Tissue Expression, Protease Specificity, and Kunitz Domain Functions of Hepatocyte Growth Factor Activator Inhibitor-1B (HAI-1B), a New Splice Variant of HAI-1*

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Hepatocyte growth factor activator inhibitor-1 (HAI-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 that 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 (IC50 = 30.5 nM), matriptase (IC50 = 16.5 nM), and trypsin (IC50 = 2.4 nM) among 16 serine proteases examined, including plasminogen activators (urokinase- and tissue-type plasminogen activators), coagulation enzymes thrombin, factors VIIa, Xa, XIa, and XIIa, and activated protein C. Relative weak inhibition was found for plasmin (IC50 = 399 nM) and plasma kallikrein (IC50 = 686 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 reverse transcription-PCR demonstrated that HAI-1B and HAI-1 were co-expressed in normal tissues and various epithelial-derived cancer cell lines. Both isoforms were up-regulated 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.

Hepatocyte growth factor activator inhibitor-1 (HAI-1) is an integral cell surface protein of 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 tumorogenesis (reviewed in Refs. 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 (19). 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 XIIa (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, 23). 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

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The abbreviations used are: HAI-1, hepatocyte growth factor activator inhibitor-1; HGF, hepatocyte growth factor; HGFA, hepatocyte growth factor activator; pro-HGF, single-chain hepatocyte growth factor; u-PA, urokinase-type plasminogen activator; CHO, Chinese hamster ovary; Ni-NTA, nickel-nitrioltriacetic acid; PBS, phosphate-buffered saline; KD1 and KD2, N- and C-terminal Kunitz domain of HAI-1B; sHAI-1B, soluble form of HAI-1B encompassing the extracellular domain.

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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, whereas matriptase was only moderately diminished (31). Elevated levels of matrkapase 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, HAIs have been postulated to 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 (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 soluble 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, Sigma); Spectrozyme® VIIa (American Diagnostica, Greenwich, CT) for HGF, Chromozym-iPA (Roche Applied Science) 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, S2314 for complement factor C1s, and S2586 for chymotrypsin. Except for bovine trypsin (Worthington, Lakewood, NJ), all of the enzymes were used 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 American Diagnostica. u-PA, acetylcholinesterase, and chymotrypsin were from Sigma. Complement factor C1s was from American Diagnostica. u-PA, acetylcholinesterase, and chymotrypsin were from Sigma. Complement factor C1s was from American Diagnostica. u-PA, acetylcholinesterase, and chymotrypsin were from Sigma.

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 5' primer GGAGGATGCGGAGCAA and 3' primer CTTATACCCATGGTTCCTTGGTGTCGAGT. A fragment containing the gene was excised from a 1% agarose gel, purified, and ligated into the pCR4-TOPO vector (Invitrogen) 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 His tag was on the C terminus. This plasmid contained a phoA promoter, the stII signal sequence, and the lam transcriptional terminator. Site-directed mutagenesis was also carried out to make the C731S mutant to avoid potential complications of an unpaired Cys in the protease domain using the oligonucleotide 5'-CGCGGATCCACGTTCGGGGCAG-3' with the QuiChange kit (Stratagene, La Jolla, CA). As used in this paper, matriptase refers to the matriptase protease domain starting with Val615 containing C731S and designated as matriptase.

E. coli strain 3D33 (W3110 ΔmukA Δ(StnA) ptrlA lac Iq lacL5 supplement Δ(nomp-fepE) degP41) was transformed with pSTII.MTSP.PD.H8. Single colonies from a LB carbenicillin plate were inoculated into 5 ml of LB medium supplemented with carbenicillin (50 μg/ml) and grown at 37°C on a culture wheel overnight. The 5-ml culture was diluted into 500 ml of phosphate-limiting medium (46). Carbenicillin was then added to the induction culture to give a concentration of 50 μg/ml, and the culture was grown for ~24 h at 30°C. E. coli pastes from 500-ml shake flasks cultures (6–10-g pellets) were resuspended in 10 volumes (v/v) of 20 mM Tris-HCl, pH 8.0, containing 7 μM guanidine HCl. Solid sodium sulfite and sodium tetrathionate were added to a final concentration of 0.1 and 0.05 mM, 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 (Uttrod 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 2–5°C. The protein was subsequently concentrated 20-fold using Vivaspin (Edgewood, NY) concentrator (molecular weight cut-off, 10,000) and dialyzed against active 25 mM Tris-HCl, pH 8.0, and 0.15 M NaCl. The refolded protein was loaded on a Superdex 75 (Amersham Biosciences) equilibrated with the same buffer. The 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. The final concentration was determined by quantitative amino acid analysis.

Reverse Transcription-PCR—Human cell lines were obtained from ATCC (Manassas, VA) or BioWhittaker, Inc. (Walkersville, MD) and were cultured in recommended serum-supplemented medium. The human normal cell lines used were mammary epithelial cells, aortic smooth muscle cells, pulmonary artery smooth muscle cells, and umbilical vein endothelial cells, and umbilical artery endothelial cells. The human tumor cell lines used were: colorectal carcinoma cell lines (Caco205, HT29, HCT 116, SW480, and DLD-1), the breast carcinoma cell line BT-474, the lung carcinoma cell lines A549 and Calu-6, the
pancreatic adenocarcinoma cell lines HPAC and HPAF-11, the bladder carcinoma cell line J82, the renal cell carcinoma cell line 786-o, the osteosarcoma cell line Saos-2, the rhabdomyosarcoma cell line A-673, and the prostate carcinoma cell line 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 Clontech (Palo Alto, CA). The RNA samples of normal ovary and ovarian adenocarcinomas were from Clontech (see Fig. 3c, samples 1 and 5). Ambion (Austin, TX; see Fig. 3c, samples 2 and 3), the University of Michigan (see Fig. 3c, samples 4 and 12), and the Cooperative Human Tissue Network (see Fig. 3c, samples 6–11). Normal and pathologic specimens were removed from patients for therapeutic procedures unrelated to this study; they were provided following appropriate Institutional Review Board review.

These total RNA samples were processed by use of oligo(dT) and SuperScript reverse transcriptase (Invitrogen) 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'-ATGAGGCTGCTTGGGCAACA-3'; HAI-1B and HAI-1 reverse, 5'-ACAGGGGCTCTGGGAGG-3'; β-actin forward, 5'-TCACCCACACTTGCCCCCATCTAGCA-3'; and β-actin reverse: 5'-CAGGGGACCGCTTATGCGGCA-3'. The PCR amplifications were carried out for 25 cycles of 45 s at 95°C, 1 min at 55°C, and 1 min at 72°C using Advantage-PC DNA polymerase mix (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 were extracted and sequenced. The obtained sequences were in full agreement with the expected sequences specific for the HAI-1B-containing 48-bp 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) and NotI site as a primer and adaptor with SacI site for the second strand. The cDNA was digested with SacI and NotI; cDNAs greater than 2.8 kb were ligated to pRK5D. Single-stranded DNA of the human placental cDNA library was generated using standard molecular biology methods. Reverse primer (5'-AAGTGGATGCGCGTCTGGCAT-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 insert size was 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 Met1–Glu360) of HAI-1B via a MalE Gly residue linker to a poly-His tag at the C terminus (MalE Gly-His). The cDNA was then inserted into the multiple cloning vector pSV17.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 phenylmethylsulfonyl fluoride were added to the filtered medium to give final concentrations of 1 and 0.5 μM, respectively. Non-specific nickel binding was also reduced by the addition of NaCl and imidazole to give final concentrations of 0.3 and 5 μM, respectively. Ni-NTA resin (3 ml/100 ml of medium) (Qiagen) 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 μM imidazole. sHAI-1B was then eluted with PBS, pH 8.0, 0.3 M NaCl, 200 μM咪多昔酸; sHAI-1B was then dialyzed against 10 mM Hepes, pH 7.5, 140 mM NaCl using a 10-kDa cut-off dialysis membrane. Dialyzed material was dialyzed 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), and sHAI-1B was eluted using a 0–0.3 M NaCl gradient in 10 mM Tris-HCl, pH 7.5. sHAI-1B fractions were concentrated, dialyzed against 10 mM Hepes, pH 7.2, 140 mM NaCl, and sterility 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 QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA). Using the cDNA of soluble HAI-1B, primers (see above) and the following nucleotides were used for generating the different mutant forms: 5'-CCT CGC ATC CAA CAA GGT TCG CTC CGG CCG TCT CTC TCC AGC C-3' for the R260A mutant, 5'-TCC AAC AAG GTG GTC GCG TGC GAG GGA TTC CCA CGC TGG TAC TAT GAA-3' for the R260E mutant, 5'-GGG TGG ACG TGC CAG CAC GAG GCC TCT GCG CCG AGA GCA TCC C-3' for the K401A mutant, and 5'-GGG TGG ACG TGC CAG CAC GAG GCC TCT GCC AGG AGA GCA TCC C-3' for the K401Q mutant. The mutations were confirmed by DNA sequencing.

Recombinant protein was produced using a transient transfection process in CHO cells. The cells were grown in 3-liter spinner flasks in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 10% fetal bovine serum (Invitrogen) and Pramdown (Sigma). The cells were transfected with DNA-cationic lipid complex preformed for 15 min in basal medium. Prior to transfection experiments the DNA to 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 membrane and concentrated to 1 ml with ethanol and diethylamine fluoride and 1 mM benzamidine and stored at –20°C. Thawed medium 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 Superflow column (Qiagen) 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, 0.3 M 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 the protein concentrations were determined by quantitative amino acid analysis.

Pro-Factor Activating Assays—HAI-1B and the Arg260 and Lys401 mutants (R260A, K401A, and K401Q) in this reaction was incubated with HGFA (5 nM) and 1% Triton X-100 for 30 min at room temperature. Substrate was added, and the change in absorbance at 405 nm was measured on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA). For assays with HGFA (5 nM), substrate was added, and the change in absorbance at 405 nm was measured on a kinetic microplate reader (Molecular Devices). Spectrozyme® ViIIa (final concentration, 200 μM; Km, 200 μM) was used for HGFA, S2765 (final concentration, 40 μM; Km, 40 μM) was used for matriptase, S2765 (final concentration, 30 μM; Km, 30 μM) was used for trypsin, and S2866 (final concentration, 200 μM; Km, 180 μM) was used for plasmin. The linear rates of the increase in absorbance at 405 nm are expressed as percentage activities (100% is calculated as the concentration of inhibitor giving 50% inhibition (IC50) 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). 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 exon 5-intron 5 boundary has the consensus boundary sequence G/GT (48) (Fig. 2). The presence of this novel splice variant is further supported by expressed sequence tag sequences (GenBank™ accession numbers CA488310, BG697670, and 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. Because of 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 reverse transcription-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, because antibody staining identified HAI-1 antigen on epithelial cells lining bile ducts in the liver (3). The 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 or in other miscellaneous cancer cell lines mainly of nonepithelial origin (Fig. 3b). Furthermore, the isoforms were detectable at very low levels in one 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, and 10) had higher levels of HAI-1B RNA as compared with 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 toward 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 (calculated, 27,512.9 Da; observed, 27,512.0 Da) for the protease domain containing three disulfide bonds, a C731S mutation, and the His8 C-terminal tag.

By screening 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 (IC50 = 30.5 nM) and matriptase (IC50 = 16.5 nM), whereas the other pro-HGF converting enzymes were either uninhibited (FXIIa, FXIa, and u-PA) or weakly inhibited (plasma kallikrein) (Table I). None of the other 10 proteases tested was inhibited by sHAI-1B, except for trypsin (IC50 = 2.4 nM) and plasmin (IC50 = 399 nM) (Table I). The protease specificity was further investigated by use of...
plasma clotting assays that 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 and prothrombin time assays (data not shown).

Roles of HAI-1B KD1 and KD2 in Enzyme Inhibition—To assess the inhibitory activities of each Kunitz domain, the P1 residues of KD1 (Arg260) and KD2 (Lys401) were individually changed to Ala residues by site-directed mutagenesis. Replacing the Kunitz domain P1 residue with Ala should largely abolish inhibitory activity because this residue is critical for interaction with respective target proteases. The purity of the sHAI-1B mutants is shown in Fig. 4, which also includes the two additional mutants sHAI-1B(R260E) and sHAI-1B(K401Q). In amidolytic assays with HGFA and matriptase, both wild type sHAI-1B and the KD2 mutant sHAI-1B(K401A) were equally potent in inhibiting HGFA or matriptase activity (Fig. 5, a and b). In contrast, the KD1 mutant sHAI-1B(R260A) was completely inactive up to a concentration of 2 μM (Fig. 5, a and b). The additionally tested mutants sHAI-1B(K401Q) and sHAI-1B(R260E) gave similar results to their respective Ala variants (Table II). Similarly, in trypsin assays the KD1 mutant sHAI-1B(R260A) was >100-fold less potent than wild type, whereas sHAI-1B(K401A) maintained wild type sHAI-1B potency (Fig. 5c). Qualitatively similar differences between KD1 and KD2 inhibitory potencies were found for the inhibition of plasmin. The results are summarized in Table II and include the IC50 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 125I-labeled pro-HGF. As illustrated in Fig. 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 α/β heterodimer (Fig. 7). However, the KD1 mutant sHAI-1B(R260A) completely lacked inhibitory activity toward HGFA and matriptase, as indicated by the unabated formation of the HGF α/β heterodimer (Fig. 7). Identical results with wild type 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 because three splice variants were found for the closely related HAI-2 (42). However, whereas 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, because both are capable of converting pro-HGF into its active form. Although 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 toward HGFA and matriptase, because it completely lacks inhibitory activity toward other examined serine proteases, including the other known pro-HGF converting enzymes u-PA, FXIa, 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 toward 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). Because 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). Because 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, because the KD2 mutants (K401A and K401Q) were as potent as wild type (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) toward chromogenic substrates were determined on a kinetic microplate reader at 405 nm. The data presented are the averages of at least three independent determinations; the lines drawn represent data fit to a four-parameter equation, from which the IC₅₀ was calculated.
The IC₅₀ values observed likely reflect inhibition caused by KD2 activity and not any remaining KD1 activity. For KD2, 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, the physiological inhibitor of the tissue factor/factor VIIa 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 bovine pancreatic trypsin inhibitor homologs, interferes with the enzyme-inhibitor docking process (55). However, HAI-1B KD2 contains the consensus residue Gly³⁶, leaving the function of KD2 unresolved.

Another possibility is that the apparent lack of KD2 anti-protease activity represents a specific property of the HAI-1B isoform, because HAI-1 KD1 mutants still inhibited trypsin and HGFA (37). In that study (37) KD2 and KD1 were equally potent for trypsin inhibition, whereas KD2 was somewhat less potent toward 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 (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 tumor necrosis factor 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.

TABLE II

| sHAI-1B mutant | HGFA | Matriptase | Trypsin | Plasmin |
|----------------|------|-----------|--------|--------|
| Wild type      | 30.8 ± 5.5 | 16.5 ± 2.2 | 2.4 ± 1.1 | 399.3 ± 147.0 |
| K401A          | 30.5 ± 4.4 | 12.8 ± 1.5 | 4.0 ± 1.7 | 197.1 ± 72.6 |
| K401Q          | 23.7 ± 4.6 | 17.7 ± 1.9 | 3.2 ± 1.7 | 240.3 ± 29.4 |
| R260A          | >2000       | >2000      | 269.9 ± 64.1 | >5000 |
| R260E          | >2000       |           | 178.3 ± 85.0 | >5000 |

a) ND, not determined.

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-α-inhibitor complex (52). The 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 FXα 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, the physiological inhibitor of the tissue factor/factor VIIa complex, also lacks any known anti-protease activity (55).

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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
sheding, 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 mass 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-1B-1 may lead to tumor growth and invasion caused by imbalanced HGFA- and/or matriptase-mediated activation of the HGF/c-Met pathway (31, 33).

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Tissue Expression, Protease Specificity, and Kunitz Domain Functions of Hepatocyte Growth Factor Activator Inhibitor-1B (HAI-1B), a New Splice Variant of HAI-1
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