Hepatocyte growth factor (HGF) activator is a serine protease responsible for proteolytic activation of HGF in response to tissue injury and thus plays an important role in the regulation of biological functions of HGF in regenerating tissue. We previously purified an inhibitor of HGF activator (HGF activator inhibitor type 1, HAI-1) from the conditioned medium of a human stomach carcinoma cell line MKN45 and cloned its cDNA. HAI-1 is a novel member of the Kunitz family of serine protease inhibitors. In the present study, we purified a second type of HGF activator inhibitor (HAI-2) from the conditioned medium of MKN45 cells and molecularly cloned its cDNA. The cDNA sequence revealed that HAI-2 is derived from a precursor protein of 252 amino acids and contains two Kunitz domains, indicating that HAI-2 is also a member of the Kunitz family of serine protease inhibitors. The primary translation product of HAI-2 has a hydrophobic sequence in the COOH-terminal region, suggesting that, like HAI-1, HAI-2 is produced in a membrane-associated form and secreted in a proteolytically truncated form. Because HAI-2 and HAI-1 are potent inhibitors specific for HGF activator, they may be involved in regulation of proteolytic activation of HGF in injured tissues. 

Hepatocyte growth factor (HGF) activator is a blood coagulation factor XII-like serine protease that converts the inactive single chain form of HGF to the active heterodimeric form in response to tissue injury and thus plays an important role in the regulation of biological functions of HGF in regenerating tissue. We previously purified an inhibitor of HGF activator (HGF activator inhibitor type 1, HAI-1) from the conditioned medium of a human stomach carcinoma cell line MKN45 and cloned its cDNA. HAI-1 is a novel member of the Kunitz family of serine protease inhibitors. In the present study, we purified a second type of HGF activator inhibitor (HAI-2) from the conditioned medium of MKN45 cells and molecularly cloned its cDNA. The cDNA sequence revealed that HAI-2 is derived from a precursor protein of 252 amino acids and contains two Kunitz domains, indicating that HAI-2 is also a member of the Kunitz family of serine protease inhibitors. The primary translation product of HAI-2 has a hydrophobic sequence in the COOH-terminal region, suggesting that, like HAI-1, HAI-2 is produced in a membrane-associated form and secreted in a proteolytically truncated form. Because HAI-2 and HAI-1 are potent inhibitors specific for HGF activator, they may be involved in regulation of proteolytic activation of HGF in injured tissues. 

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Chemical Co. Trypsin and HGF activator immobilized affinity columns were prepared by coupling 13 mg of trypsin and 1.6 mg of HGF activator with 10 and 5 ml of Affi-Gel 10, respectively, by the method described in the instruction manual.

To obtain tissue factor pathway inhibitor (TFPI), the concentrated serum-free conditioned medium of HLC-1 cells was applied to a trypsin immobilized column (1 × 13 cm) equilibrated with phosphate-buffered saline (PBS). The adsorbed fractions were eluted from the column with 10 ml of PBS containing 0.05% CHAPS and analyzed by SDS-PAGE. The gel was stained with Coomassie Blue R-250 and then applied to an anion exchange PL-SAX column (0.46 × 3 cm) equilibrated with PBS containing 0.05% CHAPS. The dialysate was applied to a hydroxyapatite HCA A-4007 column (0.35 × 7.5 cm) pre-equilibrated with the dialysis buffer. The unadsorbed fractions were collected and further incubated at 37 °C for 2 h. The mixtures were then analyzed by SDS-PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue and scanned by a Flying-Spot Scanner CS-9000 (Shimadzu).

Purification of HAI-2 from the Conditioned Medium of MKN45 Cells—Ten liters of the serum-free conditioned medium of MKN45 cells was concentrated to about 300 ml. The concentrate was applied to a heparin-Sepharose CL-6B column (2.5 × 10 cm) pre-equilibrated with PBS. The unadsorbed fractions were collected and then applied to a ConA-Sepharose column (1 × 5 cm) pre-equilibrated with PBS. The unadsorbed fractions were collected and then applied to a phenyl-Sepharose column (1 × 3 cm) pre-equilibrated with PBS. The unadsorbed fractions were collected and then applied to a hydroxyapatite column (20–40 ml) pre-equilibrated with PBS containing 0.05% CHAPS and 5 mM EDTA was first denatured by heating at 100 °C in the presence of 0.2% SDS. After denaturation, 5 ml of 2-mercaptoethanol and 1 ml of 200 units/ml N-glycosidase F were added to the mixture. The mixture was incubated at 37 °C for 20 h, and the deglycosylated product was analyzed by SDS-PAGE under reducing conditions.

Amino Acid Sequence Analysis—To determine the NH₂-terminal amino acid sequence of the purified HAI-2, the final preparation from the C8 reverse-phase chromatography was sequenced using an Applied Biosystems 470A Protein Sequencer. The internal amino acid sequences of HAI-2 were determined as described (9).

Dose Response of the Inhibitory Activity of HAI-2 against HGF Activator—Two µg/ml of HGF activator was mixed with various concentrations of HAI-2 (0–2.4 µg/ml) in 40 µl of PBS containing 0.05% CHAPS. After incubation at 37 °C for 30 min, 5 µl of 2.4 mg/ml single-chain HGF in PBS containing 0.05% CHAPS and 5 µl of 100 µg/ml dextran sulfate (molecular mass: 500 kDa) were added to the mixture and further incubated at 37 °C for 10 min. The mixture was then analyzed by SDS-PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue and scanned by a Flying-Spot Scanner CS-9000 (Shimadzu).

The inhibitory activities of HAI-2 were estimated by calculating the ratio of the single-chain to heterodimeric form of HGF.

cDNA Cloning—Total RNA was prepared from MKN45 cells by acid guanidinium thiocyanate/phenol/chloroform extraction (12), and poly(A) RNA was purified by oligo(dT) affinity chromatography. The primers 5’-AAGGT(G/T)GT(G/T)GG(G/T)(A/C)G(G/T)(T/C)(A/C)G-3’ and 5’-GGCCGT(A)/TGGG/G(T)/GGA/TA/GAT/GT/TG/GA/C’-3’ were chemically synthesized. Using the primers and poly(A) RNA as a template, DNA fragments were amplified by reverse transcription-polymerase chain reaction (PCR), and an 85-bp fragment was generated. The DNA fragment was subcloned and sequenced. The fragment was used as a probe for screening a cDNA library. To construct the cDNA library, cDNA was synthesized from poly(A) RNA of MKN45 cells using a cDNA synthesis kit with oligo(dT) primer (Pharmacia). The cDNA with EcoRI adaptors at both ends was ligated to EcoRI-digested λZAPII vector (Stratagene) and packaged in vitro using Gigapack Gold (Stratagene). Hybridization to nylon replica membranes (Hybond-N’, Amer-sham) was performed at 42 °C for 16 h with 32P-labeled probe in a solution containing 50% formamide, 5 × Denhardt’s solution (0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and 0.1% Ficoll), 0.75 M NaCl, 50 mM sodium phosphate (pH 7.4), 6 µg/ml EDTA, 0.5% SDS, and 100 µg/ml salmon testis DNA. The probe was labeled using multi-prime DNA labeling system (Amersham Life Science Inc.). The membranes were washed twice with 0.1 × SSC containing 1% SDS at 42 °C for 30 min.

Northern Blotting—Total RNA (10 µg) from MKN45 cells was denatured, electrophoresed (13), and transferred to a nylon membrane (Biond). Human adult and fetal multiple tissue Northern blot membranes were purchased from CLONTECH. The membranes were hybridized at 42 °C for 16 h with the 32P-labeled probe as described (14). The membranes were washed twice with 1 × SSC containing 1% SDS at 42 °C for 30 min.

Measurement of Relative Amounts of HAI-1 and HAI-2 in the Conditioned Medium of MKN45 Cells—The serum-free conditioned medium of MKN 45 cells was applied to a trypsin immobilized column (1 × 13 cm) equilibrated with PBS. The adsorbed proteins were eluted from the column with 10 ml HCl. The eluted fractions were neutralized immediately by 1 M Tris-HCl buffer (pH 8.0) and directly applied to an HGF activator immobilized column (1 × 6 cm) equilibrated with PBS.
Purification and Cloning of HAI-2

**A**

| Peptide no. | Amino acid sequence |
|-------------|---------------------|
| N-terminal  |                     |
| 1.          | ADERS1HDFXLVS         |
| 2.          | VVGKXASMPRWNYTVXGSXQLFVYGG |

**B**

| HAI-2 (peptide No.1) | VVGKXASMPRWNYTVXGSXQLFVYGG | identity |
|---------------------|----------------------------|----------|
| HAI-1 (first Kunitz domain) | 25C CLAVKVGNDDBSRFNYXPS0EVC35FYGGOOkKMKNLREEGY1LAC | 300 53.6% |

The partial amino acid sequence of purified HAI-2. A, amino acid sequences of the peptides derived from HAI-2. Amino acids not determined are denoted by X. B, alignment of sequence 1 of HAI-2 with the first Kunitz domain of HAI-1. Identical amino acid residues are indicated by vertical lines, and conservative amino acid residues are indicated by colons.

![FIG. 2. The partial amino acid sequence of purified HAI-2. A, amino acid sequences of the peptides derived from HAI-2. Amino acids not determined are denoted by X. B, alignment of sequence 1 of HAI-2 with the first Kunitz domain of HAI-1. Identical amino acid residues are indicated by vertical lines, and conservative amino acid residues are indicated by colons.](image)

![FIG. 3. Dose-dependence of the inhibitory activity of HAI-2 against HGF activator. Various concentrations of HAI-2 were incubated with HGF activator. After incubation at 37 °C for 30 min, single-chain HGF (sc-HGF) was added and further incubated. The reaction products were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue (A). The inhibitory activity of HAI-2 was determined as the ratio of the remaining single-chain HGF to total HGF and plotted against HAI-2 concentration (B).](image)

Adsoined proteins were eluted with 10 mM HCl, and the eluted fractions were collected and neutralized with 1 M Tris-HCl buffer (pH 8.0) and then subjected to reverse-phase YMC C4 column (0.46 × 15 cm) chromatography. Elution was with a linear gradient of 10% isopropanol (3/7) containing 0.1% TFA. The concentrations of proteins were determined using a bicinchoninic acid protein assay kit, with bovine serum albumin as a standard.

**RESULTS**

Purification of HAI-2 from the Conditioned Medium of MKN45 Cells—HAI-1 was previously purified from serum-free conditioned medium of MKN45 cells by a seven-step procedure (9). The conditioned medium was concentrated and applied to a heparin-Sepharose column. The unadsorbed fractions were applied to a ConA-Sepharose column, and the inhibitory activities were detected both in the unadsorbed and adsorbed fractions. The HAI-1 protein was recovered from the adsorbed fraction (9). In this study, we purified a protein for the activity (HAI-2) in the unadsorbed fraction by an additional five-step procedure using the columns used for the purification of HAI-1. Table I shows elution characteristics of HAI-2 and HAI-1. The elution characteristics of HAI-2 on hydrophobic, gel filtration, and reverse-phase chromatographies were distinctly different from those of HAI-1. The final preparation of HAI-2 on reverse-phase chromatography revealed a broad band of 23 to 31 kDa on SDS-PAGE (15.0% acrylamide) under reducing conditions (Fig. 1, lane 1).

The broad migration of HAI-2 on SDS-PAGE may be caused by N-glycans bound to HAI-2. We therefore deglycosylated HAI-2 using an N-glycosidase and analyzed it by SDS-PAGE. The deglycosylated HAI-2 protein migrated as two bands of 14.0 and 13.5 kDa (Fig. 1, lane 2), indicating that large N-linked sugar chains were bound to the HAI-2 protein. When the NH2-terminal amino acid sequence of the purified protein was analyzed before deglycosylation, only one sequence was obtained (Fig. 2A). Thus, the apparent heterogeneity of the deglycosylated protein on the gel might be caused by a difference in the COOH-terminal sequence. In addition to the NH2-terminal sequence, the two internal amino acid sequences were obtained from peptide fragments produced by digestion of the purified protein with *Achromobacter protease*-I (Fig. 2A). The sequence of peptide 1 showed extensive similarity (53.6% identity) to that of a part of the first Kunitz domain of HAI-1 (Fig. 2B), suggesting that HAI-2 is also a Kunitz-type serine protease inhibitor.

**Dose Dependence of the Inhibitory Activity of the Purified HAI-2—**Fig. 3 shows the dose-response curve of the inhibitory activity of HAI-2. In these reactions, HGF activator (2 μg/ml) was mixed with various concentrations of HAI-2 and incubated for 30 min to form an enzyme-inhibitor complex. Then remaining HGF-converting activity was measured. The concentration of HAI-2 for 50% inhibition was about 300 ng/ml. Considering the molecular masses of the protein portions of HAI-2 (14 kDa), HAI-2 forms about an equimolar complex with HGF activator.

Isolation of cDNA Clone and DNA Sequence Analysis—The NH2-terminal sequence, Lys-Val-Val-Gly-Arg-Cys-Arg, and the COOH-terminal sequence, Cys-Gln-Leu-Phe-Val-Tyr-Gly-Gly, in peptide 1 (Fig. 2A) were used to design degenerate oligonucleotide primers for PCR amplification of the sequence for peptide 1. A lysine residue preceding the NH2-terminal sequence was predicted because the peptide fragments were obtained by digestion with a lysylendopeptidase. The cysteine residues were not determined by the amino acid sequence analysis (Fig. 2A), but they were predicted because the sequence of peptide 1 is similar to the first Kunitz domain of HAI-1. PCR amplification of MKN45 RNA resulted in a cDNA fragment with the expected size of about 85 bp. The cDNA fragment was subcloned and sequenced. The amino acid sequence predicted from the cDNA sequence completely matched that of peptide 1. The PCR clone was thus used as a probe to screen a cDNA library constructed from MKN45 cells. Twenty-
two hybridization-positive clones were obtained from about 1.5 \times 10^5 phage. The largest clone was sequenced to determine the primary structure of human HAI-2 (Fig. 4).

**Predicted Amino Acid Sequence of HAI-2**—The amino acid sequence of HAI-2 predicted from the cDNA sequence is shown in Fig. 4. The translation initiation site was assigned to the first methionine codon because in-frame stop codons are present upstream of the methionine codon. The open reading frame that starts from the ATG codon consists of 252 amino acids, and the protein product has a calculated molecular mass of 28,169. The first methionine is followed by a hydrophobic sequence, and the NH$_2$-terminal amino acid of the purified protein is located at the 28th residue downstream of the methionine, suggesting that the hydrophobic region represents a signal peptide sequence. Excluding the signal peptide, the mature form of the protein consists of 225 amino acids and has a calculated molecular mass of 25,415. The apparent molecular mass of the protein portion of HAI-2 purified from the conditioned medium of MKN45 cells was about 14 kDa, as determined by SDS-PAGE. Thus, the protein purified from the conditioned medium appears to be a processing product cleaved at the COOH-terminal region. A hydrophobic sequence of 24 amino acids is present in the COOH-terminal region, suggesting that the primary translation product is a membrane-associated protein. There are two potential N-glycosylation sites with the canonical Asn-Xaa-(Ser/Thr). A comparison of the protein sequence of HAI-2 with sequences in the SwissProt and the National Biomedical Research Foundation protein data base revealed that two regions (residues 38–88 and 133–183) showed extensive similarity to the Kunitz-type sequence of serine protease inhibitors (Fig. 5). Thus, HAI-2 appears to be a Kunitz-type serine protease inhibitor.

**Tissue Distribution of HAI-2 mRNA**—The size and tissue distribution of HAI-2 mRNA was determined by Northern blotting with poly(A) RNAs from various human tissues (Fig. 6). A transcript of 1.6 kilobases was detected in MKN45 cells from which we purified the HAI-2 protein. The transcript was detected in a variety of human adult and fetal tissues. Among them, the expression level of HAI-2 mRNA was relatively high in the adult placenta, kidney, pancreas, prostate, testis, thyroid, and trachea.
to an HGF activator immobilized affinity column. The adsorbed fractions were analyzed by C4 reverse-phase chromatography. The inhibitory activity was quantitatively recovered in the adsorbed fractions in each affinity chromatography and did not remain in the unadsorbed fractions. On the C4 reverse-phase chromatograph, three peaks were detected (Fig. 7A). NH2-terminal amino acid sequence analysis revealed that peaks 1 (at 15.0 min) and 3 (at 24.2 min) corresponded to HAI-2 and HAI-1, respectively. When the NH2-terminal amino acid sequence of peak 2 (at 22.9 min) was analyzed, two sequences (Arg-Gln-Leu-Arg and Thr-Gln-Gly-Phe) were obtained. These sequences correspond to those of residues 150–153 and 154–157 in HAI-1, indicating that the proteins in peak 2 are processing products cleaved at the NH2-terminal region of HAI-1. These truncated HAI-1 proteins exhibited inhibitory activity toward HGF activator (data not shown). No inhibitor other than HAI-2 and HAI-1 was detected by this affinity purification procedure. Thus, the inhibitory activity toward HGF activity in the conditioned medium of MKN45 cells is derived from HAI-1 and HAI-2. The proteins in each peak were fractionated and their contents were quantified by a protein assay (Fig. 7B). The amount of HAI-2 was almost equal to that of HAI-1 in the conditioned medium of MKN45 cells.

Examination of the Inhibitory Activity of Kunitz-type Inhibitors against HGF Activator—APP, TFPI, and UTI have Kunitz domains that are responsible for the inhibitory activities of these proteins against serine proteases (15–17). Therefore, we examined whether these inhibitors exhibited an inhibitory effect on the HGF-converting activity of HGF activator (Fig. 8). The inhibitory activities of APP and TFPI against HGF activator were very weak even at high concentrations, and that of UTI was not detected although these proteins strongly inhibited the proteolytic activity of trypsin (data not shown).

DISCUSSION

We previously identified and cloned an HGF activator inhibitor (HAI-1) from MKN45 human stomach carcinoma cells (9). In the present study, we identified a second type of HGF activator inhibitor (HAI-2) from the same cells. The inhibitor protein was purified from the conditioned medium of the cells. The primary structure of the protein was predicted from the sequence of the cDNA for human HAI-2. The structure of human HAI-2 is schematically summarized in Fig. 9 together with the structure of human HAI-1. The primary translation product of HAI-2 consists of 252 amino acid residues. The NH2-terminal 27 residues may serve as a signal peptide. HAI-2 has two Kunitz domains. A Kunitz domain is typically about 60 amino acids in length and contains three disulfide bonds. It is recognized as the functional domain of serine protease inhibitors (18). Thus, one or both of the Kunitz domains in HAI-2 appears to be responsible for the inhibitory activity. The protein also has a hydrophobic sequence of about 20 amino acids in the COOH-terminal region, suggesting that it may be membrane-associated. The protein portion of HAI-2 purified from the conditioned medium of MKN45 cells has a molecular mass of about 14 kDa, which is smaller than the predicted molecular mass (25,415) of the primary translation product. Thus, HAI-2 purified from the conditioned medium appears to be a proteolytically truncated form of the membrane-associated form. HAI-1 also has two Kunitz domains and the COOH-terminal
hydrophobic region, indicating that the overall structures of the characteristic domains are similar between HAI-1 and HAI-2. However, HAI-1 has additional structures that are not found in HAI-2: a long NH₂-terminal region preceding the first Kunitz domain and a structure similar to the ligand binding domain of the low density lipoprotein receptor between the two Kunitz domains (9).

The molecular mass of the protein portion of HAI-2 purified from the conditioned medium of MKN45 cells is about 14 kDa, which corresponds to 125 amino acids, suggesting that the extracellular truncated form of HAI-2 is produced by the proteolytic cleavage that occurs within the second Kunitz domain. Similar cleavage was suggested for the generation of the extracellular truncated form of HAI-1 (9). Furthermore, the dose-dependence of the HAI-2 activity showed that HAI-2 purified from the conditioned medium forms an equimolar complex with HGF activator. Similar complex formation was observed for HAI-1 and HGF activator (9). Thus, only the first Kunitz domain in HAI-2 as well as HAI-1 may function in the inhibitory activity toward HGF activator. The first Kunitz domain of HAI-2 shows the highest similarity (54% identity) to the first Kunitz domain of HAI-1 among Kunitz domains of human serine protease inhibitors, suggesting that the conserved amino acid residues between the domains play an important role in the formation of complex between the inhibitors and HGF activator.

The treatment of HAI-2 by an N-glycosidase markedly decreased its molecular mass, indicating that large N-linked sugar chains are attached to the protein portion of HAI-2. The molecular mass of HAI-1 was also decreased by N-glycosidase treatment although the decrease (40 to 34 kDa) was less than that for HAI-2. Thus, both HAI-2 and HAI-1 are N-glycosylated proteins. HAI-1 binds to ConA-Sepharose (9), whereas HAI-2 does not. ConA is a legume lectin that specifically recognizes the trimannoside core present in all N-linked glycans (19). The N-linked sugar chains are generally classified into three main types: high mannose-, hybrid- and complex-type. In general, high mannose- and hybrid-type sugar chains are tightly bound to ConA and biantennary complex-type structures are weakly bound. In contrast, more extensively processed, highly branched or bisected forms do not bind to the lectin (20). Thus, the N-glycans bound to HAI-2 are likely to contain the complex-type structure larger than biantennary structures. There are reports that the sugar chains of glycoproteins play roles in the regulation of folding, conformational stability, protease resistance, and intracellular trafficking (21–23). The N-glycans bound to HAI-2 might have similar roles in vivo.

Both HAI-2 and HAI-1 are potent inhibitors of HGF activator, whereas the other Kunitz type serine protease inhibitors, APP, TFPI, and UTI, show little or no inhibitory activity toward HGF activator. Furthermore, serum serine protease inhibitors such as antithrombin III, C1-inhibitor, and α₂-antiplasmin did not inhibit the HGF-converting activity of HGF activator (24). These findings suggest that HAI-2 and HAI-1 function as specific inhibitors of HGF activator in vivo. Expression levels of both HAI-2 and HAI-1 mRNA are low in some tissues such as the liver. In these tissues, the low level of the inhibitors may facilitate the action of HGF activator.

Similar tissue distributions of mRNA of HAI-2 and HAI-1 were observed in various human adult and fetal tissues except adult testis, in which expression of HAI-2 mRNA is much higher than that of HAI-1 mRNA. Furthermore, about equal amounts of both inhibitors were detected in the conditioned medium of MKN45 cells. These results suggest that HAI-2 and HAI-1 simultaneously inhibit HGF activator in vivo. However, these inhibitors have some different properties. As discussed above, different N-linked sugar chains are attached to the protein portion of HAI-2 and HAI-1. Further, HAI-2 was not adsorbed by the hydrophobic column whereas HAI-1 was, indicating that HAI-2 is more hydrophilic than HAI-1. These different properties may result in different inhibitory actions toward HGF activator. Thus, further characterization of HAI-2

**Fig. 8.** Inhibitory activities of Kunitz-type inhibitors against HGF activator. A, 100 ng/ml of HGF activator was incubated with 800 ng/ml of HAI-1 (lane 1), 100 ng/ml of HAI-2 (lane 2), 800 ng/ml of APP (lane 3), 600 ng/ml of TFPI (lane 4), 200 ng/ml of UTI (lane 5), 8 μg/ml of APP (lane 6), 6 μg/ml of TFPI (lane 7), or 2 μg/ml of UTI (lane 8). Single-chain HGF was then added, and the mixtures were further incubated. The reaction products were subjected to SDS-PAGE and stained with Coomassie Brilliant Blue. B, the inhibitory activity of each protease inhibitor was determined as the ratio of the remaining single-chain HGF to total HGF. ND, not detected.

**Fig. 9.** A schematic representation of human HAI-2 together with human HAI-1.
and HAI-1 is needed to elucidate how these inhibitors act against HGF activator in vivo.

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