Tissue expression, protease specificity and Kunitz domain functions of HAI-1B, a new splice variant of hepatocyte growth factor activator inhibitor-1

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Summary

HAI-1 (hepatocyte growth factor activator inhibitor-1) is an integral membrane protein expressed on epithelial cells and contains two extracellular Kunitz domains (N-terminal KD1 and C-terminal KD2) known to inhibit trypsin-like serine proteases. In tumorigenesis and tissue regeneration, HAI-1 regulates the hepatocyte growth factor (HGF)/c-Met pathway by inhibiting the activity of HGF activator (HGFA) and matriptase, two serine proteases that convert pro-HGF into its biologically active form. By screening a placental cDNA library we discovered a new splice variant of HAI-1, designated HAI-1B, which contains an extra 16 amino acids adjacent to the C-terminus of KD1. To investigate possible consequences on Kunitz domain function, a soluble form of HAI-1B (sHAI-1B) comprising the entire extracellular domain was produced. First, we found that sHAI-1B displayed remarkable enzyme specificity by potently inhibiting only HGFA ($IC_{50} = 30.5 \text{ nM}$), matriptase ($IC_{50} = 16.5 \text{ nM}$) and trypsin ($IC_{50} = 2.4 \text{ nM}$) among 16 serine proteases examined, including plasminogen activators (u-PA, t-PA), coagulation enzymes thrombin, factors VIIa, Xa, XIa, XIIa and activated protein C. Relatively weak inhibition was found for plasmin ($IC_{50} = 399 \text{ nM}$) and plasma kallikrein ($IC_{50} = 686 \text{ nM}$). Second, the functions of the KD1 and KD2 domains in sHAI-1B were investigated using P1 residue-directed mutagenesis to show that inhibition of HGFA, matriptase, trypsin and plasmin was due to KD1 and not KD2. Furthermore, analysis by RT-PCR demonstrated that HAI-1B and HAI-1 were co-expressed in normal tissues and various epithelial-derived cancer cell lines. Both isoforms were upregulated in eight examined ovarian carcinoma specimens, three of which had higher levels of HAI-1B RNA than of HAI-1 RNA. Therefore, previously demonstrated roles of HAI-1 in various physiological and
pathological processes likely involve both HAI-1B and HAI-1.
Introduction

HAI-1 (hepatocyte growth factor activator inhibitor-1) is an integral cell surface protein of m~66 kDa expressed on epithelial cells (1-3). HAI-1 is known to inhibit the enzymatic activity of HGF activator (HGFA) (1,4) and matriptase (5-9), two trypsin-like serine proteases capable of converting the inactive single chain form of hepatocyte growth factor (pro-HGF) (10-14) into its biologically active two-chain form (HGF). When activated HGF binds to its receptor c-Met, it promotes phospho-transfer activity of the intracellular tyrosine kinase domain leading to activation of multiple intracellular signaling pathways. Therefore, as an inhibitor of HGFA and matriptase, HAI-1 may control the local generation of HGF and thus modulate the activity of the HGF/c-Met receptor system which is involved in such biological processes as tissue regeneration, morphogenesis and tumorigenesis (reviewed in (15-18)).

The activation of the HGF-converting enzymes represents yet another level of HGF/c-Met pathway regulation. Similar to the coagulation factors, HGFA is mainly produced in the liver and circulates in blood as zymogen (19), but it can also be produced by cancer cells (20). During blood coagulation HGFA is converted into its active two chain form by thrombin. Matriptase is a type II transmembrane serine protease (21) expressed as a single chain form on epithelial cell types (5,22). It has been suggested that sphingosine 1-phosphate, a serum-derived lipoprotein, is able to convert matriptase zymogen into its enzymatically active two chain form (23). In addition to HGFA and matriptase, there are a number of other serine proteases that, at least in vitro, convert pro-HGF into its active form. One of them, coagulation factor XIIa (24), is not inhibited by HAI-1 (1). The ability of HAI-1 to inhibit urokinase-type
plasminogen activator (u-PA) (12,25,26) and two newly identified pro-HGF activators plasma kallikrein and coagulation factor Xla (27) is unknown.

HAI-1 is expressed in many organs and specifically localizes to the surface of epithelial cells, particularly of the columnar epithelium (3,22,28). In addition, the expression of HAI-1 is enhanced or induced during tissue regeneration and inflammation and may regulate the HGFA-mediated activation of pro-HGF (3,29). Moreover, HAI-1 and its target proteases HGFA and matriptase are implicated in the progression of breast cancer (20,22), hepatocellular carcinoma (30) and ovarian cancer (31). In colorectal cancer, HAI-1 expression diminishes during the adenoma to adenocarcinoma transition resulting in an imbalance between HGFA and its inhibitor, which was interpreted as contributing to the invasive tumor phenotype (32,33). A similar enzyme/inhibitor imbalance was observed in ovarian cancer, in which cancer progression was associated with a marked reduction in HAI-1 antigen, while matriptase was only moderately diminished (31). Elevated levels of matriptase mRNA have also been observed in a wide variety of transformed cell lines (34). Additional studies using tissue microarrays have also implicated matriptase and HAI-1 in the progression of node-negative breast cancer (35). Therefore, HAI-1 may regulate the local generation of active HGF by HGFA or matriptase. In addition, HAI-1 may have a role in regulating matriptase-specific activities that could contribute to tumorigenicity and inflammation, such as the activation of u-PA and of G-protein coupled protease activated receptor-2 (PAR-2) (9,36).

Enzyme inhibition by HAI-1 is mediated by two Kunitz domains (N-terminal KD1 and C-terminal KD2) located in the extracellular domain. Both KD1 and KD2 can engage in protease inhibition (37), similar to the structurally
related but more promiscuous HAI-2 (38,39) (also referred to as placental bikunin (40) or kop (41)). Three splice variants were reported for HAI-2, designated as HAI-2A, HAI-2B and HAI-2C (42). They differ in RNA expression levels, tissue distribution and the number of Kunitz domains (one or two). In the present study we describe a splice variant of HAI-1, designated HAI-1B, which contains two Kunitz domains. The generation of soluble HAI-1B allowed us to study enzyme specificity as well as the contribution of each Kunitz domain to enzyme inhibition. Moreover, the expression of HAI-1B relative to HAI-1 in tissues and cells has been investigated and the implications on our current understanding of the biology of HAI-1 and HAI-1B are discussed.
Experimental procedures

Reagents

Pro-HGF, expressed in chinese hamster ovary (CHO) cells in the absence of serum and purified by HiTrap Sepharose SP chromatography, was obtained from David Kahn (Genentech, Inc., South San Francisco, CA). The following synthetic substrates were used to measure enzyme activities: Cholinesterase PTC (propionylthiocholine + DTNB) (Sigma, St. Louis, MO) for acetylcholinesterase, Spectrozyme® fVIIa (American Diagnostica, Greenwich, CT) for HGFA, Chromozym-tPA (Roche Molecular Biochemicals, Indianapolis, IN) for tissue factor/factor VIIa. The following substrates were from Diapharma, Westchester, OH: S2765 for matriptase, S2222 for factor Xa, S2302 for plasma kallikrein, S2366 for activated protein C and plasmin, S2444 for urokinase-type plasminogen activator (u-PA), S2288 for factor XIa, factor XIIa and tissue-type plasminogen activator (t-PA), S2314 for complement factor C1s and S2586 for chymotrypsin. Except for bovine trypsin (Worthington, Lakewood, NJ), all enzymes used were of human origin. Factor Xa, factor XIa, thrombin, activated protein C and plasmin were from Haematologic Technologies (Essex Junction, VT). Plasma kallikrein and factor XIIa were from American Diagnostica. u-PA, acetylcholinesterase and chymotrypsin were from Sigma. Complement factor C1s was from Calbiochem (San Diego, CA). Tissue type plasminogen activator (t-PA) was from Genentech, Inc. Soluble human tissue factor (residues 1-219) was produced in E. coli as described (43). Human recombinant factor VIIa was produced in 293 cells as described (44). All other reagents were of the highest quality available.
Cloning, expression and purification of HGFA

The active enzyme form of HGFA comprises the entire B-chain (Ile408-Ser655) disulfide-linked to the C-terminal 35 residues of the A-chain (Val373-Arg407) (4). Therefore, to produce recombinant active HGFA, the nucleotide sequence encoding amino acids 373 to 655 was cloned by PCR from an HGFA full length clone into the baculovirus expression vector pAcGP67A (Pharmingen, San Diego, CA) immediately 3’ to the gp67 secretion signal sequence. A nucleotide sequence encoding a 3 residue Ala linker and a C-terminal poly-His tag (Ala3His8) was added to the 3’-end. Isolated plasmid DNA was transfected into Spodoptera frugiperda (Sf 9) cells on plates in ESF921 media (Expression Systems, Woodland, CA) via the Baculogold Expression System according to manufacturer’s instructions (Pharmingen, San Diego, CA). Virus was amplified three times before use in protein production.

One liter of High Five™ cells (Invitrogen, San Diego, CA) growing at 5 x 10^5 cells/ml in suspension in ESF921 media was infected with 8 ml of viral stock. Cultures were incubated at 27 °C for 72 h before harvesting the culture media by centrifugation at 8,000 x g for 15 min. NiCl2, CaCl2 and Tris-HCl, pH 8.0 were added to give final concentrations of 1 mM, 5 mM and 50 mM, respectively. Precipitate was removed by filtration through a 0.2 μm filter and the media was then applied onto a 2 ml Nickel-nitrilotriacetic acid (Ni-NTA) agarose column (Qiagen, Valencia, CA). After washing with 10 column volumes of 50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 5 mM imidazole, the HGFA protein was eluted with 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 250 mM imidazole. The purity of HGFA protein (~32kDa) was greater than 95% by SDS-PAGE analysis. N-terminal
sequencing of the protein bands indicated that activation cleavage at the Arg407-Ile408 bond occurred spontaneously during the expression/purification procedures, resulting in enzymatically active two-chain HGFA. The protein concentration was determined by quantitative amino acid analysis.

**Cloning, expression and purification of matriptase**

A full length clone of matriptase was obtained by standard PCR protocols from a mixture of human cDNA libraries including those from brain, heart, liver, lung and spleen using a 5′ primer GGACCATGGGGAGCGATCG and a 3′ primer CCTATACCCCAAGGAGTTCTTCTTGTCCGT. A fragment containing the gene was excised from a 1% agarose gel, purified and ligated into the pCR4-TOPO vector (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. DNA sequencing confirmed an open reading frame of 855 residues identical to that previously described (5). The nucleotide sequence encoding amino acids 615-855 encoding the mature protease domain was cloned by PCR from the full length clone ultimately into plasmid pSTII.TIR3 variant 4 (45) such that Val615 immediately followed the stII signal sequence and a His₈ tag was on the carboxy terminus. This plasmid contained a phoA promoter, stII signal sequence and the △₉ transcriptional terminator. Site-directed mutagenesis was also carried out to make the Cys731Ser mutant to avoid potential complications of an unpaired Cys in the protease domain using the oligonucleotide 5′ CGGCCCATCTCTCCCTGCGCCGAC with the Quickchange kit (Stratagene, La Jolla, CA). As used in this paper, matriptase refers to the matriptase protease domain starting with Val615 containing Cys731Ser and a C-terminal His₈ tail.
*E. coli* strain 33D3 (W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompT Δ(nmpc-fepE) degP41 kanR) was transformed with pSTII.MTSP.PD.H8. Single colonies from a LB carbenicillin plate were inoculated into 5 ml LB medium supplemented with carbenicillin (50 µg/ml) and grown at 30 °C on a culture wheel overnight. The 5 ml culture was diluted into 500 ml of C.R.A.P. phosphate-limiting media (46). Carbenicillin was then added to the induction culture to give a concentration of 50 µg/ml and the culture was grown for approximately 24 h at 30 °C.

*E. coli* pastes from 500 ml shake flask cultures (6–10 g pellets) were resuspended in 10 volumes (w/v) of 20 mM Tris-HCl pH 8.0 containing 7 M guanidine HCl. Solid sodium sulfite and sodium tetrathionate were added to make final concentrations of 0.1 M and 0.02 M, respectively, and the solution was stirred overnight at 4 °C. The solution was clarified by centrifugation and loaded onto a 20 ml Qiagen Ni-NTA metal chelate column equilibrated in 20 mM Tris-HCl pH 8.6 containing 6 M guanidine HCl. The column was washed with additional buffer containing 50 mM imidazole (Ultrol grade; Calbiochem). The protein was eluted with buffer containing 250 mM imidazole. Fractions containing the desired protein based on SDS-PAGE were pooled and diluted to 50 µg/ml with buffer containing 20 mM Tris-Cl pH 8.6, 0.8 M arginine, 0.3 M NaCl, 20 mM glycine, 1 mM EDTA, and 1 mM cysteine. The refolding mixture was incubated overnight at 2-8 °C. The protein was subsequently concentrated 20-fold using Vivascience (Edgewood, NY) concentrator (MW cut-off 10,000) and dialyzed against 50 mM Tris-HCl pH 8.0 and 0.15 M NaCl. The refolded protein was loaded on a Superdex 75 (Amersham Pharmacia Biotech, Piscataway, NJ)
equilibrated with the same buffer. Fractions were analyzed by SDS-PAGE (>95% purity) and enzymatic activity using a chromogenic substrate (see below) and pooled. The matriptase protease domain was also analyzed by N-terminal amino acid sequencing and electrospray mass spectrometry. Protein concentration was determined by quantitative amino acid analysis.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Human cell lines were obtained from ATCC (Manassas, VA) or BioWhittaker, Inc. (Walkersville, MD) and were cultured in recommended serum-supplemented media. The human normal cell lines used were: mammary epithelial cells, aortic smooth muscle cells, pulmonary artery smooth muscle cells, pulmonary artery endothelial cells and umbilical artery endothelial cells. The human tumor cell lines used were: colorectal carcinoma cells (Colo205, HT29, HCT 116, SW480, DLD-1), the breast carcinoma cell line BT-474, the lung carcinoma cells A549 and Calu-6, the pancreatic adenocarcinoma cells HPAC and HPAF-11, the bladder carcinoma cells J82, the renal cell carcinoma cells 786-0, the osteosarcoma cells Saos-2, the rhabdomyosarcoma cells A-673 and the prostate carcinoma cells PC-3.

For RNA isolation confluent cell layers were washed with PBS and Tri-Reagent-LS (Molecular Research Center, Cincinnati, OH) was added to the cells and total RNA was extracted according to manufacturer’s protocols. Total RNA from various human tissues was purchased from BD Biosciences Clontech (Palo Alto, CA). The RNA samples of normal ovary and ovarian adenocarcinomas were from BD Biosciences Clontech (Fig. 3c, samples #1, #5), Ambion, Austin, TX (Fig. 3c, samples #2, #3), the University of Michigan (Fig. 3c, samples #4, #12)
and the Cooperative Human Tissue Network (CHTN) (samples #6-11). Normal and pathological specimens were removed from patients for therapeutic procedures unrelated to this study; they were provided following appropriate IRB review.

These total RNA samples were processed by use of oligo(dT)24 and SuperScript reverse transcriptase (Invitrogen Corp., Carlsbad, CA) to obtain cDNA. The cDNAs were subjected to PCR using the primer set for HAI-1B and HAI-1 or the primer set for β-actin (control). The sequences of the primers were as follows:

- HAI-1B and HAI-1 forward: 5’-ATGGAGGCTGCTTTGGCAACA-3’;
- HAI-1B and HAI-1 reverse: 5’-ACAGGCAGCCTCGTTCGAGG-3’;
- β-actin forward: 5’-TCACCCACACTGTGCCCATCTACGA-3’;
- β-actin reverse: 5’-CAGCGGAACCGCTCATTGCCAATGG-3’.

The PCR amplifications were carried out for 25 cycles of 45 sec at 95 °C followed by 45 sec at 55 °C, and 1 min at 72 °C using Advantage-GC cDNA polymerase mix (BD Biosciences Clontech). The PCR products were separated on a 2.5% agarose gel and then visualized by ethidium bromide staining. In some experiments the bands were excised from the gel and the PCR products extracted and sequenced. The obtained sequences were in full agreement with the expected sequences specific for HAI-1B (containing the 48bp insert region) and for HAI-1 (1), respectively.
Cloning of HAI-1B

Full length HAI-1B was obtained from a cDNA library derived from human placental RNA using oligo dT /Not I site as a primer and adatapor with Sal I site for the second strand. The cDNA was digested with Sal I and Not I; cDNAs greater than 2.8 kb were ligated to pRK5D. Single stranded DNA of the human placental cDNA/pRK5D library was generated using standard molecular biology methods. Reverse primer (5’ ACTGGATGGCGCCTTTCCATG-3’) was annealed to the single-stranded cDNA pool and extended using T7 or T4 DNA polymerase. E.coli were transformed with the synthesized double-stranded DNA and colonies were screened using standard filter hybridization methods. The insert size was analyzed by PCR and the HAI-1B full length clones were identified and confirmed by DNA sequencing.

Construction, expression and purification of soluble HAI-1B

A soluble form of HAI-1B (sHAI-1B) was produced by fusing the cDNA coding for the extracellular domain (amino acids Met-1 - Glu465) of HAI-1B via a Met-Gly residue linker to a poly-His tag at the C-terminus (Met-Gly-His\textsubscript{8}). The cDNA was then inserted into the eukaryotic expression vector pSVI7.ID.LL (47). A stable CHO cell line expressing sHAI-1B was generated using standard methods (47).

The harvested culture supernatant of the CHO stable cell line expressing sHAI-1B was filtered through a 0.2 µm filter. Sodium azide and PMSF were added to the filtered media to give final concentrations of 1 mM and 0.5 mM, respectively. Non-specific nickel binding was also reduced by addition of NaCl and imidazole to give final concentrations of 0.3 mM and 5 mM, respectively. Ni-
NTA resin (3 ml per liter medium) (Qiagen, Valencia, CA) was mixed with the medium for 2 h at 4 °C. The resin was placed into a column, washed with PBS pH 7.5, 0.3 M NaCl and with PBS pH 7.5, 0.3 M NaCl, 7.5 mM imidazole. sHAI-1B was eluted with PBS pH 8.0, 0.3 M NaCl, 250 mM imidazole. Eluted sHAI-1B was then dialyzed against 10 mM Hepes, pH 7.5, 140 mM NaCl using a 10 kDa cutoff dialysis membrane. Dialyzed material was diluted 20-fold with 10 mM Tris-HCl, and adjusted to pH 7.5. The material was then loaded onto a mono Q HR5/5 column (Amersham Biosciences, Piscataway, NJ) and sHAI-1B was eluted using a 0 - 0.3 M NaCl gradient in 10 mM Tris-HCl, pH 7.5. Pooled sHAI-1B fractions were concentrated, dialyzed against 10 mM Hepes pH 7.2, 140 mM NaCl and sterile-filtered.

Construction, expression and purification of soluble HAI-1B mutants

Changes of the P1 amino acids in KD1 and KD2 of sHAI-1B were introduced by site directed mutagenesis using the Quick change XL mutagenesis kit (Stratagene, La Jolla, CA). Using the cDNA of soluble HAI-poly-His construct (see above) as a template, the following oligonucleotides were used for generating the different mutant forms: 5’-CCT CGC ATC CAA CAA GGT TCG CTG CGC CGG CTC TTT CCC ACG C-3’ for the Arg260Ala mutant, 5’- TCC AAC AAG GTG GGT CGC TGC GAG GGA TCC CCA CGC TGG TAC TAT GA-3’ for Arg260Glu mutant, 5’-GCG TGG ACC TGC CAG ACA CAG GCC TCT GCC AGG AGA GCA TCC C-3’ for the Lys401Ala mutant and 5’-GCG TGG ACC TGC CAG ACA CAG GCC TCT GCC AGG AGA GCA TCC C-3’ for the Lys401Gln mutant. The mutations were confirmed by DNA sequencing.
Recombinant protein was produced using a transient transfection process in CHO cells. Cells were grown in 3 liter spinner flasks in F12/DMEM supplemented with ultra-low IgG serum (GibcoBRL, Carlsbad, CA) and Primatone HS (Sigma, St. Louis, MO). The cells were transfected with DNA-cationic lipid complex preformed for 15min in basal medium. Prior to transfection experiments the DNA:cationic lipid ratio as well as cell seeding density were optimized. The cultures were maintained at 33 °C for 6 days. The conditioned medium was then filtered using a 0.2 µM cellulose acetate filter and adjusted to a final concentration of 1 mM PMSF and 1 mM benzamidine and stored at –20 °C. Thawed media was adjusted to a final concentration of 50 mM NaH₂PO₄ pH 8.0, 150 mM NaCl, 10 mM imidazole and loaded onto a Ni-NTA superflo column (Qiagen, Valencia, CA) equilibrated with PBS pH 7.5, 0.3 M NaCl. After washing with PBS pH 7.5, 0.3 M NaCl and with PBS pH 8.0, 0.3 M NaCl, 15 mM imidazole, the sHAI-1B mutants were eluted with PBS pH 8.0, 0.3M NaCl, 250 mM imidazole. Pooled sHAI-1B fractions were dialyzed against 20 mM Hepes pH 7.5, 150 mM NaCl at 4 °C and then concentrated using centriprep YM- 10 (Amicon, Bedford, MA). Purity of the proteins was analyzed by SDS-PAGE and protein concentrations were determined by quantitative amino acid analysis.

Pro-HGF activation assays

¹²⁵I-labeling of pro-HGF was carried out as described using the Iodogen method (43). ¹²⁵I-labeled pro-HGF at 0.05 mg/ml in 20 mM Hepes pH 7.5, 150 mM NaCl, and 5 mM CaCl₂ (HNC buffer) was incubated with 20 nM HGFA or 20
nM matriptase in the presence of sHAI-1B at 37 °C. The concentration of wildtype sHAI-1B and the Arg260 and Lys401 mutants (R260A, K401A, K401Q) in this reaction was 1 µM. After 1 h, aliquots were removed and added to sample buffer (Bio-Rad Laboratories, Hercules, CA) with DTT (Bio-Rad). After briefly heating, samples (approx. 10^6 cpm/lane) were loaded onto a 4-20% gradient polyacrylamide gel (Invitrogen Corp., Carlsbad, CA). After electrophoresis, the dried gels were exposed on x-ray films (X-OMAT AR, Eastman Kodak Company, Rochester, NY) for 10-20 min. Films were developed (Kodak M35A X-OMAT Processor), scanned (Umax S-12, Umax Data Systems, Inc., Fremont, CA) and further processed with Adobe V.7.0 Photoshop software (Adobe Systems Inc., San Jose, CA).

**Enzyme inhibition assays**

The inhibitory activity of sHAI-1B towards a panel of serine proteases was examined. The synthetic substrates used for different enzymes are listed under ‘reagents’. In most cases the substrate concentration approximated the determined K_M values. sHAI-1B was incubated with enzyme in HBSA buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 5 mM CaCl_2) for 20 min at room temperature. Substrate was added and the change in absorbance at 405 nm measured on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

For assays with HGFA (5 nM), matriptase (0.5 nM) and trypsin (0.2 nM) increasing concentrations of wildtype sHAI-1-B and the Arg260 and Lys401 mutants (R260A, R260E, K401A, K401Q) were incubated in HNC buffer containing 0.01% Triton X-100 for 30 min at room temperature. For plasmin (4
nM) assays the mutants were incubated in HBSA buffer. Substrate was added and the change in absorbance at 405 nm measured on a kinetic microplate reader (Molecular Devices, Sunnyvale CA). Spectrozyme® fVIIa (200 µM final conc., K_M = 200 µM) was used for HGFA, S2765 (40 µM final conc., K_M = 40 µM) for matriptase, S2765 (30 µM final conc., K_M = 30 µM) for trypsin and S2366 (200 µM final conc., K_M = 180 µM) for plasmin. The linear rates of the increase in absorbance at 405 nm are expressed as percent activities (100% x v_i / v_0). Inhibitor activity was calculated as the concentration of inhibitor giving 50% inhibition (IC_{50}) of the uninhibited enzyme activity. At least three independent experiments were performed for each enzyme.
Results

Expression of HAI-1B isoform in normal tissues, cell lines and ovarian cancer

The variant HAI-1 was cloned from a placental cDNA library and coded for a 529 amino acid protein (Fig. 1), which is 16 residues longer than the previously described sequence for HAI-1 (1). In order to distinguish between these two isoforms we propose to refer to the new variant described herein as HAI-1B. This is in keeping with the nomenclature chosen for splice variants of the related HAI-2 (42). HAI-1B differs from HAI-1 in two regions (Fig. 1). First and foremost, HAI-1B has an extra 16 amino acid stretch that starts at the C-terminal end of KD1 (Fig. 1). This is due to the recognition of an alternative splice site, adding 48 nucleotides to exon 5 (Fig. 2). This new exon5/intron5 boundary has the consensus boundary sequence G/GT (48) (Fig. 2). The presence of this novel splice variant is further supported by EST sequences (Genbank accession No. CA488310, BG697670, BF749146), which span this region and contain the 48 nucleotides specific for HAI-1B. Secondly, HAI-1B differs from HAI-1 by a single amino acid in the transmembrane domain. Due to a single nucleotide change (G to A), residue Ala453 (HAI-1 numbering) becomes a Thr (residue 469, HAI-1B numbering; Fig. 1). Otherwise, both HAI-1 variants have identical amino acid sequences, including the 35 amino acid signal sequence (Fig. 1).

The expression of HAI-1B and HAI-1 was determined by RT-PCR using oligonucleotide primers, which generated PCR products of different sizes (269 bp for HAI-1B and 221 bp for HAI-1), allowing us to distinguish between the two variants. The results demonstrated that both isoforms had an identical RNA expression pattern across the examined tissues (Fig. 3a). Moreover, RNA levels for HAI-1B were about equal to those for HAI-1, except for placenta and thyroid.
which contained more HAI-1B. Both isoforms were highly expressed in placenta, prostate, salivary gland and thyroid. The absence of any detectable HAI-1B and HAI-1 RNA in the liver in all likelihood reflects limitations of the methods used, since antibody staining identified HAI-1 antigen on epithelial cells lining bile ducts in the liver (3). Results of normal and cancer cell lines were consistent with the co-expression pattern observed in tissues (Fig. 3b). Among normal cell lines, HAI-1B and HAI-1 were only found in mammary epithelial cells, but were absent in smooth muscle and endothelial cells. Both isoforms were strongly expressed in all examined colorectal cancer cell lines, the BT-474 breast cancer cells and the two pancreas cancer cell lines. No expression was found in lung cancer cells, the PC-3 prostate cancer cells or other miscellaneous cancer cell lines mainly of non-epithelial origin (Fig. 3b). Furthermore, the isoforms were detectable at very low levels in one out of four examined normal human ovary samples (Fig. 3c). In contrast, they were expressed in all examined ovarian adenocarcinoma samples (Fig. 3c). Most interestingly, three of them (Fig. 3c samples #5, #6, #10) had higher levels of HAI-1B RNA as compared to HAI-1, whereas equal expression was seen in the other cancer samples (Fig. 3c).

Specificity of enzyme inhibition by soluble HAI-1B

For inhibition studies we used a soluble form of HAI-1B protein (sHAI-1B) comprising the entire extracellular domain and a C-terminal poly-His tag. The inhibitory potency of sHAI-1B towards a panel of 16 serine proteases was measured, among them the pro-HGF converting enzymes HGFA, matriptase, FXIIa, FXIa, plasma kallikrein and u-PA. HGFA and matriptase were produced recombinantly by use of baculovirus and E.coli expression systems, respectively.
N-terminal sequencing of the purified HGFA protein (>95% purity) indicated that activation cleavage at the Arg407-Ile408 bond occurred spontaneously during the expression/purification procedures, resulting in enzymatically active two-chain HGFA. The expressed matriptase protease domain was refolded and purified to homogeneity (>95% purity) using Ni-NTA metal chelate and gel filtration chromatography. N-terminal sequencing revealed a correctly processed N-terminus, which is essential for catalytic activity, and mass spectrometry yielded the correct mass (calcd. 27,512.9 Da; obsd. 27,512.0 Da) for the protease domain containing 3 disulfide bonds, a Cys731Ser mutation and the His$_8$ C-terminal tag.

By screening of a small panel of synthetic para-nitroanilide substrates we found Spectrozyme® fVIIa and S2765 to be suitable substrates for HGFA and matriptase, respectively. sHAI-1B potently inhibited HGFA (IC$_{50}$ = 30.5 nM) and matriptase (IC$_{50}$ = 16.5 nM) while the other pro-HGF converting enzymes were either uninhibited (FXIIa, FXIa, u-PA) or weakly inhibited (plasma kallikrein) (Table 1). None of the other 10 proteases tested was inhibited by sHAI-1B, except for trypsin (IC$_{50}$ = 2.4 nM) and plasmin (IC$_{50}$ = 399 nM) (Table 1). The protease specificity was further investigated by use of plasma clotting assays which involve the activity of numerous trypsin-like serine proteases (49). sHAI-1B at relatively high concentrations (2.1 µM) did not prolong the clotting times in the activated partial thromboplastin time (APTT) and prothrombin time (PT) assays (data not shown).

Roles of HAI-1B KD1 and KD2 in enzyme inhibition
In order to assess the inhibitory activities of each Kunitz domain, the P$_1$ residues of KD1 (Arg260) and KD2 (Lys401) were individually changed to Ala residues by site-directed mutagenesis. Replacing the Kunitz domain P$_1$ residue with Ala should largely abolish inhibitory activity since this residue is critical for interaction with respective target proteases. The purity of the sHAI-1B mutants is shown in Figure 4, which also includes the two additional mutants sHAI-1B(R260E) and sHAI-1B(K401Q). In amidolytic assays with HGFA and matriptase, both wildtype sHAI-1B and the KD2 mutant sHAI-1B(K401A) were equally potent in inhibiting HGFA or matriptase activity (Fig. 5a and b). In contrast, the KD1 mutant sHAI-1B(R260A) was completely inactive up to a concentration of 2 µM (Fig. 5a and b). The additionally tested mutants sHAI-1B(K401Q) and sHAI-1B(R260E) gave similar results to their respective Ala variants (Table 2). Similarly, in trypsin assays the KD1 mutant sHAI-1B(R260A) was >100-fold less potent than wildtype, whereas sHAI-1B(K401A) maintained wildtype sHAI-1B potency (Fig. 5c). Qualitatively similar differences between KD1 and KD2 inhibitory potencies were found for inhibition of plasmin. The results are summarized in Table 2 and include the IC$_{50}$ values obtained with the two additionally examined mutants sHAI-1B(K401Q) and sHAI-1B(R260E).

The sHAI-1B mutants were further examined with HGFA and matriptase using their macromolecular substrate pro-HGF. First, the pro-HGF conversion efficiency of matriptase and HGFA was compared by use of $^{125}$I-labeled pro-HGF. As illustrated in Figure 6, both enzymes were comparably efficient in converting pro-HGF into its two chain form during a 1 h reaction period. Complete conversion of pro-HGF was achieved at 10 - 20 nM. Unlike FXIa and plasma kallikrein (27), neither HGFA nor matriptase produced the HGF 2-chain.
fragment (by cleavage at Arg424-His425) (27), even after prolonged reaction periods. Addition of 1 μM sHAI-1B completely inhibited pro-HGF conversion by either HGFA or matriptase, as did the KD2 mutants sHAI-1B(K401A) and sHAI-1B(K401Q) (Fig. 7a and b). However, the KD1 mutant sHAI-1B(R260A) completely lacked inhibitory activity towards HGFA and matriptase, as indicated by the unabated formation of the HGFα/β heterodimer (Fig. 7a and b). Identical results with wildtype sHAI-1B and the three sHAI-1B mutants were found in 125I-labeled pro-HGF activation experiments by use of 10% human serum that contains the naturally occurring form of HGFA (4) as activator (data not shown).
Discussion

HAI-1B is a newly identified HAI-1 isoform arising from alternative splicing. The HAI-1B-specific splice site defines an alternative exon 5/intron 5 boundary located 48 nucleotides downstream of the HAI-1 splice site (42). Thus, HAI-1B differs from HAI-1 by the insertion of a 16 amino acid peptide stretch C-terminal to the first Kunitz domain. The presence of alternatively spliced HAI-1 transcripts is not without precedent among Kunitz type inhibitors as three splice variants were found for the closely related HAI-2 (42). However, while two of the HAI-2 isoforms lack the second Kunitz domain (42), both HAI-1 isoforms retain the full Kunitz domain complement. Moreover, the HAI-2 isoforms differ in RNA expression levels and tissue specificity, whereas both HAI-1B and HAI-1 share similar tissue distributions and expression levels. This co-expression in conjunction with the fact that both HAI-1 isoforms potently inhibit HGFA and matriptase could mean they are involved in similar biological processes. This view is reinforced by the finding that HAI-1B is expressed along with HAI-1 in many of the examined epithelial-derived cancer cell lines, some of which also express the target proteases HGFA and matriptase (7,20,22). For instance, the co-expression of HAI-1B and HAI-1 in all tested colorectal cancer cell lines may indicate that the postulated role of HAI-1 in colorectal cancer progression (33) is in fact the result of contributions by both HAI-1B and HAI-1. Another example is the expression of HAI-1B, HAI-1 (our study) and matriptase (7,22,34) in mammary epithelial cells and breast cancer cells suggesting that HAI-1B acts as a physiologic inhibitor of matriptase in concert with the variant HAI-1, whose ability to inhibit matriptase has been shown (7,8). Consistent with this tenet is the co-expression in all examined ovarian adenocarcinoma specimens, three of which
actually had higher levels of HAI-1B RNA. A recent study by Oberst et al. (31) demonstrated the presence of HAI-1 and matriptase in ovarian tumor and the authors suggested that the enzyme/inhibitor imbalance observed in advanced stage tumors could be an underlying cause for a more aggressive phenotype. It would be of interest to understand whether differences in the HAI-1 isoform levels that were seen in three ovarian tumors have any bearing on tumor progression or are related to the tumor phenotype.

The observed tissue and cell expression of both HAI-1 variants is in general agreement with previous determinations of HAI-1 mRNA levels and HAI-1 protein staining (1,3,20,22,28). These latter studies utilized probes that may have specifically detected the expression of HAI-1 or, more likely, the combined expression of both HAI-1 and HAI-1B, such as in ovarian carcinoma. This ambiguity raises the possibility that the potential biological functions assigned to HAI-1, such as its roles in tissue regeneration, tumorigenesis and morphogenesis could in fact represent the aggregate of HAI-1 and HAI-1B functions. The further dissection of the role of each isoform for a given biological process will require the generation of specific tools capable of discriminating between these structurally similar proteins.

The HAI-1 isoform is known to inhibit three proteases, trypsin, HGFA and matriptase (1,8,37). The latter two enzymes may be functionally linked to the HGF/c-Met pathway as both are capable of converting pro-HGF into its active form. While HGFA was shown to exert strong pro-HGF converting activity (24), little is known about matriptase in this regard (9). A comparative analysis showed that both HGFA and matriptase processed pro-HGF at similar rates (Fig. 6). This result and the ability of HAI-1 and HAI-1B to inhibit them reinforces the
view that HGFA, matriptase and HAI-1/1B comprise an enzyme/inhibitor system that plays an important role in regulating the levels of active HGF available for c-Met activation. Unlike HAI-2 which inhibits numerous enzymes, the activity of HAI-1B seems almost exclusively directed towards HGFA and matriptase, since it completely lacks inhibitory activity towards other examined serine proteases, including the other known pro-HGF converting enzymes u-PA, FXIIa and FXIa (12,24-27). In addition, the finding that sHAI-1B did not inhibit any of the coagulation factors agrees with the premise that HAI-1B is important in regulating HGFA enzymatic activity, rather than interfering with the coagulation reactions that lead to the generation of active HGFA by proteolytic conversion of HGFA zymogen (19). sHAI-1B also inhibited plasmin and plasma kallikrein, albeit very weakly. Consistent with this, sHAI-1B displayed weak inhibitory activity towards plasma kallikrein-mediated pro-HGF conversion (data not shown). However, the physiological function of HAI-1B as an inhibitor of plasma kallikrein is unlikely, particularly in light of the existence of potent plasma inhibitors of kallikrein, e.g. C1 inhibitor and α2-macroglobulin (50). Since inhibition by the HAI-1 isoform has only been studied with a few enzymes, it is unknown whether the remarkable enzyme specificity of HAI-1B is a unique property of this isoform.

Both Kunitz domains of HAI-1 can engage in enzyme inhibition of HGFA and trypsin (37). Since alternative splicing results in the addition of a 16 amino acid peptide adjacent to the C-terminal end of KD1 in HAI-1B, the question arose as to whether this would impact inhibitory function of the Kunitz domains. To address this question, the Kunitz domains were separately inactivated by P1-residue directed mutagenesis. Functional analysis of the sHAI-1B mutants in
amidolytic assays demonstrated that KD1 was fully functional, since the KD2 mutants (K401A/Q) were as potent as wildtype sHAI-1B in inhibiting HGFA, matriptase, trypsin and plasmin. Therefore, despite the addition of a 16 residue peptide by alternative splicing, the functional properties of KD1 remained intact. What is more, KD1 accounted for essentially all of the inhibitory activity for sHAI-1B, since KD2 activity towards HGFA, matriptase and trypsin was ≤ ca. 1%. This conclusion is based on the premise that the measured reduction of inhibitory activities by the sHAI-1(R260A/E) mutants solely reflects KD2 function and not any remaining KD1 activity. For bovine pancreatic trypsin inhibitor (BPTI), changing the P₁ residue to Ala or Glu decreases the equilibrium binding constant for trypsin by ca. 10⁷-fold (51). Since similar affinity losses are expected for KD1 mutations of sHAI-1, the ca. 10²-fold reduction in the IC₅₀ values observed likely reflect inhibition due to KD2.

Experiments with pro-HGF, the macromolecular substrate for HGFA and matriptase, were consistent with the amidolytic assays in demonstrating that KD2 lacked inhibitory activity. This raises the question as to whether KD2 is a functional Kunitz domain. Another protein containing two Kunitz domains is bikunin, also known as urinary trypsin inhibitor, from the inter-Kunitz-inhibitor complex (52). A structure of bikunin reveals that the two Kunitz domains, which are connected by a short linker, pack closely together such that protease inhibition by the second Kunitz domain may be adversely affected (53); this has been demonstrated for FXa and kallikrein (54). In comparison, the Kunitz domains of HAI-1B are separated by the intervening LDL receptor-like domain, making a direct KD1-KD2 interaction, like that observed in bikunin, less likely to
occur. The third Kunitz domain of tissue factor pathway inhibitor-1 (TFPI-1), the physiological inhibitor of the TF/FVIIa complex, also lacks any known anti-protease activity (55). Based on structural information, it has been argued that the Ser residue at position 36, normally a Gly in BPTI homologs, interferes with the enzyme-inhibitor docking process (55). However, HAI-1B KD2 contains the consensus residue Gly36, leaving the function of KD2 unresolved at present.

Another possibility is that the apparent lack of KD2 anti-protease activity represents a specific property of the HAI-1B isoform, since HAI-1 KD1 mutants still inhibited trypsin and HGFA (37). In that study (37) KD2 and KD1 were equally potent for trypsin inhibition, while KD2 was somewhat less potent towards HGFA. Taken at face value, this suggests that the presence of the additional 16 amino acid residues in HAI-1B may have impaired the availability of KD2 for enzymatic inhibition. However, this seems unlikely and requires further evaluation. Generally, there are very few cases that clearly demonstrate different functions of protein isoforms generated by alternative splicing events (56). For instance, the two isoforms of ectodysplasin (EDA) (57), EDA-A1 and EDA-A2, differ by only two amino acids. Yet, this small difference completely changes ligand specificity for the receptors EDAR and XEDAR, both of which are members of the TNF receptor family (58). For HAI-1B, if the role of the 16 residue insert were indeed to effect suppression of KD2 function, then the KD2 fragment by itself should be devoid of any such constraint and a gain of inhibitory function should result. In the future, such studies could shed more light on the possible regulation of KD2 activity and functional differences between the HAI-1 isoforms.
We have identified the splice variant HAI-1B, which along with HAI-1, is expressed in various tissues including ovarian tumor and in epithelial-derived cancer cells. This suggests that both isoforms may be involved in similar physiological and pathological processes. This does not preclude the possibility that there are as yet unknown differences in respect to surface shedding, signaling, protease inhibition and other functions. For instance, the peptide insertion site lies within a protease-sensitive region as indicated by the finding that proteolytic cleavage between the KD1 and the LDL receptor-like domain produces a low molecular weight form of HAI-1 (40/39 kDa) with enhanced inhibitory activity (59). Thus, it is conceivable that proteolytic regulation of HAI-1B including surface shedding differs from that of HAI-1. The relatively small structural differences between HAI-1B and HAI-1 pose a significant challenge in the further investigation of such questions. In as much as the soluble form of HAI-1B is a specific and potent inhibitor of HGFA and matriptase, it has potential for use in cancer therapy. Of particular interest are colorectal and ovarian cancer, where the reduction in endogenous HAI-1/-1B might lead to tumor growth and invasion due to an imbalanced HGFA- and/or matriptase-mediated activation of the HGF/c-Met pathway (31,33).

Acknowledgments:

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References


Footnotes

1The abbreviations used are: HAI-1, HAI-1B, variants of hepatocyte growth factor activator inhibitor-1; sHAI-1B, soluble form of HAI-1B encompassing the extracellular domain; HGFA, hepatocyte growth factor activator; pro-HGF, single chain hepatocyte growth factor; HGF, two-chain hepatocyte growth factor; u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; KD1, N-terminal Kunitz domain of HAI-1/1B; KD2, C-terminal Kunitz domain of HAI-1/1B; HBSA buffer, 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.5 mg/ml BSA, 5 mM CaCl₂; HNC buffer, 20 mM Hepes pH 7.5, 150 mM NaCl, 5 mM CaCl₂; CHO cells, Chinese hamster ovary cells, APTT, activated partial thromboplastin time, PT, prothrombin time; BPTI, bovine pancreatic trypsin inhibitor; TFPI-1, tissue factor pathway inhibitor-1; Ni-NTA, Nickel-nitrilotriacetic acid.
Figure legends

Fig. 1  Alignment of amino acid sequences of HAI-1B and HAI-1. Numbering starts with residue Met1 of the signal peptide. The N-terminus of HAI-1B/HAI-1 after release of the 35 residue signal peptide is indicated by an arrow. Solid lines highlight the first and second Kunitz domains (KD1 and KD2). Also indicated are the P_1 residues of KD1 and KD2 (arrowheads) and the transmembrane domain (dashed line). HAI-1B differs from HAI-1 (1) by the 16 amino acid insert (asterisks) at the C-terminus of KD1 and by Thr469 (instead of Ala) (asterisk) located in the transmembrane region. In the region of the 16 amino acid insert, the Gly305 residue of HAI-1 can be either aligned with Gly305 of HAI-1B as shown here, or with Gly321 of HAI-1B.

Fig. 2  Schematic representation of the HAI-1B and HAI-1 splice sites at the exon5/intron5 boundary. KD1, KD2 are the Kunitz domains, LDL and TM are the LDR receptor like domain and the transmembrane domains, respectively. E5-B represents the 48 nucleotide extension of exon 5, which is specific for HAI-1B. Indicated above the codons are the deduced amino acids (single letter code). The conserved GT sequence at the intron start sites are shown in boxes. The HAI-1B sequence is on the top, HAI-1 on the bottom.

Fig. 3  RNA levels of HAI-1B and HAI-1 in tissues, cell lines and ovarian cancer. RT-PCR of total tissue and cellular RNA was carried out by use of
oligonucleotide primers that allowed amplification of the region encompassing the splice site. The two PCR products (269 base pairs for HAI-1B and 221 base pairs for HAI-1) differed by 48 base pairs.

(a) Analysis of normal tissues: 1, adrenal gland; 2, bone marrow; 3, brain (cerebellum); 4, brain (whole); 5, heart; 6, kidney; 7, liver; 8, lung; 9, prostrate; 10, salivary gland; 11, skeletal muscle; 12, spleen; 13, testis; 14, thymus; 15, thyroid; 16, trachea; 17, uterus; 18, placenta.

(b) Analysis of various cell lines. 1, mammary epithelial cells; 2 aortic smooth muscle cells; 3, pulmonary artery smooth muscle cells; 4, pulmonary artery endothelial cells; 5, umbilical artery endothelial cells; colorectal carcinoma cell lines: 6, Colo205; 7, HT29; 8, HCT 116; 9, SW480; 10, DLD-1; breast carcinoma: 11, BT-474; lung carcinoma: 12, A549; 13, Calu-6; pancreatic adenocarcinoma: 14, HPAC; 15, HPAF-11; bladder carcinoma: 16, J82; renal cell carcinoma: 17, 786-0; osteosarcoma: 18, Saos-2; rhabdomyosarcoma: 19, A-673; prostate carcinoma: 20, PC-3.

(c) Analysis of normal ovary and ovarian cancer specimens: 1-4, normal ovary; 5-12, adenocarcinoma of ovary. The figures a-c are representative of at least three independent experiments.

Fig. 4 Purified sHAI-1B mutants. Using P$_1$-residue directed mutagenesis, the KD1 mutants R260A and R260E and the KD2 mutants K401A and K401Q were produced in CHO cells. Purified proteins were analyzed by SDS-PAGE on a gradient gel followed by Coomassie-staining; WT denotes wildtype sHAI-1B. Molecular mass markers in kDa are indicated.
Fig. 5 Inhibition of amidolytic activities of (a) HGFA (b) matriptase and (c) trypsin by wildtype and mutant forms of sHAI-1B. Enzymes were incubated with the wildtype sHAI-1B (filled triangles) or the P1-residue mutants R260A (first Kunitz domain; open squares) and K401A (second Kunitz domain; open circles) for 30 min at room temperature. The enzymatic activities of HGFA (5 nM), matriptase (0.5 nM) and bovine trypsin (0.2 nM) towards chromogenic substrates were determined on a kinetic microplate reader at 405 nm. Data presented are averages of at least three independent determinations; the lines drawn represent data fit to a 4-parameter equation, from which the IC$_{50}$ was calculated.

Fig. 6 Pro-HGF converting activities of HGFA and matriptase. $^{125}$I-labelled pro-HGF (0.05 mg/ml) was incubated with decreasing concentrations (2-fold dilution steps of enzymes [E], 80 nM in lane 2 down to 1.25 nM in lane 9) of (a) HGFA and (b) matriptase at 37 °C in 20 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$. After 1 h the samples were analyzed by SDS-PAGE under reducing conditions and the dried gels were exposed to x-ray films. The positions of pro-HGF, HGF $\alpha$-chain and HGF $\beta$-chain (doublet) are indicated. Lane 1 (0) is aliquot taken at the beginning of the reaction (0 min). The figures are representative of four independent experiments.

Fig. 7 Inhibition of pro-HGF activation by mutant forms of sHAI-1B. $^{125}$I-labeled pro-HGF (0.05 mg/ml) was incubated with (a) 20 nM HGFA or (b) 20 nM matriptase for 1 h at 37 °C in the presence of 1 µM wildtype sHAI-1B (wt),
1 µM of sHAI-1B mutants R260A, K401A, K401Q or with control buffer (ctrl). The reaction mixtures were analyzed by SDS-PAGE (reducing conditions) followed by exposure to x-ray films. Lane 1 (t=0) is aliquot taken at the beginning of the reaction (0 min). The figures are representative of three independent experiments.
Table 1. Specificity of enzyme inhibition by sHAI-1B

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HGFA</td>
<td>30.5 ± 5.5</td>
</tr>
<tr>
<td>Matriptase</td>
<td>16.5 ± 2.2</td>
</tr>
<tr>
<td>Trypsin</td>
<td>2.4 ± 1.1</td>
</tr>
<tr>
<td>Plasmin</td>
<td>399 ± 147</td>
</tr>
<tr>
<td>Plasma kallikrein</td>
<td>686 ± 111</td>
</tr>
<tr>
<td>Chymotrypsin</td>
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<tr>
<td>Factor Xla</td>
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<tr>
<td>Factor XIIa</td>
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<tr>
<td>Thrombin</td>
<td>&gt;2000</td>
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<td>Tissue factor/factor VIIa</td>
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<tr>
<td>Factor Xa</td>
<td>&gt;2000</td>
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<tr>
<td>Activated Protein C</td>
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<td>Complement factor C1s</td>
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<tr>
<td>t-PA</td>
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<tr>
<td>u-PA</td>
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<tr>
<td>Acetylcholinesterase</td>
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Values are the average ± SD of at least three independent experiments
Table 2. Inhibition of HGFA, matriptase, trypsin and plasmin by sHAI-1B Kunitz domain mutants

<table>
<thead>
<tr>
<th>sHAI-1B mutant</th>
<th>HGFA IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Matriptase IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Trypsin IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>Plasmin IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
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<tr>
<td>Wildtype</td>
<td>30.8 ± 5.5</td>
<td>16.5 ± 2.2</td>
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<td>399.3 ± 147.0</td>
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<tr>
<td>K401A</td>
<td>30.5 ± 4.4</td>
<td>12.8 ± 1.5</td>
<td>4.0 ± 1.7</td>
<td>197.1 ± 72.6</td>
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<tr>
<td>K401Q</td>
<td>23.7 ± 4.6</td>
<td>17.7 ± 1.9</td>
<td>3.2 ± 1.7</td>
<td>240.3 ± 29.4</td>
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<tr>
<td>R260A</td>
<td>&gt;2000</td>
<td>&gt;2000</td>
<td>269.9 ± 64.1</td>
<td>&gt;5000</td>
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<tr>
<td>R260E</td>
<td>&gt;2000</td>
<td>N.D.</td>
<td>178.3 ± 85.0</td>
<td>&gt;5000</td>
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</table>

The P<sub>1</sub> residues of the first (KD1) and second Kunitz domain (KD2) of sHAI-1B were replaced by Ala (R260A and K401A), Glu (R260E) or Gln (K401Q). Enzyme concentrations were 5 nM for HGFA, 0.5 nM for matriptase, 0.2 nM for bovine trypsin and 4 nM for plasmin. The values are the average ± SD of at least three independent experiments.

<sup>a</sup> N.D., Not determined.
Figure 1

**KD1**

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<th>HAI-1</th>
<th>1 MAPARTMARARLAPAGIPAVAVAL WLLCTLGLQGTOAGGPPAPPGLAPAGADC 50</th>
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<td>1 MAPARTMARARLAPAGIPAVAVAL WLLCTLGLQGTOAGGPPAPPGLAPAGADC 50</td>
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<tr>
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<td>51 LNSFTAGVPGFYLDNTNASVSNAGTFTLESPTVVRGRGWDCYRACCTQCONLNA 100</td>
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<tr>
<td>HAI-1B</td>
<td>51 LNSFTAGVPGFYLDNTNASVSNAGTFTLESPTVVRGRGWDCYRACCTQCONLNA 100</td>
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<tr>
<td>HAI-1</td>
<td>101 LVELQIDRGEDAIIAACFLINCLVEQNFVCKFAPREGFINYLTVRYSYR 150</td>
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**KD2**

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<td>HAI-1</td>
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<td>HAI-1B</td>
<td>RGVQQGP LRGSSGAAQT FPQPSMMERRHPCSGTCoQPTFCRCSNGCCIDS 334</td>
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<td>-------------------------------------------------------------</td>
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<tr>
<td>HAI-1</td>
<td>351 FLECDTTPNCPDSDDEAAECYKTSGFELEDQRIHFPSDKGHCDLDPDTGLC 364</td>
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<td>HAI-1B</td>
<td>FLECDTTPNCPDSDDEAAECYKTSGFELEDQRIHFPSDKGHCDLDPDTGLC 400</td>
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</table>

**Figure 1**
Figure 2
Figure 3
Figure 4
Figure 5a

HGFA activity (% of control) vs. sHAI-1B (nM)
Figure 5b
Figure 5c
Figure 6
Figure 7

Pro-HGF α-chain

Pro-HGF β-chain

Pro-HGF γ-chain

Figure 7

<table>
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a. + sHAI-1B

b. + sHAI-1B
Tissue expression, protease specificity and Kunitz domain functions of HAI-1B, a new splice variant of hepatocyte growth factor activator inhibitor-1

Daniel Kirchhofer, Mark Peek, Wei Li, Jennifer Stamos, Charles Eigenbrot, Saloumeh Kadkhodayan, J. Michael Eliott, Racquel T. Corpuz, Robert A. Lazarus and Paul Moran

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