Matriptase is a type-II transmembrane serine protease abundantly expressed in polarized epithelia. The ectodomain of matriptase is released from the cell surface. In the present study, we found that the post-translational cleavage between Gly149 and Ser150 and the existence of catalytic domain are critical for the ectodomain release of matriptase in stable transfection experiments using the polarized Madin–Darby canine kidney epithelial cell line.

Key words: catalytic domain; ectodomain release; matriptase; post-translational cleavage; simple epithelial cells

Matriptase is a type-II transmembrane serine protease that is abundantly expressed in simple epithelial cells, in which the plasma membranes are separated into apical and basolateral domains (e.g., kidney tubular cells).1,2) Matriptase has been found to be delivered to both the apical and the basolateral side of simple epithelia.3,4) It is therefore thought that it can interact with various potential substrates, including single-chain urokinase-type plasminogen activator, pro-hepatocyte growth factor, and prostatin precursor.1,5)

It was found that in a transient-transfection experiment using monkey kidney COS-1 cells, matriptase is processed post-translationally via cleavage between Gly149 and Ser150, which are located within the sea-urchin sperm protein-enterokinase-agrin (SEA) domain (Fig. 1), and that the C-terminal fragment (CTF, Ser150–Val855), which constitutes most of the extracellular part, is released from the cell surface.5) In addition, post-translational cleavage between Gly149 and Ser150 was found to be essential for CTF release, as evidenced by the observation, in a transfection experiment using COS-7 cells, that a full-length variant of mouse matriptase (also known as epithin) in which a glycine residue (Gly149) was replaced with Asn did not undergo CTF release.6) Release of CTF would provide a mechanism allowing matriptase to gain access to non-membrane-associated substrates that occur in the vicinity of this protease-expressing cells. Our aim was to determine whether the post-translational cleavage is required for CTF release when expressed in polarized simple epithelia. We also assessed the importance of the catalytic domain for CTF release.

A plasmid for the expression of matriptase-Myc/(His)6 [pcDNA-matriptase-Myc/(His)6] was constructed using pcDNA3.1 (+) (Invitrogen, Carlsbad, CA).3,7) A plasmid for the expression of G149N-matriptase-Myc/(His)6 (Fig. 1) was created by a polymerase chain reaction (PCR)-based technique, as follows: A restriction fragment of pcDNA-matriptase-Myc/(His)6, produced by ApaI and XhoI digestion, was ligated into pBluescript SK (Stratagene, La Jolla, CA) that had been digested with the same sets of enzymes. Using this plasmid as template, to introduce the appropriate mutation, a DNA fragment was amplified with the following sequences (the nucleotides underlined indicate the introduced mutation): 5’-ACAGCGTATGGCTACTACTACT-3’ and 5’-TCTCAGGAAAGAGTTACAGT-3’. The PCR product was phosphorylated with T4 polynucleotide kinase and then self-ligated. The plasmid was digested with ApaI and XhoI, and the resulting fragment was ligated into pcDNA-matriptase-Myc/(His)6, from which the ApaI and XhoI fragments had been removed. The plasmid for the expression of ΔCD-matriptase-Myc/(His)6, which consisted of matriptase Met1 to Asp603, was created as follows: A DNA fragment was amplified by PCR using 5’-CATCTGAGGATCAGAATG-3’ and 5’-GCTCTAGAGTCAGCAGTTACTCATCGG-3’ as primers and pcDNA-matriptase-Myc/(His)6 as template. The PCR product was digested with ApaI and XbaI, and the resulting fragment was ligated into pcDNA-matriptase-Myc/(His)6, from which the ApaI and XbaI fragment had been removed. In consequence, all the recombinant matriptase variants were fused to Myc epitope/hexahistidine tag [Myc/(His)6] at their C-termini.

In the present study, we used a Madin–Darby canine kidney (MDCK) cell line as expression host. This cell line has been found to establish tight junctions and is widely used as a model of polarized epithelia.3,7) MDCK cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum. Cells stably expressing G149N-matriptase-Myc/(His)6 and ΔCD-matriptase-Myc/(His)6 were obtained, as described previously.3,7) The stably transfected cells were seeded on Transwell® (Corning Costar, Acton, MA) with polycarbonate filter inserts (24 mm in diam-
Matriptase consists of 855 amino acids (refer to Matriptase, the wild type, at the top). The N- and C-terminals are indicated by H2N and COOH. Amino acid numbering starts from the putative N-terminus of the protein. The NTF and CTF parts are indicated by lines, and their association is illustrated by three broken lines. Matriptase-Myc/[His]6 is a full-length variant of recombinant rat matriptase. The recognition site for Tmc172 is indicated by the amino acid sequence in a single-letter code with G149 at the C-terminal residue. G149N-matriptase-Myc/[His]6 is a variant of recombinant matriptase in which Gly149 is replaced with Asn. Note that the SEA domain is not cleaved in the illustration of G149N-matriptase-Myc/[His]6. ΔCD-matriptase-Myc/[His]6 is another variant of recombinant matriptase, in which the catalytic domain and its N-terminal spacer region were deleted. ΔCD-matriptase-Myc/[His]6 consists of Met1 to Asp603. These recombinant matriptase variants are fused to the Myc/[His]6 tag (MHT) at their C-terminals. The molecular tag is convenient for the detection of expression products by Western blotting with anti-Myc antibody. TM, transmembrane domain; SEA, SEA domain; CUB, complement factor C1r/C1s-urchin embryonic growth factor-bone morphogenetic protein domain; L, low-density lipoprotein receptor A module domain; CD, catalytic serine protease domain.

**Fig. 1.** Schematic Representation of Rat Matriptase and Its Expression Constructs.

Matriptase consists of 855 amino acids (refer to Matriptase, the wild type, at the top). The N- and C-terminals are indicated by H2N and COOH. Amino acid numbering starts from the putative N-terminus of the protein. The NTF and CTF parts are indicated by lines, and their association is illustrated by three broken lines. Matriptase-Myc/[His]6 is a full-length variant of recombinant rat matriptase. The recognition site for Tmc172 is indicated by the amino acid sequence in a single-letter code with G149 at the C-terminal residue. G149N-matriptase-Myc/[His]6 is a variant of recombinant matriptase in which Gly149 is replaced with Asn. Note that the SEA domain is not cleaved in the illustration of G149N-matriptase-Myc/[His]6. ΔCD-matriptase-Myc/[His]6 is another variant of recombinant matriptase, in which the catalytic domain and its N-terminal spacer region were deleted. ΔCD-matriptase-Myc/[His]6 consists of Met1 to Asp603. These recombinant matriptase variants are fused to the Myc/[His]6 tag (MHT) at their C-terminals. The molecular tag is convenient for the detection of expression products by Western blotting with anti-Myc antibody. TM, transmembrane domain; SEA, SEA domain; CUB, complement factor C1r/C1s-urchin embryonic growth factor-bone morphogenetic protein domain; L, low-density lipoprotein receptor A module domain; CD, catalytic serine protease domain.

eter, 0.4 μm pore size) at a density of 1 × 10^5 cells per well. When the transepithelial resistance of the monolayer exceeded 700 Ωcm^2, the cells were washed 3 times with phosphate-buffered saline (8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 136 mM NaCl, 2.7 mM KCl, pH 7.4), exposed to serum-free minimum essential medium (1 ml for the upper or apical chambers, and 2 ml for the bottom or basolateral chambers), and incubated for 24 h. After incubation, the apical and basolateral media were harvested. After the addition of a protease inhibitor cocktail (Complete™, Roche, Mannheim, Germany), the media were concentrated to 50 μl by ultrafiltration using Microcon®-50 (50,000 MWCO, Millipore, Bedford, MA), and stored at −20°C until use. After medium harvest, domain-selective biotinylation was conducted using a cell membrane-impermeable biotin derivative (sulfo-NHS-SS-biotin, Pierce, Rockford, IL), as described previously.3,7 Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting using a mouse anti-Myc antibody (Invitrogen) and Tmc172, a rabbit polyclonal antibody raised against a matriptase N-terminal fragment (NTF, Met1–Gly149, Fig. 1) as primary antibodies.3,5,7

We have found in MDCK cells stably expressing a full-length variant of recombinant rat matriptase [matriptase-Myc/[His]6] cultured on Transwell™ that (i) the CTF (indicated mainly by a 93-kDa band) occurs in both apical and basolateral media with preference for the basolateral side, (ii) the CTF is hardly to be detected either in the apical or the basolateral membrane domain, and (iii) the NTF containing the transmembrane domain (indicated by a 25-kDa band) localizes exclusively to the basolateral–surface membrane.3,7 These results were confirmed in the present study (Fig. 2, WT). G149N-matriptase-Myc/[His]6 is a variant of matriptase-Myc/[His]6, in which Gly149 is replaced with Asn (Fig. 1). This mutant is expected to be impaired with respect to the generation of an N-terminus at Ser150. When probed with anti-Myc antibody, no signals for matriptase CTF were detected in either an apical or a basolateral medium conditioned by the transfected cells (Fig. 2, left panel, GN). On the other hand, domain-selective biotinylation and Western blotting using anti-Myc antibody detected a 120-kDa band from the apical- and the basolateral-surface membranes (Fig. 2, left panel, GN). Judging from the size, the 120-kDa signal might represent non-processed matriptase (Met1–Val855). These results suggest that matriptase requires its post-translational cleavage for the release of CTF when expressed in polarized simple epithelia.

ΔCD-matriptase-Myc/[His]6 is a variant of recombinant rat matriptase in which the catalytic domain and its N-terminal spacer region (Cys604–Val855) are deleted from matriptase-Myc/[His]6 (Fig. 1). This variant was
also stably expressed in MDCK cells. Domain-selective biotinylation and Western blotting using an anti-Myc antibody detected a 64-kDa band in both samples labeled on the apical side or on the basolateral side (Fig. 2, left panel, ΔCD). Judging from the size, the 64-kDa band represents the Ser150 to Asp603 part of ΔCD-matriptase-Myc/(His)_6. Tmc172 detected a 25-kDa band in samples labeled on the basolateral side but scarcely in those labeled on the apical side (Fig. 2, right panel, ΔCD). These findings indicate that ΔCD-matriptase-Myc/(His)_6 is post-translationally processed via cleavage between Gly149, and Ser150 and that the catalytic domain is not required for the generation of N-terminal Ser150. No signals for the Ser150-to-Asp603 part were detected from samples of basolateral medium (Fig. 2, left panel, ΔCD). On the other hand, a weak 62-kDa signal that may represent the Ser150-to-Asp603 part was detected in the samples of apical medium (Fig. 2, left panel, ΔCD). One possible explanation for the occurrence of the Ser150-to-Asp603 part in the apical medium is that a small number of the parts failed to associate with the respective NTF molecules in the intracellular environment (possibly because of the high level of expression in MDCK cells), and hence were apically secreted. This is supported by the observation that a secreted variant of recombinant rat matriptase consisting of Tyr81 to Asp603 appeared in the apical medium but not in the basolateral medium when stably expressed in MDCK cells (Tsuzuki, Murai, Miyake, Inouye, and Fushiki, unpublished results). In addition, the Ser150-to-Asp603 part was detected in the apical-surface membrane (Fig. 2, left panel, ΔCD). These results indicate that the catalytic domain plays an important role in the release of CTF from the cell surface. However, the catalytic activity of matriptase may not be involved in facilitating CTF release. Indeed, when a full-length variant of recombinant matriptase in which the active-site Ser residue (Ser805) was replaced with an Ala residue was expressed in human embryonic kidney HEK293 cells,10 COS-1 cells,4 or MDCK cells,10 release of CTF occurred at levels similar to the respective wild-type protein.

It is unclear why the Ser150-to-Asp603 part of the ΔCD-matriptase-Myc/(His)_6 variant was detected poorly or not at all in the conditioned media. This is not because the Ser150-to-Asp603 part is susceptible to degradation in the extracellular environment. Indeed, that part was detected clearly in the cell-surface membranes. Rather, occurrence on the cell surface raises the possibility that the Ser150-to-Asp603 part nonspecifically adsorbs on certain cell-surface molecules and cannot be released into the media. Recently we obtained evidence that the N-glycan attached to Asn772 located within the catalytic domain affords solubility to the domain.10 It is thus possible that ablation of the catalytic domain results in decreased solubility of this protease (in the context of the membrane-associated form). Alternatively, the Ser150-to-Asp603 part in the context of ΔCD-matriptase-Myc/(His)_6 folds improperly due to the absence of the catalytic domain. In either case, the Ser150-to-Asp603 part can undergo adsorption to cell-surface molecules. It will be a challenge to dissect the mechanisms by which the catalytic domain is involved in CTF release.

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References