Hepatocyte Growth Factor Activator Inhibitor, a Novel Kunitz-type Serine Protease Inhibitor*

(Received for publication, August 6, 1996, and in revised form, November 7, 1996)

Takeshi Shimomura‡, Kimitoshi Denda§, Akiko Kitamura¶, Toshiya Kawaguchi¶, Masahiro Kito¶, Jun Kondo‡, Shinji Kagaya§, Li Qin§, Hiroyuki Takata¶, Keiji Miyazawa§, and Naomi Kitamura§

From the ‡Research Center, Mitsubishi Chemical Corp., Aoba-ku, Yokohama 227, the §Department of Life Science, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 226, and the ¶Institute for Liver Research, Kansai Medical University, Moriguchi, Osaka 570, Japan

Hepatocyte growth factor (HGF) activator is a serine protease that is produced and secreted by the liver and circulates in the blood as an inactive zymogen. In response to tissue injury, the HGF activator zymogen is converted to the active form by limited proteolysis. The activated HGF activator converts an inactive single chain precursor of HGF to a biologically active heterodimer in injured tissue. The activated HGF may be involved in the regeneration of the injured tissue. In this study, we purified an inhibitor of HGF activator from the conditioned medium of a human MKN45 stomach carcinoma cell line and molecularly cloned its cDNA. The sequence of the cDNA revealed that the inhibitor is a member of the Kunitz family of serine protease inhibitors. The sequence also showed that the primary translation product of the inhibitor has a hydrophobic sequence at the COOH-terminal region. Inhibitory activity toward HGF activator was detected in the membrane fraction as well as in the conditioned medium of MKN45 cells. These results suggest that the inhibitor may be produced as a membrane-associated form and secreted by the producing cells as a proteolytically truncated form.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB000095.

EXPERIMENTAL PROCEDURES

Cell Lines and Cell Culture—The following cell lines were obtained from the indicated sources: human melanoma cell lines C32 and A375, human lung carcinoma cell line A549, human lung fibroblast cell line HLF, human hepatoma cell lines Hep G2 and PLC/PRF/5, and human colon carcinoma cell line CCL229 from American Type Culture Collection (Rockville, MD); human stomach carcinoma cell lines HSC-3 and MKN45 and human lung carcinoma cell lines PC-9 and PC-3 from IBL (Gunma, Japan); human lung carcinoma cell line HLC-1 and human stomach carcinoma cell line IG-1 from Department of Physiology, Keio...
University (Tokyo, Japan); immortalized cell line of human fetal liver
NuE from Dr. N. Ishida (Tohoku University, Sendai, Japan). All cells were
grown in eRDF medium containing 5% fetal bovine serum. At
confluence, the cells were washed twice with serum-free medium
and further cultured in fresh serum-free medium for 4 days.

Assay for HGF Activator Inhibitory Activity of Conditioned Medium
and Membrane Extract—The conditioned medium was harvested, clar-
fied by centrifugation, and concentrated 20-fold by ultrafiltration
using a YM30 membrane (Amicon). The concentrates were assayed for inhib-
itory activity toward HGF activator. To prepare membrane extract, the
cells (1 × 10^7 cells) were washed three times with Dulbecco’s/sodium
phosphate-buffered saline (PBS) and harvested by a cell scraper. The
collected cells were suspended in 10 ml of 20 mM Tris-HCl (pH 8.0),
disrupted by sonication at 4°C, and centrifuged at 10,000 × g at 4°C for
10 min. The supernatant was again centrifuged at 100,000 × g at 4°C
for 1 h. The pellet was suspended in 1.5 ml of PBS containing 1% Brij
58, sonicated, and centrifuged at 100,000 × g at 4°C for 1 h. The
resultant supernatant (membrane extract) was assayed for inhibitory
activity toward HGF activator. Protein concentration in the conditioned
medium and membrane extract was measured using a bichoninic acid
protein assay kit (Pierce) with bovine serum albumin as a stand-
ard. The single chain precursor of HGF and HGF activator was pre-
pared as described (6, 7). After 10 μl of 900 mg/ml HGF activator and 40
μl of concentrated conditioned medium or membrane extract were in-
cubated at 37°C for 30 min, 10 μl of 1.5 mg/ml single chain HGF was
added as a substrate, and the mixture was further incubated at 37°C
for 2 h. The mixture was then analyzed by SDS-polyacrylamide gel
electrophoresis (PAGE) under reducing conditions. The gel was stained
with Coomasie Brilliant Blue, and the bands were scanned using a
PhastSystem (Pharmacia).

Purification of HAI from the Conditioned Medium of MKN45
Cells—To purify HAI, MKN45 cells were cultured in roller bottles (850,
 Falcon). At confluence, the cells were washed twice with serum-free
medium and further cultured in serum-free medium. After 3–6 days,
the conditioned medium was harvested. Ten liters of medium was
acentrifuged, and concentrated 20-fold by ultrafiltration using
a YM30 membrane (Amicon). The concentrates were assayed for inhib-
itory activity toward HGF activator. Protein concentration in the conditioned
medium and membrane extract was measured using a bichoninic acid
protein assay kit (Pierce) with bovine serum albumin as a stand-
ard. The single chain precursor of HGF and HGF activator was pre-
pared as described (6, 7). After 10 μl of 900 mg/ml HGF activator and 40
μl of concentrated conditioned medium or membrane extract were in-
cubated at 37°C for 30 min, 10 μl of 1.5 mg/ml single chain HGF was
added as a substrate, and the mixture was further incubated at 37°C
for 2 h. The mixture was then analyzed by SDS-polyacrylamide gel
electrophoresis (PAGE) under reducing conditions. The gel was stained
with Coomasie Brilliant Blue, and the bands were scanned using a
PhastSystem (Pharmacia).

TABLE I

| Cell line | Origin | Inhibitory activity* |
|-----------|--------|----------------------|
| C32       | Human melanoma | - |
| A379      | Human melanoma | - |
| PC-9      | Human lung carcinoma | + |
| PC-35     | Human lung carcinoma | + |
| A549      | Human lung carcinoma | + |
| HLC-1     | Human lung carcinoma | + |
| HLF       | Human lung fibroblast | ± |
| HSC-3     | Human stomach carcinoma | + |
| MKN45     | Human stomach carcinoma | + |
| IG-1      | Human stomach carcinoma | ± |
| PLC/PRF5  | Human hepatoma | ± |
| HepG2     | Human hepatoma | ± |
| NuE       | Immortalized cells from human fetal liver | ± |
| CCL229    | Human colon carcinoma | ± |

* ±, less than 10%; +, 10–50%; ++, more than 50%.

and then gel-filtrated on an Ashahipak GS520 (Asahi Chemical Indus-
tries, Ltd.) column (0.76 × 50 cm) pre-equilibrated with PBS containing
0.05% CHAPS. The HAI protein was collected at 50–30 kDa and finally
purified by reverse-phase high performance liquid chromatography
(HPLC) on a YM pack C4 (YM) column (0.46 × 15 cm). Elution was
performed with a linear gradient of 10–50% acetonitrile/isopropyl alco-
hol (3:7) containing 0.07% trifluoroacetic acid at a flow rate of 1 ml/min
for 30 min. The HAI fractions were neutralized with 1× Tris-HCl (pH
8.0), dried under a vacuum, and then dissolved in PBS containing 0.05%
CHAPS. The preparation was analyzed by SDS-PAGE and stained with
silver.

The concentration of HAI in the sample was determined by the
phenylthiocarbamyl method (12). The final preparation of the protein
was hydrolyzed with hydrochloric acid, and the resulting free amino
acids were converted to phenylthiocarbamyl derivatives (phenylthio-
carbamyl-amino acid) by phenyl isothiocyanate. Phenylthiocarbamyl-
amino acids were separated by a YM pack ODS-A column (0.46 × 15
cm), and the quantity of HAI in the sample was calculated.

Amino Acid Sequence Analysis—To determine the NH2-terminal
amino acid sequence of the purified HAI, the protein that was eluted
from the C4 column and dried was reduced with 2-mercaptoethanol in
1× Tris-HCl (pH 8.6) containing 6 M guanidine hydrochloride and 2 mM
EDTA at 40°C for 2 h. The reduced protein was then carboxymethyl-
ated with moniodoacetic acid at room temperature for 1 h, separated
by reverse-phase HPLC on a YM column, and sequenced using an
Applied Biosystems 470A Protein Sequencer. To determine the internal
amino acid sequence of HAI, the protein was digested with Achro-
mobacter protease-I. The digested peptides were separated by reverse-
phase HPLC on a YM pack C8 column (0.46 × 15 cm) and sequenced.

Dose Response of the Inhibitory Activity of HAI on HGF-converting
Activity of HGF Activator and Factor XIIa—HGF activator (18 ng) or
factor XIIa (38 ng) was mixed with various concentrations of HAI in 40
μl of PBS containing 0.05% CHAPS and incubated at 37°C for 30 min.
Five microliters of 1.5 mg/ml single chain HGF in PBS containing 0.05%
CHAPS and 5 μl of 100 μg/ml dextran sulfate (Mr cut-off, 500,000,
Sigma) were added to the mixture and further incubated (2 h for HGF
activator and 24 h for factor XIIa). The mixture was analyzed by
SDS-PAGE under reducing conditions. The amounts of single chain
HGF and the heterodimeric form were measured by Flying-Spot Scan-
er. The inhibitory activity of HAI against each protease was estimated
by calculating the ratio of the remaining single chain form to total HGF.

cDNA Cloning—Total RNA was prepared from MKN45 cells by acid
guanidinium thiocyanate/phenol/chloroform extraction (13), and
poly(A) RNA was purified by oligo(dT) affinity chromatography.
The primers, 5′-GGGGCNGAYGTGTTTAA-3′ and 5′-GGGGCNGA-
YGGYCTA-3′ (primer 1), 5′-GTRTCTAAACRAANC-3′ and 5′-GTRTCTNAGNACRAANC-3′ (primer 2), 5′-CCNCTTANACR-
AANGA-3′ and 5′-CCNCCRTANACRAACT-3′ (primer 3), 5′-
CCCCCAAYTNACYTG-3′ and 5′-CCCCCNGAYTNACYTG-3′ (primer 4) (N = A, G, C, or T, Y = C or T, and R = A or G) were
chemically synthesized. Using primers 1 and 2, and poly(A) RNA as a
template, DNA fragments were amplified by reverse transcription-
polymerase chain reaction, and a 56-bp fragment was generated. The
Novel Kunitz-type Serine Protease Inhibitor, HAI

**Table II**

| Peptide no. | Amino acid sequence* |
|------------|---------------------|
| N-terminal |                     |
| 1          | GPPPPAPPGLPGAGADClNSFTAGVGFLDVXASVSNGATF |
| 2          | VQPGQFVLVLK         |
| 3          | SFVYGGXGLNK         |
| 4          | DVENTDWRLLRGTDDVRVERK |
| 5          | AWAGIDLR            |
| 6          | DPQYVELMLK          |
| 9          | XTYLFQDITV          |

* Amino acids not determined are denoted by X.

---

**RESULTS**

Purification of an Inhibitor of HGF Activator (HAI) from the Conditioned Medium of a Stomach Carcinoma Cell Line—We screened serum-free conditioned media from a variety of human cell lines for HGF activator-inhibitory activity. Six out of 14 conditioned medium samples contained significant inhibitory activity (Table I). Among them, five cell lines (three lung and two stomach carcinoma cell lines) produced high levels of the inhibitory activity. Thus, one (MKN45) of these cell lines was used for purification of a molecule with the inhibitory activity. An inhibitor (HAI) was purified from the serum-free conditioned medium of MKN45 by a seven-step procedure described under “Experimental Procedures”. Analysis of the purified protein by SDS-PAGE revealed two bands of 39 and 40 kDa under reducing conditions (Fig. 1). When the NH2-terminal amino acid sequence of the purified protein was analyzed, only one sequence was obtained (Table II). Thus, the apparent heterogeneity of the purified protein on the gel may be caused by a difference in the COOH-terminal sequence or in glycosylation.

To determine the internal amino acid sequences, the purified protein was carboxymethylated and digested with *Acrobacter* protease-I, and the resulting peptide fragments were separated by reverse-phase HPLC. Six partial sequences were determined (Table II). None of the peptide sequences nor the NH2-terminal sequence matched those in the Swiss Prot or NBRF protein sequence data bases, indicating that HAI is a novel protein.

Properties of the Purified HAI—Fig. 2 shows the dose-response curve of the inhibitory activity of HAI. In these reactions, HGF activator (450 ng/ml) was mixed with various concentrations of purified HAI and incubated for 30 min to form an enzyme-inhibitor complex. Then remaining HGF-converting activity in the mixture was measured. The concentration of HAI for 50% inhibition was about 250 ng/ml. Considering the molecular masses of HAI and HGF activator, HAI forms about an equimolar complex with HGF activator within 30 min.

HGF activator is homologous to blood coagulation factor XIIa. Factor XIIa can activate single chain HGF in vitro, although the specific activity of factor XIIa is lower than that of HGF activator (9). We therefore examined whether or not HAI inhibits the HGF-converting activity of factor XIIa and found that it did not, even when a 5-fold molar excess of HAI was incubated with factor XIIa (Fig. 2). Thus, HAI is specific for HGF activator in HGF-converting activity.

Isolation of cDNA Clone and DNA Sequence Analysis—Two hexapeptide sequences, Gly-Ala-Asp-Cys-Leu-Asn and Gly-Phe-Val-Leu-Asp-Thr in the NH2-terminal sequence (Table II), were used to design degenerate oligonucleotide primers for PCR amplification of the sequence for the NH2-terminal region. PCR amplification of MKN45 RNA resulted in a cDNA fragment with the expected size of about 56 bp. The cDNA fragment was subcloned and sequenced. The cDNA clone encoded 19 amino acids, including 7 amino acids in the NH2-terminal amino acid sequence between the two hexapeptide sequences used for the primer design. Thirteen nucleotides from the obtained sequence were used as a 5′ primer for further PCR amplification together with a sequence corresponding to the hexapeptide Ser-Phe-Val-Tyr-Gly-Gly in peptide 2 (Table II) as a 3′ primer. The PCR amplification products were further amplified using the 5′ primer and the sequence corresponding to the hexapeptide Gly-Ala-Cys-Leu-Asp-Thr in peptide 5 (Table II) as a 3′ primer. The PCR amplification resulted in a cDNA fragment of about 480 bp. The cDNA fragment was subcloned and sequenced. The cDNA clone encoded 160 amino acids, including the sequences of the two hexapeptide sequences used for the primer design. DNA fragment was cloned and sequenced. The primer, 5′-AA-CAGCTTTACCG-3′ (primer 5), which is part of the new sequence, was chemically synthesized. Using primers 3 and 5 and poly(A) RNA as a template, DNA fragments were amplified by reverse transcription-polymerase chain reaction. The products were further amplified using primers 4 and 5, and a 480-bp fragment was generated. Using the fragment as a probe, a human placenta cDNA library (Clontech) was screened to obtain full-length cDNA.

Northern Blotting—Total RNA (10 μg) from MKN45 cells was denatured, electrophoresed (14), and transferred to a nylon membrane (Biodyne). 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 (15). The membranes were washed with 1 × SSC containing 1% SDS at 50 °C. The hybridization probe was the 480-bp PCR fragment.

---

**Fig. 2. Dose dependence of the inhibitory activity of HAI toward the HGF-converting activity of HGF activator and factor XIIa.** HGF activator or factor XIIa was incubated with various concentrations of HAI. Single chain HGF (sc-HGF) was then added and further incubated. The mixture was then analyzed by SDS-PAGE (A). The inhibitory activity toward HGF activator (○) and factor XIIa (●) was determined as the ratio of the remaining single chain HGF to total HGF (B).

DNA fragment was cloned and sequenced. The primer, 5′-AA-CAGCTTTACCG-3′ (primer 5), which is part of the new sequence, was chemically synthesized. Using primers 3 and 5 and poly(A) RNA as a template, DNA fragments were amplified by reverse transcription-polymerase chain reaction. The products were further amplified using primers 4 and 5, and a 480-bp fragment was generated. Using the fragment as a probe, a human placenta cDNA library (Clontech) was screened to obtain full-length cDNA.

---

**Table II**

| Peptide no. | Amino acid sequences |
|------------|---------------------|
| N-terminal |                     |
| 1          | GPPPPAPPGLPGAGADClNSFTAGVGFLDVXASVSNGATF |
| 2          | VQPGQFVLVLK         |
| 3          | SFVYGGXGLNK         |
| 4          | DVENTDWRLLRGTDDVRVERK |
| 5          | AWAGIDLR            |
| 6          | DPQYVELMLK          |
| 9          | XTYLFQDITV          |

* Amino acids not determined are denoted by X.
Eighty-four hybridization-positive clones were obtained from about 4 \( \times \) 10^5 phage. The largest clone was sequenced to determine the primary structure of human HAI. The determined nucleotide sequence of the cDNA is shown in Fig. 3.

Predicted Amino Acid Sequence of HAI—The amino acid sequence of HAI deduced from the cDNA sequence is also shown in Fig. 3. The translation initiation site was assigned to the first methionine codon because the sequence GCGATGG matches a favorable Kozak consensus sequence (16). This methionine is followed by a hydrophobic region (Fig. 4), and the NH\(_2\)-terminal amino acid of the purified protein is located at 36th residue downstream of the methionine. Thus, the hydrophobic region may represent a signal peptide sequence. Because the reading frame is open upstream of the first methionine codon, it is possible that translation is initiated further upstream beyond the boundary of the cDNA sequence. The open reading frame that starts from the putative ATG codon consists of 513 amino acids, and the protein product has a calculated molecular mass of 56,893. Excluding the putative signal peptide, the mature form of the protein consists of 478 amino acids and has a calculated molecular mass of 53,319. The apparent molecular mass of HAI purified from the conditioned medium of MKN45 cells was about 40 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 region of 23 amino acids is present in the COOH-terminal region (Fig. 4), suggesting that the primary translation product is a membrane-associated protein. There are three potential N-glycosylation sites with the canonical Asn-X-(Ser/Thr). A comparison of the predicted protein sequence of HAI with sequences in the Swiss Prot and NBRF protein data base revealed three regions with characteristic structural features. The two regions (residues 250–300 and 375–425) showed extensive similarity to the Kunitz-type sequence of serine protease inhibitors (Fig. 5A). Thus, HAI appears to be a Kunitz-type serine protease inhibitor. The other region (residues 319–353) located between the two
Kunitz domains showed similarity to the ligand binding domain of the low density lipoprotein (LDL) receptor and related proteins (Fig. 5). Other regions did not show clear-cut similarity to any protein sequences within the database entries.

Detection of Inhibitory Activity toward HGF Activator in Membrane Fraction of MKN45 Cells—The cDNA sequence of HAI suggests that the primary translation product is a membrane-associated protein. We therefore examined whether inhibitory activity toward HGF activator was detected in membrane fraction. Membrane extract and conditioned medium of MKN45 cells were prepared and assayed for the inhibitory activity. Protein concentration in the membrane extract and conditioned medium was 290 and 620 mg/ml, respectively. Significant activity was detected in the membrane extract (Fig. 6). Considering the protein concentration in each fraction, the activity in the membrane fraction was about 80% that in the conditioned medium. These results suggest that HAI may be produced as a membrane-associated form and is secreted as a proteolytically truncated form.

Tissue Distribution of HAI mRNA—We determined the size and tissue distribution of HAI mRNA by Northern blotting with poly(A) RNAs from various human tissues (Fig. 7). A major transcript of 2.5 kb was detected in MKN45 cells where we purified the HAI protein. In addition, a minor transcript of 5.6 kb was also detected in MKN45 cells. A transcript of 2.5 kb was detected in a variety of human adult and fetal tissues. Among them, the expression level of HAI mRNA was relatively high in the adult placenta, kidney, pancreas, prostate, and small intestine. It was also high in the fetal kidney. However, although the level was low in the adult lung, it was relatively high in the fetal lung.

DISCUSSION

In this study, we found an inhibitory activity toward HGF activator in serum-free conditioned media of various human cell lines. We purified the inhibitor protein, HAI, from the conditioned medium of MKN45 stomach carcinoma cells. The purified HAI has a molecular mass of about 40 kDa. The primary structure of the protein was predicted from the sequence of the cDNA for human HAI. The structure of human HAI is schematically summarized in Fig. 8. The primary translation product consists of 513 amino acid residues. The N-terminal 35 residues may serve as a signal sequence. The mature protein appears to be membrane-bound, because a hydrophobic region of about 20 amino acids is present at the COOH-terminal. HAI has two well-defined Kunitz domains. The Kunitz domain is typically about 60 amino acids in length and contains three disulfide bonds. It was first recognized as the functional domain of bovine pancreatic trypsin inhibitor (17). Thereafter, the domain was found in several mammalian serine protease inhibitors. Thus, one or both of the Kunitz domains found in HAI appear to be responsible for the inhibitory activity of the protein.

The first and second Kunitz domains of human HAI show the highest homology (47% identity) to those of human β-amyloid precursor protein (APP) and human APP homolog protein,
respectively. APP is the precursor protein of amyloid β-protein which is present in neurtic plaque and cerebrovascular deposits in individuals with Alzheimer’s disease and Down’s syndrome (18). The Kunitz domain is located in the middle of APP (19–21), and it efficiently functions as an inhibitor of several serine proteases (22). The primary translation product of APP has a hydrophobic sequence at the COOH-terminal region, and thus it appears to be a membrane-bound protein. Oltersdorf et al. (23) and Van Nostrand et al. (24) reported that protease nexin II (PNII) is a secreted form of APP. PNII is a protease inhibitor that forms SDS-resistant inhibitory complexes with epidermal growth factor-binding protein, the γ-subunit of nerve growth factor, and trypsin. Smith et al. (25) reported that an inhibitor of coagulation factor Xa purified from the serum-free conditioned medium of HepG2 liver cells is also a secreted form of APP. Truncated forms of APP are derived from their cognate membrane-associated forms by proteolysis and have apparently lost the cytoplasmic and transmembrane domains (26). Thus, PNII and factor Xa inhibitor are proteolytically truncated forms of the transmembrane form of APP. HAI purified from the serum-free conditioned medium of HepG2 liver cells is also a secreted form of APP. HAI has two Kunitz domains interrupted by another domain. Two serine protease inhibitors with two or three Kunitz domains have been identified. Inter-α-trypsin inhibitor (IαTI) found in mammalian plasma is a high molecular weight glycoprotein with two tandemly repeated Kunitz domains in the light chain, which is one of three polypeptide chains linked by a glycosaminoglycan (27). Tissue factor pathway inhibitor (TFPI), which was also found in mammalian plasma, has three tandemly repeated Kunitz domains (28). TFPI inhibits activated factor X(a) directly and, in a Xa-dependent manner, inhibits VII(a)/tissue factor activity by forming a quaternary Xa-TFPI-VII(a)/tissue factor complex. The Kunitz domains in these inhibitors are not interrupted by another domain. Trypsin and chymotrypsin form equimolar complexes with IαTI. Furthermore, a protease inhibitor, which consists of only the second Kunitz domain of the light chain of IαTI, has been identified from mast cells (30). This inhibitor, named tryptstatin, markedly inhibits factor Xa and tryptase and also inhibits trypsin and chymase. Thus, the second Kunitz domain in the light chain of IαTI is required for the inhibition of serine proteases, whereas the first domain does not seem to be required for this function. Site-directed mutagenesis of TFPI has revealed that the second Kunitz domain is required for the efficient binding and inhibition of Xa, that both Kunitz domains 1 and 2 are required for the inhibition of VII(a)/tissue factor activity, but that the third Kunitz domain does not seem to be required for these functions (31). The dose-response curve of the HAI activity showed that HAI purified from the conditioned medium seems to form an equimolar complex with HGF activator. Furthermore, the proteolytic cleavage to produce the extracellular truncated form of HAI appears to occur within the second Kunitz domain, because the size (about 40 kDa) of the protein implies that it consists of about 360 amino acids. Thus, the first Kunitz domain in HAI may be sufficient for it to exert activity.
The other characteristic structural domain in HAI is located between the two Kunitz domains. It consists of about 40 amino acid residues and bears a close resemblance to the “cysteine domain” repeat of the LDL receptor and related proteins. The LDL receptor has seven repeats of the domain (32). Each repeat has six cysteine residues, all of which are involved in disulfide bonds, and in addition it has several negatively charged amino acid residues at the COOH-terminal region. The clustering of cysteine residues in the domain of HAI is similar to that in the repeat of the LDL receptor. Furthermore, the domain of HAI has 8 negatively charged but only 2 positively charged amino acid residues. The negatively charged domain in the LDL receptor is believed to be the binding site for its positively charged apoprotein ligand (32). Although the significance of the negatively charged domain in HAI remains to be established, it may be involved in formation of the inhibitor-enzyme complex, because HGF activator shows high affinity to negatively charged substances.

HGF activator is produced mainly in the liver, and it normally circulates in the blood as an inactive zymogen. In response to tissue injury, the zymogen is activated by proteolytic processing exclusively in the injured tissue (10). The activated HGF activator acquires strong affinity for heparin, which may ensure the localization of the enzyme in injured tissue (10). This localized HGF activator activates single chain HGF that is also associated with a heparin-like molecule on the cell surface. Thus, the activity of HGF activator may be regulated by tissue-derived inhibitors. Because human HAI mRNA is expressed in various tissues, the HAI protein produced by cells of these tissues may be responsible for inhibiting HGF activator. However, HAI mRNA expression is low in some tissues, including the liver and lung. HGF is thought to play a crucial role in repair of liver and lung following injury (1, 33). In these tissues, production of HAI could be induced during tissue repair. Alternatively, another inhibitor(s) may function in these tissues. Characterizations of these inhibitors in injured tissues are needed to understand mechanisms for regulating the activation of HGF.

REFERENCES
1. Miyazawa, K., Shimomura, T., Naka, D., and Kitamura, N. (1994) J. Biol. Chem. 269, 8966–8970
2. Naldini, L., Tamagnone, L., Vigna, E., Sachs, M., Hartmann, G., Birchmeier, W., Daikuhara, Y., Tsubouchi, H., Blasi, F., and Comoglio, P. M. (1992) EMBO J. 11, 4825–4833
3. Chu, M.-L., Zhang, R.-Z., Pan, T.-C., Stokes, D., Conway, D., Kuo, H.-J., Miletich, J. P., and Broze, G. J., Jr. (1989) J. Biol. Chem. 264, 6001–6004
4. Kawarabayasi, Y., and Yamamoto, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 415–419
5. Gohda, E., Tsubouchi, H., Nakamura, T. (1991) Biochem. Biophys. Res. Commun. 174, 831–838
6. Shimomura, T., Ochiai, M., Kondo, J., and Morimoto, Y. (1992) Cytotechnology 8, 219–229
7. Miyazawa, K., Shimomura, T., Kitamura, A., Kondo, J., Morimoto, Y., and Kitamura, N. (1993) J. Biol. Chem. 268, 10024–10028
8. Mars, W. M., Zarnegar, R., and Michalopoulos, G. K. (1993) Am. J. Pathol. 143, 949–958
9. Shimomura, T., Miyazawa, K., Komiyama, H., Hiraoka, H., Naka, D., Morimoto, Y., and Kitamura, N. (1995) Eur. J. Biochem. 229, 257–261
10. Miyazawa, K., Shimomura, T., and Kitamura, N. (1996) J. Biol. Chem. 271, 3615–3618
11. Shimomura, T., Kondo, J., Ochiai, M., Naka, D., Miyazawa, K., Morimoto, Y., and Kitamura, N. (1993) J. Biol. Chem. 268, 22972–22977
12. Stone, K. L., and Williams, R. K. (1986) J. Chromatogr. 359, 203–212
13. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
14. Lehrach, H., Diamond, D., Wozney, J. M., and Boedeker, H. (1977) Biochemistry 16, 4745–4751
15. Miyazawa, K., Kitamura, A., Naka, D., and Kitamura, N. (1991) Eur. J. Biochem. 197, 15–22
16. Kozak, M. (1987) Nucleic Acids Res. 15, 8125–8148
17. Laskowski, M., Jr., and Kato, I. (1988) Annu. Rev. Biochem. 49, 593–626
18. Kang, J., Lemaire, H.-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Grzeschik, K.-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. (1987) Nature 325, 733–736
19. Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. (1988) Nature 331, 525–527
20. Tanzi, R. E., McClatchey, A. I., Lamperti, E. D., Villa-Komaroff, L., Gusella, J. F., and Neve, R. L. (1988) Nature 331, 528–530
21. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shioji, S., and Ito, H. (1988) Nature 331, 530–532
22. Sinha, S., Dovey, H. F., Seubert, P., Ward, P. J., Blacher, R. W., Blaber, M., Brashadaw, R. A., Arici, M., Mohley, W. C., and Lieberburg, I. (1990) J. Biol. Chem. 265, 8863–8865
23. Oltersdorf, T., Fritz, L. C., Schenk, D. B., Lieberburg, I., Johnson-Wood, K. L., Beattie, E. C., Ward, P. J., Blacher, R. W., Dovey, H. F., and Shinya, S. (1989) Nature 341, 144–147
24. Van Nostrand, W. E., Wagner, S. L., Suzuki, M., Choi, B. H., Farrow, J. S., and Cunningham, D. D. (1989) Nature 341, 546–549
25. R. H., Higuchi, D. A., and Broze, G. J., Jr. (1990) Science 248, 1126–1128
26. Weidemann, A., Konig, G., Bunke, D., Fischer, P., Salbaum, J. M., Masters, C. L., and Beyreuther, K. (1989) Cell 57, 115–126
27. Sailer, J.-P. (1990) Trends Biochem. Sci. 15, 435–439
28. Wun, T.-C., Kawarabayasi, Y., and Yamamoto, T. (1994) J. Biol. Chem. 269, 6001–6004
29. Itoh, H., Ide, H., Ishikawa, N., and Nawa, Y. (1994) J. Biol. Chem. 269, 3818–3822
30. Kido, H., Yokogoshi, Y., and Katunuma, N. (1988) J. Biol. Chem. 263, 18104–18107
31. Girard, T. J., Warren, L. A., Novotny, W. F., Likert, K. M., Brown, S. G., Mileitch, J. P., and Broze, G. J., Jr. (1988) Nature 338, 518–520
32. Sudhof, T. C., Goldstein, J. L., Brown, M. S., and Russell, D. W. (1985) Science 228, 815–822
33. Yanagita, K., Matsutomo, K., Sekiguchi, K., Ishibashi, H., Niyo, Y., and Nakamura, T. (1993) J. Biol. Chem. 268, 21212–21217
34. Kyte, J., and Dodotille, R. F. (1982) J. Mol. Biol. 157, 105–132
35. Sprecher, C. A., Grant, P. J., Grimm, G., O’Hara, P. J., Norris, F., Norris, K., and Foster, D. C. (1993) Biochemistry 32, 4481–4486
36. Kammeyer, J. P., Polaz, J. O., and Kottick, M. P. (1986) Nucleic Acids Res. 14, 7839–7850
37. Chu, M.-L., Zhang, R.-Z., Pan, T.-C., Stokes, D., Conway, D., Kue, H.-J., Gianvillo, R., Mayer, U., Mann, K., Deutzmann, R., and Timpl, R. (1990) EMBO J. 9, 365–375
38. Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., and Beyreuther, K. (1989) J. Biol. Chem. 264, 8983–8985
39. Kawarabayasi, Y., and Yamamoto, T. (1994) J. Biol. Chem. 269, 2173–2182
40. Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausseph, H., and Stanley, K. K. (1988) EMBO J. 7, 4119–4127
41. Murdoch, A. D., Dodge, G. R., Cohen, I., Tuan, R. S., and Joza, R. V. (1992) J. Biol. Chem. 267, 8544–8557