J. Biochem. 2010;148(6):721–732 doi:10.1093/jb/mvq108

JB THE JOURNAL OF BIOCHEMISTRY

A recombinant catalytic domain of matriptase induces detachment and apoptosis of small-intestinal epithelial IEC-6 cells cultured on laminin-coated surface

Received July 20, 2010; accepted September 7, 2010; published online September 19, 2010

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Matriptase is a type-II transmembrane serine protease that is expressed strongly in the epithelial elements of various organs. In the small intestine, it is expressed prominently at the villus tip where aged epithelial cells undergo shedding and/or apoptosis. This observation, together with the ability of matriptase to cleave laminin (a basement membrane component critical for epithelial cell attachment), prompted us to hypothesize that it plays an important part in the removal of aged epithelial cells in the small intestine. We tested this hypothesis by determining whether a recombinant catalytic domain of rat matriptase (His_6t-S-CD) causes detachment and/or apoptosis of small-intestinal epithelial IEC-6 cells. His_6t-S-CD caused detachment of cells attached to laminin-coated plates but did not detach cells attached to fibronectin- or type-IV collagen-coated plates. Pre-treatment of laminin-coated plates with His_6t-S-CD decreased the attachment of cells, suggesting that the recombinant matriptase caused detachment through a mechanism involving a direct effect on laminin. His_6t-S-CD was also found to induce apoptosis in the cells cultured on laminin-coated plates, as assessed by annexin-V staining, DNA fragmentation and caspase-3 activity assays. These findings support our hypothesis regarding the role of matriptase in the small intestine.

Keywords: apoptosis/cell shedding/laminin/matriptase/small-intestinal epithelial cells.

Abbreviations: Ac, acetyl; Boc, *t*-butyloxycarbonyl; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; MCA, 4-methylcoumaryl-7-amide; MDCK cells, Madin-Darby canine kidney cells; MEM, minimal essential medium; MMP, matrix metalloprotease; r-EK, recombinant enterokinase; RT, reverse transcription; SFM, serum-free medium; SIECs, small-intestinal epithelial cells; uPA, urokinase-type plasminogen activator.

The cells of the epithelial lining of the small intestine [hereinafter small-intestinal epithelial cells (SIECs)] play a major part in nutrient absorption. SIECs are generated by stem cells at the crypt base and migrate towards the lumen, during which time many differentiate into absorptive cells (*1*). Because of repeated exposure to a variety of foreign bodies, SIECs of the upper villus undergo exhaustion and/or injury. At the villus tip, SIECs (especially injured cells) are thought to undergo apoptosis followed by loss of adhesion to surrounding cells and the basement membrane, which is composed of extracellular matrix (ECM) proteins (*2*, *3*). Alternatively, SIECs may first detach from the basement membrane and then undergo death (detachment-induced apoptosis or anoikis) (*4*). In either case, SIECs are finally shed into the lumen (*1*–*5*). Shedding of epithelial cells may be an important mechanism for maintaining function and integrity, particularly in the context of the small intestine, because failure of continual epithelial renewal could result not only in nutrient malabsorption but also in impaired defence against microorganisms. Although various extracellular proteolytic events may be involved in the shedding of SIECs, details of the underlying mechanisms are largely unknown.

Matriptase is a type-II transmembrane serine protease (*6*–*8*). This protease is first synthesized as a zymogen comprising 855 amino acid residues in human, mouse and rat enzymes, which requires processing by cleavage between Arg614 and Val615 (activation cleavage) to generate the disulphide-linked two-chain fully active enzyme with trypsin-like specificity of cleavage (tc-matriptase) (Fig. 1A) (*9*–*14*). In addition, matriptase appears to function in a plasma membrane-associated form and in a shed (soluble) form (*6*, *10*, *11*). The release of ectodomain would provide a mechanism for enabling matriptase to gain access to non-plasma-membrane-associated substrates that occur in the vicinity of the sites of expression of this protease.

Matriptase is expressed by the epithelial elements of almost all organs examined to date (*15*, *16*). We have found that (i) of the normal tissues examined, mRNA for rat matriptase is expressed most strongly in the small intestine; (ii) the mRNA signal is most prominent in the epithelium of the villus tip in the small intestine; (iii) the matriptase protein localizes to the basolateral surface membrane of SIECs; and (iv) a recombinant form of tc-matriptase produced in a mammalian cell line cleaves laminin, a basement membrane

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component critical for anchorage of SIECs (*12*, *17*). In addition, tc-matriptase purified from human milk (*18*), bacterially expressed recombinant forms of the catalytic domain of matriptase (*19*, *20*) and our recombinant tc-matriptase (*12*) have potent activity for converting single-chain urokinase-type plasminogen activator (hereinafter pro-uPA) to two-chain uPA (hereinafter uPA), which drives activation of ECM-degrading enzymes [*e.g.*, matrix metalloproteases (MMPs)] through its ability to convert plasminogen to plasmin (*21*). These findings suggest that matriptase makes a substantial contribution to the shedding of SIECs at the villus tip through direct and indirect modification and/or proteolysis of the basement membrane components.

The purpose of this study was to obtain more definitive evidence for the involvement of matriptase in the shedding of SIECs. To achieve this, we conducted experiments using IEC-6, a non-transformed rat SIEC line. This cell line has been used in numerous studies to clarify the mechanisms of various events in SIECs, including proliferation, differentiation and death (*22–24*). In the present study, we describe that a recombinant catalytic domain of rat matriptase causes detachment and apoptosis of IEC-6 cells. To our knowledge, this is the first cell-based evidence that matriptase could contribute to the shedding of SIECs.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), high glucose, supplemented with L-Glu, minimal essential medium (MEM), supplemented with L-Glu and non-essential amino acids), penicillin–streptomycin mixture and synthetic oligonucleotides were from Invitrogen (Carlsbad, CA, USA). The DNA ligation kit, KOD Dash® and KOD$_{-plus-}$® DNA polymerases, used for PCR, restriction endonucleases, recombinant RNase inhibitor and T4 polynucleotide kinase, were purchased from Toyobo (Osaka, Japan). Moloney murine leukaemia virus reverse transcriptase (M-MLV RTase) used for the reverse transcription (RT) reaction and Ni^{2+}-chelating resin (HisLink™ resin) were purchased from Promega (Madison, WI, USA). Bovine recombinant enterokinase (hereinafter r-EK) was purchased from Novagen (Madison). A chromogenic substrate, methylsulphonyl-D-cyclohexyl-Tyr–Gly–L-Arg-*p*-nitroanilide acetate named Spectrozyme tPA® (hereinafter Sp-tPA) was purchased from American Diagnostica (Stanford, CA, USA). BSA (protease free), ECM gel from Engelbreth–Holm–Swarm mouse sarcoma (hereinafter called ECM gel), fibronectin (human plasma), insulin (bovine pancreas), laminin (Engelbreth–Holm–Swarm murine sarcoma basement membrane), trypsin (type III, bovine pancreas) and type-IV collagen (human placenta) were purchased from Sigma (St Louis, MO, USA). Acetyl (Ac)-L-Asp–L-Glu–L-methyl-coumaryl-7-amide (MCA) (Ac-DEVD-MCA) and *t*-butyloxycarbonyl (Boc)-L-Gln–L-Ala–L-Arg–MCA (Boc-QAR-MCA) were purchased from the Peptide Institute (Osaka). GM6001, MMP-2/MMP-9 Inhibitor II, MMP-9 Inhibitor I and UK122 were purchased from Calbiochem (San Diego, CA, USA). All other reagents were of analytical grade and were purchased from Nacalai Tesque (Kyoto, Japan).

Cell culture

IEC-6 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 13, and were maintained in DMEM supplemented with 5% foetal bovine serum, 4 μg/ml insulin, 100 IU/ml penicillin and 100 IU/ml streptomycin at 37°C in 5% CO_2. Madin–Darby canine kidney (MDCK) epithelial cells were purchased from RIKEN Bioresource Center (Ibaraki, Japan) and were maintained in MEM supplemented with 10% foetal bovine serum.

Expression constructs

A plasmid for expression of His$_6$t-S-CD pseudozymogen (pPICZ-His$_6$t-S-CD) in the methylotrophic yeast *Pichia pastoris* has been constructed using the pPICZαC vector (Invitrogen) (*25*). A plasmid for *P. pastoris* expression of a variant of the His$_6$t-S-CD pseudozymogen bearing an alanine substitution at the residue corresponding to the active site of matriptase (Ser805) was created as follows. A DNA fragment was amplified by PCR using KOD$_{-plus-}$® DNA polymerase, 5′-CCGGTGGCCCCTTGTC-3′ and 5′-CATCA CCCTGGCAGGAGTC-3′ as primers (the underlined nucleotide indicates the introduced mutation) (*26*, *27*) and pPICZ-His$_6$t-S-CD as the template. The amplified fragment was phosphorylated with T4 polynucleotide kinase and then self-ligated. The circular plasmid DNA was designated pPICZ-S805A-His$_6$t-S-CD. The nucleotide sequence was determined in both directions by the dideoxynucleotide chain-termination method, as described earlier (*12*).

Secreted expression in P. pastoris, purification and treatment with r-EK of the His$_6$t-S-CD and S805A-His$_6$t-S-CD pseudozymogens

We have established a system for expression and secretion of the His$_6$t-S-CD pseudozymogen in a strain of *P. pastoris*, KM71H (Invitrogen) (*25*). The S805A-His$_6$t-S-CD pseudozymogen was also expressed in and secreted by KM71H. Procedures for the introduction of plasmid DNA into the yeast and the selection of a clone with the highest expression were the same as reported previously for the His$_6$t-S-CD pseudozymogen (*25*). The yeast clone with the highest expression of the S805A-His$_6$t-S-CD pseudozymogen (and the His$_6$t-S-CD pseudozymogen) was grown in 300 ml of medium [100 mM KH_2PO_4–K_2HPO_4 (pH 8.0) buffer containing 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 400 μg/l biotin and 1% methanol] by shaking as described earlier (*25*). After 72 h of culture, cells were precipitated by centrifugation at 1500*g* for 15 min at 22°C. The resulting supernatants were filter sterilized, and the recombinant proteins of interest were precipitated by 60% ammonium sulphate saturation. The precipitants obtained by centrifugation at 10,000*g* for 20 min at 4°C were suspended in 2.5 ml of 25 mM HEPES (pH 7.5) buffer containing 145 mM NaCl and 0.1% Triton X-100 (HEPES buffer A) and the suspensions were subjected to gel filtration in HEPES buffer A using a PD-10 column (GE Healthcare, Tokyo, Japan) with an elution volume of 3.5 ml. Then, the recombinant proteins were purified by Ni^{2+}-chelating chromatography, followed by treatment with r-EK, as described earlier (*25–28*). Procedures for determination of concentration (by SDS–PAGE and silver staining) and of specific activity (using Sp-tPA as the substrate) were described in detail earlier (*25*). HEPES buffer A solutions containing the r-EK-treated His$_6$t-S-CD and S805A-His$_6$t-S-CD pseudozymogens were stored at −20°C until use.

Preparation of recombinant matriptase variants used for cultured cell experiments

His$_6$t-S-CD and S805A-His$_6$t-S-CD pseudozymogens treated with r-EK were repurified by Ni^{2+}-charged chromatography and subjected to gel filtration for use in cultured cell experiments, as follows: 500 μl of HEPES buffer A containing r-EK-treated His$_6$t-S-CD and S805A-His$_6$t-S-CD pseudozymogens was incubated with Ni^{2+}-chelating resins (100 μl) in a 1.5 ml microcentrifuge tube, as described earlier (*25*). Ni^{2+}-chelating resins, to which the proteins of interest bound, were washed five times with 1 ml of 25 mM HEPES (pH 7.5) buffer containing 145 mM NaCl, 5 mM imidazole and 0.1 mg/ml BSA. After washing, the proteins of interest were eluted with 500 μl of 25 mM HEPES (pH 7.5) buffer containing 145 mM NaCl, 500 mM imidazole and 100 μg/ml BSA. Eluates were subjected to gel filtration in serum-free DMEM containing 4 μg/ml insulin and 0.1 mg/ml BSA (hereinafter D-SFM) or serum-free MEM containing 0.1 mg/ml BSA (hereinafter M-SFM) using an NAP-5 column (GE Healthcare) with an elution volume of 1 ml. The gel filtrates were re-subjected to gel filtration in D-SFM or M-SFM using an NAP-10 column (GE Healthcare) with an elution volume of 1.5 ml. A 1 μl portion of gel filtrate containing the r-EK-treated His$_6$t-S-CD pseudozymogen was added to 39 μl of

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D-SFM (or M-SFM) or HEPES buffer A containing 125 μM of Boc-QAR-MCA. After incubation for 30 min at 37°C, the reactions were terminated by adding 400 μl of 0.1 M monochloroacetic acid in 0.1 M sodium acetate (pH 4.3) buffer, and the absorbance of the product, 4-methyl-coumaryl-7-amine, was measured at 370 nm. The remainder of the gel filtrate was diluted with D-SFM (or M-SFM), filter sterilized and stored at 4°C until use. Samples were used for cultured cell experiments within 10 days.

Preparation of and treatments of cells with protease inhibitors

GM6001, MMP-2/MMP-9 Inhibitor II, MMP-9 Inhibitor I and UK122 were individually dissolved in dimethylsulphoxide at a concentration of 10 mM and stored at −20°C until use. When used for cultured cell experiments, each of the inhibitors was included in the media at a concentration of 10 μM (unless otherwise noted).

Coating of multiwell plates with ECM gel or pure matrices

ECM gel (protein concentration, ~10 mg/ml) was diluted 1 : 100 with phosphate buffered saline (PBS, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 136 mM NaCl, 2.7 mM KCl, pH 7.4). Laminin, fibronectin and type-IV collagen were diluted with PBS: the final concentration of each ECM protein was 10 μg/ml. Wells of polystyrene multiwell plates (Asahi Techno Glass, Tokyo) were exposed to diluted ECM gel or pure matrices (for instance, 250 μl per well of a 24-well plate) and incubated for 1 h at 37°C. After incubation, each well was washed three times with PBS (1.5 ml for a well of a 24-well plate). Each well was then incubated for 30 min at 37°C with PBS containing 0.5 mg/ml BSA (250 μl per well of a 24-well plate) to block sites not bound by the ECM gel or matrices. After incubation, wells were washed three times with PBS and used for experiments.

Cell detachment assay

Passaged cultures of IEC-6 and MDCK cells were detached by incubation for 5 min at 37°C with 0.25% trypsin-1 mM EDTA solution, immediately washed four times with PBS and suspended in D-SFM or M-SFM, respectively. Aliquots of 2.5×10^5 cells were incubated for 4 h at 37°C to enable them to adhere to the wells of 24-multiwell plates coated with ECM gel or matrices. After removing non-adherent cells by washing the wells twice with PBS, cells were exposed to D-SFM (for IEC-6) or M-SFM (for MDCK) with or without test substances and incubated for an additional 36 h. After microscopic observation, cell detachment was assessed as follows. Conditioned media were collected into a 1.5 ml microcentrifuge tube. Detached cells included in the conditioned media were precipitated by centrifugation at 5,000*g* for 2 s at 22°C, washed twice with PBS and lysed with 50 μl of 0.5 N NaOH solution. Cells that remained attached were washed twice with PBS, and then lysed with 50 μl of 0.5 N NaOH solution. These lysates were centrifuged at 10,000*g* for 10 min at 4°C to remove insoluble debris. The protein contents of the resulting supernatants were determined using a BCA protein assay kit (Pierce Chemical, Rockford, IL, USA) with BSA as the standard. Cell detachment was expressed as the protein content of the detached cells as a percentage of the total protein content; *i.e.* the protein content of the detached cells plus that of the attached cells.

IEC-6 cell attachment to laminin-coated plates pre-treated with the recombinant catalytic domain of matriptase

Wells of a 24-well microplate were coated with laminin as described earlier. After washing with PBS, the wells were exposed to 250 μl of D-SFM or D-SFM containing 1 μM r-EK-treated His_6t-S-CD pseudozymogen and incubated for an additional 24 h. After removal of the medium, each well was washed four times with D-SFM. Then IEC-6 cells detached by the trypsin-EDTA solution and suspended in D-SFM were added to the wells. After 4 h of incubation, the protein contents of cells in the supernatants and the attached cells were determined. Cell attachment was expressed as the protein content of the attached cells as a percentage of the total protein content; *i.e.* the protein content of the cells in the supernatants plus that of the attached cells.

RT–PCR

IEC-6 cells were seeded into 12-well plates coated with laminin at a density of 2×10^5 cells/well. After attachment, the cells were washed with PBS, exposed to D-SFM alone or D-SFM containing 1 μM r-EK-treated His_6t-S-CD pseudozymogen and incubated for an additional 24 h. Using the RNeasy Mini Kit (Qiagen, Tokyo), total RNA was extracted from each well before (0 h) and after 3, 6, 12 and 24 h of incubation. Total RNA was also extracted from cells that attached to plates in the presence of and grew in DMEM containing serum (*i.e.* cells cultured under passage conditions). The RT reaction was performed for 50 min at 42°C with 2 μg of total RNA, 1.6 U/μl of RNase inhibitor and 8 U/μl of M-MLV RTase according to the manufacturer's protocol. Oligo $(dT)_{12–18}$ (Invitrogen) was used as a primer for this reaction. After incubation, samples were heated at 70°C for 15 min to terminate the reaction. The cDNA obtained from 0.5 μg of total RNA was used as the template for PCR amplification with KOD Dash® DNA polymerase. The PCR amplification consisted of 27 or 35 cycles, each consisting of 94°C for 30 s, 60°C for 5 s and 74°C for 30 s. PCR products were analysed by agarose gel electrophoresis (1.5% agarose) and ethidium bromide staining.

Annexin-V labelling

IEC-6 cells were seeded into 48-well plates coated with laminin at a density of 1×10^5 cells per well. After attachment, the cells were washed twice with PBS, exposed to D-SFM with or without test substances and incubated for an additional 48 h. Cells that were incubated with D-SFM alone or D-SFM containing 1 μM r-EK-treated S805A-His_6t-S-CD pseudozymogen were washed twice with PBS and harvested by scraping in PBS. Cells detached by incubation with SFM containing 1 μM of the r-EK-treated His_6t-S-CD pseudozymogen were harvested with the medium. These cells were precipitated by centrifugation at 5,000*g* for 2 s at 22°C and washed twice with PBS. Annexin-V/propidium iodide labelling of the cells was performed using the Annexin-V-FLUOS staining kit (Roche Diagnostics, Manheim, Germany) according to the manufacturer's instructions. Fluorescence images were acquired by confocal laser scanning microscopy (Fluoview FV300, Olympus, Tokyo).

DNA fragmentation assay

IEC-6 cells were seeded into 12-well plates coated with laminin at a density of 2×10^5 cells per well. After attachment, the cells were washed twice with PBS, exposed to D-SFM containing 1 μM r-EK-treated His_6t-S-CD pseudozymogen and incubated for an additional 48 h. Cells were also exposed to D-SFM containing 500 nM trypsin and incubated for an additional 12 h. Cells that remained attached during culture under passage conditions were collected by scraping in PBS. These cells and those that became detached during incubation with proteases were precipitated by centrifugation at 5,000*g* for 2 s at 22°C, washed twice with PBS, suspended in 100 μl of 10 mM Tris–HCl (pH 8.0) buffer containing 100 mM NaCl, 25 mM EDTA, 5 mg/ml SDS and 0.1 mg/ml proteinase K and incubated for 24 h at 50°C. RNaseA was added to the mixtures at a final concentration of 1 mg/ml, and the mixtures were incubated for 1 h at 37°C. After incubation, genomic DNA was extracted using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Extracted DNA was analysed by agarose gel electrophoresis (1.5% agarose) and ethidium bromide staining.

Caspase-3 activity assay

IEC-6 and MDCK cells were attached to 12-well plates coated with laminin, as described earlier. After attachment, these cells were washed twice with PBS, exposed to D-SFM (for IEC-6) or M-SFM (for MDCK) with or without test substances and incubated for an additional 24 h. Detached cells included in the conditioned media were precipitated by centrifugation at 5,000*g* for 2 s at 22°C. The precipitated cells were washed twice with PBS. The cells that remained attached were washed twice with PBS. Both the precipitated and attached cells were exposed to 100 μl of 50 mM HEPES (pH 7.4) buffer containing 0.2% CHAPS (HEPES buffer B) and incubated for 30 min on ice with occasional agitation. The extracts were pooled and centrifuged at 10,000*g* for 20 min at 4°C to remove insoluble material. The resulting supernatants (CHAPS extracts) were collected, and the protein concentration was determined as described earlier. Each CHAPS extract was diluted with HEPES buffer B to a final protein concentration of 0.15 mg/ml and placed on ice.

Ac-DEVD-MCA (a substrate for caspase-3) and Ac-DEVD-CHO (an inhibitor for caspase-3) had been individually dissolved in dimethylsulphoxide at a concentration of 10 mM and stored at −20°C until use. The reactions were initiated by adding 20 μl of

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diluted CHAPS extract to 80 μl of substrate mixture [25 mM PIPES (pH 7.3) buffer containing 2 mM EGTA, 2 mM $MgCl_2$, 5 mM dithiothreitol and 62.5 μM Ac-DEVD-MCA] and of the mixture containing 250 μM Ac-DEVD-CHO. After incubation for 30 min at 22°C, fluorescence of 4-methyl-coumaryl-7-amine liberated from Ac-DEVD-MCA was measured using a plate reader (PowerScan, Dainippon Sumitomo Pharma, Osaka) at an excitation wavelength of 380 nm and an emission wavelength range of 460 nm. Under these conditions, no fluorescence was produced in any sample in which Ac-DEVD-CHO was included.

Statistical analysis

Differences between means were determined using the Student's *t*-test and Kruskal–Wallis analysis of variance with Dunn's multiple comparison test using Instat statistical software (GraphPad, San Diego, CA, USA). A $P<0.05$ was considered to be statistically significant.

Results

Preparation of proteolytically active and inactive variants of the recombinant catalytic domain of matriptase for cultured cell experiments

We have produced a pseudozymogen form of recombinant rat matriptase consisting of the catalytic domain (Val615–Val855) and the N-terminal spacer region (Cys604–Arg614) in a *P. pastoris* expression system (His_6t-S-CD pseudozymogen) (*25*). In this pseudozymogen, the five-amino acid sequence before a valine residue corresponding to the N-terminus of the matriptase catalytic domain is Asp-Asp-Asp-Asp-Lys (Fig. 1A). This enables generation of an N-terminus at Val615 (*i.e.* activation cleavage) by treatment with r-EK *in vitro*. The His_6t-S-CD pseudozymogen was purified to homogeneity (>95%) by Ni^{2+}-chelating chromatography and activated by cleavage by r-EK (Fig. 1B). In HEPES buffer A, the r-EK-treated His_6t-S-CD pseudozymogen exhibited catalytic activity and substrate specificity similar to that of a recombinant tc-matriptase produced in mammalian cells (*25*). For treatment of IEC-6 and MDCK cells, r-EK-treated His_6t-S-CD pseudozymogen was subjected to gel filtration in serum-free culture media free of the non-ionic detergent Triton X-100 (D-SFM and M-SFM, respectively). The Boc-QAR-MCA-hydrolysing activity of His_6t-S-CD in D-SFM (or M-SFM) was similar to that in HEPES buffer A. A recombinant tc-matriptase has been found to cleave fibronectin and laminin but not type-IV collagen *in vitro* (*12*). r-EK-treated His_6t-S-CD pseudozymogen gave similar results (Supplementary Fig. S1).

In the present study, a site-directed variant of His_6t-S-CD in which a Ser residue corresponding to the active site of matriptase (Ser805) was changed to an Ala residue (S805A-His_6t-S-CD pseudozymogen) was prepared using the *P. pastoris* expression system. Like the His_6t-S-CD pseudozymogen, the purified S805A-His_6t-S-CD pseudozymogen migrated to positions corresponding to 32 and 30 kDa under reducing and non-reducing SDS–PAGE conditions, respectively (Fig. 1B). Like the r-EK-treated His_6t-S-CD pseudozymogen, when treated with r-EK the S805A-His_6t-S-CD pseudozymogen migrated to the 30 and 32 kDa positions under reducing and non-reducing SDS–PAGE conditions, respectively (Fig. 1B), indicating that the S805A-His_6t-S-CD pseudozymogen was cleaved by r-EK at the desired position. A minor band is visible in each lane (*e.g.* a 30-kDa band in lane 3). In His_6t-S-CD and the S805A-His_6t-S-CD variant, this band was shown to represent the non-glycosylated form. When treated with r-EK, the S805A-His_6t-S-CD pseudozymogen exhibited no detectable activity towards Sp-tPA or Boc-QAR-MCA in HEPES buffer A. Moreover, the r-EK-treated S805A-His_6t-S-CD did not cleave laminin, fibronectin or Type-IV collagen in HEPES buffer A (Supplementary Fig. S1). The r-EK-treated S805A-His_6t-S-CD was also subjected to gel filtration in D-SFM (or M-SFM) for cultured cell experiments.

Hereinafter, the r-EK-treated His_6t-S-CD and S805A-His_6t-S-CD pseudozymogens are referred to as His_6t-S-CD and S805A-His_6t-S-CD, respectively.

His_6t-S-CD-induced detachment of IEC-6 cells attached to ECM gel-coated plates

ECM gel is a commercially available mixture of basement membrane components and contains laminin, the major component, Type-IV collagen, heparan sulphate proteoglycan, entactin and other minor components. We first assessed whether His_6t-S-CD causes detachment of IEC-6 cells attached to ECM gel-coated plates. Cell number was evaluated according to the protein content of harvested cells.

Attached cells were incubated with D-SFM alone, D-SFM containing His_6t-S-CD or D-SFM containing S805A-His_6t-S-CD. After 3 h of incubation with D-SFM alone, ~10% of cells detached (Fig. 2A). However, the percentage of cell detachment remained unchanged for up to 36 h of incubation (Fig. 2A). This cell detachment may have been caused by mechanical trauma during exposure to D-SFM. Incubation with D-SFM containing His_6t-S-CD increased cell detachment in a time-dependent manner (Fig. 2A). After 36 h of incubation, >90% of cells had detached (Fig. 2A). After 36 h of incubation, the percentage cell detachment in wells incubated with D-SFM alone was similar to that in wells incubated with D-SFM containing S805A-His_6t-S-CD (Fig. 2A). These results indicate that His_6t-S-CD causes detachment of IEC-6 cells *via* a mechanism that requires catalytic activity. The concentration of His_6t-S-CD required for 50% cell detachment was ~300 nM (Fig. 2B). The morphological appearances of cells after 24 h of incubation with D-SFM alone (left) and with D-SFM containing 1 μM His_6t-S-CD (right) are shown in Fig. 2C.

Importance of laminin as a target for His_6t-S-CD in inducing IEC-6 cell detachment

In the small intestine, laminin is present in the basement membrane underlying SIECs and serves as an important anchorage site of epithelial cells (*29–32*). We hypothesized that His_6t-S-CD would cause detachment of IEC-6 cells attached to ECM gel-coated plates mainly through its ability to impair cell–laminin adhesion. The cells were efficiently attached to laminin-coated plates and >90% remained attached after 24 h of incubation with D-SFM alone (Fig. 3A). Similar results were obtained when cells were incubated with D-SFM containing 1 μM S805A-His_6t-S-CD (data not

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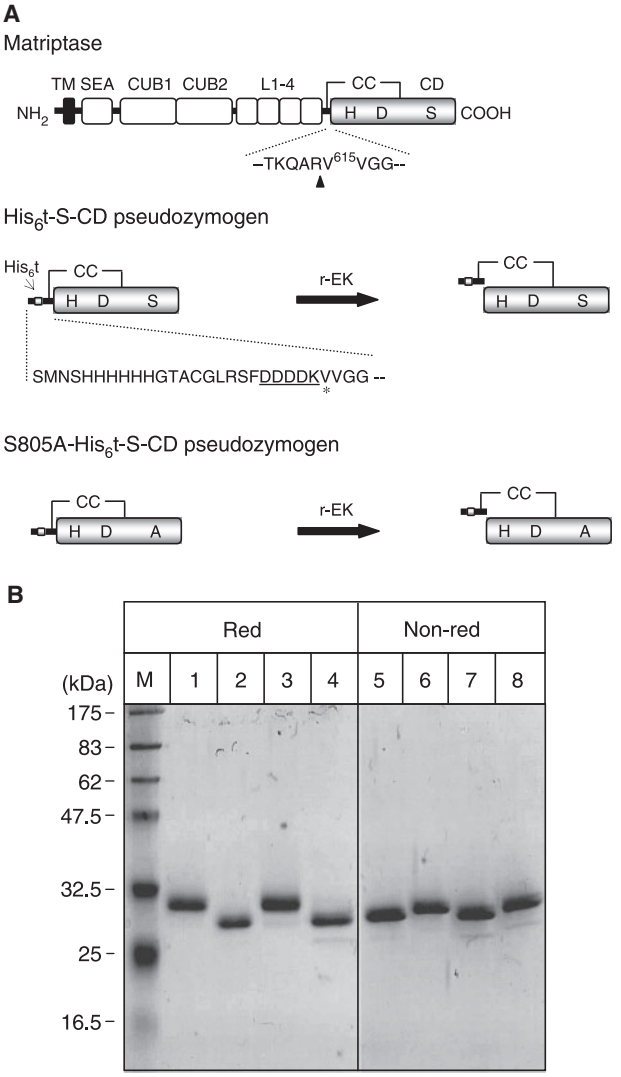

Fig. 1 Preparation of the recombinant catalytic domain of rat matriptase used in cultured cell experiments. (A) Schematic illustration of rat matriptase and expression constructs. The initial translation product of rat matriptase (Matriptase) is shown at the top. The amino acid numbering starts from the putative N-terminus. The amino acid sequence around the activation-cleavage site is indicated using the single-letter code with amino acid numbering at the N-terminal valine residue of the catalytic domain (Val615). The activation-cleavage site is indicated by the arrowhead. His_6t-S-CD pseudozymogen is converted to an activated form by incubation with r-EK. For the His_6t-S-CD pseudozymogen, the predicted N-terminal sequence of secretion product, including the enterokinase recognition sequence (DDDDK, underlined), is shown in single-letter code. A valine residue corresponding to matriptase Val615 is marked with an asterisk. The catalytic triad, His656, Asp711 and Ser805 are indicated as H, D and S, respectively, in the catalytic domain of the full-length matriptase, the His_6t-S-CD pseudozymogen and the r-EK-treated His_6t-S-CD pseudozymogen. The S805A-His_6t-S-CD pseudozymogen is a site-directed mutant of the His_6t-S-CD pseudozymogen in which a serine residue corresponding to matriptase S805 is replaced with an alanine residue. S805A-His_6t-S-CD pseudozymogen is also converted to a form that may have an N-terminal valine residue (corresponding to matriptase Val615) by incubation with r-EK. Note that the position corresponding to matriptase S805 is indicated in single-letter code for alanine in the S805A-His_6t-S-CD pseudozymogen and the r-EK-treated S805A-His_6t-S-CD. The predicted disulphide linkages between two cysteine residues corresponding to Cys604 and Cys731 are shown as C–C. TM, transmembrane domain; SEA, sea-urchin sperm protein–enterokinase–agrin domain; CUB1 and CUB2, the first and second complement factor C1r/C1s–urchin embryonic growth factor–bone morphogenetic protein domains, respectively; L1-4, four repeats of low-density lipoprotein receptor class A domain; CD, catalytic domain. (B) Analysis of recombinant variants of the catalytic domain of matriptase by SDS–PAGE and Coomassie staining. Samples were subjected SDS–PAGE (12% gels) under reducing (Red) (lanes 1–4) and non-reducing (Non-red) (lanes 5–8) conditions and Coomassie staining: lanes 1 and 5, His_6t-S-CD pseudozymogen; lanes 2 and 6, r-EK-treated His_6t-S-CD pseudozymogen; lanes 3 and 7; S805A-His_6t-S-CD pseudozymogen; lanes 4 and 8; r-EK-treated S805A-His_6t-S-CD pseudozymogen. A molecular-mass standard was also separated by SDS–PAGE (lane M). The sizes are indicated on the left in kilo dalton.

shown). When incubated with D-SFM containing 1 μM His_6t-S-CD, ~27% of cells detached after 12 h ($P < 0.05$ versus cells incubated for 12 h with D-SFM alone) and ~38% of cells detached after 24 h (Fig. 3A). Note that confluent IEC-6 cells (attached to laminin-coated plates) were rarely detached by D-SFM containing 1 μM His_6t-S-CD for up to 24 h of incubation. This suggests that the components of tight junction are resistant to the recombinant matriptase.

For comparison, we determined whether His_6t-S-CD causes detachment of IEC-6 cells attached to plates coated with other components of the basement membrane (fibronectin and Type-IV collagen). Fibronectin is present in the basement membrane underlying crypts and lower parts of villi (*i.e.* the proliferation zone) and is likely to play a role in mediating proliferation of SIECs (*1*, *29*, *30*). Type-IV collagen is uniformly present in the small-intestinal epithelial basement membrane and may serve as an anchorage (*29*, *32*). Cells were attached to fibronectin- or Type-IV collagen-coated plates and then incubated for 24 h with D-SFM alone or D-SFM containing His_6t-S-CD. With both matrices, the percentages of cell detachment were similar between wells incubated with D-SFM alone and those incubated with D-SFM containing His_6t-S-CD (Fig. 3A). His_6t-S-CD does not appear to gain access to or cleave fibronectin molecules attached to plates or the molecules that are already associated with their specific integrins.

We next determined whether His_6t-S-CD causes detachment of IEC-6 cells attached to laminin-coated plates through a mechanism involving a direct action on the ECM component. Laminin-coated plates were incubated for 24 h with D-SFM alone or D-SFM containing His_6t-S-CD and IEC-6 cells were then placed on the plates. The percentage of attached cells was significantly lower in wells incubated with D-SFM containing His_6t-S-CD than with D-SFM alone (Fig. 3B). In the laminin-coated wells pre-treated with His_6t-S-CD, many round cells likely to be poorly attached were evident (Fig. 3B).

The results shown in Fig. 3 suggest that His_6t-S-CD causes detachment of IEC-6 cells *via* a mechanism involving direct modification and/or proteolysis of laminin. The subsequent experiments with IEC-6 cells were performed using cells attached to laminin-coated plates.

Effects of protease inhibitors on His_6t-S-CD-induced detachment of IEC-6 cells

We examined whether His_6t-S-CD-induced detachment of IEC-6 cells occurs *via* a mechanism requiring

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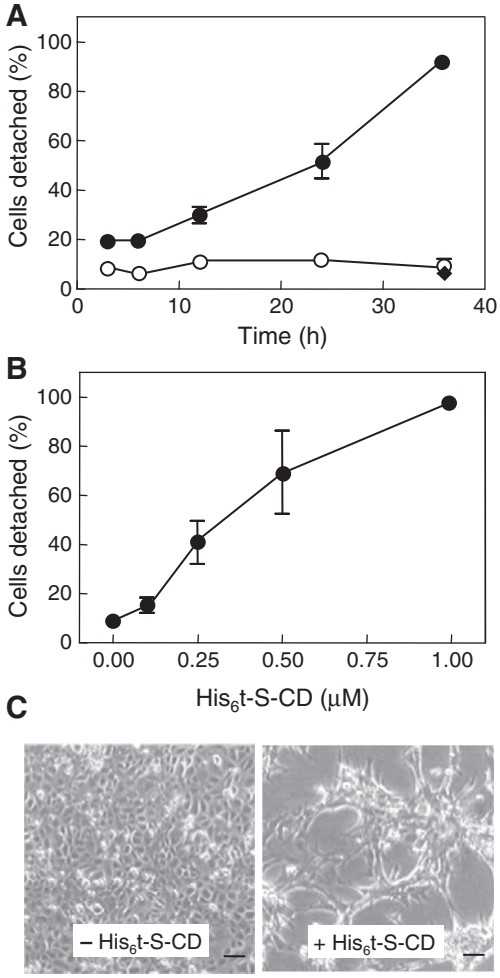

Fig. 2 His_6t-S-CD-induced detachment of IEC-6 cells attached to ECM gel-coated plates. (A) The time courses of detachment of IEC-6 cells incubated with D-SFM alone (open circles), SFM containing 1 μM His_6t-S-CD (solid circles) or 1 μM S805A-His_6t-S-CD (solid diamond). Detached cell numbers were assessed at the time points indicated. (B) Concentration dependency of His_6t-S-CD-induced detachment of IEC-6 cells. Numbers of detached cells were assessed after 36 h of incubation with D-SFM alone or D-SFM containing His_6t-S-CD. The concentrations of His_6t-S-CD are indicated. In (A) and (B), data are expressed as the mean ± SD from at least three independent wells. (C) Morphology of IEC-6 cells after incubation for 24 h with D-SFM alone (−His_6t-S-CD) or D-SFM containing 1 μM His_6t-S-CD (+His_6t-S-CD). Bar = 100 μm.

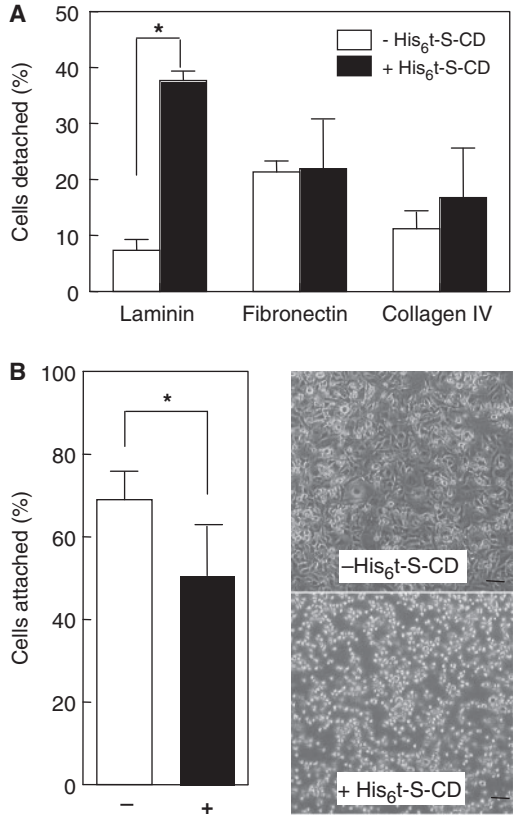

Fig. 3 Identification of laminin as a potential target for His_6t-S-CD in causing detachment of IEC-6 cells. (A) The effect of His_6t-S-CD on the detachment of IEC-6 cells attached to plates coated with laminin, fibronectin or Type-IV collagen (Collagen IV). After attachment, the cells were incubated for 24 h with D-SFM alone or D-SFM containing 1 μM His_6t-S-CD. Cell detachment was assessed, as described in 'Materials and Methods' section. (B) Attachment of IEC-6 cells to laminin-coated plates pre-treated with His_6t-S-CD. Wells of a laminin-coated plate were incubated for 24 h with D-SFM alone (−) or D-SFM containing 1 μM His_6t-S-CD (+). IEC-6 cells were placed on both plates and incubated for 4 h. Cell attachment was assessed, as described in 'Materials and Methods' section. In (A) and (B) (left graph), data are expressed as the mean ± SD from at least three wells. $*P < 0.05$ (Student's *t*-test). Morphological appearances of IEC-6 cells after 4 h of incubation with D-SFM alone (−His_6t-S-CD) and D-SFM containing His_6t-S-CD (+His_6t-S-CD) are also shown in (B). Bar = 100 μm.

the uPA/plasmin/MMPs system. His_6t-S-CD-induced IEC-6 cell detachment was assessed in the presence of the following synthetic protease inhibitors: (i) UK122 [a uPA inhibitor (IC_{50} = 200 nM)]; (ii) GM6001 (a broad-spectrum zinc metalloprotease inhibitor); (iii) MMP-2/MMP-9 Inhibitor II [a potent inhibitor for MMP-2 (IC_{50} = 17 nM) and MMP-9 (IC_{50} = 30 nM)]; and (iv) MMP-9 Inhibitor I [a potent and selective inhibitor for MMP-9 (IC_{50} = 5 nM)]. None of the four inhibitors (at concentrations up to 10 μM) inhibited the hydrolysis of Sp-tPA catalysed by 10 nM His_6t-S-CD.

UK122 had no effect on His_6t-S-CD-induced detachment of cells (Fig. 4). GM6001 weakly but significantly inhibited it (Fig. 4) and MMP-2/MMP-9 Inhibitor II inhibited it to a similar extent as GM6001 (Fig. 4). MMP-9 Inhibitor I had no effect on it (Fig. 4). Similar results were also observed when each of the inhibitors was included at a concentration of 0.1 or 1 μM (data not shown). Both GM6001 and MMP-2/MMP-9 Inhibitor II exhibited no effects on the detachment of cells incubated for 24 h with D-SFM alone or D-SFM containing 1 μM S805A-His_6t-S-CD (data not shown).

RT–PCR analysis of mRNAs from IEC-6 cells to identify protease zymogens potentially involved in His_6t-S-CD-induced detachment

We used RT–PCR to detect expression of mRNAs in IEC-6 cells for protease zymogens activated by tc-matriptase or recombinant variants of the catalytic domain of matriptase (matriptase zymogen, pro-MMP-3, pro-prostasin and pro-uPA) (*6*, *12*, *18–20*, *34*, *35*) and protease zymogens potentially involved in

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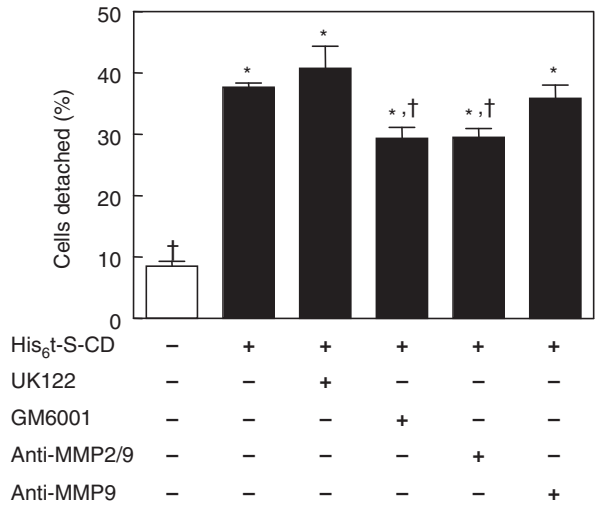

Fig. 4 Effects of protease inhibitors on His_6t-S-CD-induced detachment of IEC-6 cells. After attachment to laminin-coated plates, the cells were incubated for 24 h with D-SFM alone or D-SFM containing 1 μM His_6t-S-CD with or without UK122, GM6001, MMP-2/MMP-9 Inhibitor II (anti-MMP2/9) or MMP-9 Inhibitor I (anti-MMP9). After incubation, cell detachment was assessed. The '−' and '+' symbols indicate the absence and the presence of the indicated substances, respectively. Data are expressed as the mean ± SE from at least three independent experiments performed in triplicate wells. $^*P < 0.05$ versus IEC-6 cells treated with D-SFM alone; $^\dagger P < 0.05$ versus IEC-6 cells treated with D-SFM containing His_6t-S-CD (Kruskal–Wallis analysis of variance with Dunn's multiple comparison test).

cell detachment (pro-MMP-2, pro-MMP-9 and plasminogen) (*21*). The expression of mRNA for β-actin was also evaluated as an internal control for RNA degradation in cells. Total RNA was extracted before (0 h) and after 3, 6, 12 or 24 h of incubation with D-SFM or D-SFM containing 1 μM His_6t-S-CD. Total RNA was also extracted from cells cultured under passage conditions. The target cDNAs of interest were amplified by PCR using the specific primer sets shown in Supplementary Table S1.

Using a 27-cycle PCR, no products for protease zymogens were detected in samples from any of the culture conditions examined (data not shown). Using a 35-cycle PCR, the products for matriptase zymogen and pro-MMP-2 were detected, whereas those for pro-MMP-3, pro-MMP-9, pro-prostasin, pro-uPA and plasminogen were not (Fig. 5). It is noteworthy that expression of mRNAs for matriptase zymogen and pro-MMP-2 was induced by attachment of cells onto the laminin-coated plates. The PCR products for matriptase zymogen and pro-MMP-2 decreased during the later stage of incubation with D-SFM alone. These decreases may be associated with decreased viability of cells possibly in relation to serum deprivation. This is supported by the observation that the level of PCR product for β-actin decreased slightly during incubation with D-SFM alone. Moreover, the PCR products for matriptase zymogen and pro-MMP-2 became undetectable during the later stage of incubation with D-SFM containing His_6t-S-CD. These losses may be due to decreased viability of cells in relation to serum deprivation and exposure to His_6t-S-CD (Fig. 5).

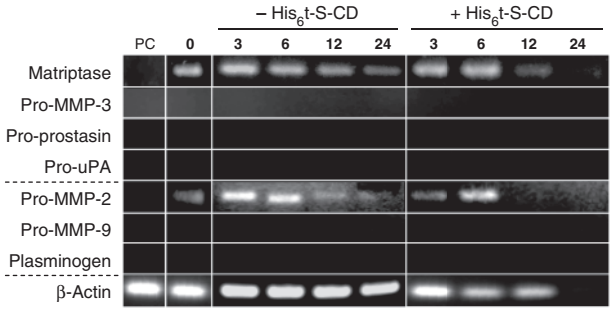

Fig. 5 RT–PCR detection of mRNAs in IEC-6 cells. After attachment to laminin-coated plates, the cells were incubated with D-SFM alone (−His_6t-S-CD) or D-SFM containing 1 μM His_6t-S-CD (+His_6t-S-CD). Total RNAs were extracted before (0 h) and after 3, 6, 12 and 24 h of incubation. Total RNAs were also extracted from cells cultured under passage conditions (PC). RT–PCR products were analysed by agarose gel electrophoresis (1.5% agarose) and ethidium bromide staining.

His_6t-S-CD-induced apoptotic death of IEC-6 cells

Detachment of SIECs from the basement membrane is thought to trigger an apoptotic death program (*2–4*). We assessed whether His_6t-S-CD causes apoptotic death of IEC-6 cells.

We used annexin-V labelling to evaluate the apoptosis of IEC-6 cells detached by incubation with His_6t-S-CD. Almost all IEC-6 cells detached after 48 h of incubation with D-SFM containing 1 μM His_6t-S-CD. The detached cells were collected, suspended in a solution containing fluorescein-labelled annexin-V (for detection of phosphatidylserine in apoptotic and necrotic cells) and propidium iodide (for detection of DNA of leaky necrotic cells) and analysed by confocal laser scanning microscopy. Some but not all cells produced green fluorescence derived from fluorescein-labelled annexin-V (Fig. 6A, right panel and Supplementary Fig. S2, left panel). Detached cells (even cells that produced green fluorescence) did not produce the red fluorescence derived from propidium iodide (Supplementary Fig. S2, right panel). This indicates that His_6t-S-CD causes IEC-6 cell apoptosis, but not necrosis. Note that cells that were associated with other cells (*i.e.* cell aggregates) hardly produced green fluorescence (Fig. 6A, right panel and supplementary Fig. S2, left panel). IEC-6 cells that remained attached to the plates after incubation for 24 h with D-SFM alone were collected by scraping and incubated with fluorescein-labelled annexin-V and propidium iodide. These cells produced neither green (Fig. 6A, left panel) nor red fluorescence (data not shown).

Apoptotic DNA fragmentation (*i.e.* DNA laddering at 200 bp intervals) was detected by agarose gel electrophoresis and ethidium bromide staining of extracts from IEC-6 cells detached by incubation for 48 h with D-SFM containing 1 μM His_6t-S-CD (Fig. 6B, lane 1). High-molecular-weight DNAs that did not undergo fragmentation were also visible (Fig. 6B, lane 1). A similar result was obtained for extracts of cells detached by incubation for 12 h with 500 nM trypsin (Fig. 6B, lane 2). No DNA laddering was detected in extracts of cells cultured under passage conditions (Fig. 6B, lane 3).

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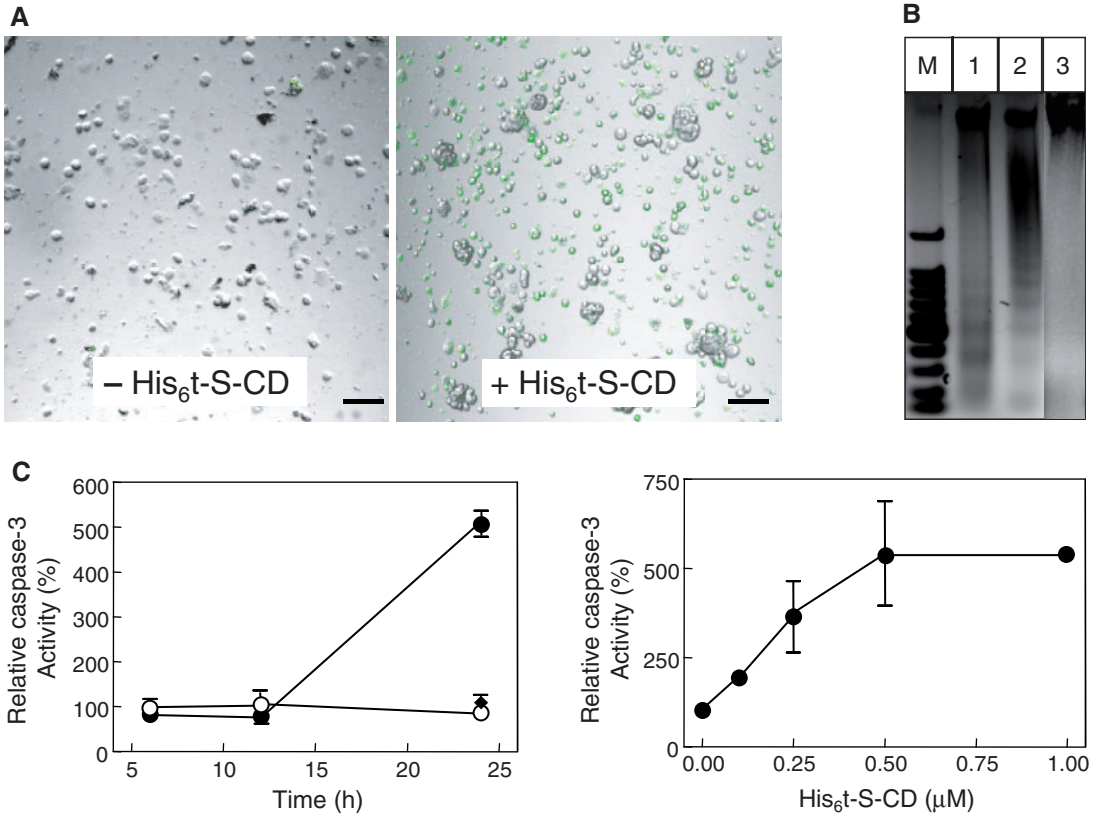

Fig. 6 Apoptotic death of IEC-6 cells by incubation with His_6t-S-CD. (A) Annexin-V labelling. Cells that remained attached to the plates after 24 h of incubation with D-SFM alone (−His_6t-S-CD) and cells that detached from laminin-coated plates within 48 h of incubation with 1 μM His_6t-S-CD (+His_6t-S-CD) were collected and incubated with an annexin-V/propidium iodide solution. After incubation, the cells were analysed by confocal laser scanning microscopy. Bar = 100 μm. (B) DNA fragmentation assay. IEC-6 cells were attached to laminin-coated plates. Genomic DNAs were extracted from cells that detached from laminin-coated plates within 48 h of incubation with D-SFM containing 1 μM His_6t-S-CD (lane 1), cells that detached within 12 h of incubation with D-SFM containing 500 nM trypsin (lane 2) and cells cultured under passage conditions (lane 3). DNA samples were analysed by agarose gel electrophoresis and ethidium bromide staining. A bright-field image is shown. Lane M shows the positions of molecular-weight markers (100 bp DNA ladder, New England Biolabs, Beverly, MA, USA). (C) Caspase-3 activity assay. IEC-6 cells attached to laminin-coated plates were used for experiments. The left panel shows time-dependent changes in caspase-3 activity in IEC-6 cells incubated with D-SFM alone (open circles), D-SFM containing 1 μM His_6t-S-CD (solid circles) or D-SFM containing 1 μM S805A-His_6t-S-CD (solid diamond). Caspase-3 activity was assessed at the time points indicated. Caspase-3 activity in cells incubated for 6 h with D-SFM alone was assigned a value of 100%. Right panel shows concentration dependency of the His_6t-S-CD-induced increase in caspase-3 activity. Caspase-3 activity was assessed after 24 h of incubation. The concentrations of His_6t-S-CD are indicated. Caspase-3 activity in cells incubated with D-SFM alone was assigned a value of 100%. Data are expressed as the mean ± SD from at least three independent wells.

To further validate that His_6t-S-CD induces apoptotic death of IEC-6 cells, we measured the activity of caspase-3 (an apoptotic effector caspase) in cells. The level of caspase-3 activity remained unchanged from 6 to 24 h of incubation with D-SFM alone (Fig. 6C, left panel). After 24 h of incubation, the levels of caspase-3 activity were similar between wells incubated with D-SFM alone and those incubated with D-SFM containing 1 μM S805A-His_6t-S-CD (Fig. 6C, left panel). After 6 and 12 h of incubation, the levels of caspase-3 activity were similar between wells incubated with D-SFM alone and those incubated with D-SFM containing His_6t-S-CD (Fig. 6C, left panel). After 24 h of incubation with D-SFM containing His_6t-S-CD, the level of caspase-3 activity increased drastically (Fig. 6C, left panel). At 24 h of incubation, the EC_{50} value for increasing caspase-3 activity was about 300 nM (Fig. 6C, right panel). None of the four protease inhibitors (UK122, GM6001, MMP-2/MMP-9 Inhibitor II and MMP-9 Inhibitor I) significantly reduced the increase in caspase-3 activity caused by incubation of IEC-6 cells for 24 h with 1 μM His_6t-S-CD (results not presented), indicating that the uPA/plasmin/MMPs system does not participate in the recombinant matriptase-induced apoptosis of the cells.

His_6t-S-CD-induced detachment and apoptosis of MDCK cells

We assessed whether the effects of His_6t-S-CD are specific to IEC-6 cells by also examining whether the recombinant matriptase causes detachment and apoptosis of MDCK cells. MDCK is a non-transformed epithelial cell line that originated from distal tubular cells and has been widely used as a simple epithelial cell model (*36*, *37*).

MDCK cells were attached to laminin-coated plates. The cells were tightly attached to the plates and >95% remained attached after 24 h of incubation with

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M-SFM alone (Fig. 7A). After 24 h of incubation with M-SFM containing 1 μM His_6t-S-CD, ~20% of cells detached (Fig. 7A). After 24 h of incubation, the level of caspase-3 activity was significantly higher in cells incubated with M-SFM containing 1 μM His_6t-S-CD than in those incubated with M-SFM alone (Fig. 7B). The S805A-His_6t-S-CD variant caused neither detachment nor increase in caspase-3 activity when at concentrations of 0.02–1 μM. These findings suggest that His_6t-S-CD causes apoptosis of MDCK cells.

His_6t-S-CD also caused morphological changes in the cells (Fig. 7C, right panel). Some cells became abnormally elongated and cell aggregates twisted around bands, especially at the junctions of the bands. Similar morphological patterns emerged even when cells were incubated with lower concentrations of His_6t-S-CD (<50 nM). The morphological appearance of MDCK cells incubated with M-SFM alone is shown in Fig. 7C (left panel). The S805A-His_6t-S-CD variant did not cause morphological changes of the cells when at concentrations of 0.02–1 μM (data not shown).

Detachment (Supplementary Fig. S3) and morphological changes (results not presented) of MDCK cells induced by 1 μM His_6t-S-CD were strongly inhibited by the presence of GM6001 and MMP-2/MMP-9 Inhibitor II. On the other hand, UK122 and MMP-9 Inhibitor I had no apparent effects on the His_6t-S-CD-induced detachment (Supplementary Fig. S3) and morphological changes (results not presented). This suggests that the effects of His_6t-S-CD highly depend on MMP-2 in the MDCK cell model.

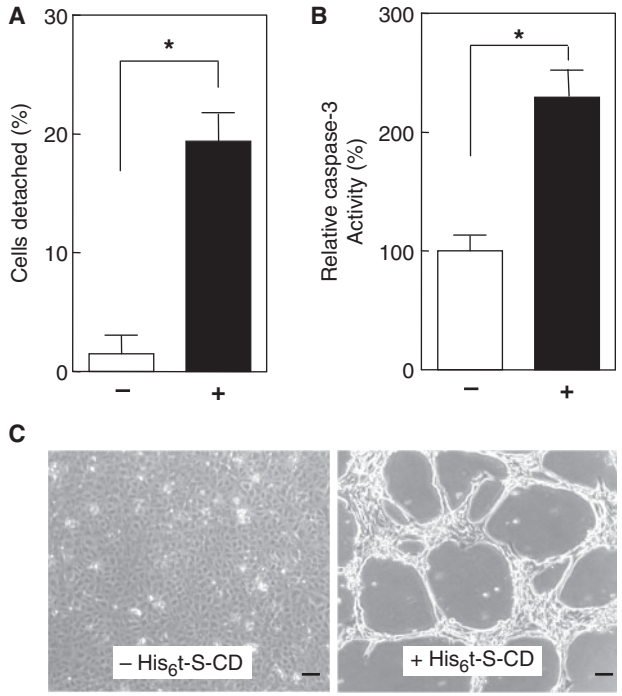

Fig. 7 Effects of His_6t-S-CD on the detachment, apoptosis and morphological appearance of MDCK cells. (A) Cell detachment assay. (B) Caspase-3 activity assay. MDCK cells were attached to laminin-coated plates. After attachment, the cells were incubated for 24 h with M-SFM alone (−) or M-SFM containing 1 μM His_6t-S-CD (+). Cell detachment and caspase-3 activity were assessed, as described in 'Materials and Methods' section. Data are expressed as the mean ± SD from three independent wells. $*P < 0.05$ versus MDCK cells treated with M-SFM alone (Student's *t*-test). (C) Light microscopic observation. After 24 h of incubation with M-SFM alone (−His_6t-S-CD) or M-SFM containing 1 μM His_6t-S-CD (+His_6t-S-CD), cells were photographed. Bar = 100 μm.

Discussion

In the present study, we found that His_6t-S-CD caused detachment of IEC-6 cells when they were attached to laminin-coated plates but not when they were attached to fibronectin- or Type-IV collagen-coated plates. The behaviour towards IEC-6 cell detachment of His_6t-S-CD differs from those of certain other serine proteases with trypsin-like specificity of cleavage. For example, in contrast to His_6t-S-CD, a recombinant form of granzyme A, a serine protease abundantly expressed by intra-epithelial lymphocytes scattered through the epithelial layer in the small intestine, caused detachment of the cells attached to fibronectin- or Type-IV-coated plates but not those attached to laminin-coated plates (*38*, *39*). Laminin is particularly important for attachment of simple epithelial cells, including SIECs of upper villi (*29–32*). The substrate specificity of His_6t-S-CD supports our hypothesis that matriptase contributes to the shedding of SIECs.

We obtained evidence that His_6t-S-CD acts directly on laminin (Fig. 3B). However, direct modification or proteolysis of laminin appears to be insufficient to totally account for His_6t-S-CD-induced detachment. We found that MMP-2/MMP-9 Inhibitor II significantly inhibited the His_6t-S-CD-induced detachment of IEC-6 cells, whereas MMP-9 Inhibitor I did not and that the mRNA for pro-MMP-2 was detected in the cells, whereas that for pro-MMP-9 was not. Together with evidence that MMP-2 but not MMP-9 directly cleaves laminin (*21*), these findings suggest that MMP-2 plays a part in the His_6t-S-CD-induced IEC-6 cell detachment. Zinc metalloproteases other than MMP-2 do not appear to have substantial effects on the His_6t-S-CD-induced IEC-6 cell detachment. This idea is supported by the observation that MMP-2/MMP-9 Inhibitor II inhibited the detachment to a similar extent as GM6001. The direct actions of His_6t-S-CD and MMP-2 on laminin may substantially account for the detachment.

It appeared that pro-MMP-2 expressed by IEC-6 cells was activated following exposure to D-SFM containing His_6t-S-CD. We found that His_6t-S-CD-induced detachment was unaffected by the presence of UK122 and that the mRNAs for pro-uPA and plasminogen were undetectable. It is therefore unlikely that the uPA/plasmin system is responsible for the activation of pro-MMP-2 in our experimental model. We hypothesize that His_6t-S-CD directly activates pro-MMP-2 or activates unknown proteases that activate pro-MMP-2. Also, we found that the expression of mRNA for matriptase zymogen as well as that for pro-MMP-2 was induced by attachment of cells onto the laminin-coated plates. However, endogenously expressed matriptase zymogen and pro-MMP-2 may be insufficient to induce detachment of cells. It seems that they participated in detachment after activation through a mechanism involving His_6t-S-CD.

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His_6t-S-CD caused apoptotic death of IEC-6 cells attached to laminin-coated plates. The recombinant matriptase also caused a statistically significant increase in caspase-3 activity in the cells attached to ECM gel-coated plates (Mochida, S., Tsuzuki, S., Inouye, K., and Fushiki, T., unpublished data). However, it remains uncertain whether the apoptotic death is secondary to detachment. We have found that (i) after incubation for 24 h with D-SFM containing 1 μM His_6t-S-CD, the activity of caspase-3 was produced largely by the detached cells but poorly by the cells that remained attached to the plates and that (ii) the levels of caspase-3 activity were similar between the cells that remained attached to the plates even after incubation with His_6t-S-CD and those that remained attached to the plates after incubation with D-SFM alone (Mochida, S., Tsuzuki, S., Inouye, K., and Fushiki, T., unpublished data). These results suggest that detachment is a prerequisite for apoptotic death in the IEC-6 cell model. It is however unlikely that detachment is sufficient for the apoptotic progression of this cell line. This is supported by observations that (i) after 12 h of incubation with His_6t-S-CD, cell detachment was evident, whereas no significant increase in caspase-3 activity was observed and (ii) cells detached by incubation with His_6t-S-CD did not uniformly exhibit characteristics of apoptosis. Of the detached cells, those that remained associated with other cells exhibited little labelling with annexin-V (Fig. 6A). We previously reported that a recombinant granzyme A-treated IEC-6 cells detached as aggregates with no changes characteristic of apoptosis (*39*). Taken together, we suggest that loss of cell–cell adhesion is an important prerequisite for IEC-6 cell apoptosis. It will be a challenge to identify molecules responsible for the loss of cell-cell adhesion.

Detachment and increase in caspase-3 activity by His_6t-S-CD were also found in the MDCK cell model, suggesting that the epithelial cell-shedding function of matriptase may not be restricted to the small intestine. However, the degrees of detachment and increase in caspase-3 activity with MDCK cells were lower than those with IEC-6 cells. Unexpectedly, His_6t-S-CD caused dramatic morphological changes in MDCK cells. Judging from their morphological appearance, His_6t-S-CD-treated cells may undergo branching morphogenesis or tubulogenesis (*36*, *37*). In addition, the morphological appearance of cells was found for up to at least 36 h of incubation with 1 μM His_6t-S-CD. We speculate that during the process of His_6t-S-CD-induced morphogenesis, some of the MDCK cells inadvertently detached and died. In other words, His_6t-S-CD is thought to act primarily as a morphogen in the MDCK cell model. To date, the function of matriptase has been postulated to be organ specific. For instance, matriptase expressed by gastric chief cells is thought to serve as an intracellular pro-protein convertase (*40*). Studies using matriptase-deficient mice revealed that this enzyme plays essential roles in the terminal differentiation of keratinocytes and in the survival of thymocytes (*41*, *42*). When using IEC-6 cells, higher concentrations of His_6t-S-CD (*e.g.* 100 nM) were required to apparently cause detachment and apoptosis (Figs 2B and 6C). Taken together, we suggest that the epithelial cell-removing function of matriptase, if any, is restricted to tissue sites with high levels of this protease, such as the villus tip in the small intestine. Buzza *et al.* (*43*) have recently provided evidence that matriptase colocalizes with *E*-cadherin to apical junctional complexes in differentiated polarized Caco-2 (an enterocyte-like cell line) and plays a pivotal role in the formation and integrity of the intestinal epithelial barrier. We suggest that matriptase contributes to the maintenance of intestinal epithelial integrity when expressed at relatively low levels, while it exhibits an enhanced ectodomain shedding, gains access to the basement membrane and contributes to the cell shedding when expressed at high levels.

Bugge and co-workers showed using mice in which the matriptase gene had been temporarily disrupted by the Cre-lox P recombination system that no histological defects occurred in the small-intestinal epithelium, even when severe morphological defects in other epithelia (*e.g.* large-intestinal epithelium) were evident (*44*). One explanation for this is that certain proteases compensate for the shedding of aged epithelial cells in the small intestine of mice. For instance, pancreatic trypsin (Fig. 6B) and granzyme A (*38*, *39*), which are abundant in the vicinity of the villus tip, could participate in the shedding of SIECs. Nonetheless, our results on matriptase, together with its abundant expression at the villus tip, indicate that it makes a contribution to the shedding of aged SIECs.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We sincerely acknowledge helpful advices from Drs Shigenobu Matsumura and Ai Eguchi (Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University) in use of confocal laser scanning microscope and in RT–PCR procedures, respectively.

Funding

Grants-in-Aid for Scientific Research (18580118 and 21580138 to S. Tsuzuki) from the Japan Society for the Promotion of Science.

Conflict of interest

None declared.

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