both activities.

A mutant (Δ FS) with a deletion in both the IGFBP and KI domains (here we combine these two domains and refer to them as FS) bound to both Bmp4 and Gdf5 (Fig. 3B,a) with affinities equivalent to those of wild-type HtrA1. Δ FS inhibited Bmp4 signaling quite efficiently (Fig. 4C). The deletion mutant (Δ linker/SP/PDZ), retaining only the FS domain, did not show binding activity (Fig. 3B,c) or inhibitory activity on Bmp4 signaling (Fig. 4C). The PDZ domain was not involved in the binding; a deletion mutant in both the FS domain and the PDZ domain (Δ FS/PDZ) retained binding activity to Bmp4 and Gdf5, as well as inhibitory activity on Bmp4 signaling (Fig. 3B,b; Fig. 4C). Results with other mutants are summarized in Fig. 5. When the N-terminal deletion was extended to the linker region (Δ FS/linker), binding and inhibitory activities were both diminished. The mutant (Δ SP/PDZ) lacking the SP domain was completely devoid of binding and inhibitory activities. These results indicate that the linker and SP domains are essential for binding to and inhibition of Tgf β proteins.

We then examined whether serine protease activity was necessary for binding and inhibition of Tgf β family proteins. The serine residue at position 328 in the catalytic center was substituted with alanine. This mutant, S328A, exhibited no protease activity (data not shown) (see also Hu et al., 1998). S328A showed binding activity with an affinity comparable to that of wild-type HtrA1 (Fig. 3B,d). The serine protease activity, therefore, is not essential for the binding activity. By contrast, this mutant was completely inactive with regard to inhibition of Bmp4 signaling (Fig. 4C).

Taken together, these results indicated that the linker region and the SP domain are necessary for both binding and inhibitory activities, and that the serine protease activity is required for the inhibitory activity of HtrA1 on Tgf β -mediated signaling. We examined the expression levels of all mutants, except for Δ FS/PDZ, which our antisera did not detect, in the C2C12 culture media by western blotting (see insets in Fig. 4C). Although some variations in the production levels for each mutant were observed, they were not significant and thus do not affect our conclusion. It was noticeable, however, that the expression levels observed with the mutant Δ FS in the C2C12 medium were less than one third of those observed with wild type (data not shown). Taking into account these expression levels, the inhibitory activity of Δ FS may be higher than that of wild type. We also measured the protease activities of the mutants used in this study (M.Y., unpublished) and found that protease activity correlates well with signal inhibition.

Inhibition of chick eye development in ovo

In an attempt to determine whether HtrA1 inhibits signaling by Tgf β proteins in vivo, we investigated the effects of exogenous HtrA1 during in ovo chick eye differentiation. Another purpose of this experiment was to examine whether the secreted HtrA1 protein inhibited Tgf β proteins extracellularly. Both *Bmp4* and *Bmp7* play important roles in early stages of lens development (Dudley et al., 1995; Wawersik et al., 1999; Furuta and Hogan, 1998) (for a review, see Chow and Lang, 2001). Noggin

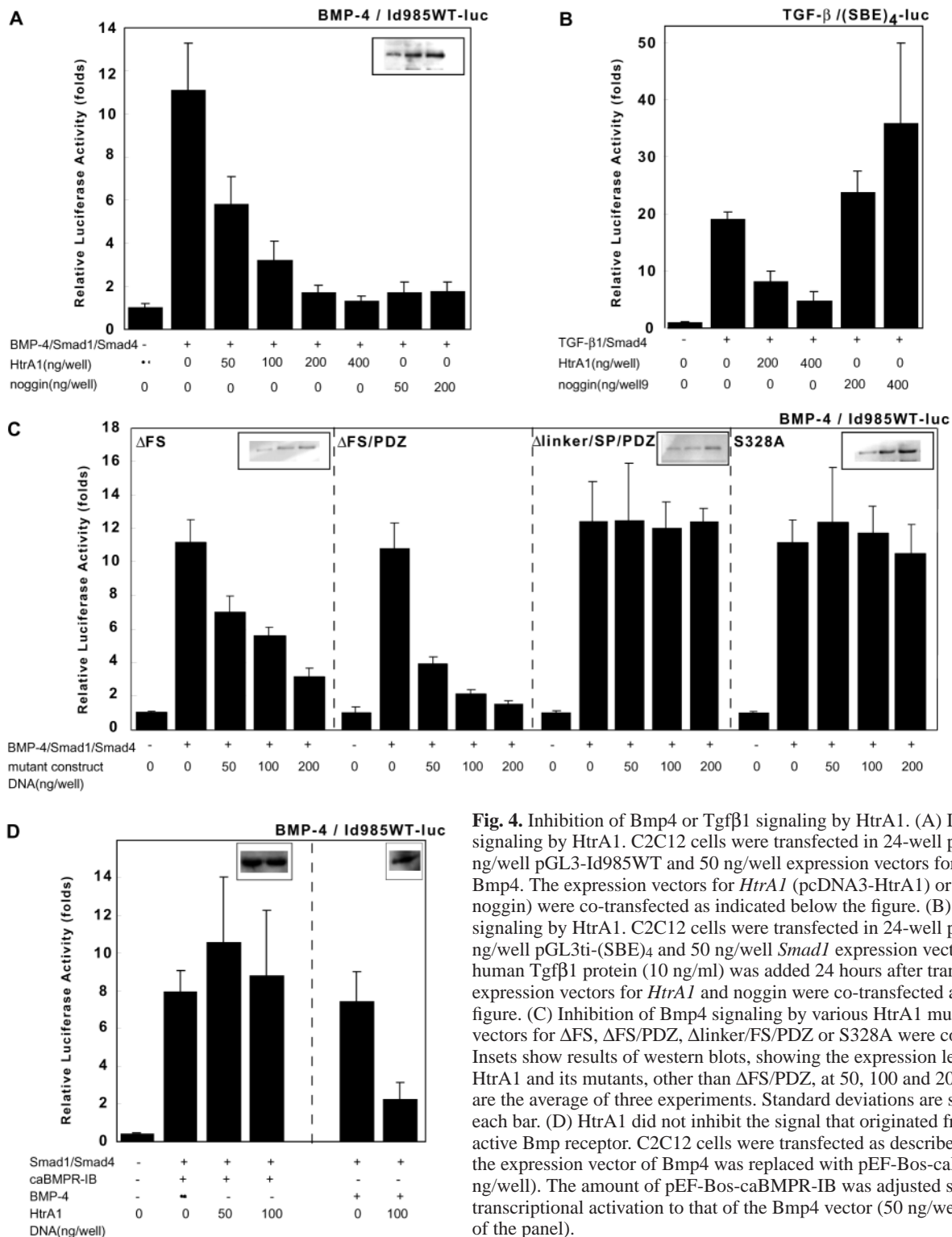


Fig. 4. Inhibition of Bmp4 or Tgf β 1 signaling by HtrA1. (A) Inhibition of Bmp4 signaling by HtrA1. C2C12 cells were transfected in 24-well plates with 250 ng/well pGL3-Id985WT and 50 ng/well expression vectors for Smad1, Smad4 and Bmp4. The expression vectors for *HtrA1* (pcDNA3-HtrA1) or noggin (pEF-Bos-noggin) were co-transfected as indicated below the figure. (B) Inhibition of Tgf β 1 signaling by HtrA1. C2C12 cells were transfected in 24-well plates with 250 ng/well pGL3ti-(SBE)₄ and 50 ng/well *Smad1* expression vector. Recombinant human Tgf β 1 protein (10 ng/ml) was added 24 hours after transfection. The expression vectors for *HtrA1* and noggin were co-transfected as indicated below the figure. (C) Inhibition of Bmp4 signaling by various HtrA1 mutants. The expression vectors for Δ FS, Δ FS/PDZ, Δ linker/SP/PDZ or S328A were co-transfected as in A. Insets show results of western blots, showing the expression levels of wild-type HtrA1 and its mutants, other than Δ FS/PDZ, at 50, 100 and 200 ng DNA/well. Data are the average of three experiments. Standard deviations are shown as a line on each bar. (D) HtrA1 did not inhibit the signal that originated from the constitutively active Bmp receptor. C2C12 cells were transfected as described in A, except that the expression vector of Bmp4 was replaced with pEF-Bos-caBMPR-IB (3 ng/well). The amount of pEF-Bos-caBMPR-IB was adjusted so that it gave similar transcriptional activation to that of the Bmp4 vector (50 ng/well; see the right half of the panel).

misexpression at the optic vesicle was shown to inhibit endogenous Bmp signaling, severely suppress the development of the lens and retina, and result in microphthalmia (Adler and Belecky-Adams, 2002).

Cultured 293T cells were transiently transfected with either a mouse *HtrA1* expression vector or a control vector expressing the antisense strand of *HtrA1* cDNA. A suspension of 293T cells

was injected into the extraocular mesenchyme near the optic vesicle of chick embryos at stage (St.) 9-10. A *GFP* expression vector was co-transfected in order to monitor the injected 293T cells (see Fig. 6A,J). At St. 23-24, eye morphology of the embryo was examined. Twenty-three percent of chick embryos that were injected with *HtrA1*-expressing 293T cells had smaller lenses in the injected side than in the untreated side ($n=43$) (Fig.

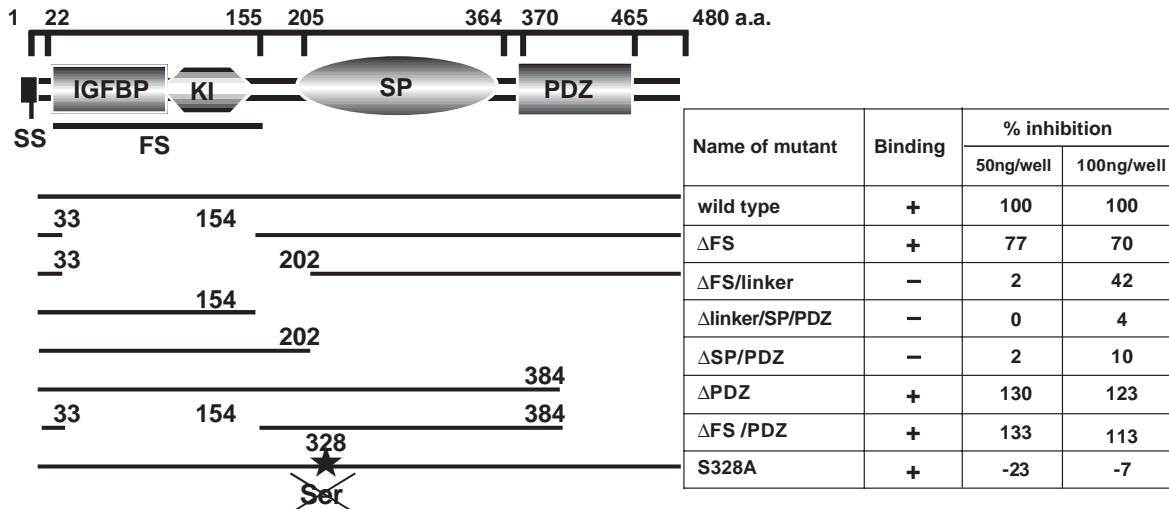


Fig. 5. Binding to and inhibition of Bmp4 signaling by HtrA1 mutants. Schematic presentation of each HtrA1 mutant is shown on the left. Numbers indicate the amino acid residues. The table on the right summarizes the activities of HtrA1 mutants to bind and inhibit Bmp4. The binding activity was designated as '+' when higher than 3% but less than 10% of input HtrA1 mutant protein was recovered by the GST pull-down assay, and '-' when the recovery was less than 1%. Inhibitory activity of the wild-type HtrA1 was defined as 100% for each amount of vector DNA (50 or 100 ng) added to the well. SS, signal sequence.

6B,C), whereas none of the chick embryos that were injected with the control 293T cells exhibited this phenotype ($n=25$). As for the retina, 33% of embryos injected with *HtrA1*-expressing cells had smaller pigmented retinas (Fig. 6B,C). On the contrary, only 8% of embryos injected with the control 293T cells showed smaller pigmented retinas than the control side, and this may be due to physical damage caused by the injection procedure. When noggin-expressing 293T cells were injected, 80% of embryos developed smaller lenses and 87% of embryos showed smaller pigmented retinas ($n=15$; Fig. 6K,L). The effects of HtrA1 were thus apparently weaker than those of noggin. To estimate the potency of HtrA1, we diluted noggin-expressing cells with the control 293T cells. An approximate 3- to 10-fold dilution of noggin-expressing cells gave rise to the same degree of inhibition as *HtrA1*-expressing cells (data not shown), suggesting that the potency of HtrA1 is several-fold lower than that of noggin.

The frontal section of the eye clearly showed that the size of the HtrA1-treated eyecup was much smaller than that of the control (Fig. 6D,E). In the eye of the injected side, the lens diameter was smaller, and the lens was also thinner, when compared with the control side. This was because extension of the primary lens fiber was significantly suppressed (Fig. 6F,G). The phenotypes seen in the HtrA1-treated eye were indistinguishable from those seen in the noggin-treated eye (Fig. 6M-P) (Faber et al., 2002; Belecky-Adams et al., 2002).

Retinal development was also abnormal in HtrA1- or noggin-treated eyes; the neural retina was not arranged in the correct layers, was detached from the retinal pigment epithelium (Fig. 6E,I,N,R) and, in some cases, retinal cells were aggregated (data not shown). In the ventral optic cup of the HtrA1-treated eye, a neuroepithelium-like tissue replaced the normal retinal pigment epithelium (Fig. 6H,I). This abnormal transition of epithelial cell types was observed in noggin-treated eyes in our experiment (Fig. 6Q,R) and was also reported previously (Adler and Belecky-Adams, 2002).

In conclusion, all abnormalities observed in the HtrA1-treated eyes were indistinguishable from those caused by noggin, but differed in severity. This result strongly suggests that secreted HtrA1 is able to extracellularly inhibit Bmp signaling in vivo.

Induction of *Vax* expression by *HtrA1* overexpression

We further examined whether expression of *Vax* was affected by *HtrA1* misexpression in vivo. Expression of *Vax*, which ventralizes the retina, is restricted to the ventral half of the retina, whereas expression of *Tbx5*, which dorsalizes the retina, is restricted to the dorsal half. Bmp4 signaling regulates the determination of dorsoventral polarity in the neural retina by regulating the expression of these two transcription factor genes (Schutle et al., 1999; Koshiba-Takeuchi et al., 2000). Overexpression of a Bmp antagonist, like noggin or ventroptin, leads to upregulation of *Vax* expression and downregulation of *Tbx5* expression (Koshiba-Takeuchi et al., 2000; Sakuta et al., 2001). We electroporated an *HtrA1* expression vector into the optic vesicle at St. 10-11 and examined *Vax* expression after incubation for 24-28 hours at St. 17-18. Expression of *Vax* was restricted to the ventral half of the retina in the untreated eye (Fig. 6T). However, in the retina electroporated with the *HtrA1* expression vector, *Vax* expression was upregulated in the ventral half and expanded into the dorsal half (Fig. 6U). Electroporation of a *GFP* expression vector alone had no effect on *Vax* expression (data not shown). These data indicate that *HtrA1* misexpression ventralizes the retina by inhibiting Bmp4 signaling. Taken together, the results obtained from in ovo experiments showed that HtrA1 is a novel inhibitor that attenuates Bmp signaling extracellularly.

Discussion

We have shown in this paper that HtrA1 binds to a broad range

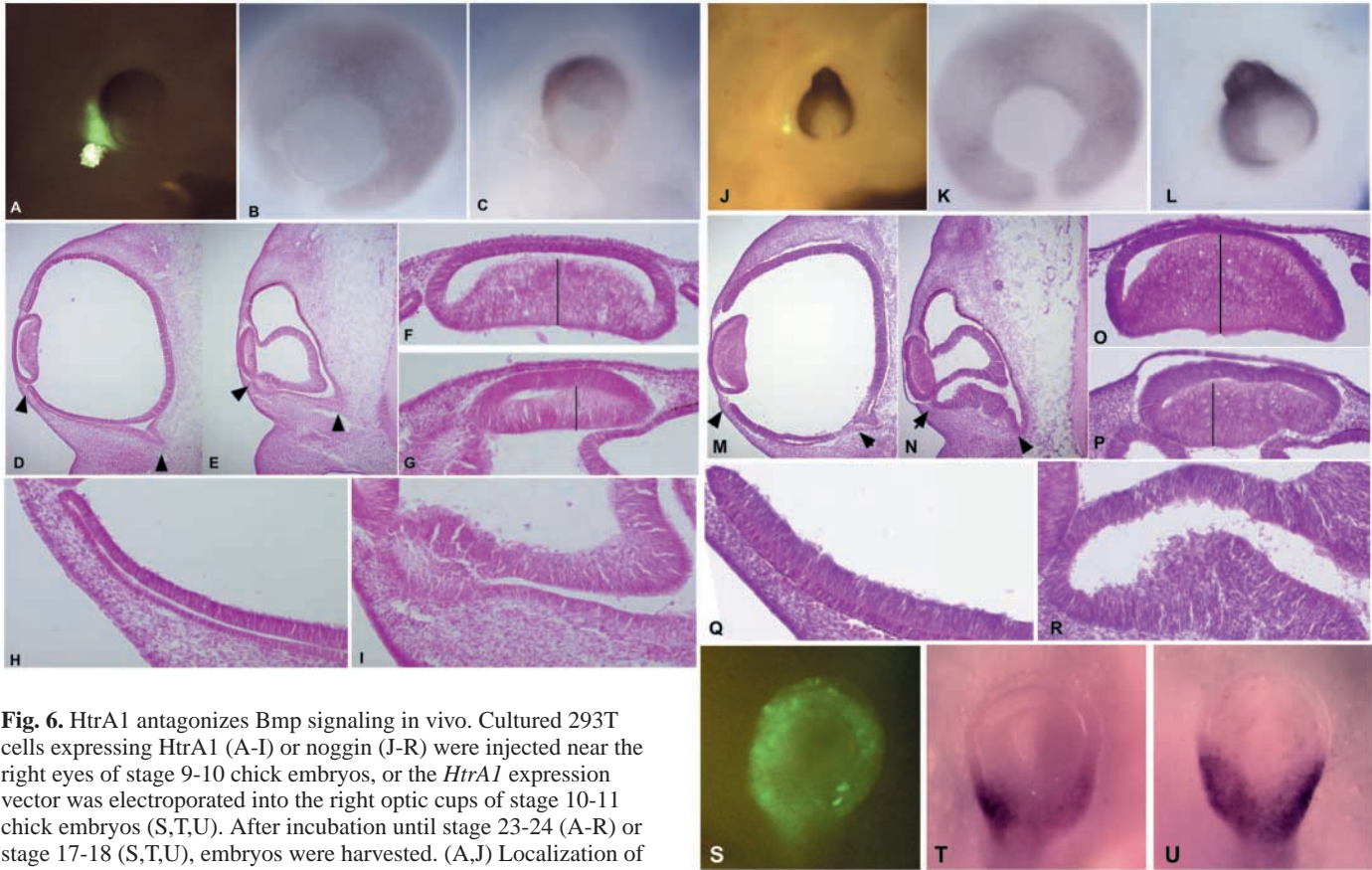


Fig. 6. HtrA1 antagonizes Bmp signaling in vivo. Cultured 293T cells expressing HtrA1 (A-I) or noggin (J-R) were injected near the right eyes of stage 9-10 chick embryos, or the *HtrA1* expression vector was electroporated into the right optic cups of stage 10-11 chick embryos (S,T,U). After incubation until stage 23-24 (A-R) or stage 17-18 (S,T,U), embryos were harvested. (A,J) Localization of injected cells as monitored by GFP fluorescence. Pictures of the eyes were taken at a constant magnification and image-analyzed for size. The lens or retina was judged to be small when the area of the lens or the pigmented retina in the picture was less than 70% of the untreated side. (B,K) Eyes in un-injected sides. (C,L) Eyes injected with HtrA1- or noggin-expressing cells. (D,M) Frontal sections of control eyes. (E,N) Frontal sections of injected eyes. (F,H,O,Q) Magnified views of D and M. (G,I,P,R) Magnified views of E and N, showing abnormalities of the lens and ventral retina caused by HtrA1 and noggin, respectively. Note that the retinal pigment epithelium (regions between arrowheads in D and M) was replaced by a neuroepithelium-like tissue (regions between arrowheads in E and N). Vertical lines in F, G, O and P indicate the thickness of the primary lens fibers. (S) Retina showing expression of co-electroporated *GFP*. (T) *Vax* expression in the untreated eye. (U) Upregulated *Vax* expression in the retina caused by overexpression of *HtrA1*. Eyes tended to be smaller after electroporation with the HtrA1 expression vector. Upregulation of *Vax* expression was seen in eyes of normal or slightly small size.

of Tgf β family members, including members of the Bmp, Gdf and Tgf β subfamilies. We have also shown that HtrA1 is able to inhibit signaling of Tgf β proteins extracellularly, both in vitro and in vivo. Based on the expression patterns during development, HtrA1 appears to participate physiologically in the regulation of signaling by various Tgf β family members in a range of tissues. Of particular importance is that HtrA1 is a likely inhibitor of Tgf β subfamily members, such as Tgf β 1 and Tgf β 2 (Figs 3, 4). There are only a few antagonists, for example, latency-associated protein (LAP) and decorin (Massagué and Chen, 2000; Yamaguchi et al., 1990), reported so far for Tgf β subfamily proteins.

Expression of *HtrA1* during morphogenesis

In the brain, *HtrA1* expression was observed in regions where the choroid plexus arises from the neuroepithelium. The importance of the Bmp signaling in choroid plexus development was recently demonstrated in a study using conditional knockout mouse of the type I Bmp receptor (*Bmpr1a*) gene; disruption of *Bmpr1a* in the telencephalon

led to defects in choroid plexus formation (Hebert et al., 2002).

In the developing heart and gonad, expression of HtrA1 seems to be closely associated with EMT, a process regulated by Tgf β signaling. The relationship between HtrA1 and EMT was also proposed from several studies on differentially expressed genes in malignant cancers. Downregulation of *HtrA1* expression has been repeatedly observed during the late stages of progression or metastasis of melanomas (Baldi et al., 2002) and ovarian cancers (Shridhar et al., 2002). The process of cancer invasion and metastasis has been shown to recapitulate EMT, and, in certain types of cancer, is enhanced by Tgf β signaling (Moustakas et al., 2002). Therefore, it is plausible that HtrA1 suppresses EMT of cancer cells by antagonizing Tgf β signaling.

During the development of bones and their appending tissues, HtrA1 is likely to suppress chondrocyte differentiation and divert the fate of mesenchyme to non-chondral types, such as periosteum, perichondrium, joints, tendons and ligaments. This is consistent with the fact that Bmps and Tgf β s enhance

differentiation of chondrocytes from mesenchymal cells (Capdevila and Belmonte, 2001). Development of tendons and ligaments also depend on signaling by Bmp/Gdf proteins. Bmp signaling seems to specify regions from which tendon progenitors arise, whereas additional signals, for example Gdf6, Gdf7 and Tgfb β 2, may be required for further maturation of tendons (Schweitzer et al., 2001; Wolfman et al., 1997; Merino et al., 1998). Because HtrA1 is expressed in tendons at later stages of development, it is possible that HtrA1 may regulate the maturation of tendons.

HtrA1 is expressed in joint interzones and may regulate joint development, a process closely regulated by Bmp/Gdf signaling (Brunet et al., 1998; Francis-West et al., 1999). It has also been proposed that signaling mediated by the Tgfb β subfamily participates in the maintenance of adult joints. Tgfb β is required to keep articular chondrocytes in their normal, undifferentiated state. Transgenic mice carrying a dominant-negative, kinase-defective Tgfb β type II receptor gene (Serra et al., 1997), or *Smad3* knockout mice (Yang et al., 2001), showed phenotypes very similar to human osteoarthritis. In these mice, suppression of Tgfb β signaling probably promoted terminal differentiation of articular chondrocytes, leading to osteoarthritis-like phenotypes. Interestingly, expression of human *HTRA1* increased substantially in articular cartilage cells of patients with osteoarthritis (Hu et al., 1998). It is possible that HtrA1 antagonizes Tgfb β and aggravates osteoarthritis.

Functional domains of HtrA1

Unexpectedly, we found that the linker region and the SP domain, but none of the IGFBP and KI (or follistatin-like) domains, were required for HtrA1 binding to Tgfb β family members.

The linker regions are highly conserved among the four HtrA members (Li et al., 2002). The linker region of HtrA2 is essential for trimer formation. This suggests that HtrA1 may form a homotrimer, similar to HtrA2. The binding activity of each HtrA1 mutant to Tgfb β family proteins seems to closely correlate with its ability to form a possible trimeric structure. For example, the mutant Δ FS/linker, which lacked the linker region, was incapable of binding to Tgfb β family proteins. By contrast, S328A, which did not possess protease activity but was possibly able to form the trimer (Li et al., 2002), demonstrated binding activity to Tgfb β family proteins, though it lacked inhibitory activity.

The PDZ domain of HtrA1 was not required for either binding or inhibitory activity. The PDZ domain of HtrA2 negatively regulates proteolytic activity. A hypothetical trimeric apoptosis receptor is thought to bind to the PDZ domain and enhances the proteolytic activity of HtrA2 (Li et al., 2002). The PDZ domain of HtrA1 may have a similar regulatory function. Indeed, the two mutants that lacked PDZ domains, Δ PDZ and Δ FS/PDZ, seemed to have stronger inhibitory activity on Tgfb β signaling than wild-type HtrA1 (Fig. 5). Our yeast two-hybrid screening isolated trimeric extracellular matrix (ECM) proteins most frequently as binding factors for the PDZ domain of HtrA1 (Murwantoko, unpublished). Tgfb β signaling has a close and mutual correlation with the ECM; production of ECM is regulated by Tgfb β signaling and vice versa (Kresse and Schönherr, 2001). Secreted HtrA1 may bind to ECM around the cell, become

fully active and, subsequently, regulate Tgfb β signaling. The inhibiting activity of HtrA1 was 3- to 10-fold weaker than that of noggin, based on in vitro (Fig. 4A) and in vivo experiments (Fig. 6). One explanation is that HtrA1 must be bound to and activated by ECM proteins, and thus it functions fully as a semi-autocrine factor.

The function of the IGFBP and KI domains remains to be elucidated. Δ FS mutants lacking these domains seemed to have enhanced inhibitory activity on Tgfb β signaling (see Fig. 5 and Results). The N-terminal FS domain may have another regulatory function on the protease activity of HtrA1.

The way in which HtrA1 protease activity inhibits signaling by Tgfb β proteins has yet to be clarified. During the GST pull-down assay we did not detect any degradation of GST-Tgfb β proteins by HtrA1. Furthermore, untagged human recombinant Tgfb β 1 and Bmp2, as well as myc-tagged Bmp4 produced by 293T cells, were all resistant to HtrA1 (data not shown). This does not exclude the possible degradation of Tgfb β proteins in vivo, because degradation of Tgfb β proteins may require co-factors, such as ECM proteins that bind to the PDZ domain. Alternatively, HtrA1 may degrade the receptors for Tgfb β family proteins or the extracellular proteins that positively regulate Tgfb β signaling. In these cases, the specific and high-affinity binding of HtrA1 to Tgfb β may bring HtrA1 into close vicinity of the substrate proteins, and may thus facilitate efficient degradation.

Another possibility we must consider is that binding to Tgfb β proteins is irrelevant to the apparent inhibition of their signaling. So far, we have not obtained evidence indicating that binding is essential for the inhibition of Tgfb β signaling. It is possible that HtrA1 digests ECM, indirectly inhibiting Tgfb β signaling. In fact, we found that major proteoglycans of ECM, decorin and biglycan, were digested by HtrA1 in vitro (M.Y., unpublished). These two proteoglycans bind to Tgfb β s, and at least decorin can modulate physiological signaling of Tgfb β s. In order to understand the biological functions of HtrA1, it will first be necessary to identify the physiological substrates of this interesting serine protease.

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