Matrix metalloproteinase-7 (MMP-7) plays important roles in tumor progression and metastasis. Our previous studies have demonstrated that MMP-7 binds to colon cancer cells via cell surface–bound cholesterol sulfate and induces significant cell aggregation by cleaving cell-surface protein(s). These aggregated cells exhibit a dramatically enhanced metastatic potential. However, the molecular mechanism inducing this cell–cell adhesion through the proteolytic action of MMP-7 remained to be clarified. Here, we explored MMP-7 substrates on the cell surface; the proteins on the cell surface were first biotinylated, and a labeled protein fragment specifically released from the cells after MMP-7 treatment was analyzed using LC-MS/MS. We found that hepatocyte growth factor activator inhibitor type 1 (HAI-1), a membrane-bound Kunitz-type serine protease inhibitor, is an MMP-7 substrate. We also found that the cell-bound MMP-7 cleaves HAI-1 mainly between Gly451 and Leu452 and thereby releases the extracellular region as soluble HAI-1 (sHAI-1). We further demonstrated that this sHAI-1 can induce cancer cell aggregation and determined that the HAI-1 region corresponding to amino acids 141–249, which does not include the serine protease inhibitor domain, has the cell aggregation–inducing activity. Interestingly, a cell-surface cholesterol sulfate–independent proteolytic action of MMP-7 is critical for the sHAI-1–mediated induction of cell aggregation, whereas cholesterol sulfate is needed for the MMP-7–catalyzed generation of sHAI-1. Considering that MMP-7–induced cancer cell aggregation is an important mechanism in cancer metastasis, we propose that sHAI-1 is an essential component of MMP-7–induced stimulation of cancer metastasis and may therefore represent a suitable target for antimetastatic therapeutic strategies.

This work was supported in part by the grant for Research Development Fund (No. 5G2802) of Yokohama City University, Japan (to S. H.), an Extra-mural Collaborative Research Grant of the Cancer Research Institute, Kanazawa University, Japan (to S. H.), and Grants-in-Aid for Challenging Exploratory Research 16K12900 (to S. H.) and the fund for Creation of Innovation Centers for Advanced Interdisciplinary Research Areas Program in the Project for Developing Innovation Systems (to S. H.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan. The authors declare that they have no conflicts of interest with the contents of this article.

1 To whom correspondence should be addressed: Graduate School of Nanobioscience, Yokohama City University, 22-2, Seto, Kanazawa-ku, Yokohama 236-0027, Japan. Tel.: 81-45-787-2380; Fax: 81-45-787-2413; E-mail: shigashi@yokohama-cu.ac.jp.

2 The abbreviations used are: MMP, matrix metalloproteinase; HAI-1, hepatocyte growth factor activator inhibitor type 1; CS, cholesterol sulfate; CM, conditioned medium; CBB, Coomassie Brilliant Blue R-250; sHAI-1, soluble HAI-1; Mβ-CD, methyl-β-cyclodextrin; KD, Kunitz-type inhibitor domain; LDLR, low-density lipoprotein receptor; MANSC, motif at N terminus with seven cysteines; PKD, polycystic kidney disease; MT1-MMP, membrane type-1 MMP; DME/F12, Dulbecco’s modified Eagle’s/Ham’s F12; ACN, acetonitrile; HEMA, 2-hydroxyethyl methacrylate.
been found that the latter step is mediated by E-cadherin, the mechanism of the initial cell aggregation has remained to be clarified.

In this study, we identified hepatocyte growth factor activator inhibitor type 1 (HAI-1) as a novel substrate of membrane-bound MMP-7, and we demonstrated that the fragment of HAI-1 released from colon cancer cells upon MMP-7 cleavage has the ability to induce cell aggregation. We also determined a region of HAI-1 essential for induction of the homotypic cell adhesion.

**Results**

**Identification of HAI-1 as a cell-surface protein cleaved by membrane-bound MMP-7**

To explore the cell-surface proteins, which are released from WiDr human colon carcinoma cells by the CS-dependent proteolytic action of MMP-7, surface proteins of WiDr cells were first biotinylated. The surface protein-labeled cells were then treated with MMP-7 or MMP-7(29,33,51,55/M2)ΔC3, the variant of MMP-7 lacking affinity for CS (10). When the proteins in the conditioned medium (CM) of the treated cells were analyzed by ligand blotting, using avidin-conjugated alkaline phosphatase as a probe, we found that several biotinylated protein fragments were released from the cells treated with MMP-7 or MMP-7(29,33,51,55/M2)ΔC3, and a 44-kDa fragment was released only from the MMP-7-treated cells (Fig. 1A). The biotinylated proteins released from the MMP-7-treated cells were collected using an avidin-Sepharose column, which were then subjected to SDS-PAGE followed by Coomassie Brilliant Blue R-250 (CBB) staining. The protein band of the 44-kDa fragment was excised from the gel. LC-MS/MS analysis of the tryptic peptides of the 44-kDa fragment revealed that the fragment was derived from HAI-1, a type I membrane protein, suggesting that the extracellular region of HAI-1 is proteolytically released as “soluble HAI-1” (sHAI-1). When the CMs of the WiDr cells were analyzed by immunoblotting under non-reduced conditions, the HAI-1-derived 44-kDa fragment in the culture medium was indeed increased upon treatment of the cells with MMP-7 (Fig. 1B). The immunoreactive protein in the CM migrated as a 51-kDa protein under reduced conditions. The intramolecular disulfide bonds of sHAI-1 probably cause the difference of mobilities between reduced and non-reduced conditions. We found that HAI-1 in the lysate of WiDr cells also showed different mobilities in the immunoblot analysis under non-reduced (51 kDa) and reduced (60 kDa) conditions.

**Binding of MMP-7 to CS is important for cleavage of HAI-1 localized in the raft region and determination of the peptide bond of HAI-1 cleaved by MMP-7**

To examine whether cell-surface HAI-1 is shed by MMP-7 in a CS-dependent manner, MMP-7(29,33,51,55/M2)ΔC3 and wild-type MMP-7 were compared for their abilities to release the soluble fragment of HAI-1. As shown in Fig. 2A, treatment of WiDr cells with the variant of MMP-7 led to a slight release of the 44-kDa HAI-1-derived fragment, but the level was almost the same as that released from the non-treated cells, suggesting that the variant of MMP-7 hardly sheds HAI-1. In contrast, wild-type MMP-7 effectively released the HAI-1 fragment. Furthermore, when WiDr cells were treated with methyl-β-cyclo- dextrin (MB-CD), release of the HAI-1 fragment by MMP-7-catalyzed cleavage was decreased significantly (Fig. 2B). Therefore, it is likely that binding of MMP-7 to CS is crucial for the shedding of HAI-1. We next examined localization of HAI-1 on the cell membrane. We prepared the membrane fraction from Colo201 human colon carcinoma cells by the differential centrifugation method as described under “Experimental procedures,” and the membrane fraction was solubilized with
the non-ionic detergent Triton X-100 at 4 °C. As shown in Fig. 2C, HAI-1 was mainly partitioned into the detergent-insoluble fraction when the membrane fraction prepared from the non-treated cells was analyzed. In contrast, HAI-1 was efficiently solubilized when the membrane fraction was prepared from Mβ-CD–treated cells. Consistent with our previous study (9), when the membrane fraction prepared from Colo201 cells incubated with MMP-7 was analyzed, MMP-7 was also detected in the detergent-insoluble fraction, whereas this MMP did not bind to the Mβ-CD–treated cells; hence, MMP-7 was detected neither in the detergent-insoluble fraction nor in the soluble fraction. These data suggest that both HAI-1 and MMP-7 are colocalized in distinctive microdomains such as rafts.

We next constructed a mammalian expression vector of N-terminally FLAG-tagged HAI-1 (named nFL-HAI-1) and stably-transfected it into DLD-1 human colon carcinoma cells. When the nFL-HAI–transfected cells were treated with MMP-7 and the resultant CM was analyzed by immunoblotting under reduced conditions (Fig. 2D), a 52-kDa fragment was

Figure 2. Binding of MMP-7 to CS is important for cleavage of HAI-1 localized in raft region. A, WiDr cells were incubated in serum-free medium without (none) or with 50 nm MMP-7 or MMP-7(29,33,51,55/M2)/Δ3 (MMP-7V) at 37 °C for 2 h. B, WiDr cells were preincubated without (−) or with (+) 10 mM Mβ-CD (+) at 37 °C for 30 min, and then the cells were further incubated without (−) or with (+) 50 nm MMP-7 at 37 °C for 2 h. C, Colo201 cells were preincubated without (−) or with (+) 10 mM Mβ-CD at 37 °C for 30 min, and then the cells were further incubated without (top two panels) or with (bottom two panels) 50 nm MMP-7 at 37 °C for 3 h. The membrane fraction prepared from the incubated cells was dissolved in Triton X-100 at 4 °C and analyzed by immunoblotting. D, construction of nFL-HAI-1 is schematically represented. The numbers in the scheme indicate the position of amino acid residues. The number in parentheses represents the deduced molecular mass in Da of the polypeptide moiety of nFL-HAI-1. CHO represents the potential site of Asn-linked glycosylation (top). The nFL-HAI–transfected DLD-1 cells or the mock-transfected cells were treated with 50 nm MMP-7 at 37 °C for 24 h. The resultant CM corresponding to 5 × 10^5 mock-transfected cells or that corresponding to 1 × 10^6 nFL-HAI–transfected cells was analyzed by immunoblotting (IB) under reduced conditions with the anti-HAI-1 pAb (bottom left). 52-kDa arrow and 51-kDa arrow represent the FLAG-tagged sHAI-1 and non-tagged sHAI-1, respectively. The nFL-HAI–transfected DLD-1 cells were treated without (−MMP-7) or with 50 nm MMP-7 (+MMP-7) at 37 °C for the indicated length of time. The N-terminally tagged fragments of HAI-1 released into the medium were analyzed by immunoblotting under reduced conditions with the anti-FLAG M2 mAb or anti-HAI-1 pAb (bottom left). 52-kDa arrow and 51-kDa arrow represent the FLAG-tagged sHAI-1 and non-tagged sHAI-1, respectively. The nFL-HAI–transfected DLD-1 cells were treated without (−MMP-7) or with 50 nm MMP-7 (+MMP-7) at 37 °C for 3 h. The CM and cell lysate prepared from the incubated cells were examined for their contents of FLAG-tagged proteins by the immunoblotting with the anti-FLAG M2 mAb. β-Actin in the cell lysate was also detected by immunoblotting and used as an internal loading control.
mainly detected by anti-HAI-1 pAb and anti-FLAG M2 mAb. When the CM of the MMP-7–treated mock-transfected DLD-1 cells was analyzed, a 51-kDa fragment was mainly detected by anti-HAI-1 pAb but not by anti-FLAG M2 mAb. These data suggest that non-tagged HAI-1 expressed endogenously in the mock-transfected cells and the nFL-HAI-1 in the expression vector–transfected cells are cleaved at the same specific site by MMP-7, and the fragments of which difference in molecular mass corresponding to that of FLAG tag moiety (~1 kDa) are released. Therefore, it is likely that the 52-kDa soluble fragment is the FLAG-tagged form of the 51-kDa sHAI-1. The 51-kDa reduced form of non-tagged sHAI-1 corresponds to the 44-kDa non-reduced form of sHAI-1 in Fig. 1.

We also examined the time course of release of HAI-1 fragments from the nFL-HAI-1–transfected cells after MMP-7 treatment (Fig. 2D), and we found that in addition to the major 52-kDa fragment, minor 45- and 38-kDa fragments were released from the transfected cells after a 48-h incubation. Because the 38-kDa fragment was also released from the DLD-1 cells without MMP-7 treatment, it is likely that the 52- and 45-kDa HAI-1 fragments are generated by MMP-7–catalyzed cleavage. To determine the sites of HAI-1 cleaved by MMP-7, the FLAG-tagged HAI-1 fragments released into the medium were collected, using an anti-FLAG M2 mAb-conjugated agarose column, which were then subjected to SDS–PAGE under reduced conditions. The band of 52- or 45-kDa proteins (Fig. 2E) was excised from the gel and subjected to arginyl endopeptidase or Asp-N digestion followed by LC-MS/MS analysis, respectively. We found that the arginyl endopeptidase digests of the 52-kDa protein contained a fragment corresponding to amino acid residues 443–451 of HAI-1, which does not include the arginine residue. The Asp-N digests of the 45-kDa protein contained fragments corresponding to amino acid residues 365–375 and that corresponding to residues 365–378 of HAI-1, both of which had C termini followed by non-aspartic acid residues in the HAI-1 sequence. These data suggest that MMP-7 cleaves HAI-1 mainly at the peptide bonds between Gly375 and Leu452, and the peptide bonds corresponding to Gly375–Phe376 and Glu378–Leu379 in HAI-1 are slightly susceptible to MMP-7 cleavage. To verify this, we constructed expression vectors of a variant of nFL-HAI-1 that had Leu452 of HAI-1 replaced with glycine (named HAI-1 L452/G), and another variant that had three residues Phe376, Leu379, and Leu452 of HAI-1 replaced with glycine (named HAI-1 F376/G, L379/G, L452/G). The vectors of these variants or a wild-type FLAG-tagged HAI-1 were transiently transfected into DLD-1 cells. These transfectants were then treated with MMP-7, and the release of FLAG-tagged fragments was examined by immunoblotting. As shown in Fig. 2F, 52- and 45-kDa FLAG-tagged fragments were released from the wild-type HAI-1–transfected cells, whereas the 52-kDa fragment was not released from the HAI-1 L452/G–transfected cells. Neither the 52-kDa nor the 45-kDa FLAG-tagged fragment was released from the HAI-1 F376/G, L379/G, L452/G–transfected cells. Therefore, it is likely that the Gly375–Phe376, Glu378–Leu379, and Gly451–Leu452 bonds of HAI-1 are the sites of cleavage by MMP-7.

Figure 3. Soluble HAI-1 binds to cell surface in a metal ion-dependent manner. Colo201 cells were treated with 50 nM MMP-7 at 37 °C for 3 h, and the membrane-bound MMP-7 was removed by treating the cells with 2 μM TAPI-1. These cells were washed two times with serum-free medium supplemented without or with 5 mM EDTA and were then washed with serum-free medium. A, cells were further incubated in the serum-free medium containing 5 mM TAPI-1 at 37 °C for 3 h and photographed. Scale bar, 100 μm. B, washed cells were homogenized and fractionated by centrifugation as described under “Experimental procedures.” HAI-1-derived fragments in the membrane fraction were detected by immunoblotting under non-reduced conditions. The intact arrowhead and the soluble arrowhead represent the immunoreactive bands of HAI-1 and sHAI-1, respectively. Ordinate, molecular mass in kDa.

Induction of colon cancer cell aggregation by sHAI-1

It is known that several cell adhesion proteins, such as E-cadherin and integrins, work in a metal ion-dependent manner. We next examined whether metal ions are involved in cell aggregation induced by MMP-7. Consistent with our previous study (13), when the aggregated cells were freed from MMP-7 by the treatment of the cells with synthetic MMP inhibitor TAPI-1, and then dispersed into single cells by pipetting, these cells were re-aggregated during further incubation in the presence of TAPI-1. However, when the aggregated cells were treated both with TAPI-1 and EDTA, these cells were not re-aggregated during further incubation (Fig. 3A), suggesting that metal ions are required for the MMP-7–induced cell aggregation.

To examine whether the cleaved-HAI-1 fragments bind to the cell surface in a metal ion–dependent manner, Colo201 cells were incubated with MMP-7 and then washed with serum-free medium supplemented without or with 5 mM EDTA. As shown in Fig. 3B, the 44-kDa sHAI-1 (non-reduced form) generated by MMP-7 treatment was detected in the membrane fraction prepared from the cells without the EDTA treatment, whereas the cell-bound HAI-1 fragment was diminished by washing the cells with the EDTA-containing medium. Therefore, metal ions are also essential for the binding of the 44-kDa sHAI-1 to the cell surface. We also determined the ratio of amounts of HAI-1 and sHAI-1 in the membrane fraction and sHAI-1 in CM by comparing their band intensities of immunoblotting, and we found that the ratio of cell-associated sHAI-1/cell-associated HAI-1/sHAI-1 in CM was ~1:210:30, suggesting that small
fraction of sHAI-1 is associated with the surface of the aggregated cells.

To examine whether sHAI-1 is involved in the MMP-7–induced cell aggregation, Colo201 cells were treated with MMP-7 to allow the cells to form aggregation, and then the cell-associated MMP-7 and the cleaved-HAI-1 fragment were removed by sequential treatments with TAPI-1 and EDTA. These cells were dispersed by pipetting and then further incubated with or without recombinant sHAI-1. As shown in Fig. 4A, the cells were re-aggregated only in the presence of sHAI-1, suggesting that sHAI-1 has the ability to induce cell aggregation. The cell aggregation was enhanced in an sHAI-1 concentration-dependent manner (Fig. 4A). When the binding of exogenous sHAI-1 to the cell surface was examined by the fluorescence staining, using biotinylated sHAI-1 as a probe, the labeled protein was localized on the cell surface, including regions of intercellular contact, suggesting that sHAI-1 behaves as a cell-adhesion molecule. When the binding of biotinylated sHAI-1 to MMP-7–treated or non-treated cells was tested by the fluorescence staining, the extent of sHAI-1 bound to non-treated cells was lower than that bound to MMP-7–treated cells. The cell ELISA analysis also demonstrated that the MMP-7 treatment facilitated the binding of sHAI-1 to the cells (Fig. 4B). The binding of sHAI-1 to MMP-7–treated cells was metal ion-dependent, and the bound sHAI-1 was released upon the treatment of the cells with EDTA, as expected (Fig. 4C).

**HAI-1 expression is necessary for MMP-7–induced cell aggregation**

To verify that HAI-1 expression is necessary for WiDr cells to be aggregated upon MMP-7 treatment, we prepared WiDr cells stably transfected with a short hairpin RNA (shRNA) targeting the hain-1 gene or non-targeting shRNA. The sHAI-1 was hardly released from WiDr cells of which the expression of HAI-1 was prevented by the shRNA (Fig. 5A). When the HAI-1 expression-prevented WiDr cells were treated with MMP-7, they were hardly aggregated (Fig. 5B). However, the MMP-7 induction of cell aggregation was restored (Fig. 5C) when the HAI-1 expression was rescued by transient transfection of the HAI-1 expression vector (Fig. 5D). These data strongly suggest that HAI-1 expression is necessary for the MMP-7–induced cell aggregation.

**MMP-7 induces aggregation of HT1080 fibrosarcoma cells transfected with HAI-1**

We found that human fibrosarcoma-derived HT1080 cells did not express HAI-1 (Fig. 6A), and they were hardly aggregated upon MMP-7 treatment under suspended cell culture conditions (Fig. 6B). When the MMP-7–induced aggregation...
Shed HAI-1 fragment has cell aggregation–inducing activity

Figure 5. HAI-1 expression is necessary for MMP-7–induced cell aggregation. A, cell lysates of WiDr cells stably transfected with the expression vector of the shRNA targeting HAI-1 (shHAI-1) or non-targeting shRNA (NT) were subjected to immunoblotting (IB), using the anti-HAI-1 pAb. β-Actin in the cell lysate was also analyzed by immunoblotting (top). The WiDr cells transfected with shRNA against hai-1 (shHAI-1) or non-targeting shRNA (NT) in suspended conditions were incubated incubated without (−) or with (+) 50 nM MMP-7 at 37 °C for 4 h. Fragments of HAI-1 released into the culture medium were analyzed by immunoblotting (IB) under reduced conditions with the anti-HAI-1 pAb (bottom). Ordinate, molecular mass in kDa. B, WiDr cells transfected with shRNA against HAI-1 (shHAI-1) or non-targeting shRNA (NT) in suspended conditions were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-2-hydroxyethyl methacrylate (poly-HEMA)-coated 35-mm dishes with serum-free medium supplemented with 0.5 mg/ml DNase I at 37 °C for 4 h, and the cells were photographed. Scale bar, 100 μm (top). The degree of cell aggregation was quantified as described under “Experimental procedures.” Error bars represent mean ± S.D.; n = 3 (bottom). C, WiDr cells stably transfected with shRNA against HAI-1 were further transfected transiently with empty vector (mock) or expression vector of HAI-1 (HAI-1). The transfected cells in suspended conditions were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-HEMA coated 35-mm dishes in serum-free medium supplemented with 0.5 mg/ml DNase I at 37 °C for 4 h, and the cells were photographed. Scale bar, 100 μm (top). The degree of cell aggregation was quantified. Error bars represent mean ± S.D.; n = 3 (bottom). D, 48 h after the transfection as described in C, the cell lysates were examined for their contents of HAI-1 proteins by the immunoblotting with an anti-HAI-1 pAb under reduced conditions. β-Actin in the cell lysate was also detected by immunoblotting and used as an internal loading control.

of HT1080 cells stably transfected with HAI-1 was tested, they were significantly aggregated (Fig. 6B). To examine whether the MMP-7–catalyzed cleavage of HAI-1 is necessary for the cell aggregation, expression vectors of the HAI-1 variants HAI-1-L452/G and HAI-1-F376/G, L379/G, L452/G were transiently transfected HT1080 cells, and expression of HAI-1 and the two variants on the cell surface was examined under fluorescence-activated cell-sorting analysis. These transfectants were then treated with MMP-7, and the release of HAI-1 fragments was examined by immunoblotting. As shown in Fig. 6C, both the variants and wild-type HAI-1 were expressed on surface of HT1080 cells, and HAI-1-F376/G, L379/G, L452/G–transfected cells did not release any soluble fragment of HAI-1 upon MMP-7 treatment. When the MMP-7–induced aggregation of HT1080 cells expressing wild-type HAI-1 or HAI-1-F376/G, L379/G, L452/G were tested, the cells expressing the cleavage-resistant HAI-1 variant were hardly aggregated (Fig. 6D), suggesting that cleavage of HAI-1 is critical for the MMP-7–induced cell aggregation.

CS-independent proteolytic action of MMP-7 on the cell surface is necessary for the sHAI-1–mediated induction of cell aggregation

In the studies in Fig. 4, we also tested whether sHAI-1 induces aggregation of Colo201 cells previously treated without MMP-7, and we found that the non-treated cells were not aggregated even in the presence of sHAI-1 (data not shown). Therefore, it is likely that proteolytic action of MMP-7 on the cell surface, other than HAI-1 cleavage, is required for the sHAI-1–mediated induction of cell aggregation.

To examine whether the CS-dependent proteolytic action of MMP-7 is also needed for the sHAI-1–mediated cell aggregation, we first prepared the CM of WiDr cells, which were pretreated without or with Mβ-CD and then incubated with MMP-7 under the suspended cell culture condition. We found that the WiDr cells pretreated without Mβ-CD were aggregated but those pretreated with Mβ-CD were not, and the Mβ-CD treatment of the cells caused significant decrease of the
MMP-7–catalyzed release of sHAI-1, as expected (Fig. 7A). The CM from WiDr cells treated as described above was then incubated without or with mock-transfected HT1080 cells in suspended condition were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-HEMA-coated 35-mm dishes in serum-free medium at 37 °C for 2 h, and the cells were photographed. Scale bar, 100 μm. C, HT1080 cells were transfected transiently with the empty vector (Mo) or the expression vectors of nFL-HAI-1 (WT), the single amino acid residue-substituted variant nFL-HAI-1 L452/G (variant 1, V1), or the triple amino acid residue-substituted variant nFL-HAI-1 F376/G, L379/G, L452/G (variant 2, V2). Forty eight hours after transfection, cell-surface expressions of these variants of HAI-1 were measured by fluorescence-activated cell sorting. Gray or black histograms represent empty vector (mock) or expression vectors of HAI-1 variant-transfected HT1080 cells, respectively (left). Forty eight hours after transfection, cells were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7) at 37 °C for 3 h. The CM and cell lysate prepared from the incubated cells were examined for their contents of HAI-1 or its fragments by the immunoblotting with the anti-HAI-1 pAb under reduced conditions. β-Actin in the cell lysate was also detected by immunoblotting and used as an internal loading control (right). D, HAI-1 or the HAI-1 F376/G, L379/G, L452/G-transfected HT1080 cells in suspended conditions were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-HEMA-coated 35-mm dishes in serum-free medium at 37 °C for 2 h, and the cells were photographed. Scale bar, 100 μm.

Figure 6. MMP-7 treatment induces aggregation of HT1080 fibrosarcoma cells stably transfected with HAI-1. A, cell lysates of HT1080 cells stably transfected with the expression vector of nFL-HAI-1 or empty vector (mock) were subjected to immunoblotting (IB) with the anti-HAI-1 pAb under reduced conditions. Ordinate, molecular mass in kDa. B, nFL-HAI-1 or mock-transfected HT1080 cells in suspended condition were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-HEMA-coated 35-mm dishes in serum-free medium at 37 °C for 2 h, and the cells were photographed. Scale bar, 100 μm. C, HT1080 cells were transfected transiently with the empty vector (Mo) or the expression vectors of nFL-HAI-1 (WT), the single amino acid residue-substituted variant nFL-HAI-1 L452/G (variant 1, V1), or the triple amino acid residue-substituted variant nFL-HAI-1 F376/G, L379/G, L452/G (variant 2, V2). Forty eight hours after transfection, cell-surface expressions of these variants of HAI-1 were measured by fluorescence-activated cell sorting. Gray or black histograms represent empty vector (mock) or expression vectors of HAI-1 variant-transfected HT1080 cells, respectively (left). Forty eight hours after transfection, cells were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7) at 37 °C for 3 h. The CM and cell lysate were also analyzed by immunoblotting. Ordinate, molecular mass in kDa. D, nFL-HAI-1 or mock-transfected HT1080 cells in suspended condition were incubated without (−MMP-7) or with 50 nM MMP-7 (+MMP-7), in poly-HEMA-coated 35-mm dishes in serum-free medium at 37 °C for 2 h, and the cells were photographed. Scale bar, 100 μm.

Shed HAI-1 fragment has cell aggregation–inducing activity

J. Biol. Chem. (2017) 292(50) 20769 –20784

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Mβ-CD followed by MMP-7 treatment was slightly faster than that treated with MMP-7 alone, and as compared with wild-type MMP-7, the variant of MMP-7 lacking the affinity for CS induced the cell aggregation more effectively (Fig. 7B). These data suggest that CS-independent action of MMP-7 on the cell surface is critical for the sHAI-1–mediated induction of cell aggregation.

**Region of HAI-1 corresponding to amino acid residues 141–249 is essential for cell aggregation–inducing activity**

To explore the region of HAI-1 essential for induction of the homotypic cell aggregation, we constructed mammalian expression vectors for various domains-deleted variants of sHAI-1 (Fig. 8A). These vectors were stably transfected into CHO cells (Fig. 8B), and each of the variants of sHAI-1 secreted from the transfected cells was purified to homogeneity. As shown in Fig. 8C, all of the variants lacking two Kunitz-type inhibitor domains (KID), the low-density lipoprotein receptor (LDLR)-like domain, or the motif at the N terminus with seven cysteines (MANSC) domain of HAI-1 induced the cell aggregation, suggesting that these domains are not essential for the cell aggregation–inducing activity. These data also suggest that the region of HAI-1 corresponding to Leu141–Tyr249 is essential for the induction of homotypic cell adhesion. The sHAI-1 variants that do not include the region between Leu141 and Tyr249, such as sHAI-1(245–465) and sHAI-1(Δ141–249), however, were not secreted from CHO cells (Fig. 8B); therefore, further analysis was infeasible.

We then constructed an *Escherichia coli* expression vector for the region corresponding to Leu141–Tyr249 of HAI-1, named HAI-1(141–249), and the protein was expressed in *E. coli*, refolded, and purified to homogeneity (Fig. 8C). The HAI-1(141–249) showed significant cell aggregation–inducing activity (Fig. 8D), suggesting that this region of HAI-1 is sufficient for the induction of homotypic cell aggregation. When the binding of exogenous HAI-1(141–249) to the cell surface was examined by fluorescence staining, using biotinylated-HAI-1(141–249) as a probe, the labeled protein was localized on the cell surface (Fig. 8D).

When the time course of binding of biotin-labeled HAI-1(141–249) to the surface of MMP-7–treated Colo201 cells was examined (Fig. 9A), the recombinant HAI-1 fragment rapidly bound to the cells, and the amount of the fragment bound to cells reached a constant after a 30-min incubation. Neither degradation nor decrease of the cell-bound fragment during the 5-h incubation was observed. The amount of HAI-1 fragment bound to the MMP-7–treated cells was much higher than that bound to the non-treated cells, suggesting that MMP-7 modifies cell-surface protein(s) and facilitates the binding of the HAI-1 fragment to the cell surface.
Cell ELISA revealed that HAI-1(141–249) bound to the MMP-7–treated Colo201 cells in a concentration-dependent and saturable manner (Fig. 9B). The half-maximal binding was observed at ~30 nM HAI-1(141–249). We found that HAI-1(141–249) bound to Colo201 cells without MMP-7 treatment, in a concentration-dependent manner, and the half-maximal binding was observed also at 30 nM HAI-1(141–249). At the saturating concentrations of HAI-1(141–249), the amount of the protein bound to the MMP-7–treated cells was much higher than that bound to the non-treated cells, suggesting that the MMP-7 treatment leads to an increase of the sites for HAI-1(141–249) binding on the cell surface. The binding of HAI-1(141–249) to the cells was also metal ion-dependent (Fig. 9C).

To examine whether sHAI-1 and HAI-1(141–249) compete with each other to bind to common site on the cell surface, a fixed concentration of biotin-labeled sHAI-1 or HAI-1(141–249), and various concentrations of non-labeled HAI-1(141–249) were incubated with the MMP-7–treated Colo201 cells, and the amount of labeled sHAI-1 or labeled HAI-1(141–249) bound to the cells was measured by cell ELISA. As shown in Fig. 9D, non-labeled HAI-1(141–249) partially inhibited the binding of the labeled HAI-1(141–249) to the cells, as expected. In contrast, HAI-1(141–249) partially inhibited the binding of the labeled sHAI-1 to the cells, suggesting that HAI-1(141–249) and sHAI-1 share, at least in part, a common binding site on the cells.

**Discussion**

In this study, we identified HAI-1, a type I membrane protein, as a novel substrate of membrane-bound MMP-7; the
Figure 9. Exogenously added HAI-1(141–249) binds to cell surface in an MMP-7 treatment- and a metal ion-dependent manner. A, MMP-7–treated or non-treated Colo201 cells (8 × 10^5 cells) were incubated with 50 nM biotin-labeled HAI-1(141–249) in 800 μl of serum-free medium supplemented with 5 μM TAPI-1 and 1 mg/ml BSA at 37 °C for the indicated length of time. The cells were washed three times with serum-free medium and lysed to analyze the cell-bound HAI-1(141–249) by SDS-PAGE under reduced conditions followed by ligand blotting (LB) with the avidin-conjugated horseradish peroxidase (HRP-avidin) as a probe. The arrowhead represents the band of the cell-bound biotin-labeled HAI-1(141–249). B, the cell lysate was prepared from the Colo201 cells without incubation with the labeled HAI-1 fragment. Ordinate, molecular mass in kDa. β-Actin in the cell lysate was also detected by immunoblotting and was used as an internal loading control. C, 50 nM biotin-labeled HAI-1(141–249) was incubated with MMP-7–treated or non-treated Colo201 cells at room temperature for 1 h. The cell-bound labeled protein was visualized by fluorescent staining. Scale bar, 20 μm. D, the indicated concentrations of biotin-labeled HAI-1(141–249) was incubated with MMP-7–treated or non-treated Colo201 cells at 37 °C for 1 h. The amount of the labeled protein bound to the cells was measured by cell ELISA. Error bars in B–D represent mean ± S.D.; n = 3.
MMP-7–catalyzed cleavage of HAI-1 on the surfaces of human colon cancer cells led to release of a 51-kDa fragment covering the extracellular region of the membrane protein. We also demonstrated that the released HAI-1 fragment acts as a cell-adhesion molecule.

It has been reported that a variant of MMP-7 lacking affinity for CS does not induce the cell aggregation (10), and binding of MMP-7 to CS is essential for MMP-7–catalyzed modulation of cell-surface proteins (9). This study showed that the variant of MMP-7 lacking CS-binding ability failed to shed HAI-1. Our data further suggest that active MMP-7 and a part of HAI-1 are localized in the lipid raft region of the cell membrane. These data are consistent with the view that colocalization of MMP-7 and HAI-1 in the CS-containing lipid rafts facilitates the cleavage of HAI-1.

The extracellular region of HAI-1 consists of an N-terminal MANSC domain (14), a polycystic kidney disease (PKD)-like domain (15), KD1, LDLR-like domain, and KD2. The KD1 of HAI-1 has inhibitory activities against various trypsin-type serine proteases, whereas the KD2 does not (16). HAI-1 was initially identified as an inhibitor of hepatocyte growth factor activator, a trypsin-type serine protease (17); and further analyses revealed that it has potent inhibitory activities against matriptase, hepsin, plasmin, and trypsin (18–21). The expression of HAI-1 is increased during tissue remodeling and inflammation (22, 23), and it is thought to regulate activation of hepatocyte growth factor precursor. It has been reported that the extracellular domain of HAI-1 is cleaved at several sites, and the pattern of cleavage changes in the presence or absence of EDTA, suggesting that metalloproteinases are involved in the cleavage (24). A recent study has reported that membrane type-1 MMP (MT1-MMP) cleaves HAI-1 at a peptide bond between Gly431 and Leu452 in the membrane-proximal external region, and at a site between KD1 and LDLR domain (25). We showed that the former site of HAI-1 cleaved by MT1-MMP is also cleaved by cell-associated MMP-7. HAI-1 is not known as a metal ion-containing protein; however, sHAI-1 binds to the cell surface in a metal ion-dependent manner, suggesting that metal ions stabilize the functional structure of sHAI-1 or that of unidentified sHAI-1 receptor(s).

This study for the first time revealed that sHAI-1, generated by MMP-7–catalyzed cleavage, binds to the cell surface and plays a role in homotypic cell aggregation. As the MMP-7 treatment led to an increase of the sHAI-1-binding capacity of the cells, MMP-7 may modify and activate an unidentified cell-surface receptor(s) of sHAI-1. This study also demonstrated that the CS-independent proteolytic action of MMP-7 on cell surface is critical for the sHAI-1-mediated induction of cell aggregation. Considering that the CS-dependent and the CS-independent actions of MMP-7 are essential for the generation of sHAI-1 and sHAI-1–mediated induction of cell aggregation, respectively, it seems likely that MMP-7 acts as a specific inducer of the cell aggregation due to having the dual activities. For instance, some metalloproteinases other than MMP-7 that can shed HAI-1 will not be able to induce the cell aggregation if they do not have the activity corresponding to the CS-independent action of MMP-7 on cell surface. Further studies are needed to clarify the detailed mechanism.

We determined a region of sHAI-1 essential for the cell aggregation–inducing activity; the region of HAI-1 corresponding to amino acid residues Leu441–Tyr449, including the PKD-like domain, had the activity. A previous study reported that polyclystin-1, which is membrane protein having multiple PKD domains, forms homodimer via its PKD domains, thereby contributing to cell–cell adhesion (26, 27). Because the concentration of HAI-1(141–249) required for half-maximal induction of cell aggregation was lower than that of sHAI-1, the cell aggregation–inducing activity of HAI-1(141–249) is likely higher than that of sHAI-1. A recent report suggests that the PKD-like domain interacts with the neighboring KD1 in HAI-1, thereby modulating the protease inhibitor activity (15). The inter-domain interaction may partially hamper the binding of the 141–249 region of sHAI-1 to cell-surface receptor(s), thereby lowering their affinity. In addition to the 141–249 region, some other region(s) of sHAI-1 is likely involved in the interaction with cell-surface molecules, because the binding of sHAI-1 to the cells was only partially competed by the HAI-1(141–249) fragment. Although contribution of the additional region(s) of sHAI-1 is currently unknown, our present data strongly suggest that interaction between the region of HAI-1(141–249) and its corresponding receptor(s) on the cell surface is directly involved in the induction of homotypic cell adhesion.

HAI-1 has been considered to down-regulate hepatocyte growth factor activity, thus suppressing cancer malignancy and metastasis. It has been reported that knockdown of HAI-1 induces epithelial to mesenchymal transition (28), and cleavage of HAI-1 by MT1-MMP induces invasive growth of oral squamous cell carcinoma cells via increasing proteolytic activity of matriptase (25). This study further suggests that cleavage of HAI-1 promotes cancer metastasis via production of the cell adhesion molecule. Therefore, it is likely that MMP-7 converts the cancer-suppressive molecule into a cancer-promoting one. Our finding also provides the potential to develop sHAI-1–targeted novel anti-cancer drugs that block the MMP-7–promoted cancer metastasis.

Experimental procedures

Materials

The sources of materials used are as follows: EZ-Link Sulfo-NHS-LC-biotin, pSecTag2B, and pSecTagA were from Thermo Fisher Scientific (Waltham, MA); SoftLink™ Soft Release Avidin Resin and Asp-N from were from Promega (Madison, WI); the synthetic MMP inhibitor TAPI-1 was from Peptides Institute, Inc. (Osaka, Japan); pAb against HAI-1–eCTD was from R&D Systems (Minneapolis, MN); polymyxin B-agarose, mAb against β-actin, mAb against FLAG epitope, and pFLAG-CTC were from Sigma; mAb 11B4G against MMP-7 was from Oriental Yeast Co. (Shiga, Japan); biotin–AC5-Osu, puromycin, and G418 sulfate solution were from Wako Pure Chemical Industries (Osaka, Japan); Zeocin, Dulbecco’s modified Eagle’s/Ham’s F-12 (DME/F12) medium, and Lipofectamine LTX reagent were from Life Technologies, Inc.; deoxyribonucleoside 5′(DNase I) was from Worthington; arginyl endopeptidase, pBasi-hU6 Neo DNA, and PrimeSTAR
Max DNA were from Takara Bio Inc. (Shiga, Japan); pEAK8-HAI-1 previously constructed (25) was a generous gift from Dr. Hiroshi Sato (Kanazawa University, Japan). The recombinant wild-type MMP-7 and MMP-7(29,33,51,55/M2)ΔC3, a variant of MMP-7 lacking affinity for CS, were prepared as described previously (10).

### Construction of expression vector for HAI-1 variants or shRNA targeting HAI-1 gene

In this study, gene constructions were carried out using PCR with PrimeSTAR Max DNA polymerase. Oligonucleotide sequences used as primers and inserts are listed in Table 1.

To construct a mammalian expression vector for the C-terminally tagged sHAI-1, PCR was first carried out, using a pair of primers pEAK EcoRI- and sHAI EcoRI- and the pEAK8-HAI-1 as a template. The primers having a 15-base overlapped sequence, including a mutagenic one, were designed in inverted tail-to-tail directions to amplify the cloning vector together with the extracellular region of the HAI-1 sequence and to introduce an EcoRI site in the C-terminal side of the part of HAI-1 sequence. The resultant PCR product having adhesive tails due to the overlapped sequence was used directly for transformation, according to the manufacturer’s instruction. The resultant pEAK8-shHAI-1 vector was cleaved with EcoRI and ligated with annealed oligonucleotides cFL+ and cFL-.

The resultant pEAK8-HAI-1/cFL vector was used for the following constructions. To fuse the sequence encoding sHAI-1 and that of the FLAG tag and to add a linker sequence consisting of three tandem glycine residues, PCR was carried out, using a pair of primers SHAIcFLj and SHAIcFLj, and the pEAK8-HAI-1/cFL as a template. The resultant pEAK8-shHAI-1-ΔGly3-cFL vector was used for expression of the recombinant protein.

To construct the N-terminally truncated sHAI-1 variant sHAI-1(141–465) or sHAI-1(245–465), PCR was carried out using a pair of primers HA1-141-HindIII+ and cFL EcoRI- or HA1-245-HindIII+ and cFL EcoRI-, respectively, and the pEAK8-shHAI-1-ΔGly3-cFL as a template.

### Table 1

| Name | Oligonucleotide sequence |
|------|--------------------------|
| **Primer**a | **Primer**a |
| pEAK EcoRI+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| pEAK EcoRI+: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |
| sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' | sHAI EcoRI-: 5'-CTGCTGTCATCCACTCAGAGGCTGCCGTCGAG-3' |

| **Inserts** | **Inserts** |
|-------------|-------------|
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |
| cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' | cFL+: 5'-GAGGAGCTAAGAGTGGTCGCTGTAACACGGCGCTGAG-3' |

**a** The italic letters represent the restriction enzyme site corresponding to the name of each oligonucleotide.
To construct a mammalian expression vector for the shRNA targeting the hαι-1 gene, a pair of oligonucleotides HAI shRNA+ and HAI shRNA− were annealed and ligated with pBAsi-hU6 Neo DNA cleaved with BamHI and HindIII. To construct a vector for the non-targeting shRNA, a pair of oligonucleotides NT shRNA+ and NT shRNA− were annealed and ligated with pBAsi-hU6 Neo DNA as described above.

To construct an E. coli expression vector for the region of HAI-1 corresponding to amino acid residues 141–249 with an N-terminal FLAG tag, PCR was first carried out, using a pair of primers pnFL1st+ and pnFL1st−, and the pFLAG-N-APP-IP-MMP-2cat-FLAG, which was constructed in the previous study (29), as a template. The resultant PCR product was further amplified by PCR with a pair of primers pnFL2nd+ and pnFL2nd−. The PCR product having adhesive tails was used directly for transformation. The cloning vector together with the N-terminal FLAG tag region of the resultant pnFL-APP-IP-MMP-2cat-FLAG was amplified by PCR with a pair of primers pnFL EcoR+ and pnFL−, and the resultant PCR product was cleaved with EcoRI. A part of cDNA encoding the amino acid residues 141–249 of HAI-1 was also amplified by PCR with a pair of primers HAI 141+ and HAI 249 EcoRI−, and the pEAK8-HAI-1 as a template, and the resultant PCR product was cleaved with EcoRI. These two PCR products both cleaved with EcoRI were combined and ligated. The resultant pnFL-HAI-1(141–249) vector was used for expression of the recombinant protein in E. coli.

Cell lines and culture conditions

Human colon carcinoma cell lines WiDr, DLD-1, Colo201, human fibrosarcoma cell line HT1080, and CHO cell line were maintained in DME/F12 medium supplemented with 10% FBS, penicillin G, and streptomycin sulfate at 37 °C in a humidified atmosphere. E. coli (29), as a template. The resultant PCR product was further amplified by PCR with a pair of primers pnFL EcoR+ and pnFL−, and then washed twice with 100 mM ammonium bicarbonate (pH 8.0), 60% acetonitrile (ACN). After completely dried, the gel pieces were incubated with 100 μl of 50 mM ammonium bicarbonate (pH 8.0) in the presence of 10 mM DTT and 0.2 M guanidine HCl at 60 °C for 1 h and were subsequently alkylation with an equal volume of 50 mM ammonium bicarbonate (pH 8.0) containing in 108 mM iodoacetamide at 37 °C for 30 min in the dark. Next, the gel pieces were washed with 50 mM ammonium bicarbonate (pH 8.0), 60% ACN for 70 min (with a buffer change every 10 min) to remove the excess salt. After the gel pieces were completely dried, in-gel digestion was performed using 100 ng of mass spectrometry grade trypsin (Promega, Madison, WI) in 50 mM ammonium bicarbonate (pH 8.0) at 37 °C overnight. For MS analysis, the resulting peptides were desalted and enriched using a self-packed SDB/C18 tip column (Stage tip). Stage tips were prepared by packing Empore SDB XD (3M, Tokyo, Japan) and Empore C18 (3M) into a 200-μl pipette tip as described previously (31). First, the columns were washed with 100 μl of 80% ACN, 0.1% TFA by centrifuging at 3000 × g and then equilibrated with 100 μl of A buffer (2% ACN, 0.1% TFA) by centrifuging at 3000 × g. After sample-loading by centrifugation at 1000 × g, flow-through samples were reloaded, centrifuged, and then washed twice with 100 μl of A buffer and were eluted with 100 μl of 30% ACN, 0.1% TFA and then with 100 μl of 60% ACN, 0.1% TFA. Eluted samples were completely dried and stored at −20 °C.

LC-MS/MS analysis was performed on a LTQ Orbitrap Velos hybrid-mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) using Xcalibur version 2.0.7 software. UltiMate® 3000 LC system ( Dionex, LC Packings, Sunnyvale, CA) was used to provide the gradient for on-line reversed-phase nano-LC at a flow of 300 nL/min. C18 Pepmap™ column (100-μm inner diameter × 2 cm) (LC Packings) and nanoscale C18 Pepmap™ capillary column (75-μm inner diameter × 15 cm) (LC Packings) were used as analytical columns. The mobile phases consisted of the following: A, 2% ACN, 98% water, 0.1% formic acid; B, 95% ACN, 5% water, 0.1% formic acid. Peptides were separated using a 60-min gradient program consisting of 2–41% B in 40 min. The full-scan mass spectra were measured from m/z 350 to 1200 in the positive ion electrospray ionization mode on a mass spectrometer operated in the data-dependent mode. The general mass spectrometric conditions were as follows: spray voltage, 1.8 kV; capillary temperature, 250 °C; normalized collision energy, 35.0%; isolation width, 2 m/z; activation time, 10 ms; activation Q, 0.25; dynamic exclusion, 180 s; resolution, 30,000; data-dependent mode, TOP15 strategy.

To identify peptides, peak lists were created using a Proteome Discoverer software version 1.4 (Thermo Fisher Scientific) and were searched against the human protein sequences in the UniProt Knowledgebase (UniProtKB/Swiss-Prot) database (version May, 2013; 538,849 entries) using MASCOT (version 2.4.1, Matrix Science, London, UK). The search parame-
Shed HAI-1 fragment has cell aggregation–inducing activity

ters were as follows: trypsin digestion with three missed cleavages permitted; variable modifications, protein N-terminal acetylation, oxidation of methionine, propionamidation of cysteine, and biotin of lysine and N terminus; peptide-mass tolerance for MS data, ±5 ppm; and fragment mass tolerance, ±0.5 Da. We used significance threshold (p < 0.05) as a cutoff to export results from the analysis by MASCOT. In addition, peptides that yielded a peptide ion score, which was greater than or equal to 30, were considered positive identifications.

**Preparation and fractionation of cell membrane by the differential centrifugation method**

Colo201 cells (1 × 10^7 cells) were washed two times with serum-free medium, and then homogenized in 1 ml of 20 mM HEPES buffer (pH 7.5) containing 250 mM sucrose by a Potter-Elvehjem-type homogenizer. The homogenates were centrifuged at 800 g for 7 min to remove nuclei and cellular debris. The supernatant was centrifuged at 21,000 × g for 30 min, and resultant precipitate was used as the cell membrane fraction. To separate detergent-soluble and -insoluble fractions, the membrane fraction was suspended in 20 mM Tris-HCl (pH 7.5) containing 150 mM NaCl (TBS) supplemented with 10 mM CaCl_2 and 1% non-ionic detergent Triton X-100, and then incubated at 4 °C. After incubation, the sample was centrifuged at 21,000 × g at 4 °C for 15 min. The resultant precipitate and supernatant were dissolved in an SDS sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 20 µg/ml bromphenol blue and subjected to SDS-PAGE.

**Knockdown of HAI-1**

The vector for expression of shRNA targeting the hai-1 gene and non-targeting shRNA constructed as described above were transfected into WiDr cells using Lipofectamine LTX and Plus reagent according to the manufacturer’s instructions. Stable transfectants were selected with G418. The selection was performed by culturing the cells for 3 weeks in DME/F12 medium containing 600 µg/ml G418. After selection, the G418-resistant cells were cultured DME/F12 medium containing 600 µg/ml G418.

**Fluorescence-activated cell-sorting analysis**

The various expression vectors were transfected into HT1080 cells using Lipofectamine LTX and Plus reagent. Forty-eight hours after transfection, the cells were prepared for cell-surface labeling of HAI-1 as follows: the cells were washed in PBS and then the cells were removed from the culture dish with trypsin and EDTA. The cells were washed twice with PBS supplemented with 0.02% EDTA (PBSE). After 5 min of centrifugation at 800 × g, they were suspended in PBSE containing 3% BSA. Cells were counted, diluted, and aliquoted into 100-µl fractions that contained 5 × 10^5 cells. Each sample was then incubated on ice for 1 h with a 1:50 dilution of anti-HAI-1 pAb. After repeated washings, each cell pellet was resuspended in ice-cold PBSE containing 1% BSA. Cells were then labeled with a 1:1000 dilution of rabbit anti-goat FITC in the dark, on ice, for 30 min. After extensive washing in ice-cold PBSE containing 1% BSA, the cells were suspended in 1 ml of PBSE containing 1% BSA and subjected to flow cytometric analysis using a JSAN cell sorter (Bay Bioscience, Kobe, Japan).

**Expression and preparation of shHAI-1 variants tagged with FLAG epitope**

The various expression vectors were transfected into CHO cells using Lipofectamine LTX and Plus reagent according to the manufacturer’s instructions. Stable transfectants expressing the recombinant protein were selected with puromycin or Zeocin. The selection was performed by culturing the cells for 3 weeks in DME/F12 medium containing 5 µg/ml puromycin or 800 µg/ml Zeocin. The transfected CHO cells were grown to confluence in 25 ml of the growth medium in 150-mm dishes. To prepare the CMs, cells were rinsed with PBS, and the culture was continued in 15 ml of serum-free medium for 24 h. After incubation, the CM was harvested, clarified by centrifugation, and stored at −40 °C until used for purification of recombinant proteins.

The frozen CM was thawed and added with ammonium sulfate to make an 80% saturated ammonium sulfate solution and stirred at 4 °C for 15 h. The sample was then centrifuged at 13,000 × g at 4 °C for 30 min. The resultant precipitates were dissolved in TBS and dialyzed extensively against the same buffer. After dialysis, the sample was clarified by centrifugation and then loaded onto an anti-FLAG M2 mAb-conjugated agarose column equilibrated previously with TBS. The column was washed with the equilibration buffer, and soluble variants of HAI-1 tagged with FLAG were eluted with 100 µg/ml FLAG peptide dissolved in TBS. The eluted sample from the anti-FLAG antibody column was dialyzed against 50 mM HEPES (pH 7.5), containing 150 mM NaCl.

**Expression and purification of HAI-1(141–249)**

The E. coli expression vector pNFl-HAI-1(141–249) constructed as described above was used for transformation of E. coli strain DH5α competent cells. The transformant was cultured in 2× YT medium (0.08% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.25% (w/v) NaCl) at 37 °C, and the recombinant protein was induced by the addition of 1.0 mM isopropyl β-d-thiogalactopyranoside. After a 5-h induction, E. coli cells were broken in 50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and 5 mM EDTA by sonication, and the resultant inclusion bodies were collected by centrifugation. The inclusion bodies were solubilized in 50 mM Tris-HCl (pH 8.0) containing 6 µM guanidine HCl and 100 mM DTT with gentle stirring at 25 °C for 2 h. The solubilized sample was first clarified by centrifugation and then refolded by the rapid dilution method using a refolding buffer consisting of 1 M arginine, 50 mM Tris-HCl, 150 mM NaCl, and 5 mM CaCl_2 in which the pH was adjusted to 7.5. The refolded protein was dialyzed extensively against TBS, and concentrated using a Centriprep-10 centrifugal filter device (Merck Millipore Ltd., Darmstadt, Germany). After concentration, the recombinant protein was purified with an anti-FLAG antibody column as described above. To remove lipopolysaccharide potentially included in the sample, the fraction eluted from the anti-FLAG antibody column was loaded onto a polymyxin B-agarose column equilibrated previously with TBS containing 10 mM CaCl_2, and the flow-through fraction was collected. The collected
sample from the polymyxin B-agarose column was dialyzed against 50 mm HEPES (pH 7.5) and 150 mm NaCl, containing 10 mm CaCl₂.

**Biotinylation of sHAI-1 or HAI-1(141–249)**

Two micromolar recombinant sHAI-1 or 5 μM HAI-1(141–249) was incubated with 100 μM biotin-AC₅-Osu in 50 mm HEPES (pH 7.5), containing 150 mm NaCl at 25 °C for 1 h. The biotinylation reaction was terminated by adding with 100 mm ethanolamine (pH 8.0) and incubated at 25 °C for 15 min. The sample was then dialyzed against 50 mm HEPES (pH 7.5), containing 150 mm NaCl.

**SDS-PAGE and immunoblotting analysis**

SDS-PAGE was performed on polyacrylamide gel under non-reduced or reduced conditions. In immunoblotting analysis, proteins separated by SDS-PAGE were transferred onto nitrocellulose or PVDF membranes and visualized by the ECL method (GE Healthcare, Buckinghamshire, UK).

**Assay of cell aggregation-inducing activities of variants of sHAI-1**

Ccolo201 cells were incubated with 50 nm MMP-7 at 37 °C for 3 h and then with 2 μM TAPI-1 and 5 mm EDTA in the serum-free DME/F12 medium. These cells were dispersed by pipetting and washed two times with PBS. The cell density was adjusted to 5 × 10⁵ cells/ml, and the cells were further incubated without or with variants of sHAI-1 (each 50 nm) in serum-free DME/F12 medium containing 5 μM TAPI-1 and 0.01% BSA at 37 °C for 5 h. The degree of cell aggregation was quantified by following equation: (aggregated cells/total cell) × 100, where the aggregates formed by over four cells are defined as aggregated cells.

**Fluorescence staining**

Ccolo201 cells treated without or with 50 nm MMP-7 as described above were plated on poly-L-lysine-coated plastic plates, fixed with ice-cold acetone/methanol for 15 min, and washed three times with PBS. After blocking the non-specific binding sites with 3% BSA in PBS, the cells were incubated with 50 nm biotinylated sHAI-1 or 50 nm biotinylated HAI-1(141–249) in TBS containing 10 mm CaCl₂ at 37 °C for 1 h. Biotinylated proteins bound to the cell surface were incubated with HRP-conjugated streptavidin (Vector Laboratories, Burlingame, CA) by incubating at 37 °C for 30 min. The cells were washed three times and then added with 180 μl of chromogenic reaction mixture consisting of 75 mm citrate/phosphate buffer (pH 5.0), containing 3.7 mm α-phenylenediamine and 0.01% H₂O₂. The reaction was terminated by adding with 50 μl of 2 M H₂SO₄, and the intensity of the color developed was measured at 485 nm.

**Statistical analysis**

All experiments were carried out independently at least three times. Comparisons between the two groups were performed using Student’s t test, with p < 0.05 considered to be significant.

**Author contributions**—T. I. and S. H. conceived and coordinated the study and wrote the manuscript. T. I. conducted experiments on the roles of HAI-1 fragments. S. H. designed and constructed the plasmid vectors for recombinant proteins. Y. K. and H. H. performed MS analysis. S. H. obtained project funding.

**Acknowledgments**—We thank Dr. Hiroshi Sato for providing the plasmid encoding cDNA of HAI-1. We are grateful to Tomoko Akiyama, Yuta Sasaki, and Yuki Kondo for technical assistance and helpful discussions.

**References**

1. Nagase, H., Visse, R., and Murphy, G. (2006) Structure and function of matrix metalloproteinases and TIMPs. *Cardiovasc. Res.* 69, 562–573
2. Overall, C. M., and Kleifeld, O. (2006) Tumour microenvironment-opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy. *Nat. Rev. Cancer* 6, 227–239
3. Werb, Z. (1997) ECM and cell surface proteolysis: regulating cellular ecology. *Cell* 91, 439–442
4. Egeland, M., and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat. Rev. Cancer* 2, 161–174
5. Miyazaki, K., Hattori, Y., Umenishi, F., Yasumitsu, H., and Umeda, M. (1990) Purification and characterization of extracellular matrix-degrading metalloproteinase, matrin (pump-1), secreted from human rectal carcinoma cell line. *Cancer Res.* 50, 7758–7764
6. Nagashima, Y., Hasegawa, S., Koshikawa, N., Taki, A., Ichikawa, Y., Kita-mura, H., Misugi, K., Khira, Y., Matuo, Y., Yasumitsu, H., and Miyazaki, K. (1997) Expression of matrilysin in vascular endothelial cells adjacent to matrilysin-producing tumors. *Int. J. Cancer* 72, 441–445
7. Adachi, Y., Yamamoto, H., Itoh, F., Hinoeda, Y., Okada, Y., and Imai, K. (1999) Contribution of matrilysin (MMP-7) to the metastatic pathway of human colorectal cancers. *Gut* 45, 252–258
8. Ichikawa, T., Ichikawa, Y., Mitutushia, M., Momiyama, N., Chishima, T., Tanaka, K., Yamaoka, H., Miyazaki, K., Nagashima, Y., Akitaya, T., and Shimada, H. (1996) Matrilysin is associated with progression of colorectal tumor. *Cancer Lett.* 107, 5–10
9. Yamamoto, K., Higashi, S., Kioi, M., Tsunezumi, I., Honke, K., and Miyazaki, K. (2006) Binding of active matrilysin to cell surface cholesterol sulfate is essential for its membrane-associated proteolytic action and induction of homotypic cell adhesion. *J. Biol. Chem.* 281, 9170–9180
10. Higashi, S., Oeda, M., Yamamoto, K., and Miyazaki, K. (2008) Identification of amino acid residues of matrix metalloproteinase-7 essential for binding to cholesterol sulfate. *J. Biol. Chem.* 283, 35735–35744
11. Fidler, I. J. (2003) The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited. *Nat. Rev. Cancer* 3, 453–458
Shed HAI-1 fragment has cell aggregation-inducing activity

12. Farahani, E., Patra, H. K., Jangamreddy, J. R., Rashedi, I., Kawai, M., Rao Pariti, R. K., Batakis, P., and Wiehevec, E. (2014) Cell adhesion molecules and their relation to (cancer) cell stemness. Carcinogenesis 35, 747–759

13. Kioi, M., Yamamoto, K., Higashi, S., Koshikawa, N., Fujita, K., and Miyazaki, K. (2003) Matripsy (MMP-7) induces homotypic adhesion of human colon cancer cells and enhances their metastatic potential in nude mouse model. Oncogene 22, 8662–8670

14. Guo, J., Chen, S., Huang, C., Chen, L., Studholme, D. I., Zhao, S., and Yu, L. (2004) MANSC: a seven-cysteine-containing domain present in animal membrane and extracellular proteins. Trends Biochem. Sci. 29, 172–174

15. Hong, Z., De Meulemeester, L., Jacobi, A., Pedersen, J. S., Morth, J. P., Andreasen, P. A., and Jensen, J. K. (2016) Crystal structure of a two-domain fragment of hepatocyte growth factor activator inhibitor-1. J. Biol. Chem. 291, 14340–14355

16. Kojima, K., Tsuzuki, S., Fushiki, T., and Inouye, K. (2008) Roles of functional and structural domains of hepatocyte growth factor activator inhibitor type 1 in the inhibition of matriptase. J. Biol. Chem. 283, 2478–2487

17. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo, J., Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor. J. Biol. Chem. 272, 6370–6376

18. Oberst, M., Anders, J., Xie, B., Singh, B., Osandon, M., Johnson, M., Dickson, R. B., and Lin, C. Y. (2001) Matriptase and HAI-1 are expressed by normal and malignant epithelial cells in vitro and in vivo. Am. J. Pathol. 158, 1301–1311

19. Kirchhofer, D., Peek, M., Lipari, M. T., Billeci, K., Fan, B., and Moran, P. (2005) Hepsin activates pro-hepatocyte growth factor and is inhibited by hepatocyte growth factor activator inhibitor-1B (HAI-1B) and HAI-2. FEBS Lett. 579, 1945–1950

20. Shia, S., Stamos, J., Kirchhofer, D., Fan, B., Wu, J., Corpuz, R. T., Santell, L., Lazarus, R. A., and Eigenbrot, C. (2005) Conformational liability in serine protease active sites: structures of hepatocyte growth factor activator (HGFA) alone and with the inhibitory domain from HGFA inhibitor-1B. J. Mol. Biol. 346, 1335–1349

21. Szabo, R., Hobson, J. P., List, K., Molinolo, A., Lin, C. Y., and Bugge, T. H. (2008) Potent inhibition and global co-localization implicate the transmembrane Kunitz-type serine protease inhibitor hepatocyte growth factor activator inhibitor-2 in the regulation of epithelial matriptase activity. J. Biol. Chem. 283, 29495–29504

22. Kataoka, H., Suganuma, T., Shimomura, T., Itoh, H., Kitamura, N., Nabeshima, K., and Koono, M. (1999) Distribution of hepatocyte growth factor activator inhibitor type 1 (HAI-1) in human tissues. Cellular surface localization of HAI-1 in simple columnar epithelium and its modulated expression in injured and regenerative tissues. J. Histochem. Cytochem. 47, 673–682

23. Itoh, H., Kataoka, H., Tomita, M., Hamasuna, R., Nawa, Y., Kitamura, N., and Koono, M. (2000) Upregulation of HGF activator inhibitor type 1 but not type 2 along with regeneration of intestinal mucosa. Am. J. Physiol. Gastrointest. Liver. Physiol. 278, G635–G643

24. Shimomura, T., Denda, K., Kawaguchi, T., Matsumoto, K., Miyazawa, K., and Kitamura, N. (1999) Multiple sites of proteolytic cleavage to release soluble forms of hepatocyte growth factor activator inhibitor type 1 from a transmembrane form. J. Biochem. 126, 821–828

25. Domoto, T., Takino, T., Guo, L., and Sato, H. (2012) Cleavage of hepatocyte growth factor activator inhibitor-1 by membrane-type MMP-1 activates matriptase. Cancer Sci. 103, 448–454

26. Bycroft, M., Bateman, A., Clarke, J., Hamill, S. J., Sandford, R., Thomas, R. L., and Chothia, C. (1999) The structure of a PKD domain from polycystin-1: implications for polycystic kidney disease. EMBO J. 18, 297–305

27. Ibraghimov-Beskrovnaya, O., Bukanov, N. O., Donohue, L. C., Dackowski, W. R., Klinger, K. W., and Landes, G. M. (2000) Strong homophilic interactions of the Ig-like domains of polycystin-1, the protein product of an autosomal dominant polycystic kidney disease gene, PKD1. Hum. Mol. Genet. 9, 1641–1649

28. Cheng, H., Fukushima, T., Takahashi, N., Tanaka, H., and Kataoka, H. (2009) Hepatocyte growth factor activator inhibitor type 1 regulates epithelial to mesenchymal transition through membrane-bound serine proteinases. Cancer Res. 69, 1828–1835

29. Higashi, S., and Miyazaki, K. (2008) Identification of amino acid residues of the matrix metalloproteinase-2 essential for its selective inhibition by B-amyloid precursor protein-derived inhibitor. J. Biol. Chem. 283, 10068–10078

30. Takakura, D., Hashii, N., and Kawasaki, N. (2014) An improved in-gel digestion method for efficient identification of protein and glycosylation analysis of glycoproteins using guanidine hydrochloride. Proteomics 14, 196–201

31. Rappsilber, J., Ishihama, Y., and Mann, M. (2003) Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal. Chem. 75, 663–670