Hepatocyte growth factor activator inhibitor type 1 is a specific cell surface binding protein of hepatocyte growth factor activator (HGFA) and regulates HGFA activity in the pericellular microenvironment

Hiroaki Kataoka, Takeshi Shimomura, Toshiya Kawaguchi, Hiroshi Itoh, Naomi Kitamura, Keiji Miyazawa, and Masashi Koono

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Hepatocyte growth factor activator (HGFA) is responsible for proteolytic activation of the precursor form of hepatocyte growth factor in injured tissues. To date, two specific inhibitors of HGFA have been identified, namely HGFA inhibitor type 1 (HAI-1) and type 2 (HAI-2)/placental bikunin (PB). Both inhibitors are first synthesized as integral membrane proteins having two Kunitz domains and a transmembrane domain, and are subsequently released from cell surface by shedding. Here we show that an active form of HGFA is specifically complexed with membrane-form HAI-1, but not with HAI-2/PB, on the surface of epithelial cells expressing both inhibitors. This binding required the enzyme activity of HGFA. The selective binding of HGFA to the cell surface HAI-1 was further confirmed in an engineered system using Chinese hamster ovary cells, in which only the cells expressing HAI-1 retained exogenous HGFA. The binding of HGFA to HAI-1 was reversible, and no irreversible modifications affecting the enzyme activity occurred during the binding. Importantly, HAI-1 and the HGFA-HAI-1 complex were quickly released from the cell surface by treatment with phorbol 12-myristate 13-acetate or interleukin 1β accompanying the generation of 58-kDa fragments of HAI-1, which are less potent against HGFA, as well as significant recovery of HGFA activity in the culture supernatant. This regulated shedding was completely inhibited by BB3103, a synthetic zinc-metalloproteinase inhibitor. We conclude that HAI-1 is not only an inhibitor but also a specific acceptor of active HGFA, acting as a reservoir of this enzyme on the cell surface. The latter property appears to ensure the concentrated pericellular HGFA activity in certain cellular conditions, such as tissue injury and inflammation, via the up-regulated shedding of HGFA-HAI-1 complex. These findings shed light on a novel function of the integral membrane Kunitz-type inhibitor in the regulation of pericellular proteinase activity.

Hepatocyte growth factor (HGF), also known as scatter factor, is a pleiotropic factor that functions as a mitogen, motogen, and/or morphogen for a variety of cells, particularly epithelial cells, bearing c-Met receptor tyrosine kinase (1, 2). Because HGF is secreted as an inactive precursor form, proteolytic activation of the precursor form in the extracellular milieu is a critical limiting step in the HGF-induced signaling pathway. HGFA activator (HGFA) is a factor XII-like serine proteinase having a critical role in the activation of HGF in injured tissue (3–7). Because active HGF is not inhibited by serum proteinase inhibitors (8), it has been suggested that local synthesis of HGFA inhibitors could have a critical regulatory role for HGFA activity in the injured tissue. HGFA inhibitor type 1 (HAI-1) was initially identified as a potent inhibitor of HGFA present in a culture-conditioned medium of MKN45 gastric adenocarcinoma cell line (9). A second type of HGFA inhibitor (HAI-2) was subsequently identified (10). HAI-2 was also independently isolated as placental bikunin (PB) (11, 12) and as a protein overexpressed in pancreatic cancer (13). Both HAI-1 and HAI-2/PB have two well-defined Kunitz inhibitor domains and a presumed transmembrane domain. In addition, HAI-1 has an LDL receptor-like domain between two Kunitz domains (9, 10).

HAI-1 protein is predominantly expressed on the basolateral surface of columnar epithelial cells covering the mucosal surfaces and duct lumina (14). Its localization on the cell surface as a transmembrane form integrated in the plasma membrane was also confirmed in a cultured epithelial cell line MKN45 (15). HAI-1 was first purified as a 40-kDa secreted protein from the conditioned medium of MKN45 cells (9). Therefore, mature membrane-form HAI-1 can be proteolytically cleaved, and the resultant truncated form with Kunitz domain(s) is released into the extracellular space as a secreted form of HAI-1 (sHAI-1). Our recent observation indicated that there are multiple

The abbreviations used are: HGF, hepatocyte growth factor; HGFA, HGF activator; HAI-1, HGFA inhibitor type 1; HAI-2/PB, HGFA inhibitor type 2/placental bikunin; sHAI-1, secreted form of HAI-1; scHGF, single-chain HGF; CHO, Chinese hamster ovary; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PMA, phorbol 12-myristate 13-acetate; Sulfo-DST, disulfosuccinimidyl tartrate; IL-1β, interleukin-1β; IgG-FITC, fluorescein 5-isothiocyanate-conjugated IgG; mAb, monoclonal antibody; PBS, fetal bovine serum; PBS, phosphate-buffered saline; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; TNFα, tumor necrosis factor-α; TACE, TNFα-converting enzyme; ADAM, a disintegrin and metallopeptase; LDL, low density lipoprotein; BSA, bovine serum albumin; bp, base pair(s); APP, amyloid β protein precursor.

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sites of proteolytic cleavage to release sHAI-1 and two major secreted forms, 40 and 58 kDa in size, were identified (15). The 40-kDa sHAI-1 contains only the first Kunitz domain, whereas the 58-kDa form contains both Kunitz domains. Interestingly, the 58-kDa sHAI-1 showed a markedly lower affinity for HGFA than the initially identified 40-kDa sHAI-1 (9, 15), although the pathophysiologic significance of these differentially processed forms of sHAI-1 remains to be clarified. To date, the precise functions of HAI-1 and HAI-2/PB in vivo are undefined. However, previous studies have shown that the cellular surface expression of HAI-1 is significantly up-regulated in the epithelial cells in response to tissue injury and regeneration, suggesting a potential role of HAI-1 in the survival and regeneration of the epithelial cells in vivo (14, 16). In contrast, HAI-2/PB expression was not altered in the injured tissue (16).

HGFA is produced mainly by the liver in which hepatocytes being responsible (4, 17). In recent reports indicate that gastrointestinal tissues also express the HGFA gene (18–20). HGFA is secreted as an inactive single-chain 96-kDa zymogen (pro-HGFA) and is activated by thrombin in injured tissues via a cleavage at the bond between Arg407 and Ile408 that gastrointestinal tissues also express the HGFA gene (18–20). HGFA activation is a process requiring de novo synthesis of HLCKEs (14, 15). C76-18 and 1N7 recognized the 58-kDa form containing both Kunitz domains. Interestingly, the 58-kDa form contains both Kunitz domains. Recently, we identified a monoclonal antibody that detected the 58-kDa form of HGFA (15). The 58-kDa form of HGFA was shown to have a markedly lower affinity for HAI-1, although the pathophysiologic significance of these differentially processed forms of sHAI-1 remains to be clarified.

In this report, we describe evidence showing that active HGFA selectively binds to the membrane-form HAI-1, but not to HAI-2/PB, of epithelial cells, and that this binding is reversible. Further investigation revealed that membrane-form HAI-1 acts not only as a cellular surface inhibitor of HGFA but also as a reservoir for this enzyme. The latter function of HAI-1 paradoxically contributed to ensure the pericellular HGFA activity in certain cellular conditions via up-regulated shedding of the HGFA-HAI-1 complex from the cell surface followed by the recovery of HGFA activity. These results propose a novel important role of the membrane-type Kunitz inhibitor in the regulation of pericellular proteolysis.

## EXPERIMENTAL PROCEDURES

### Materials—Cell lines and reagents were obtained as follows: human lung carcinoma cell line HLC-1 from the Department of Physiology, Keio University; human colon carcinoma cell lines WiDr and SW837 from Daihbon Seiyaku Co., Ltd.; Chinese hamster ovary (CHO) cell line from Riken Cell Bank; 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), phenylmethylsulfonyl fluoride (PMSF) and phorbol 12-myristate 13-acetate (PMA) from Sigma; apro tinin from Pentapharm Ltd.; 6-amidino-2-naphthyl p-guanidinobenzo ate dimethanesulfonate (nafamostat mesylate) from Torii Co., Ltd.; heparitinase II from Seikagaku Co.; disulfosuccinimidyl tartrate (Sulfo- dTST) from Pierce Chemical Co.; recombinant human interleukin-1 (IL-1b) from Becton Dickinson; nonspecific mouse IgG and fluorescein 5-isothiocyanate-conjugated sheep IgG anti-mouse IgG (anti-mouse IgG-FTTC) from Organo Teknis; protein A/G-agarose from Santa Cruz Biotechnologies. A synthetic metalloproteinase inhibitor, BB3103, was kindly provided from British Biotech Pharmaceuticals. Preparation of mouse monoclonal antibodies (mAbs), C76-18 and 1N7, against human HAI-1 was as described previously (14, 15). C76-18 and 1N7 recognized around the first and second Kunitz domains, respectively, of HAI-1 protein. Preparation of mAbs against human HGFA (A1, A23, and 7E10) was as described previously (7). Anti-human HAI-2/PB mAb (2N9) was prepared by the method described previously (25). Antiserum against cytokeratin polyclonal antibodies were purchased in Shull glass chamber slides in Ham’s F-12 medium containing 5% fetal bovine serum (FBS). When semiconfluent, the cells were washed with cold phosphate-buff ered saline (PBS) containing 0.1% gelatin (washing buffer) three times, and incubated with 50 µg/ml anti-HAI-1 mAb (C76-18) or 500 µg/ml control mouse IgG for 60 min on ice. After the incubation, the cells were washed with washing buffer and incubated with anti-mouse IgG-FTTC for 30 min at 4 °C. After the incubation, the cells were washed with washing buffer and visualized by use of fluorescence microscope. For the detection of bound HGFA on cell surface, the HLC-1 cells were maintained in Ham’s F-12 medium containing bovine HGFA-depleted FBS (5%) that was prepared by anti-HGFA immunopentrifugation column. When semiconfluent, the cells were washed with cold washing buffer, washed HGFA in recombinant HGFA in serum-free Ham’s F-12 medium containing 0.1% bovine serum albumin (BSA) for 50 min at 4 °C. Then the cells were washed four times with cold washing buffer and cultured for the indicated periods at 37 °C in the serum-free medium containing 0.1% BSA. After incubation, the bound HGFA proteins were visualized by incubating the cells with 10 µg/ml anti-HGFA mAb (A23) followed by incubation with anti-mouse IgG-FTTC as described above. The method for the immunohistochemical detection of HAI-1 protein using formalin- fixed paraffin embedding tissue specimen was described previously (14).

### Immunoblot Analysis and Immunoprecipitation—For the extraction of cellular proteins, cultured cells were washed three times with PBS and immediately scraped in 2 ml of 10% trichloroacetic acid on ice. The precipitated proteins were harvested by centrifugation, and the pellet was extracted with 200 µl of 7 M urea/2% Triton X-100/50% 2-mercaptoethanol, followed by centrifugation. The resultant supernatants were mixed with SDS sample buffer and boiled for 3 min. Immunoblot analysis was done according to the method described previously (14). Briefly, SDS-polycrylamide gel electrophoresis (PAGE) was performed under a reducing condition using 4–12% gradient gel. After electrophoresis, the proteins were transferred electrophoretically onto an Immobilon membrane (Millipore). After blocking the nonspecific binding sites with 5% nonfat dry milk in 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20, the membrane was incubated with primary antibody diluted in the buffer containing 1% BSA at 4 °C overnight, followed by washing in the buffer four times and incubation with peroxidase-conjugated secondary antibody diluted in the buffer with 1% BSA for 1 h at room temperature. The labeled proteins were visualized by chemiluminescence reagent (PerkinElmer Life Sciences). Immunoprecipitation of HGFA or HAI-1 was done using A23 anti-HGFA mAb or C76-18 anti-HAI-1 mAb, respectively. Confuent HLC-1 cells in 6-well plate were maintained overnight in serum-free medium. Then the cells were washed three times with PBS and incubated with 2 µg/ml two-chain 34-kDa form of HGFA for 50 min at 4 °C. Then the cells were washed with cold PBS four times and cultured in a serum-free medium for 30 min at 37 °C in the presence or absence of 0.1 µM PMA. Then the culture supernatant (1 ml/well) was collected and used for the immunoprecipitation study. For preparation of cell extracts, the cells were harvested mechanically by cell scraping. After brief centrifugation, the cellular proteins were extracted in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 1% Triton X-100, for 1 h on ice. After centrifugation, the supernatant was used for the immunoprecipitation assay. Before immunoprecipitation, samples were precleared with nonspecific mouse IgG (1 µg/ml) and protein A/G-agarose (Santa-Cruz Biotechnologies) according to the manufacturer’s instruction. Then the samples were immunoprecipitated with 2 µg/ml immunoprecipitating antibody at 4 °C overnight. Nonspecific mouse IgG was used as a control. After incubation, the samples were washed three times with preimmune rabbit serum and processed according to the manufacturer’s instruction. The immunoprecipitated sample was eluted in SDS sample buffer, boiled, and analyzed by immunoblot under the reducing condition. For the detection of HGFA, anti-HGFA mAb (A1) was used, and for HAI-1, biotinylated anti-HAI-1 mAb (1N7) was used.

### RNA Blot Analysis—RNA blot analyses for human HAI-1 and HAI-2/PB were done according to the method described previously (25). Briefly, total RNA was isolated from the cells as described previously (25). RNA samples were treated with a protective solution and electrophoresed on a 1% agarose gel. The blots were hybridized with probes for human HAI-1 and HAI-2/PB, respectively. The blots were washed three times with 0.1% SDS at 60 °C, followed by exposure to X-ray films.
2/PB mRNA were done according to the methods previously described (25, 26). Total cellular RNA was extracted by Trizol reagent (Life Technologies, Inc.). The NaCl-Xhol fragment (1221 bp) from human HAI-1 cDNA or the NcoI fragment (648 bp) from human HAI-2 cDNA was used as a probe. For internal control of loading, the blots were subsequently hybridized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (CLONTECH). The probes were radiolabeled by random priming with [32P]CTP. For quantification of the RNA blot analysis, the radioactivity of mRNA signals for HAI-Ls were directly measured by a Bioimage Analyzer, FUJIX BAS2000 system (Fuji Photo Film), normalized by division through those of the corresponding G3PDH mRNA signals.

Constitution and Expression of a Plasmid Encoding Human HAI-1 or HAI-2/PB—A cDNA encoding the whole coding region of HAI-1 or HAI-2/PB was constructed by polymerase chain reaction using full-length HAI-1 cDNA or HAI-2 cDNA as a template. Primers used were, 5'-TTGGAATTCGCGATGGCCCCTGCGAGGAC-3' and 5'-TTATCTGCACTCATCGCACTGGTGCTGCT-3' for HAI-1, and 5'-AGCTTCGAC GCCATTGCGACCTGTCCCGG-3' and 5'-TTATCTGCACTCAAGGACTATATGTGGTTTCTC-3' for HAI-2/PB. The polymerase chain reaction products were subcloned into the EcoRI/SalI site (HAI-1) or XbaI/SalI site (HAI-2/PB) of an expression plasmid pCM0 (Promega). The plasmid was linearized by XmnI and transfected into CHO cells by the LipofectAMINE method (Life Technologies, Inc.). After transfection, the cells were cultured in Ham’s F-12 medium containing 0.5 mg/ml G418 (25, 26). Total cellular RNA was extracted by Trizol reagent (Life Technologies, Inc.). The expression of HAI-1 or HAI-2/PB mRNA were done according to the methods previously described (25, 26).

RESULTS

Specific Binding of Active HGFA to the Surface of Epithelial Cells—Initially we attempted to test the cellular binding of HGFA. When various molecular forms of HGFA (5) were added into the culture medium of HLC-1 epithelial cell line, only active forms (34-kDa and 96-kDa two-chain forms) of HGFA were bound to the cell surface as judged by immunocytochemistry (Fig. 1), suggesting that the binding of HGFA to HLC-1 cells was its active conformation-dependent form. Because a previous study has shown that the active two-chain form of HGFA acquired enhanced heparin affinity (7), it suggested the possibility that the active forms of HGFA are bound to the cell-surface proteoglycans through their heparin-binding properties. However, pretreatment of HLC-1 cells with heparitinase II (0.015 units/ml), or the co-presence of heparin or dextran sulfate (1 mg/ml) with HGFA did not affect the binding significantly (data not shown). A subsequent study using serine protease inhibitors having potent inhibitory activity against HGFA indicated the critical involvement of the active site of this enzyme in the binding to the cell surface. As shown in Fig. 2, pretreatment of HGFA with the inhibitors significantly suppressed the cellular binding of HGFA. Complete inhibition caused by the synthetic low molecular weight inhibitors (nafamostat mesylate and PMSF) strongly suggested the essential requirement of the active site of HGFA in the binding of active HGFA to cell surface. Among the inhibitors tested, benzamidine showed the weakest effect. This is in accordance with the fact that the inhibitory activity of benzamidine against HGFA is the lowest among the inhibitors tested in this study (3). Taken altogether, it was concluded that the binding of HGFA to the surface of HLC-1 cell is primarily mediated by the active site of HGFA.
Identification of HAI-1 but Not HAI-2/PB as a Cell Surface HGFA Binding Protein—The above observations raised the possibility that the binding of HGFA to the epithelial cell surface is mediated by membrane-form HAI-1, as a result of protease-proteinase inhibitor interaction. In fact, HLC-1 cells expressed both HAI-1 and HAI-2/PB mRNAs (Fig. 3A). To identify the cell-surface protein complexed with HGFA, a chemical cross-linking study using intact cultured cells was performed. In this experiment, two other epithelial cell lines (WiDr and SW837), both of which concomitantly expressed high levels of endogenous HAI-1 and HAI-2/PB (Fig. 3A), were also tested in addition to HLC-1. Water-soluble, membrane-impermeable cross-linker, sulfo-DST, was used to ensure cellsurface labeling. As shown in Fig. 3B, two specific bands cross-linked to 32-kDa heavy chain of two-chain active form HGFA were identified with an anti-HGFA antibody in all three cell lines, which migrated at the molecular mass positions of 34 kDa and around 100 kDa under the reducing condition. The former band represented HGFA showing intramolecular cross-linking (32-kDa heavy chain, which also served as an internal control of the cross-linking efficiency. The latter 100-kDa band was an HGFA-HAI-1 complex according to its molecular mass (34- or 32-kDa HGFA + 66-kDa membrane-form HAI-1) as well as to cross-reactivity to the anti-HAI-1 antibody (Fig. 3B). The absence of other specifically cross-linked bands suggested that only HAI-1 could be complexed with the exogenously added HGFA on the cell surface. In fact, no specifically cross-linked band was observed with immunoblot analysis using the anti-HAI-2/PB antibody (data not shown). Although the amounts of cross-linked HGFA-HAI-1 complex were low, according to the low cross-linking efficiency estimated from the level of intramolecular cross-linking of two-chain HGFA (34-kDa band), it was suggested that most of HGFAs were complexed with HAI-1. When the 96-kDa two-chain form of HGFA was used for the cross-linking study, a specifically cross-linked band of approximately 160 kDa was observed under nonreducing condition (Fig. 3C), again suggesting the specific binding of active HGFA to the membrane-form HAI-1.

To further confirm the specific binding of HGFA to membrane-form HAI-1, the engineered system overexpressing either HAI-1 or HAI-2/PB was used. After CHO cells were stably transfected with an expression vector harboring a whole coding region of human HAI-1 or HAI-2/PB cDNA, the expression of each HAI was examined. Immunoblot analysis using cell extracts indicated that the cells in fact expressed membrane-form HAI-1 (66 kDa) or HAI-2/PB (broad smear of glycosylated forms between 30 and 40 kDa with two main vague bands that were probably differentially glycosylated forms) (Fig. 4A). Then, these cells were processed for the HGFA binding and cross-linking assays. Interestingly, the 34-kDa two-chain active form of HGFA was able to associate with the cells only when the CHO cells expressed human HAI-1 (Fig. 4B). Neither mock-transfected control nor HAI-2/PB-expressing CHO cells could retain exogenous HGFA. These observations clearly indicated that the membrane-form HAI-1 selectively acts as a binding protein for the active HGFA on the epithelial cell surface.

Up-regulated Shedding of HAI-1 and HGFA-HAI-1 Complex by PMA and Subsequent Recovery of HGFA Activity—We have previously reported multiple secreted forms of HAI-1 (sHAI-1),...
and among them, 58-kDa sHAI-1 is less potent against HGFA than is 40-kDa sHAI-1, which was initially identified as an HGFA inhibitor present in a culture supernatant (15). When HLC-1 cells were treated with PMA, considerable amounts of 58-kDa sHAI-1 were quickly released into the culture supernatants within 30 min (Fig. 5). This up-regulated shedding was completely inhibited by BB3103, indicating the involvement of a zinc-metalloproteinase activity in this phenomenon (Fig. 5). Without PMA treatment, small amounts of 58-kDa sHAI-1 were slowly released from the cells, and this shedding was also BB3103-sensitive. Similar results were obtained in WiDr and SW837 cells (data not shown).

We then examined the effects of PMA treatment on the shedding of HGFA/HAI-1 complex. Interestingly, when HLC-1 cells were pretreated with active HGFA, a significant level of HGFA activity was recovered in the culture supernatant very quickly within 30 min after PMA treatment (Fig. 6A, a)). Because this recovery of HGFA activity in the supernatant was completely inhibited by BB3103 treatment of HLC-1 cells (Fig. 6A, a)), and BB3103 itself did not inhibit HGFA activity at all (data not shown), the shedding of HAI-1 complexed with HGFA by metalloproteinase is prerequisite to the recovery of HGFA activity. As shown in Fig. 6, A (b), more than 60% of the cell-associated HGFA activity appeared to be released within 30 min. It should be emphasized that the cell-surface HGFA proteins complexed with membrane-form HAI-1 were able to be recovered by brief acid treatment, suggesting that the binding between HGFA and HAI-1 was reversible. The total activity of HGFA released into the medium and remained on the cell surface after 30-min treatment with PMA appeared to be very similar to that of HGFA initially associated with the cell surface (Fig. 6A, b)). Because the PMA-induced release of HGFA activity was accompanied by the shedding of considerable amounts of 58-kDa sHAI-1, it could be postulated that, after regulated shedding of the HGFA-HAI-1 complex, the 58-kDa sHAI-1 tended to dissociate from HGFA, eventually resulting in a considerable recovery of HGFA activity. In fact, less than 30% of the HGFA proteins in the supernatant of PMA-treated cells appeared to be tightly complexed with 58-kDa sHAI-1 proteins, which were immunoprecipitated by the anti-HAI-1 antibody (Fig. 6B). In contrast, more than 80% of HGFA, in the extract of cells without PMA treatment, was co-immunoprecipitated with membrane-form HAI-1 (66 kDa) by anti-HAI-1 antibody (Fig. 6B). Similar results were obtained in the engineered CHO cells (Fig. 6C). Upon PMA treatment, the release of HGFA activity was observed only in HAI-1-expressing CHO cells pretreated with HGFA, but not in HAI-2/PB-expressing and mock-transfected CHO cells, indicating that the above-mentioned phenomena were HAI-1-dependent (Fig. 6D).

Absence of Irreversible Modification of HGFA during the Binding with Membrane-form HAI-1—Time-course study for the release of HGFA activity after the binding of active 34-kDa HGFA to membrane-form HAI-1 was performed. In accordance with the above described observations, considerable amounts of HGFA activity were quickly released upon PMA treatment, and this regulated shedding was inhibited by BB3103 (Fig. 7A). The low level of shedding of HGFA activity was also observed in the cells without PMA treatment when the cells were incubated for longer periods, which was also inhibited by BB3103 (Fig. 7A). Subsequent quantitative analysis of the cell surface-associated HGFA also supported the above observations. In this experiment, cell-surface HGFA proteins complexed with mem-

![Figure 4. Specific binding of active HGFA to HAI-1-transfected CHO cells but not to HAI-2/PB-transfected and mock-transfected CHO cells. CHO cells stably transfected either with expression vector carrying human HAI-1 cDNA (CHO/HAI-1) or human HAI-2/PB cDNA (CHO/HAI-2), or with vector only (CHO/mock) were cultured in 6-well plates under serum-free condition. A, immunoblot analysis for HAI-1 or HAI-2/PB using cell extracts. B, cell binding study of active HGFA followed by cross-linking analysis. Each cell line was pretreated with or without 5 μg/ml HGFA for 50 min at 4 °C. After washing, the cell extracts were prepared and subjected to the immunoblot analysis using anti-HGFA mAb (A1), anti-HAI-1 mAb (1N7), or anti-HAI-2 mAb (2N9). Note that only HAI-1-expressing cells retained exogenous HGFA proteins. The asterisk indicates the specifically cross-linked HGFA/HAI-1 complex.

![Figure 5. Enhanced shedding of HAI-1 upon PMA treatment. Cultured HLC-1 cells were treated with 0.1 μM PMA for indicated periods at 37 °C in the presence or absence of BB3103 (2 μM). HAI-1 proteins in the culture supernatants (A) or cell extracts (B) were then analyzed by immunoblot using C76-18 mAb. Immunocytochemistry for cellular HAI-1 protein (30 min after the treatment) is also shown (C).]
brane-form HAI-1 were recovered by brief acid treatment at the indicated time points, and the amounts of recovered HGFA were measured. As shown in Fig. 7, B (a), the bound HGFA to HLC-1 cell surface in fact decreased significantly by PMA treatment very quickly, and the amounts of cell-associated HGFA were inversely correlated with the HGFA activity released into the supernatant observed in Fig. 7A. The HGFA proteins recovered from the cell surface at each time point still retained their HGFA-processing activity, and the activities were in fact correlated with the protein amounts of HGFA recovered (Fig. 7B, b). Importantly, if the shedding of HAI-1 was inhibited by BB3103, the total activity and protein amounts of HGFA retained on the cell surface remained unchanged (Fig. 7B). Thus, neither irreversible modification affecting the activity of HGFA nor apparent internalization occurred during the binding of HGFA to membrane-form HAI-1.

Up-regulated Shedding of HGFA/HAI-1 Complex and Subsequent Recovery of HGFA Activity Induced by IL-1β—In an attempt to test the in vivo relevance of the above described observations, we examined the effects of an inflammatory cytokine, IL-1β, on the shedding of HAI-1 and the HGFA-HAI-1 complex as well as subsequent recovery of HGFA activity in the supernatant. This was based on the observation that epithelial cells in injured and inflamed tissue showed up-regulated expression of HAI-1 on their surface (14, 16). An example is shown in Fig. 8. In this ulcerated gastric mucosa tissue, the epithelial cells at the injured edge strongly expressed HAI-1 compared with the epithelial cells distant from the injured site in the same specimen (Fig. 8B). In contrast, the epithelial cells at the injured site showed significantly decreased E-cadherin expression, which may reflect migratory responses of these cells for the repair of the ulcer surface (Fig. 8, A and B). Mild immunoreactivity was also observed in the stromal tissue of the injured lesion suggesting the up-regulated shedding of HAI-1, in addition to the up-regulated expression. However, co-localization of HAI-1 and active HGFA proteins could not be evaluated immunohistochemically, because the antibody specific for the active form of HGFA was not available.

As shown in Fig. 9A, treatment of the cultured cells with IL-1β in fact quickly induced up-regulated shedding of 58-kDa sHAI-1. This was also significantly inhibited by BB3103, indicating a similar shedding mechanism to that induced by PMA. Consequently, similar to the PMA treatment, the HGFA-HAI-1 complex was also simultaneously released upon IL-1β treatment when the cells were pretreated with HGFA (Fig. 9B), resulting in the recovery of HGFA activity in the culture supernatant (Fig. 9C). These observations indicate that membrane-form HAI-1 is able to work as a reservoir of active HGFA and recruits the activity via regulated shedding in response to extracellular stimuli induced by inflammatory cytokines such as IL-1β, as well as in response to activation of protein kinase C in injured and inflamed tissue. Interestingly, although IL-1β induced the shedding of HAI-1 as early as 30 min after the treatment, it also induced up-regulated HAI-1 mRNA level when the cells were treated for a longer period (Fig. 9D).
Alternatively, HAI-2/PB may be very quickly released from the cell surface. It was stained on the basolateral cellular surface (14, 15, 25, 26). The staining was intracellular in the epithelial cells, whereas HAI-1 was not complexed with the cellular HAI-2/PB. Thus, the membrane-form HAI-1 is a specific inhibitor of active HGFA acting on the epithelial cell surface. Rather surprisingly, although the molecular structure of HAI-2/PB is similar to HAI-1 having a transmembrane domain and two Kunitz domains, active HGFA was not complexed with the cellular HAI-2/PB. Thus, the membrane-form HAI-2/PB does not act as an HGFA inhibitor on the cell surface, although the secreted form of HAI-2/PB shows potent anti-HGFA activity in vitro (10, 28). These observations support a hypothesis that HAI-1 and HAI-2/PB acquire distinct biological roles in vivo during their evolution, although they might be derived from the same ancestor gene (29). HAI-1 has an LDL receptor-like domain that is absent in HAI-2/PB between two Kunitz inhibitor domains. Thus, it may be that the LDL receptor-like domain is somehow involved in the accessibility of active HGFA to the membrane-form HAI-1. Another possibility is that a Kunitz-containing part of HAI-2/PB is not exposed to the extracellular milieu but is exposed to a lumen of a vesicular compartment depending on its post-Golgi trafficking pathway. Although convincing evidence supporting this possibility is lacking at present, our previous immunohistochemical study showed that HAI-2/PB was preferentially stained intracellularly in the epithelial cells, whereas HAI-1 was stained on the basolateral cellular surface (14, 15, 25, 26). Alternatively, HAI-2/PB may be very quickly released from the cell surface.

**FIG. 7.** Time-course study for the regulating shedding of HGFA-HAI-1 complex. HLC-1 cells in 6-well plate were pretreated with 2 μg/ml two-chain 34-kDa HGFA at 4 °C for 50 min under serum-free condition. After repeated washing with cold PBS, the cells were incubated in 1 ml of serum-free medium with or without PMA (0.1 μM) in the presence or absence of BB3103 (2 μM) for indicated periods at 37 °C. A, single-chain HGFA-processing activity in the culture supernatant (30 μl for each assay) was measured and plotted. B, the bound HGFA was stripped by acid treatment from the cell surface at the indicated time points, and the amounts of HGFA recovered from the cell surface were measured by enzyme-linked immunosorbent assay (a). HGF-processing activities in the recovered samples were also measured (b).

**DISCUSSION**

In the present study, we showed that only an active form of HGFA was bound to the epithelial cell surface. This binding was mediated by membrane-form HAI-1 and was considered to be a proteinase-proteinase inhibitor interaction, because the pretreatment of active HGFA with low molecular weight synthetic serine proteinase inhibitors abolished the binding completely. Therefore, it can be postulated that the membrane-form HAI-1 is a specific inhibitor of active HGFA acting on the epithelial cell surface. Rather surprisingly, although the molecular structure of HAI-2/PB is similar to HAI-1 having a transmembrane domain and two Kunitz domains, active HGFA was not complexed with the cellular HAI-2/PB. Thus, the membrane-form HAI-2/PB does not act as an HGFA inhibitor on the cell surface, although the secreted form of HAI-2/PB shows potent anti-HGFA activity in vitro (10, 28). These observations support a hypothesis that HAI-1 and HAI-2/PB acquire distinct biological roles in vivo during their evolution, although they might be derived from the same ancestor gene (29). HAI-1 has an LDL receptor-like domain that is absent in HAI-2/PB between two Kunitz inhibitor domains. Thus, it may be that the LDL receptor-like domain is somehow involved in the accessibility of active HGFA to the membrane-form HAI-1. Another possibility is that a Kunitz-containing part of HAI-2/PB is not exposed to the extracellular milieu but is exposed to a lumen of a vesicular compartment depending on its post-Golgi trafficking pathway. Although convincing evidence supporting this possibility is lacking at present, our previous immunohistochemical study showed that HAI-2/PB was preferentially stained intracellularly in the epithelial cells, whereas HAI-1 was stained on the basolateral cellular surface (14, 15, 25, 26). Alternatively, HAI-2/PB may be very quickly released from the cell surface.

The most important aspect of this study is the demonstration that HGFA proteins complexed with membrane-form HAI-1 were released into the extracellular milieu by regulated shedding of the membrane-form HAI-1 followed by a considerable recovery of HGFA activity. This shedding was significantly enhanced by PMA or IL-1β in a metalloproteinase-dependent manner, accompanying the generation of 58-kDa sHAI-1 fragments. Previous study has revealed that this 58-kDa sHAI-1 shows a significantly lower affinity for HGFA than the 40-kDa one that was initially identified as an HGFA inhibitor in a conditioned medium of MKN45 cells (9, 15). Indeed, immunoprecipitation study indicated that only a minor portion of HGFA proteins was tightly complexed with 58-kDa sHAI-1 in the PMA-treated culture supernatant. Therefore, it is conceivable that, after regulated shedding of the HGFA-HAI-1 complex, an equation among the inhibitor (58-kDa sHAI-1), enzyme (active HGFA), and substrate (single-chain HGF) shifts in favor of the enzyme-substrate complex, eventually resulting in an efficient generation of an active two-chain form of HGF. Currently, the precise biochemical mechanism underlying the decreased affinity of 58-kDa sHAI-1 to HGFA is undefined. Because the NH₂-terminal amino acid sequence of the 58-kDa form is identical to those of membrane-form HAI-1 and 40-kDa sHAI-1 (15), the 58-kDa form appears to be generated by the cleavage at the COOH-terminal region of the extracellular part of membrane-form HAI-1 (15). The similar immunoreactivity of 58-kDa sHAI-1 to C76-18 mAb and 1N7 mAb, recognized around the first and second Kunitz domains, respectively, confirmed the existence of both Kunitz domains in this form. The presence of the second Kunitz domain and free COOH terminus may somehow interfere with the binding of HGFA to the first...
domain that is responsible to the inhibition of HGFA (9) in a soluble phase. For instance, bikunin (the light chain of inter-α-trypsin inhibitor) also has two Kunitz domains, and proteinase binding to the second domain is affected by the presence of the first domain (30, 31). Although the molecular mechanism involved in the dissociation of 58-kDa sHAI-1 and HGFA after regulated shedding remains to be clarified, these observations propose a novel and unexpected mechanism for the regulation of serine proteinase activity in the pericellular microenvironment, as well as on the cell surface, mediated by an integral membrane Kunitz-type serine proteinase inhibitor. Importantly, it seems likely that the distance of cleavage site for the shedding of HGFA may aid in terminating the regulated shedding of HAI-1 by the regulated shedding of 58-kDa sHAI-1, which would be necessary to clarify the mechanism of decrease in affinity of this secreted form for active HGFA.

2 H. Kataoka, R. Hamasuna, H. Itoh, and M. Koono, unpublished observation.
HAI-1 Regulates Pericellular HGF Activation

A: Resting condition

B: Tissue injury and inflammation

FIG. 10. Hypothetical model for the regulation of pericellular HGF activity by HAI-1 in injured tissue. In a resting condition (A), HGF molecules are primarily of proform, which are circulating in the blood or locally produced by epithelial cells (5–7, 19). Spontaneous release of 40-kDa sHAI-1 containing only the first Kunitz domain (closed square), which is processed by yet unknown proteases, may efficiently inhibit the spontaneous activated HGF (9, 15). In the injured tissue (B), activation of the coagulation cascade results in thrombin generation, which in turn activates the pro-HGF (5–7). The excess active HGF would be concentrated on the epithelial cell surface through the binding to membrane-form HAI-1 that is up-regulated in the epithelial cells of injured tissue (14, 16). The bound HGF remains on the cellular surface complexed with HAI-1, and no irreversible modifications affecting the enzyme activity occur during the complex formation. Once the epithelial cells are further activated by cytokines and/or growth factors to enter the regenerating cycle, the membrane-form HAI-1 complexed with HGF is cleaved by a TACE-like proteinase, quickly generating considerable amounts of 58-kDa sHAI-1 and IL-1f peptide. 

Roles in the repair and regeneration of various epithelial tissues (22–24). HAI-1 would contribute to this process via the regulation of pericellular HGF activity that is critical in the activation of HGF (Fig. 10). Using the integral membrane HAI-1 proteins, the epithelial cells that are going to enter the regenerating process will be able to utilize active HGF efficiently in the pericellular microenvironment upon stimulation of IL-1β in injured and inflamed tissues. Although little is known about the regulatory mechanisms of HAI-1 gene expression in injured tissue, we have recently observed that consensus binding sites of several early responsive factors expressed in case of tissue injury, such as HSFs, NF-κB, and Egrs, are present in the 5′-flanking region of the HAI-1 gene (29). Indeed, IL-1β treatment induced not only up-regulated shedding of HAI-1 but also an increased level of HAI-1 mRNA. Other inflammatory stimuli also may possibly induce the enhanced expression of the HAI-1 gene.

In summary, this work shows that membrane-form HAI-1 is a specific cellular surface binding protein for active HGF acting not only as the inhibitor but also as a reservoir to ensure the pericellular HGF activity, particularly in injured and inflamed tissue where HGF could have important roles in the subsequent repair process. To the best of our knowledge, this is the first case of an integral membrane Kunitz-type inhibitor that, in fact, works on the mammalian cell surface. The dual role of HAI-1, as both inhibitor and reservoir of active enzyme, would propose a novel mechanism of the regulation of serine protease activity in a pericellular microenvironment, mediated by the integral membrane Kunitz-type inhibitor.

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vivo, because HAI-1 expression is up-regulated in the epithelial cells of injured and regenerating tissues (14, 16). Recently, a number of studies have shown that HGF could have crucial roles in the repair and regeneration of various epithelial tissues (22–24). HAI-1 would contribute to this process via the regulation of pericellular HGF activity that is critical in the activation of HGF (Fig. 10). Using the integral membrane HAI-1 proteins, the epithelial cells that are going to enter the regenerating process will be able to utilize active HGF efficiently in the pericellular microenvironment upon stimulation of IL-1β in injured and inflamed tissues. Although little is known about the regulatory mechanisms of HAI-1 gene expression in injured tissue, we have recently observed that consensus binding sites of several early responsive factors expressed in case of tissue injury, such as HSFs, NF-κB, and Egrs, are present in the 5′-flanking region of the HAI-1 gene (29). Indeed, IL-1β treatment induced not only up-regulated shedding of HAI-1 but also an increased level of HAI-1 mRNA. Other inflammatory stimuli also may possibly induce the enhanced expression of the HAI-1 gene.

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Hiroaki Kataoka, Takeshi Shimomura, Toshiya Kawaguchi, Ryouichi Hamasuna, Hiroshi Itoh, Naomi Kitamura, Keiji Miyazawa and Masashi Koono

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