Matriptase is a type II transmembrane serine protease. Paradoxically, activation of this protease is thought to require its physiological inhibitor, hepatocyte growth factor activator inhibitor type-1 (HAI-1). In the present study, however, we obtained evidence in a stable transfection experiment using Madin–Darby canine kidney cells that matriptase activation does not require HAI-1.

Key words: activation of matriptase; C-terminal fragments; hepatocyte growth factor activator inhibitor type-1; Madin–Darby canine kidney cells; simple epithelial cells

Matriptase is a type II transmembrane serine protease that is strongly expressed in simple epithelial cells such as kidney tubular cells. It is first synthesized as a zymogen comprising 855 amino-acid residues that requires processing by cleavage after Arg614 to generate the disulfide-linked-two-chain active enzyme. This processing is defined as the activation of matriptase. The activation of matriptase is generally thought to occur via a mechanism by which a zymogen interacts with another zymogen, resulting in activation cleavage of each zymogen (known as transactivation). In addition, matriptase activation appears to occur at the cell surface, and then the ectodomain is shed from the surface. Two-chain active matriptase cleaves and activates a number of macromolecules, including pro-hepatocyte growth factor (pro-HGF) and prostasin zymogen. These characteristics suggest that matriptase plays a key role in maintaining epithelial integrity.

HGF activator inhibitor type-1 (HAI-1), a Kunitz-type protease inhibitor, is a physiological inhibitor of matriptase. HAI-1 is first synthesized in membrane-bound form (Fig. 1B). Like matriptase, the ectodomain of this inhibitor is shed from the cell surface. HAI-1 was considered to play an important role in the activation of matriptase, because co-expression of HAI-1 was required for the detection of activated matriptase in transient-transfection experiments using human breast cancer BT549 cells. A transient interaction between matriptase zymogen and HAI-1 might be essential for activation. We have found in transient-transfection experiments using monkey kidney COS-1 cells that co-expression of HAI-1 is required for the extracellular appearance of activated matriptase. It is uncertain, however, whether detection of activated matriptase means HAI-1 is required for activation. It should be noted that an activated form of matriptase was detected when expressed alone in a human embryonic kidney cell line HEK293 that expresses neither matriptase nor HAI-1 endogenously. In addition, in a human monocytic cell line, THP-1, that expresses matriptase but not HAI-1, this protease was detected as the active form. In the present study, we attempted to determine whether HAI-1 is required for the appearance of activated matriptase by stable-transfection experiments using Madin–Darby canine kidney epithelial cells (widely used as a model of simple epithelial cells).

Plasmids for the expression of full-length rat matriptase (pcDNA-WT-matr) and full-length rat HAI-1 with an S-tag at its N-terminus (pSec-Stag-HAI-1) have been constructed using pcDNA3.1(+) (containing neomycin resistance gene) and pSecTag2/HygroB (containing hygromycin resistance gene) vectors (Invitrogen, Carlsbad, CA) respectively. MDCK cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. The plasmids were co-transfected into MDCK cells as described previously. Forty-eight h post-transfection, the cells were trypsinized, diluted with fresh MEM, and re-plated on 96-well plates to isolate single clones. Clones resistant to a medium containing 0.5 mg/ml of G418 (Invitrogen) and 0.2 mg/ml of hygromycinB (Invitrogen) were propagated until they were 80% confluent. Growing clones were trypsinized and plated on two wells of a 48-well plate. After reaching confluence, one of two wells for each clone was replaced with serum-free MEM. After 24 h of incubation, the serum-free media were harvested and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and Western blotting using a rabbit anti-rat matraptase catalytic domain antibody (Spr992, for the analysis of WT-matr) or S-protein conjugated with horseradish peroxidase (for the analysis of Stag-HAI-1).
We have found in stable-transfection studies using MDCK cells that (i) a full-length rat matriptase with Myc-epitope/hexahistidine tag at its C-terminus, matriptase-Myc/[His]$_6$, is processed post-translationally via cleavage between Gly149 and Ser150, and that (ii) the C-terminal fragment (CTF, Ser150–Val855), which constitutes the longest part of the extracellular domain, including the C-terminal catalytic domain (Fig. 1A), occurs abundantly in the conditioned medium.\(^{14}\) Hence, detection in media is convenient in evaluating the expression of WT-matriptase.

In the present study, we obtained 24 G418- and hygromycinB-resistant clones. Of these, seven clones (M12, M15, M16, M22, MH14, MH26, and MH210) were found to secrete CTF of WT-matriptase (hereinafter called WT-matriptase-CTF) (Fig. 2). Note that proteolytically-inactive, single-chain WT-matriptase-CTF was detected at the position corresponding to 90 kDa, whereas the catalytic domain of proteolytically-active WT-matriptase-CTF migrate are indicated on the right by white and black arrowheads respectively. Stag-HAI-1 occurring in the conditioned medium was detected as a 58-kDa band (indicated by grey arrowhead). Note that 40-kDa and 36-kDa bands were visualized in samples of MH210. The molecular sizes of the protein markers are indicated on the left in kilodaltons (kDa).

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**Fig. 1.** Schematic Representation of Expression Constructs and Processing of Them.

A. Structure and processing of WT-matriptase. WT-matriptase is a recombinant form of full-length rat matriptase. The N- and C-termini are indicated by NH$_2$ and COOH. The N-terminal fragment (NTF) and CTF are indicated by lines, and their association is illustrated by broken lines. Amino acid numbering starts from the putative N-terminus of the protein. The predicted disulfide linkages between the two cysteine residues corresponding to Cys604 and Cys731 in matriptase are shown as C-C. The amino acid sequence around the matriptase activation cleavage site is indicated in a single-letter code with amino acid numbering at the N-terminal Val residue of the catalytic domain (Val615). The CTF of WT-matriptase (WT-matriptase-CTF) is known to be shed from the cell membrane in the proteolytically-inactive and proteolytically-active forms. The positions at which inactive CTF and the catalytic domain of active CTF migrate on SDS–PAGE are indicated. TM, transmembrane domain; SEA, sea-urchin sperm protein–enterokinase–agrin domain; K1, first protease inhibitory Kunitz domain; L, low-density lipoprotein receptor A1R–urchin embryonic growth factor–bone morphogenetic protein agrin domain; Cytosolic domain; Cyto, cytoplasmic domain; K1, the first protease inhibitory Kunitz domain; L, low-density lipoprotein receptor A1R domain; L1-4, four repeats of low-density lipoprotein receptor A1R domain; C1 and C2, the first and second complement factor inhibitor; SEA, sea-urchin sperm protein–enterokinase–agrin domain; C1 and C2, the first and second complement factor inhibitor; TM, transmembrane domain; Cyto, cytoplasmic domain; Cyto, cytoplasmic domain.

B. Stag-HAI-1 (one of shed forms)

Stag-HAI-1 (membrane-anchored form) is indicated by an arrow. B, Structure and processing of Stag-HAI-1. Stag-HAI-1 is a recombinant form of full-length rat matriptase for activation in this expression model. N, N-terminal domain; I, internal domain; K1, the first protease inhibitory Kunitz domain; L, low-density lipoprotein receptor A1R domain; K2, the second protease inhibitory Kunitz domain; TM, transmembrane domain; Cyto, cytoplasmic domain.

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**Fig. 2.** Western Blots Showing the Occurrence of WT-Matriptase-CTF in Conditioned Media. M12, M15, M16, M22, MH14, MH26, and MH210 exhibited the expression of WT-matriptase. MH14, MH26, and MH210 exhibited the expression of Stag-HAI-1. WT-matriptase-CTF and Stag-HAI-1 were probed with an anti-rat matriptase catalytic domain antibody (Spr992) and S-protein conjugated with horseradish peroxidase (S-protein HRP). The positions to which proteolytically-inactive WT-matriptase-CTF and the catalytic domain of proteolytically-active WT-matriptase-CTF migrate are indicated on the right by white and black arrowheads respectively. Stag-HAI-1 occurring in the conditioned medium was detected as a 58-kDa band (indicated by grey arrowhead). Note that 40-kDa and 36-kDa bands were visualized in samples of MH210. The molecular sizes of the protein markers are indicated on the left in kilodaltons (kDa).
Simple epithelial cells form tight junctions, and the plasma membranes are separated into apical and basolateral domains. Matriptase has been found to occur preferentially on the basolateral side of intact simple epithelia.5,6) Matriptase-Myc/(His)6 also occurs preferentially on the basolateral side of transfected MDCK cells cultured in a two-chamber system.13,14) It is important to determine whether activated matriptase molecules are present in conditioned media of MDCK clones cultured in the two-chamber system. In the present study, we seeded M12, MH26, and MH210, in which matriptase expression was fairly abundant, on Transwell polycarbonate filter inserts (24-mm diameter, 0.4 μm pore size; Costar Corning, Acton, MA), and determined whether activated matriptase was present. Cells were plated at a density of 1 × 105 cells per well. When the transepithelial resistance of the monolayer exceeded 650 W/cm2, the cells were exposed to serum-free medium and incubated for 24 h. After incubation, the media were harvested. After the addition of a protease inhibitor cocktail (Complete, Roche, Manheim, Germany), the various media were concentrated to 50 μl by ultrafiltration using Microcon 30 (30,000 MWCO, Millipore, Bedford, MA), and analyzed as described above. In M12, WT-matriptase-CTF occurred preferentially in the basolateral medium (Fig. 3). This is consistent with observations of MDCK cells expressing matriptase-Myc/(His)6.13,14) More importantly, WT-matriptase-CTF in the basolateral medium was detected mainly as the activated form (Fig. 3). In MH26 and MH210, the activated forms of WT-matriptase-CTF and Stag-HAI-1 were detected in the apical and basolateral media (Fig. 3). In MH26, WT-matriptase-CTF occurred preferentially in the apical medium (Fig. 3). In MH210, the corresponding WT-matriptase-CTF occurred in a non-polarized fashion (Fig. 3). It has been postulated that matriptase is transcytosed from the basolateral to the apical side with the aid of HAI-1 in simple epithelia.6,10) It is tempting to speculate that HAI-1 assists in the apical delivery of matriptase in transfected MDCK cells.

It is still unclear whether matriptase requires HAI-1 for activation. In expression experiments of matriptase using BT549 and COS-1 cells, only inactive molecules were poorly produced.4,7) We found recently that extracellular HAI-1 and aprotinin (known to inhibit matriptase) did not correct for the poor production of this protease in COS-1 cells transfected with pcDNA-WT-matriptase alone.8) We speculate that when expressed alone in these cell lines, matriptase undergoes activation inadvertently in intracellular environments, leading to self-degradation, and that when co-expressed with HAI-1, intracellular activation and/or degradation are prevented by the inhibitor. Alternatively, HAI-1 might inhibit unknown proteases that degrade matriptase in the intracellular environment. On the other hand, in HEK293, THP-1, and MDCK cells, there might be mechanisms that do not allow the intracellular activation and/or degradation of matriptase even in the absence of HAI-1. We propose that matriptase does not require HAI-1 for activation (i.e., interaction betweenzymogen molecules).

Acknowledgment

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science.

References