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

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Hepatocyte growth factor activator inhibitors (HAI)-1 and -2 are recently identified and closely related Kunitz-type transmembrane serine protease inhibitors. Whereas HAI-1 is well established as an inhibitor of the serine proteases matriptase and hepatocyte growth factor activator, the physiological targets of HAI-2 are unknown. Here we show that HAI-2 displays potent inhibitory activity toward matriptase, forms SDS-stable complexes with the serine protease, and blocks matriptase-dependent activation of its candidate physiological substrates pro-prostasin and cell surface-bound pro-urokinase plasminogen activator. To further explore the potential functional relationship between HAI-2 and matriptase, we generated a transgenic mouse strain with a promoterless β-galactosidase marker gene inserted into the endogenous locus encoding HAI-2 protein and performed a global high resolution mapping of the expression of HAI-2, matriptase, and HAI-1 proteins in all adult tissues. This analysis showed striking co-localization of HAI-2 with matriptase and HAI-1 in epithelial cells of all major organ systems, thus strongly supporting a role of HAI-2 as a physiological regulator of matriptase activity, possibly acting in a redundant or partially redundant manner with HAI-1. Unlike HAI-1 and matriptase, however, HAI-2 expression was also detected in non-epithelial cells of brain and lymph nodes, suggesting that HAI-2 may also be involved in inhibition of serine proteases other than matriptase.

Recent mining of vertebrate genomes uncovered an unexpectedly large number of new membrane-associated trypsin-like serine proteases. The biochemical and physiological functions of most of these new serine proteases are undefined and the subject of active investigation. Trypsin-like serine proteases are typically synthesized as inactive zymogens that are irreversibly activated by a single endoproteolytic cleavage within a highly conserved activation site. They are subsequently inactivated by specific serine protease inhibitors that bind directly to the active site (1–3). Three functionally distinct classes of serine protease inhibitors, termed serpin-, Kazal-, and Kunitz-type inhibitors, have been identified in vertebrates. Whereas the serpin-type inhibitors have been extensively studied due to their preeminent role in regulating coagulation and fibrinolysis (3), the Kazal- and Kunitz-type serine protease inhibitors in vertebrates are comparatively less explored.

Hepatocyte growth factor activator inhibitor (HAI)-2 and HAI-2 (also known as placental bikunin), encoded by 

**Supplemental Figs. S1–S3.**

2 The abbreviations used are: HAI, hepatocyte growth factor activator inhibitor; β-gal, fusion of β-galactosidase and neomycin phosphotransferase; IC₅₀, half-maximal (50%) inhibitory concentration; PAI, plasminogen activator inhibitor; uPA, urokinase plasminogen activator.
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FIGURE 1. HAI-2 is a potent inhibitor of matriptase. A, alignment of the amino acid sequences of Kunitz domains (KD) 1 (top panel) and 2 (bottom panel) of mouse and human HAI-2 and HAI-1. Identical residues are highlighted in green and indicated by an asterisk. Homologous substitutions are highlighted in yellow and indicated by a colon. Residues of the reactive site loop and the secondary binding segment that are involved in binding of the protease target are boxed in red. B and C, matriptase forms stable inhibitory complexes with HAI-2. 20 (B) or 100 (C) ng of human recombinant matriptase serine protease domain was incubated with 50 (B) or 300 (C) ng of human recombinant HAI-1 or HAI-2 for 30 min at room temperature. The formation of protease-inhibitor complexes was analyzed by silver staining (B) or Western blot using an antibody that recognizes both HAI-1 and HAI-2 (C). Positions of non-complexed HAI-1, non-complexed HAI-2, matriptase-HAI-1 complexes, and matriptase-HAI-2 complexes are indicated by arrows. The positions of molecular weight markers in kilodaltons are shown at the left. D, HAI-2 inhibits matriptase peptidolytic activity. 1 nM human recombinant matriptase serine protease domain was incubated in the presence of 0–500 nM of human recombinant HAI-1 or HAI-2 for 20 min at 37 °C. Matriptase activity toward the chromogenic peptide Glu-Gly-Arg-p-nitroanilide was measured as an increase in absorbance at 405 nm. Measurements were performed in triplicate. E and F, matriptase forms stable inhibitory complexes with HAI-2. 50 ng of human recombinant matriptase serine protease domain and 50 ng of human recombinant HAI-1 or HAI-2 for 30 min at room temperature. The formation of protease-inhibitor complexes was analyzed by silver staining (E) or Western blot using an antibody that recognizes both HAI-1 and HAI-2 (F). Positions of non-complexed HAI-1, non-complexed HAI-2, matriptase-HAI-1 complexes, and matriptase-HAI-2 complexes are indicated by arrows. The positions of molecular weight markers in kilodaltons are shown at the left. G, HAI-2 inhibits activation of the prostasin zymogen by matriptase. 100 nM human recombinant prostasin are indicated by arrows. The positions of molecular weight markers in kilodaltons are shown at the left.

EXPERIMENTAL PROCEDURES

Chromogenic Peptide Hydrolysis Assay—The chromogenic peptide Glu-Gly-Arg-p-nitroanilide was purchased from Bachem Bioscience (King of Prussia, PA), the soluble recombinant human HAI-1 and HAI-2 extracellular domains were from R&D Systems (Minneapolis, MN). To study the inhibition of matriptase proteolytic activity, 1 nM recombinant human matriptase serine protease domain (16) was incubated with 100 μM chromogenic peptide substrate in 50 mM Tris-HCl, pH 8.0, 1 mM CaCl₂, 0.1% Tween 20 for 20 min at 37 °C in the presence of 0–500 nM HAI-1 or HAI-2 proteins. Changes in absorbance at 405 nm were monitored over time on a SAFIRE2™ Microplate Reader (Tecan, Durham, NC). All measurements were performed in triplicate.

Formation of HAI/Matriptase Inhibitory Complexes—100 ng of the recombinant active human matriptase serine protease domain in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl buffer was incubated with 300 ng of human recombinant HAI-1 or HAI-2 for 30 min at room temperature. Protein complexes were analyzed by 4–12% reducing SDS-PAGE and Western blotting using a polyclonal anti-mouse HAI-2 primary antibody (R&D Systems) and anti-goat secondary antibody conjugated with alkaline phosphatase (EMD Chemicals-Calbiochem, La Jolla, CA) and a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium detection system (Roche Applied Science). For non-immunological detection of HAI-matriptase complex formation, the assay was performed using 20 ng of active human matriptase serine protease domain and 50 ng of human endogenous Spint2 locus, we show that HAI-2 co-localizes with matriptase and HAI-1 in the epithelia of all major organ systems. Collectively, these new data strongly implicate HAI-2 as a physiologically relevant inhibitor of matriptase, possibly acting in a redundant or partially redundant manner with HAI-1 to regulate epithelial cell surface proteolysis in adult tissues.
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Recombinant HAI-1 or HAI-2, followed by 4–12% reducing SDS-PAGE and silver staining using a SilverQuest kit (Invitrogen) according to the manufacturer’s instructions.

Prostasin Zymogen Activation Assay—0.1 μM human soluble prostasin zymogen prepared as described (17) was incubated with 5 nM recombinant active human matriptase serine protease domain for 1 h at 37 °C in 50 mM Tris/HCl, pH 8.0, 100 mM NaCl buffer. To evaluate the effect of HAI-1 and HAI-2 on matriptase-mediated activation of pro-prostasin, matriptase was preincubated with 100 nM human recombinant HAI-1 or HAI-2, followed by 4–12% reducing SDS-PAGE and silver staining using a SilverQuest kit (Invitrogen). The mixture was incubated for 30 min at 37 °C. Cells were washed twice with serum-free RPMI 1640, then resuspended at 10^6 cells/ml in 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 0.01% Tween 20 with 0.5 mM Spectrozyme UK (American Diagnostica, Stamford, CT), and incubated at 37 °C for 4 h. uPA substrate hydrolysis was measured by the increase in absorbance at 405 nm using a Victor3V spectrophotometer (PerkinElmer Life Sciences).

Tissue Acquisition—All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following Institutional Guidelines and standard operating procedures. Spint2 knock-in mice containing a promoterless β-galactosidase gene trap inserted into intron 1 of the mouse Spint2 gene were generated from the embryonic stem cell line KST272 obtained from Bay Genomics (San Francisco, CA) (15). The generation of the Spint1 null mice and β-galactosidase-tagged ST14 knock-in mice have previously been described (12, 19, 20). The Spint2 knock-in mice were genotyped for the presence of the β-galactosidase gene trap by PCR using HAI-2-geo51 (5'-ATCTGCAACCTCAAGCTAGC-3') and HAI-2-geo31 (5'-CAGAAACCAGCAGAATCTGAGG-3') primers. The Spint1 null mice and ST14 knock-in mice were genotyped by PCR as described previously (12, 19, 20).

Whole Mount X-gal Staining—Six-month-old wild-type or heterozygous β-galactosidase-tagged Spint2 knock-in mice or β-galactosidase-tagged ST14 knock-in mice were euthanized by CO₂ inhalation. Organs were excised, and slices of each tissue were placed in 4% paraformaldehyde in phosphate-buffered...
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saline for 30 min, rinsed in phosphate-buffered saline, and stained overnight at 37 °C with a β-galactosidase staining kit (Roche Applied Science). The tissues were post-fixed for 16 h in 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections were counterstained with nuclear fast red and subsequently examined for HAI-2 or matriptase expression. All microscopic images were acquired on a Zeiss AxioImager Z1 light microscope using an AxioCam HRc digital camera system (both Carl Zeiss AG, Gottingen, Germany).

Immunohistochemistry—Six-month-old wild-type or Spint1 null mice were euthanized by CO2 inhalation. Tissues were fixed overnight in 4% paraformaldehyde, embedded in paraffin, and cut into sections 5 μm thick. Antigens were retrieved by incubation for 10 min at 37 °C with 10 μg/ml proteinase K (Fermentas, Hanover, MD). The sections were blocked for 1 h at room temperature with 2% bovine serum albumin in phosphate-buffered saline and incubated overnight at 4 °C with the goat anti-mouse HAI-1 primary antibody (R&D Systems). Bound antibodies were visualized using a biotin-conjugated anti-goat secondary antibody (Vector Laboratories, Burlingame, CA) and a Vectastain ABC kit (Vector Laboratories) using 3,3’-diaminobenzidine substrate (Sigma).

In Situ Hybridization—In situ hybridization of digoxigenin-labeled probes to paraformaldehyde-fixed, paraffin-embedded sections was carried out using the Link-Label ISH Core Kit II (BioGenex, San Ramon, CA) following the manufacturer’s instructions. The Spint2-specific digoxigenin-labeled RNA probes were prepared by in vitro transcription of the +40- to +347-bp fragment of the mouse Spint2 open reading frame cloned into a pCRII-TOPO vector using the DIG RNA Labeling Kit (Roche Applied Science) according to the manufacturer’s instructions. 100 ng of sense or antisense RNA probe was applied on parallel pCRII-TOPO vector using the DIG RNA Labeling Kit (Roche Applied Science) as described in the instruction manual, and the sections were counterstained with nuclear fast red.

RESULTS

HAI-2 Is a Potent Inhibitor of Matriptase That Blocks the Proteolytic Activation of Its Candidate Physiological Targets the Prostasin Zymogen, and Cell Surface Pro-uPA—HAI-2 consists of two Kunitz-type inhibitor domains followed by a single span transmembrane domain and lacks the additional N-terminal region of unknown function found in HAI-1 (7). Although this accounts for the significant difference in the overall molecular weight between the two inhibitors (28.2 and 56.9 kDa for nascent unmodified proteins, respectively), the two proteins nevertheless exhibit a high degree of homology in their inhibitory domains. Comparing the two Kunitz-type inhibitor domains of the human and murine forms of HAI-1 and HAI-2 revealed a 39–56% overall amino acid identity and a 57–67% amino acid homology (Fig. 1A). The most highly conserved motifs included the reactive site loop and the secondary binding segment of the Kunitz domain (Fig. 1A), which were previously shown to be involved in the binding of the Kunitz domain to target pro tease, including matriptase (16). Because HAI-1 is an essential inhibitor of matriptase in both mice and zebrafish (12, 14), this suggested that HAI-2 could also be a relevant inhibitor of this widely expressed membrane serine protease. HAI-1 has been shown previously to form SDS-stable complexes with matriptase that can be visualized by SDS-PAGE under non-boiling conditions (9). Therefore, to investigate the possible interaction between matriptase and HAI-2, we incubated the recombinant matriptase serine protease domain with recombinant soluble extracellular domain of either HAI-2 or HAI-1. Protease-inhibitor complex formation was analyzed by reducing SDS-PAGE followed by either silver staining or Western blotting using an HAI-2 antibody that cross-reacts with HAI-1. As reported previously, a novel protein species appeared with a molecular weight predicted for the matriptase-HAI-1 complex when matriptase was incubated with HAI-1 (Fig. 1, B and C, lane 3). This was associated with a decrease in the amount of non-complexed HAI-1 (Fig. 1, B and C, compare lanes 2 and 3). Interestingly, the formation of similar matriptase-HAI-2 complexes was readily detected when matriptase was incubated with HAI-2 (Fig. 1, B and C, lane 5), also associated with a decrease in non-complexed HAI-2 (Fig. 1, B and C, compare lanes 4 and 5). Next, we determined if HAI-2 displayed inhibitory activity against matriptase by determining the IC50 of the soluble HAI-2 toward the matriptase serine protease domain. In agreement with the high amino acid identity of the Kunitz-type inhibitor domains of the two transmembrane serine protease inhibitors, HAI-2 potently inhibited matriptase, displaying an IC50 that was indistinguishable from that of HAI-1 (0.5 nM and 0.63 nM, respectively) (Fig. 1D).

HAI-2 Blocks Matriptase-mediated Activation of Its Candidate Substrates Pro-prostasin and Cell Surface-bound Pro-uPA—Matriptase likely promotes oral epithelial and epithelial differentiation by proteolytically activating the prosta-

### TABLE 1

| Tissue                  | Cell population | HAI-2 | HAI-1 | Matriptase |
|------------------------|-----------------|-------|-------|------------|
| Skin                   | Interfollicular epidermis | – | +/− | – |
| Basal layer            |                 | – | +/− | – |
| Spinous layer          |                 | – | +/− | – |
| Granular layer         |                 | – | +/− | – |
| Hair follicles         |                 | – | +/− | – |
| Outer root sheath      |                 | – | +/− | – |
| Inner root sheath      |                 | + | +/− | + |
| Medulla                |                 | – | +/− | – |
| Cortex                 |                 | – | +/− | – |
| Cuticle                |                 | – | +/− | – |
| Sebaceous gland        |                 | + | +/− | + |
| Oral cavity            | Tongue epithelium | – | +/− | – |
| Oral mucosal epithelium|                 | – | +/− | – |
| Lip epithelium         |                 | + | +/− | + |

Dietogenic tract:  
Esophagus, Epithelium, suprabasal layer | – | +/− | – |
Foremostach, Epithelium, suprabasal layer | – | +/− | – |
Glandular stomach, Surface epithelium | – | +/− | – |
Parietal cells | + | +/− | + |
Zymogenic cells | – | – | – |
Jejunum/ileum, Crypts | – | +/− | – |
Villous epithelium | +/− | +/− | + |
Colon, Crypts | + | +/− | + |
Goblet cells | + | +/− | + |
Surface epithelium | + | +/− | + |
Liver, Hepatocytes | – | +/− | – |
Bile duct epithelium | – | +/− | – |
Pancreas, Acinar cells | – | – | – |
Islets of Langerhans | + | +/− | + |
sin (PRSS8/CAP1) zymogen, because ST14- and PRSS8-ablated epidermis are phenocopies of each other and active prostanin is absent in ST14 null mice (17, 21). To determine if HAI-2 can block prostanin activation by matriptase, recombinant prostanin zymogen was expressed in HEK293T cells and released from the cell surface by phosphatidylinositol-specific phospholipase C. As reported previously (17), incubation of soluble prostanin zymogen with low amounts of matriptase lead to the formation of active two-chain prostanin, which displayed a slightly increased electrophoretic mobility in high percentage SDS-PAGE after reduction of the single disulfide bridge that links the two chains (Fig. 1E, compare lanes 2 and 3). This activation of prostanin by matriptase was completely blocked when matriptase was preincubated with either HAI-1 (Fig. 1E, compare lanes 3 and 4) or HAI-2 (Fig. 1E, compare lanes 3 and 5).

We next determined if HAI-2 was capable of inhibiting matriptase activity in a physiologically relevant setting. Recent studies have shown that matriptase is required for the conversion of pro-uPA to active two-chain uPA on the surface of the acute monocytic leukemia cell line THP-1, ultimately leading to plasminogen activation on the cell surface (18). To investigate if HAI-2 could regulate matriptase activity under physiologically relevant conditions, we therefore measured uPA activity on the surface of THP-1 cells that were preincubated with either pro-uPA or active high molecular weight uPA in the presence or absence of soluble HAI-2 protein. HAI-2 displayed no inhibitory activity toward active two-chain uPA when assayed in vitro (data not shown). In agreement with this, the presence of HAI-2 had no effect on cell surface uPA activity when THP-1 cells were preincubated with active uPA (Fig. 1F). However, HAI-2 almost completely abolished uPA activity on the surface of cells that were preincubated with pro-uPA (Fig. 1F).

In contrast, when the known uPA inhibitor plasminogen activator inhibitor-1 was added to THP-1, ultimately leading to plasminogen activation on the cell surface (18). To investigate if HAI-2 could regulate matriptase activity under physiologically relevant conditions, we therefore measured uPA activity on the surface of THP-1 cells that were preincubated with either pro-uPA or active high molecular weight uPA in the presence or absence of soluble HAI-2 protein. HAI-2 displayed no inhibitory activity toward active two-chain uPA when assayed in vitro (data not shown). In agreement with this, the presence of HAI-2 had no effect on cell surface uPA activity when THP-1 cells were preincubated with active uPA (Fig. 1F). However, HAI-2 almost completely abolished uPA activity on the surface of cells that were preincubated with pro-uPA (Fig. 1F). In contrast, when the known uPA inhibitor plasminogen activator inhibitor-1 was added to THP-1 cells...
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The data presented above potentially implicated HAI-2 in the regulation of matriptase proteolytic activity. However, for a protease inhibitor to serve as a physiological inhibitor of a cognate protease requires the physical proximity of the two proteins in tissues. HAI-2 and matriptase are both membrane-anchored proteins, making it likely that HAI-2 must be expressed by the same cells that express matriptase to inhibit the protease. Therefore, we next set out to delineate the potential co-localization of HAI-2 and matriptase in cell lineages that form adult murine tissues. Furthermore, we mapped the expression of HAI-2 relative to HAI-1 to determine the possible functional overlap between the two membrane serine protease inhibitors.

An initial test of several commercially available anti-mouse HAI-2 antibodies revealed prohibitively high nonspecific immunohistochemical background using a variety of staining conditions, as well as cross reactivity with HAI-1 (data not shown). A similar lack of specificity was observed with all commercially available and in-house-generated matriptase antibodies. To analyze the expression of HAI-2 in mouse tissues, we therefore employed the technique of enzymatic gene trapping. A search of ES cell clones available through the International Gene Trap Consortium (www.genetrap.org) revealed one ES cell clone, KST272, with a promoterless β-galactosidase-neomycin phosphotransferase fusion gene (β-geo) inserted into intron 1 of the mouse Spint2 gene, which encodes HAI-2 (15) (HAI-2/β-geo allele, Fig. 2A). The insertion results in the expression of a β-geo reporter protein from the endogenous promoter of the Spint2 gene, thus allowing identification of HAI-2-expressing cells in situ by X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. This technique has previously been shown to be a reliable alternative to immunohistochemistry in the absence of suitable antibodies or when proteins are expressed below the level of immunological detection (20, 22, 23).

FIGURE 4. Expression profiles of HAI-2, HAI-1, and matriptase in gallbladder, pancreas, respiratory, and urinary tissues. A–F, X-gal staining of HAI-2 expression (blue). High levels of HAI-2 were observed in epithelium of gallbladder (A, arrowheads), respiratory epithelium of the trachea (C, arrowheads), and bronchioles (D, arrowheads), distal and collecting tubules in kidney (E, arrowheads), and in epithelium of urinary bladder (F, arrowheads). Low expression was detected in pancreas in islets of Langerhans (B, arrowheads) and in proximal tubules in kidney (E, open arrowhead), whereas no HAI-2 expression was observed in exocrine pancreas (B, open arrowhead) or in glomeruli (E, arrow). A’–F’, immunohistochemical staining of HAI-1 expression (brown), A’–F’, X-gal staining of matriptase expression (blue). Expression of HAI-1 (A’, arrowheads), but not matriptase (A”, arrowhead) was detected in epithelium of gallbladder. Both proteins co-localized in islets of Langerhans in pancreas (B’ and B”, arrowheads), respiratory epithelium of the trachea (C’ and C”, arrowheads) and bronchioles (D’ and D”, arrowheads), distal and collecting tubules in kidney (E’ and E”, arrowheads), and to epithelia of urinary bladder (F’ and F”, arrowheads). Low expression of HAI-1 (E’, open arrowhead) but not matriptase (E”, open arrowhead) was also observed in kidney proximal tubules, and neither HAI-1 nor matriptase were expressed in glomeruli (E’ and E”, arrows). The immunostaining seen in some endothelial cells within pancreas (B”, open arrowhead) is nonspecific, because the same signal was present in tissues of animals expressing no HAI-1 protein (inset of B”, open arrowhead). Scale bars: 30 μm (A’–A”), 50 μm (B’–F’).
To validate the use of Spint2 β-gal–targeted mice to delineate HAI-2 expression, staining with X-gal for detection of β-galactosidase activity and in situ hybridization of Spint2 mRNA was performed on parallel sections. In several tissues, including kidney, colon, trachea, and gallbladder, the pattern of β-galactosidase expression closely matched that of Spint2 mRNA (Fig. 2, B–D, and data not shown). This preliminary analysis established that the expression pattern of the β-galactosidase protein is a faithful representation of the expression of the endogenous HAI-2 in mouse tissues.

For the detection of HAI-1, we performed immunohistochemistry using a previously validated polyclonal antibody (12). Sections of the corresponding tissues from adult HAI-1-deficient mice3 were used as negative controls in all experiments. To locate the expression of matriptase, we used a knock-in mouse strain with an insertion of a promoterless gene that was previously validated for detecting matriptase expression in adult tissues (20, 24).

**HAI-2 Co-localizes with Matriptase and HAI-1 in Sebaceous Glands and Vibrissal Inner Root Sheath Cells of the Epidermis**—The expression of HAI-2 in the integumentary system appeared to be confined to the cells of sebaceous glands and the inner root sheath of vibrissal, but not pelage, hair follicles (Table 1, Fig. 3 A and B, and data not shown). In contrast, examination of adult skin confirmed the previously reported widespread and overlapping expression of HAI-1 and matriptase in the keratinocytes of the suprabasal layer of the interfollicular epidermis, and the inner root sheath and matrix of pelage and vibrissal hair follicles, as well as the sebaceous glands (Fig. 3, A′, A′′, B′, and B′′). None of the three proteins were expressed in the basal layer of the epidermis or in any of the cell types in the dermis. Similar patterns of expression were found in keratinized stratified epithelia of several other organs, including the oral cavity, tongue, esophagus, and forestomach. Although all these tissues showed expression of both HAI-1 and matriptase in the keratinocytes of their respective suprabasal, but not basal layers, none of them showed any detectable expression of HAI-2 (Table 1 and supplemental Fig. S1).

**Widespread and Coordinated Expression of HAI-2 with Matriptase and HAI-1 in the Digestive Tract**—Unlike the stratified epithelium of the upper digestive tract, the simple epithelium of the glandular stomach and the entire intestinal tract showed a high level of HAI-2 expression (Table 1). In the glandular stomach, HAI-2 was detected in the parietal cells, whereas no expression was observed in the surface mucous cells or the chief cells (Fig. 3C). Matriptase was also found exclusively in the parietal cells, especially in the region proximal to the surface of the gastric gland (Fig. 3C′). On the other hand, the expression of HAI-1 was highly restricted to the surface mucous cells, with only very low levels of HAI-1 detectable in the parietal cells (Fig. 3C′′), thus restricting the expression of the two inhibitors to non-overlapping populations of gastric epithelial cells (Fig. 3D). In the small intestine, both HAI-2 and HAI-1 showed widespread expression in the surface epithelium of both the intestinal villi and the crypts of Lieberkühn (Fig. 3, E and E′). Matriptase, on the other hand, was expressed predominantly in the stem cells and proliferating regions of the crypt epithelium, with progressively decreased expression toward the mature absorptive cells of the villous epithelium (Fig. 3E). Similarly, the two inhibitors were strongly expressed in both goblet cells and the surface epithelial cells of the colon, whereas matriptase showed high expression in the cells at the base of the colonic crypts and in the goblet cells and only much lower expression in the surface mucosal cells (Fig. 3, F–F′).

No HAI-2 was detected in the liver, whereas HAI-1 and matriptase showed specific expression in the bile ducts (supplemental Fig. S2). HAI-1 was also detected in a subpopulation of periportal cells, but the presence of an equally strong signal in tissues from HAI-1-deficient animals indicates that this is a result of a cross-reactivity of anti-HAI-1 antibody with a different protein target (supplemental Fig. S2). Columnar epithelium of the gallbladder stained positively for both HAI-2 and HAI-1, but not for matriptase (Fig. 4, A–A′). In the pancreas, the expression of all three proteins was restricted to the islets of Langerhans that showed significant expression of HAI-1 and low, but clearly identifiable expression of HAI-2 and matriptase (Fig. 4, B–B′). Neither the protease nor the inhibitors were present in the acinar or ductal cells of the exocrine portion of the pancreas (Fig. 4, B and B′, and data not shown).

**Coordinated Expression of HAI-2 with Matriptase and HAI-1 in the Respiratory System**—HAI-2 and HAI-1, as well as matriptase, showed a widespread expression throughout the epithelia of respiratory tissues (Table 2). In the trachea, the proteins were expressed in the ciliated columnar epithelial cells (Fig. 4, C–C′), and the same pattern of expression was maintained also in bronchi and bronchioles (Fig. 4, D–D′, and supplemental Fig. S2). In addition, only HAI-1 was detected in lung alveoli, where it localized specifically to type II alveolar cells (Fig. 4D). None of the proteins was observed in supporting cartilage, fibroblast stroma, or smooth muscle cells or capillary endothelium of the lungs.

**HAI-2, HAI-1, and Matriptase Co-localize in the Urogenital System**—High levels of HAI-2 expression were detected in the kidney (Table 2). In this organ, HAI-2 specifically localized to the epithelium of the distal convoluting ducts and collecting

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3 R. Szabo, and T. H. Bugge, unpublished data.

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**TABLE 2**

| Tissue                        | Cell population          | HAI-2 | HAI-1 | Matriptase |
|-------------------------------|--------------------------|-------|-------|------------|
| **Respiratory system**        |                          |       |       |            |
| Trachea                       | Epithelium               | +     | +     | +          |
| Bronchi/bronchioles           | Epithelium               | +     | +     | +          |
| Lungs                         | Alveolar type II cells    | –     | –     | –          |
| **Urinary system**            |                          |       |       |            |
| Kidney                        | Glomeruli                | –     | –     | –          |
|                                | Proximal tubules          | +/−   | +/−   | +/−        |
|                                | Distal/collecting tubules| +     | +     | +          |
| Bladder                       | Epithelium               | +     | +     | +          |
| **Reproductive system**       |                          |       |       |            |
| Mammary gland                 | Ductal epithelium        | −     | −     | +          |
| Uterus                        | Surface epithelium       | +/−   | +     | +/−        |
|                                | Uterine glands            | +     | +     | +/−        |
| Ovary                         | Granulosa cells, antral   | +     | +     | –          |
|                                | follicles                | –     | –     | –          |
| Testis                        | Corpus luteum            | +     | –     | –          |
|                                | Undifferentiated         | –     | –     | –          |
|                                | spermatagonia            | +     | –     | –          |
| Seminal vesicle               | Epithelium               | +     | +     | –          |

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ducts (Fig. 4E). Proximal tubules displayed much weaker staining, and no signal was detected in the glomeruli. This pattern of expression was identical to that of HAI-1, whereas matriptase expression appeared to be restricted to the distal and collecting ducts (Fig. 4, E′ and E″). All three proteins were also expressed in the epithelium of the urinary bladder. Whereas the expression of HAI-2 and matriptase was restricted to the surface (suprabasal) layer, HAI-1 displayed a more general distribution and was detected in both basal and suprabasal cells of this transitional epithelium (Fig. 4, F–F″).

Although most of the mouse reproductive tissues showed expression of either HAI-1, HAI-2, or matriptase, each of the proteins exhibited a unique pattern of expression (Table 2). In females, HAI-2 was detected in the surface epithelium of the uterus and in the uterine glands, whereas no expression was observed in adult virgin mammary gland (supplemental Fig. S2). In ovary, HAI-2 expression was detected both in the granulosa cells of the antral follicles and in the progesterone-producing cells of the corpora lutea (Fig. 5A). Matriptase was also present in the antral follicles (Fig. 5A″), whereas HAI-1 did not show detectable levels of expression in this organ (Fig. 5A′). In the male reproductive system, HAI-2 was present in the columnar epithelium of the seminal vesicle, but not in the testis (Fig. 5B and supplemental Fig. S2). The expression of the HAI-2/β-geo fusion protein in prostate could not be evaluated due to prominent endogenous β-galactosidase activity in this tissue, which resulted in a strong X-gal signal even in tissues from control wild-type mice (data not shown). Similar to HAI-2, HAI-1 was also expressed in seminaliferous tubules of the adult testis, where it localized specifically to undifferentiated spermatogonia that are in contact with the basement membrane (supplemental Fig. S2). Unlike HAI-1 or HAI-2, no expression of matriptase was detected in testis or in seminal vesicle (Fig. 5B″ and supplemental Fig. S2).

**HAI-2, HAI-1, and Matriptase Co-localization in Thymic Epithelium**—Thymic epithelial matriptase plays a critical role in thymocyte maturation (19). HAI-2 expression was detected in the medullary epithelial cells of the thymus, where it precisely co-localized with the expression of HAI-1 and matriptase (Table 3 and Fig. 5C–C″). No expression of HAI-2 was found in any of the salivary glands from adult male or female mice (supplemental Fig. S3, and data not shown). This was in contrast to HAI-1 and matriptase that showed high levels of expres-
HAI-2 as Novel Candidate Inhibitor of Matriptase

The Kunitz-type inhibitor domains of HAI-1 and HAI-2 have been reported to have broad inhibitor specificity toward trypsin-like serine proteases (25–31), and the efficient inhibition of matriptase by HAI-2 in vitro would be of little significance unless the membrane serine protease and protease inhibitor pair were in physical proximity in tissues. To address this issue in the absence of suitable antibodies, we created a mouse strain that expressed a β-galactosidase marker gene under the control of the endogenous Spint2 regulatory sequences and performed a global high resolution mapping of the co-localization of HAI-2, HAI-1, and matriptase. Overall, the HAI-2 expression data generated this way were in good agreement with previous reports of HAI-2 mRNA and protein expression in individual human and mouse tissues (5, 6, 32–36). This analysis identified multiple epithelial cell lineages that expressed both HAI-2 and matriptase. These included the sebaceous glands and inner root sheath cells of the epidermis, the parietal cells of the glandular stomach, the transitional region between crypt and surface epithelium of the small intestine, the goblet cells of the colon, the ciliated columnar cells of the trachea, bronchi, and bronchioles, the collecting ducts of the kidney, the suprabasal urinary bladder epithelium, the antral follicles of the ovary, and the thymic epithelium (Tables 1–3). This widespread co-localization of matriptase indicated that HAI-2 would have the capacity to inhibit matriptase in multiple tissues.

Most of the cell lineages expressing HAI-2 and matriptase also expressed HAI-1. Exceptions included the parietal cells of the glandular stomach and the antral follicle cells of the ovary. This suggests that HAI-2 regulation of matriptase may be mostly redundant or partially redundant with HAI-1, but in a few cell lineages, HAI-2 would be the principal matriptase inhibitor. Finally, a few epithelial tissues, as well as the lymphatic and central nervous systems, displayed prominent expression of HAI-2 in the absence of matriptase, suggesting that HAI-2 may have additional matriptase-independent substrates.

In summary, our biochemical analysis and high resolution expression analysis strongly suggests that HAI-2 is a physiologically relevant inhibitor of matriptase in multiple adult epithelial tissues.

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REFERENCES

1. Puente, X. S., Sanchez, L. M., Overall, C. M., and Lopez-Otin, C. (2003) Nat. Rev. Genet. 4, 544–558
2. Rawlings, N. D., and Barrett, A. J. (1994) Methods Enzymol. 244, 19–61
3. Rau, J. C., Beaulieu, L. M., Huntington, J. A., and Church, F. C. (2007) J. Thromb. Haemost. 5, Suppl. 1, 102–115
4. Marlbor, C. W., Delaria, K. A., Davis, G., Muller, D. K., Greve, J. M., and Tamburini, P. P. (1997) J. Biol. Chem. 272, 12202–12208
5. Itoh, H., Kataoka, H., Hamasuna, R., Kitamura, N., and Koono, M. (1999) Biochem. Biophys. Res. Commun. 255, 740–748
6. Muller-Pillsch, F., Wallrapp, C., Bartels, K., Varga, G., Friess, H., Buchler, M., Adler, G., and Gress, T. M. (1998) Biochem. Biophys. Acta 1395, 88–95
7. Kawaguchi, T., Qin, L., Shimomura, T., Kondo, J., Matsumoto, K., Denda, K., and Kitamura, N. (1997) J. Biol. Chem. 272, 27558–27564
8. Shimomura, T., Denda, K., Kitamura, A., Kawaguchi, T., Kito, M., Kondo,

**TABLE 3**

Expression of HAI-2, HAI-1, and matriptase in other organs

| Tissue               | Cell population | HAI-2 | HAI-1 | Matriptase |
|----------------------|-----------------|-------|-------|------------|
| Salivary glands      |                 |       |       |            |
| Submandibular gland  | Ductal epithelium | –     | +     | +          |
| Sublingual gland     | Macous/serous acini | –     | –     | –          |
| Parotid gland        | Ductal epithelium | –     | +     | +          |
|                      | Macous acini     | +     | +     | –          |
|                      | Serous acini     | –     | –     | –          |
| Thymus               | Muscular epithelium | +     | +     | +          |
| Lymph nodes          | Lymphatic endothelium | –     | –     | –          |
| Brain                |                 |       |       |            |
| Olfactory bulb       | Glomerular layer | –     | –     | –          |
| Cerebrum             | Cortex           | –     | –     | –          |
|                     | Striatum         | –     | –     | –          |
| Cerebellum           | Inner granular layer | +     | –     | –          |
| Spleen               | Lymphoid/endothelial cells | –     | –     | –          |
| Skeletal muscle      | Myocytes         | –     | –     | –          |
| Heart                | Cardiomyocytes   | –     | –     | –          |
| Vascular system      | Arteries, veins, and capillaries | –     | –     | –          |
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J. Kagaya, S., Qin, L., Takata, H., Miyazawa, K., and Kitamura, N. (1997) J. Biol. Chem. 272, 6370–6376
9. Lin, C. Y., Anders, J., Johnson, M., and Dickson, R. B. (1999) J. Biol. Chem. 274, 18237–18242
10. Tanaka, H., Nagaie, K., Takeda, N., Itoh, H., Kohama, K., Fukushima, T., Miyata, S., Uchiyama, S., Shimomura, T., Miyazawa, K., Kitamura, N., Yamada, G., and Kataoka, H. (2005) Mol. Cell Biol. 25, 5687–5698
11. Fan, B., Brennan, J., Grant, D., Peale, F., Rangell, L., and Kirchhofer, D. (2007) Dev. Biol. 303, 222–230
12. Szabo, R., Molinolo, A., List, K., and Bugge, T. H. (2007) Oncogene 26, 1546–1556
13. Mathias, J. R., Dodd, M. E., Walters, K. B., Rhodes, J., Kanki, J. P., Look, A. T., and Huttenlocher, A. (2007) J. Cell Sci. 120, 3372–3383
14. Carney, T. J., von der Hardt, S., Sonntag, C., Amsterdam, A., Topczewski, J., Hopkins, N., and Hammerschmidt, M. (2007) Development 134, 3461–3471
15. Mitchell, K. J., Pinson, K. L., Kelly, O. G., Brennan, J., Zapicich, J., Scherz, P., Leighton, P. A., Goodrich, L. V., Lu, X., Avery, B. J., Tate, P., Dill, K., Pangilinan, E., Wakenight, P., Tessier-Lavigne, M., and Skarnes, W. C. (2007) Nat. Genet. 28, 241–249
16. Friedrich, R., Fuentes-Prior, P., Ong, E., Coombs, G., Hunter, M., Oehler, R., Pierson, D., Gonzalez, R., Huber, R., Bode, W., and Madison, E. L. (2002) J. Biol. Chem. 277, 2160–2168
17. Netzelt-Arnett, S., Currie, B. M., Szabo, R., Lin, C. Y., Chen, L. M., Chai, K. X., Antalis, T. M., Bugge, T. H., and List, K. (2006) J. Biol. Chem. 281, 32941–32945
18. Kilpatrick, L. M., Harris, R. L., Owen, K. A., Bass, R., Ghorayeb, C., Bar-Or, A., and Ellis, V. (2006) Blood 108, 2616–2623
19. List, K., Haudenschild, C. C., Szabo, R., Chen, W., Wahl, S. M., Swaim, W., Engelholm, L. H., Behrendt, N., and Bugge, T. H. (2002) Oncogene 21, 3765–3779
20. List, K., Szabo, R., Molinolo, A., Nielsen, B. S., and Bugge, T. H. (2006) Am. J. Pathol. 168, 1513–1525
21. Leyvraz, C., Charles, R. P., Rubera, I., Guitard, M., Rotman, S., Breiden, B., Sandhoff, K., and Hummler, E. (2005) J. Cell Biol. 170, 487–496
22. Voss, A. K., Thomas, T., and Gruss, P. (1998) Dev. Dyn. 212, 171–180
23. Wurst, W., and Gessler, A. (2000) in Gene Targeting: A Practical Approach, 2nd Ed. (Joyner, A., ed) pp. 207–254, Oxford University Press, New York
24. List, K., Hobson, J. P., Molinolo, A., and Bugge, T. H. (2007) J. Cell. Physiol. 213, 237–245
25. Denda, K., Shimomura, T., Kawaguchi, T., Miyazawa, K., and Kitamura, N. (2002) J. Biol. Chem. 277, 14053–14059
26. Kirchhofer, D., Peek, M., Lipari, M. T., Billeci, K., Fan, B., and Moran, P. (2005) FEBS Lett. 579, 1945–1950
27. Kirchhofer, D., Peek, M., Li, W., Stamos, J., Eigenbrot, C., Kadkhodayan, S., Elliott, J. M., Corpuz, R. T., Lazarus, R. A., and Moran, P. (2003) J. Biol. Chem. 278, 36341–36349
28. Herter, S., Piper, D. E., Aaron, W., Gabriele, T., Cutler, G., Cao, P., Bhatt, A. S., Choe, Y., Craik, C. S., Walker, N., Meininger, D., Hoey, T., and Austin, R. J. (2005) Biochem. J. 390, 125–136
29. Delaria, K. A., Muller, D. K., Marlor, C. W., Brown, J. E., Das, R. C., Roczniak, S. O., and Tamburini, P. P. (1997) J. Biol. Chem. 272, 12209–12214
30. Fan, B., Wu, T. D., Li, W., and Kirchhofer, D. (2005) J. Biol. Chem. 280, 34513–34520
31. Qin, L., Denda, K., Shimomura, T., Kawaguchi, T., and Kitamura, N. (1998) FEBS Lett. 436, 111–114
32. Yamauchi, M., Kataoka, H., Itoh, H., Seguchi, T., Hasui, Y., and Osada, Y. (2004) J. Urol. 171, 890–896
33. Yamauchi, M., Itoh, H., Naganuma, S., Koono, M., Hasui, Y., Osada, Y., and Kataoka, H. (2002) Biol. Chem. 383, 1953–1957
34. Kataoka, H., Itoh, H., Uchino, H., Hamasuna, R., Kitamura, N., Nabeshima, K., and Koono, M. (2000) Cancer Lett. 148, 127–134
35. Parr, C., Watkins, G., Mansel, R. E., and Jiang, W. G. (2004) Clin. Cancer Res. 10, 202–211
36. Itoh, H., Kataoka, H., Tomita, M., Hamasuna, R., Nawa, Y., Kitamura, N., and Koono, M. (2000) Am. J. Physiol. 278, G635–G643