Crystal Structures of Matriptase in Complex with Its Inhibitor Hepatocyte Growth Factor Activator Inhibitor-1*

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Background: Matriptase requires very strict regulation by its inhibitor, hepatocyte growth factor activator inhibitor-1 (HAI-1). It has been demonstrated that the Kunitz domain I (KD1) but not Kunitz domain II (KD2) of HAI-1 is responsible for the inhibitory activity of HAI-1 against matriptase. To investigate the molecular basis of inhibition of matriptase by HAI-1, we solved several crystal structures of matriptase serine protease domain in complex with the fragments of HAI-1. Based on these structures, we found that the binding of KD1 was different from previously predicted binding mode. The P3 arginine residue occupies the S3 specificity pocket of matriptase, but not the S4 pocket as in the cases of hepatocyte growth factor activator inhibitor (HGFA) KD1 and matriptase-sunflower trypsin inhibitor (HAI-1)-complexes. The long 60-loop of matriptase makes direct contact with HAI-1 but remains flexible even in the complexes, and its apex does not bind with KD1 tightly. The interactions between this unique 60-loop and KD1 may provide an opportunity to increase the specificity and inhibitory activity of KD1 for matriptase. Furthermore, comparison between KD1 and a homology model of HAI-1 KD2 rationalizes the structural basis of why KD1 but not KD2 is responsible for the inhibitory activity of HAI-1 against matriptase.

Conclusion: These structures elucidate the structural basis of inhibition of matriptase by HAI-1 KD1.

Significance: This work provides important structural insights for the future design of small molecular inhibitors.

Matriptase, a type II trans-membrane serine protease of the S1 trypsin-like family, is expressed on the surface of nearly all normal human epithelium and found in biological fluid-like human milk. Matriptase overexpression has been implicated in tumor progression in certain epithelium-derived cancer cells. Matriptase is tightly regulated by its cognate inhibitor hepatocyte growth factor activator inhibitor-1 (HAI-1). It has been demonstrated that the Kunitz domain I (KD1) but not Kunitz domain II (KD2) of HAI-1 is responsible for the inhibitory activity of HAI-1 against matriptase. To investigate the molecular basis of inhibition of matriptase by HAI-1, we solved several crystal structures of matriptase serine protease domain in complex with the fragments of HAI-1. Based on these structures, we found that the binding of KD1 was different from previously predicted binding mode. The P3 arginine residue occupies the S3 specificity pocket of matriptase, but not the S4 pocket as in the cases of hepatocyte growth factor activator inhibitor (HGFA) KD1 and matriptase-sunflower trypsin inhibitor (HAI-1)-complexes. The long 60-loop of matriptase makes direct contact with HAI-1 but remains flexible even in the complexes, and its apex does not bind with KD1 tightly. The interactions between this unique 60-loop and KD1 may provide an opportunity to increase the specificity and inhibitory activity of KD1 for matriptase. Furthermore, comparison between KD1 and a homology model of HAI-1 KD2 rationalizes the structural basis of why KD1 but not KD2 is responsible for the inhibitory activity of HAI-1 against matriptase.

Results: Crystal structures of matriptase serine protease domain and its complex with HAI-1 were determined.

Conclusion: These structures elucidate the structural basis of inhibition of matriptase by HAI-1 KD1.

Significance: This work provides important structural insights for the future design of small molecular inhibitors.

Matriptase, a type II trans-membrane serine protease that is expressed in all types of epithelium (1–5) and plays critical roles in the establishment and maintenance of epithelial integrity. Matriptase is also found on mast cells, peripheral blood monocytes, and B cells, implicating that the protease might also participate in immune response (6, 7). Indeed, knockdown studies in mice have shown that matriptase is important in postnatal survival, the development of the epidermis, hair follicles, and cellular immune system (8). Matriptase is also recognized as a cancer-associated protease because its substrates prourokinase plasminogen activator and prohepatocyte growth factor (3, 9) have both been implicated in cancer invasion and metastasis. Furthermore, the expression of matriptase has been demonstrated to be up-regulated in various cancer forms such as breast, cervix, ovaries, prostate, esophagus, and liver cancers (10–19). Some of these cancers have been shown to be the results of the loss of balance between the protease and its cognate inhibitor hepatocyte growth factor activator inhibitor-1 (HAI-1),3 suggesting that matriptase requires very strict regulation by its inhibitor and serves as a potential anti-cancer therapeutic target.

Matriptase consists of an N-terminal cytoplasmic domain, a signal anchor trans-membrane domain that tethers it to the cell surface, followed by a sea urchin sperm protein, enterokinase, agrin domain, two complement C1r/C1s, urchin embryonic growth factor, bone morphogenic protein 1 domains, four tandem low-density lipoprotein receptor class A (LDLRA) domains and a C-terminal serine protease domain. Similar to most serine proteases, matriptase is expressed as an inactive zymogen that requires endoproteolytic cleavage for activation. However, matriptase does not depend on other active protease for activation but instead undergoes autoactivation, endowing matriptase the potential as an initiator for downstream signaling cascade (20–23). Detailed mechanism of the autoactivation of matriptase remains unresolved but is believed to be dependent on its own catalytic triad as well as interactions between its non-catalytic domains and HAI-1.

HAI-1 is a trans-membrane epithelial-derived Kunitz-type serine protease inhibitor originally identified as a potent inhibitor of hepatocyte growth factor activator (HGFA) (24–26). HAI-1 comprises 513 amino acids and consists of an N-termi-

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3 The abbreviations used are: HAI-1, hepatocyte growth factor activator inhibitor-1; KD, Kunitz domain; BPTI, bovine pancreatic trypsin inhibitor; APPI, amyloid protein precursor inhibitor; TFPI, tissue factor pathway inhibitor; HGFA, hepatocyte growth factor activator; LDLRA, LDL receptor class A; PDB, Protein Data Bank.
n al domain, an internal domain, a LDLRA domain that is inserted between two Kunitz domains, and a C-terminal transmembrane domain. The extracellular domain can be cleaved at several sites and released as soluble ectodomains by at least two proteases (27). Cleavage of HAI-1 results in not less than two soluble species of HAI-1, 58 and 40 kDa, respectively, in the conditioned media of MKN45 cells and transfected COS-1 cells (24, 27, 28). The 58-kDa species contains the entire extracellular domain after shedding, whereas the 40-kDa species lacks the LDLRA and Kunitz II domains. Soluble HAI-1 species of 40 and 25 kDa were isolated from human milk in complex with matriptase (29). Co-expression of HAI-1 and matriptase are found in most epithelial tissues (5, 26) and on the surfaces of cultured breast cancer cells (30), further confirming that HAI-1 is a physiological inhibitor of matriptase. Enzymatic studies on the soluble fragments of HAI-1 suggest that HAI-1 domains interplay in the regulation of the inhibitory effect of Kunitz domain I (KD1) toward different proteases, including HGFA and matriptase (31–33). For instance, Kojima et al. (33) reported that the removal of the C-terminal Kunitz domain II (KD2) of rat HAI-1 dramatically lowers the $K_i$, from 647 pm (NIK$^1$LK$^2$) to 53 pm (NIK$^1$L) for inhibition of rat matriptase. Truncation of the N-terminal domain in addition to the KD2 of HAI-1 (IK$^1$L) further lowers the $K_i$ to 1.6 pm, suggesting that these two domains, instead of facilitating the inhibition of matriptase by HAI-1, might obstruct the interaction between HAI-1 and matriptase. This is in agreement to the in vivo finding that only the shorter 40- and 25-kDa species of HAI-1 but not the full-length HAI-1 are found to associate with matriptase in human milk (29). However, the exact physiological roles of the different molecular size HAI-1 remain largely unclear.

Several structures of the catalytic domain of matriptase in complex of different inhibitors, including the prototypic Kunitz-type inhibitor bovine pancreatic trypsin inhibitor (BPTI), have been solved (34). The structure of HAI-1 KD1 in complex with another protease target HGFA has also been reported (32). However, structural and mechanistic details on the inhibition of matriptase by KD1 but not KD2 of HAI-1, and the role of the long 60-loop in matriptase substrate specificity remain largely unknown. Here, we generated recombinant proteins of the human matriptase serine protease domain with a mutation N164Q in its active (β-matriptase-N164Q) and inactive (β-matriptase-N164Q/S195A) forms, and the KD2-truncated HAI-1 (HAI-1-NIK1L) independently. However, when we formed complexes of HAI-1-NIK1L and β-matriptase, HAI-1-NIK1L underwent degradation into KD1 only as revealed by our LC-MS/MS results. Eventually, we obtained the crystal structures of the inactive β-matriptase-N164Q/S195A in a ligand-free form and in complex with HAI-1 KD1, as well as two different forms of β-matriptase-N164Q-KD1 complex at 1.48, 2.29, 2.45, and 2.01 Å, respectively. These crystal structures provide insights into the inhibition mechanism of matriptase by HAI-1 KD1, which was different from previously predicted mechanism. Furthermore, homology modeling of HAI-1 KD2 based on KD1 structure elucidates the structural basis of selective inhibition of matriptase by KD1 but not KD2.

### Crystal Structures of Matriptase-HAI-1 Complex

#### EXPERIMENTAL PROCEDURES

**Recombinant Protein Expression and Purification**—The serine protease domain of matriptase, β-matriptase (Swiss-Prot accession no. Q9Y5Y6, residues 615–855, corresponds 16–244 in chymotrypsin numbering, which will be used throughout this work) with a N164Q mutation to eliminate potential glycosylation was expressed in X-33 Pichia pastoris (P. pastoris, Komagataella) cells and purified as described previously (35). The active site-mutated β-matriptase-N164Q (β-matriptase-N164Q/S195A) was generated by site-directed mutagenesis using the plasmid of pPICZαA containing the β-matriptase-N164Q gene. Then, it was expressed in X-33 Pichia pastoris according to the β-matriptase-N164Q expression procedure (35). The culture supernatant was concentrated to 50 ml using a Millipore concentrator (8000 Da Molecular Weight Cut-Off membrane) and then diluted to 500 ml with 30 mM Tris-HCl, pH 8.5. The diluted medium was applied onto a Q Sepharose Fast Flow column (GE Healthcare) equilibrated with 30 mM Tris-HCl, pH 8.5, and eluted with a NaCl gradient (0–0.5 M). Fractions containing β-matriptase-N164Q/S195A based on SDS-PAGE analysis were concentrated and further purified by a gel filtration chromatography (Superdex 75 HR 10/30, Pharmacia Biotech) equilibrated with 30 mM Tris-HCl, pH 8.5, 150 mM NaCl. Fractions containing β-matriptase-N164Q/S195A were collected and concentrated.

To express soluble human HAI-1-NIK1L protein (Swiss-Prot accession no. O43278-2, residues Glh$^{36}$(H) to Ser$^{386}$(H), the amino acid numbering of HAI-1 starts from the putative N terminus of the protein), the cDNA was generated by PCR using the full-length cDNA (kindly provided by Dr. Chen-Yong Lin). The fragments were digested with MluI and cloned into the expression vector pMT/Bip/V5-His-A (Invitrogen), which was digested with Smal and MluI to yield expression vector pMT/Bip/HAI-1-NIK1L-His. The final expression vector sequence was confirmed by DNA sequencing. 2 μg of expression vector of pMT/Bip/HAI-1-NIK1L-His and selection vector pCoBlast (Invitrogen) at a ratio of 19:1 were co-transfected into Drosophila S2 cells using Cellfectin® II Reagent (Invitrogen) according to the manufacturer’s instructions. Selection for stable cell lines was performed by the addition of 25 μg/ml of blasticidin S-HCl (Invitrogen). The selected stable cells with HAI-1-NIK1L construct were grown in EX-CELL 420 serum-free medium (Sigma-Aldrich), and the target protein expression was induced by adding 500 μM CuSO$_4$ to secrete HAI-1-NIK1L into the cultured medium. The conditioned medium was applied to a nickel-nitrotriacetic acid column equilibrated with equilibrium buffer (150 mM NaCl in 50 mM Tris-HCl, pH 8.0). After washing, the target protein was eluted with 300 mM imidazole in the equilibrium buffer. The eluent was dialyzed against 50 mM Tris-HCl at pH 8.0 overnight and applied to an anion exchange column (MonoQ, Pharmacia Biotech) and eluted with a NaCl gradient (0–0.5 M) in 50 mM Tris-HCl, pH 8.0. The fractions containing the target protein were concentrated and applied to a gel filtration chromatography (Superdex 75 HR 10/30, Pharmacia Biotech) equilibrated with the equilibrium buffer. Fractions containing the target protein were collected and concentrated.
Crystallization and Data Collection of the Ligand-free Matriptase and Matriptase-HAI-1 Complex—The ligand-free β-matriptase-N164Q/S195A was concentrated to 10 mg/ml in 10 mM Tris-HCl, pH 8.5, and crystallized by using sitting drop vapor diffusion at room temperature. Tabular crystals were obtained with a precipitant solution of 0.1 M Tris-HCl, pH 8.5, 1.7 mM ammonium sulfate. For x-ray data collection, crystals were dipped into a precipitant solution of 0.1 M Tris-HCl, pH 8.5, 2.2 mM ammonium sulfate containing 20% (v/v) glycerol and then quickly immersed in liquid nitrogen.

The complexes were formed by mixing matriptase and HAI-1 at a molar ratio of 1:5.1 and purifying by a gel filtration chromatography (Superdex 75 HR 10/30, Pharmacia Biotech), respectively. The complexes were concentrated to 10 mg/ml in 10 mM Tris-HCl, pH 8.0, and subjected to crystallization trials using the method of sitting drop vapor diffusion at room temperature. Diamond shape protein crystals were obtained with a precipitant solution of 20% (w/v) polyethylene glycol 8000 in 0.1 M Tris-HCl at pH 8.5. For x-ray data collection, crystals were dipped into a cryoprotectant solution containing 30% (w/v) polyethylene glycol 8000, 20% (v/v) glycerol, and 0.1 M Tris-HCl, pH 8.0, and then quickly immersed in liquid nitrogen. X-ray diffraction data were collected at Shanghai Synchrotron Radiation Facility (Table 1).

Structure Determination and Refinement—The ligand-free β-matriptase-N164Q/S195A and the complex crystal structures were solved by molecular replacement using Molrep of the CCP4 program suite (36) and the published β-matriptase-N164Q structure (PDB code 3P8G) as the search model, yielding clear rotational and translation peaks. After the first rigid body refinement and restrained refinement using the REFMAC program (37), the Fo − Fc electron density map revealed clear extra electron density at the active site of matriptase in the complexes. The KD1 model (PDB code 1YCO) was positioned into the electron density by molecular replacement method of CCP4 program suite (36), which was then manually adjusted by COOT (38) and refined by REFMAC (37). At the same time, an extra electron density near residue Cys122 of matriptase was observed and a reduced glutathione, which might have covalently linked to Cys122 during protein synthesis in yeast (39), was built into the density based on the contour of the extra electron density. In the final cycles of refinement using REFMAC of CCP4 and adjustment using COOT, there were no residues in the disallowed region of the Ramachandran plot. Coordinates of the crystal structures have been deposited in the Protein Data Bank under codes 4IS5, 4ISL, 4ISN, and 4ISO.

Homology Modeling of HAI-1 KD2—SWISS-MODEL was used for building the KD2 model. A fully automated homology modeling approach was adapted. Briefly, coordinates of KD1 were used as the template; the amino acid sequence of KD2 was submitted to the server for alignment and modeling. The model obtained was then assessed by Procheck for conformational quality.

RESULTS AND DISCUSSION

Crystals of the Ligand-free β-Matriptase and β-Matriptase-HAI-1 Complexes—Previous works by Inouye and others (33, 40) have demonstrated that the KD1 but not KD2 of HAI-1 potently inhibits matriptase. To study the mechanism of recognition and inhibition of matriptase by HAI-1, we constructed a KD2-deleted construct of HAI-1 (HAI-1-NIK1L) (Fig. 1A) and established a stable Drosophila S2 cell line to produce the recombinant protein in large quantity for biophysical study. On the other hand, β-matriptase-N164Q and its inactive mutant β-matriptase-N164Q/S195A (Fig. 1A) were expressed in Pichia pastoris as secreted proteins. The single site mutation of Ser195 to alanine dramatically increased the final yield of β-matriptase from 1 mg/liter to 10 mg/liter, suggesting matriptase proteolytic activity could be toxic to Pichia pastoris and exerted inhibitory effect on its expression, and the result was in accordance with previous experiment (21). However, this S195A mutant could not be purified by a benzamidine column and required a purification protocol different from that of the active form (see “Experimental Procedures”). This suggests that the hydroxyl group of Ser195, which directly interacts with the phenyl group of benzamidine, might play an important stabilizing role in the binding of the small molecule inhibitor.

The two mutant forms of β-matriptase were mixed with HAI-1-NIK1L under native conditions, respectively, and the complexes were isolated by gel filtration chromatography. SDS-PAGE analysis of the gel filtration fractions, however, revealed that HAI-1-NIK1L had been degraded. Only a fragment of ~7 kDa remained in complex with β-matriptase. LC-MS/MS analysis of the fragment (data not shown) identified it as solely KD1 spanning residues 244–307 of HAI-1 (Fig. 1A). We reasoned this was the result of limited nonspecific proteolysis mediated by trace amount of endogenous proteases from Pichia pastoris culture because HAI-1-NIK1L was not degraded in Drosophila S2 cell culture.4

We further formed the crystals of the complexes purified through gel filtration chromatography. Dissolution of the crystals on 15% SDS-PAGE confirmed that only a fragment of HAI-1 ~7 kDa remained in the complex crystals (data not shown). Our structural determination also unveiled that only KD1 can be modeled based on the observed electron density and there is no extra space in the crystal packing to accommodate other domains of HAI-1-NIK1L. This tendency to proteolysis suggests that HAI-1 domains flanking KD1 may interact with other non-protease domains of matriptase. The sole matriptase serine protease domain used in our studies could not protect the other domains of HAI-1 from proteolysis.

Using the purified recombinant proteins, we first obtained two types of protein crystals of β-matriptase-N164Q-KD1 complex. Both crystals had the same space group but exhibited slightly different unit cell parameters. The electron densities also revealed different conformations at the protease 60-loop. Therefore we solved two structures of this complex and refined them to 2.45 Å (β-matriptase-N164Q-KD1-A) and 2.01 Å (β-matriptase-N164Q-KD1-B) respectively for comparison. To further investigate the conformational difference at the 60-loop, we formed two more types of diffracting crystals, including the ligand-free inactive apo enzyme (β-matriptase-N164Q/S195A) and its complexes with HAI-1 KD1 (β-matriptase-N164Q-KD1-A).

4 Baoyu Zhao, Cai Yuan, Rui Li, Dan Qu, Mingdong Huang, and Jacky Chi Ki Ngo, unpublished data.
tase-N164Q/S195A-KD1), which were solved by molecular replacement and refined to 1.48 and 2.29 Å, respectively, for further analysis (Table 1).

Overall Structures of β-Matriptase-KD1 Complexes and Flexibility of 60-Loop—In the two models of β-matriptase-N164Q-KD1 complex, the conformations of both matriptase and KD1 are nearly identical except their 60-loops. Therefore, we first described their overall structures as a whole in this section and focused on their 60-loop conformations below.

KD1 adopts the canonical compact pear-shaped conformation that is stabilized by three disulfide bonds (Fig. 1B). Several residues at the N and C terminus of KD1 could not be observed based on the electron density maps. We reasoned that these residues at the terminal are flexible. Matriptase binds KD1 at its substrate binding cleft and the 60-loop region. The matriptase substrate-binding cleft adopts the conventional substrate/inhibitor-binding conformation where KD1 docks in with full shape complementarities. The binding mode of KD1 to the active site cleft of matriptase resembles those of other serine protease-Kunitz-type inhibitors complexes, matriptase-BPTI (PDB code 1EAW), HGFA-KD1 (PDB code 1YCO), trypsin-APPI (orange, PDB code 1TAW), and HGFA-HAI-1-KD1 (blue, PDB code 1YCO) complexes. D, Arg260 of HAI-1 KD1 inserts deeply into the S1 pocket of matriptase. The catalytic triad and oxyanion hole of the protease are denoted.
Indeed, we solved the crystal structure of matriptase to study matriptase pocket, suggesting it is appropriate to use this construct in addition to the active form to study matriptase.

The 60-loop of matriptase has different conformations in the two KD1 complex models except that the KD1 complex models except that the KD1-B complex, the guanidinium group interacts with the side chains of Asp199 and Ser190, as well as the backbone carbonyl of Ser190 and Gly219 (Fig. 1D). The main chain interactions between the P1 residue with matriptase also agree with other Kunitz-type inhibitor-protease complex structures where the Gly193, Asp194, and Ser195 backbone amines, which line the cavity of the oxyanion hole, are in proper conformation and can readily donate hydrogen atoms to form hydrogen bonds with the P1 backbone carbonyl oxygen. This suggests the inhibitor-bound matriptase is in active conformation to carry out hydrolysis.

As mentioned previously, we have also solved the crystal structures of the inactive protease in its ligand-free and KD1-bound forms. Our model of the ligand-free β-matriptase-N164Q/S195A structure was refined to an r.m.s.d. value of 0.159 and 0.180, respectively, suggesting that the atomic model has high accuracy. The model includes residues 16–244 of matriptase, which comprise the entire protease domain. This model is identical with the structures of matriptase serine protease domain in the matriptase-HAI-1 (Arg258(H)) may adopt a “kinked” position to fit into the negatively charged depression at the S4 subsite, which is further stabilized by the backbone carbonyls of the 99-loop of matriptase and cation-stabilizing interaction contributed by the side chains of Phe99 and His57. Kunitz-type inhibitors such as BPTI and APPI usually contain a proline at P3 position (Fig. 2A) and mediate van der Waals interaction with Phe99 as seen in the matriptase-BPTI structure (34). KD1, on the other hand, contains an arginine instead of the commonly found proline. In contrast to the previous prediction (34) that the P3 arginine of HAI-1 contains a phenylalanine (Phe263(H)) at the P3’ position instead of the commonly found isoleucine in other Kunitz-type inhibitors (Fig. 2A). This larger hydrophobic side chain, together with the small P1’ glycine, fits snugly into the hydrophobic S1’/S3’ cavity formed by the disulfide bridge between Cys42 and Cys58, the side chains of Tyr59, Trp64, and Ile41. This fit is better compared with the isoleucine of BPTI in the matriptase-BPTI complex. Phe263(H) mediates extra van der Waals interactions with matriptase side chains of Ile41, Tyr64G, and the Ca of Gly219(H), which further stabilize the protease-inhibitor complex (Fig. 2A).

Matriptase strongly prefers substrate with basic residues at P4 position and accepts either glutamine or basic residues at P3; yet P3 and P4 should not be basic residues simultaneously (3).

Structures of matriptase bound to either BPTI or the bicyclic peptidyl inhibitor sunflower trypsin inhibitor-1 suggest that both preferences for P3 or P4 basic residues are created by a negatively charged depression at the S4 subsite, which is formed by the backbone carbonyls of the 99-loop of matriptase and cation-stabilizing interaction contributed by the side chains of Phe99 and Phe99. Kunitz-type inhibitors such as BPTI and APPI usually contain a proline at P3 position (Fig. 4A) and mediate van der Waals interaction with Phe99 as seen in the matriptase-BPTI structure (34). KD1, on the other hand, contains an arginine instead of the commonly found proline. In contrast to the previous prediction (34) that the P3 arginine of HAI-1 (Arg258(H)) may adopt a “kinked” position to fit into the S4 subsite of matriptase, our structures revealed that the P3 arginine does not interact with the acidic S4 subsite due to steric hindrance created by the side chains of Leu284(H) of KD1, which caps the top of the S4 subsite and the Phe99 of matriptase side chains of Phe99 and His57. Kunitz-type inhibitors such as BPTI and APPI usually contain a proline at P3 position (Fig. 4A) and mediate van der Waals interaction with Phe99 as seen in the matriptase-BPTI structure (34). KD1, on the other hand, contains an arginine instead of the commonly found proline. In contrast to the previous prediction (34) that the P3 arginine of HAI-1 (Arg258(H)) may adopt a “kinked” position to fit into the S4 subsite of matriptase, our structures revealed that the P3 arginine does not interacts with Phe99 and His57 of the catalytic triad (Fig. 2A).

### Table 1

| Diffraction data | β-Matriptase-N164Q/S195A | β-Matriptase-N164Q/S195A-KD1 | β-Matriptase-N164Q/KD1-A | β-Matriptase-N164Q/KD1-B |
|------------------|---------------------------|-----------------------------|--------------------------|--------------------------|
| **Space group**  | C222                      | P4,2,2                      | P4,2,2                   | P4,2,2                   |
| **Cell parameters (Å)** | 66.59, 140.96, 51.57 | 61.93, 61.93, 178.99 | 64.53, 64.53, 172.39 | 61.37, 61.37, 178.36 |
| **R<sub>merge</sub> (%)** | 0.074 (0.111)  | 0.057 (0.149)  | 0.046 (0.157)  | 0.079 (0.283)  |
| **Completeness (%)** | 99.5 (100.0)  | 98.3 (83.0)  | 94.9 (70.6)  | 97.5 (97.3)  |
| **Average I/σI** | 54.2 (25.6)  | 55.9 (16.2)  | 47.2 (6.2)  | 34.9 (7.0)  |
| **Data redundancy** | 5.8 (5.3)  | 8.0 (6.9)  | 6.4 (3.1)  | 4.1 (3.4)  |
| **Statistics of x-ray diffraction data collection and structure refinement** | | | | |
| **Refinement** | Resolution (Å) | 1.48 | 2.29 | 2.45 | 2.01 |
| **R<sub>merge</sub>/R<sub>free</sub> (%)** | 15.9/17.9 | 18.4/23.2 | 21.0/25.9 | 17.2/19.9 |
| **r.m.s.d.** | 0.014 | 0.007 | 0.007 | 0.014 |
| **Bond angles** | 1.6° | 1.1° | 1.1° | 1.4° |
| **Mean B factors (Å²)** | 11.7 | 21.7 | 36.8 | 25.2 |
| **Ramachandran plot, % residues in regions** | | | | |
| **Preferred regions** | 95.6 | 95.9 | 96.0 | 95.9 |
| **Allowed regions** | 4.4 | 4.1 | 4.0 | 4.1 |
| **Outliers** | 0 | 0 | 0 | 0 |
| **PDB code** | 4IS5 | 4ISL | 4ISN | 4ISO |

<sup>a</sup> Numbers in parentheses are for the highest resolution shells.

<sup>b</sup> r.m.s.d. indicates root mean square deviation.
Crystal Structures of Matriptase-HAI-1 Interface (60-Loop Exosite)—Interaction between matriptase and KD1 buried an average of 1800 Å² of solvent accessible area in the three complexes. This size of the buried area is significantly larger than other protease Kunitz-type inhibitor interfaces. For example, 1580 Å² of solvent exposed surface is lost at the interface of HGFA and KD1, and only 1293 Å² is lost upon trypsin and APPI binding. This significant increase of buried surface area is contributed by an extensive network of interactions between KD1 and a secondary substrate binding site formed by the 60-loop of matriptase. The 60-loop is capable of undergoing interconversion between different forms as shown in our three complex structures. This is presumably because the presence of a glycine residue at the 60-loop apex of matriptase. In the β-matriptase-N164Q-KD1-A complex, the 60-loop of matriptase is similar to that in the matriptase-BPTI complex and provides additional interactions with a secondary binding region of KD1 that comprises Arg60C, Asn286(H), and Asn289(H). A network of interactions is formed between the protease and inhibitor including a salt bridge between Asp60B and Arg265(H), hydrogen bonding between Tyr60G and Arg265(H), as well as Arg60C with the backbone carbonyl of Asn286(H) and Asn289(H) (Fig. 3A, top panel). This network of interaction is further stabilized by a water molecule that bridges the N atom of Arg265(H) with the side chain of Gln38, which adopts a different conformation in the apo enzyme. In the structure of β-matriptase-N164Q-KD1-B complex, the 60-loop moves away from KD1 and the side chains of Asp60B and Arg60C bend outward into the solution. Although the network of interactions between the 60-loop and KD1 in β-matriptase-N164Q-KD1-B complex is similar with that in β-matriptase-N164Q-KD1-A complex, the hydrogen bonds between Arg60C and the backbone carboxyls of Asn286(H) and Asn289(H) are lost (Fig. 3A, second panel from the top). However, in the structure of β-matriptase-N164Q/S195A-KD1 complex, the calculated electron density map revealed that the 60-loop adopts two alternate conformations in the crystal (Fig. 3B). One resembles the conformation in β-matriptase-N164Q-KD1-A complex and orients the residue Arg60C toward KD1 to form hydrogen bonds with the backbone carbonyl of Asn286(H) and Asn289(H) (Fig. 3A, third panel from the top). Another resembles the conformation in β-matriptase-N164Q-KD1-B complex and bends outward into the solution, demonstrating the 60-loop may adopt two dominant conformations upon binding KD1. As aforementioned, the slightly depressed S4 subsite of matriptase is partially occupied by Leu284(H), which is close to this secondary binding region. The hydrophobic side chain mediates van der Waals interaction with the phenyl group of Phe97 and form a cap that tops the S4 subsite flanked by Phe97 and Phe99.

Based on observations from our three complex structures, the 60-loop, despite its relatively long sequence, adopts a rather rigid overall conformation to provide a binding scaffold for its substrate/inhibitor while having some flexibility at its apex. This flexibility generates large alteration of Arg60C conformation (Fig. 3A, bottom panel), which might serve a regulatory function during the recognition and binding with different substrates or inhibitors.

Because the amino acid sequence of the 60-loop is largely unique, we suggest that this secondary interaction site may provide new opportunity to develop potent matriptase inhibitor by engineering KD1 to increase its specificity and inhibitory activity against matriptase. In particular, specific interaction with Arg60C in its “inward” position might improve specificity and further stabilize the matriptase-KD1 complex. At the same time, this region can be used as a new target for designing small molecule inhibitor specific for matriptase. It is interesting to note that thrombin, which also has a long 60-loop, does not bind to Kunitz-type inhibitor BPTI even at high concentration (micromolar range) (41). Yet, a single mutation at residue 192 (E192Q) induces a large conformational change in the 60-loop of thrombin and renders the protease to bind BPTI with high affinity (41). This finding supports that the long 60-loops in some trypsin-type proteases might play critical roles for their specificities.

Specificity toward HAI-1 KD1—HAI-1 contains two Kunitz domains that share high homology in sequence. KD1 exhibits highly potent inhibitory activity toward matriptase, whereas KD2 does not, suggesting KD1 is responsible for the inhibitory effect of HAI-1 (33). To understand how matriptase discriminates KD1 against KD2 of HAI-1, we constructed a homology model of KD2 by the Swiss model (42) using our structure of KD1 as template. According to the model, nearly all amino acids of the secondary binding region of KD1 are conserved in...
KD2, except that Leu\textsuperscript{284}(H) is now replaced by a tyrosine at the equivalent position. Based on the model, the intermolecular interaction pattern between the 60-loop of matriptase and the secondary binding region should be conserved, suggesting that this binding site does not involve in the discrimination of KD2. This is no surprise as the inhibitory activity of Kunitz-typed inhibitor is usually determined by the sequence of the reactive loop region. The sequences of P4 to P3\textsuperscript{+} residues in KD1 and KD2 are Gly-Arg-Cys-Arg\textsuperscript{260}-Gly-Ser-Phe and Gly-Leu-Cys-Lys\textsuperscript{385}-Glu-Ser-Ile, respectively (Fig. 4A). Similar to most trypsin-like serine proteases, structural studies of matriptase bound to different inhibitors such as BPTI and sunflower trypsin inhibitor-1 reveal that the protease S1 pocket can accept Lys equally well as Arg (34, 35). However, our structures show that Arg\textsuperscript{260}(H) of KD1 likely extends deeper into the S1 pocket than Lys\textsuperscript{385}(H) of KD2 to make better interactions with the side chain of Asp\textsuperscript{189} and Ser\textsuperscript{190}, as well as the backbone carbonyl of Ser\textsuperscript{190} and Gly\textsuperscript{219} (Fig. 4B). Similar observation has been observed in the study of mesotrypsin, which is a tryptic protease that cleaves a subset of Kunitz-type inhibitor like BPTI and APPI with substrate-like kinetics (43). Kinetic measurements of mesotrypsin activity against BPTI and APPI show that the pro-

**FIGURE 3.** *Alternating conformations of 60-loop.* A, comparison of the overall structures of the three matriptase-HAI-1 complexes and the apo matriptase model obtained in this study. The 60-loop of matriptase provides large interaction surface for HAI-1 KD1 (yellow) binding and stabilizes the complex with an extensive network of interaction at the 60-loop. In the β-matriptase-N164Q-KD1-A complex (top panel), Arg\textsuperscript{60C} of the 60-loop interacts directly with HAI-1 KD1. β-Matriptase-N164Q/KD1-B (second panel) shows that the apex of 60-loop adopts a different conformation and bends outward into the solution. β-Matriptase-N164Q/S195A-KD1 (third panel) shows that the 60-loop adopts two alternate conformations; one resembles that of β-matriptase-N164Q-KD1-A, whereas the other resembles the conformation in β-matriptase-N164Q-KD1-B complex. Apo β-matriptase-N164Q/S195A (bottom panel) shows that the 60-loop adopts a rigid conformation in the absence of HAI-1 KD1, yet the side chain of Arg\textsuperscript{60C} remains flexible. Intermolecular interactions between matriptase and HAI-1 KD1 are depicted by dotted lines. B, the 60-loops are well ordered as shown by the $2F_o - F_c$ electron density maps (contoured at 1σ) in the structures of the three matriptase-HAI-1 complexes and the apo matriptase.
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FIGURE 4. Comparison of KD1 and KD2 of HAI-1. A, sequence alignment of KD1 and KD2 of HAI-1 reveals key differences within the reactive loop regions that might contribute to their different specificities. The putative cleavage site by matriptase is indicated by an arrow. Fully conserved reactive loop residues are denoted in green. Non-conserved reactive loop residues in KD1 are denoted by a star. P2 residue that contributes to the different specificities of KD1 and KD2 of HAI-1 is denoted by the # symbol. B, P1 arginine of KD1 (yellow) could make stronger interaction with Asp\(^{189}\) of matriptase compared with Lys of KD2 (purple) and might help to discriminate KD1 from KD2. C, Glu\(^{386}\) of KD2 (purple) causes steric hindrance and charge repulsion at the S1'/S3' cavity of matriptase. D, the positively charged reactive loop region of KD1 favors binding at the negatively charged catalytic site of matriptase, whereas the slightly acidic KD2 does not. Solvent-accessible surfaces of matriptase, HAI-1 KD1, and KD2 are colored by electrostatic potential (−7 to +7 kT/e in blue to red) computed by the APBS program.

Unlike KD1, KD2 of HAI-1 does not contain the preferred basic residue at neither P3 nor P4 position. Instead, the P3 Arg\(^{258}\) (in KD1 is substituted by Leu\(^{383}\) (in KD2, which might only make weak van der Waals interaction with Phe\(^{99}\). The lost of hydrogen bond with HGFA Ser\(^{99}\) is compensated in the matriptase-KD1 complexes by the new hydrogen bond and salt bridge formed with Gln\(^{175}\) at the S3 subsite and Asp\(^{217}\), respectively (Fig. 5A). For instance, Arg\(^{258}\) of KD1, which contributes to the second largest inter-residue contact (in addition to the P1 residue) in HGFA-HAI-1 KD1 complex by stacking with the highly conserved Trp\(^{215}\) of HGFA and hydrogen-bonded to Ser\(^{99}\) of HGFA, is projected away from the 99-loop in the matriptase-KD1 complexes due to steric hindrance with Phe\(^{99}\). The lost of hydrogen bond with HGFA Ser\(^{99}\) is compensated in the matriptase-KD1 complexes by the new hydrogen bond and salt bridge formed with Gln\(^{175}\) at the S3 subsite and Asp\(^{217}\), respectively (Fig. 5B). Another major difference between the interfaces of human HGFA and matriptase with KD1 detects that KD2 fails to inhibit matriptase.

Comparison of HAI-1 Interaction with HGFA and Matriptase—To gain further insights on how HAI-1 KD1 recognizes different proteases, we overlaid and compared the KD1-bound HGFA structure (PDB code 1YC0 (32)) with our structures. The overlaid structures revealed several major differences between the two proteases at the substrate/inhibitor interaction sites. Nearly all specificity-determining loops in HGFA, i.e., the 60-loop, 37-loop, 147-loop, and 99-loop adopt different conformations and show interactions quite different from matriptase (Fig. 5A). For instance, Arg\(^{258}\) of KD1, which contributes to the second largest inter-residue contact (in addition to the P1 residue) in HGFA-HAI-1 KD1 complex by stacking with the highly conserved Trp\(^{215}\) of HGFA and hydrogen-bonded to Ser\(^{99}\) of HGFA, is projected away from the 99-loop in the matriptase-KD1 complexes due to steric hindrance with Phe\(^{99}\). The lost of hydrogen bond with HGFA Ser\(^{99}\) is compensated in the matriptase-KD1 complexes by the new hydrogen bond and salt bridge formed with Gln\(^{175}\) at the S3 subsite and Asp\(^{217}\), respectively (Fig. 5B). Another major difference between the interfaces of human HGFA and matriptase with KD1 is the conformation of the 60-loop. The longer 60-loop of matriptase provides extra and strong interactions with KD1 (see above). These additional interactions may have contributed to the slightly lower IC\(_{50}\) of KD1 toward matriptase (1.7 ± 0.5 nm versus 4.0 ± 0.3 nm toward HGFA (32)). In addition to these differences, the interactions between KD1 with HGFA and matriptase at the S1 and S1' are identical. Because HAI-1 KD1 inhibits both proteases with similarly high affinities, this analysis suggest S1 and S1' subsites are the key structural determinants that confer the strong inhibitory effect.
FIGURE 5. Comparison of HAI-1 KD1 interactions with HGFA (purple) and matriptase (green). A, the conformations of the specificity-determining loops in both proteases are different. B, P3 arginine of HAI-1 KD1 is stabilized by different networks of interaction upon binding to HGFA and matriptase.

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