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Hepatocyte growth factor activator inhibitor type 1 inhibits protease activity and proteolytic activation of human airway trypsin-like protease

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Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a Kunitz-type transmembrane serine protease inhibitor initially identified as a potent inhibitor of hepatocyte growth factor activator (HGFA), a serine protease that converts pro-HGF to the active form. HAI-1 also has inhibitory activity against serine proteases such as matriptase, hepsin and prostasin. In this study, we examined effects of HAI-1 on the protease activity and proteolytic activation of human airway trypsin-like protease (HAT), a transmembrane serine protease that is expressed mainly in bronchial epithelial cells. A soluble form of HAI-1 inhibited the protease activity of HAT *in vitro*. HAT was proteolytically activated in cultured mammalian cells transfected with its expression vector, and a soluble form of active HAT was released into the conditioned medium. The proteolytic activation of HAT required its own serine protease activity. Co-expression of the transmembrane full-length HAI-1 inhibited the proteolytic activation of HAT. In addition, full-length HAI-1 associated with the transmembrane full-length HAT in co-expressing cells. Like other target proteases of HAI-1, HAT converted pro-HGF to the active form *in vitro*. These results suggest that HAI-1 functions as a physiological regulator of HAT by inhibiting its protease activity and proteolytic activation in airway epithelium.

Keywords: hepatocyte growth factor activator inhibitor type 1 (HAI-1)/Kunitz-type inhibitor/ human airway trypsin-like protease (HAT)/type-II transmembrane serine protease/proteolytic activation.

Abbreviations: FBS, fetal bovine serum; HAI-1, hepatocyte growth factor activator inhibitor type 1; HAT, human airway trypsin-like protease; HGFA, hepatocyte growth factor activator; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is a Kunitz-type serine protease inhibitor initially identified as a potent inhibitor of hepatocyte growth factor activator (HGFA), a blood coagulation factor XII-like serine protease that converts pro-HGF to the active form (*1*). Subsequently, HAI-1 has been shown to also have inhibitory activity against serine proteases such as matriptase/MT-SP1, hepsin and prostasin (*2–5*). Physiological roles of HAI-1 were determined by analyzing knockout mice. Homozygous deletion of HAI-1 in mice resulted in embryonic lethality due to impaired formation of the placental labyrinth layer, thus HAI-1 is essential for placental development (*6*, *7*). Matriptase/HAI-1 double-deficient mice formed the placental labyrinth and developed to term, indicating that HAI-1 is an essential matriptase inhibitor during placental development in the mouse embryo (*8*). Moreover, although HAI-1-deficient neonates were successfully delivered on rescue of the placental formation, they died within 16 days after delivery with skin abnormalities, indicating that HAI-1 also has critical roles in the regulated keratinization of the epidermis (*9*).

HAI-1 is predominantly expressed in the simple columnar epithelium of the ducts, tubules and mucosal surface of various tissues (*10*). Thus, HAI-1 might regulate the catalytic activity of each serine protease expressed by these epithelial cells. Recently, a detailed immunohistochemical analysis of HAI-1 in human pulmonary tissue revealed that HAI-1 is expressed in bronchial respiratory epithelial cells, and this expression is upregulated in ciliated epithelial cells in response to tissue injuries. Possible target proteases of HAI-1 in bronchial epithelial cells have also been proposed (*11*). One attractive candidate is human airway trypsin-like protease (HAT), also known as TMPRSS11D. HAT is located on ciliated cells of the bronchial epithelium and its distribution is quite similar to that of HAI-1 (*12*). Moreover, mRNAs of HAI-1 and HAT were detected in primary bronchial epithelial cells (*13*). Thus, HAI-1 might regulate the protease activity of HAT in airway tissues.

HAT is a serine protease purified from the sputum of patients with chronic airway diseases such as chronic bronchitis and bronchial asthma (*14*). Several substrate proteins of this protease have been identified. First, HAT proteolytically activates protease-activated receptor-2 (PAR-2) (*15*). This activation stimulates human bronchial fibroblasts to proliferate (*16*), and upregulates mucin gene expression in airway epithelial cells (*17*), suggesting that HAT is responsible for fibrosis and remodelling in chronic airway disease as well as mucus hypersecretion. Second, HAT proteolytically

converts the full-length urokinase receptor (uPAR) to the truncated form, thus HAT is likely to modulate cell adherence and motility, as well as tissue remodelling during the inflammatory response in the airways (*18*). Third, HAT cleaves the precursor of the haemagglutinin protein (HA) of influenza viruses into disulphide-linked subunits (*19*). Since the cleavage of HA is essential for infection, HAT may be responsible for infections of influenza viruses in the respiratory tract.

HAT belongs to the type-II transmembrane serine protease family (*20*), the members of which are characterized by an N-terminal transmembrane domain and a C-terminal extracellular serine protease domain (*21*). HAT has a SEA domain between the transmembrane domain and the seine protease domain. The SEA domain has been found in a number of transmembrane proteins, and serves as a site for proteolytic cleavage (*22*). HAT is proteolytically converted from the transmembrane precursor into a soluble form corresponding to the C-terminal extracellular catalytic domain, and this soluble form has protease activity (*20*). However, a protease responsible for the conversion has not been identified, and the regulation of the proteolytic activation of HAT has not been clarified. Matriptase also belongs to the type-II transmembrane serine protease family. Matriptase is proteolytically activated by conversion of a single-chain zymogen to the two-chain form, and this activation depends on its own active site triad (*23*). In addition to the inhibition of protease activity, HAI-1 is required for the proteolytic activation of matriptase in cells co-expressing matriptase and HAI-1 (*23*). Thus, it is possible that HAI-1 regulates the proteolytic activation of HAT in cells co-expressing HAI-1 and HAT.

In this study, we first demonstrated that HAI-1 has an inhibitory effect on the protease activity of HAT. Then, we examined effects of the co-expression of HAI-1 and HAT in cultured cells on the proteolytic activation of HAT. The results demonstrated that the transmembrane full-length HAI-1 inhibits the proteolytic activation of HAT by associating with the protease.

Materials and Methods

Materials

Reagents were obtained as follows: the recombinant HAT, a monoclonal antibody which recognizes the protease domain of HAT and an anti-HAI-1 ectodomain monoclonal antibody from R&D systems (Minneapolis, MN, USA); the substrate Boc-Ile-Gln-Gly-Arg-MCA (Boc-IQGR-MCA) from Peptide Institute (Osaka, Japan); a horseradish peroxidase (HRP)-conjugated anti-goat IgG antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); an anti-FLAG-HRP antibody, anti-FLAG M2 affinity resin and aprotinin from SIGMA (St Louis, MO, USA); and protein G sepharose and an anti-mouse IgG-HRP antibody from GE Healthcare UK Ltd. (Buckingamshire, England). The recombinant pro-HGF was prepared as described previously (*24*).

Construction

The cDNA clone for full-length HAT was obtained from a human lung cDNA library (Takara, Kyoto, Japan) by PCR, tagged with 3 × FLAG at the N-terminal end (N-FLAG-HAT), and subcloned into an expression vector, pEF6-mycHis-A (Invitrogen, Carlsbad, CA, USA). The cDNA clones for the full-length HAI-1, NK1 and NK1LK2 were obtained as described previously (*25*). These cDNAs were subcloned into an expression vector, pcDNA3.1-mycHis-A (Invitrogen). Point mutations were introduced with a Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) using primers containing appropriate nucleotide changes. All site-directed mutations were confirmed by DNA sequencing.

Cell culture and transfection

The human embryonic kidney cell line HEK293 was purchased from ATCC (Manassas, VA, USA), and maintained in Dulbecco's modified eagle medium (SIGMA) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Cells were seeded on 6-well collagen-coated plates (IWAKI, Chiba, Japan) 1 day prior to transfection. Transfection was accomplished using Lipofectamine with Plus reagent (Invitrogen) in serum-free medium (500 µl). After incubation for 3 h, medium containing 2% FBS (500 µl) was added, and the cells were cultured at 37°C overnight. For co-transfections, the ratio of pEF6-N-FLAG-HAT to pcDNA3.1-HAI-1 was kept at 4 : 1 (wt/wt).

Enzyme inhibition assay

HAI-1-NK1 and HAI-1-NK1LK2 were prepared as follows. The expression vectors encoding HAI-1-NK1 and HAI-1-NK1LK2 were introduced into CHO cells using Superfect transfection reagent (Qiagen, Hilden, Germany). Transfected cells were cultured at 37°C overnight. The medium was replaced with fresh medium containing Geneticin (G418). Neomycin-resistant colonies were selected and further cultured in a roller bottle. When the cells became confluent, the medium was replaced with serum-free medium, and the cells were further cultured for 5 days. The proteins were purified from the conditioned medium by column chromatography using nickel-nitrilotriacetic acid and anti-c-Myc antibody resins.

The hydrolytic activity of HAT was measured as follows. The recombinant HAT (0.12 nM) and a series of concentrations of inhibitors were mixed and incubated in the assay buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl and 0.05% Brij 35] for 5 min at room temperature. Then Boc-IQGR-MCA was added to the mixture at a final concentration of 100 µM. The final volume of each mixture was 200 µl. After 30 min at 37°C, the fluorescence (Ex: 360 nm/Em: 460 nm) of the mixture was measured using a fluorometer (ARVO, Perkin Elmer Life Science, Boston, MA, USA). The enzyme activity without inhibitors was used as uninhibited control. The IC_{50} was defined as the concentration of inhibitors which inhibited the enzyme activity by 50% compared with uninhibited control. The percentage of uninhibited control was plotted versus the log of inhibitor concentrations, and the IC_{50} values were calculated using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Immunoblot analysis

Cell lysate for immunoblotting and immunoprecipitation was prepared as follows. Cells were washed in phosphate-buffered saline (pH 7.4, PBS), then lysed with 500 µl of RIPA buffer [0.1% Nonidet-P40, 0.5% sodium deoxycholate and 0.1% SDS in PBS (pH 7.4)] containing a protease inhibitor cocktail (Thermo Fisher Scientific, Rockford, IL, USA) at 4°C overnight. After centrifugation for 10 min at 15,000 rpm, the soluble fraction was collected. The concentration of protein in the cell lysate was determined using micro BCA protein assay reagents (Thermo Fisher Scientific). The conditioned medium was harvested, and the supernatant was obtained by removing cell debris. The cell lysate and conditioned medium were mixed with 5 × sample buffer [125 mM Tris–HCl (pH 6.8), 4.3% SDS, 30% glycerol, 10% 2-mercaptoethanol and 0.01% bromophenol blue], and heated for 5 min at 95°C. Aliquots of the samples were separated by SDS–PAGE, and the proteins in the gel were transferred electrophoretically to a PVDF membrane (Invitrogen). The blotted membrane was treated with blocking solution (5% non-fat dry milk in PBS). For the detection of N-terminal HAT, the membrane was incubated with a HRP-conjugated anti-FLAG M2 mouse monoclonal antibody in blocking solution. For the detection of the HAT protease domain, the membrane was incubated with an anti-HAT mouse monoclonal antibody, then washed with PBST (0.05% Tween-20 in PBS), and incubated with an anti-mouse IgG-HRP antibody. For the detection of HAI-1, the membrane was incubated with an anti-HAI-1 goat antibody, followed by an anti-goat

IgG-HRP antibody. After washing with PBST, the proteins were visualized with an ECL chemiluminescence system (GE Healthcare).

Immunoprecipitation

Cell lysate (100 µl) diluted 10-fold with PBS was reacted with the anti-HAI-1 antibody (2 µg) or anti-FLAG M2 affinity resin (30 µl) at 4°C overnight. The samples incubated with the anti-HAI-1 antibody were then mixed with protein G-sepharose and incubated at 4°C for 3 h. The samples were washed twice with PBST, once with PBS and once with 150 mM NaCl at 4°C. The immunoprecipitate was eluted with 20 mM HCl, neutralized with a 1/10 volume of 1M Tris–HCl (pH 8.0), and then mixed with sample buffer. The samples were subjected to immunoblotting as described above.

Assay for HGF-activating activity of HAT

Four micrograms of pro-HGF was mixed with various amounts of recombinant HAT in 20 µl of PBS containing 0.1% CHAPS and incubated at 37°C for 30 min. The mixture was separated by SDS–PAGE under reducing conditions. To examine the inhibitory effect of HAI-1-NK1 on the HGF-converting activity of HAT, HAT (10 ng) was incubated with HAI-1-NK1 (100 ng) in PBS containing 0.1% CHAPS at room temperature for 5 min. Then, pro-HGF (4 µg) was added to the mixture. The final volume of the mixture was 20 µl. After incubation at 37°C for 30 min, the reaction mixture was analysed by SDS–PAGE. Proteins in the gel were stained with 2.5% Coomassie Brilliant Blue in a water/methanol/acetic acid solution (6 : 3 : 1, v/v). The biological activity of the activated HGF was assessed by cell scattering using HepG2 hepatoma cells (*26*). HepG2 cells were treated with the reaction mixture at 50 ng/ml pro-HGF and then cultured for 4 days. The morphology of the cells was analysed by light microscopy.

Results

Inhibition of HAT protease activity by soluble HAI-1

Inhibition of the protease activity of HAT by HAI-1 was assessed using soluble forms of HAI-1. HAI-1 is first produced as a 66-kDa transmembrane form, and subsequent ectodomain shedding releases two major soluble forms from the cell surface into the extracellular space. The sizes of the soluble forms are 40 and 58 kDa. The 40-kDa HAI-1 consists of the N-terminal region (N) and one Kunitz domain (K1), and shows strong inhibitory activity against the HGF-converting activity of HGFA. The 58-kDa HAI consists of the N-terminal region (N), two Kunitz domains (K1 and K2) and the LDL receptor class A domain (L) between the two Kunitz domains, and shows weaker inhibitory activity against HGFA (*27*). We constructed expression vectors encoding HAI-1-NK1 and -NK1LK2 (Fig. 1A) corresponding to the 40- and 58-kDa HAI-1, respectively, and stably transfected CHO cells with them. The proteins were purified from the conditioned medium of the transfected cells, and subjected to an assay of the inhibitory activity against HAT. A commercially available HAT was used for the assay, and the protease activity of HAT was measured using a synthetic substrate (Boc-IQGR-MCA). HAI-1-NK1 inhibited the protease activity ($IC_{50} = 0.85$ nM) (Fig. 2), and its inhibitory effect was more potent than that of aprotinin ($IC_{50} = 9.86$ nM), previously reported as an inhibitor of HAT (*14*). In contrast, HAI-1-NK1LK2 showed almost no inhibitory activity against HAT (Fig. 2).

Proteolytic activation of HAT by the catalytic activity of its own serine protease domain

HAT is first produced as a 48-kDa transmembrane form. Proteolytic cleavage generates an active form of HAT with a molecular size of 27 kDa, and releases it into the extracellular space (*20*). However, the proteolytic activation of HAT in cultured mammalian cells has not been well characterized. Therefore, we first examined the activation in cultured HEK293 cells transiently transfected with an expression vector encoding an N-terminally FLAG-tagged HAT (Fig. 1B). The cell lysate and conditioned medium were subjected to SDS–PAGE under reducing conditions, followed by an immunoblot analysis. The immunoblot analysis of the cell lysate using an anti-FLAG antibody showed three bands of 52, 25 and 15 kDa (Fig. 3A). The 52-kDa band corresponded to the full-length HAT. The detection of the 25- and 15-kDa bands indicated at least two cleavage sites

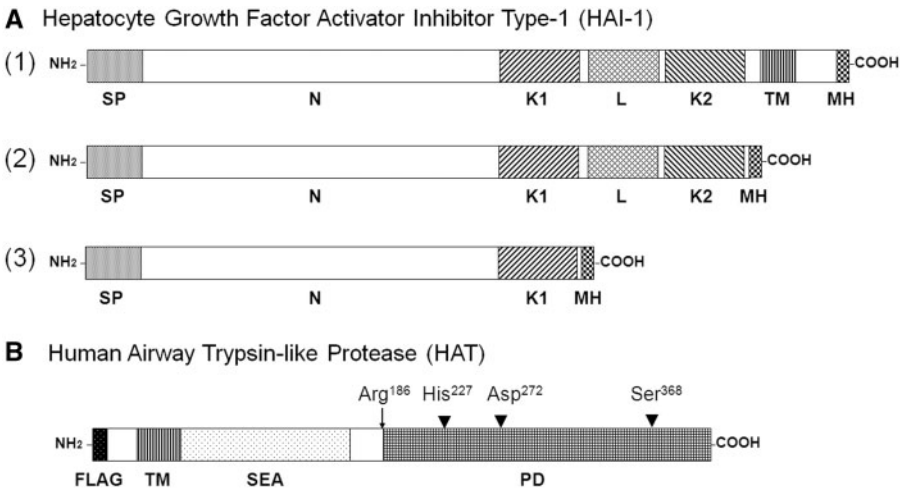

Fig. 1 Schematic structures of HAI-1 and HAT used for transfection experiments. (A) The structures of full-length HAI-1 (1), HAI-1-NK1LK2 (2) and HAI-1-NK1 (3) tagged C-terminally by mycHis. SP, signal peptide; N, N-terminal region; K1, Kunitz domain 1; L, LDL receptor class A domain; K2, Kunitz domain 2; TM, transmembrane domain; MH, mycHis tag. (B) The structure of HAT tagged N-terminally by 3 × FLAG. FLAG, 3 × FLAG; TM, transmembrane domain; SEA, SEA domain; PD, serine protease domain. The activation cleavage site (Arg 186) and the catalytic triad (His 227, Asp 272 and Ser 368) are indicated by an arrow and arrowheads, respectively.

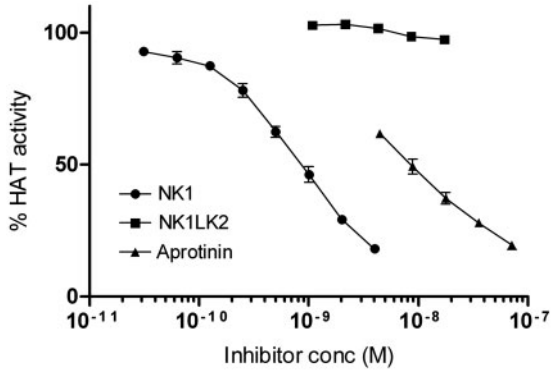

Fig. 2 Dose dependence of the inhibitory activity of soluble forms of HAI-1 against the protease activity of HAT. HAT was incubated with various concentrations of HAI-1-NK1 (filled circles), HAI-1-NK1LK2 (filled squares) or aprotinin (filled triangles). Then, Boc-IQGR-MCA was added and after further incubation, the fluorescence of the reaction mixtures was measured. Each value represents the mean ± SD of triplicate measurements.

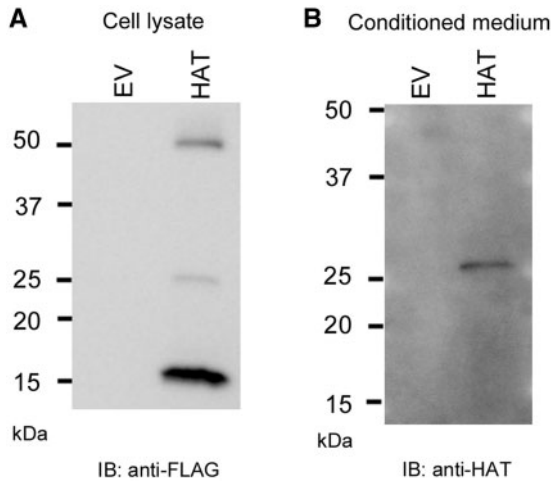

Fig. 3 Proteolytic activation of HAT in mammalian cells. The expression vector encoding the N-terminal FLAG-tagged HAT or empty vector (EV) was transfected into HEK293 cells. (A) Cell lysate was analysed by immunoblotting with the anti-FLAG antibody, which recognizes the N-terminal fragment of HAT. (B) Conditioned medium was analysed by immunoblotting with the anti-HAT antibody, which recognizes the catalytic domain of HAT.

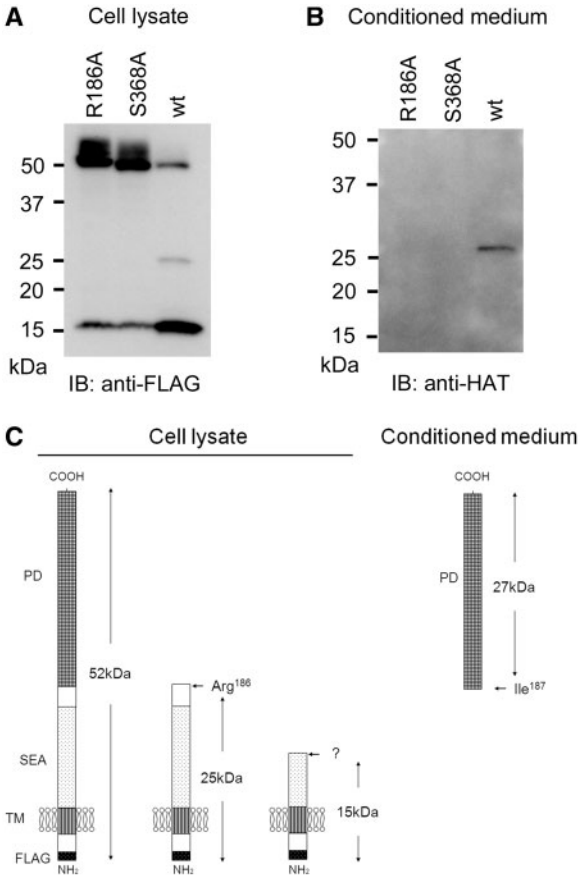

Fig. 4 Effect of point mutations at the proteolytic activation site and the catalytic triad on the proteolytic activation of HAT. The expression vector encoding wild-type HAT (wt), HAT R168A or HAT S368A was transfected into HEK293 cells. (A) Cell lysate was analysed by immunoblotting with the anti-FLAG antibody. (B) Conditioned medium was analysed by immunoblotting with the anti-HAT antibody. (C) Schematic representation of proteolytic cleavage of HAT exogenously expressing in HEK293 cells.

within the HAT protein. The immunoblot analysis of the conditioned medium using an anti-HAT antibody, which recognizes the catalytic domain, showed a 27-kDa band (Fig. 3B). The 27-kDa protein seems to be an active form of HAT, because it is quite similar in size to the active HAT purified from the sputum of patients with chronic airway diseases.

The active HAT is produced from its precursor by proteolytic cleavage between Arg 186 and Ile 187 (*20*). To examine whether the 27-kDa protein detected in the conditioned medium is a product of the cleavage, we constructed an expression vector encoding a mutant HAT, in which Arg 186 was replaced with Ala (R186A). HEK293 cells were transiently transfected with the vector, and the expressed HAT was subjected to an immunoblot analysis. The 25-kDa band in the lysate and the 27-kDa band in the conditioned medium were not detected (Fig. 4A and B). These results further support that the 27-kDa protein is the active form of HAT, and suggests that the 25-kDa protein in the lysate is the N-terminal counterpart product of the cleavage (Fig. 4C).

Next, to examine whether production of the 27-kDa protein requires the catalytic activity of the serine protease domain, we constructed an expression vector encoding a mutant HAT, in which a serine residue (Ser 368) of the catalytic triad was replaced with Ala (S368A), and introduced it into HEK293 cells. The 25-kDa band in the lysate and the 27-kDa band in the conditioned medium were not detected (Fig. 4A and B), suggesting the activation of HAT by the catalytic activity of the serine protease domain of HAT.

The 15-kDa protein detected in the lysate is likely to be an N-terminal fragment produced by proteolytic cleavage within the SEA domain (Fig. 4C). The 15-kDa fragment was detected in cells expressing HAT R186A or HAT S368A, but was much weaker than that in cells expressing wild-type HAT (Fig. 4A). These results suggest that the cleavage generating the active HAT is dispensable for the N-terminal cleavage

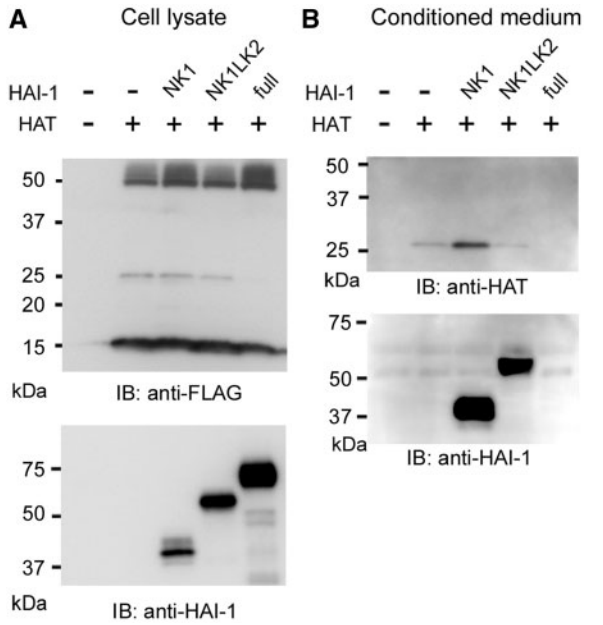

Fig. 5 Effect of co-expression of HAI-1 on the proteolytic activation of HAT. The expression vector encoding HAT and expression vector encoding HAI-1-NK1, HAI-1-NK1LK2 or full-length HAI-1 were co-transfected into HEK293 cells. Cell lysate (A) and conditioned medium (B) were analysed by immunoblotting with the anti-FLAG antibody (A, upper) or anti-HAI-1 antibody (A, lower) and with the anti-HAT antibody (B, upper) or anti-HAI-1 antibody (B, lower).

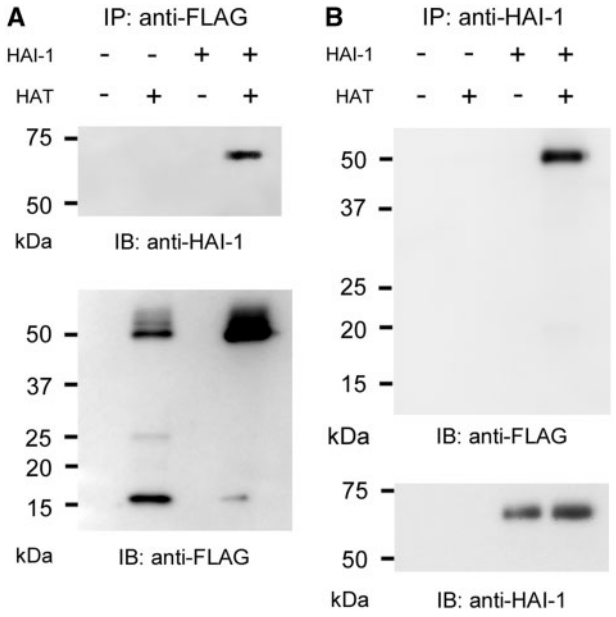

Fig. 6 Association of HAT with HAI-1. The expression vector encoding HAT was transfected with (+) or without (−) the expression vector encoding HAI-1 into HEK293 cells. Cell lysate was immunoprecipitated (IP) with the anti-FLAG antibody (A) or anti-HAI-1 antibody (B). The immunoprecipitates were immunoblotted with the anti-HAI-1 antibody (A, upper) or anti-FLAG antibody (B, upper). The immunoprecipitates were also analysed by immunoblotting with the anti-FLAG antibody (A, lower) or anti-HAI-1 antibody (B, lower) to verify the expression of HAT and HAI-1.

generating the 15-kDa fragment, but enhances the cleavage. The C-terminal counterpart product of the cleavage, the size of which is calculated as 37 kDa, was not detected in the conditioned medium of the cells expressing wild-type HAT, HAT R186A or HAT S368A (Figs 3B and 4B). These results suggest that the 37-kDa product may be unstable, probably because it has an extra N-terminal sequence.

Inhibition of proteolytic activation of HAT by HAI-1

To examine whether HAI-1 regulates the proteolytic activation of HAT, we transiently co-transfected HEK293 cells with the expression vector encoding N-FLAG-HAT and expression vector encoding the full-length HAI-1, HAI-1-NK1 or HAI-1-NK1LK2. Co-expression of NK1 or NK1LK2 did not affect the generation of the 25- and 15-kDa bands in the cell lysate (Fig. 5A). Whereas co-expression of NK1LK2 did not significantly affect the generation of the 27-kDa band in the conditioned medium, co-expression of NK1 increased the intensity of the 27-kDa band (Fig. 5B). These results suggest that co-expression of the soluble forms of HAI-1 (NK1 and NK1LK2) did not affect the proteolytic activation of HAT. The increase in the 27-kDa band in the conditioned medium of cells co-expressing NK1 suggests that NK1 forms a strong complex with the activated HAT and stabilizes it. In contrast, the 25-kDa band in the cell lysate and the 27-kDa band in the conditioned medium were not detected on co-expression of the full-length HAI-1 (Fig. 5A and B), suggesting that the full-length HAI-1 inhibits the proteolytic activation of HAT. The 15-kDa band in the lysate was observed in the cells co-expressing the full-length HAI-1, although it was weaker (Fig. 5A), suggesting that co-expression of the full-length HAI-1 does not inhibit the cleavage generating the 15-kDa fragment.

Association of HAT with HAI-1

The inhibitory effect of the full-length HAI-1 on the proteolytic activation of HAT suggested that HAT is associated with HAI-1 in co-expressing cells. To test this possibility, the association of HAT with HAI-1 was examined by co-immunoprecipitation. HEK293 cells were co-transfected with expression vectors encoding N-FLAG-HAT and the full-length HAI-1. Lysate of the cells was immunoprecipitated with the anti-FLAG antibody, and the immunoprecipitates were immunoblotted with the anti-HAI-1 antibody. HAI-1 was detected in the immunoprecipitates from the co-transfected cells (Fig. 6A). Similarly, the full-length HAT was detected in the immunoprecipitates obtained with the anti-HAI-1 antibody, by immunoblotting with the anti-FLAG antibody (Fig. 6B). These results indicate that the full-length form of HAT is associated with the full-length HAI-1.

Proteolytic activation of pro-HGF by HAT

Pro-HGF is activated by limited proteolysis (*28*). Several serine proteases have been shown to activate pro-HGF. Among them, the protease activity of HGFA, matriptase and hepsin is inhibited by HAI-1 (*1–4*). Thus, it is possible that pro-HGF is also activated by HAT. To test this possibility, we incubated

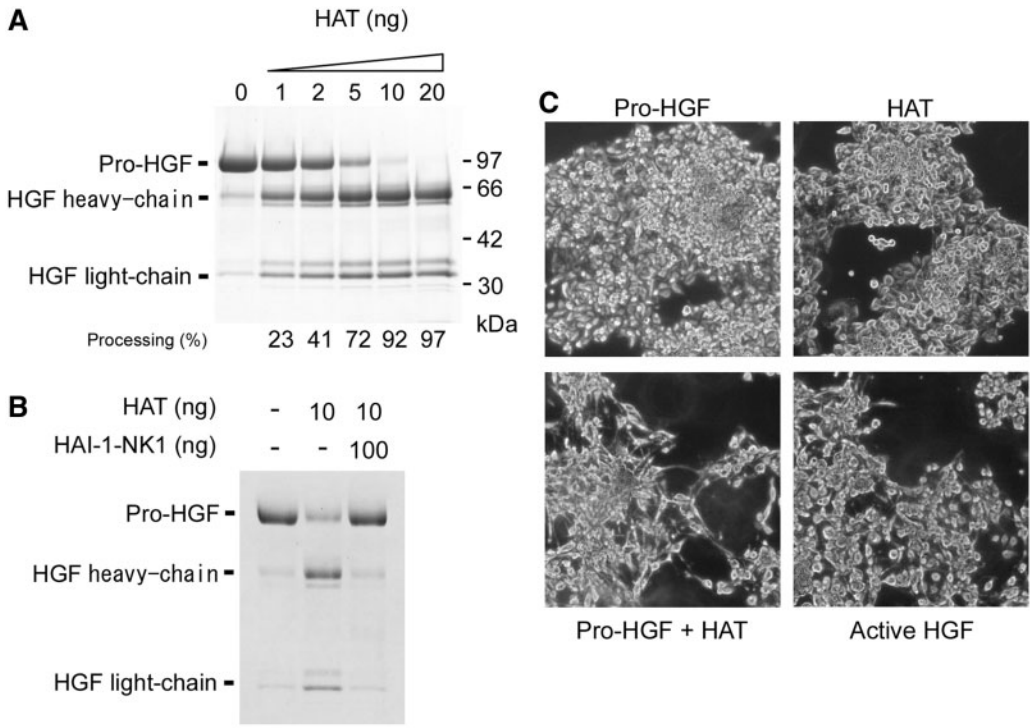

Fig. 7 Proteolytic activation of pro-HGF by HAT. (A) Four micrograms of pro-HGF was incubated with various concentrations of HAT. The reaction mixtures were separated by SDS–PAGE under reducing conditions. The gel was stained with Coomassie Brilliant Blue. Extent of processing (%) is shown below the stained gel. (B) Four micrograms of pro-HGF was incubated with HAT (10 ng) pretreated with or without HAI-1-NK1 (100 ng). The reaction mixtures were analysed as described in (A). (C) HepG2 cells were treated with reaction mixtures of pro-HGF alone (Pro-HGF), HAT alone (HAT) or pro-HGF and 20 ng of HAT (Pro-HGF + HAT) at 50 ng/ml pro-HGF. Cells were also treated with purified active HGF at 50 ng/ml (Active HGF). Then, cells were cultured for 4 days. The morphology of the cells was analysed by light microscopy.

pro-HGF with a commercially available active HAT, and analysed the products by SDS–PAGE under reducing conditions. The incubation generated two main bands of ~60 and 32 kDa (Fig. 7A). The sizes corresponded to the heavy chain and light chain of activated HGF. We then analysed the effect of HAI-1-NK1 on the pro-HGF converting activity of HAT. The pro-HGF (4 μg) was incubated with HAT (10 ng) pretreated with or without HAI-1-NK1 (100 ng). The pretreatment of HAT with HAI-1-NK1 did not generate the 60- and 32-kDa bands (Fig. 7B), indicating that HAI-1-NK1 inhibits the pro-HGF converting activity of HAT.

To examine whether the reaction product of pro-HGF incubated with HAT is biologically active, the reaction mixture was subjected to a scattering assay using HepG2 human hepatoma cells. Addition of the reaction mixture led to scattered morphology of HepG2 cells (Fig. 7C). These results indicate that HAT has activity to convert pro-HGF to the active form *in vitro*.

Discussion

A soluble HAI-1 (NK1) with only the N-terminal Kunitz domain (Kunitz 1) had an inhibitory effect on the protease activity of HAT, which was more potent than that of aprotinin previously described as a strong inhibitor of HAT (Fig. 2). Active HAT was originally purified from the sputum of patients with chronic airway diseases (*14*), and HAT is expressed mainly in ciliated cells of bronchial epithelium (*12*). Bronchial epithelial cells are also HAI-1-producing cells (*11*). Thus, HAI-1 could function as an endogenous inhibitor of HAT in airway tissues. Another soluble HAI-1 (NK1LK2) with two Kunitz domains showed almost no inhibitory activity against HAT (Fig. 2), suggesting that the C-terminal Kunitz domain (Kunitz 2) interferes with the binding of Kunitz 1 to the protease domain of HAT. Similar interference was observed on the binding of Kunitz 1 to the protease domain of HGFA or matriptase (*25*, *29*). Thus, proteolytic cleavage to generate HAI-1 with only Kunitz 1 is required for full inhibitory activity against HAT, similar to that against HGFA and matriptase.

The proteolytic cleavage to generate the active form of HAT has been shown by exogenous expression of the full-length HAT in insect cells (*20*). The detection of the active HAT in lysate of insect cells suggests that the proteolytic activation of HAT occurs within the cells and releases the active protease into the extracellular space. In this study, we demonstrated similar proteolytic activation of HAT in mammalian cells exogenously expressing the full-length HAT. We detected the active HAT mainly in the conditioned medium (Fig. 3), and hardly detected it in the cell lysate (data not shown), suggesting a rapid release of the active HAT into the extracellular space of mammalian cells. A point mutation of the catalytic triad (S368A) revealed that the proteolytic activation of

HAT requires its own serine protease activity (Fig. 4). Proteolytic activation of matriptase also requires its own protease activity. A transactivation mechanism has been proposed for the activation of matriptase, but an autocatalytic mechanism whereby matriptase activates itself in an intramolecular fashion is excluded because of the structural inaccessibility of the activation cleavage site to the active site in the catalytic domain (*23*). Since the activation cleavage site in HAT is located at a similar position to that in matriptase, the proteolytic activation of HAT probably occurs by the transactivation mechanism.

In addition to activation cleavage, cleavage to generate the N-terminal 15-kDa fragment was observed (Fig. 3). The fragment was generated in cells expressing HAT S368A with a point mutation of the catalytic triad (Fig. 4), indicating that the cleavage does not require the enzyme's own serine protease activity. The fragment was also generated in cells expressing HAT R168A with a point mutation of the activation cleavage site (Fig. 4), suggesting that the cleavage occurs independently of the activation cleavage. However, there was much less of the 15-kDa fragment in the cells expressing the HAT mutants than in the cells expressing wild-type HAT (Fig. 4), suggesting that the activation cleavage enhances the N-terminal cleavage. Based on molecular size, the cleavage site is probably located within the SEA domain between the transmembrane region and the catalytic domain. The SEA domain has been found in a number of transmembrane proteins, and serves as a site for proteolytic cleavage. A consensus sequence Gly-Ser-Val-Val-Val within the SEA domain was assigned as the exact cleavage site, and the cleavage takes place just downstream of the glycine residue (*22*). However, the SEA domain of HAT does not contain the consensus sequence. Further analyses are needed to determine the cleavage site and characterize the role of the cleavage in the activation of HAT.

Co-expression experiments revealed that the transmembrane full-length HAI-1 inhibits the proteolytic activation of HAT (Fig. 5). Furthermore, the full-length HAI-1 was co-immunoprecipitated with the transmembrane form of HAT in the lysate of cells co-expressing the proteins (Fig. 6). These results suggest that in co-expressing cells, the transmembrane full-length HAI-1 associates with HAT, and inhibit the catalytic activity of the protease domain required for the proteolytic activation of HAT. As described above, Kunitz 2 in a soluble HAI-1 (NK1LK2) seems to interfere with the binding of Kunitz 1 to the protease domain of HAT. However, the transmembrane full-length HAI-1, which also has two Kunitz domains, inhibited the proteolytic activation of HAT, suggesting that Kunitz 2 in the transmembrane full-length HAI-1 does not interfere with the binding of Kunitz 1 to HAT. Similar effects of Kunitz 2 were observed on the binding of Kunitz 1 to the protease domain of HGFA: the transmembrane full-length HAI-1 had a high affinity for HGFA, whereas the soluble 58-kDa HAI-1, which corresponds to NK1LK2, had a weak affinity for HGFA (*30*).

A detailed analysis of the intracellular trafficking of HAI-1 in Madin-Darby Canine Kidney cells revealed that after its biosynthesis, HAI-1 is exocytosed mainly to the basolateral plasma membrane, and then a portion is transcytosed to the apical plasma membrane, suggesting that HAI-1 plays a role in the trafficking of associated proteins within the cells (*31*). It has been shown that HAI-1 regulates the intracellular trafficking of matriptase (*32*). Thus, it is likely that HAI-1 is involved in the intracellular trafficking of HAT in co-expressing cells. During such trafficking, the proteolytic activation of HAT could be inhibited by the Kunitz domain of HAI-1. Co-expression of HAI-1 and HAT has been suggested in bronchial epithelium (*11–13*). Moreover, the epithelial expression of HAI-1 is augmented in response to tissue injury such as cancer invasion and inflammation (*11*). Thus, the intracellular trafficking and proteolytic activation of HAT in bronchial epithelial cells might be regulated by the expression level of HAI-1, which changes with the physiological status of airway epithelium. Co-expression of soluble forms of HAI-1 (NK1 and NK1LK2) with HAT did not inhibit the proteolytic activation of HAT (Fig. 5), although NK1 inhibited the protease activity of HAT *in vitro* (Fig. 2). These results suggest that the soluble forms of HAI-1 do not associate with the transmembrane form of HAT in co-expressing cells. Because the soluble forms of HAI-1 lack the C-terminal region including the transmembrane domain, the region may be required for the association with the transmembrane form of HAT. Alternatively, only the transmembrane form of HAI-1 could be co-localized with the transmembrane form of HAT during intracellular trafficking.

Based on the results presented in this study and from previous reports, we propose the model displayed in Fig. 8 to illustrate the inhibitory effect of HAI-1 on proteolytic activation and protease activity of HAT. In co-expressing cells, the transmembrane full-length HAI-1 associates with the transmembrane full-length HAT, and inhibits the proteolytic activation of HAT (Fig. 8A). Ectodomain shedding of the transmembrane form of HAI-1 releases two major soluble forms, NK1 and NK1LK2, into the extracellular space (*27*). The soluble forms of HAI-1 do not associate with the transmembrane form of HAT, thus the ectodomain shedding results in the proteolytic activation of HAT. The activated HAT is released into the extracellular space. The protease activity of the released HAT is inhibited by HAI-1-NK1, but not by HAI-1-NK1LK2 (Fig. 8B). HAI-1 is required for the proteolytic activation of matriptase in cells co-expressing matriptase and HAI-1 (*23*). In this study, we showed that HAI-1 inhibits the proteolytic activation of HAT. Thus, our study revealed that HAI-1 has different effects on the proteolytic activation of members of the type-II transmembrane serine protease family.

Several endogenous substrates for the protease activity of HAT have been proposed (*15–19*). In this study, we demonstrated that HAT also has activity to convert pro-HGF to the active form *in vitro*, and HAI-1-NK1 inhibits the converting activity (Fig. 7).

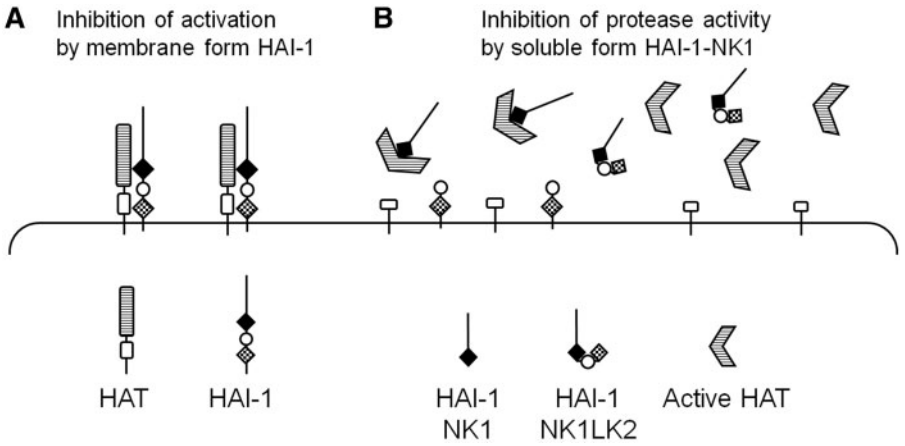

Fig. 8 Proposed model for the inhibitory effect of HAI-1 on proteolytic activation and protease activity of HAT. In co-expressing cells, the transmembrane full-length HAI-1 associates with the transmembrane full-length HAT, and inhibits the proteolytic activation of HAT (A). Ectodomain shedding of the transmembrane form of HAI-1 releases two major soluble forms, NK1 and NK1LK2, into the extracellular space. The soluble forms of HAI-1 do not associate with the transmembrane form of HAT, thus the ectodomain shedding results in the proteolytic activation of HAT. The activated HAT is released into the extracellular space. The protease activity of the released HAT is inhibited by HAI-1-NK1, but not by HAI-1-NK1LK2 (B).

HGF is a mesenchymal-derived growth factor, and functions as a potent mitogen for various epithelial cells (*33*, *34*). HGF is thought to play an important role in the regeneration of injured tissues (*35*). HGF is proteolytically activated in response to tissue injury, such as liver and kidney injury, and this activation is mediated by an enzymatic activity which is induced exclusively in the injured tissues (*35*). Thus, the proteolytic activation system may function as a mechanism for localizing HGF activation to injured tissue. Because active HAT is isolated from the sputum of patients with chronic airway diseases (*14*), HAT may be responsible for HGF activation in response to injury of airway tissues. HAI-1 could regulate the activity of HGF through inhibition of the protease activity of HAT in the tissues.

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Conflict of interest

None declared.

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