ROLES OF FUNCTIONAL AND STRUCTURAL DOMAINS OF HEPATOZYTE GROWTH FACTOR ACTIVATOR INHIBITOR TYPE 1 IN THE INHIBITION OF Matriptase

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Hepatocyte growth factor (HGF) activator inhibitor type 1 (HAI-1) is a membrane-bound, Kunitz-type serine protease inhibitor. HAI-1 inhibits serine proteases that have potent pro-HGF-converting activity, such as the membrane-type serine protease, matriptase. HAI-1 comprises an N-terminal domain, followed by an internal domain, first protease inhibitory domain (Kunitz domain I), low-density lipoprotein receptor A module (LDLRA) domain, and a second Kunitz domain (Kunitz domain II) in the extracellular region. Our aim was to assess the roles of these domains in the inhibition of matriptase. Soluble forms of recombinant rat HAI-1 mutants made up with various combinations of domains were produced, and their inhibitory activities toward the hydrolysis of a chromogenic substrate were analyzed using a soluble recombinant rat matriptase. Kunitz domain I exhibited inhibitory activity against matriptase, but Kunitz domain II did not. The N-terminal domain and Kunitz domain II decreased the association rate between Kunitz domain I and matriptase, whereas the internal domain increased this rate. The LDLRA domain suppressed the dissociation of the Kunitz domain I–matriptase complex. Surprisingly, an HAI-1 mutant lacking the N-terminal domain and Kunitz domain II showed an inhibitor constant of 1.6 pm, and the inhibitory activity was 400 times higher in this HAI-1 mutant than in the mutant with all domains. These findings, together with the known occurrence of an HAI-1 species lacking the N-terminal domain and Kunitz domain II in vivo, suggest that the domain structure of HAI-1 is organized in a way that allows HAI-1 to flexibly control matriptase activity.

Hepatocyte growth factor (HGF) activator inhibitor type 1 (HAI-1) is an epithelial-derived, serine protease inhibitor with multiple domains, including two protease-inhibiting Kunitz domains (1,2). HAI-1 was isolated originally from the conditioned medium of a human stomach carcinoma MKN45 cell line as a potent inhibitor of HGF activator, a 53-kDa serine protease responsible for proteolytic activation of the inactive single chain precursor of HGF (pro-HGF) (1,3). HAI-1 is believed to play a crucial role in growth factor-mediated biological processes such as tissue regeneration by counteracting the HGF activator activity (2,4).

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 the C-terminal region (Fig. 1A). This suggests that HAI-1 is produced first as a type I membrane protein (1). Indeed, the transmembrane form of HAI-1, which has a molecular mass of 66 kDa, was detected in extracts of MKN45 cells (5) and monkey kidney COS-1 cells transiently transfected with a rat HAI-1 cDNA (6). The extracellular domain can be released by cleavage with certain proteases (5). At least two HAI-1 species of 58 and 40 kDa are found in conditioned media of MKN45 cells (1,5) and transfected COS-1 cells (6). The 58-kDa species (58-kDa HAI-1) has all of the HAI-1 extracellular region and comprises the N-terminal domain with eight cysteine residues, followed by the internal domain rich in acidic amino acid residues, the first Kunitz domain (Kunitz domain I), the low-density lipoprotein receptor A module (LDLRA)-like domain (LDLRA domain), and the second Kunitz domain (Kunitz domain II) (Fig. 1A). The 40-kDa species (40-kDa HAI-1) lacks Kunitz domain II (5,7), but is thought to be responsible for inhibiting...
target proteases in vivo because it exhibits higher inhibitory activity against HGF activator than does 58-kDa HAI-1 (5,7).

Soluble HAI-1 species with molecular masses of 40 and 25 kDa are found as complexes with matriptase (also known as membrane-type serine protease 1 or MT-SP1) in biological fluids such as human milk (8). Matriptase is produced first as a membrane-bound form (type II transmembrane serine protease) (9), which comprises multiple domains in the extracellular region, including a trypsin-like serine protease domain at the C-terminus (Fig. 2A), and the ectodomain is then released by an unknown mechanism (6,8,10). This protease is expressed by epithelial elements of almost all organs examined so far (11,12), and because it digests various potential substrates, including pro-HGF (13,14) and single-chain urokinase-type plasminogen activator (sc-uPA) (11,13,15), is thought to play broad roles in the physiology of epithelial cells. The cellular sites of matriptase production are consistent with those of HAI-1 (2,12). Importantly, matriptase activity is inhibited in vitro by HAI-1 (14,16), but not (or poorly) by other endogenous serine protease inhibitors such as α1-antitrypsin (17). These observations suggest that HAI-1 functions as a physiological inhibitor of matriptase. The biological importance of the inhibition is supported by evidence showing that the ratio of matriptase to HAI-1 increases with increasing malignancy in cancers such as ovarian cancer (18,19). This might be explained by overproduction of active HGF, two-chain uPA, and active matrix metalloproteinases, which are characteristic of malignant tissues (20–24). Furthermore, matriptase zymogen occurs in a single-chain form, and one zymogen molecule is converted into an active two-chain form by another zymogen molecule in an activation mechanism known as transactivation. This may allow matriptase to act as an initiator protease for cancer invasion and metastasis (25). Therefore, inhibition of matriptase activity by exogenous HAI-1 could be one strategy to retard cancer progression.

Little is known about the mechanism by which HAI-1 inhibits matriptase. Kirchhofer and co-workers previously reported the existence of an alternative splicing variant of HAI-1 which contains an extra 16-amino acid sequence adjacent to the C-terminus of Kunitz domain I and designated it as HAI-1B. (14). Using soluble HAI-1B mutants that comprise all extracellular region, they showed that Kunitz domain I but not Kunitz domain II is responsible for the inhibition of matriptase and HGF activator (14). However, there is a possibility that the availability of Kunitz domain II in HAI-1B for enzymatic inhibition is impaired because of the additional amino acid residues. Indeed, Kunitz domain II in 58-kDa HAI-1 inhibits HGF activator (7). Therefore, the ability of Kunitz domain II to inhibit matriptase is not understood fully. In addition, the occurrence of 25-kDa HAI-1 lacking the N-terminal domain in complex with matriptase in human milk suggests that the structural domains can also affect the inhibitory activity of HAI-1 against matriptase.

Our goal is to elucidate the roles of the functional and structural domains of HAI-1 in the inhibition of matriptase. For this, we prepared soluble recombinant rat HAI-1 mutants made of various combinations of domains, and we characterized their inhibitory activities against a soluble recombinant species of rat matriptase. We showed that Kunitz domain I is responsible for the inhibitory activity of HAI-1 against matriptase, but that Kunitz domain II is not. We also showed that both the N-terminal domain and Kunitz domain II attenuate the inhibitory activity of Kunitz domain I, whereas the internal domain and LDLRA domain enhance the inhibitory activity. We suggest possible roles of the structural domains and Kunitz domain II in inhibition of matriptase by HAI-1. Our results might lead to the development of HAI-1 derivatives that may prove useful for cancer therapy.

EXPERIMENTAL PROCEDURES

Materials – A Chinese hamster ovary cell line (CHO-K1) was obtained from Health Science Research Resources Bank (Osaka, Japan). All synthetic deoxyribonucleotides, pSecTag2/Hygro vector, and mouse anti-myc epitope antibody conjugated with horseradish peroxidase (HRP) were purchased from Invitrogen (Carlsbad, CA). PT7blue-2 vector, recombinant bovine enterokinase, immobilized S-protein, S-protein conjugated with HRP, and Perfect Protein Marker™ were purchased from Novagen (Madison, WI). Goat anti-rabbit IgG secondary antibody was from Dako Japan (Tokyo). Prestained protein markers (broad range) and restriction endonucleases were purchased from New England BioLabs (Beverly, MA). KODplus-
DNA polymerase used for polymerase chain reaction (PCR), T4 polynucleotide kinase, and a DNA ligation kit were from Toyobo (Osaka, Japan). Various peptidyl-4-methylcoumaryl-7-amide (MCA) substrates were purchased from Peptide Institute (Osaka, Japan). Spectrozyme tPA® (Sp-tPA, methylsulfonyl-d-cyclohexyltyrosyl-glycyl-arginine-p-nitroaniline acetate) was purchased from American Diagnostica (Stanford, CT), and bovine trypsin (type III) was from Sigma (St. Louis, MO). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

**Construction of Expression Plasmids** – Expression plasmids for the production of soluble recombinant rat HAI-1 mutants were created by a PCR-based technique with an expression plasmid containing the sequence of rat HAI-1 (Pro$^4$–Leu$^{517}$) with an S-tag at the N-terminus in pSecTag2/Hygro B (designated as pSecHAI-1) as the template (the amino acid numbering of HAI-1 starts from the putative N-terminus of the protein) (6). An expression plasmid (pSecNIK$^1$LK$^2$) for the production of a recombinant HAI-1 mutant designated HAI-1 NIK$^1$LK$^2$ (Pro$^{41}$–Ser$^{441}$) was constructed as follows. In the designation of the HAI-1 mutants, the abbreviations of the domains were placed according to their order in the mutants from the N-terminus to C-terminus (refer to Fig. 1A). A DNA fragment amplified with 5′-TAATACGACTCACTATAGGGGACC$^{-}$′ and 5′-GCTCTAGAGCTTTCCTTCCGAG$^{-}$′ as a forward primer and 5′-GCTCTAGAGCTTTCCTTCTGGGACC$^{-}$′ as a reverse primer was digested with KpnI and XbaI; and the resulting fragment was ligated into pSecHAI-1 in which the KpnI and XbaI fragment had been removed. Expression plasmids for the HAI-1 NIK$^1$L (Pro$^{41}$–Ser$^{370}$) and NIK$^1$(Pro$^{41}$–Ser$^{107}$) mutants were made using the same method except that 5′-GCTCTAGAGGAAGTTAGTACCTCAGA$^{-}$′ and 5′-GCTCTAGAGATTCTTTTACATCCTTGGA$^{-}$′, respectively, were used as reverse primers. Plasmids for the HAI-1 IK$^1$LK$^2$ (Thr$^{354}$–Ser$^{441}$), IK$^1$L (Thr$^{354}$–Ser$^{370}$), IK$^1$(Thr$^{354}$–Ser$^{370}$), K$^1$L (Gln$^{245}$–Ser$^{441}$), K$^1$(Gln$^{245}$–Ser$^{370}$), and K$^2$(Ser$^{370}$–Ser$^{441}$) mutants were made as follows. DNA fragments were amplified with appropriate forward primers phosphorylated at their 5′ ends, including 5′-AATCTCGAGGCTTCCGAGTTC-3′, 5′-GCAGACGGGAGTATTTGC-3′, and 5′-CAGTGACAAAGGGTGACTGT-3′, and with the appropriate reverse primers as described above. The PCR products were digested with XbaI, and the resulting fragments were ligated once into the PT7blue-2 vector predigested with Smal and XbaI; fragments of these plasmids digested with KpnI and XbaI were ligated into pSecHAI-1 as described above. Plasmids for the HAI-1 NIK$^1$LK$^2$ (Leu$^{152}$–Ala$^{243}$-truncated NIK$^1$LK$^2$) and NIK$^1$LK$^3$ (Pro$^{308}$–Ser$^{370}$-truncated NIK$^1$LK$^3$) mutants were created with pSecNIK$^1$LK$^2$ as the template. To create pSecNIK$^1$LK$^2$, forward primers used to create pSecK$^1$LK$^2$ and 5′-TCGCAGAGGAGTTGTAAGG-3′ were used as the forward and reverse primers, respectively. For pSecNIK$^1$LK$^2$, forward primers for pSecK$^2$ and 5′-ATTCCCTTTTACATCCTTGCAAGG-3′ were used as the forward and reverse primers, respectively. The amplified DNA fragments were self-ligated. Plasmids for the HAI-1 R260A, D349Y, and K385A mutants were made as follows. A restriction fragment of pSecNIK$^1$LK$^2$ produced by Xhol and XbaI was ligated into pBluescriptSK (Stratagene, La Jolla, CA) that had been predigested with the same sets of enzymes. Using this plasmid as the template, DNA fragments of about 3.8 kbp were amplified with the following sequences (the nucleotides underlined indicate the introduced mutations): 5′-CGGGCTCTCTTTCCAC-CG-3′ and 5′-CGCAGCAGGCCCCACCTT-3′, 5′-AGAGGACCCTGGTTGAAAAATATAGG-3′ and 5′-AGGGAGCTCGAGGCAA-3′, and 5′-CGGAGAACATCCCCAGGCT-3′ and 5′-CGCAAAAATC-CAGTGCTCTGGCA$^{-}$GAG-3′ to introduce appropriate mutations. These PCR products were phosphorylated with T4 nucleotide kinase and then self-ligated. The plasmids were digested with Xhol and XbaI, and the resulting fragments were ligated into pSecNIK$^1$LK$^2$ from which the Xhol and XbaI fragment had been removed. **Establishment of Stable Clones for the Production of Soluble Recombinant Rat Matriptase and Its Purification and Activation** – CHO-K1 cells were maintained in Ham’s F12 medium containing 5% fetal bovine serum. An expression plasmid, designated as pSec-ekMT-SP1s, for the production of soluble recombinant rat pro-matriptase (Fig. 2A) (17) was transfected into the cell line as described previously (6). Clones resistant to Ham’s F12 medium containing 400 µg/ml hygromycin B were obtained, and the expression level in the conditioned media by each clone was determined by Western blotting using S-protein conjugated
with HRP as the probe. T3245, a clone with the highest expression of soluble recombinant pro-matriptase, was propagated and stored in liquid nitrogen until use. Aliquots of the frozen cells were thawed and cultured in 75-cm² flasks until reaching confluence. Cells were washed three times with ice-cold phosphate-buffered saline (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 136 mM NaCl, and 2.7 mM KCl, pH 7.4), 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 x g for 10 min at 4°C, and the resulting supernatants were stored −70°C until use. The procedures for purification using immobilized S-protein and activation of recombinant pro-matriptase by recombinant enterokinase were the same as described previously (17) except that Amicon Ultra-15 (50,000 MWCO, Millipore, Cork, Ireland) was used to concentrate the conditioned media and that a lower concentration (3.4 units/ml) of recombinant enterokinase was used (17). The concentration of the activated form of recombinant matriptase was determined as described previously (6). Briefly, a part of the activated recombinant matriptase was incubated with 500 µM of t-butyloxy carbonyl (Boc)-L-Gln-L-Ala-L-Arg-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 final volume of 80 µl. The reaction was terminated by adding 350 µl of 0.1 M sodium acetate buffer, pH 4.0, containing 100 mM monochloroacetic acid, and the absorbance at 370 nm was measured. The hydrolysis of other peptidyl-MCA substrates by recombinant matriptase was measured as described above.

**Production of HAI-1 Mutants Using CHO-K1 Cells** – CHO-K1 cells transfected with an expression plasmid for the production of the HAI-1 NIK¹LK², K¹, K², R260A, D349Y, or K385A mutants were cultured in Ham’s F12 medium containing 5% fetal bovine serum and 400 µg/ml hygromycin B. The cells resistant to the antibiotic were pooled, propagated, and stored. The procedures for harvest of conditioned media (serum-free media), concentrating the media, and gel filtration in HEPES buffer were the same as used in the production of recombinant matriptase.

**Production of HAI-1 Mutants in COS-1 Cells** – COS-1 cells were maintained as described previously (11, 17). The procedures for transient expression in COS-1 cells, harvest of conditioned media (serum-free media), and gel filtration in HEPES buffer were described previously (6).

**Preparation of HAI-1 Mutants and Determination of their Concentrations** – Preparation of HAI-1 mutants from gel filtrates was performed using Ni²⁺-charged resin (HisLink™ resin, Promega, Madison, WI) as described previously (6). Because of possible occurrence of cleavage between Kunitz domain I and Kunitz domain II during production in COS-1 cells (17), HAI-1 NIK¹LK² was subsequently treated with an immobilized S-protein. The HAI-1 mutant bound to the immobilized S-protein was eluted with 0.2 M sodium citrate buffer, pH 2.0, and the eluate was immediately neutralized with 2 M Tris solution. All HAI-1 mutants were subjected finally to gel filtration in HEPES buffer. Various amounts of HAI-1 mutants and Perfect Protein Marker™ protein size marker, in which each protein is fused to an S-tag and the concentration of each marker was known, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western transfer. The blots were probed with S-protein conjugated with HRP. The X-ray films were scanned digitally, and the signal intensities of protein bands were analyzed by densitometry as described previously (6). The concentrations of the HAI-1 NIK¹LK², NIK¹L, IK¹LK², NK¹LK², NIK²K², R260A, D349Y, and K385A mutants were calculated by comparing the signal density of the bands of interest with the 50-kDa protein contained in the protein marker. The HAI-1 NIK¹L, IK¹L, and K¹LK² mutants were compared with the 35-kDa protein, the HAI-1 IK¹ and K¹L mutants with the 25-kDa protein, and the HAI-1 K¹ and K² mutants with the 15-kDa protein (see Fig. 1B).

**Kinetic Studies with the Chromogenic Substrate, Sp-tPA** – All enzyme and inhibitor assays using Sp-tPA were carried out in HEPES buffer at 25°C in a final volume of 100 µl in 96-well plates. Sp-tPA was dissolved in the same buffer just before each experiment. In steady-state kinetic studies, various concentrations of HAI-1 mutants were preincubated for 2 h in the assay mixture (90 µl) containing 0.12 nM recombinant matriptase. After the preincubation, hydrolysis was initiated by adding 10 µl of 5 mM Sp-tPA (500 µM) to the assay solution. The hydrolysis was followed by monitoring the increase in absorbance at 405 nm.
resulting from the liberation of the product p-nitroaniline at 30 s intervals for 20 min with a plate reader (Powerscan HT, Dainippon Pharmaceutical, Tokyo, Japan). The initial rate of hydrolysis was determined using the molar absorption coefficient ε405 of 9650 M⁻¹ cm⁻¹ for p-nitroaniline. Inhibition of trypsin (0.05 nM) by HAI-1 NIK’LK², K², and R260A was analyzed under the same conditions. In pre–steady-state kinetic studies, various concentrations of the HAI-1 mutants were incubated for 5 min in the assay mixture (90 µl) containing 555 µM Sp-tPA, and the hydrolysis was initiated by adding 10 µl of 1.2 nM recombinant matriptase (0.12 nM). The kinetic analysis was also performed with various concentrations of Sp-tPA at a fixed concentration of the HAI-1 mutants. Absorbance at 405 nm was monitored at 30 s intervals for 90 min with a plate reader, and the rate of hydrolysis was determined, as described above.

Analysis of Progress Curves – The apparent first-order rate constant (kobs) for the transition of the initial rate (v0) to the steady-state rate (vs) observed in the matriptase-catalyzed hydrolysis of Sp-tPA in the presence of the HAI-1 mutants was determined by nonlinear regression analysis of the progress curve using Equation 1 (26,27) as follows.

\[ [P] = v_s t + (v_0 - v_s) [1 - \exp(-k_{obs}t)] / k_{obs} \]  
(Eq. 1)

where [P] is the concentration of p-nitroaniline at time t, vs is the reaction rate at the steady state, and v0 is the initial reaction rate. The values of kobs, vs, and v0 were determined by fitting the progress curve to the theoretical curve using Equation 1 with the aid of commercially available software (KaleidaGraph, Synergy Software, Reading, PA). Equations 2 and 3 (26–28) can be used to calculate the rate constant (koff) for association of the enzyme and inhibitor, the rate constant (kon) for dissociation of the enzyme–inhibitor complex, and dissociation constant of the complex or the inhibitor constant (Ki) when kobs increases linearly with increases inhibitor concentration and diminishes hyperbolically with increasing substrate concentration as follows.

\[ k_{obs} = k_{off} + k_{on}[I] / (1 + [S] / K_m) \]  
(Eq. 2)

\[ K_i = k_{off} / k_{on} \]  
(Eq. 3)

where [I] and [S] are the concentrations of the inhibitor and substrate, respectively, and Km is the Michaelis constant. Km for Sp-tPA was determined separately by fitting the initial rate data to the Michaelis–Menten equation. The value of koff can also be calculated using Equation 4 (28,29) as follows.

\[ k_{off} = k_{obs} v_s / v_0 \]  
(Eq. 4)

Binding of Recombinant Matriptase to the Immobilized HAI-1 Mutants – One hundred picomoles of the HAI-1 K¹, K², R260A, or K385A mutants dissolved in 200 µl of HEPES buffer was re-treated with a 20-µl slurry of Ni²⁺-charged resin in a microcentrifuge tube as described previously (6). The resin was washed several times with buffer, and 1 pmol of recombinant matriptase dissolved in 300 µl of the buffer was added. The mixture was incubated for 2 h at 25°C with rocking. The proteins bound to the resin were eluted by boiling and analyzed by SDS-PAGE and Western blotting with Spr992 or anti-myc antibody as described previously (6).

Statistical Analysis – Differences in the kon and koff values between any two HAI-1 mutants were determined by the two-tailed t test using Instat statistical software (GraphPad, San Diego, CA). P < 0.05 was considered significant.

RESULTS

Design of the HAI-1 Mutants – The domain structures of the HAI-1 mutants produced in this study are illustrated in Fig. 1A. The largest HAI-1 mutant, NIK’LK², comprises the N-terminal domain, internal domain, Kunitz domain I, LDLRA domain, and Kunitz domain II. This mutant corresponds to 58-kDa HAI-1 released into the conditioned medium of the cultured cells (1,5,6). To examine the roles of the structural domains and functional domains in the inhibition of matriptase, deletion mutants were produced and designated according to the domains comprised. For instance, the role of the N-terminal domain in matriptase inhibition could be predicted by comparing kinetic parameters between HAI-1 NIK’LK² and I K’LK².

We produced three HAI-1 mutants by site-directed mutagenesis in the context of HAI-1 NIK’LK², which we designated HAI-1 R260A, D349Y, and K385A. HAI-1 R260A and K385A are mutants in which Arg²⁶⁰ (in Kunitz domain I) and Lys³⁸⁵ (in Kunitz domain II) are replaced with Ala (7,14).
These residues are considered essential for the potential inhibitory activities of the respective Kunitz domains, so these mutants are expected to be impaired with respect to the inhibitory activities of Kunitz domains I and II, respectively. The HAI-1 R260A and K385A mutants may be useful for examining the ability of all HAI-1 domains to bind with and inhibit matriptase. In HAI-1 D349Y, Asp349 (in the LDLRA domain) is replaced with Tyr. This position of HAI-1 corresponds to the Asp500 residue in the fifth LDLRA domain of the LDL receptor (30,31). Replacing Asp306 in the LDL receptor with Tyr strongly inhibits the binding of LDL to the receptor (32). Accordingly, the mutation in the LDLRA domain of HAI-1 might affect the interaction of HAI-1 with matriptase. All HAI-1 mutants were produced as fusion proteins with an S-tag at their N-termini. The fusion proteins were convenient for purification of the resin-immobilized S-protein and for measuring their concentrations using HRP-conjugated S-protein. The HAI-1 mutants were also fused to the myc epitope and hexahistidine tag at their C-termini for convenient detection with an anti-myc antibody and for purification with Ni2+-charged resin.

Characterization of the HAI-1 Mutants Produced in COS-1 or CHO-K1 Cells – In this study, HAI-1 mutants except for HAI-1 K2 and R260A were produced by a transient expression system using COS-1 cells. The cell line secreted at least 10 µg of the respective HAI-1 mutants into the cultivation medium for two weeks. Because HAI-1 K2 and R260A mutants were produced poorly in the COS-1 system, we prepared them in a stable expression system using CHO-K1 cells.

All HAI-1 mutants were prepared from the conditioned media by means of single-step affinity chromatography using Ni2+-charged resin. An unidentified protein with a mass of 40 kDa was mostly evident in each HAI-1 mutant preparation using this resin (the silver staining result of HAI-1 NIK1LK2 is shown in Fig. 1B, lane 1). Subsequent treatment of the HAI-1 NIK1LK2 preparation with an immobilized S-protein resulted in the removal of this and other minor contaminating proteins (a 59-kDa band of HAI-1 NIK1LK2 is only visible) (Fig. 1B, lane 2). Preliminary experiments revealed that there was no difference in the inhibitory activities against matriptase between an aliquot of the HAI-1 NIK1LK2 preparation using Ni2+-charged resin alone and that using Ni2+-charged resin and immobilized S-protein. The contaminating proteins included if any in the preparation with Ni2+-charged resin alone could neither affect the enzyme–inhibitor interaction nor hydrolyze Sp-tPA. Because of the low recovery of HAI-1 NIK1LK2 and the other mutants after treatment with the immobilized S-protein, the preparations with Ni2+-charged resin alone were used for the kinetic studies.

SDS-PAGE and Western blotting with the S-protein conjugated with HRP were also performed to confirm the absence of degraded products in the preparations and to measure the concentrations of the HAI-1 mutants (Fig. 1C). The preparations of HAI-1 mutants produced signals at the following positions: HAI-1 NIK1LK2, 59 kDa; NIK1L, 46 kDa; NIK1, 41 kDa; IK1LK2, 47 kDa; IK1L 38 kDa; IK1, 26 kDa; KL, 22 kDa; K1, 16 kDa; K2, 17 kDa; NK1LK2, 45 kDa; NIK1K2, 49 kDa; R260A, 59 kDa; D349Y, 59 kDa; and K385A, 59 kDa. The HAI-1 K1LK2 preparation gave a signal at the position of 34 kDa with an extra 80-kDa band. Similar results were obtained when the blots were probed with anti-myc antibody (the Western blot results of HAI-1 K1, K2, R260A, and K385A are shown in Fig. 4, bottom panel). When the mass of a sugar chain on the Asn235 residue in internal domain (approximately 5 kDa) was taken into consideration, the sizes of the respective HAI-1 mutants evaluated from SDS-PAGE were similar to the calculated masses. This indicates the absence of degraded products in each HAI-1 preparation. The absence of degraded products in the NIK1LK2 preparation also suggests that an HAI-1 species of 40 kDa found in the conditioned media of transfected COS-1 cells (6) is generated from the membrane-bound HAI-1 but not from 58-kDa HAI-1 (5,7).

We confirmed that the N- and C-terminal tags do not affect the inhibitory activities of the HAI-1 mutants. The IC50 value for the inhibitory activity of HAI-1 NIK1LK2 against trypsin is 1.5 nM, which is similar to that of 58-kDa HAI-1 purified from the conditioned medium of MKN45 cells (7).

Characterization of Soluble Recombinant Matriptase Produced in CHO-K1 Cells – We have previously produced a secreted soluble form of recombinant rat matriptase using COS-1 cells (17). This recombinant protein is cleaved artificially in vitro before Val615 by recombinant enterokinase to form a disulfide-linked form, in which two polypeptides formed by the cleavage are
linked by a disulfide bond (Fig. 2A). This disulfide-linked form exhibits proteolytic activity, and the activity agreed with that of soluble matriptase purified from human milk (13,17). We raised a stable CHO-K1 cell line that strongly expresses the soluble form of recombinant promatriptase, and we established the procedures for its purification and activation. The expression product was analyzed on Western blots probed with an antibody designated Spr992 (6). The antibody recognizes the serine protease domain of rat matriptase (Val615–Val855) that migrates at the position corresponding to 28 kDa on SDS-PAGE under reducing conditions (6,11). The recombinant matriptase produced in CHO-K1 cells produced signals at 95 kDa and 28 kDa before and after recombinant enterokinase treatment, respectively (Fig. 2B). The recombinant matriptase after recombinant enterokinase treatment hydrolyzed peptidyl-MCA substrates having an Arg residue at the P1 position. The order of the substrates from the highest to lowest activity was: Boc–L-Glu–L-Ala–L-Arg–MCA, Boc–benzyl-L-Asp–L-Pro–L-Arg–MCA, Boc–benzyl-L-Glu–L-Ala–L-Arg–MCA, Boc–benzyl-L-Glu–L-Gly–L-Arg–MCA, Boc–L-Leu–L-Gly–L-Arg–MCA, Boc–L-Phe–L-Ser–L-Arg–MCA, and Boc–L-Val–L-Pro–L-Arg–MCA. The relative activity for these substrates was similar to that of the recombinant matriptase preparation produced in COS-1 cells (17). The $K_m$ value of recombinant matriptase for Sp-tPA substrate (10) was determined to be 221 ± 40 µM (mean ± SD of triplicate sample) by an initial rate method.

**Steady-State Kinetic Analysis** – The HAI-1 mutants except for HAI-1 K2 and R260A inhibited the recombinant matriptase-catalyzed hydrolysis of Sp-tPA substrate, and the steady-state kinetic studies were done to overview their inhibitory activities. The order of their inhibitory activities evaluated by the $IC_{50}$ values was: HAI-1 IK1L (IC$_{50}$ = 0.065 nM) > D349Y (0.18 nM) > NIK1 (0.23 nM) > IK1 (0.32 nM) > K1L (0.52 nM) > K1L (0.65 nM) > NIK1K2 (0.75 nM) > K385A (1.2 nM) = NIK1K2 (1.5 nM) = K1 (1.6 nM) ≈ NK1L (16 nM). The steady-state fractional rates ($v_i/v$) at which $v$ and $v_i$ are the initial reaction rates in the presence and absence of the HAI-1 mutant, respectively, of the representative HAI-1 mutants, NIK1K2, NIK1L, IK1L, and R260A, were plotted against their concentrations (Fig. 3). Mixing recombinant matriptase with these HAI-1 mutants inhibited matriptase activity completely at the inhibitor concentration equal to that of the enzyme. Thus, these HAI-1 mutants appear to be tight-binding inhibitors. In contrast, the lack of inhibitory activity of HAI-1 K2 and R260A may not arise from their misfolding because they inhibited trypsin with IC$_{50}$ values of 0.25 and 4.0 nM, respectively. Consequently, Kunitz domain I must be solely responsible for the matriptase inhibition by HAI-1, but Kunitz domain II is not directly involved in the inhibition.

**Involvement of Kunitz Domain I in the Interaction of HAI-1 with Matriptase** – We examined whether the contribution of domains other than Kunitz domain I to the matriptase inhibition by HAI-1. Recombinant matriptase was precipitated with the respective resins attached to HAI-1 K385A and K1, but it did not precipitate with resins attached to HAI-1 R260A and K2 (Fig. 4). No serious degradation of these HAI-1 mutants was observed (Fig. 4), suggesting that no domains other than Kunitz domain I interact with matriptase and that Arg260 in Kunitz domain I is critical for both the inhibition and interaction.

**Pre-Steady-State Kinetic Analysis** – Pre-steady-state kinetics for the interaction of recombinant matriptase with HAI-1 mutants was examined by measuring the inhibition of the matriptase-catalyzed hydrolysis of Sp-tPA. The hydrolysis was initiated by the addition of enzyme to a mixture of the HAI-1 mutant and substrate. The kinetic parameters, the association rate constants ($k_{on}$) of recombinant matriptase with the HAI-1 mutants, the dissociation rate constant ($k_{off}$) of the matriptase-HAI-1 mutant complexes, and the inhibitor constants ($K_i$) of the HAI-1 mutants for the inhibition of matriptase were determined (Table I). Preliminary experiments revealed that the HAI-1 K2, NK1L, and R260A mutants showed no inhibition under the conditions examined.

Progress curves for the recombinant matriptase-catalyzed hydrolysis of Sp-tPA in the presence of increasing concentrations of the HAI-1 NK1L, K2, and IK1L mutants are shown in Fig. 5A. The degree of inhibition increased in a manner related to the reaction time and inhibitor concentration. Nonlinear regression analysis of the curves using Equation 1 revealed that $k_{obs}$ increased linearly with increasing concentrations of HAI-1 NK1L, K2, or IK1L (Fig. 5B). $k_{obs}$ diminished hyperbolically with increasing Sp-tPA concentration at fixed concentrations of HAI-1.
NIK1LK2 and IK1L (Fig. 5C), indicating that the interaction of these two HAI-1 mutants with recombinant matriptase is competitive with respect to the substrate (28). All the other HAI-1 mutants that exhibited inhibitory activity also showed reaction time- and inhibitor concentration-dependent competitive inhibition (data not shown). Therefore, the $k_{on}$ values for the interaction of recombinant matriptase with the HAI-1 mutants can be determined according to Equation 2. In contrast, the intercepts on the vertical axis in the plots of $k_{abs}$ versus [HAI-1 mutant] for all HAI-1 mutants examined were too small to estimate the $k_{off}$ values correctly (Fig. 5B). Instead, the $k_{off}$ values were calculated according to Equation 4. Linear correlation analysis revealed that the order of the inhibitory activities of HAI-1 mutants (which are expressed by the $1/K_i$ values determined according to Equation 3) were consistent with that under steady-state conditions ($P = 0.0002, r = 0.874$). This strongly supports the validity of the $k_{off}$ values determined by Equation 4. The kinetic parameters for the interaction of recombinant matriptase with HAI-1 mutants are summarized in Table I. It is noteworthy that the inhibitory activity assessed by $1/K_i$ of HAI-1 IK1L is 400 times higher than that of HAI-1 NIK1LK2, whereas the inhibitory activity of HAI-1 K1 is similar to that of HAI-1 NIK1LK2. Table I also shows that any two HAI-1 mutants with similar inhibitory activity do not necessarily give similar $k_{on}$ and $k_{off}$ values. For instance, the $K_i$ values of HAI-1 NIK1L and IK1 are similar, but their $k_{on}$ and $k_{off}$ values differ considerably.

Roles of N-terminal Domain, Internal Domain, LDLRA Domain, and Kunitz Domain II in the Inhibition of Matriptase – The $k_{on}$, $k_{off}$, and $K_i$ values (Table I) were compared between any two HAI-1 mutants to evaluate the roles of the respective domains. The differences in these values, described below, were significant.

The $k_{on}$ value was 6.6 times higher in HAI-1 NIK1L than in HAI-1 NIK1LK2, whereas $k_{off}$ was similar in HAI-1 NIK1L and NIK1LK2, suggesting that Kunitz domain II decreases the rate of association between Kunitz domain I and matriptase. Lys385 is unlikely to be essential for the effect of Kunitz domain II because there was no difference in the $k_{on}$ values between HAI-1 K385A and NIK1LK2. In the absence of the N-terminal domain, the deletion effect of Kunitz domain II was still observed, although slightly weaker. The $k_{on}$ value was 3.4 times higher in HAI-1 IK1L than in HAI-1 IK1LK2. In the absence of both the N-terminal and internal domains, the deletion effect of Kunitz domain II was not so evident, and the $k_{on}$ was 2.6 times higher in HAI-1 K1L than in HAI-1 K1LK2.

The $k_{on}$ value was 18 times higher in HAI-1 IK1LK2 than in HAI-1 NIK1LK2, whereas their $k_{off}$ values were similar, suggesting that the N-terminal domain as well as Kunitz domain II decreases the association rate without affecting the dissociation rate. In the absence of Kunitz domain II, the deletion effect of the N-terminal domain was still observed, although weaker, and the $k_{on}$ was 9.4 times higher in HAI-1 IK1L than in HAI-1 NIK1L. In the absence of the LDLRA domain and Kunitz domain II, the N-terminal domain did not severely decrease the association rate, and the difference in $k_{on}$ between HAI-1 NIK1 and IK1 was only 2.2 times.

The $k_{on}$ values of HAI-1 IK1LK2, IK1L, and IK1 were 4.0, 5.3, and 4.7 times higher than those of HAI-1 K1LK2, K1L, and K1, respectively. The $k_{off}$ was 4.9 times lower in HAI-1 IK1L than in K1L, and 1.6 times lower in HAI-1 IK1 than in K1. These results suggest that the internal domain increase the rate of association between Kunitz domain I and matriptase, and decreases the dissociation rate of the Kunitz domain I–matriptase contact in the enzyme–inhibitor complex. These association and dissociation rates show that the internal domain contributes to the stability of the interaction between Kunitz domain I and matriptase. However, the $k_{off}$ values did not differ between HAI-1 K1LK2 and IK1LK2, indicating that the internal domain decreases effectively the dissociation of the Kunitz domain I–matriptase complex only in the absence of Kunitz domain II.

The $k_{on}$ values were 10 times higher in HAI-1 NIK1LK2 and 4.9 times higher in D349Y than in HAI-1 NIK1LK2. The $k_{on}$ value was 3.3 times higher in HAI-1 NIK1L than in HAI-1 NIK1LK2. This shows that the LDLRA domain decreases the association rate between Kunitz domain I and matriptase, and that Asp349 in the LDLRA domain is critical to this effect. However, the $k_{on}$ value was similar in HAI-1 K1L and K1, suggesting that the LDLRA domain exhibits its effect only when it is accompanied by the N-terminal domain or Kunitz domain II. The $k_{off}$ values were 3.3 times lower in HAI-1 NIK1L than in HAI-1 NIK1, 17 times lower in IK1L than in IK1, and 6.3 times lower in K1L
than in K1. These results suggest that the LDLRA domain decreases the dissociation rate of the Kunitz domain I–matrkapase complex and that the effect of the LDLRA domain is maximized when the domain is accompanied by the internal domain in the absence of the N-terminal domain and Kunitz domain II.

**DISCUSSION**

The HAI-1 mutants we examined exhibited time-dependent inhibition of matriptase (Fig. 5A), and some of them, such as HAI-1 IK1L, interacted tightly with the enzyme (Fig. 3). Some Kunitz-type inhibitors interact with their target proteases in a time-dependent manner. Tissue factor protease inhibitor, an anti-factor Xa, is one type of such inhibitors, and acts through a mechanism involving the rapid interaction between the inhibitor and enzyme to form an initial collision complex with a low affinity, followed by a slow isomerization to a tightened enzyme–inhibitor complex (28,33). In such cases, the plots of $k_{obs}$ versus [I] give hyperbolic curves, and this type of inhibitors is defined as a slow-binding inhibitor. In the interaction of recombinant matriptase with HAI-1 mutants, however, the plots of $k_{obs}$ versus [I] were linear, although the kinetics of inhibition was slow in all cases examined (Fig. 5B). We observed no evidence supporting the formation of an initial low-affinity complex. Such time-dependent reactions have been described in the inhibition of blood coagulation factors Xa and Xa by protease nexin-2 and a tick anticoagulant peptide, respectively, each of which has a Kunitz-like domain (34,35). The physiological significance of the time-dependent binding process of protease inhibitors is unclear. Matriptase and HAI-1 might be coexpressed in tissues (2,12). If so, the enzyme is hardly active if the inhibitor binds with the enzyme rapidly to form the tight and stable complex. On the other hand, prolonged matriptase activity might be harmful to the cells (36).

Therefore, the time-dependent process associated with the binding between matriptase and HAI-1 may allow the enzyme to be active temporarily.

The precise roles of the Kunitz domains in the matriptase inhibition remain to be determined (14). Our study demonstrated that Kunitz domain I has inhibitory activity against the enzyme, but Kunitz domain II does not. The inhibitory activity of the Kunitz domains is characterized by their sequences from the P4 to P1 positions in the protease-binding site. The sequences of Kunitz domains I and II are Gly−Arg−Cys−Arg260 and Gly−Phe−Cys−Lys385, respectively. Kunitz I is preferred by matriptase (15). Another important finding regarding Kunitz domain II is that the deletion of the domain from HAI-1 NIK1LK2 enhanced the rate of association between Kunitz domain I and the enzyme. Kunitz domain II may obstruct the protease-binding site of Kunitz domain I (Fig. 6). This idea is supported by the observation that HGF activator binding of each Kunitz domain is affected by the presence of other Kunitz domain (7). Because Kunitz domain II has inhibitory activity against HGF activator and trypsin (7), the occurrence of soluble HAI-1 species lacking Kunitz domain II may be more important for the inhibition of matriptase than for that of HGF activator (1,6–8).

The deletion of the N-terminal domain, like that of Kunitz domain II, increased the association rate between Kunitz domain I and matriptase. Because the N-terminal domain does not bind specifically to matriptase (Fig. 4), this effect of the N-terminal domain may also be caused by obstruction of the protease-binding site of Kunitz domain I (Fig. 6). A synergistic effect was observed when both N-terminal domain and Kunitz domain II were deleted: the $k_{on}$ values were 18 and 6.6 times higher in HAI-1 IK1LK2 and NIK1L than in HAI-1 NIK1LK2, respectively, and the values were 62 times higher in HAI-1 IK1L than in HAI-1 NIK1LK2. This suggests that the N-terminal domain and Kunitz domain II act cooperatively to obstruct enzyme activity (Fig. 6). We suggest that 58-kDa HAI-1 may not inhibit matriptase in vivo because the corresponding HAI-1 mutant NIK1LK2 had very low inhibitor activity (Table I). This suggestion is supported by the fact that 58-kDa HAI-1 is not found as a complex with the enzyme in human milk. Thus, the 40- and 25 kDa HAI-1 species may be responsible for the matriptase inhibition in vivo.

No reports have described the occurrence of HAI-1 variants lacking the internal domain, which suggests the importance of the domain. We examined the effect of the internal domain on the inhibitory activities of HAI-1 IK1LK2, IK1L, and IK1. The internal domain increased the association rate $k_{on}$, suggesting that the domain improves the availability of Kunitz domain I for the enzyme inhibition. Unexpectedly, however, HAI-1 NK1LK2 showed inhibitory activity too weak to evaluate the $k_{on}$ value, and this result cannot be accounted for.
only by the lack of this putative effect of the internal domain. The most probable explanation is that the N-terminal domain moves closer to Kunitz domain I by the deletion of the internal domain, thereby severely suppressing the association between Kunitz domain I and the enzyme.

The cysteine-rich LDLRA domain is about 40 amino acids, is biologically ubiquitous, and is found in more than 100 proteins. This domain is thought to be involved in protein–protein interaction (37). However, no apparent role of the LDLRA domain has been implicated in the inhibition of HGF activator and trypsin by HAI-1 (7). We found dual effects of this domain on the matriptase inhibition—the domain decreased both the association rate and the dissociation rate. A crystallographic study of the fifth LDLRA domain in the LDL receptor showed that this domain contains six amino acids that coordinate a Ca\(^{2+}\) ion in an octahedral arrangement, termed the calcium cage (30,31). Site-directed mutagenesis at Asp\(^{336}\), one of the six amino acid residues comprising the cage, strongly suppresses the LDL binding to this domain (32), suggesting that the three-dimensional structure essential for LDL binding involves the binding of Ca\(^{2+}\) to it. In this study, HAI-1 D349Y showed a higher association rate than did HAI-1 NIK\(^1\)LK\(^2\). This suggests that the Ca\(^{2+}\) binding to the LDLRA domain may be important in HAI-1 to maintain the correct protein conformation and that the conformation is critical for suppressing the association between Kunitz domain I and matriptase. However, the LDLRA domain per se is unlikely to suppress the association. The conformation may be essential for the correct positioning and orientation of Kunitz domain II in 58-kDa HAI-1 (Fig. 6). The higher association rate of HAI-1 D349Y than NIK\(^1\)LK\(^2\) can be explained by the altered position of Kunitz domain II in HAI-1 D349Y. In contrast, the correct conformation of the LDLRA domain is probably not essential for its effect on the dissociation, because the \(k_{\text{off}}\) value is lower than in HAI-1 D349Y that in HAI-1 NIK\(^1\)LK\(^2\). The reason for the decrease in the dissociation rate of Kunitz domain I–matriptase complex caused by the presence of the LDLRA domain in HAI-1 (i.e., the considerably lower dissociation rate of HAI-1 IK\(^1\)L–matriptase complex than HAI-1 IK\(^2\)–matriptase complex) is unclear. It seems unlikely that a specific interaction, which stabilizes the Kunitz domain I–matriptase complex, occurs between the LDLRA domain and enzyme (see Figs. 4 and 5B). One explanation is that the LDLRA domain in HAI-1 mutants such as HAI-1 IK\(^1\)L and K\(^L\) is positioned so that it can obstruct the dissociation of matriptase from the Kunitz domain I.

In summary, our results demonstrate that Kunitz domain I is responsible for the inhibition of matriptase and that domains other than Kunitz domain I affect the inhibitory activity considerably. Together with evidence of the occurrence of soluble HAI-1 species in vivo, our data lead us to propose that the domain structure of HAI-1 is organized in a way that allows HAI-1 to flexibly control the activity of matriptase, possibly in relation to the protease activity in tissues. Another striking finding of our study is that HAI-1 IK\(^1\)L showed potent inhibitory activity. It remains unknown whether 25-kDa HAI-1 in human milk corresponds to the HAI-1 IK\(^1\)L mutant and whether the inhibitory activity is stronger in 25-kDa HAI-1 than in 40-kDa HAI-1. Regardless, HAI-1 IK\(^1\)L could be a powerful tool for suppressing cancer invasion and metastasis, for which matriptase is responsible.

REFERENCES

1. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) J. Biol. Chem. 272, 6370–6376
2. Kataoka, H., Suganuma, T., Shimomura, T., Itoh, H., Kitamura, N., Nabeshima, K., and Koono, M. (1999) J. Histochem. Cytochem. 47, 673–682
3. Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., and Kitamura, N. (1993) J. Biol. Chem. 268, 10024–10028
4. Itoh, H., Kataoka, H., Tomita, M., Hamasuna, R., Nawa, Y., Kitamura, N., and Koono, M. (2000) Am. J. Physiol. Gastrointest. Liver Physiol. 278, G635–G643
5. Shimomura, T., Denda, K., Kawaguchi, T., Matsumoto, K., Miyazawa, K., and Kitamura, N. (1999) J. Biochem. (Tokyo) 126, 821–828
6. Tsuzuki, S., Murai, N., Miyake, Y., Inouye, K., Hirayasu, H., Iwanaga, T., and Fushiki T. (2005) Biochem. J. 388, 679–687
7. Denda, K., Shimomura, T., Kawaguchi, T., Miyazawa, K., and Kitamura, N. (2002) J. Biol. Chem. 277, 14053–14059
8. Lin, C. Y., Anders, J., Johnson, M., and Dickson, R. B. (1999) J. Biol. Chem. 274, 18237–18242
9. Hooper, J. D., Clements, J. A., Quigley, J. P., and Antalis, T. M. (2001) J. Biol. Chem. 276, 857–860
10. Takeuchi, T., Shuman, M. A., and Craik, C. S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11054–11061
11. Satomi, S., Yamasaki, Y., Tsuzuki, S., Hitomi, Y., Iwanaga, T., and Fushiki, T. (2001) Biochem. Biophys. Res. Commun. 287, 995–1002
12. Oberst, M. D., Singh, B., Ozdemirli, M., Dickson, R. B., Johnson, M. D., and Lin, C. Y. (2003) J. Histochem. Cytochem. 51, 1017–1025
13. Lee, S. L., Dickson, R. B., and Lin, C. Y. (2000) J. Biol. Chem. 275, 36720–36725
14. Kirchhofer, D., Peek, M., Li, W., Stamos, J., Eigenbrot, C., Kadkhodayan, S., Elliott, J. M., Corpuz, R. T., Lazarus, R.A., and Moran, P. (2003) J. Biol. Chem. 278, 36341–36349
15. Takeuchi, T., Harris, J. L., Huang, W., Yan, K. W., Coughlin, S. R., and Craik, C. S. (2000) J. Biol. Chem. 275, 26333–26342
16. Benaud, C., Dickson, R. B., and Lin, C. Y. (2001) Eur. J. Biochem. 268, 1439–1447
17. Yamasaki, Y., Satomi, S., Murai, N., Tsuzuki, S., and Fushiki, T. (2003) J. Nutr. Sci. Vitaminol. (Tokyo) 49, 27–32
18. Oberst, M. D., Johnson, M. D., Dickson, R. B., Lin, C. Y., Singh, B., Stewart, M., Williams, A., al-Nafussi, A., Smyth, J. F., Gabra, H., and Sellar, G. C. (2002) Clin. Cancer Res. 8, 1101-1107
19. Vogel, L. K., Saeb, M., Skjelbred, C. F., Abell, K., Pedersen, E. D., Vogel, U., and Kure, E. H. (2006) BMC Cancer 4, 176
20. Mazzone, M., and Comoglio, P. M. (2006) FASEB J. 20, 1611–1621
21. Nozaki, S., Endo, Y., Nakahara, H., Yoshizawa, K., Ohara, T., and Yamamoto, E. (2006) Anticancer Drugs 17, 1109–1117
22. Deryugina, E. I., and Quigley, J. P. (2006) Cancer Metastasis Rev. 25, 9–34
23. Rosmann, S., Hahn, D., Lottaz, D., Kruse, M. N., Stocker, W., and Sterchi, E. E. (2002) J. Biol. Chem. 277, 40650–40658
24. Bond, J. S., Matters, G. L., Banerjee, S., and Dusheck, R. E. (2005) FEBS Lett. 579, 3317–3322
25. Oberst, M. D., Williams, C. A., Dickson, R. B., Johnson, M. D., and Lin, C. Y. (2003) J. Biol. Chem. 278, 26773–26779
26. Morrison, J. F., and Walsh, C. T. (1988) Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–203
27. Dharmasena, S. P., Wimalasena, D. S., and Wimalasena, K. (2002) Biochemistry 41, 12414–12420
28. Copeland, R. A. (2005) in Evaluation of Enzyme Inhibitors in Drug Discovery: A Guide for Medical Chemists and Pharmacologists, pp. 141–177, John Wiley & Sons, Inc., Hoboken, New Jersey
29. Lu, D., Yuan, X., Zheng, X., and Sadler, J. E. (1997) J. Biol. Chem. 272, 31293–31300
30. Brown, M. S., Herz, J., and Goldstein, J. L. (1997) Nature 388, 629–630
31. Fass, D., Blacklow, S., Kim, P. S., and Berger, J. M. (1997) Nature 388, 691–693
32. Esser, V., Limbird, L. E., Brown, M. S., Goldstein, J. L., and Russel D. W. (1988) J. Biol. Chem. 263, 13282–13290
33. Huang, Z. F., Wun, T. C., and Broze, G. J. Jr. (1993) J. Biol. Chem. 268, 26950–26955
34. Scandura, J. M., Zhang, Y., Van Nostrand, W. E., and Walsh, P. N. (1997) Biochemistry 36, 412–420
35. Rezaie, A. R. (2004) Biochemistry 43, 3368–3375
36. Oberst, M. D., Chen, L. Y., Kiyomiya, K., Williams, C. A., Lee, M. S., Johnson, M. D., Dickson, R. B., and Lin, C. Y. (2005) Am. J. Physiol. Cell Physiol. 289, C462–C470
37. Moestrup, S. K. (1994) Biochem. Biophys. Acta 1197, 197–213
FOOTNOTES

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1The abbreviations used are: Boc, t-butylxocarbonyl; CHO, Chinese hamster ovary; HGF, hepatocyte growth factor, HAI-1, HGF activator inhibitor type 1; HRP, horseradish peroxidase; LDLRA, low-density lipoprotein receptor type A module; MCA, 4-methylcoumaryl-7-amine; MT-SP1, membrane-type serine protease 1; sc-uPA, single-chain urokinase-type plasminogen activator.

FIGURE LEGENDS

FIGURE 1. Schematic domain construction of rat HAI-1 variants and their characterization. A. schematic representation of the structure of rat HAI-1 and diagram of expression constructs. Domain structure of the primary translation product of rat HAI-1 is indicated as HAI-1 at the top. A soluble HAI-1 occurring naturally is indicated as 58-kDa HAI-1. HAI-1 NIK1LK2 represents a recombinant form of 58-kDa HAI-1 in which the N-terminal region containing the signal peptide (Met1–Pro46) is replaced by the human immunoglobulin K-chain signal peptide and S-tag (ST) and the myc epitope and hexahistidine tag (MHT) is fused after Ser441. The deletion mutants are shown below the HAI-1 NIK1LK2 mutant. The truncated regions in each deletion mutant are indicated by the dotted line. The positions at which amino acid substitution was introduced for HAI-1 R260A, D349Y, and K385A are indicated on the top. TM, transmembrane domain; N, N-terminal domain; I, Internal domain; KI, Kunitz domain I; L, LDLRA domain; KII, Kunitz domain II. B, silver staining of HAI-1 NIK1LK2 preparations. The concentrated conditioned medium was treated with Ni2+-charged resin and the proteins bound to the resin were eluted (lane 1). The eluate was subsequently treated with an immobilized S-protein (lane 2). The eluates were analyzed SDS-PAGE (12% polyacrylamide) under reducing conditions and silver staining. Lane M shows the protein size markers with each mass indicated on the left in kilodaltons (kDa). Note that an unidentified protein with a mass of 40 kDa is mostly evident in the preparation with Ni2+-charged resin alone. C, Western blot analysis of all HAI-1 mutants prepared in this study. The HAI-1 mutants treated with Ni2+-charged resin were separated by SDS-PAGE (12% polyacrylamide) under reducing conditions, and the Western blot was probed with S-protein conjugated with HRP. No cleaved products were evident in each HAI-1 mutant preparation. Lane Marker shows the signals given by proteins to which S-tag was fused at their N-terminus (Perfect Protein Marker™). Note that longer exposure was required to detect the marker protein with a mass of 15 kDa.

FIGURE 2. Production and characterization of recombinant rat matriptase. A, schematic representation of the structure of rat matriptase and an expression construct. This protease comprises 855 amino acids. The amino acid numbering starts from the putative N-terminus of the protein. The domain structure is indicated as matriptase (wild type) at the top. The predicted disulfide linkages are shown as S–S. The activation cleavage site (indicated by the arrow) and its surrounding sequence (single-letter code) are shown in matriptase (wild type). Recombinant pro-matriptase is a secreted form of matriptase in which the cytoplasmic domain and the signal anchor (Met1–His80) are replaced by the human immunoglobulin K-chain signal peptide, S-tag (ST), and the enterokinase recognition sequence (DDDKK, underlined). In addition, TKQAR615 in the wild-type enzyme was replaced with the enterokinase recognition sequence for in vitro cleavage before Val615 (activation cleavage). The disulfide-linked, two-chain recombinant matriptase (active recombinant matriptase) is illustrated at the bottom. Note that the S-tag at the N-terminus can also be removed with recombinant enterokinase. TM, transmembrane domain; N, N-terminal domain; I, internal domain; KI, Kunitz domain I; L, LDLRA domain; KII, Kunitz domain II; SEA, sea urchin sperm protein–enterokinase–agrin domain; C1 and C2, the first and second complement protein subcomponents.
C1r/C1s–urchin embryonic growth factor–bone morphogenetic protein 1 domains, respectively; L1–4, four tandem repeats of LDLRA domain; SPD, serine protease domain. B, Western blot analysis of recombinant matriptase produced in CHO-K1 cells. Recombinant matriptase treated with an immobilized S-protein was eluted by boiling (lane 1) as described previously (6) or eluted with enterokinase (lane 2) as described in Experimental Procedures. Samples were separated by SDS-PAGE (12% polyacrylamide) under reducing conditions, and the Western blot was probed with rabbit anti-rat matriptase catalytic domain antibody (Spr992). The molecular masses of the standards are indicated on the left in kDa.

FIGURE 3. Plots of the steady-state fractional rate as a function the concentrations of HAI-1 mutants. The steady-state initial reaction rates in the presence (v) and absence (v′) of HAI-1 mutants were measured as described in Experimental Procedures. The reaction mixtures contained various concentrations (indicated) of HAI-1 NIK1LK2 (open squares), HAI-1 NIK1LK (closed circles), HAI-1 IK1L (open circles), and HAI-1 R260A (closed squares), and 0.12 nM recombinant matriptase. Reactions were initiated by addition of Sp-tPA at a final concentration of 500 µM.

FIGURE 4. Exclusive responsibility of Kunitz domain I in the interaction of HAI-1 with matriptase. The Ni2+-charged resin bound to HAI-1 R260A, K385A, K1, or K2 was incubated with HEPES buffer alone or with HEPES buffer containing recombinant matriptase. After being washed with HEPES buffer, the precipitants were eluted by boiling. The eluates were separated by SDS-PAGE (12% polyacrylamide) under reducing conditions, blotted, and probed with rabbit anti-rat matriptase catalytic domain antibody (Spr992) (upper panel) or anti-myc antibody (bottom panel). The catalytic domain of matriptase is visualized at the position corresponding to 28 kDa (indicated by arrowhead). The HAI-1 R260A, K385A, K1, and K2 mutants are visualized at the positions corresponding to 59, 59, 16, and 17 kDa, respectively.

FIGURE 5. Pre–steady-state kinetic analysis of the inhibition of recombinant matriptase by the HAI-1 NIK1LK2 and IK1L mutants. A, progress curves for the reaction of recombinant matriptase in the presence of increasing concentrations of HAI-1 NIK1LK2 and IK1L. The enzymatic reactions were initiated by the addition of 0.12 nM recombinant matriptase, and the product formation was monitored as detailed in Experimental Procedures. The concentrations (from top to bottom) for HAI-1 NIK1LK2 are 0, 20, 30, 40, 50, 60, 70, and 80 nM; those for HAI-1 IK1L are 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 nM. B, dependence of kobs on the concentration of inhibitor. The progress curve data were analyzed using Equation 1 to obtain kobs as a function of the concentrations of HAI-1 NIK1LK2 (closed circles) and HAI-1 IK1L (open circles). C, dependence of kobs on the substrate concentration. Assays were performed under the same conditions as described in Experimental Procedures except that the reaction mixtures contained 0.2–1.0 mM Sp-tPA and 60 nM HAI-1 NIK1LK2 (closed circles) or 1.0 nM HAI-1 IK1L (open circles).

FIGURE 6. Proposed domain structure of 58-kDa HAI-1. Putative sites for protease binding in Kunitz domain I and Kunitz domain II are indicated by small black rectangles. Spacer regions are indicated by dotted curves. In 58-kDa HAI-1, HGF activator binding of each Kunitz domain is affected by the presence of other Kunitz domain, suggesting that the two protease-binding sites in the molecule are close to each other (7). In the interaction of HAI-1 NIK1LK2 with matriptase, Kunitz domain II is postulated to function only in the obstruction of the protease-binding site of Kunitz domain I. The N-terminal domain and Kunitz domain II may collaborate to severely obstruct this site. The presence of the LDLRA domain appears to be critical for the correct positioning of Kunitz domain II. These can account for the low association rate of HAI-1 NIK1LK2 (58-kDa HAI-1) with matriptase. N, N-terminus domain; I, internal domain; K1, Kunitz domain I; L, LDLRA domain; K2, Kunitz domain II.
Table I. Kinetic parameters for reaction of recombinant matriptase with HAI-1 mutants*

*Assays were performed as described in Experimental Procedures. NIK\(^{1}\)LK\(^{2}\) and IK\(^{1}\)L were included in the reaction mixture over the concentration range as described in the legend for Fig. 4B: K385A, 20-80 nM, the other HAI-1 mutants, 4-20 nM. The values of \(k_{\text{on}}\) were determined by fitting the data to Equations 1 and 2. The value of \(k_{\text{off}}\) was determined from each progress curve according to Equation 4. The mean of the seven \(k_{\text{off}}\) values obtained in a separate experiment was taken as the \(k_{\text{off}}\) value. The values of \(K_i\) were calculated according to Equation 3. The values shown are means ± SD of 3–4 separate experiments. N.D., kinetic parameters cannot be determined.

| HAI-1 mutants | \(k_{\text{on}}\) \(\times 10^9\) M\(^{-1}\) s\(^{-1}\) | \(k_{\text{off}}\) \(\times 10^{-6}\) s\(^{-1}\) | \(K_i\) pM |
|---------------|-----------------|------------------|---------|
| NIK\(^{1}\)LK\(^{2}\) | 3.5 ± 2.0 | 23 ± 14 | 647 ± 62 |
| NIK\(^{1}\) | 23 ± 3 | 12 ± 3 | 53 ± 18 |
| NIK\(^{1}\) | 75 ± 9 | 40 ± 10 | 45 ± 11 |
| IK\(^{1}\)LK\(^{2}\) | 64 ± 6 | 15 ± 4 | 24 ± 7 |
| IK\(^{1}\)L | 217 ± 10 | 3.5 ± 2.1 | 1.6 ± 1.0 |
| IK\(^{1}\) | 168 ± 37 | 61 ± 8 | 38 ± 3 |
| K\(^{1}\)LK\(^{2}\) | 16 ± 3 | 18 ± 10 | 42 ± 17 |
| K\(^{1}\)L | 41 ± 30 | 17 ± 2 | 54 ± 27 |
| K\(^{1}\) | 36 ± 9 | 107 ± 37 | 328 ± 181 |
| K\(^{2}\) | N.D. | N.D. | N.D. |
| NK\(^{1}\)LK\(^{2}\) | N.D. | N.D. | N.D. |
| NIK\(^{1}\)K\(^{2}\) | 35 ± 15 | 28 ± 7 | 86 ± 28 |
| R260A | N.D. | N.D. | N.D. |
| D349Y | 17 ± 1 | 5.9 ± 2.0 | 37 ± 11 |
| K385A | 6.3 ± 0.7 | 18 ± 5 | 278 ± 93 |
Figure 1

A

HAI-1

58-kDa HAI-1

(Soluble HAI-1 mutants)

NIK'LK²

NIK'L

IK'LK²

IK'L

K'LK²

K'L

K'

K²

NK'K²

NIK'K²

B

(kDa) M 1 2

175 173 17

33 33 33

48 48 48

83 83 83

100 100 100

C

(kDa)

100 75 50 25 15

75 75 75

50 50 50

25 25 25

15 15 15

Marker

Arg²⁶⁰ Asp³⁴⁹ Lys³⁸⁵ (Soluble HAI-1 mutants)

R²⁶⁰A D³⁴⁹Y K³⁸⁵A
Figure 2

A

Matriptase (wild type)

Recombinant pro-matriptase

Recombinant enterokinase

B

(kDa)

175  150  125  100  75  50  25  17

1  2
Figure 3
Figure 4

Blot
Spr992

Blot
anti-myc
Figure 5

A

B

C

NIK\textsuperscript{1}LK\textsuperscript{2}

IK\textsuperscript{1}L

0
20
40
60
80
100
Time (min)

0
20
40
60
80
100
Time (min)

0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
\textit{k}_\text{obs} (\text{min}^{-1})

[Inhibitor]_0 (nM)

[Sp-tPA]_0 (mM)

\textit{k}_\text{obs} (\text{min}^{-1})
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