Serase-1B, a new splice variant of polyserase-1/TMPRSS9, activates urokinase-type plasminogen activator and the proteolytic activation is negatively regulated by glycosaminoglycans

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Polyserase-1 (polyserine protease-1)/TMPRSS9 (transmembrane serine protease 9) is a type II transmembrane serine protease (TTSP) that possesses unique three tandem serine protease domains. However, the physiological function of each protease domain remains poorly understood. We discovered a new splice variant of polyserase-1, termed Serase-1B, which contains 34 extra amino acids consisting a SEA module (a domain found in sea urchin sperm protein, enterokinase and agrin) adjacent to the transmembrane domain and the first protease domain with a mucin-like box at the C-terminus. The tissue distribution of this enzyme by RT (reverse transcription)–PCR analysis revealed high expression in the liver, small intestine, pancreas, testis and peripheral blood CD14⁺ and CD8⁺ cells. To investigate the role of Serase-1B, a full-length form recombinant protein was produced. Interestingly, recombinant Serase-1B was partly secreted as a soluble inactive precursor and it was also activated by trypsin. This

activated enzyme selectively cleaved synthetic peptides for trypsin and activated protein C, and it was inhibited by several natural serine protease inhibitors, such as aprotinin, α_2 -antiplasmin and plasminogen activator inhibitor 1. In addition, Serase-1B efficiently converted pro-uPA (urokinase-type plasminogen activator) into active uPA and this activation was strongly inhibited by these natural inhibitors. Furthermore, this activation was also negatively regulated by glycosaminoglycans. Our results indicate that Serase-1B is a novel member of TTSPs that might be involved in uPA/plasmin-mediated proteolysis and possibly implicated in biological events such as fibrinolysis and tumour progression.

Key words: glycosaminoglycan, pericellular proteolysis, polyserase-1, pro-urokinase/plasminogen-activation system, Serase-1B, TTSP (type II transmembrane serine protease).

INTRODUCTION

Serine proteases are involved in a variety of biological processes, such as food digestion, blood coagulation, tissue remodelling and embryonic development [1–3]. Most of these enzymes are trypsin-type serine proteases secreted by cells or found in biological fluids. However, in the last decade, a number of cell-surface-anchored trypsin-like serine proteases have been identified and their roles have been examined. Among them, the most rapidly expanding family is the TTSPs (type II transmembrane serine proteases) including enteropeptidase (enterokinase) [4], hepsin [5], membrane-bound arginine-specific serine protease [6], HAT (human airway trypsin-like protease) [7], corin/LRP4 [LDL (low-density lipoprotein) receptor-related protein 4] [8,9], MT-SP1 (membrane-type serine protease 1)/epithin/matriptase [10–12], TADG-12 (tumour-associated differentially expressed gene-12) [13], MSPL/MSPS (mosaic serine protease long-form/short-form) [14], TMPRSS2 (transmembrane serine protease 2)/epitheliasin [15,16], TMPRSS3 [17], TMPRSS4 [18], spinesin/TMPRSS5 [19], matriptase-2/TMPRSS6 [20], polyserase-1

(polyserine protease-1)/TMPRSS9 [21], DESC1 (differentially expressed squamous cell carcinoma gene 1) [22] and, most recently, matriptase-3 [23]. These TTSPs have a common structure: a short cytoplasmic domain and a transmembrane domain at the N-terminus, and a trypsin-like protease domain at the extracellular C-terminus. The TTSPs also contain multiple structural domains, including a Group A scavenger receptor domain, LDL receptor class A domain, CUB (C1s/C1r, urchin embryonic growth factor and bone morphogenetic protein-1) domain, SEA (sea urchin sperm protein, enterokinase and agrin) domain, MAM (meprin, A5 antigen and receptor protein phosphatase μ) domain and Frizzled domain in their stem regions that may modulate protein–protein interactions [24].

Although the expression profiles and enzymatic characterization of TTSPs have been reported, their physiological functions and natural substrates and inhibitors are currently less well substantiated. The most well-characterized TTSP is matriptase/MT-SP1/epithin. Matriptase was originally identified as a matrix-degrading enzyme with gelatinolytic activity from human breast cancer cells [25]. In *in vitro* assays, PAR-2 (protease-activated

Abbreviations used: AP, adaptor primer; Boc, t-butoxycarbonyl; Bz, benzoyl; DESC1, differentially expressed squamous cell carcinoma gene 1; E, ekon; GAG, glycosaminoglycan; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Glt, glutaryl; GST, glutathione S-transferase; HEK, human embryonic kidney; HGF, hepatocyte growth factor; LDL, low-density lipoprotein; LMWH, low-molecular-mass heparin; MCA, 4-methylcoumaryl-7-amide; MMP, matrix metalloproteinase; MT-SP1, membrane-type serine protease 1; OBzI, benzyloxy; PAI, plasminogen activator inhibitor; polyserase-1, polyserine protease-1; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SEA domain, a domain found in sea urchin sperm protein, enterokinase and agrin; SFCM, serum-free culture medium; SLPI, secretory leukoprotease inhibitor; TBS, Tris-buffered saline; TMPRSS, transmembrane serine protease; TTSP, type II transmembrane serine protease; uPA, urokinase-type plasminogen activator.

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The nucleotide sequences for rat, human and mouse Serase-1B have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AB109392, AB109390 and AB109391 respectively.

receptor-2), pro-uPA (urokinase-type plasminogen activator), a single-chain HGF (hepatocyte growth factor) and Trask (transmembrane and associated with Src kinases) have been shown to be potential matriptase substrates [26–28]. By activating these substrates, matriptase seems to play a role in epithelial development, extracellular matrix degradation and mitotic regulation of cell adhesion. Moreover, mice deficient in matriptase/MT-SP1/epithin died within 48 h of birth owing to a disorder of the epidermal barrier function, thus suggesting that matriptase plays a pivotal role in epidermal development [29,30]. To our knowledge, there are no studies of knockout mice in other members of the matriptase subfamily (matriptase-2, matriptase-3 and polyserase-1).

Polyserase-1/TMPRSS9, a member of the matriptase subfamily, was first identified and cloned from human liver cDNA by Cal et al. [21]. This protease has a unique structure with three tandem serine protease domains and the ability to generate three independent serine proteases (i.e. Serase-1, -2 and -3). The third protease domain, Serase-3, is thought to be catalytically inactive because it contains an alanine residue instead of a serine residue in its active site. Although Serase-1 and Serase-2 contain catalytic serine residues in their active sites and have trypsin-like specificity for the synthetic substrates with a basic amino acid residue in the P1 position, their natural substrates and inhibitors have not yet been elucidated. In addition, the presence of two splice variants with 3.8 and 2.4 kb identified using Northern blot analysis has been reported, although without detailed analysis [21].

In the present paper, we describe the identification and enzymatic characterization of Serase-1B, which is a new splice variant of polyserase-1/TMPRSS9. We show that Serase-1B encodes a TTSP that has a SEA module in the stem region and a single protease domain with a mucin-like box at the C-terminus. This architecture of Serase-1B is well conserved in the sequences of its mouse and rat orthologues. We also show that Serase-1B has a divergent expression style compared with that of polyserase-1/TMPRSS9 and other members of the matriptase subfamily. Furthermore, we demonstrate that Serase-1B activates pro-uPA and that its activation is negatively regulated by GAGs (glycosaminoglycans).

MATERIALS AND METHODS

Proteins and chemicals

MCA (4-methylcoumaryl-7-amide) peptide substrates were purchased from the Peptide Institute (Osaka, Japan). Human PAI-1 (plasminogen activator inhibitor-1) was purchased from Molecular Innovations. All other protease inhibitors and heparin were obtained from Sigma. GM 6001 and LMWH (low-molecular-mass heparin) were provided from Calbiochem. Hyaluronic acid was purchased from MP Biomedicals, and heparan sulfate was purchased from Celsus Laboratories.

Cell lines and culture conditions

HEK-293T cells (human embryonic kidney cell line) were purchased from GenHunter Corporation and cultured at 37 °C in 5% CO₂ in DMEM (Dulbecco's modified Eagle's medium) (Invitrogen) containing 10% FBS (foetal bovine serum) (Roche Molecular Biochemicals) and 50 µg/ml gentamicin (Invitrogen).

Molecular cloning of Serase-1B cDNA

We initially identified rat Serase-1B from rat testis Marathon-Ready cDNA (Clontech Laboratories) by PCR using degenerate oligonucleotides designed from the conserved sequence of various

trypsin-type serine proteases. Oligonucleotide primers used were sense, 5'-CCCTGGCAGGT(G/C)AGCCTG(A/C)G(A/T/G)-3', and antisense, 5'-CC(A/T/G)GAGTC(A/T/G)CCCTGGCAGG-A(A/G)TC-3'. For PCR, 33 cycles were run, each consisting of a 30 s denaturing step at 94 °C, and a 90 s annealing and extension step at 68 °C, and a final extension step at 72 °C for 5 min. The amplified DNA fragment was subcloned into pGEM-T Easy vector (Promega) and sequenced. Based on the deduced nucleotide sequence of the PCR fragment and the results of RnBLAST (<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10116>) analysis, gene specific primers (primer 1, 5'-GTCAGCCTCCGAGAGAATCACGAACA-3'; primer 2, 5'-GAGCTGGGAATCGGCTGTGCAGAA-3'; primer 3, 5'-GGGT-CCTGGAATTCGTTGAAGCAGT-3'; primer 4, 5'-GCTCGGAGCAGAAAGTGTAGCCGGAA-3') were synthesized and 5'- and 3'-RACE (rapid amplification of cDNA ends) approaches were carried out using a Marathon-Ready cDNA according to the instruction manual. For 3'-RACE, nested PCR with primer 2 and adaptor primer 2 (AP2, 5'-ACTCACTATAGGGCTCGAGCGGC-3') was performed using the PCR products with primer 1 and adaptor primer 1 (AP1, 5'-CCATCCTAATACGACTCACTATAG-3') as a template. For 5'-RACE, nested PCR with AP2 and primer 4 was performed using the PCR products with AP1 and primer 3 as a template. For further cloning of the 5' end, nested PCR with AP2 and primer 5 (5'-GCATATGTGGCCCTCTTCTCTGTCTCT-3') was performed using the PCR products with AP1 and primer 3 as a template. The nucleotide sequence has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AB109392. Based on the rat Serase-1B cDNA sequence, a BLAST analysis was performed using the NCBI human and mouse genome database (<http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9606> and <http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=10090> respectively). These bioinformatic searches showed a structural organization similar to that of the gene encoding rat Serase-1B. Then, a PCR-based strategy was used to clone the human and mouse Serase-1B cDNA from human liver and mouse testis Marathon-Ready cDNA respectively. For human sequences, specific oligonucleotides designed from the identified genome sequences were: hS1B-sense-1, 5'-GCGTGTCTCTGAGCCATGGAGCCC-3'; hS1B-antisense-1, 5'-CCCACGTAGGCCACCCACTTCGT-3'; hS1B-sense-2, 5'-GGCAGGATCGTGGGCGGCATGGAAGCA-3'; and hS1B-antisense-2, 5'-GGCACGTACATCATCTTCTACCTTTCT-3'. For the PCR, 35 cycles were run, each consisting of a 15 s denaturing step at 95 °C, a 90 s annealing and extension step at 60 °C, and a final extension step at 72 °C for 7 min. For mouse sequences, specific oligonucleotides designed from the identified genome sequences were: mS1B-sense-1, 5'-GTTGATCAGGAGTCCAGGGCCAAGTGT-3'; mS1B-antisense-1, 5'-GAGCAGAAAGTGTAGCCGGAATGT-3'; mS1B-sense-2, 5'-GCAGAGCTACATGGGATCCGTTTCA-3'; mS1B-antisense-2, 5'-GGGTCCTGGAATTCGTTGAAGCAGT-3'; mS1B-sense-3, 5'-GGTCAGCCTGCGAGAGAATCACGAACA-3'; and mS1B-antisense-3, 5'-GGCAGGCATTAATAGTCAATAGAGT-3'. For PCR, 35 cycles were run, each consisting of a 30 s denaturing step at 95 °C, a 30 s annealing step at 62 °C, a 1 min extension step at 72 °C and a final extension step at 72 °C for 7 min. The amplified DNA fragments were subcloned into pGEM-T Easy vector and ten independent clones were sequenced. To remove the overlapped regions of each PCR fragment, they were then digested by restriction endonucleases, purified and ligated. Finally, full-length cDNA was obtained by PCR amplification using primers hS1B-sense-1 and h1B-antisense-2, and mS1B-sense-1 and mS1B-antisense-3 respectively. The nucleotide

sequences thus obtained have been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession numbers AB109390 for human Serase-1B and AB109391 for mouse Serase-1B.

Analysis of Serase-1B expression in human and mouse tissues

To characterize and compare the mRNA expression levels of human Serase-1B and polyserase-1, Human Multiple Tissue cDNA panels were obtained from BD Biosciences and analysed by RT (reverse transcription)-PCR: 35 cycles of PCR were performed with the gene-specific primers (hSerase-1B-sense, 5'-GGCTACGTGACTGGATCCTGGAGG-3', and hSerase-1B-antisense, 5'-GGCAGGTACATCATCTTCTACCTTTCT-3'; hpolyserase-1-sense, 5'-CGCTTCTACCCAGTGCAGATCA-3', and hpolyserase-1-antisense, 5'-GTGACCCAGTTAGCACC-CACC-3'). Each cycle for human Serase-1B consisted of a 30 s denaturing step at 94°C, a 30 s annealing step at 64°C and a 30 s extension step at 72°C. Each cycle for human polyserase-1 consisted of a 30 s denaturing step at 94°C, a 30 s annealing and extension step at 68°C. To analyse the expression levels of Serase-1B in mice, total RNA was extracted from tissue specimens of 8-week-old male and female C57BL/6J mice using a Sepasol-RNA I super reagent (Nacalai Tesque). First-strand cDNA templates were synthesized from total RNA (1 µg each) using random primer and MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Promega). The expression of the mRNA levels was also analysed by RT-PCR: 35 cycles of PCR were performed with the gene-specific primers (mSerase-1B-sense, 5'-CGGACGGGTCAGATTAGGCCAG-3', and mSerase-1B-antisense, 5'-GGAGCCAATGAGACAGATGTGGA-3'; mpolyserase-1-sense, 5'-GGCAGGCCCCACTTCCCAGGT-3', and mpolyserase-1-antisense, 5'-GGGCAATCATTCCTGGATGTTCTG-3'). Each cycle for mouse Serase-1B consisted of a 30 s denaturing step at 94°C, a 30 s annealing step at 60°C and a 30 s extension step at 72°C. Each cycle for mouse polyserase-1 consisted of a 30 s denaturing step at 94°C, and a 30 s annealing and extension step at 68°C. Human and mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific primers (hGAPDH-sense, 5'-TGAAGGTCGGAGTCAACGGATTTG-GT-3', and hGAPDH-antisense, 5'-CATGTGGGCCATGAGG-TCCACCAC-3'; mGAPDH-sense, 5'-CATCACCATCTTCCAG-GA-3', and mGAPDH-antisense, 5'-GAGGGGCCATCCACAG-TCTTC-3') were used as a positive-control reaction (22 cycles of PCR for human GAPDH and 25 cycles of PCR for mouse GAPDH). Each cycle for human GAPDH consisted of a 30 s denaturing step at 94°C, and a 2 min annealing and extension step at 68°C. Each cycle for mouse GAPDH consisted of a 30 s denaturing step at 94°C, a 30 s annealing step at 63°C and a 30 s extension step at 72°C. The PCR products were applied on a 2% agarose/TAE (Tris/acetate/EDTA) gel for electrophoresis and were stained with ethidium bromide solution. The specificity of the amplified PCR products was confirmed by sequencing.

Expression and purification of recombinant Serase-1B

Expression clones were constructed from human and mouse Serase-1B full-length cDNA by standard PCR amplification methods. For protein expression, PCR-amplified products were cloned into p3xFLAG-CMV14 vector (Sigma) at the EcoRI and XbaI sites for human clones and the KpnI and XbaI sites for mouse clones. For the construction of an active-site mutant (Ser → Ala) clone, mutated primers were designed and standard PCR amplification methods were performed using a full-length expression clone as a template. All constructs were verified by sequence analysis.

To establish a stable cell line expressing Serase-1B, full-length Serase-1B cDNA with a FLAG tag sequence was subcloned into pIRES-puro vector (BD Bioscience) at the NotI site and transfected into HEK-293T cells, and cells were screened for resistance to puromycin. For purification of recombinant Serase-1B, conditioned SFCM (serum-free culture medium) was collected from pools of stably transfected HEK-293T cells and concentrated by ultrafiltration on a Biomax-10 membrane (Millipore Corporation). Tris buffer (pH 7.4) and NaCl were added to the concentrated conditioned medium to a final concentration of 50 and 150 mM respectively. The solution was applied to an anti-FLAG M2-agarose affinity gel column, and bound proteins were eluted with 0.1 M glycine/HCl, pH 3.5. The purified recombinant Serase-1B was dialysed against PBS and stored at -30°C until use.

Preparation of antibody against Serase-1B

An immunogen peptide corresponding to human Serase-1B, VQIVKHPLYNADTADFVDA (amino acids 310-328) was synthesized using the solid-phase method on an automated peptide synthesizer (Applied Biosystems model 430A) according to the instructions provided by the manufacturer. The peptides were conjugated to a keyhole-limpet haemocyanin and used for the immunization of rabbits. The resulting antisera against Serase-1B were purified with immunogen peptide-coupled epoxy-activated Sepharose 6B (Amersham Biosciences) as described previously [31].

SDS/PAGE, immunoblotting and amino acid sequence analysis

SDS/PAGE was performed using gradient gels (10-20%) under reducing conditions according to the method of Laemmli [32]. For immunoblotting analysis, proteins in the gels were transferred electrophoretically on to PVDF membranes. Excess sites on the membranes were blocked by incubation with 3.5% (w/v) non-fat dried skimmed milk in TBS (Tris-buffered saline; 20 mM Tris/HCl, pH 7.5, and 150 mM NaCl) for 2 h at room temperature (23°C). After washing once with TBS, the membranes were probed with 5 µg/ml anti-human Serase-1B antibody in 3.5% (w/v) non-fat dried skimmed milk in TBS or with 3.5 µg/ml anti-FLAG M2 monoclonal antibody in TBS/Tween 20 by incubation overnight at 4°C. After washing three times with TBS/0.05% Tween 20, the membranes were incubated for 1 h at room temperature in a 1:2500 dilution of horseradish-peroxidase-labelled anti-rabbit IgG or a 1:4000 dilution of horseradish-peroxidase-labelled anti-mouse IgG in 3.5% (w/v) non-fat dried skimmed milk in TBS. Immunoreactive proteins were visualized using the ECL® (enhanced chemiluminescence) detection system (Amersham Biosciences). The N-terminal amino acid sequence of the soluble form of the enzyme was also determined using an Applied Biosystems model 492 gas-phase sequencer/140C system following the instructions provided by the manufacturer.

Proteolytic activation of Serase-1B by trypsin

A 2 µg amount of Serase-1B was incubated with 10 µl of immobilized Tos-Phe-CH₂Cl (tosylphenylalanylchloromethane)-trypsin (Pierce) in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 2 h at 37°C. After incubation, trypsin beads were removed from the digestion mixture by centrifugation at 20 400 g for 2 min, and the amidolytic activities of the supernatant were analysed. In another experiment, 100 ng of Serase-1B was incubated directly with 1 ng of trypsin in 0.1 M Tris/HCl, pH 8.5, for 0-30 min at 37°C. After incubation, the enzyme preparations were applied to SDS/PAGE and the amidolytic activities were analysed by substrate zymography.

Table 1 Substrate specificities of recombinant soluble Serase-1B

$V_{\max}/K_m$ ratios were derived from the individual K_m (μM) and $V_{\max}$ ($\mu\text{mol}/\text{min}$ per mg) values. Boc, t-butoxycarbonyl; ND, not determined; Suc, succinyl.

Substrate	$V_{\max}/K_m$	
	Human Serase-1B	Mouse Serase-1B
Boc-Gln-Ala-Arg-MCA	3.33	5.00
Boc-Gln-Gly-Arg-MCA	1.25	1.11
Boc-Leu-Arg-Arg-MCA	0.25	0.25
Boc-Val-Leu-Lys-MCA	0.50	0.50
Boc-Phe-Ser-Arg-MCA	0.50	0.50
Boc-Leu-Thr-Arg-MCA	3.33	5.00
Boc-Leu-Ser-Thr-Arg-MCA	3.33	5.00
Bz-Arg-MCA	ND	ND
Boc-Glu(OBzl)-Ala-Arg-MCA	3.33	5.00
Boc-Glu(OBzl)-Gly-Arg-MCA	1.00	1.00
Pro-Phe-Arg-MCA	1.67	2.00
Suc-Ala-Pro-Ala-MCA	ND	ND
Suc-Ala-Ala-Pro-Phe-MCA	ND	ND
Suc-Leu-Leu-Val-Try-MCA	ND	ND

Zymography

Substrate zymography was performed as described previously [33]. Briefly, the samples were first mixed with the SDS/PAGE sample buffer in the absence of a reducing agent and incubated for 10 min at room temperature. Electrophoresis was performed on 10 % polyacrylamide gels in the presence of 0.1 % SDS and 200 μM fluorogenic substrates. After electrophoresis, the gels were washed with 2.5 % Triton X-100 for 30 min at room temperature, then washed twice with the reaction buffer (0.1 M Tris/HCl, pH 8.5) for 15 min at room temperature. After washing, the gels were incubated with the same buffer for 2 h at 37 °C. The clear bands with amidolytic activity were monitored using a UV lamp and then photographed.

Enzyme and inhibitor assays

The amidolytic activities of the enzyme preparations were analysed toward various synthetic peptides listed in Table 1. The amount of MCA liberated from the substrate was determined fluorimetrically with excitation and emission wavelengths of 370 and 460 nm respectively using a MS fluorescence spectrophotometer (Hitachi model 650-10). One unit of enzyme activity was defined as the amount that degraded 1 μmol of substrate per min. The kinetic parameters (K_m , $V_{\max}$ and $V_{\max}/K_m$ ratio) were determined from the double-reciprocal plots by using different concentrations of fluorogenic peptides (10–100 μM). To determine the inhibitor specificities, enzyme preparations were pre-incubated for 5 min with various inhibitors listed in Table 2 at 37 °C, and the residual enzyme activities were measured towards Boc-Gln-Ala-Arg-MCA (where Boc is t-butoxycarbonyl), one of the efficient substrates tested.

Serase-1B-inhibitor complex formation

Purified human Serase-1B (0.5 μg) was incubated with 10 μl of trypsin-immobilized beads in 0.1 M ammonium bicarbonate buffer, pH 8.0, for 1 h at 37 °C. After incubation, trypsin beads were removed by centrifugation at 20400 g for 2 min, and the supernatants were incubated for 30 min at 37 °C with PAI-1 (final concentration 500 nM) or α_2 -antiplasmin (final concentration 500 nM). The proteins were separated by SDS/PAGE under reducing conditions and the protease-inhibitor complex formation was analysed by Western immunoblotting with antibodies against human Serase-1B as described above.

Table 2 Inhibitor specificities of recombinant soluble Serase-1B

The value measured in the absence of inhibitors was considered to be 100 % enzyme activity. E-64c, synthetic E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] analogue; $i\text{Pr}_2\text{P-F}$, di-isopropyl fluorophosphate; UTI, urinary trypsin inhibitor.

Inhibitor	Final concentration (mM)	Relative activity (%)	
		Human Serase-1B	Mouse Serase-1B
None		100.0	100.0
PMSF	5.0	63.5	43.1
$i\text{Pr}_2\text{P-F}$	1.0	62.5	75.7
Aprotinin	0.01	0.0	0.0
Leupeptin	0.01	17.7	16.4
Benzamidine	1.0	12.5	16.7
Soybean trypsin inhibitor	0.01	41.7	17.5
Elastatinal	0.01	97.7	87.3
Chymostatin	0.01	114.6	94.5
E-64c	0.01	101.0	136.4
Pepstatin A	0.01	122.0	127.3
Phosphoramidon	0.01	129.2	62.9
EDTA	1.0	89.6	100.0
UTI	0.01	47.9	20.7
α_1 -Antitrypsin	0.01	95.8	94.5
Antithrombin III	0.01	78.1	72.6
α_2 -Antiplasmin	0.001	27.2	27.2
PAI-1	0.001	0.0	0.0
SLPI	0.01	24.0	1.8

Activation of pro-uPA

A 1 μg amount of pro-uPA (single-chain uPA; American Diagnostica and Technoclone) was incubated with 130 ng of activated Serase-1B in the presence of synthetic substrate for uPA (Glt-Gly-Arg-MCA, where Glt is glutaryl) in the reaction buffer (0.1 M Tris/HCl, pH 8.5) at 37 °C. The uPA activity was monitored using the method described above. In some experiments, pro-uPA incubated with activated Serase-1B was analysed by either substrate zymography or Western immunoblotting with a monoclonal antibody against the A-chain of uPA (5 $\mu\text{g}/\text{ml}$) (product #3921; American Diagnostica). In the experiments to determine the effect of natural inhibitors for Serase-1B on the activation of pro-uPA, either SLPI (secretory leukoprotease inhibitor) (final concentration, 10 μM) or α_2 -antiplasmin (final concentration, 1 μM) was pre-incubated for 5 min with 130 ng of activated Serase-1B in the reaction buffer at 37 °C. Next, it was incubated with 1 μg of pro-uPA in the same buffer at 37 °C, and then the uPA activity was measured.

Effect of GAGs on pro-uPA activation

Increasing concentrations of GAGs (hyaluronic acid, 0–50 $\mu\text{g}/\text{ml}$; heparan sulfate, 0–50 ng/ml; heparin, 0–50 ng/ml; LMWH, 0–5.0 ng/ml) were pre-incubated with 43.5 ng of activated Serase-1B in a reaction buffer (0.1 M Tris/HCl, pH 8.5) for 5 min at 37 °C. Next, the reaction mixture was incubated with 0.5 μg of pro-uPA in the same buffer containing the fluorogenic substrate for uPA (Glt-Gly-Arg-MCA). The amidolytic activity of uPA was monitored using the same method as described above.

Ethical considerations

All experimental protocols involving animals described in this study were approved by the Ethics Review Committee for Animal Experimentation of Tokushima University.

RESULTS

Cloning and sequence analysis of Serase-1B

We cloned and identified Serase-1B, a membrane-associated serine protease, from human liver and mouse/rat testis cDNA. The predicted human Serase-1B cDNA sequence covers 1593 bp with a single open reading frame (Figure 1A). The deduced amino acid sequence of its cDNA showed that human Serase-1B translated a protein of 531 amino acids with a calculated molecular mass of 57.5 kDa. This amino acid sequence also showed that human Serase-1B has an active form sequence of 295 amino acids starting with the Ile-Val-Gly-Gly motif, the typical catalytic triad His²⁷⁷, Asp³²⁶, Ser⁴²¹ of serine protease and an S1-binding pocket (Asp⁴¹⁵, Gly⁴⁴³, Gly⁴⁵³), suggesting that Serase-1B is a member of the trypsin-type serine proteases. Based on the nucleotide and amino acid sequences, Serase-1B is a splice variant for the previously reported polyserase-1/TMPRSS9 [21]. A comparison of the splice site of human Serase-1B with polyserase-1/TMPRSS9 showed human Serase-1B to have an additional 68 nucleotides in EIIB (where E is exon) and 34- and 23-nucleotide extensions in EIII and EIXB respectively (Figure 1C). Thus human Serase-1B differs from polyserase-1 by the extra 34-amino-acid insert (from Phe⁹¹ to Gln¹²⁴) and the seven-amino-acid extension at the C-terminus (from Gly⁵²⁵ to Arg⁵³¹) (Figure 1B). The schematic representation of Figure 1(D) shows that this enzyme possesses a transmembrane domain at the N-terminus and a catalytic domain in the C-terminus, suggesting that Serase-1B is a member of the TTSPs.

Tissue distribution of Serase-1B mRNA

RT-PCR was carried out to analyse the gene expression profiles of Serase-1B and polyserase-1 in human and mouse tissues (Figure 2). Although there does appear to be some loading inequity in the human samples based on the GAPDH loading controls, both isoforms had a similar expression pattern in the examined tissues and were expressed prominently in the liver, pancreas, small intestine and testis. In addition, human Serase-1B was highly expressed in the prostate and peripheral blood leucocytes (CD14⁺ and CD8⁺ cells) compared with the expression of polyserase-1. This expression pattern in human tissues is thus different from the distribution of mouse Serase-1B and polyserase-1. Although mouse polyserase-1 was expressed ubiquitously in all examined tissues, mouse Serase-1B was highly expressed in the testis, but scarcely expressed in the liver or small intestine. These results indicate that the splicing of mRNA or the transcriptional regulation of Serase-1B may differ among organs and species.

Expression and characterization of recombinant Serase-1B

For the expression of recombinant protein, we constructed the full-length form with a FLAG tag at the C-terminus, and then established the cells that expressed the protein stably. Flow-cytometric analysis using anti-FLAG antibodies showed significant expression of recombinant protein on the cell surface (results not shown). However, the recombinant Serase-1B purified from conditioned SFCM of the stable transfectant cells showed diffuse protein bands corresponding to the expected Serase-1B sizes (Figure 3A). To clarify how Serase-1B was secreted into the culture medium, we first analysed the N-terminal amino acid sequence of soluble recombinant Serase-1B (Figure 3B). This result revealed that the cleavage occurred between Gly⁵⁴ and Phe⁵⁵ (Gly⁵⁶ and Ala⁵⁷ in mouse). In addition, human Serase-1B was cleaved further between Lys¹⁸⁴ and Ser¹⁸⁵, suggesting that human Serase-1B seems to exist as a short form lacking the SEA module. Shedding of an ectodomain of the membrane protein

by a member of the MMP (matrix metalloproteinase) family was reported in epithin, the mouse homologue of matriptase [34,35]. To determine the role of MMPs in this shedding of Serase-1B, we analysed the effect of GM 6001, an inhibitor of MMPs, and the cleavage site mutation (Gly → Asn) on the secretion of Serase-1B. Neither treatment with MMP inhibitor nor the presence of a cleavage site mutation affected the secretion of Serase-1B (results not shown). This result suggests that the mechanism of ectodomain processing for Serase-1B is different from that of epithin, and it seems to be processed by unknown proteases located in the intracellular compartment. In addition, the recombinant Serase-1B in the SFCM fraction did not show any enzyme activity in this state. Therefore Serase-1B appears to be secreted in an inactive precursor form (pro-Serase-1B). To activate this precursor protein, pro-Serase-1B was incubated with trypsin. As shown in Figure 4, Serase-1B was efficiently converted from the inactive precursor into its active form by trypsin in a time-dependent manner. These results suggest that recombinant Serase-1B was synthesized as a transmembrane protein, but it was also partly secreted as a soluble inactive precursor and, as a result, it might be activated by trypsin or other trypsin-type enzymes.

Substrate and inhibitor specificities

The recombinant Serase-1B purified from the SFCM fraction was used to analyse the substrate specificity after activation of the enzyme preparation by trypsin. Among the substrates of trypsin-type proteases tested, Serase-1B hydrolysed preferentially Boc-Gln-Ala-Arg-MCA, Boc-Glu(OBzl)-Ala-Arg-MCA (where OBzl is benzyloxy), Boc-Leu-Thr-Arg-MCA, and Boc-Leu-Ser-Thr-Arg-MCA (Table 1). Unlike for trypsin, however, Bz-Arg-MCA (where Bz is benzoyl) was not a suitable substrate for Serase-1B. In addition, no significant activity was observed on substrates for furin/PACE, the paired basic amino acid cleaving enzymes, or the substrates for elastase and chymotrypsin-type proteases. It is noteworthy that substrates with arginine in position P1 and alanine or threonine in position P2 were effectively hydrolysed, whereas the examined substrates with lysine in the P1 position were minimally hydrolysed.

The effects of various protease inhibitors on the activity of Serase-1B are shown in Table 2. Aprotinin, leupeptin and benzamidine markedly inhibited the enzymatic activity. However, unlike trypsin, α_1 -antitrypsin did not inhibit its activity. No effect of any other types of protease inhibitors, such as cysteine protease and metalloproteinase inhibitors, was observed. Among the physiological serine protease inhibitors, PAI-1, α_2 -antiplasmin and SLPI also strongly inhibited Serase-1B activity. PAI-1 and α_2 -antiplasmin formed covalent complexes with active Serase-1B as determined by a molecular-mass shift of the enzyme after incubation with these inhibitors by SDS/PAGE and Western blot analysis (Figure 5). These observations imply that Serase-1B is the target protease, which regulates its stability and proteolytic activity by serpin-type inhibitors as reported previously in DESC1 protease and matriptase-3 [22,23].

Serase-1B activates pro-uPA

To identify the physiological substrate for Serase-1B, we examined the ability of Serase-1B to convert several pro-enzymes into active proteases. As shown in Figures 6(A) and 6(B), Serase-1B efficiently converted pro-uPA into active uPA. All other pro-proteases tested in this experiment including trypsinogen, plasminogen, Factor IX, Factor X, Factor XII and pro-HGF activator were not suitable substrates for Serase-1B (results not shown). In addition, the active-site mutant, Serase-1B S421A, did not activate pro-uPA and the natural inhibitors for Serase-1B,

such as SLPI and α_2 -antiplasmin strongly inhibited this pro-uPA activation (Figures 6A and 6C). These results confirmed that pro-uPA is one of the physiological substrates for Serase-1B.

Inhibition of pro-uPA activation

Since GAGs have many negatively charged groups, ionic bonds are likely to be formed upon GAG–protein interactions, resulting

A	10	20	30	40	50	60	70	80	90
	ATGGAGCCCACTGTGGCTGACGTACACCTCGTGCCAGGACAAACAGGAAGTCCCGGCTCTGGATGCCGCGTCTGTGAGCGGCCACC								
	M E P T V A D V H L V P R T T K E V P A L D A A C C R A A T								
	100	110	120	130	140	150	160	170	180
	ATTGGCGTGGTGCCACAGCCCTGTGCTCTCACCTGGGAGTCTTTTGGCTTCTCTCTACACAGGGCTTCCAGCTGGACACACG								
	I G V V A T S L V V L T L G V L L A F L S T Q G F H V D H T								
	190	200	210	220	230	240	250	260	270
	GCCGAGCTGGGGAAATCCGGTGACAGCAGTTTGGCGGGAGACCTCGGAATACCCGACGCTGACGCCACCTCGAGGCACTG								
	A E L R G I R W T S S L R R E T S D Y H R T L T P T L E A L								
	280	290	300	310	320	330	340	350	360
	TTTGTAAATGTTTCAAGAGACAGAGTTAGAGGCAAGCTGCTGGGTGCTCGTACTGAATTATAGGATGGAACTCCAGTGTCTCT								
	F V S S F Q K T E L E A S C V G C S V L N Y R D G N S S V L								
	370	380	390	400	410	420	430	440	450
	GTACATTTCCAGCTGCATTTCTGCTGCGACCCCTCCAGACGCTGAGCCTGGGCTGGAGGAGGATTTGACGAGGAGTCCGGCA								
	V H F Q L H F L L R P L Q T L S L G L E E E L L Q R G I R A								
	460	470	480	490	500	510	520	530	540
	AGGCTGCGGGAGCAGGCACTCCCTGGCTGCCTATGGCAATTTGTGCGCTGAGCTACAGGAGGACATAAGGAACTTGGCAAGAA								
	R L R E H G I S L A A Y G T I V S A E L T G R H K G P L A E								
	550	560	570	580	590	600	610	620	630
	AGAGACTTCAATCAGGCGCTGTCAGGGAACCTCTTTCTGCGGGAACAGCAGTGTGTGACCAAGTGAACCGGAGTGTGACGAC								
	R D F K S G R C P G N S F S C G N S Q C V T K V N P E C D D								
	640	650	660	670	680	690	700	710	720
	CAGGAGGACTGCTCGATGGGTCGAGAGGCGCACTGCGAGTGTGGCTTGAGCCTGCTGGAGGATGGCGGAGGATCGTGGCGGC								
	Q E D C S D G S D E A H C E C G L Q P A W R M A G R A I V G G								
	730	740	750	760	770	780	790	800	810
	ATGGAGCATCCCGGGGAGTTTCCGTGGCAAGCAGCCTTCGAGAGAACAGGAGCACTTCTGTGGGCGCCATCATCAAGGCCAGG								
	M E A S P G E F P W Q A S L R E N K E H F C G A A I I N A R								
	820	830	840	850	860	870	880	890	900
	TGGCTGGTGTCTGCTGCTCACTGCTTCAATGAGTTCGAAGACCCGACGAAGTGGGCTGAGCTGGGTGGGCTACCTCAGCGGCTCG								
	W L V S A A [H] C F N E F Q D P T K W V A Y V G A T Y L S G S								
	910	920	930	940	950	960	970	980	990
	GAGGECAGCACCTGCGGGCCAGTGGTGCAGATGTCAGACCCCTGTACAACGCGGACAGGCGGCTTTGAGTGGCTGTGCTG								
	E A S T V R A Q V V Q I V K H P L Y N A D T A D F [D] V A V L								
	1000	1010	1020	1030	1040	1050	1060	1070	1080
	GAGCTGACAGCCTCTGCTCTTGGCGGACATCCAGCCCGTGTGCTCCCGGCTGCCACACATCTTCCACCCAGCAAGTGC								
	E L T S P L P F G R H I Q P V C L P A A T H I F P P S K K C								
	1090	1100	1110	1120	1130	1140	1150	1160	1170
	CTGATCTCAGGCTGGGCTACCTCAAGGAGGACTTCTGCTCAAGCAGAGTGTGCTGAGAAAGCACTGTGGAGTGTGCTGGACGAGCA								
	L I S G W G Y L K E D F L V K P E V L Q K A T V E L L D Q A								
	1180	1190	1200	1210	1220	1230	1240	1250	1260
	CTGTGTGACGCTGTGACGGCATTCACTGACAGGATGGTGTGCTGGCTACCTGGAGCGGAAGTGGACTCTTGGCAGGCTGAC								
	L C A S L Y G H S L T D R M V C A G Y L D G K V D S C Q G D								
	1270	1280	1290	1300	1310	1320	1330	1340	1350
	TGAGGAGGACCCCTGGTCTGCGAGGAGCCCTCTGGCGGTTCTTTCTGGCTGGCATCTGAGCTGGGAATCGGTGTGCGGAAGCCGG								
	[S] G G P L V C E E P S G R F F L A G I V S W G I C A E A R								
	1360	1370	1380	1390	1400	1410	1420	1430	1440
	CGTCCAGGGCTATGCGCGAGTCACAGGCTACGTGACTGGATCTGGAGGCGCACCAAGCCAGATGCTTGGCCCCACCATG								
	R P G V Y A R V T R L R D W I L E A T T K A S M P L A P T M								
	1450	1460	1470	1480	1490	1500	1510	1520	1530
	GCTCCTGCCCTGCCGCCCGACAGCCTGGCCACAGCTCTGAGAGCCCTGTGGTCAAGCCCCACCAATCGATGAGGCCCCCTC								
	A P A P A A P S T A W P T S P E S P V V S T P T K S M Q A L								
	1540	1550	1560	1570	1580	1590			
	AGTACCGTGCCTTGAATGGGTACCGTTCTAAGCTACAAGGTATTTTCGGGGCAGAAAGGTAG								
	S T V P L D W V T V P K L Q G I F G A E R *								
B									
Polyserase-1	1	MEPTVADVHLVPRITKEVPALDAACRAA	*						60
Serase-1B	1	MEPTVADVHLVPRITKEVPALDAACRAA	TIQVVATSLVLTILGVLLAFSLTQGFHVDHT						60
Polyserase-1	61	AELRGIRWTSLLRRETSYHRTLPTLEAL	*****						90
Serase-1B	61	AELRGIRWTSLLRRETSYHRTLPTLEAL	FVSSFFQKTELEASCVGSVLNRYDGNSSVL						120
Polyserase-1	91	---LHFLRLPLQLSLGLEEELLQRIARLREHGISLAAYGTIVSAELTGRHKGPLAE							146
Serase-1B	121	VHFLHFLRLPLQLSLGLEEELLQRIARLREHGISLAAYGTIVSAELTGRHKGPLAE							180
Polyserase-1	147	RFKSGRCPGNSFCGNSQCVTKVNPEDDQEDCSOGSDEAHCEGLQPAWRMAGRIVGG							206
Serase-1B	181	RFKSGRCPGNSFCGNSQCVTKVNPEDDQEDCSOGSDEAHCEGLQPAWRMAGRIVGG							240
Polyserase-1	207	MEASPGFEPWQASLRENKEHFCGAATINARWLVSAAHCFNEFDPTKWVAYVGYATYLSGS							266
Serase-1B	241	MEASPGFEPWQASLRENKEHFCGAATINARWLVSAAHCFNEFDPTKWVAYVGYATYLSGS							300
Polyserase-1	267	EASTVRAQVVQIVKHPLYNADTADFVAVLEITSPLPFGRHIQPVCLPAATHIFPPSKKC							326
Serase-1B	301	EASTVRAQVVQIVKHPLYNADTADFVAVLEITSPLPFGRHIQPVCLPAATHIFPPSKKC							360
Polyserase-1	327	LISGWYLKEDFLVKPEVLQKATVELLDQALCASLYGHSITDRMVCAGYLDGKVDSCQGD							386
Serase-1B	361	LISGWYLKEDFLVKPEVLQKATVELLDQALCASLYGHSITDRMVCAGYLDGKVDSCQGD							420
Polyserase-1	387	SGGPLVCEEPSGRFFLAGIVSWGIGCAEARPGVYARVTRLRDWILEATTKASMLAPTMT							446
Serase-1B	421	SGGPLVCEEPSGRFFLAGIVSWGIGCAEARPGVYARVTRLRDWILEATTKASMLAPTMT							480
Polyserase-1	447	APAPAAPSTAWPTSPESPVSPTKSMQALSTVPLDWTVPKLCGCGARPAMEKPTRVVG							506
Serase-1B	481	APAPAAPSTAWPTSPESPVSPTKSMQALSTVPLDWTVPKLCGCGARPAMEKPTRVVG	*****						531

Figure 1 For legend see facing page

Figure 1 The predicted nucleotide sequence, alignment of amino acid sequence and schematic representation of the human Serase-1B splice site

(A) Nucleotide and amino acid sequences of the full-length human Serase-1B transcript. Components of the catalytic triad are boxed. The arrow points to the putative cleavage site for an active serine protease. (B) Alignment of the amino acid sequences of human Serase-1B and polyserase-1. Numbering starts with Met¹ of the signal peptide. Serase-1B differs from polyserase-1 at Thr³⁰ (instead of serine), the 34-amino-acid insert (from Phe⁹¹ to Gln¹²⁴) and the seven-amino-acid extension at the C-terminus (from Gly⁵²⁵ to Arg⁵³¹) (all indicated with asterisks). (C) Schematic representation of the human Serase-1B and polyserase-1 splice site. EIIB represents the additional 68 nucleotides. EII and EIX represent the 34- and 23-nucleotide extensions respectively. The conserved GT sequence at the intron start sites is indicated by the asterisk. The polyserase-1 (P-1) is on the top and Serase-1B (S-1B) is on the bottom of each pair. (D) Schematic representation of the predicted domain structure of human Serase-1B and corresponding genomic organization of the coding region. N, N-terminus; TM, transmembrane domain; LDLA, LDL receptor domain class A; SPD, serine protease domain; MB, mucin-like box; C, C-terminus.

Figure 2 RNA levels of Serase-1 and polyserase-1 in human and mouse tissues

RT-PCR analysis was carried out using Serase-1B and polyserase-1 specific primers. As a template, Human Multiple Tissue cDNA panels and first-strand cDNA synthesized from mouse total RNA (1 µg each) were used. The expression level of GAPDH mRNA in each sample was used as an internal control. (A) Analysis of human Serase-1B and polyserase-1 expression. Lane 1, no template control; lane 2, brain (whole); lane 3, heart; lane 4, lung; lane 5, thymus; lane 6, liver; lane 7, spleen; lane 8, pancreas; lane 9, kidney; lane 10, small intestine; lane 11, colon; lane 12, prostate; lane 13, testis; lane 14, ovary; lane 15, skeletal muscle; lane 16, placenta; lane 17, leucocyte/peripheral blood cells; lane 18, mononuclear cells; lane 19, CD14⁺ cells; lane 20, CD4⁺ cells; lane 21, CD8⁺ cells; lane 22, CD19⁺ cells. (B) Analysis of expression levels of mouse Serase-1B and polyserase-1. The examined mouse tissues in lanes 1–15 correspond to human tissues described in (A).

in changes in their activities; probably due to changes in their conformations and substrate accessibility to their active sites. To investigate the effects of GAGs on Serase-1B activity, we next examined whether GAGs regulate pro-uPA activation by Serase-1B. Interestingly, although Serase-1B activity for the synthetic peptide substrate was not affected by GAGs, pro-uPA activation by Serase-1B was inhibited by GAGs in a dose-dependent manner (Figure 7). Especially, LMWH showed the strongest inhibition among GAGs (IC₅₀: hyaluronic acid, 15.7 µg/ml; heparan sulfate, 10.9 ng/ml; heparin, 3.8 ng/ml; and LMWH, 0.9 ng/ml). These results suggest that Serase-1B is a newly discovered protease that

is negatively regulated by GAGs. In addition, a human Serase-1B variant (hSerase-1B^{185–467}; from the LDL receptor class A domain to the serine protease domain lacking a mucin-like box) also activated pro-uPA, and the activation was inhibited by GAGs (results not shown). These results suggest that GAGs seem to interact with the region of Serase-1B through the LDL receptor class A domain to the serine protease domain lacking the mucin-like box. However, there is no common heparin-binding site reported in the primary amino acid sequence of Serase-1B; some of the basic amino acids at the C-terminal side of the active-site serine residue may participate in the binding. Similarly, the

Figure 3 Gel electrophoretic and amino acid sequence analyses of recombinant soluble Serase-1B

(A) Each 0.5 µg of purified recombinant Serase-1B or each 20 µl sample obtained from conditioned SFCM of Serase-1B stable transfectant was subjected to SDS/PAGE and was analysed by silver staining or Western immunoblotting with anti-FLAG antibodies respectively. Lanes 1 and 3, human Serase-1B (hSerase-1B); lanes 2 and 4, mouse Serase-1B (mSerase-1B). Molecular-mass sizes are indicated on the left in kDa. (B) Amino acid sequence of the soluble Serase-1B (shown in A, a–d) was performed as described in the text. The predicted amino acid sequences of human and mouse Serase-1B (hSerase-1B and mSerase-1B respectively) are on the top and the results of amino acid sequences are on the bottom of each panel.

basic residues in thrombin, Factor Xa and Factor IXa consist of heparin-binding sites [36–38]. Therefore the positive-charged fields of the surface of the Serase-1B molecule might be affected by GAGs.

DISCUSSION

The completion of human and mouse genome sequences and the development of systematic bioinformatic analysis resulted in an explosive increase in the discovery of novel genes with the potential to encode functional serine proteases. Especially, the number of functional TTSP genes has markedly increased and over 20 family members have been identified to date. However, the contribution of each of these genes to physiological and pathological conditions remains poorly understood.

In the present study, we described the identification and characterization of Serase-1B, a new polypserase-1/TMPRSS9 isoform

Figure 4 Proteolytic activation of Serase-1B by trypsin

A 100 ng amount of human Serase-1B (A) or mouse Serase-1B (B) was directly incubated with 1 ng of trypsin in 0.1 M Tris/HCl, pH 8.5, for 0–30 min at 37 °C. After incubation, the enzyme preparations were applied to SDS/PAGE containing fluorogenic substrate (Boc-Glu-Ala-Arg-MCA) and the amidolytic activities were analysed by zymography. The increased amidolytic protein corresponding to an activated Serase-1B in each panel is indicated by an arrowhead. Molecular-mass sizes are indicated on the left of each panel in kDa.

Figure 5 Human Serase-1B forms inhibitory complexes with PAI-1 and α_2 -antiplasmin

Purified human Serase-1B (hSerase-1B; lane 1) was activated using trypsin-immobilized beads. After incubation, trypsin beads were separated by centrifugation. The supernatants (lane 2) were then incubated with PAI-1 (lane 3) or α_2 -antiplasmin (α_2 -AP; lane 4). The proteins were separated by SDS/PAGE under reducing conditions, and the blots were probed with antibodies against human Serase-1B. PAI-1 alone or α_2 -antiplasmin alone controls are in lanes 5 and 6 respectively. The positions of the pro-Serase-1B, active Serase-1B, Serase-1B–serpin complexes, degradation product, α_2 -antiplasmin and PAI-1 are indicated. Molecular-mass sizes are indicated on the left in kDa.

Figure 6 Pro-uPA is proteolytically activated by Serase-1B

(A) Pro-uPA was incubated with BSA (Δ), plasmin ($\square$), activated human Serase-1B ($\diamond$), human Serase-1B S421A ($\bullet$), mouse Serase-1B ($\blacktriangle$) or mouse Serase-1B S421A ($\blacksquare$) in the presence of synthetic substrate for uPA (Glt-Gly-Arg-MCA) in 0.1 M Tris/HCl, pH 8.5, at 37 °C. The uPA activity was monitored using the method described above. The enzyme activity of pro-uPA itself ($\circ$) was taken as a background control. (B) Pro-uPA incubated with or without activated Serase-1B was analysed by SDS/PAGE followed by Western immunoblotting with an antibody against the A-chain of urokinase (left-hand panel) or by substrate zymography (right-hand panel). In each panel, lane 1, pro-uPA without activation; lane 2, pro-uPA incubated with BSA; lane 3, pro-uPA incubated with human Serase-1B; lane 4, pro-uPA incubated with mouse Serase-1B; lane 5, pro-uPA incubated with plasmin. Molecular-mass sizes are indicated on the left in kDa. (C) Pro-uPA was incubated with plasmin ($\circ$), plasmin pre-incubated with SLPI (Δ), plasmin pre-incubated with α_2 -antiplasmin ($\square$), human Serase-1B ($\diamond$), human Serase-1B pre-incubated with SLPI ($\times$), human Serase-1B pre-incubated with α_2 -antiplasmin ($\bullet$), mouse Serase-1B ($\blacktriangle$), mouse Serase-1B pre-incubated with SLPI ($\blacksquare$) or mouse Serase-1B pre-incubated with α_2 -antiplasmin ($\blacklozenge$), in 0.1 M Tris/HCl, pH 8.5, containing the fluorogenic substrate for uPA (Glt-Gly-Arg-MCA) at 37 °C. The amidolytic activity of uPA was monitored by the above-described method.

arising from alternative splicing. The Serase-1B-specific splice site defines an additional EIIB, alternative EIII boundary located 34 nucleotides upstream of the polyserase-1 splice site and an extended EIXB. Thus Serase-1B differs from polyserase-1 due to the insertion of 34 amino acids encoding the SEA module in the stem region and a first single protease domain. The detection of alternatively spliced polyserase-1 transcripts has been reported as minor transcripts of 3.8 and 2.4 kb by Northern blotting without detailed analysis [21]. The 2.4 kb band could correspond to Serase-1B. In addition, we also identified another 3.8 kb splice

variant that contains two serine protease domains, namely Serase-2B (results not shown). These results indicate that, in addition to polyserase-1, there are splice variants containing one or two serine protease domains adjacent to their stem region (i.e. Serase-1B and -2B). Although the functional advantages of Serase-1B and -2B are still unclear, the architecture is well conserved in the sequence of mouse and rat orthologues.

A previously reported gene expression analysis of another matriptase subfamily showed high transcript levels of matriptase/MT-SP1/epithin in the rectum, colon and small intestine [39], whereas matriptase-2 expression is restricted to the liver [20] and matriptase-3 is highly expressed in the testis, ovary and trachea [23]. In contrast, the mRNA expression profiles of Serase-1B tend to vary among organs and species. From the panels of human tissues tested by RT-PCR, the highest relative expression of the human Serase-1B mRNA was found in the liver, small intestine, pancreas, testis and CD14⁺ and CD8⁺ cells in peripheral blood. Therefore it appears that the pattern of expression of Serase-1B is different from that of other matriptase subfamilies, thus suggesting that Serase-1B seems to have unique biological functions.

Although Serase-1B was originally predicted to be a membrane-anchored trypsin-type protease from its amino acid sequence, we found a soluble form in conditioned medium from cells transfected with the full-length Serase-1B cDNA. In addition, recombinant Serase-2B was also partly secreted into the culture medium (results not shown). Among other TTSPs, matriptase/MT-SP1/epithin [32] and hepsin [40] were also released from the cell surface in soluble forms. In both cases, the possible protease involved in their ectodomain shedding is a membrane-anchored ADAM (a disintegrin and metalloproteinase) family protein. However, unlike these TTSPs, no effect was observed on the shedding of Serase-1B by treatment with MMP inhibitor or cleavage-site mutation. On the basis of these results, we presume that the secretion of Serase-1B is regulated by other unknown processing proteases. In addition, ectodomain processing itself does not seem to be necessary for such activation, because we could not detect any protease activity of expressed Serase-1B in HEK-293T cells.

Most of the trypsin-type serine proteases are synthesized as a zymogen and cleaved at an activation site to exhibit their proteolytic activity. In order for Serase-1B to become an active protease, the cleavage at its activation site by exposure to trypsin or another trypsin-like protease appears to be required. In contrast, in the case of polyserase-1, GST (glutathione S-transferase)-fusion protease domain proteins (e.g. GST-Serase-1 and GST-Serase-2) expressed in *Escherichia coli* were apparently autoactivated after incubation at 37 °C [21]. Although it is not clear at present whether the extracellular stem region is essential for the maturation and function of Serase-1B, it seems to modulate the conformation of the catalytic domain. Moreover, since a soluble Serase-1B exists, its inactive short form, at least the region from the LDL receptor class A domain to its activation site, thus appears to be required for it to become an active enzyme.

Like other TTSPs, activated Serase-1B showed trypsin-like substrate specificities. Although GST-Serase-1 preferentially hydrolysed the substrates with glycine in the P2 position instead of alanine [21], Serase-1B hydrolysed the substrates in preference to those with small side-chain amino acids, e.g. alanine, and with polar but uncharged amino acids, e.g. threonine, in the P2 position. Therefore the extracellular stem region is most likely to affect not only the maturation of the protease domain, but also the substrate specificity. In addition, the present study suggests that pro-uPA is one of the potential physiological substrates for Serase-1B, although other serine proteases including matriptase/MT-SP1

Figure 7 GAGs inhibit Serase-1B-induced pro-uPA activation

Increasing concentrations of GAGs [(A) hyaluronic acid (HA): 0 ($\circ$), 5 (Δ), 10 ($\square$), 25 ($\bullet$) and 50 ($\blacktriangle$) $\mu\text{g/ml}$; (B) heparan sulfate (HS): 0 ($\circ$), 5 (Δ), 10 ($\square$), 25 ($\bullet$) and 50 ($\blacktriangle$) ng/ml ; (C) heparin: 0 ($\circ$), 5 (Δ), 10 ($\square$), 25 ($\bullet$) and 50 ($\blacktriangle$) ng/ml ; (D) LMWH: 0 ($\circ$), 0.5 (Δ), 1.0 ($\square$), 2.5 ($\bullet$) and 5.0 ($\blacktriangle$) ng/ml] were first pre-incubated with 43.5 ng of activated Serase-1B in the reaction buffer (0.1 M Tris/HCl, pH 8.5) for 5 min at 37°C. Next, the reaction mixture was incubated with 0.5 μg of pro-uPA in the same buffer containing the synthetic substrate for uPA (Glt-Gly-Arg-MCA). The uPA activity was monitored as described in the Materials and methods section. The enzyme activity of pro-uPA itself ($\blacksquare$) was considered to be the background control.

are also involved in pro-uPA activation with functional redundancy. Further studies are required to find whether Serase-1B can activate or degrade some other macromolecules, such as growth factors, GPCRs (G-protein-coupled receptors) and extracellular matrix components in a biologically relevant manner as reported previously in other TTSPs [20,26,27]. Studies of the regulation of Serase-1B activities demonstrated that Serase-1B efficiently forms inhibitory complexes with the serpins, PAI-1 and α_2 -antiplasmin. This finding, combined with the studies of mouse DESC1 [41] and matriptase-3 [23], suggests that the serpin-type serine protease inhibitors are likely to be the regulators of TTSPs and TTSP-mediated physiological processes.

It is widely recognized that GAGs exert important biological functions through binding via their negatively charged sulfate groups. In our studies, GAGs showed negative effects on Serase-1B-induced pro-uPA activation. Thrombin, Factor Xa and Factor IXa are also known to have a heparin-binding exosite and their proteolytic activities are negatively regulated by heparin [36–38]. Among these proteases, the positive-charged fields of the surface of the molecule composed of basic residues located at the C-terminal side of the active-site serine residue are critical for binding to heparin. The basic residues of this region are also conserved at similar locations in Serase-1B. Although more detailed studies are necessary, one possible explanation for the effect of GAGs on pro-uPA activation by Serase-1B is that GAGs interact directly with the positively charged fields on the surface of Serase-1B, resulting in conformational changes and substrate accessibility to the active site.

TTSPs are a rapidly expanding protease family with attention currently focused on elucidation of their functions in biological events. To date, knockout mice of three TTSP genes (hepsin, corin and matriptase) have been reported [28,42,43]. These studies

presented the biological significance of TTSPs in basic development and homeostasis, including epidermal development, blood pressure regulation and liver homeostasis. In addition, the congenital deficiency of enteropeptidase can lead to a severe failure to thrive [44], and mutations of the *TMPRSS3* gene have been demonstrated to be responsible for a congenital or childhood-onset form of deafness [17]. These findings also suggest that TTSPs play a role in the development and maintenance of organ function. Therefore identifying the natural substrates of TTSPs may eventually lead to a better understanding of their function in physiological and pathological conditions.

In summary, we have identified and enzymatically characterized Serase-1B, a novel member of the TTSPs, which has a different expression pattern to that of polyserase-1 and other members of the matriptase subfamily. Our study also demonstrated Serase-1B to be an activator of pro-uPA and its activation is negatively regulated by GAGs. *In vivo* studies using null mutant mice are currently being carried out in our laboratories to help us understand further the physiological and pathological roles of Serase-1B and polyserase-1/TMPRSS9.

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