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Role of the Stem Domain of Matriptase in the Interaction with its Physiological Inhibitor, Hepatocyte Growth Factor Activator Inhibitor Type I

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Matriptase is a type II transmembrane serine protease containing the non-catalytic domains (stem domain) and catalytic domain in the extra-cellular region. Our aim is to address the role of the stem domain in the interaction between matriptase and its physiological inhibitor, hepatocyte growth factor activator inhibitor type I (HAI-1). We prepared secreted variants of recombinant matriptase containing the entire extra-cellular domain (HL-matriptase) or only the catalytic domain (L-matriptase), and compared the inhibition activities of a cell membrane-anchored form of recombinant HAI-1 (maHAI-1) against the matriptase variants in the hydrolysis of peptidyl–4-methyl-coumaryl-7-amide (MCA) substrates. HL-matriptase and L-matriptase were inhibited by purified maHAI-1 with a similar extent when *t*-butyloxycarbonyl (Boc)-Gln-Ala-Arg-MCA (1) and acetyl-Lys-Thr-Lys-Gln-Leu-Arg-MCA (2) were used as substrates. However, HL-matriptase was inhibited more strongly than L-matriptase by maHAI-1 in the hydrolysis of Boc-[(2S)-2-amino-3-(benzyloxycarbonyl)propionyl]-Pro-Arg-MCA (3). These results show that the stem domain of matriptase facilitates the inhibitory interaction of this protease with maHAI-1 in the hydrolysis of substrate 3, although it has no effect in the hydrolysis of substrates 1 and 2. To our knowledge, this is the first evidence that the stem domain of matriptase can affect the interaction between this protease and HAI-1.

Key words: hepatocyte growth factor activator inhibitor type I, inhibitory interaction, matriptase, membrane-type serine protease, stem domain.

Abbreviations: BEK, bovine enterokinase; Boc, *t*-butyloxycarbonyl; CHO, Chinese hamster ovary; CUB domain, complement factor 1R–urchin embryonic growth factor–bone morphogenetic protein domain; HAI-1, hepatocyte growth factor activator inhibitor type I; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; LDLRA domain, low-density lipoprotein receptor A module domains; MCA, 4-methyl-coumaryl-7-amide; r-EK, recombinant enterokinase; sc-uPA, single-chain urokinase-type plasminogen activator; SEA domain; sea-urchin sperm protein–enterokinase–agrin domain.

Matriptase (also known as membrane-type serine protease 1, epithin, suppression of tumorigenecity 14, *etc*.) is a type II transmembrane serine protease consisting of 855-amino-acid residues (*1–5*). Hydrolysis of the peptide bond between Arg614 and Val615 of a latent matriptase molecule is catalysed by another matriptase molecule (latent or activated form), and the enzyme is activated, and this process is named transactivation (*6*, *7*). The activated enzyme has trypsin-like activity, and is known to cleave to activate a variety of proteins, including single-chain urokinase-type plasminogen activator (sc-uPA) (*8–11*), pro-hepatocyte growth factor (HGF) (*9*, *12*), and the precursor form of prostasin, a glycosyl-phosphatidylinositol-linked serine protease (*3*, *13*). These characteristics, together with the abundant expression in surface-lining epithelial cells such as enterocytes (*10*, *14*), lead to a proposal that matriptase is a key upstream regulator of the epithelial-cell turnover, including proliferation, migration, differentiation and exfoliation.

Type II transmembrane serine proteases are characterized by the N-terminal cytoplasmic domain, a signal anchor transmembrane domain, and an extra-cellular C-terminal serine protease catalytic domain. In addition, members of this family with a few exceptions (*e.g*. hepsin) have the extra-cellular non-catalytic domains (hereinafter called stem domain) consisting of various structural motifs (*15*, *16*). For instance, the matriptase stem domain comprises a sea-urchin sperm protein–enterokinase–agrin (SEA) domain, two complement factor 1R–urchin embryonic growth factor–bone morphogenetic protein (CUB) domains, and four low-density lipoprotein receptor A module (LDLRA) domains (Fig. 1A). Of interest is the finding that the activity of enteropeptidase (also called enterokinase), a member of this family, is greatly dependent on the stem domain (*17*). Indeed, a secreted variant of recombinant (or r-) bovine enteropeptidase containing the entire extra-cellular domain (HL-BEK) cleaves the substrate trypsinogen with a catalytic efficiency 520 times higher than does another variant consisting only of the catalytic

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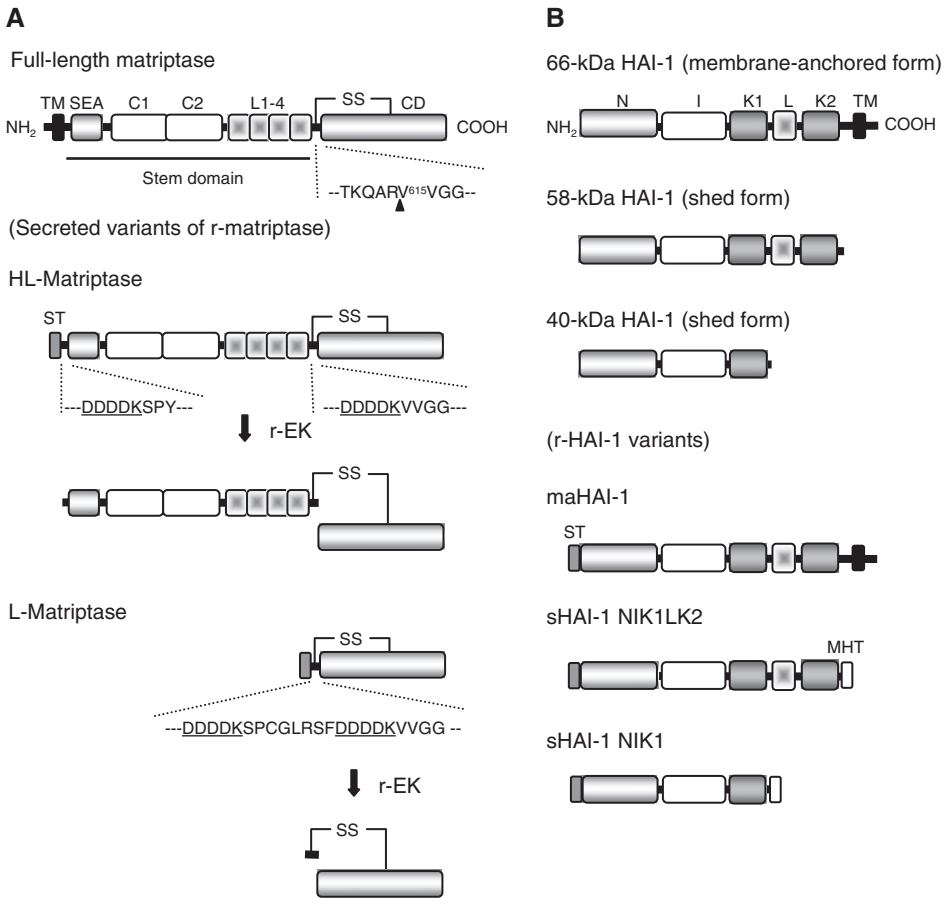

Fig. 1. **Schematic illustration of the structure of naturally occurring matriptase and HAI-1 species and their expression constructs.** (A) Domain structures of full-length rat matriptase (top), HL-matriptase, and L-matriptase. The amino acid numbering starts from the putative N-terminus of the protein. The N- and C-termini are indicated by NH_2 and COOH, respectively in full-length matriptase. The predicted disulphide linkages between two cysteine residues corresponding to Cys604 and Cys731 in matriptase are shown as SS. The amino acid sequence around the matriptase activation cleavage site is indicated in the single-letter code with amino acid numbering at the N-terminal Val residue of the catalytic domain (Val615). The activation cleavage site is indicated by arrowhead. The stem domain of matriptase is indicated with underline. HL-matriptase is a secreted form of r-matriptase in which the cytosolic domain and the signal anchor (Met1–His80) are replaced with the human immunoglobulin κ-chain signal peptide and S-tag (ST). L-matriptase is another r-matriptase variant in which Tyr81–Cys602 is truncated from HL-matriptase. Enteropeptidase recognition sequences (DDDDK, underlined) and their surrounding sequences are shown in both r-matriptase variants. Both HL-matriptase and L-matriptase are activated *in vitro* by incubation with r-EK. Note that S-tag at the N-terminus of each variant can also be removed by incubation with r-EK. TM, transmembrane domain; SEA, SEA domain; C1 and C2, the first and second CUB domain; L1–4, four LDLRA repeats; CD, catalytic domain. (B) Domain structures of 66-kDa HAI-1, 58-kDa HAI-1, 40-kDa HAI-1, maHAI-1, sHAI-1 NIK1LK2 and sHAI-1 NIK1. The N- and C-termini are indicated by NH_2 and COOH, respectively, in the 66-kDa HAI-1. maHAI-1 is a cell membrane-anchored form of r-HAI-1 in which S-tag (ST) is fused at its N-terminus. sHAI-1 NIK1LK2 and sHAI-1 NIK1 are secreted variants of r-HAI-1 corresponding to 58-kDa HAI-1 and 40-kDa HAI-1, respectively. S-tag and *Myc*-hexahistidine tag (MHT) are fused at their N- and C-termini, respectively. N, N-terminal domain; I, internal domain; K1, Kunitz domain I; L, LDLRA domain; K2, Kunitz domain II; TM, transmembrane domain.

domain (L-BEK) (*17*). Later, the N-terminal fragment 118–465 [consisting of a mucin-like domain, a first LDLRA domain, a first CUB domain, and the N-terminal half of an MAM domain (named for motifs found in Meprin, *Xenopus laevis* A5 protein, and protein tyrosine phosphatase μ)] in the stem domain was found to contain a secondary substrate-binding site that interacts directly with trypsinogen (*18*). On the other hand, we found no evidence that the stem domain of matriptase affects its activity (*11*). The activities of secreted variants of r-matriptase containing the entire extra-cellular domain (HL-matriptase) or only the catalytic domain (L-matriptase) were similar toward various peptidyl–4-methyl-coumaryl-7-amide (MCA) substrates containing an Arg residue at the P1 site and sc-uPA (*11*). Therefore, the significance of the stem domain in matriptase remains to be determined.

HGF activator inhibitor type I (HAI-1) is a physiological inhibitor of matriptase (*1*). HAI-1 is a Kunitz-type serine protease inhibitor isolated originally from the conditioned medium of a human stomach carcinoma MKN45 cell line as a potent inhibitor of a serine

protease, HGF activator (*19*, *20*). The primary translation product of HAI-1 predicted from the cDNA sequence comprises 513 amino acid residues, including a putative N-terminal signal peptide sequence and a hydrophobic region at its C-terminal region, suggesting that HAI-1 is produced first as a type I transmembrane protein (*19*). An HAI-1 species with a molecular mass of 66 kDa detected in extracts of MKN45 cells and monkey kidney COS-1 cells transiently transfected with a full-length rat HAI-1 cDNA is thought to be the membrane-anchored form (hereinafter 66-kDa HAI-1) (*21*, *22*). The extracellular domain of HAI-1 comprises an N-terminal domain, followed by an internal domain, first protease inhibitory domain (Kunitz domain I), an LDLRA domain and a second Kunitz domain (Kunitz domain II) (Fig. 1B). The extra-cellular domain is shed by cleavage with certain proteases. At least two HAI-1 species of 58 and 40 kDa are found in conditioned media of MKN45 cells (*19*, *21*). The 58-kDa species (58-kDa HAI-1) has the N-terminal domain to Kunitz domain II, whereas the 40-kDa species (40-kDa HAI-1) lacks Kunitz domain II and presumably the LDLRA domain (Fig. 1B) (*19*).

We previously produced secreted variants of r-HAI-1 using COS-1 cells, and found that Kunitz domain I was responsible for the inhibition of HL-matriptase but Kunitz domain II was not (*23*). We also found that a secreted variant of r-HAI-1 (designated sHAI-1 NIK1 in the present study, Fig. 1B), which might correspond to 40-kDa HAI-1, inhibited HL-matriptase more efficiently than did another secreted variant (designated sHAI-1 NIK1LK2, Fig. 1B) corresponding to 58-kDa HAI-1 (*23*). Taken together, we suggested that the matriptase-binding site in Kunitz domain I could be obstructed by Kunitz domain II, and thus, the inhibitory activity of sHAI-1 NIK1LK2 was lower than that of sHAI-1 NIK1 against HL-matriptase.

The stem domain of enteropeptidase was also shown to drastically affect the inhibitory interaction of this protease with macromolecule inhibitors (*17*). For instance, L-BEK was inhibited with a Kunitz-type inhibitor from soybean, whereas HL-BEK was not. This led us to examine whether the stem domain of matriptase affects the interaction of this protease with HAI-1. In the present study, we compare the inhibitory activities of the r-HAI-1 variants against HL-matriptase and L-matriptase, and provide evidence that the stem domain of matriptase can facilitate the inhibitory interaction of this protease with the HAI-1.

MATERIALS AND METHODS

Materials—A bovine r-enteropeptidase catalytic domain (Recombinant Enterokinase® and hereinafter designated r-EK), an immobilized S-protein (S-protein Agarose®), a horseradish peroxidase (HRP)-conjugated S-protein (S-protein HRP Conjugate®), and a protein size marker (Perfect Protein™ Markers, 15–150 kDa) were purchased from Novagen (Madison, WI). A mouse anti-*Myc* epitope-tag antibody conjugated with HRP and cell culture reagents, including fetal bovine serum, were purchased from Invitrogen (Carlsbad, CA). *t*-Butyloxycarbonyl (Boc)-L-glutamyl-L-alanyl-L-arginine-MCA (Boc-QAR-MCA), acetyl-L-lysyl-L-threonyl-L-lysyl-L-glntaminyl-L-leucyl-L- arginine-MCA (Ac-KTKQLR-MCA), and Boc-[(2*S*)-2-amino-3-(benzyloxycarbonyl) propionyl]-L-prolyl-L-arginine-MCA [Boc-D(OBzl)-PR-MCA] were purchased from Peptide Institute (Osaka, Japan). Bovine trypsin (type III) was from Sigma (St. Louis, MO, USA). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

Cell Culture—A Chinese hamster ovary cell line (CHO-K1) was obtained from Health Science Research Resources Bank (Osaka, Japan), and were maintained in Ham's F12 medium supplemented with 5% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in 5% CO_2.

Preparation of Secreted Variants of r-Matriptase—Using CHO-K1 cells, we have produced secreted variants of rat r-matriptase consisting of the entire extra-cellular domains (Tyr81–Val855) (HL-matriptase) and of the catalytic domain (and its N-terminal spacer region) (Asp603–Val855) (L-matriptase) (Fig. 1A) (*11*, *23*). They are activated *in vitro* by r-EK. Hereinafter, the secreted variants of r-matriptase activated by incubation with r-EK are referred to as simply HL-matriptase and L-matriptase (or r-matriptase variants). The procedures for the production, purification using immobilized S-protein and activation with r-EK were described previously (*11*, *23*). The active sites of the r-matriptase variants were titrated in the procedures as follows: A part of them was incubated with 1 mM of Boc-QAR-MCA in 25 mM HEPES buffer (pH 7.5) containing 145 mM NaCl and 0.1% Triton X-100 (hereinafter called HEPES buffer), for 10 min at 37°C in a volume of 80 μl. The reaction was terminated by adding 350 μl of 0.1 M monochloroacetic acid in 0.1 M sodium acetate buffer, pH 4.3 (stop solution), and the absorbance at 370 nm was measured. One unit of activity was defined as the amount of the enzyme that librates 0.5 nmol of 4-methyl-coumaryl-7-amine/min from Boc-QAR-MCA, and the enzyme concentration was estimated by sodium dodecyl sulphate–polyacrylamide electrophoresis (SDS–PAGE) followed by silver staining with bovine trypsin as the standard. One enzyme unit was given by 1 pmol enzyme.

Preparation of r-HAI-1 Variants—A plasmid for expression of an HAI-1 variant harbouring the amino acid residues from Pro41 to Leu513 (the amino acid numbering of HAI-1 starts from the putative N-terminus of the protein), including those for the transmembrane and cytoplasmic domains, has already been constructed using the pSecTag/hygroB vector (Invitrogen) (*22*). In the present study, the expression plasmid and product were designated pSec-maHAI-1 and maHAI-1, respectively (Fig. 1B). Expression plasmids designated pSecNIK1LK2 and pSecNIK1 for the respective secreted r-HAI-1 variants, sHAI-1 NIK1LK2 and sHAI-1 NIK1, have already been constructed using this vector (*23*). In the designation of the secreted r-HAI-1 variants, the abbreviations of the domains were placed according to their order in the variants from the N-terminus to C-terminus (refer to Fig. 1). The plasmids were transfected into CHO-K1 cells using LipofectAMINE2000™ as described previously (*10*). Clones resistant to

Ham's F12 medium containing 400 μg/ml hygromycin B were obtained, and the expression levels in the conditioned media by each clone was determined by western blotting using HRP-conjugated S-protein as described previously (*23*). S2125, N1403 and N1515, clones with the highest expression of maHAI-1, sHAI-1 NIK1LK2 and sHAI-1 NIK1, respectively, were propagated and stored in liquid nitrogen until use.

maHAI-1 was prepared from Triton extracts of S2125 as follows: Cells were cultured on 100-cm^2 dishes (Asahi Techno Glass, Tokyo, Japan). After reaching confluence, cells were washed three times with ice-cold phosphate-buffered saline (PBS; 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4), and then exposed to 1 ml of ice-cold PBS containing 1% Triton X-100. After incubation for 5 min on ice, cells were scraped with Triton, transferred to a 1.5 ml microcentrifuge tube, and incubated for 1 h on ice with occasional agitation. After incubation, the mixture was centrifuged at 10,000*g* for 30 min at 4°C. The resulting supernatant (Triton extract) was incubated for 30 min with 30 μl of a slurry of immobilized S-protein at 25°C with rocking. After centrifugation at 5,000*g* for 2 s at 25°C, the resulting supernatant was incubated again with fresh immobilized S-protein as described above. After centrifugation at 5,000*g* for 2 s at 25°C, the resulting supernatant was discarded. Slurries were pooled, washed extensively with PBS containing 0.1% Triton X-100 (PBST) and washed once with 0.1% Triton X-100 solution. Then, maHAI-1 bound to the slurry was eluted with 375 μl of 0.2 M sodium citrate buffer (pH 2.0), and the eluate was immediately neutralized with 125 μl of 2 M Tris. The r-HAI-1 variant was subjected to gel filtration in HEPES buffer using NAP-5 column (GE Healthcare, Tokyo blanch) with an elution volume of 1.0 ml. The gel filtrate was stored at –20°C until use.

sHAI-1 NIK1LK2 and NIK1 were prepared as follows: N1403 and N1515 were cultured on 75-cm^2 flasks. After reaching confluence, cells were washed three times with ice-cold PBS, and 10 ml of serum-free Ham's F12 was added to each flask. After 48 h, the conditioned medium was collected, and fresh serum-free medium was added. This was repeated until half of the cells were peeled off. The collected media were centrifuged immediately at 3,000*g* for 10 min at room temperature, and the resulting supernatants were stored at –20°C until use. For purification, 120 ml of the conditioned media was collected using two flasks. After thawing, the media were pooled and concentrated to 2.5 ml by ultrafiltration using Amicon® Ultra-15 (10,000 MWCO, Millipore, Cork, Ireland). The concentrated medium was subjected to gel filtration in PBST using a PD-10 column (GE Healthcare) with an elution volume of 3.5 ml. sHAI-1 NIK1LK2 and NIK1 were purified from 1 ml of the gel filtrates using immobilized S-protein as described above. The remainder of the gel filtrate was stored at –20°C until purification. sHAI-1 NIK1LK2 and NIK1 samples eluted from the S-protein column were subjected to gel filtration in HEPES buffer and stored at –20°C until use.

Determination of Concentrations of r-HAI-1 Variants—A portion of the sample of each r-HAI-1 variant in HEPES buffer was added to an equal volume of 2 × Laemmli protein sample buffer (or Laemmli buffer) [1 × Laemmli buffer: 0.05 M Tris–HCl (pH 6.8), 12 mM dithiothreitol, 10% glycerol, 2% SDS and 0.005% bromophenol blue] (*24*). Perfect Protein™ Marker, in which each protein is fused to an S-tag and the concentration of each marker was known, was diluted with 1 × Laemmli buffer (1:25 dilution). After boiling for 3 min, various amounts of diluted samples were subjected to SDS–PAGE followed by western transfer. The blots were probed with HRP-conjugate S-protein, and the X-ray films were scanned digitally, and the signal intensities of protein bands were analysed by densitometry as described previously (*22*). The concentration of maHAI-1 was calculated by comparing the signal density of the bands of interest with the 75-kDa protein contained in the protein marker. The sHAI-1 NIK1LK2 and NIK1 were compared with the 50-kDa and 35-kDa proteins, respectively (see Fig. 2).

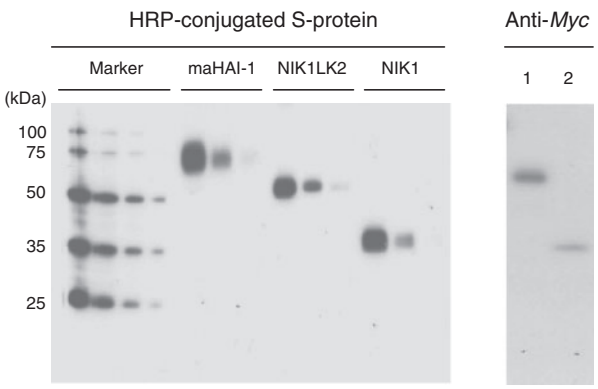

Fig. 2. **Western blot analysis of r-HAI-1 variants.** Various amounts of a protein size marker (Marker) diluted with Laemmli buffer (16, 8, 4 and 2 μl) and those of r-HAI-1 variants [maHAI-1, sHAI-1 NIK1LK2 (NIK1LK2) and sHAI-1 NIK1 (NIK1)] in 1 × Laemmli buffer (20, 10 and 5 μl) were subjected to SDS–PAGE (12% polyacrylamide). After electronic transfer, the blot was probed with an HRP-conjugated S-protein (left panel). The molecular masses of the marker proteins are indicated on the left in kilodaltons (kDa). sHAI-1 NIK1LK2 (lane 1) and NIK1 (lane 2) were also probed with an anti-*Myc* epitope-tag antibody (Anti-*Myc*, right panel).

Inhibition Assays—Peptidyl-MCA substrates used were dissolved in dimethylsulphoxide at the concentration of 40 mM and stored at –20°C until use. Inhibition assays were carried out in HEPES buffer at 37°C in a final volume of 80 μl in a 0.5 ml microcentrifuge tube. The r-matriptase variants (at 2.5 nM each) were pre-incubated with or without r-HAI-1 variants in HEPES buffer (78 μl). After pre-incubation (with various incubation time), the enzyme reaction was initiated by adding 2 μl of 40 mM peptidyl-MCA substrates to the assay mixture. After further incubation for 60 min, the reaction was terminated by adding 350 μl of stop solution. The following assay was also conducted: The r-HAI-1 variants (or HEPES buffer alone) were incubated for 2 min in 72 μl of HEPES buffer containing 1.1 mM peptidyl-MCA substrates, and the hydrolysis was initiated by adding 8 μl of 25 nM r-matriptase variants. After further incubation for 60 min, the reaction was terminated as

described above. In each kinetic study, the absorbance at 370 nm of the product 4-methyl-coumaryl-7-amine was measured. The initial reaction rate (v_o) of the hydrolysis was determined using the molar absorption coefficient of 7.7 $mM^{-1}\,cm^{-1}$ of 4-methyl-coumaryl-7-amine.

RESULTS

Characterization of r-HAI-1 Variants Produced in CHO-K1 Cells—In the present study, we produced a cell membrane-anchored form of r-HAI-1 with S-tag at its N-terminus (maHAI-1) and secreted variants of r-HAI-1 with S-tag and *Myc*-hexahistidine-tag at their N- and C-termini, respectively (sHAI-1 NIK1LK2 and sHAI-1 NIK1) in a stable expression system using CHO-K1 cells (Fig. 1B). These r-HAI-1 variants were purified by means of single-step affinity chromatography using an immobilized S-protein. The variants were purified to homogeneity (higher than 95%) as judged by SDS–PAGE and silver staining (data not shown). A representative western blot data of the rHAI-1 variants probed with HRP-conjugated S-protein is shown in Fig. 2 (left panel). maHAI-1, sHAI-1 NIK1LK2 and sHAI-1 NIK1 produced signals at the positions corresponding to 66 kDa, 59 kDa and 41 kDa, respectively. sHAI-1 NIK1LK2 and NIK1 also produced 59-kDa and 41-kDa signals, respectively, when probed with an anti-*Myc* epitope-tag antibody (Fig. 2, right panel). When the mass of a sugar chain on the Asn235 residue in the internal domain (~5 kDa) was taken into consideration, the molecular masses of the r-HAI-1 variants evaluated from SDS–PAGE were in good agreement with the calculated ones.

Inhibition of HL-Matriptase and L-Matriptase by maHAI-1—To address the role of the stem domain of matriptase in its interaction with HAI-1, we compared the inhibitory activities of r-HAI-1 variants against HL-matriptase and L-matriptase in the hydrolysis of peptidyl-MCA substrates. Boc-QAR-MCA and Boc-D(OBzl)-PR-MCA were used as substrates, because they were preferred by the r-matriptase variants (*11*). Ac-KTKQLR-MCA, a substrate for proteases that have pro-HGF converting activity was also examined (*7*, *12*, *20*).

At first, we investigated the inhibition of r-matriptase variants by the purified preparation of maHAI-1. It is known that the secreted r-HAI-1variants inhibit HL-matriptase as slow-binding inhibitors (*23*). To monitor inhibition evidently, the enzyme was incubated with the inhibitor prior to adding the substrate. Preliminary experiments using Boc-QAR-MCA showed that 2.5 nM HL-matriptase was inhibited 90% or more by 15 nM maHAI-1 after they were pre-incubated for 15 min. Under the kinetic conditions, HL-matriptase and L-matriptase were inhibited by maHAI-1 with a similar extent when Boc-QAR-MCA and Ac-KTKQLR-MCA were used (Fig. 3, upper and middle panels, grey bars). HL-matriptase was inhibited more than 90% in the hydrolysis of Boc-D(OBzl)-PR-MCA (Fig. 3, bottom panel, left grey bar), whereas L-matriptase was not much inhibited and only (45 ± 5)% was observed (Fig. 3, bottom panel, right grey bar).

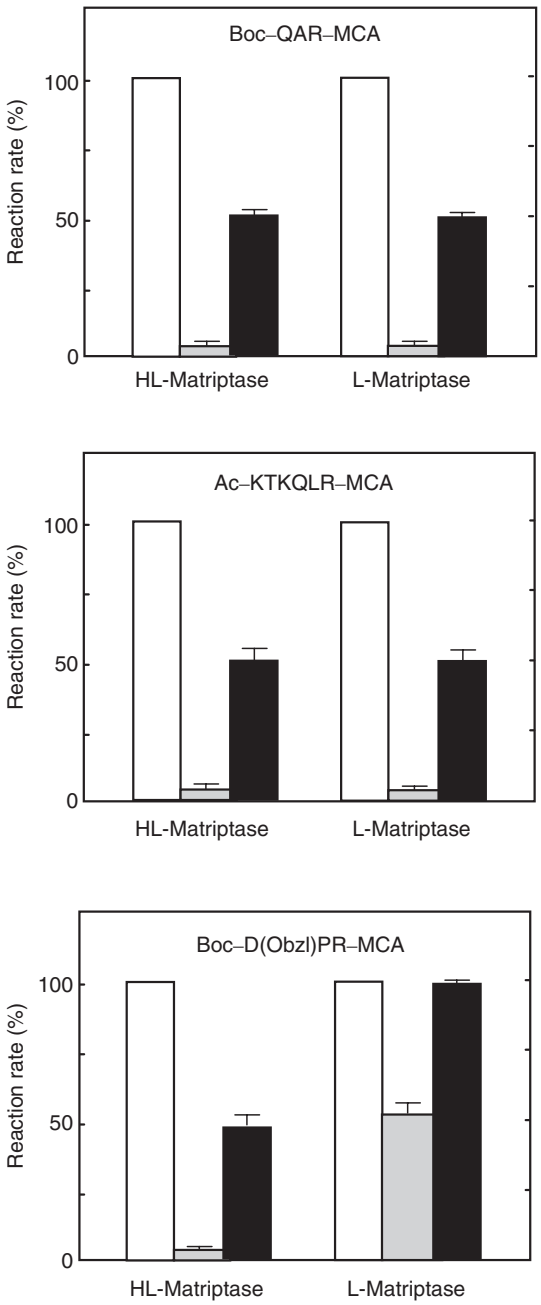

Fig. 3. **Inhibition of r-matriptase variants by maHAI-1.** HL-matriptase and L-matriptase (2.5 nM each) were incubated with Boc-QAR-MCA, Ac-KTKQLR-MCA and Boc-D(OBzl)-PR-MCA (1 mM each) for 60 min. The v_0 values for the hydrolysis of Boc-QAR-MCA, Ac-KTKQLR-MCA and Boc-D(OBzl)-PR-MCA by HL-matriptase were 12 $nM\,s^{-1}$, 2.0 $nM\,s^{-1}$ and 11 $nM\,s^{-1}$, respectively, and they were comparable to those by L-matriptase (*11*). The rates (in the absence of inhibitor) were taken as 100% (white bars). HL-matriptase and L-matriptase (2.5 nM each) were incubated for 15 min with maHAI-1 (15 nM), and the reaction was initiated by adding peptidyl-MCA substrates at a final concentration of 1 mM (grey bars). Black bars indicate the reaction initiated by adding HL-matriptase and L-matriptase at a final concentration of 2.5 nM to mixture containing 17 nM maHAI-1 and 1.1 mM peptidyl–MCA substrates. All values are expressed as means ± standard deviations (SD) of at least two separate experiments.

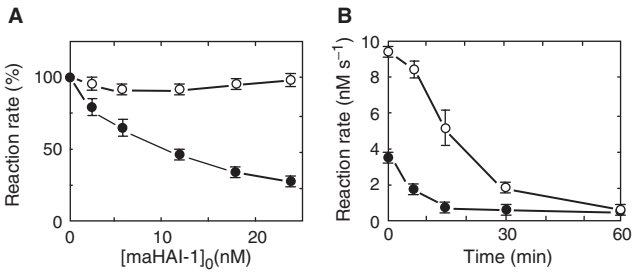

Fig. 4. **Further characterization of inhibition of r-matriptase variants by maHAI-1 in the hydrolysis of Boc-D(OBzl)-PR-MCA.** (A) Concentration dependency of inhibition of r-matriptase variants by maHAI-1. The reaction was initiated by the addition of 8 µl of HEPES buffer containing 25 nM matriptase variants to 72 µl of the buffer containing maHAI-1 and 1.11 mM Boc-D(OBzl)-PR-MCA. The final concentrations of maHAI-1 were 0, 3, 6, 12, 18 and 24 nM (from left to right). (B) The effect of pre-incubation on the inhibition of r-matriptase variants by maHAI-1. Each r-matriptase variant (2.5 nM) was incubated with maHAI-1 (15 nM) for the times indicated in 78 µl of HEPES buffer, and then 2 µl of 40 mM Boc-D(OBzl)-PR-MCA was added to initiate the reaction. In (A) and (B), the values for HL-matriptase and L-matriptase are indicated with closed and open circles, respectively. All values are expressed as means ± SD of at least two separate experiments.

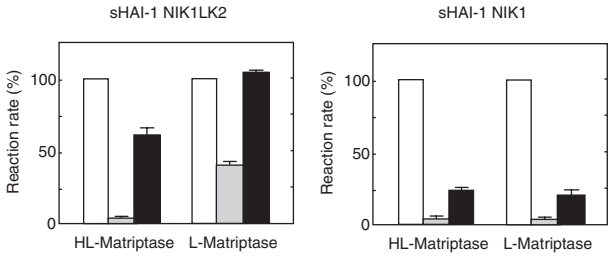

Fig. 5. **Inhibition of r-matriptase variants by secreted variants of r-HAI-1 in the hydrolysis of Boc-D(OBzl)-PR-MCA.** HL-matriptase and L-matriptase (2.5 nM each) were incubated with Boc-D(OBzl)-PR-MCA (1 mM) for 60 min. The initial reaction rate for the hydrolysis of the substrate (in the absence of inhibitor) was taken as 100% (white bars). HL-matriptase and L-matriptase (2.5 nM each) were incubated for 15 min with sHAI-1 NIK1LK2 (15 nM) or sHAI-1 NIK1 (8 nM), and the reaction was initiated by adding the substrate at a final concentration of 1 mM (grey bars). Black bars indicate the reaction initiated by adding HL-matriptase and L-matriptase at a final concentration of 2.5 nM to HEPES buffer containing 17 nM sHAI-1 NIK1LK2 (or 9 nM sHAI-1 NIK1) and 1.1 mM substrate. All values are expressed as means ± SD of at least two separate experiments.

We also conducted kinetic assay where the enzyme reaction was initiated by adding enzyme to the mixture containing inhibitor and substrate. In the hydrolysis of Boc-QAR-MCA and Ac-KTKQLR-MCA, HL-matriptase and L-matriptase were inhibited by 15 nM maHAI-1 with a similar extent (Fig. 3, upper and middle panels, black bars). However, in the hydrolysis of Boc-D(OBzl)-PR-MCA, HL-matriptase was inhibited moderately [(52 ± 3)% inhibition], and L-matriptase was hardly inhibited (10% inhibition or less) (Fig. 3, bottom panel, black bars). These results indicate that the stem domain of matriptase facilitates the inhibitory interaction of this protease with maHAI-1 in the specified hydrolysis of Boc-D(OBzl)-PR-MCA, although it has no effect in the hydrolysis of Boc-QAR-MCA and Ac-KTKQLR-MCA.

To validate further the difference in the inhibitory activities of maHAI-1 against the hydrolysis of Boc-D(OBzl)-PR-MCA by HL-matriptase and L-matriptase, HL-matriptase and L-matriptase were added to the mixture containing various concentrations of maHAI-1 and a fixed concentration of the substrate. The degree of inhibition of HL-matriptase increased with increasing concentrations of the inhibitor (Fig. 4A, black circles). On the other hand, no significant inhibition of L-matriptase was observed at any inhibitor concentrations examined (Fig. 4A, white circles). Figure 4B shows inhibition as a function of pre-incubation time at fixed concentrations of the enzyme (2.5 nM) and inhibitor (15 nM). HL-matriptase was inhibited 90% or more when it was pre-incubated with maHAI-1 for 15 min. Pre-incubation for 60 min was required for the strong inhibition of L-matriptase. Results shown in Fig. 4 clearly indicate that HL-matriptase is inhibited with maHAI-1 more efficiently than L-matriptase in the hydrolysis of the Boc-D(OBzl)-PR-MCA substrate.

Inhibition of HL-Matriptase and L-Matriptase by the Secreted Variants of r-HAI-1—The inhibitory activities of sHAI-1 NIK1LK2 and sHAI-1 NIK1 against r-matriptase variants were examined in the hydrolysis of Boc-D(OBzl)-PR-MCA. HL-matriptase and L-matriptase were inhibited by sHAI-1 NIK1LK2 similarly by maHAI-1 (Fig. 5, left panel). On the other hand, there was no difference in the inhibitory activity of sHAI-1 NIK1 against HL-matriptase and L-matriptase (Fig. 5, right panel).

DISCUSSION

We previously produced secreted variants of r-HAI-1 using COS-1 cells and demonstrated their inhibitory activities against HL-matriptase (*23*). However, the inhibitory activity of membrane-anchored form of HAI-1 (66-kDa HAI-1) toward matriptase has been unknown. In the present study, we purified maHAI-1 from the Triton extracts of CHO-K1 cells stably transfected with pSec-maHAI-1. maHAI-1 migrated at the position corresponding to 66 kDa in SDS–PAGE (Fig. 2), suggesting that this variant corresponds to the membrane-anchored form of HAI-1. Likewise, the sHAI-1 NIK1LK2 and sHAI-1 NIK1 variants produced in CHO-K1 cells might correspond to 58-kDa and 40-kDa HAI-1 species, respectively. maHAI-1 and sHAI-1 NIK1LK2 exhibited the similar inhibitory activity towards the r-matriptase variants (Figs 3 and 5). These findings lead to an assumption that 66-kDa HAI-1 may behave against matriptase similarly to 58-kDa HAI-1 *in vivo*.

The activation cleavage sequence of latent matriptase is P4(Lys)-P3(Gln)-P2(Ala)-P1(Arg) (see Fig. 1A); and that of pro-HGF is P4(Lys)-P3(Gln)-P2(Leu)-P1(Arg). Hence, the hydrolysis of Ac-KTKQLR-MCA and Boc-QAR-MCA is expected to be as indicated by the activation cleavage of latent matriptase and pro-HGF.

However, we found no difference between the inhibitory activities of maHAI-1 against HL-matriptase and L-matriptase when these substrates were used (Fig. 2). In addition, HL-matriptase and L-matriptase were inhibited with a similar extent by sHAI-1 NIK1LK2 (*23*) in the conversion of sc-uPA into two-chain uPA (Kojima, K., Tsuzuki, S., Fushiki, T., and Inouye, K., unpublished observation). These results suggest that the stem domain of matriptase has no significant effect on the inhibitory interaction of this protease with HAI-1 (*e.g.*, 66-kDa and 58-kDa HAI-1 species) in the activation cleavage of some substrates, including latent matriptase, pro-HGF and sc-uPA.

In the hydrolysis of Boc-D(OBzl)-PR-MCA, HL-matriptase was inhibited more efficiently than L-matriptase by maHAI-1 and sHAI-1 NIK1LK2 (Figs 3–5). Matriptase was previously shown to cleave and activate the precursor form of prostasin, which activation cleavage sequence is P4(Ile)-P3(Gln)-P2(Pro)-P1(Arg) (*13*). Note that proline is in the P2 position. It is, therefore, tempting to speculate that the matriptase-catalyzed hydrolysis of Boc-D(OBzl)-PR-MCA is an indicative of the activation cleavage of prostasin zymogen. It has recently been shown that HAI-1 (corresponding to our sHAI-1 NIK1LK2) inhibits the activation of prostasin zymogen catalysed by a recombinant catalytic domain of matriptase (*25*). Here, we could give a possibility that the stem domain of matriptase may facilitate the inhibitory interaction of this protease with HAI-1 in a specific cleavage of substrates, including prostasin zymogen.

We reported previously that sHAI-1 NIK1 associated with HL-matriptase faster than did sHAI-1 NIK1LK2, and suggested that the Kunitz domain II might obstruct the matriptase-binding site in Kunitz domain I (*23*). In the present study, we have found that there is no difference in the inhibition between HL-matriptase and L-matriptase by sHAI-1 NIK1 in the hydrolysis of Boc-D(OBzl)-PR-MCA (Fig. 5, right panel). Taken together, we present a model explaining the stronger inhibition of HL-matriptase than L-matriptase by maHAI-1 (or sHAI-1 NIK1LK2) in the hydrolysis of the substrate (Fig. 6, upper). In this model, the stem domain of HL-matriptase is postulated to interact with the LDLRA domain and/or Kunitz domain II of maHAI-1, thereby alleviating the Kunitz domain II-mediated obstruction of Kunitz domain I. L-matriptase is illustrated to bind with Kunitz domain I very slowly, because of the lack of the stem domain (*i.e.*, the lack of alleviation of obstruction). On the other hand, HL-matriptase and L-matriptase are inhibited by sHAI-1 NIK1 with a similar extent, because the HAI-1 variant lacks LDLRA domain and Kunitz domain II (Fig. 6, bottom). The reason that the stem domain has no significant effect on the interaction with maHAI-1 in the hydrolysis of Boc-QAR-MCA and Ac-KTKQLR-MCA is unclear. One explanation is that these substrates themselves alleviate the obstruction of Kunitz domain I by Kunitz domain II to an extent that matriptase catalytic domain could bind to Kunitz domain I without the aid of the stem domain.

In summary, we found that the stem domain of matriptase facilitates the inhibitory interaction of this protease with maHAI-1 in a specific cleavage of Boc-D(OBzl)-PR-MCA. To our knowledge, this is the first report showing that the stem domain of matriptase can affect the interaction between this protease and HAI-1. At present, it is unknown what parts of the stem domain is important for facilitating the interaction with HAI-1. A study is underway to produce additional r-matriptase variants, and will provide further insights into the role of the stem domain of matriptase.

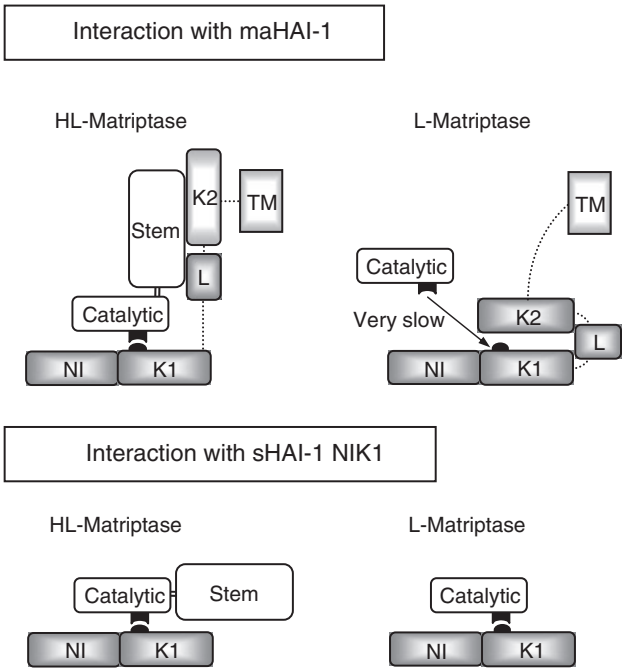

Fig. 6. **A schematic model for interaction of r-matriptase variants with maHAI-1 and sHAI-1 NIK1 in the hydrolysis of Boc-D(OBzl)-PR-MCA.** Spacer regions of maHAI-1 are indicated by dashed line or curve. The disulphide bond between the stem domain and the catalytic domain of HL-matriptase is indicated by double line. The matriptase binding site in Kunitz domain I and the active site of matriptase are indicated (black). The N-terminal and internal domains of maHAI-1 are illustrated as one rectangle (NI). Catalytic, catalytic domain; K1, Kunitz domain I; K2, Kunitz domain II; L; LDLRA domain; NI, N-terminal and internal domains; Stem, stem domain; TM, transmembrane domain.

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CONFLICT OF INTEREST

None declared.

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