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Activation of a Membrane-Bound Serine Protease Matriptase on the Cell Surface

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Matriptase is a type II transmembrane serine protease. The activation (*i.e.* conversion of the single-chain pro-form to the disulphide-linked-two-chain active form) of this enzyme is known to occur *via* a mechanism requiring its catalytic triad. We reported previously that the activated enzyme was produced in the conditioned medium when full-length rat matriptase was expressed in monkey kidney COS-1 cells. The present study aimed to address when and where the matriptase activation occurs. COS-1 cells expressing matriptase were labelled with a membrane-impermeable biotin derivative and then solubilized with Triton. Both activated and non-activated matriptase molecules were detected in the avidin precipitants of Triton extracts, whereas only the non-activated molecules were detected in the flow-through fraction of avidin-precipitation procedure. Single-chain matriptase has been thought to have an inherent activity. Indeed, a secreted single-chain variant of recombinant matriptase bearing mutation at the activation-cleavage site was found to exhibit the activity in hydrolyzing a synthetic peptide substrate at pH 7.5. However, the variant had little activity at pH 5.5, as found in the lumen of post-Golgi secretory vesicles. Altogether, it is concluded that the activation of matriptase may occur when the enzyme reaches the cell surface.

Key words: cell surface, enzyme activation, intracellular trafficking, matriptase, membrane-type serine protease.

Abbreviations: CHO, Chinese hamster ovary; CTF, C-terminal fragment; ER, endoplasmic reticulum; HAI-1, hepatocyte growth factor activator inhibitor type I; HGF, hepatocyte growth factor; HRP, horseradish peroxidase; NTF, N-terminal fragment; PAR-2, protease-activated receptor-2; rEK, recombinant enterokinase.

Matriptase (also known as membrane-type serine protease 1, epithin, suppression of tumorigenecity 14, *etc.*) is a member of type II transmembrane serine proteases, which are characterized by the N-terminal cytoplasmic domain followed by a signal anchor transmembrane domain and the extra-cellular domain, including a serine protease catalytic domain at the C-terminus (Fig. 1) (*1–5*). The primary translation product predicted from the cDNA sequence comprises 855 amino acid residues in human, mouse, and rat enzymes (*4–6*). When the Arg^{614}–Val^{615} bond (or activation-cleavage site) of single-chain pro-form matriptase is hydrolysed, disulphide-linked-two-chain matriptase (or activated matriptase) is formed (*6–8*). The two-chain form exhibits protease activity with trypsin-like specificity, and is known to cleave and activate single-chain urokinase-type plasminogen activator (*6*, *9*, *10*), pro-hepatocyte growth factor (HGF) (*9*), insulin-like growth factor binding protein-related protein-1 (*11*), and stromelysin (*12*) and to digest extra-cellular matrix components such as fibronectin and laminin (*6*). The catalytic domain of matriptase over-expressed in *Escherichia coli*. activates a G-protein-coupled receptor named protease-activated receptor-2 (PAR-2) possibly *via* cleavage of its extra-cellular N-terminal region (*13*, *14*), cleaves to activate the precursor form of prostasin, a glycosyl-phosphatidylinositol-linked membrane serine protease (*15*), and cleaves a transmembrane protein Trask associated with src kinase (*16*). These enzymatic characteristics, together with the abundant expression in the epithelial elements of mucosal tissues (*e.g.* in enterocytes) and in keratinocytes (*6*, *17*, *18*), lead to a speculation that matriptase plays pleiotropic roles in epithelial and epidermal homeostasis. To date, analysis using matriptase gene-disrupted mice showed the importance of the enzyme for postnatal survival, epidermal barrier function, hair follicle development and thymic homeostasis (*19*, *20*).

On the other hand, the aberrant and excessive activity of matriptase resulting from its uncontrolled expression could contribute to cancer progression. This is exemplified by its existence in a number of cancer cell lines and malignant tissues (*1–3*, *21*), increased ratio of this protease to the physiological inhibitor, HGF activator inhibitor type I (HAI-1) in cancer cells (*22*, *23*), and the malignant transformation by transgenic expression in the epidermis (*24*). The dysregulated enzyme also appears to participate in chronic inflammation, probably

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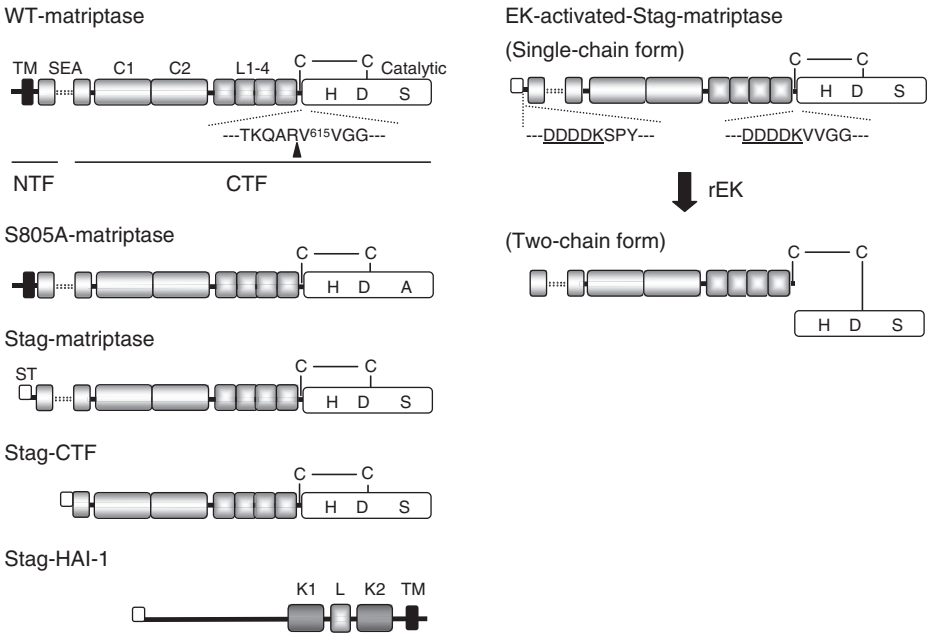

Fig. 1. **Schematic illustration of the expression constructs for rat matriptase and HAI-1.** The amino acid numbering of full-length rat matriptase (WT-matriptase) starts from the putative N-terminus. WT-matriptase was shown to be processed post-translationally *via* cleavage after Gly^{149}. The NTF and CTF parts are underlined and the association is illustrated with double broken lines. The amino acid sequence around the matriptase activation cleavage site is indicated in the single-letter code with amino acid numbering at the N-terminal valine residue of the catalytic domain (Val^{615}). The activation cleavage site is indicated by arrowhead. The predicted disulphide linkages between two cysteine residues corresponding to Cys^{604} and Cys^{731} in all the expression constructs for matriptase are shown as C–C. The catalytic triad, His^{656}, Asp^{711} and Ser^{805} are indicated as H, D and S, respectively, in the catalytic domain of constructs except S805A-matriptase. S805A-matriptase is a full-length rat matriptase variant in which the active-site serine residue (Ser^{805}) was changed to an alanine residue. Stag-matriptase is a secreted variant of recombinant matriptase in which the cytoplasmic and signal anchor transmembrane domains (Met^{1}–His^{80}) are replaced with the human immunoglobulin κ-chain signal peptide and S-tag (ST). Stag-CTF was another secreted variant consisting of S-tag and matriptase CTF. Stag-HAI-1 is a full-length rat HAI-1 with S-tag at the N-terminus. EK-activated-Stag-matriptase is a mutant in the context of Stag-matriptase. In this variant, the five amino-acid residues at the activation-cleavage site have been changed to those for enterokinase recognition (DDDDK, underlined). The surrounding sequences are also shown in the variant. The variant (single-chain form) is converted to two-chain form *in vitro* by incubation with rEK. Note that S-tag at the N-terminus can also be removed by incubation with rEK. TM, transmembrane domain; SEA, sea-urchin sperm protein–enterokinase–agrin domain; C1 and C2, the first and second complement factor 1R–urchin embryonic growth factor–bone morphogenetic protein domains; L1–4, four low-density lipoprotein receptor class A domains; Catalytic, catalytic domain. K1, Kunitz domain I (the first protease inhibitory domain); L, low-density lipoprotein receptor class A domain, and K2, Kunitz domain II (the second protease inhibitory domain).

through its ability to activate PAR-2 (*13*, *14*, *25*). Of importance is the finding that full-length human pro-form matriptase underwent activation cleavage when it was co-expressed with HAI-1 in human breast carcinoma BT549 cells, although the variant bearing mutation in its catalytic triad (His^{656}, Asp^{711} and Ser^{805}) did not (*26*). This suggests that the activation cleavage occurs *via* an autoactivation mechanism or a transactivation mechanism in which interaction between the single-chain pro-form molecules leads to generation of two-chain activated molecules. This characteristic might allow matriptase to serve as the most upstream protease for cancer progression and inflammation. Therefore, inhibition of the matriptase activation could be an effective strategy to retard progression of these disorders. The reason that HAI-1 is required for activation of matriptase (*i.e.* detection of activated matriptase) is unclear. It has been hypothesized that interaction between HAI-1 and single-chain matriptase *via* their low-density lipoprotein class A domains (Fig. 1) induces the conformational changes of enzyme that transiently allow it to permanently activate another (*26*).

It is unclear when and where the matriptase activation occurs. Lin and co-workers (*27*, *28*) demonstrated sphingosine 1-phosphate-induced accumulation of activated matriptase at cell-cell contacts of a human mammary epithelial line 184 A1N4 by immunocytochemical approach. This finding suggests that matriptase activation occurs when the enzyme reaches the cell surface. A recent *in vitro* study using homogenates of 184 A1N4 cells showed that the optimal pH for matriptase activation was around six and the activation was strongly inhibited by fairly high ionic strength (140 mM NaCl) (*29*). These findings led to the suggestion that the matriptase activation might occur during its intracellular trafficking to cell surfaces, particularly during its transit through the Golgi apparatus. However, it is unclear if this event is truly occurred in living cells.

When full-length rat matriptase (hereinafter designated WT-matriptase, Fig. 1) was co-expressed with

a recombinant rat HAI-1 (Stag-HAI-1, Fig. 1) in a transformed cell line derived from monkey kidney epithelium (COS-1), the activated enzyme was detected in samples of conditioned media but not in those of cell membrane-enriched fraction (*30*). This suggests that, at least in this cell line, matriptase is shed as a single-chain molecule, after which it undergoes activation. However, a possibility cannot be excluded that the lack of signal for activated matriptase in the cell-membrane fraction is due to artificial shedding from cell membranes during membrane preparation (*30*). Indeed, activated matriptase was found in lysates of BT549 cells transfected with the cDNA (*26*). The aim of the present study is to address when and where matriptase activation occurs in living cells. Using an approach combining *in cellulo* and *in vitro* experiments, we provide evidence that the activation occurs on the cell surface.

MATERIALS AND METHODS

Materials—KOD_{plus}® DNA polymerase used for polymerase chain reaction (PCR), restriction endonucleases, T4 polynucleotide kinase and a DNA ligation kit were purchased from Toyobo (Osaka, Japan). Synthetic oligonucleotides and cell culture reagents were from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP)-conjugated S-protein, an agarose-immobilized S-protein, pT7blue2 vector, and recombinant enterokinase (rEK) were purchased from Novagen (Madison, WI). A protease inhibitor cocktail (Complete™, EDTA-free) was purchased from Roche Diagnostics (Mannheim, Germany). A membrane-impermeable biotin derivative (sulpho-NHS-SS-biotin) and an agarose-immobilized avidin (Neutravidin™ agarose) were purchased from Pierce (Rockford, IL). A pre-stained protein marker (broad range) was from New England Biolabs (Beverly, MA). A synthetic 15-amino-acid peptide corresponding to the sequence of S-tag (KETAAAKF ERQHIDS) was obtained from BEX (Tokyo, Japan). Spectrozyme tPA® (Sp-tPA, methylsulphonyl-D-cyclohexyltyrosyl-glycyl-L-arginine–*p*-nitroanilide acetate) was purchased from American Diagnostica (Stanford, CA). Bovine trypsinogen was from Sigma (St. Louis, MO). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

Anti-Rat Matriptase Catalytic Domain Antibody—The procedure for producing a rabbit polyclonal anti-matriptase antibody that recognizes a site within the catalytic domain (Ser^{682}–Arg^{696}) (Spr992) was described previously (*30*).

Expression Constructs—A plasmid for expression of WT-matriptase has been constructed using pcDNA3.1(+) vector (Invitrogen) (*30*), and was designated pcDNA-WT-matriptase in the present study. The plasmid for expression of a secreted matriptase variant consisting of the entire extracellular domain (Tyr^{81}–Val^{855}) with an S-tag at the N-terminus has been constructed using the pSectag2/HygroB vector (Invitrogen) (*6*). The plasmid and product were described as pSec-Stag-matriptase and Stag-matriptase, respectively, in this study (Fig. 1). The procedure for constructing a plasmid for producing another secreted variant (designated EK-activated-Stag-matriptase) was described previously (*31*). The plasmid for a rat HAI-1 (Pro^{41}–Leu^{513}) with an S-tag at the N-terminus (Stag-HAI-1, Fig. 1) has also been constructed using pSectag2/HygroB (*30*).

A plasmid for expression of full-length rat matriptase bearing a mutation in the active site serine residue (Ser^{805}) (S805A-matriptase, Fig. 1) was constructed as follows. A restriction fragment of pcDNA-WT-matriptase produced by XhoI and SmaI was ligated into the pBluescriptSK vector (Stratagene, La Jolla, CA) that had been pre-digested with the same sets of enzymes. Using this plasmid as the template, a DNA fragment was amplified by PCR with KOD_{plus}® DNA polymerase and oligonucleotides: 5′-CCGGTGGCCCCTTGTC-3′ and 5′-CATCACCCTGGCAGGAGTC-3′, to introduce appropriate mutation (the nucleotide underlined indicates the introduced mutation). The PCR product was phosphorylated with T4 nucleotide kinase and then self-ligated. The plasmid was digested with XhoI and SmaI, and the resulting fragment was ligated into pcDNA/WT-matriptase from which a fragment produced by digestion with XhoI and SmaI had been removed.

The plasmid for a secreted variant of matriptase consisting of CTF (Ser^{150}–Val^{855}) (Stag-CTF, see Fig. 1) was created as follows. A DNA fragment was amplified by PCR using 5′-CAGCGTCATTGCCTACTACTGG-3′ and 5′-CTCGAGCCTGTTTGGTAAAGG-3′ as primers and pSec-Stag-matriptase as a template. The DNA fragment was ligated into the SmaI-linearlized pT7blue2 vector (pT7-CTF). pT7-CTF was digested with KpnI and XhoI, and the resulting fragment was ligated into pSec-Stag-matriptase from which a fragment produced by digestion with KpnI and XhoI had been removed. The sequences of expression plasmids constructed in the present study were determined in both directions by the dideoxynucleotide chain-termination method (*6*).

Transient Expression Experiments Using COS-1 Cells—COS-1 cells were maintained as described previously (*6*). The trypsinized cells were plated on a plastic 100-mm dish (Asahi Techno Glass, Tokyo, Japan) for transient expression experiments of COS-1 cells. The transfection of constructs into the cells using Lipofectamine2000™ (Invitrogen) was done according to the procedure described previously (*30*). Cells were left undistributed for 24 h after transfection. The transfected cells were then washed three times with phosphate-buffered saline (PBS) [8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136 mM NaCl and 2.7 mM KCl (pH 7.4)], and then cultured for an additional 24 h in 10 ml of Dulbecco's modified Eagle's medium without fetal bovine serum. After incubation, the conditioned medium was harvested and transferred to a centrifuge tube (15 ml) in which 1 ml of Complete™ (10 × concentrate) was included. After centrifugation at 3,000 *g* for 5 min at 22°C, the resulting supernatant was concentrated to about 1 ml by ultra-filtration using an Amicon® Ultra-15 membrane filter (50,000 MWCO, Millipore, Bedford, MA). The concentrated medium was transferred to a 1.5-ml microcentrifuge tube in which 100 µl of Complete™ (10× concentrate) was included, and was centrifuged at 10,000 *g* for 10 min at 22°C. The resulting supernatant

was further concentrated to 80 μl using a Microcon®-50 membrane filter (Millipore). The ultra-filtrate was stored at –20°C until use after the addition of 20 μl of 5× Laemmli protein sample buffer (Laemmli buffer) [1× Laemmli buffer, 0.05 M Tris–HCl (pH6.8), 10% glycerol, 2% sodium dodecylsulphate (SDS) and 0.005% Bromophenol Blue with dithiothreitol at a final concentration of 12 mM] (*32*). After the conditioned media were collected, the cells transfected with pcDNA-WT-matriptase and pcDNA-S805A/matriptase (with pSec-Stag-HAI-1) were washed three times with ice-cold PBS, mechanically scraped and collected by centrifugation at 3,000*g* for 3 min at 4°C. The cells were re-suspended in 5 ml of hypo-osmotic buffer [50 mM Tris–HCl (pH 7.0) containing Complete™] and quickly frozen in liquid nitrogen. After thawing and centrifugation at 500*g* for 5 min at 4°C, the post-nuclear supernatants were re-centrifuged at 48,000*g* for 30 min at 4°C. The resulting pellets (crude membranes) were solubilized with 100 μl of 1× Laemmli buffer and then stored at –20°C until use.

Samples were thawed, heated to 95°C for 5 min and subjected to SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (12% polyacrylamide). A 30 μl sample was loaded onto each lane. After separation, the proteins were transferred by electroblotting onto a polyvinylidene fluoride membrane (Fluorotrans W; Nihon Genetics, Tokyo, Japan), and then the blots were incubated with PBST (PBS containing 0.1% Tween-20) and the primary antibody Spr992 (1:20 dilution). Protein bands were visualized using a goat anti-rabbit IgG secondary antibody (Dako Japan, Kyoto, Japan) and ECL® detection system (GE healthcare, Tokyo, Japan). S-tagged proteins were visualized using HRP-conjugated S-protein (1:3000 dilution).

Biotinylation of WT-matriptase Expressed in COS-1 Cells—COS-1 cells cultured on a plastic 150-mm dish (Asahi Techno Glass) were transfected with pcDNA-WT-matriptase and pSec-Stag-HAI-1 as described above. After incubation for 24 h, the cells were washed three times with PBS, exposed to 20 ml of PBS containing 0.5 mg/ml sulpho-NHS-SS-biotin and incubated for 30 min on ice. After the incubation, 1 ml of PBS containing 200 mM glycine was added to terminate biotinylation reaction. After removing the solution, cells were washed extensively with ice-cold PBS, exposed to 2 ml of PBS containing 1% Triton X-100 and then incubated for 5 min on ice. Cells were scraped with Triton, transferred to a centrifuge tube (5 ml), and incubated for 1 h on ice with occasional agitation. After the incubation, the mixture was centrifuged at 10,000*g* for 30 min at 4°C. The resulting supernatant (Triton extract) was transferred to two 1.5-ml microcentrifuge tubes in each of which 12 μl slurry of immobilized avidin was included and then incubated for 30 min at 22°C with rocking. After precipitation of slurry by a brief centrifugation, the supernatants were incubated again with the immobilized avidin as described above. The mixtures were briefly centrifuged, and the supernatants (flow-through fraction of the avidin-precipitation procedure) were pooled and concentrated to 100 μl by ultra-filtration using a Microcon® YM30 membrane filter. Proteins in the ultra-filtrate were precipitated using an SDS–PAGE Clean-Up kit (GE healthcare) according to the manufacturer's instruction, solubilized with 48 μl of 1 × Laemmli buffer, and stored at –20°C until use. Precipitated immobilized avidin were pooled (48 μl), washed extensively with PBST, exposed to 48 μl of 2 × Laemmli buffer and heated at 95°C for 3 min. After a brief centrifugation, the supernatant (avidin precipitants) was stored at −20°C until use. The samples of avidin precipitant and of the flow-through fraction of avidin precipitation procedure were analyzed by means of SDS–PAGE and Wwstern blotting using Spr992 and HRP-conjugated S-protein as described above.

Preparation of Single-Chain EK-Activated-Stag-Matriptase—We have established a CHO (Chinese hamster ovary)-K1 line expressing a high level of EK-activated-Stag-matriptase (*33*). The clonal line was cultured in a 75-cm^2 plastic flask (Asahi Techno Glass) as described previously (*33*). After reaching confluence, cells were washed three times with PBS and 10 ml of Ham's F12 without fetal bovine serum was added to the flask. After 48 h, the conditioned medium was collected, and fresh serum-free medium was added. This was repeated until half of the cells were peeled off. The collected media were centrifuged immediately at 3,000*g* for 10 min at 22°C, and the resulting supernatants were stored at –20°C until use. For purification, 200 ml of the conditioned media was collected using three flasks. After thawing, the media were pooled and concentrated to 2.5 ml by ultra-filtration using an Amicon® Ultra-15 membrane filter (50,000 MWCO, Millipore). The concentrated medium was subjected to gel filtration in a buffer [25 mM HEPES (pH7.5) containing 145 mM NaCl and 0.1% Triton X-100] (hereinafter HEPES buffer A) using a PD-10 column (GE Healthcare) with an elution volume of 3.5 ml. The gel filtrate was divided equally, and each part was transferred to a 2.0-ml microcentrifuge tube in which 62.5-μl slurry of immobilized S-protein was included. The tubes were incubated for 30 min at 22°C with rocking. After brief centrifugation, the supernatants were incubated again with immobilized S-protein as described above. The precipitated slurries were pooled (250 μl) and washed four times with HEPES buffer A and three times with 5 mM HEPES (pH7.5) containing 5 mM NaCl and 0.01% Triton X-100 (hereinafter HEPES buffer B). To elute single-chain EK-activated-Stag-matriptase, the slurry washed was transferred with 250 μl of HEPES buffer B containing 200 μM synthetic S-tag peptide to a spin column (Attoprep™, 0.22 μm pore size, Atto, Tokyo, Japan) and centrifuged at 10,000*g* for 1 min at 22°C. The eluate was subjected to gel filtration in HEPES buffer B and stored at −20°C until use.

The concentration of single-chain EK-activated-Stag-matriptase was measured semi-quantitatively as follows. A part of purified EK-activated-Stag-matriptase and bovine trypsinogen (10, 20, 40 and 80 ng) were subjected to SDS–PAGE (12% polyacrylamide) under reducing conditions followed by silver staining as described previously (*6*). After drying, the gels were photographed digitally, and the signal intensities of protein bands were analysed by densitometry as described previously (*30*). The concentration was calculated by comparing

the signal density of a 90-kDa band with that of a 25-kDa band (for trypsinogen) (see Supplementary Fig. 1).

Preparation of Two-Chain EK-activated-Stag-Matriptase—To generate two-chain EK-activated-Stag-matriptase (Fig. 1), a 500 µl portion of HEPES buffer B containing 50 nM single-chain EK-activated-Stag-matriptase was incubated for 24 h at 22°C with rEK. The final concentration of rEK was 1 U/ml. After incubation, the solution (activation mixture) was stored at –– 20°C until use. The concentration of two-chain enzyme occurred in the activation mixture was evaluated by means of SDS–PAGE under reducing conditions and western blotting using Spr992. Practically, the signal density of a 28-kDa band produced from two-chain enzyme was compared with that of a 90-kDa band produced from single-chain enzyme (*10*, *33*). We have confirmed that no 90-kDa band was produced but that the molar concentration of EK-activated-Stag-matriptase is almost unchanged, after incubation with rEK (*10*, *33*).

Kinetic Analysis of EK-Activated-Stag-Matriptase—Sp-tPA was dissolved in distilled water at the concentration of 5 mM and stored at –20°C until use. The assays were carried out at 37°C in a final volume of 40 µl in a 0.5-ml microcentrifuge tube. The reaction using single-chain enzyme was initiated by the addition of 20 µl of HEPES buffer B containing 50 nM single-chain EK-activated-Stag-matriptase (or HEPES buffer B alone) to 20 µl of HEPES buffer B containing Sp-tPA (final concentration: 0, 25, 50, 100, 200, 300, 400 and 500 µM), and the reaction mixtures were incubated for 24 h. To prevent water evaporation within the tube, the incubation was conducted using a temperature controller (PC805-MI, ASTEC, Fukuoka, Japan). The reaction using two-chain enzyme was initiated by the addition of 2 µl of activation mixture (see above) (or HEPES buffer B alone) to 38 µl of HEPES buffer B containing Sp-tPA (final concentration of two-chain enzyme, 2.5 nM; final concentrations of substrate, 0, 25, 50, 100, 200, 300, 400 and 500 µM), and the reaction mixtures were incubated for 30 min in a water bath. The reactions using the single-chain and two-chain enzymes were terminated by adding 400 µl of 0.1 M monochloroacetic acid in 0.1 M sodium acetate buffer (pH 4.0), and the absorbance at 405 nm was measured. In order to evaluate the enzyme-catalysed reaction rate, the non-enzymatic spontaneous reaction rate of absorbance increase was subtracted from the reaction rate observed in the presence of the enzyme. The initial rate of hydrolysis was determined using the molar absorption coefficient ε_{405} of 9.65 $mM^{-1}\,cm^{-1}$ for *p*-nitroaniline. The Michaelis constant (K_m) and molecular activity (k_{cat}) for Sp-tPA hydrolysis were determined by fitting the rate data to the Michaelis–Menten equation using KaleidaGraph (Synergy Software, Reading, PA). The kinetic values are expressed as mean ± SD of three independent experiments. We did not remove rEK from the activation mixture, because rEK alone (0.05 U/ml) exhibited no activity toward Sp-tPA.

pH-Dependence of Activity of the Single-Chain and Two-Chain EK-Activated-Stag-Matriptase Molecules—The reactions using single-chain enzyme were conducted as described above, except that 20 µl of 200 mM MES (pH 5.0, 5.5, 6.0, 6.5 and 7.0) and 200 mM HEPES (pH 7.5) containing 5 mM NaCl, 0.01% Triton X-100 and 1 mM Sp-tPA were added to initiate the reaction. The reactions using two-chain enzyme were conducted as described above with minor modification. The activation mixture (see above) was diluted with HEPES buffer B (1:10 dilution). The reactions were initiated by the addition of a 20 µl part of diluted activation mixture to 20 µl of 200 mM MES (pH 5.0, 5.5, 6.0, 6.5 and 7.0) and 200 mM HEPES (pH 7.5) containing 5 mM NaCl, 0.01% Triton X-100 and 1 mM Sp-tPA. Note that rEK alone (0.05 U/ml) exhibited no activity toward Sp-tPA at any pH.

RESULTS

Activation of Matriptase through Its Active-Site Serine Residue-Dependent Mechanism in COS-1 Cells—We previously found that, when transiently expressed alone in COS-1 cells, WT-matriptase was post-translationally processed by cleavage between Gly^{149} and Ser^{150} and that some of the C-terminal fragment (CTF) molecules (Ser^{150}–Val^{855}) remained in the cells by association with the N-terminal fragment (NTF) molecules (Met^{1}–Gly^{149}) while the other CTF molecules were released into the culture medium in the non-activated form (*i.e.* single-chain CTF, refer to illustration for WT-matriptase in Fig. 1) (*30*). When co-expressed with HAI-1, the post-translational processing also occurred. More importantly, both non-activated and activated forms were found in the medium upon co-expression with HAI-1 (*30*). Single-chain CTF was visualized as a 90-kDa band by SDS–PAGE under reducing conditions and western blotting with an anti-rat matriptase catalytic-domain antibody (Spr992) (*30*). The activation of WT-matriptase (*i.e.* the occurrence of disulphide-linked-two-chain CTF) could be assessed by the appearance of a 28-kDa band in this analysis. This was confirmed in the present study (refer to Fig. 2A, a lane for a medium sample of WT-matriptase). The absence of 28-kDa signal in a cell membrane-enriched fraction was also reproduced (Fig. 2A). Although HAI-1 is a type I transmembrane protein, this inhibitor undergoes ectodomain shedding (*7*). We reported previously that Stag-HAI-1 occurs in the conditioned medium as a 58-kDa protein and in the cell membrane-enriched fraction as a 66-kDa protein when expressed in COS-1 cells (*30*). This was confirmed in the present study (Fig. 2B).

It is important to address whether the activation cleavage of this protease found in COS-1 cells occurs through a mechanism requiring its catalytic triad. We produced a full-length rat matriptase variant bearing mutation in which its active-site serine residue (Ser^{805}) was changed to an alanine residue (S805A-matriptase). S805A-matriptase was co-expressed with Stag-HAI-1. When probed with Spr992, 28-kDa band was not produced from either medium or cell-membrane samples (Fig. 2A). Co-expression of Stag-HAI-1 was confirmed (Fig. 2B). These results suggest that the activation cleavage of WT-matriptase in COS-1 cells occurs *via* a mechanism requiring its active site.

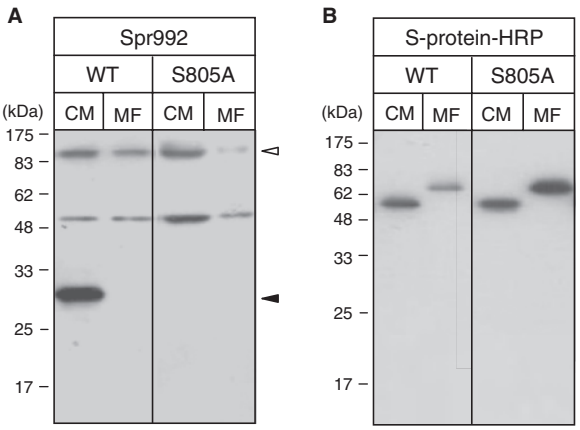

Fig. 2. **Representative western blot showing the occurrence of activation cleavage of WT-matriptase through a mechanism requiring its active site serine residue in COS-1 cells.** pcDNA-WT-matriptase and pSectag-Stag-HAI-1 (WT) or pcDNA-S805A-matriptase and pSectag-Stag-HAI-1 (S805A) were transfected into the cells. Samples of medium (CM) and cell membrane-enriched fraction (MF) were separated by SDS–PAGE under reducing conditions; western blot was probed with Spr992 (A). The positions at which the non-activated enzyme (*i.e.* single-chain CTF) and activated enzyme (*i.e.* catalytic domain part of two-chain CTF) migrate are indicated on the right by open and closed arrowheads, respectively. A protein with a mass of ~50 kDa visible in the blot is a non-specific band representing an unknown protein. Another western blot was probed with HRP-conjugated S-protein (S-protein-HRP) to address the expression of Stag-HAI-1 (B). The molecular-mass standards are indicated on the left of each blot in kilo Daltons.

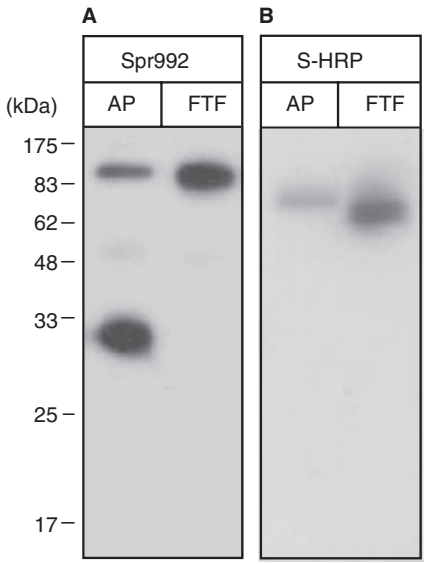

Fig. 3. **Representative western blot showing the occurrence of activated matriptase on the surface of COS-1 cells.** Cells were transfected with pcDNA-WT-matriptase and pSectag-Stag-HAI-1. Cells were labelled with a biotin derivative and solubilized using Triton X-100. The avidin precipitants (AP) and the flow-through fraction of avidin-precipitation procedure (FTF) were prepared as described in the MATERIALS AND METHODS section. Samples were separated by SDS–PAGE under reducing conditions, and western blots were probed with Spr992 (A) or HRP-conjugated S-protein (S-HRP) (B). The molecular-mass standards are indicated on the left of each blot in kilo Daltons. Note that the biotinylated proteins migrated more slowly than those in the flow-through fraction because of the addition of the sulpho-NHS-SS-biotin spacer.

Localization of Activated Matriptase in COS-1 Cells—COS-1 cells transfected with pcDNA-WT-matriptase and pSec-Stag-HAI-1 were incubated with a cell membrane-impermeable biotin derivative (for biotinylation of cell-surface proteins) and then solubilized with Triton. The avidin precipitants of the Triton-soluble fraction and the flow-through fraction of the avidin-precipitation procedure should contain the cell surface and intracellular enzymes, respectively. When probed with Spr992, 95-kDa and 30-kDa bands were produced from samples of the avidin precipitants, whereas only a 90-kDa band was produced from those of the flow-through fraction (Fig. 3A). The 95- and 30-kDa signals are thought to represent single-chain CTF and the catalytic-domain part of two-chain CTF, respectively, to each of which the spacer of the biotin derivative remained bound. Upon SDS–PAGE, the biotinylated Stag-HAI-1 molecules (*i.e.* those in the avidin precipitants) also migrated more slowly than the non-biotinylated molecules (*i.e.* those in the flow-through fraction of the avidin-precipitation procedure) (Fig. 3B). The presence of the activated enzyme in the avidin precipitant and its absence in the flow-through fraction suggested that matriptase is activated when it reaches the cell surface.

Kinetic Analysis of EK-Activated-Stag-Matriptase—Although single-chain matriptase has been thought to have an inherent protease activity (*26*, *34*), the activity has not been determined. We have produced a secreted variant of matriptase consisting of the entire extracellular domain (EK-activated-Stag-matriptase) using CHO-K1 cells (*32*). In this variant, five amino-acid residues at the activation-cleavage site of matriptase (Thr-Lys-Gln-Ala-Arg^{614}) were changed to those suitable for the cleavage by enterokinase (Asp-Asp-Asp-Asp-Lys) (Fig. 1). This variant was purified by means of single-step affinity chromatography using immobilized S-protein and visualized as a 90-kDa band in SDS–PAGE and silver staining (see Supplementary Fig. 1). The purified enzyme can be converted to an active form (two-chain EK-activated-Stag-matriptase) by treatment with rEK (*33*). Two-chain EK-activated-Stag-matriptase has been found to display protease activity comparable to two-chain enzyme purified from human milk (*9*, *33*). In the present study, we used EK-activated-Stag-matriptase without rEK treatment as a model of single-chain matriptase, and hereinafter we designated this as pseudo-single-chain matriptase. Pseudo-single-chain matriptase was neither converted to the two-chain form nor severely degraded by incubation at 37°C for 72 h in HEPES buffer B (Supplementary Fig. 1).

The matriptase catalytic domain produced in *E. coli*. (*34*) and two-chain EK-activated-S-tag-matriptase (*33*) were found to effectively hydrolyze a peptidyl substrate, Sp-tPA. For this reason, we examined the activity of

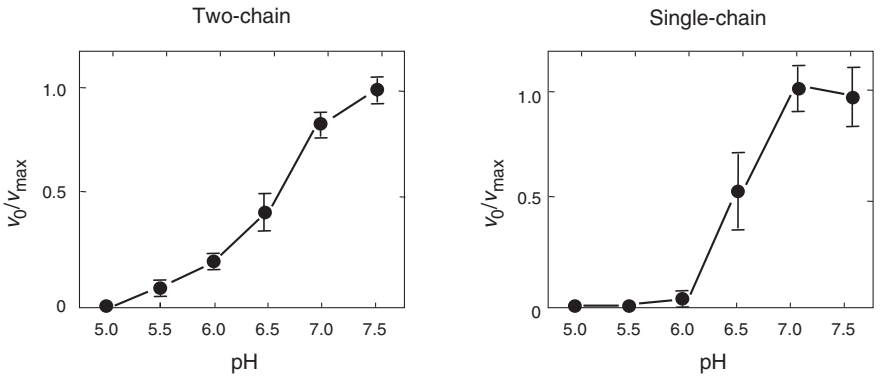

Fig. 4. **Effect of pH on the initial reaction rate in the hydrolysis of Sp-tPA catalysed by recombinant two-chain and single-chain matriptase molecules.** Two-chain EK-activated-Stag-matriptase (Two-chain) and pseudo-single-chain matriptase (Single-chain) were incubated with substrate at pH ranges of 5.0–7.5. The initial reaction rate (v_o) observed at the optimal pH is denoted by v_{max}. The average of triplicate determination with standard error (SE) value is shown.

pseudo-single-chain matriptase towards this substrate. In HEPES buffer B, the two-chain EK-activated-S-tag-matriptase hydrolyzed the substrate with K_m of (33 ± 8) μM, k_{cat} of (45 ± 3.2) s^{-1} and k_{cat}/K_m of $(1.4 \times 10^6 \pm 4.0 \times 10^5)$ $M^{-1}\,s^{-1}$. Pseudo-single-chain matriptase did it with K_m of (149 ± 50) μM, k_{cat} of $(1.3 \pm 0.6) \times 10^{-2}\,s^{-1}$ and k_{cat}/K_m of (87 ± 12) $M^{-1}\,s^{-1}$. It is notable that the k_{cat}/K_m value of the single-chain molecule was about 16,000 times lower than that of the two-chain molecule.

The activities of the two-chain and pseudo-single-chain molecules were determined in a pH range of 5.0–7.5 (Fig. 4). In the reaction catalysed by the two-chain molecule, the initial reaction rate (v_0) decreased with decreasing pH (Fig. 4). The pH dependence of the hydrolysis catalysed by the pseudo-single-chain matriptase was considerably different from that by the two-chain matriptase (Fig. 4). The maximum reaction rate [$v_{max} = (0.20 \pm 0.05)$ nM s^{-1}] was observed at pH 7.0. The v_0 value at pH 6.5 was significantly lower than that at pH 7.0. Although a trace activity was found at pH 6.0, almost no activity was observed at pH 5.5, which is considered to be the physiological pH level inside the secretory vesicles (Fig. 4) (*35*). These lines of evidence support that pseudo-single-chain matriptase has an inherent activity and that matriptase can be autoactivated or transactivated. The v_0 value of the pseudo-single-chain matriptase in the Sp-tPA hydrolysis at pH 7.5 in 100 mM HEPES buffer containing 145 mM NaCl and 0.01% Triton X-100 was 0.18 ± 0.05 nM s^{-1}, indicating that the enzyme activity was not much affected by the addition of 145 mM NaCl.

The Processing of Secreted Variants of Recombinant Matriptase in COS-1 Cells—Secreted variants of recombinant matriptase were co-expressed with Stag-HAI-1 in COS-1 cells, and the occurrence of activated enzyme in the conditioned media was examined. Stag-matriptase is a secreted variant consisting of the intact entire extracellular domain with S-tag at the N-terminus (Fig. 1) (*6*). A 90-kDa band was produced when probed with Spr992 (Fig. 5A, lane 1). When probed with HRP-conjugated S-protein, a 22-kDa band (with a 58-kDa band for Stag-HAI-1) was produced (Fig. 5B, lane 3). The 22-kDa band may represent S-tag and the N-terminal half of the sea-urchin sperm protein–enterokinase–agrin domain of

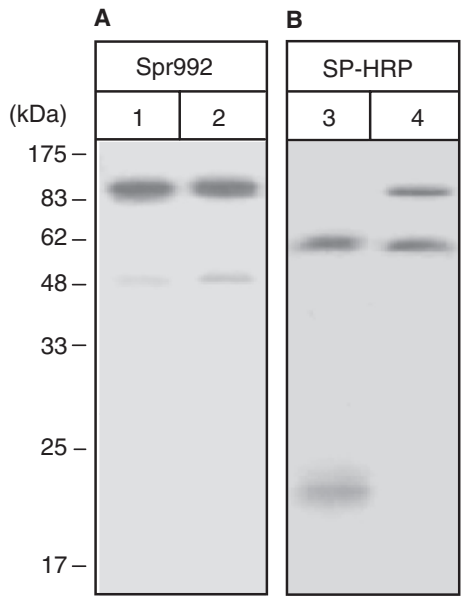

Fig. 5. **Representative western blot showing the secretion of Stag-matriptase and Stag-CTF as a non-activated form into the medium of COS-1 cells.** pSectag-Stag-matriptase and pSectag-Stag-HAI-1 (lanes 1 and 3) or pSectag-Stag-CTF and pSectag-Stag-HAI-1 (lanes 2 and 4) were transfected into COS-1 cells. Medium samples were separated by SDS–PAGE under reducing conditions, and western blots were probed with Spr992 (A) or HRP-conjugated S-protein (S-HRP) (B). The molecular-mass standards are indicated on the left of each blot in kilo Daltons.

Stag-matriptase (see Fig. 1). This result suggested that the post-translational cleavage after Gly^{149} could occur without membrane-anchoring of this protease. On the other hand, the activation cleavage after Arg^{614} appeared to require membrane anchoring of the single-chain molecule, because no 28-kDa band was observed (Fig. 5A, lane 1).

S-tag CTF is another secreted variant consisting of S-tag and matriptase CTF (Fig. 1). When probed with

Spr992, a 90-kDa band was produced but a 28-kDa band was not (Fig. 5A, lane 2). The absence of the activation cleavage in this variant was further supported by the fact that only a 90-kDa band (with a 58-kDa band for Stag-HAI-1) was visualized when the blot was probed with HRP-conjugated S-protein (Fig. 5B, lane 4).

DISCUSSION

To address when and where matriptase activation occurs in living cells, we conducted an *in cellulo* experiment using COS-1 cells. First we obtained evidence that the activation of WT-matriptase in this cell line occurs through a mechanism requiring the active-site serine residue (Fig. 2A). Cell-surface biotinylation analysis of COS-1 cells expressing WT-matriptase showed that the activated enzyme occurs on the cell surface but not in intracellular environments. The non-activated enzyme was also evident on the cell surface. These findings strongly suggest that matriptase is activated on the cell surface and then shed extra-cellularly. The lack of 28-kDa band in the crude membrane fraction [refer to Fig. 2A and reference (*30*)] may be due to a possibility that the activated enzymes are artificially shed during the preparation. The non-activated enzyme (*i.e*. single-chain CTF) was also present in the conditioned medium of COS-1 cells expressing WT-matriptase [refer to Fig. 2A and reference (*30*)], implying that some of matriptase molecules are shed from cell surface as single-chain molecules and then converted to two-chain molecules in the medium. However, this seems unlikely, because Stag-CTF was unable to undergo activation cleavage (Fig. 5A). Our interpretation for the occurrence of non-activated enzyme in the medium of COS-1 cells expressing WT-matriptase is that, upon the high level of expression from exogenous cDNA under the control of strong promoters, considerable single-chain CTF molecules dissociate from NTF on the cell surface and/or during intracellular trafficking.

HAI-1 has been thought to be required for the activation of matriptase in BT549 cells (*26*, *28*). No activated matriptase could also be detected when expressed alone in COS-1 cells (*30*). In the study by Désilets *et al*. (*36*), however, activated matriptase occurred when a cDNA for full-length human enzyme was transfected alone into a human embryonic kidney cell line (HEK293) that expresses neither matriptase nor HAI-1 endogenously. This finding indicates that interaction with HAI-1 *per se* is not necessarily required for matriptase activation. In the present study, pseudo-single-chain matriptase was found to exhibit activity toward hydrolysis of Sp-tPA under slightly alkaline, neutral and mildly acidic pH conditions (Fig. 4), suggesting that single-chain matriptase can exhibit protease activity in endoplasmic reticulum (ER) and Golgi, the insides of which are slightly basic (pH 7.2) and slightly to mildly acidic (pH 6.0–6.7), respectively (*35*). In addition, when expressed alone in BT549 cells, matriptase was retained in the perinuclear region (*e.g*. in the ER or Golgi) (*28*). Taken together, we suggest that HAI-1 interacts with single-chain matriptase to prevent the premature activation in the intracellular organelles of BT549 cells and COS-1 cells. In the absence of HAI-1, the activated matriptase might degrade other matriptase molecules and/or adaptor molecules required for transit of the enzyme to secretory vesicles. This could be a plausible reason that matriptase was poorly detected when expressed alone in BT549 and COS-1 cells (*28*, *30*). In HEK293 cells, there may be additional mechanisms that prevent premature activation of matriptase in ER and Golgi.

In an *in vitro* experiment using homogenates of 184 A1N4 cells, matriptase was activated under acidic to neutral pH conditions (pH 5.2–7.0) but not under slightly alkaline pH conditions (pH 7.2 and 7.4) (*29*). More surprisingly, the degree of activation was high and similar at a pH range of 5.4–6.2 (*29*). This implied that matriptase could undergo activation even in the secretory vesicles. On the other hand, the activity of pseudo-single-chain matriptase could not be detected at pH 5.5. This finding, together with the lack of signal for activated matriptase in the intracellular region of 184 A1N4 cells stimulated by sphingosine 1-phosphate (*27*, *28*) and of the transfected COS-1 cells (Fig. 3), leads to the suggestion that matriptase activation does not occur in the secretory vesicles. The occurrence of matriptase activation on the cell surface can be supported by the observation that pseudo-single-chain matriptase exhibited a protease activity in a buffer with pH and sodium levels of the extra-cellular milieu. In the cell homogenates, however, matriptase rarely underwent activation at pH 6.0 in the presence of 140 mM NaCl (*29*). We assume that the activation of matriptase under acidic pH conditions found in the cell homogenates is not through a mechanism requiring its catalytic triad.

In this study, we present a model for the activation of matriptase in COS-1 cells (Fig. 6). In ER, the catalytic domain of single-chain matriptase is thought to interact with the protease inhibitory domain of HAI-1 (*34*). This idea is supported by the fact that pseudo-single-chain matriptase exhibited protease activity at pH 7.0 and 7.5. Interaction of low-density lipoprotein receptor class A domains of matriptase with that of HAI-1 may stabilize single-chain matriptase–HAI-1 complexes (*26*). In any event, the complex formation might be critical for preventing the premature activation of matriptase within ER and Golgi (*28*). When delivered to the secretory vesicles, the single-chain matriptase–HAI-1 complexes may dissociate. This is suggested by the lack of protease activity of pseudo-single-chain matriptase at pH 5.0 and 5.5. The dissociation could enable a single-chain matriptase to activate itself or cleave another single-chain molecule when reaching to cell surface. Membrane anchoring of single-chain matriptase molecules appears to be essential for the activation. This is evidenced by the fact that secreted variants of matriptase were unable to undergo activation cleavage (Fig. 5A). Activated matriptase on the cell surface would be inactivated by HAI-1 and then shed extra-cellularly (*1*).

In summary, we provided *in cellulo* and *in vitro* evidence for the occurrence of matriptase activation on the cell surface. This finding suggests that exogenous administration of specific inhibitors for single-chain matriptase might effectively suppress the activation. However, it is unclear whether the cell-surface activation of matriptase

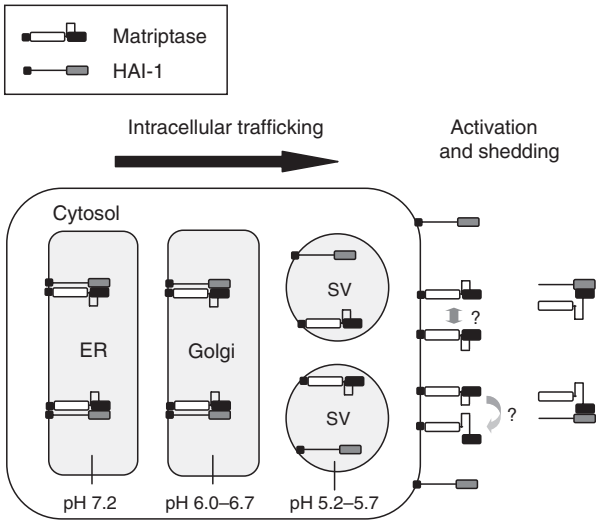

Fig. 6. **A schematic model for activation of matriptase in cultured cells.** In ER and Golgi, a single-chain matriptase molecule forms a complex with an HAI-1 molecule. When delivered to the secretory vesicles (SV), the complex dissociates. The dissociation allows the activation of matriptase when they reach the cell surface. The activated enzymes may be inactivated by HAI-1 on the cell surface and then shed extra-cellularly. In this illustration, the catalytic domain of matriptase and the protease inhibitory domain of HAI-1 are indicated by black and grey rectangles, respectively. The pH values in the organelles of secretory pathway are indicated.

is the ubiquitous mechanism. Indeed, the mechanism by which matriptase is activated *in vivo* is largely unknown. Regardless, the present findings obtained from COS-1 cells might lead to development of strategies at least for preventing the progression of epithelial-derived cancers in which matriptase is involved.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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