

HAI-2 stabilizes, inhibits, and regulates SEA-cleavage-dependent secretory transport of matriptase

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Abstract

It has recently been shown that HAI-2 is able to suppress carcinogenesis induced by overexpression of matriptase, as well as cause regression of individual established tumors in

a mouse model system. However, the role of HAI-2 is poorly understood.

In the present study we describe three mutations in the binding loop of the HAI-2

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Kunitz domain 1 (K42N, C47F, and R48L) that cause a delay in the SEA domain cleavage of matriptase, leading to accumulation of non-SEA domain cleaved matriptase in the ER.

We suggest that, like other known SEA domains, the matriptase SEA domain auto-cleaves and reflects that correct oligomerization, maturation, and/or folding has been obtained. Our results suggest that the HAI-2 Kunitz domain 1 mutants influence the flux of matriptase to the plasma membrane by affecting the oligomerization, maturation, and/or folding of matriptase, and

as a result the SEA domain cleavage of matriptase.

Two of the HAI-2 Kunitz domain 1 mutants investigated (C47F, R48L, C47F/R48L) also displayed a reduced ability to proteolytically silence matriptase. Hence, HAI-2 separately stabilizes matriptase, regulates the secretory transport, possibly via maturation/oligomerization, and inhibits the proteolytic activity of matriptase in the ER, and possible throughout the secretory pathway.

Matriptase (also known as MT-SP1, epithin, TADG-15 and SNC19) is a type II transmembrane serine protease that is expressed in most epithelia and is known to have pleiotropic roles in epithelial development and homeostasis¹⁻⁵. Knockout studies of matriptase in mice have shown that the protease has a principal and global function in promoting and/or restoring paracellular permeability barriers in simple and stratified epithelia^{2,4,6}. A mutation in the *ST14* gene encoding matriptase (matriptase G827R) is the underlying cause of a type of congenital ichthyosis⁷. Matriptase is known to be regulated by the two hepatocyte growth factor activator inhibitors (HAI) -1 and -2⁸. Genetic inactivation of either HAI-1 or HAI-2 in mice leads to failure of placental labyrinth formation^{9,10}. However, this defect can be completely rescued by simultaneously reducing or eliminating matriptase expression^{10,11}.

A wealth of evidence has coalesced within the last decade to indicate that matriptase is highly oncogenic unless kept under strict post-translational regulation by HAI-1 and/or HAI-2^{12,13}. In addition, deregulated matriptase has been shown to single-handedly cause squamous cell carcinoma formation in transgenic mice overexpressing wild type matriptase in the epidermis^{14,15}. A simultaneous increase in either HAI-1 or HAI-2 expression completely negates the oncogenic potential caused by matriptase overexpression^{14,15}. Furthermore, upregulation of HAI-2 expression has been shown to cause regression of already established individual tumors in mice¹⁴. However, the way by which HAI-1 and HAI-2 exerts this post-translational control of matriptase is still poorly understood.

Matriptase is a modular, approximately 95 kDa, protease that consists of a short cytoplasmic N-terminal peptide, a signal anchor that functions as a single-pass transmembrane domain, a sea urchin sperm protein, enteropeptidase, and agrin (SEA) domain (residues 86-201), two complement C1r/s urchin embryonic growth factor and

bone morphogenetic protein-1 (CUB) domains (residues 214-334), four low-density lipoprotein receptor class A (LDLA) domains (residues 452-604), and a trypsin-like serine protease domain (SPD) (residues 614-855)¹⁶⁻¹⁹ (Figure 1 A). Matriptase is synthesized as a single chain pro-form. The newly synthesized matriptase, expressed in the absence of HAI-2 and HAI-1, can only be detected at very low levels in cells, whereas matriptase co-expressed together with HAI-1 or HAI-2 can be detected in high amounts on the plasma membrane of cells in culture²⁰, and for HAI-2 also in genetically engineered mice²¹. Thus, suggesting that the membrane bound form of matriptase expressed without HAI-1 and HAI-2 becomes destabilized by an unknown mechanism²⁰. It has previously been shown that matriptase mutated in either G827R (causing congenital ichthyosis) or S805A (active site mutated matriptase) is able to escape this unknown mechanism²² and consequently, matriptase G827R and matriptase S805A can be readily detected independently of HAI-1 and HAI-2 by SDS-PAGE and Western blotting.

Newly synthesized matriptase is, by hydrolysis of the Gly149-Ser150 peptide bond, located within the SEA domain, converted into the SEA domain cleaved form of matriptase. The cleaved matriptase remains attached to the membrane by non-covalent interactions within the SEA domain²³. The SEA domain cleavage takes place in the secretory pathway, as only the SEA domain cleaved form of the matriptase mouse orthologue, epithin, has been found on the surface of cells²⁴. It is generally believed, that the SEA domain cleaved matriptase subsequently becomes proteolytically cleaved after Arg614 in the SPD domain (zymogen conversion)^{25,26}, generating the Arg614 cleaved form (also denoted as the activated form or the two chain form) (Figure 1 A). The Arg614 cleaved matriptase rapidly makes a complex with HAI-1, whereby it becomes proteolytically silent²⁷⁻²⁹.

HAI-1 and HAI-2 are closely related transmembrane serine protease inhibitors, each consisting of two extracellular Kunitz-

type protease inhibitor domains, followed by a C-terminal transmembrane domain^{30,31} (Figure 1 A). In addition, HAI-1 also contains an extracellular LDLR domain and a MANEC domain, recently shown to have a PAN/apple domain-type fold^{30,32}. HAI-2 primarily resides within the endoplasmic reticulum (ER)²⁰. In contrast to HAI-2, HAI-1 is exocytosed to the basolateral plasma membrane and subsequently transcytosed to the apical plasma membrane²⁹. A missense mutation in HAI-2, Y163C, is known to cause an autosomal recessive form of congenital sodium diarrhea³³ by an unknown mechanism.

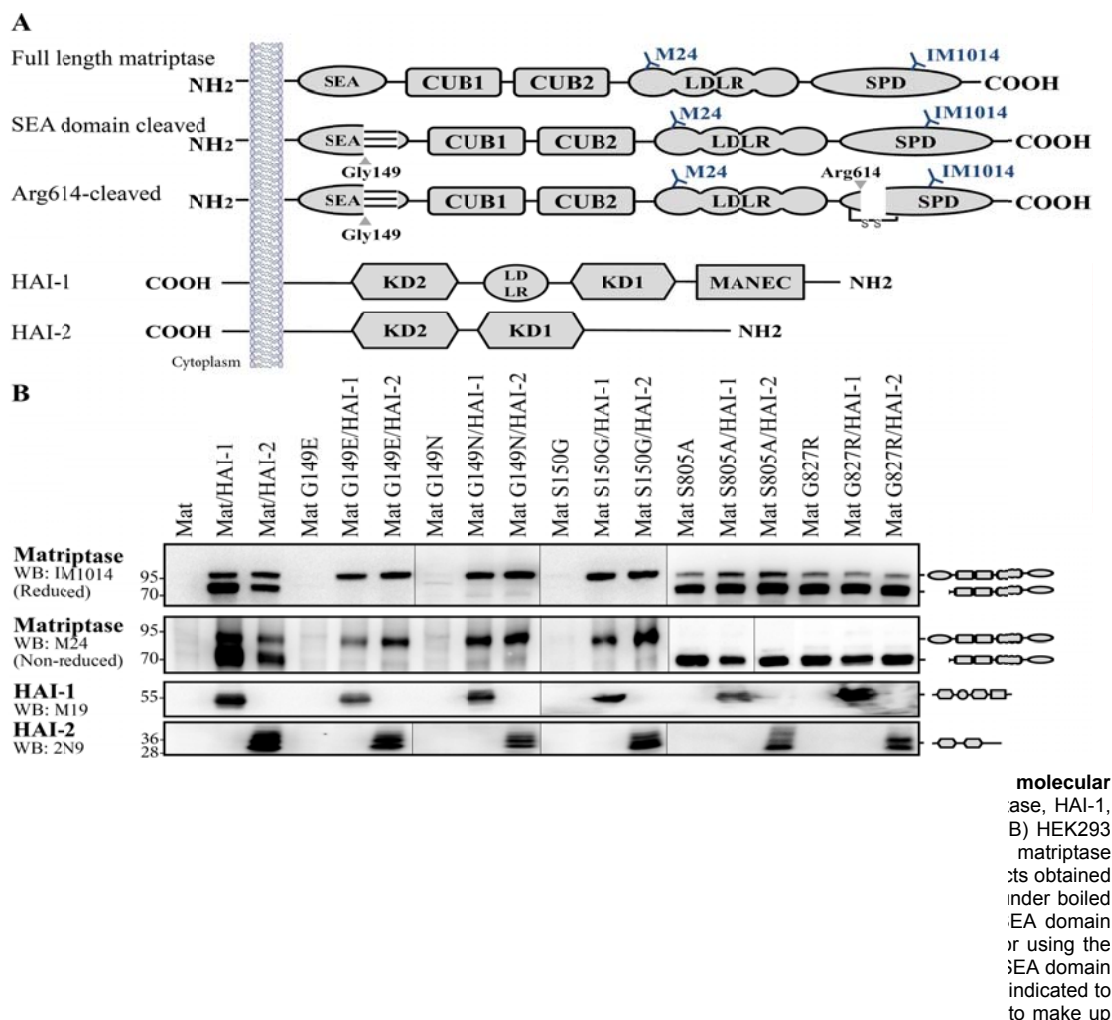
In the present study, we analyzed the functions of HAI-2 towards matriptase, using transient expression in HEK293 cells. None or very low levels of HAI-1, HAI-2, and matriptase are endogenously expressed in these cells, as tested by immunohistochemistry, SDS-PAGE and Western blotting (data not shown). In accordance, none/low levels of recombinant expressed matriptase could be detected, unless when stabilized by co-expression with either HAI-1 or HAI-2 (Figure 1 B). In order to focus on the interactions of newly synthesized matriptase taking place in the early secretory pathway, matriptase mutated in position Gly149 or Ser150, flanking the SEA domain cleavage site, were studied. It has previously been shown, that HAI-2 favors binding to this early non-SEA domain cleaved form of matriptase, as compared to the SEA domain cleaved form²¹. In this paper, we describe three individual mutations of the HAI-2 Kunitz domain 1 that results in delayed SEA domain cleavage and accumulation of matriptase in the ER, and of these three, two also displayed a reduced ability to proteolytically silence matriptase.

Results

Mutation of amino acids Gly149 and Ser150, flanking the matriptase SEA domain cleavage site, generates a high molecular weight non-SEA domain cleaved form of matriptase

In the present study, we aim to investigate the interactions between matriptase and HAI-

2 in the secretory pathway. Thus, our focus is mainly on the non-SEA domain cleaved form of matriptase. SEA domain cleavage of matriptase occurs between amino acid residues Gly149 and Ser150 (Figure 1 A). To prevent SEA domain cleavage, the amino acids on either side of the cleavage site were mutated, generating matriptase cDNAs encoding matriptase G149E, matriptase G149N and matriptase S150G. To confirm that these three mutated forms of matriptase are unable to SEA domain cleave, HEK293 cells were transiently transfected with expression plasmids for matriptase, matriptase G149E, matriptase G149N, and matriptase S150G, alone, or co-expressed together with either HAI-1 or HAI-2. The extracts obtained by lysis were analyzed by SDS-PAGE and Western blotting (Figure 1 B). The results showed that both wild type matriptase, and matriptase mutated to G149E, G149N or S150G were all undetectable, when expressed alone. However, all variants of matriptase were detectable in the non-SEA domain cleaved form, of approximately 100 kDa under reducing conditions and 90 kDa under non-reducing conditions, when co-expressed together with either HAI-1 or HAI-2. Additionally, only wild type matriptase was detected in the SEA domain cleaved form of approximately 80 kDa under reducing conditions and 70 kDa under non-reducing conditions, when co-expressed together with HAI-1 or HAI-2. Thus, confirming that the SEA domain cleavage of matriptase is prevented by inserting any one of the analyzed cleavage site mutations. Furthermore, these results confirmed the stabilizing roles of HAI-1 and HAI-2 towards matriptase, in this setup. In the same fashion, the expression of the catalytically inactive matriptase S805A, as well as a mutant known to cause autosomal recessive congenital ichthyosis, matriptase G827R with a strongly reduced proteolytic activity³⁴, were analyzed (Figure 1 B). The analysis showed that matriptase S805A and matriptase G827R were detectable in both the non-SEA domain cleaved form and the SEA domain cleaved form, regardless of co-expression with HAI-1 or HAI-2. Showing that matriptase activity is not a prerequisite for



SEA domain cleavage, and that it takes place independently of HAI-1 and HAI-2. Similar results have previously been obtained by others²².

Mutations in the Kunitz domain 1 of HAI-2 affect the SEA domain cleavage of matriptase

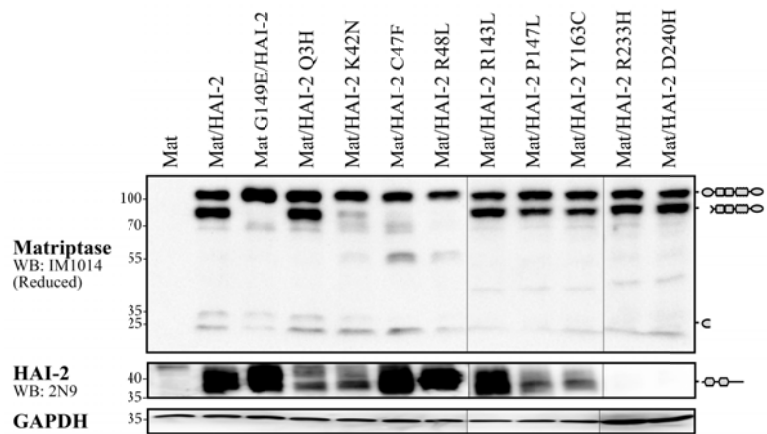
To investigate the importance of HAI-2 in the SEA domain cleavage of matriptase, a range of HAI-2 mutants were generated. Some of these mutations were based on reported single nucleotide polymorphisms within the coding region of HAI-2, and included Q3H, K42N, C47F, P147L, R233H, and D240H. Furthermore, the rare HAI-2 mutation Y163C was included, as it has been shown to give rise to an autosomal recessive syndromic form of congenital sodium diarrhea³³. In addition, mutations of the putative inhibitory sites in the HAI-2 Kunitz domain 1, R48L, and

in the Kunitz domain 2, R143L, previously described^{20,35}, were also investigated. Extracts of HEK293 cells transiently co-expressing wild type matriptase together with either wild type HAI-2 or any one of the mutated HAI-2 variants, were analyzed by SDS-PAGE and Western blotting (Figure 2). Extracts of cells expressing matriptase alone were included as a control. Furthermore, extracts from cells transiently co-expressing matriptase G149E with wild type HAI-2 were included, to indicate the position of non-SEA domain cleaved matriptase. The analysis showed that matriptase co-expressed with the HAI-2 mutants K42N, C47F, and R48L, all exhibited reduced levels of SEA domain cleaved matriptase, as compared to matriptase co-expressed with wild type HAI-2, or any one of the remaining HAI-2 mutants. As a control, all cell extracts were analyzed using

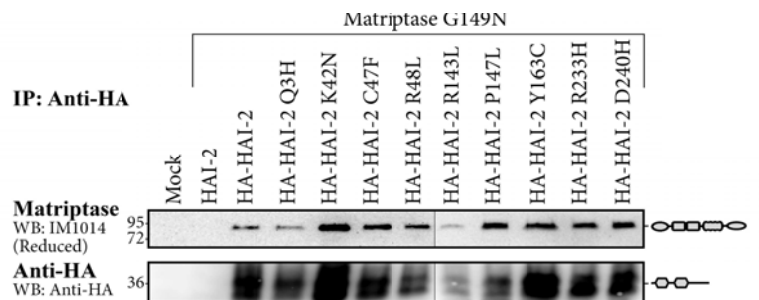
the 2N9 antibody directed against HAI-2. The 2N9 antibody was able to detect all the HAI-2 mutants, except for HAI-2 R233H and D240H. However, we have no reason to suspect that the HAI-2 R233H and D240H were not expressed, as matriptase was clearly detected in both of these extracts, unlike cells expressing matriptase alone. All the HAI-2 mutants examined were thus able to stabilize matriptase, as matriptase became detectable in the cell extracts when co-expressed with all of the HAI-2 variants investigated. These data suggest that HAI-2 directly or indirectly affects the SEA domain cleavage of matriptase, and that this process involves the Kunitz domain 1 of HAI-2, where K42N, C47F, and R48L are all located.

Non-SEA domain cleaved matriptase co-immunoprecipitates with HAI-2, regardless of the HAI-2 Kunitz domain 1 mutations K42N, C47F, and R48L

To investigate whether the HAI-2 mutants affect matriptase-HAI-2 interaction early on in the secretory pathway, the matriptase mutant G149N was co-immunoprecipitated with HA-HAI-2 wild type, or with HA-HAI-2 mutants; Q3H, K42N, C47F, R48L, R143L, P147L, Y163C, R233H, and D240H, from transiently expressing HEK293 cell extracts. Mock cell extracts were included as a control. The precipitates were analyzed by SDS-PAGE and Western blotting, using antibodies against the HA-tag and matriptase (Figure 3). The analysis showed that the HA-tag could be detected in all samples, except for the control cells, expressing untagged wild



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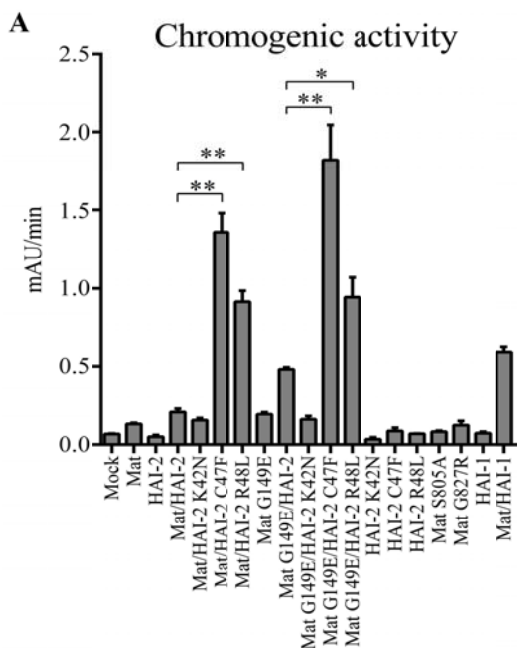


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type HAI-2, or mock transfected cells. Matriptase was detected in the precipitates when co-expressed with both HA-HAI-2 wild type and all of the investigated HA-HAI-2 mutations; Q3H, K42N, C47F, R48L, R143L, P147L, Y163C, R233H, and D240H. The same analysis was also done for matriptase G149E and matriptase S150G, with similar results (data not shown), showing that HAI-2 interacts with early matriptase despite the presence of any of the mutations analyzed,

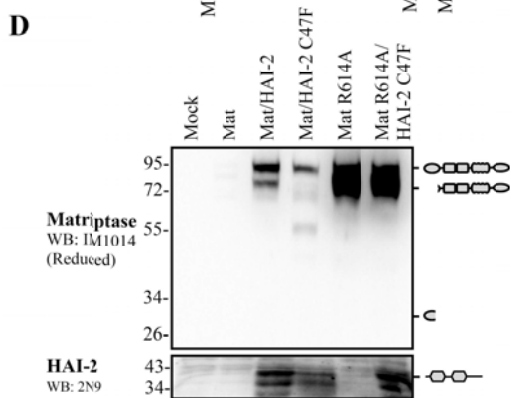
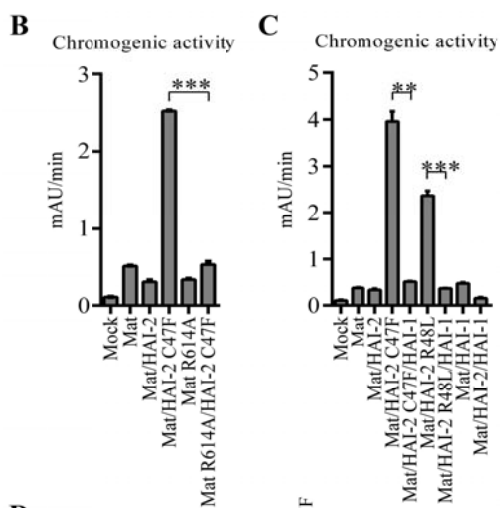
including the HAI-2 Kunitz domain 1 mutants, affecting the SEA domain cleavage of matriptase, K42N, C47F, and R48L. Thus, suggesting that the interaction between

matriptase and HAI-2 involves areas not investigated by our HAI-2 mutants, and that this binding may depend on more than one site.

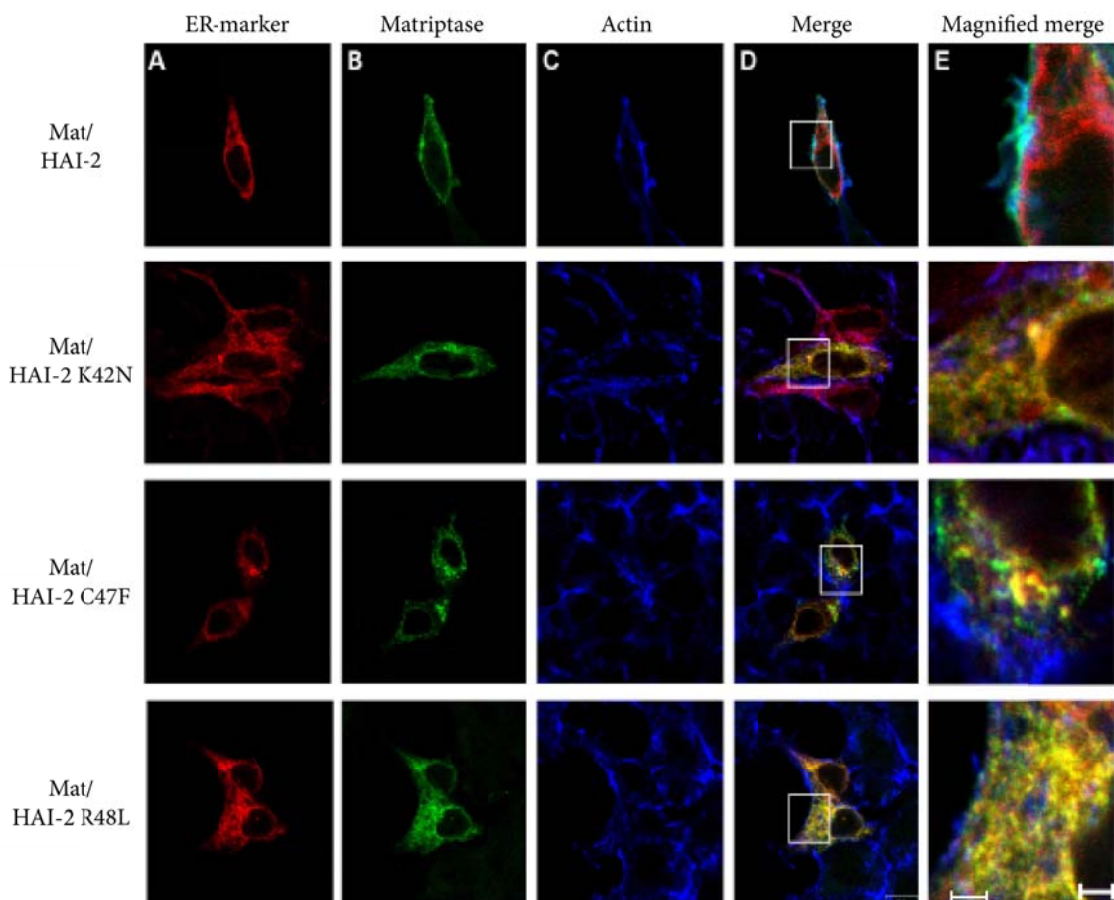


Co-expression of matriptase with HAI-2 Kunitz domain 1 mutants C47F or R48L increase the chromogenic activity of cell extracts

The HAI-2 mutations K42N, C47F, and R48L are all located in the binding loop of the Kunitz domain 1, and could potentially interfere with the ability of HAI-2 to proteolytically silence matriptase. However, no specific substrates or inhibitors are available for analysis of matriptase enzymatic activity. As an alternative, we consider how the general serine protease activity level of HEK293 cell extracts, transiently co-expressing matriptase and HAI-2, is affected by these HAI-2 mutations. The proteolytic turnover rate of extracts, from cells co-expressing matriptase with either HAI-2 wild type or any one of the different HAI-2 Kunitz domain 1 mutations, K42N, C47F or R48L, were determined using the peptide based chromogenic substrate, isoleucil-prolyl-arginine-p-nitroaniline (S-2288) (Figure 4 A). This substrate is known to be readily cleaved by a broad spectrum of serine proteases, including the serine protease domain of



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matriptase²⁰. Extracts from mock transfected cells and cells expressing matriptase, HAI-2, HAI-2 K42N, HAI-2 C47F, and HAI-2 R48L alone, were included as controls. Strikingly, a significant increase in chromogenic activity was observed for the cell extracts co-expressing matriptase with HAI-2 C47F or HAI-2 R48L, as compared to the activity level of cells co-expressing matriptase with HAI-2 wild type.

As HAI-2 favors binding to the early non-SEA domain cleaved form of matriptase, a similar experiment was performed using the non-SEA domain cleaving matriptase mutant G149E. Extracts of cells expressing matriptase G149E, matriptase S805A, matriptase G827R, HAI-1 alone or matriptase together with HAI-1, were

included as controls (Figure 4 A). These results also showed a significant increase in chromogenic activity in the cell extracts co-expressing matriptase G149E with HAI-2 C47F or HAI-2 R48L, as compared to the activity level of cell extracts co-expressing matriptase G149E with HAI-2 wild type. Thus, suggesting that HAI-2 is able to proteolytically silence both matriptase wild type and matriptase G149E, and that this ability can be compromised by the HAI-2 Kunitz domain 1 mutations C47F and R48L, but not K42N.

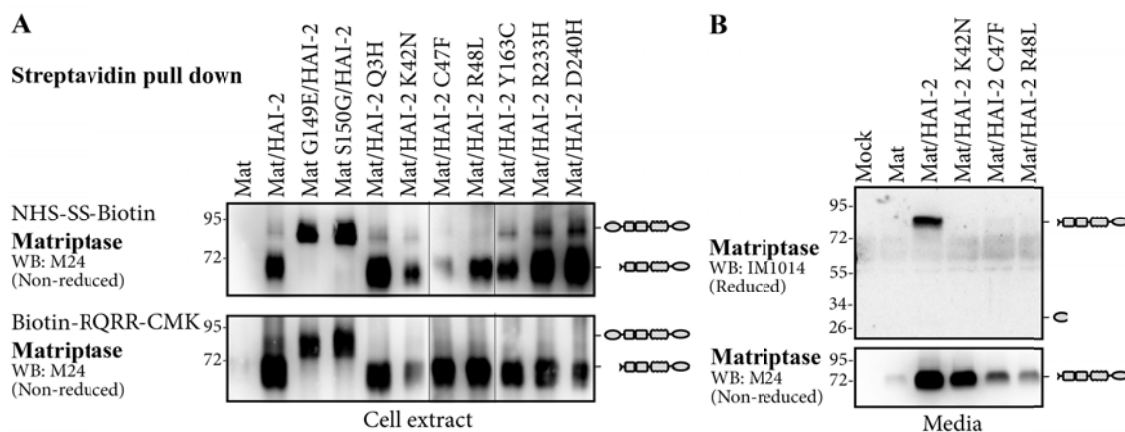
Another experiment was carried out to confirm that the chromogenic activity detected, directly or indirectly, represent the presence of Arg614 cleaved matriptase. The chromogenic activity of extracts from cells co-

expressing matriptase wild type with HAI-2 C47F and matriptase R614A, rendering matriptase unable to become active site cleaved³⁶, with HAI-2 C47F, was compared. Extracts from mock transfected cells and cells expressing matriptase and matriptase R614A alone, as well as matriptase together with HAI-2, were included as controls (Figure 4 B). The results showed a significantly lower activity for the cell extracts co-expressing matriptase R614A with HAI-2 C47F, as compared to wild type matriptase together with HAI-2 C47F. Thus, suggesting that the increase in activity detected directly or indirectly represents the presence of Arg614 cleaved matriptase. Furthermore, the extracts were analyzed by SDS-PAGE and Western blotting, to confirm protein expression and stability of matriptase R614A (Figure 4 D). Both matriptase R614A expressed alone and together with HAI-2 C47F were detected in both the SEA and non-SEA domain cleaved form, suggesting that matriptase SEA domain cleave independently of HAI-1, HAI-2 and matriptase Arg614 cleavage. Mock transfected cells, cells expressing matriptase alone, and cells co-expressing matriptase together with either HAI-2 or HAI-2 C47F were included as controls.

In order to investigate if the increased chromogenic activity, observed for cells co-expressing matriptase with HAI-2 mutants C47F or R48L, can be inhibited by HAI-1, an additional experiment was performed, where the chromogenic activity of extracts from cells co-expressing matriptase with HAI-2 C47F or R48L, with or without co-expressed HAI-1, was compared (Figure 4 C). Extracts from mock transfected cells, cells expressing matriptase alone, and cells co-expressing matriptase either with HAI-2, with HAI-1, or with HAI-2 and HAI-1, were included as controls. A significant decrease in total chromogenic activity was observed when HAI-1 was co-expressed with matriptase and HAI-2 C47F, as well as matriptase and HAI-2 R48L, as compared to the samples without HAI-1 expression. Thus, suggesting that HAI-1 is able to proteolytically silence the matriptase activity presented by co-expression of matriptase with a compromised HAI-2 Kunitz domain 1.

Matriptase co-expressed with HAI-2 Kunitz domain 1 mutants K42N, C47F, or R48L is retained in the ER

We have previously shown that HAI-2 mainly co-localizes with an ER marker²⁰, the product



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of the plasmid vector pDsRed2-ER, expressing a fluorescence protein fused to both the ER retention sequence KDEL and to the ER targeting sequence of calreticulin. To investigate the subcellular localization of matriptase when co-expressed with a Kunitz domain 1 mutated HAI-2, HEK293 cells transiently expressing wild type matriptase in combination with either wild type HAI-2 or HAI-2 mutated in K42N, C47F, or R48L together with the plasmid vector pDsRed2-ER were grown for 48 h before fixation, and immunocytochemically stained with M24 to detect matriptase, and phalloidin to detect F-actin, as a surface marker (Figure 5). In agreement with previous findings²⁰, confocal microscopy showed that matriptase co-expressed with wild type HAI-2 mainly co-localized with the cell surface marker F-actin (Figure 5, upper panel). However, matriptase co-expressed with HAI-2 K42N, C47F, or R48L mainly co-localized with the ER-marker (Figure 5, lower panels). Mock transfected cells and cells transfected with wild type matriptase alone were included as controls, and both displayed a low background staining (data not shown). This experiment suggests an increase in the steady-state level of matriptase within the ER, when matriptase is co-expressed with HAI-2 Kunitz domain 1 mutants K42N, C47F, or R48L. Matriptase mutated in the SEA domain cleavage site, G149E, G149N, or S150G co-expressed with wild type HAI-2, mainly co-localized with the ER-marker as well (data not shown), supporting the use of these non-SEA domain cleaved matriptase mutants to investigate interactions taking place in the early secretory pathway.

The SEA domain cleavage and shedding of matriptase co-expressed with HAI-2 Kunitz domain 1 mutants K42N, C47F, or R48L is reduced

Non-SEA domain cleaved matriptase is normally not present on the plasma membrane²⁸, correlating with the majority of the matriptase, exhibiting delayed SEA domain cleavage when co-expressed with HAI-2 mutants K42N, C47F or R48L, being located predominantly in the ER. To examine, whether any matriptase, when co-expressed with either of the HAI-2 Kunitz domain 1

mutants, still reaches the plasma membrane, and whether this matriptase is SEA domain cleaved, we used a cell-surface biotinylation approach. Using either NHS-SS-Biotin, which in this setup labels all membrane bound proteins, or Biotin-RQRR-CMK, which in this setup detects active matriptase (both in the zymogen form and the Arg614 cleaved form³⁷), in combination with streptavidin pull down, SDS-PAGE and Western blotting, using an antibody against matriptase. This experiment was designed to analyze the status regarding SEA domain cleavage and not the quantity of matriptase bound to the plasma membrane. HEK293 cells transiently expressing wild type matriptase in combination with HAI-2 wild type or mutated HAI-2 were cell-surface biotinylated using either the NHS-SS-Biotin or Biotin-RQRR-CMK. Extracts were obtained by lysis and streptavidin precipitated. The precipitates were analyzed by SDS-PAGE and Western blotting, using the antibody M24 against matriptase, under boiled non-reducing conditions (Figure 6 A). As a control, matriptase G149E and S150G co-expressed with HAI-2 were analyzed in the same manner. The analysis showed that matriptase mutated in the SEA domain cleavage site e.g. matriptase G149E and S150G, could be detected on the plasma membrane in the non-SEA domain cleaved form, whereas matriptase wild type co-expressed with HAI-2 wild type or HAI-2 mutated to Q3H, K42N, C47F, R48L, Y163C, R233H, and D240H were all detected on the plasma membrane, mainly in the SEA domain cleaved form. Thus, suggesting that the SEA domain cleavage of matriptase, when co-expressed with HAI-2 Kunitz domain 1 mutants K42N, C47F, or R48L, is not absent but rather delayed and/or inefficient.

We have previously shown in MDCK cells (endogenously expressing canine HAI-1 and HAI-2), that matriptase, when co-expressed with both HAI-2 wild type and HAI-2 Kunitz domain 1 mutant R48L, sheds to the media, and can be detected after immunoprecipitation²⁰. To investigate how co-expression of matriptase with HAI-2 Kunitz domain 1 mutants K42N, C47F, or R48L affects

HAI-2, HAI-2 C47F, HAI-2 R48L, the double mutant HAI-2 C47F/R48L, or with HAI-1. All transfections were done in triplicates, where one set was cell surface labeled with NHS-SS-Biotin, one was cell surface labeled with Biotin-RQRR-CMK, and one control set was treated with PBS++ (denoted untreated) before being extracted by lysis, streptavidin pull down, and the level of chromogenic activity was determined. Both NHS-SS-Biotin and Biotin-RQRR-CMK are non-cell membrane permeable. The NHS-SS-Biotin labels plasma membrane proteins in a non-specific manner, whereas the Biotin-RQRR-CMK specifically labels and inhibits active serine proteases, including matriptase³⁷. For each transfection a sample of the total extract (Figure 7, total extract), a pull down fraction of surface biotinylated proteins (Figure 7, pull down) and the remaining fraction (Figure 7, supernatant) representing the intracellular part of the cell were analyzed. Nothing was done to release bound proteins from the beads in the pull down fractions. The levels of chromogenic activity were obtained as previously described, and the level of GAPDH was determined by SDS-PAGE and Western blotting, to ensure a consistent cell quantity for all transfections (Figure 7).

All samples have been analyzed by SDS-PAGE and Western blotting using antibodies against matriptase and HAI-2, to ensure correct expression (data not shown). The double HAI-2 mutant C47F/R48L did not differ from the single mutants (HAI-2 C47F or R48L) when blotted. Also, matriptase co-expressed with the new double HAI-2 mutant (C47F/R48L), depicted a significant level of unopposed matriptase activity (Figure 7).

In cells labeled with NHS-SS-Biotin the majority of chromogenic activity, in cells co-expressing matriptase and a compromised HAI-2 (C47F, R48L, C47F/R48L), was found in the supernatant fraction, representing the non-plasma membrane part of the cells, suggesting that the chromogenic activity is located mainly intracellularly (Figure 7, supernatant). Only a small amount of chromogenic activity was detected in the pull down fractions (Figure 7, pull down).

Treatment with Biotin-RQRR-CMK, that is known to bind and inhibit active matriptase in the plasma membrane³⁷, did not clearly reduce the level of chromogenic activity in the supernatants (Figure 7, supernatant) as compared to cells treated with NHS-SS-Biotin. The non-cell permeant agent Biotin-RQRR-CMK is thus, unable to react with the majority of the unopposed matriptase activity in cells co-expressing matriptase and a compromised HAI-2, supporting that the chromogenic activity has an intracellular location.

Discussion

We have previously shown that co-expression with HAI-1 or HAI-2 is necessary for the stability of matriptase *in vitro*²⁰. None of the HAI-2 mutants analyzed in this study affected the ability of HAI-2 to stabilize matriptase (Figure 2).

Based on the sequence homology between the Kunitz domains of HAI-2 and Kunitz domains found in other known protease inhibitors, it is generally presumed that HAI-2 has a biological function as a protease inhibitor. *In vitro*, HAI-2 has been shown to inhibit the enzymatic activity of a soluble matriptase serine protease domain⁸, hepatocyte growth factor activator³⁵, trypsin, plasma kallikrein, tissue kallikrein, and plasmin³⁸, supporting its role as a protease inhibitor. The HAI-2 mutations K42N, C47F, and R48L are all located in the Kunitz domain 1 that displays the typical pear-shaped Kunitz domain fold, with the binding loop of canonical conformation at the top³⁹. It is believed that Kunitz type inhibitors competitively prevent access of physiologically relevant substrates to a serine protease, by inserting its P1 residue, located in the binding loop, into the active site cleft⁴⁰. The binding loop mimics the substrate and forms an interaction resembling an enzyme-substrate Michaelis complex⁴¹, ultimately blocking out any physiological relevant substrates. The HAI-2 mutations K42N, C47F, and R48L are all located within the binding loop of the Kunitz domain 1. Three conserved disulfide bonds³⁹, one of which is disrupted by the mutation C47F, stabilize the compact

structure of the HAI-2 Kunitz domain 1. It has previously been shown that the two other disulphide bonds, but not the one disrupted by C47F, are required for the maintenance of native conformation of a Kunitz domain⁴². The HAI-2 R48-A49 bond is positioned in a way that mimics an expected cleavage site (P1-P1') targeted by the catalytic S residue and other catalytic triad residues of the protease. Concerning peptide substrates, matriptase prefers an R in the P1 position, as found in both the Kunitz domain 1 (R48) and Kunitz domain 2 (R143) of HAI-2, and its S2 position is shaped to fit a small hydrophobic amino acid residue^{43,44}. Thus, the HAI-2 mutation C47F, at the predicted P2 position, and R48L, at the predicted P1 position, would be expected to disfavor insertion of the HAI-2 binding loop into the active site cleft of matriptase, and as a result have reduced ability to proteolytically silence matriptase.

Matriptase resembles most other enzymes, where zymogen conversion results in an activated form with a higher catalytic activity. However, the difference in activity between the zymogen form (not cleaved after Arg614) and the activated form (Arg614 cleaved) is unusually small for matriptase, only in the order of 27 fold (the zymogenicity factor)^{37,45,46}. There are no known specific substrates or inhibitors of matriptase available. Therefore, the general level of proteolysis was assessed using a chromogenic substrate, recognized by most serine proteases. The increase in chromogenic activity observed in the cell extracts co-expressing matriptase with the HAI-2 Kunitz domain 1 mutants C47F or R48L (Figure 4 A) appears to stem directly from, or at least require, the presence of activated matriptase (Arg614 cleaved), as no/lower increase in activity was observed using the matriptase mutant R614A (the activation cleavage site mutation) together with HAI-2 C47F (Figure 4 B). It is possible that the observed activity does not stem directly from matriptase, but rather from other proteases activated by matriptase. However, it is safe to conclude that the observed increase in chromogenic activity in the extracts reflects, either directly or indirectly, the catalytic activity of matriptase.

In this setup, detection of the matriptase Arg614 cleaved SPD fragment could not be used to indicate activation of matriptase, as no clearly defined matriptase SPD band was observed (app. 30 kDa) (Figure 2, 4 and 6). This may be because only a minor fraction of the matriptase molecules were activated, under the conditions used in this study.

We observed three HAI-2 Kunitz domain 1 mutants, K42N, C47F and R48L that lead to reduced levels of SEA domain cleavage, when co-expressed with matriptase (Figure 2). The observed reduced levels of SEA domain cleaved matriptase could be explained by an accelerated activation of matriptase, diminishing the cellular pool of SEA domain cleaved matriptase. However, only two out of the three HAI-2 mutants affecting matriptase SEA domain cleavage, also affected matriptase activity (Figure 4 A), suggesting that the reduced levels of SEA domain cleaved matriptase observed with the HAI-2 mutants, are not the result of accelerated matriptase activation. Moreover, we observed a decrease in the shedding of matriptase to the media, from cells co-expressing matriptase with all three HAI-2 Kunitz domain 1 mutants (Figure 6 B), suggesting that the reduced levels of SEA domain cleaved matriptase, are not the result of increased shedding. Overall, these findings suggest that the reduced levels of SEA domain cleaved matriptase, observed with the HAI-2 Kunitz domain 1 mutants, are a result of delayed matriptase SEA domain cleavage, rather than the result of increased matriptase activation and/or shedding.

The increased unopposed matriptase activity, observed when matriptase is co-expressed with a compromised HAI-2 (C47F, R48L or C47F/R48L), was primarily observed with an intracellular localization (Figure 7) and was somewhat unaffected by extracellular inhibition with Biotin-RQRR-CMK (Figure 7). Furthermore, matriptase co-expressed with these compromised HAI-2s was found to co-localize with an ER marker (Figure 5) and to be mainly in the non-SEA domain cleaved form (Figure 2). Based on these findings, we suggest that a Kunitz domain 1 compromised HAI-2 leads to an increase in intracellular

unopposed non-SEA domain cleaved matriptase, likely predominantly located in the ER. The subcellular localization, where matriptase is Arg614 cleaved under non-pathological conditions, is at present unclear. Thus, it is unknown whether HAI-2 under normal conditions prevents the auto-activation of zymogen matriptase in the secretory pathway before it possibly becomes Arg614 cleaved on the plasma membrane, or whether matriptase auto-activates in the ER, followed by prompt inhibition by HAI-2, resulting in proteolytic silencing of matriptase during secretory transport. Either way, we propose that wild type HAI-2 functions to keep matriptase proteolytically silent in the ER and possibly throughout the secretory pathway.

The effect of HAI-1 co-expression was investigated in cells co-expressing matriptase with a compromised HAI-2 (C47F or R48L), the analysis showed that HAI-1 co-expression significantly decreased the level of unopposed matriptase activity caused by a compromised HAI-2 (Figure 4 C). Thus, suggesting that HAI-1 contributes to keeping matriptase proteolytically silent in the ER and possibly the secretory pathway, and can compensate for inefficient HAI-2 inhibition.

None of the HAI-2 mutations analyzed prevented the binding between non-SEA domain cleaved matriptase and HAI-2, including the Kunitz domain 1 binding loop mutations K42N, C47F and R48L, as shown by immunoprecipitation of matriptase with all HAI-2 mutants (Figure 3). Thus, suggesting that matriptase and HAI-2 interacts both at the binding loop of the HAI-2 Kunitz domain 1, as well as at one or more ectosites. It has previously been shown that the binding of Kunitz type inhibitors to a protease involves, not only insertion of the binding loop into the active site cleft, but also an interaction at a secondary binding segment, as observed for the serine protease domain of matriptase and HAI-1⁴⁷, and for matriptase and pancreatic trypsin inhibitor⁴³. An unusually large binding interface was observed between the serine protease domain of matriptase and HAI-1, in the order of 1800 Å²⁴⁷, which corresponds

well with the strong binding observed between matriptase and HAI-1, as the complex can withstand separation on an SDS-PAGE gel⁴⁸. Likewise, the matriptase-HAI-2 complex has been reported to withstand separation on an SDS-PAGE gel⁴⁹. Thus, supporting the presence of at least one unknown ectosite where matriptase and HAI-2 interacts. It is possible, that a HAI-2-matriptase interaction at an undiscovered ectosite is responsible for the stabilization of the membrane-bound form of matriptase, however it is at present time unclear where this ectosite is located.

Generally, each domain in a multi-domain protein like matriptase is a stable globular structure of polypeptide chain(s), representing an autonomous folding unit⁵⁰. SEA domains are found in a range of proteins, the structure is well known⁵¹ and they often have the ability to auto-cleave⁵². If the SEA domain of matriptase is able to auto-cleave, we expect it to take place independently of e.g. HAI-1 and HAI-2. Accordingly, this study and others⁵³ have observed that matriptase S805A, matriptase G827R^{22,53}, and matriptase R614A (this study) SEA domain cleave independently of HAI-1 and HAI-2 (Figure 1 and 4 D). We suggest that the matriptase SEA domain auto-cleaves, and that it reflects when the correct three-dimensional fold of the domain has been obtained.

It is well known, that for some proteins transport out of the ER is retained until proper oligomerization and folding have taken place^{50,54}. In the present study, we observed that co-expression of matriptase with HAI-2 Kunitz domain 1 mutants K42N, C47F or R48L delayed the SEA domain cleavage of matriptase, and caused matriptase to be retained predominantly in the ER (Figure 1 and 5). When labeling matriptase at the plasma membrane, mainly SEA domain cleaved matriptase was observed, and only SEA domain cleaved matriptase was detected in the media, when matriptase was co-expressed with the three HAI-2 Kunitz domain 1 mutants (Figure 6 A, B). Suggesting, that only SEA domain cleaved matriptase is competent for transport to the plasma

membrane and subsequently shedding. Thus, HAI-2 may influence matriptase folding and/or oligomerization and thereby control the flux of matriptase to the plasma membrane, where matriptase can cleave several extracellular substrates^{55,56}. However, it seems that the retention of non-SEA domain cleaved matriptase can be exhausted, as matriptase mutated in the SEA domain cleavage site (matriptase G149N, matriptase G149E, and matriptase S150G) co-expressed together with wild type HAI-2, was detected on the plasma membrane in the non-SEA domain cleaved form.

HAI-2 K42N and C47F were designed based on SNP's (rs35896127 and rs1804770) that occur at very low frequencies. No phenotype has been associated with these SNP's. However, it would be highly interesting to know whether carriers of C47F (rs1804770) have an affected cancer incidence, since unopposed matriptase has been reported to be highly oncogenic^{14,15}.

In conclusion, our results suggest that HAI-2 play an important role in the stabilization of matriptase, regulation of matriptase activity and in obtaining the correct fold/oligomeric state necessary to allow transport of matriptase from the ER to the plasma membrane.

Most proteases are synthesized in the zymogen form, then transported to wherever they perform their action, and becomes activated there, where also inhibitors are located to keep them under control. This scheme, that is valid for a large number of protease-inhibitor pairs, does not seem to apply for matriptase and HAI-2, as HAI-2 appears to have multiple roles towards matriptase, of both a stabilizing as well as a regulating nature. The newly synthesized non-SEA domain cleaved form of matriptase appears to interact with HAI-2 already in the ER, as HAI-2 favors binding to the early non-SEA domain cleaved matriptase, and only in exceptional cases has HAI-2 been located on the plasma membrane⁴⁹. We suggest that the purpose of this interaction is to ensure the following three events; 1) inhibition of the proteolytic activity and/or inhibition of

activation/auto-activation of matriptase within the secretory pathway, to ensure safe passage of other proteins, especially relevant due to the relatively low zymogenicity factor of matriptase, 2) control of the folding, maturation and/or oligomerization of matriptase, possibly reflected by SEA domain cleavage of matriptase, making it transport competent, as mainly SEA domain cleaved matriptase is found on the plasma membrane and in the media, and 3) the previously described stabilization of the membrane bound form of matriptase^{20,21}.

Materials and Methods

Cell culture

The human embryonic kidney cell line HEK293 was grown in minimal essential medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin at 37 °C in an atmosphere of 5% CO₂. For experiments, cells were seeded one day prior into either 6- or 12-well Corning® Costar® cell culture plates (cat. no. CLS3512 or CLS3516, Sigma) and grown to 80% confluence.

Transfections and DNA constructs

For transient expression, adherent HEK293 cells were transfected using Lipofectamine™ 2000 (Invitrogen), according to the protocol supplied by the manufacturer. For co-transfections the same overall quantity of plasmid was used. The cDNA coding for full-length human wild type matriptase, HAI-1 and HAI-2 were incorporated into pcDNA3.1 plasmid vectors. The HAI-2 cDNA used in the present study contains a naturally occurring SNP resulting in the amino acid substitution V200L. Construction of the HA-HAI-2 plasmid has previously been described²¹. Empty expression plasmids were used for mock transfections. Mutations in the cDNA encoding matriptase, HAI-2 and HA-HAI-2 were introduced by site-directed mutagenesis using the GeneArt Site-Directed Mutagenesis System (cat. no. A13282, Life technologies) according to the manufacturers recommendations and verified by sequencing. The mutations R48L and R143L in HAI-2 have previously been described²⁰.

Media from transiently transfected cells was collected 48 hours post-transfection. Cell extracts were obtained by lysis in phosphate buffered saline (PBS), containing 1% Triton X-100, 0.5% deoxycholate (lysis buffer) 48 hours post-transfection, spun and the supernatant was saved at -20°C.

HA-Immunoprecipitation

EZview Red Anti-HA Affinity Gel (cat. no. E6779, Sigma-Aldrich) was prepared according to the manufacturer's instructions and added to the supernatant from lysis treated HEK293 cells (spun 20,000 x g, 15 min). Samples were incubated with end over end rotation for 2 h at 4°C and spun (8000 x g, 30 s) to precipitate the affinity gel. The affinity gel was washed 3 times with 25 mM Tris-HCl, 500 mM NaCl, 0.5% Triton-X-100 pH 7.8 and 4 times with 10 mM Tris-HCl, 150 mM NaCl pH 7.8 and eluted in 2 x SDS sample buffer and boiled for 10 min. The eluate was analyzed by SDS-PAGE and Western blotting.

SDS-PAGE and Western blotting

Samples were prepared by addition of 2 x SDS sample buffer (1:1) and for reducing conditions 0.2 M dithiothreitol (DTT) was added. Proteins were separated on either 7% or 10% SDS polyacrylamide gels and transferred to Immobilon-P PVDF membranes (Millipore). The blots were blocked with 10% non-fat dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 h. The individual PVDF membranes were probed with primary antibodies diluted in 1% non-fat dry milk in PBS-T at 4°C overnight, followed by 3 x 5 min wash in PBS-T and 1 h incubation with secondary antibodies diluted in 1% non-fat dry milk in PBS-T. After 3 x 5 min wash in PBS-T the signal was developed using ECL[®] (enhanced chemiluminescence) reagent Super Signal West Femto Maximum Sensitivity Substrate (cat. no. 34095, Thermo scientific) or Pierce ECL Western Blotting Substrate (cat. no. 32106, Pierce) according to the protocol supplied by the manufacturer, and visualized with a Fuji LAS-1000 camera and Intelligent DarkBox II (FujiFilm Sweden AB), using the program LAS1000 Lite v1.5.

Antibodies

Blots were probed with primary monoclonal mouse anti-human matriptase antibodies,

M24⁵⁷ (1:2000) recognizing both non-SEA and SEA domain cleaved matriptase under non-reducing, boiled conditions, polyclonal rabbit anti-human matriptase/MT-SP1 (cat. no. IM1014, Calbiochem[®]) (1:1000) recognizing the serine protease domain of matriptase under reducing, boiled conditions, monoclonal mouse anti-human HAI-2 2N9⁵⁸ (1:1000) recognizing an epitope in the intracellular domain of HAI-2 under reducing, boiled conditions, M19⁵⁷ (1:2000) recognizing HAI-1 under non-reducing, non-boiled conditions, Anti-HA High Affinity (cat. no. 1867423, Roche) (1:1000) recognizing the HA-tag under reducing, boiled conditions, and antibodies against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (cat. no. MAB374, Zymed laboratories inc.) (1:2000) as loading controls. Blots were probed with secondary horseradish peroxidase (HRP)-conjugated antibodies (1:5000) Pierce goat anti rabbit (cat. no. 1858415, Thermo Scientific), polyclonal rabbit anti rat (cat. no. P0162, Dako) and goat anti mouse (cat. no. T30953, Invitrogen).

Cell surface labeling of matriptase with Biotin-RQRR-CMK and NHS-SS-Biotin

Transiently transfected HEK293 cells in 6 well culture plates were washed twice with ice-cold PBS++ (PBS supplemented with 0.7 mM CaCl₂ and 0.25 mM MgCl₂). For labeling and inhibition of active matriptase, the cells were incubated with 50 μM biotin-Arg-Gln-Arg-Arg-chloromethyl ketone (Biotin-RQRR-CMK) peptide inhibitor (American Peptide) dissolved in serum-free minimal essential medium (MEM) eagle with Earle's salt supplemented with 0.2% NaHCO₃ or left untreated (incubated with PBS⁺⁺) for 90 min at 4°C with gentle rotation, as previously described³⁷. Peptides were prepared as 50 mM stock in DMSO and stored at -20°C. For labeling of surface proteins, cells were biotinylated with 1 mg/ml EZ-link[™] Sulfo-NHS-SS-Biotin (prod. no. 21331, Thermo) dissolved in PBS++ for 30 min at 4°C and washed with PBS++. Residual biotin was quenched in 50 mM glycine/PBS++ for 5 min at 4°C and washed with PBS++. Cells were then extracted by lysis (PBS containing 1% Triton-X-100 and 0.5% deoxycholate) and insoluble material was precipitated at 20,000

x g for 20 min at 4°C, for the chromogenic activity assay a sample of the supernatant was saved at -20°C for less than 24h (total extract fraction). The supernatant was incubated over night with end-over-end rotation at 4°C with 75 µL streptavidin-coated resin (prod. no. 20349 Thermo), prepared as described by the manufacturer. After incubation, the streptavidin-coated resin was pulled down (2,000 x g, 30 s), for the chromogenic activity assay a sample of the supernatant was saved for instant analysis (supernatant fraction). The pull down for Western blotting was washed 4 times with 25 mM Tris-HCL, 500 mM NaCl, 0.5% Triton-X-100 pH 7.8 and 3 times with 10 mM Tris-HCl, 150 mM NaCl pH 7.8. Proteins for Western blotting were eluted from the streptavidin-coated resin by boiling in 2 x SDS sample buffer and analyzed by SDS-PAGE and Western blotting. The pull down for chromogenic activity analysis was washed 1 time with 25 mM Tris-HCL, 500 mM NaCl, 0.5% Triton-X-100 pH 7.8 and instantly used for the chromogenic activity assay (pull down fraction).

Immunofluorescence

Transiently transfected HEK293 cells grown on glass coverslips for two days were fixed in 4% paraformaldehyde in PBS for 10 minutes at room temperature. Blocking and permeabilization was performed by a 30 min incubation with 0.2% fish skin gelatin in PBS-T. The cells were incubated for 1h in primary antibodies in PBS-T, washed 3 times in PBS-T and incubated for 45 min in secondary antibodies in PBS-T. The coverslips were mounted in Prolong Gold (Invitrogen). Confocal laser scanning microscopy was performed using the Zeiss LSM 710 confocal system. Original images were acquired using a 63x oil immersion objective, NA 1.4 with a pinhole size of 1 and a pixel format of 1756x1756. Presented images were cropped to a pixel format of 768x768. Line averaging was used to reduce noise. For quadruple-labeling experiments sequential scanning was employed to allow the separation of signals from the individual channels. The images were treated using the Zen 2011 Black edition software (Zeiss). Mouse anti-Matriptase (M24, 1:100) was employed as primary

antibody. As secondary antibody an Alexa Fluor®488-conjugated donkey anti-mouse IgG (1:200) was employed. Alexa®Fluor 647 Phalloidin (1:200) was used to stain actin filaments. All Alexa Fluor®-coupled reagents were purchased from Invitrogen (Glostrup, Denmark).

Chromogenic activity assay

Extracts obtained by lysis of transiently transfected HEK293 cells, prepared as previously described, were used for a S-2288 chromogenic substrate activity assay. For this assay, 60 µl cell extract was diluted with 134 µl 20mM HEPES pH 7.4, 140 mM NaCl supplemented with 0.1% BSA (Sigma) (HBS buffer), and 6 µl 10 mM chromogenic substrate H-d-Isoleucyl-l-prolyl-l-arginine-p-nitroaniline (cat.no. S-2288, Chromogenix) in HBS buffer to a final volume of 200 µl in a 96 well plate and heated to 37 °C for 15 min. Color development was measured at 405nm (A_{405}) continuously every 5 min for 5h in a standard plate reader (Biotek Synergy HT) at 37 °C. For detection of chromogenic substrate activity on streptavidin-coated resin, HBS buffer containing 300 µM chromogenic substrate S-2288 was added to the pull down and samples were incubated at 37°C with end-over-end rotation for 4h. Samples were spun (2,000 x g, 1 min) and 150 µl was removed 5 times during the incubation and A_{405} was measured and the mean velocity of the substrate reactions (mAU/min) was calculated for each reaction. The rate of substrate turnover was determined from color development resulting from the pseudo-first order reaction due to a substrate concentration far greater than the expected pM range of protease content, as judged by semi-quantitative Western blot analysis. All measurements were adjusted for the optical path length and a background sample prepared with lysis buffer and indicated as mAU/min.

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