Activation of Hepatocyte Growth Factor and Urokinase/Plasminogen Activator by Matriptase, an Epithelial Membrane Serine Protease*

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Matriptase is an epithelial-derived, integral membrane serine protease. The enzyme was initially isolated from human breast cancer cells and has been implicated in breast cancer invasion and metastasis. In the current study, using active matriptase isolated from human milk, we demonstrate that matriptase is able to cleave various synthetic substrates with arginine or lysine as their P1 sites and prefer small side chain amino acids, such as Ala and Gly, at P2 sites. For the most reactive substrates, N-t-butylcarboxyamido (N-t-Boc)-γ-phenyl-Glu-Ala-Arg-7-amino-4-methylcoumarin (AMC) and N-t-Boc-Gln-Ala-Arg-AMC, the $K_m$ values were determined to be 3.81 and 4.89 μM, respectively. We further demonstrated that matriptase can convert hepatocyte growth factor/scattering factor to its active form, which can induce scatter of Madin-Darby canine kidney epithelial cells and can activate c-Met tyrosine phosphorylation in A549 human lung carcinoma cells. In addition, we noted that matriptase can activate urokinase plasminogen activator but has no effect on plasminogen. These results suggest that matriptase could act as an epithelial, upstream membrane activator to recruit and activate stromal-derived downstream effectors important for extracellular matrix degradation and epithelial migration, two major events of tissue remodeling, cancer invasion, and metastasis.

Tissue remodeling is observed both in physiological and pathologic processes. These include organ development, morphogenesis, wound healing, cancer invasion, and metastasis. Degradation of extracellular matrix (ECM) and cellular migration are two prominent steps in tissue remodeling. Considering that the majority of the ECM-degrading proteases, such as the plasmin/urokinase type plasminogen activator system (1), and the major motility factor, hepatocyte growth factor (HGF)/scattering factor (SF) (2) are mainly produced by the stromal components in vivo, tissue remodeling is likely to be an event that depends entirely upon stromal-epithelial collaboration (3). A search for epithelial-derived proteases, which may interact both with stromal-derived ECM-degrading protease systems and with motility factors, could provide a missing link in our understanding of tissue remodeling and cancer invasion and metastasis.

To investigate the epithelial role in tissue remodeling and in many aspects of tumor behavior, including growth and metastasis, we have carried out our studies on an epithelial-derived, integral membrane, trypsin-like, serine protease (matriptase) and its cognate, Kunitz-type serine protease inhibitor (hepatocyte growth factor activator inhibitor-1, HAI-1) (4–7). Matriptase is a type 2, integral membrane, trypsin-like serine protease with two putative regulatory modules: two tandem repeats of a CUB (C1r/s, Uegf, and Bone morphogenetic protein-1) domain and four tandem repeats of a low density lipoprotein (LDL) receptor domain (also see updated sequence in the GenBank®/EBI Data Bank with accession number AF118224). Matriptase was independently cloned by others, and termed membrane-type serine protease 1 (MT-SP1) (8). The mouse homologue of matriptase was also cloned and termed epithin (9). The cognate inhibitor of matriptase is a type 1 integral membrane protein, containing two Kunitz domains, separated by an LDL receptor domain (7). The inhibitor was independently characterized by others as an inhibitor (HAI-1) of hepatocyte growth factor activator, an enzyme identified in serum (10).

Considering that matriptase exhibits trypsin-like activity and presents on the surfaces of epithelial cells, and that activation of the uPA system and HGF/SF requires cleavage at Arg or Lys, we hypothesize that matriptase could act as an upstream, epithelial membrane activator of the downstream, stromal-derived effectors of tissue remodeling. In the current study, we set out to investigate the potential collaboration between epithelial and stromal cells by examining if matriptase is able to activate HGF/SF and the protease components of the uPA system. Using the 70-kDa active matriptase, purified from human milk, we are able to demonstrate that matriptase can activate pro-uPA and pro-HGF, but not plasminogen. These results reveal that a novel mechanism involving both an upstream epithelial membrane activator as well as downstream stromal effectors may play an important role in tissue remodeling.

MATERIALS AND METHODS

Antibodies—Polyclonal antibodies to hepatocyte growth factor (HGF) α-chain (C-20) and β-chain (N-19) were purchased from Santa Cruz Biotechnology, Inc.

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1 The abbreviations used are: ECM, extracellular matrix; HGF, hepatocyte growth factor; SF, scattering factor; HAI-1, hepatocyte growth factor activator inhibitor-1; LDL, low density lipoprotein; MT-SP1, membrane-type serine protease 1; uPA, urokinase plasminogen activator; pro-uPA, single-chain form of human uPA; MDCK, Madin-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; FCS, fetal calf serum; PBS, phosphate-buffered saline; AMC, 7-amino-4-methylcoumarin; N-t-Boc, N-t-butylcarboxyamido.
Biotecnologies (Calne, Wiltshire, UK). Monoclonal anti-human Met antibodies (clones IX-21 and IX-24) were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). The recombinant anti-phosphotyrosine antibody (RC20: HRPO) was from Transduction Laboratory (Lexington, KY).

Cell Lines and Protein Substrates—Human lung carcinoma cell line A549 was from the ATCC. Madin-Darby canine kidney (MDCK II) epithelial cell lines and the single-chain form HGF protein were the generous gifts from Dr. George Vande Woude (Van Andel Research Institute, Grand Rapids, MI). The single-chain form of human urokinase plasminogen activator (pro-uPA) was purchased from American Diagnostics Inc. (Greenwich, CT). Plasminogen, plasmin, and fluorescently labeled peptides N-terbutoxycarbonyl (N-t-BOC)-Glu-Ala-Arg-AMC, -Bz-Glu-Ala-Arg-AMC, -Glu-Ala-Arg-AMC, -Succinyl (S)-Ala-Phe-Lys-AMC, and -Suc-Leu-Leu-Val-Tyr-AMC were from Sigma Chemical Co. (St. Louis, MO).

Purification of Active Matriptase—The 70-kDa active matriptase and its endogenous inhibitor HAI-1 were purified from human liver by immunoaffinity chromatography and maintained in their uncomplexed status in glycine buffer, pH 2.4, as described previously (7). Matriptase and HAI-1 were further separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was stained using a zin stain kit (Bio-Rad, Hercules, CA). The 70-kDa active matriptase was sliced out and then eluted from the gel using Electro-EIuter (Bio-Rad) under non-denatured conditions (Tris-glysine buffer, pH 8.3). Alternatively, the active matriptase was purified by a high pressure liquid chromatography C18 column (Ydac VHP5B). The mobile phase was A = 0.1% trifluoroacetic acid in water; B = 95% acetonitrile in water containing 0.1% trifluoroacetic acid. The flow rate was 1 ml/min. The gradient was set from 0 to 60% B over 8 min and at 60% B for next 7 min. The 70-kDa active matriptase was composed of two major and one minor cleaved products of the membrane-bound matriptase. The cleavage sites of the two major bands were identified to be Lys189Ser190 and Lys204Thr205 (the numbering of amino acid residues are based on the updated cDNA sequence of matriptase). The 70-kDa, active matriptase contains two CUB domains, four LDL receptor domains, and the serine protease domain but lacks the transmembrane domain.

Determination of Matriptase Activity—The enzyme activity of matriptase was measured at room temperature in a reaction buffer containing 100 mM Tris-HCl (pH 8.5) and 100 μg/ml bovine serum albumin, using a fluorescent peptide as substrate. In brief, 10 μl of enzyme solution and 10 μl of peptide substrate were added to a cuvette containing 180 μl of the reaction buffer. The mixture was mixed well, placed back into a fluorescent spectrophotometer (Hitachi F4500), and the release of fluorescence resulting from hydrolysis of the peptide substrate was recorded with excitation at 360 nm and emission at 480 nm.

Determination of Kinetic Parameter—Substrate concentration versus initial reaction velocity were analyzed by the Michaelis-Menten equation and plotted using Sigmaplot software. Double reciprocal (Lineweaver-Burk) plots thus derived were used to determine V_{max} and K_{m} values.

Cleavage of Protein Substrates—Single chain HGF protein, plasminogen, or pro-uPA was incubated with various amounts of matriptase in 100 mM Tris-HCl (pH 8.5) overnight at room temperature. Incubation was stopped by boiling the mixture in SDS sample buffer. The cleaved products were then separated on SDS-PAGE and analyzed by Western blot hybridization or by silver stain.

Scattering Assay—The MDCK II cell line was maintained at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS). The scatter assay was carried out in the 96-well culture plate (11). To each well was added 150 μl of Dulbecco's modified Eagle's medium supplemented with 5% FCS and leupeptin at 100 μg/ml; HGF or matriptase-cleaved HGF was added into one well and serial 2-fold dilutions were made with sequential 150-μl aliquots of medium transferred from well to well. About 3000 MDCK II cells in 150 μl of medium were added to each well, and the plate was placed in a 37 °C incubator for 24 h. Thereafter, the supernatant was fixed and stained with 15% crystal violet in 50% methanol. Cell scattering (spreading and dispersion of epithelial colonies) was examined under light microscopy.

c-Met Phosphorylation Detection—A549 cells were grown confluent in RPMI medium supplemented with 10% FCS. After 3-hour serum starvation, cells were incubated 5 min at 37 °C with 450 ng/ml HGF or matriptase-cleaved HGF in RPMI medium supplemented with 5% FCS.

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**Table I**

| Peptide substrate | P4-P5-P2-P1-AMC | K_{m} | V_{max} |
|------------------|----------------|------|--------|
| 1 | Gln-Ala-Arg<sup>a</sup> | 4.89 μM | 654 AMC/min |
| 2 | Glu-Ala-Arg<sup>a</sup> | 3.81 μM | 76.3 |
| 3 | Leu-Gly-Arg<sup>a</sup> | 13.6 μM | 309 |
| 4 | Gln-Gly-Arg<sup>a</sup> | 33.5 μM | 528 |
| 5 | Glu-Gly-Arg | 47.5 μM | 170 |
| 6 | Ala-Phe-Lys | 69.9 μM | 524 |
| 7 | Leu-Leu-Val-Tyr<sup>b</sup> | 887 μM | 92 |
| 8 | Ala-Ala-Pro-Phe<sup>b</sup> | 1.3 μM | 24 |
| 9 | Ala-Ala-Ala<sup>b</sup> | 1.3 μM | 24 |

*Gln-Ala-Arg is a standard substrate for trypsin, Glu-Ala-Arg is a substrate for factor XIa, Leu-Gly-Arg is a substrate for uPA, Glu-Gly-Arg is a substrate for XIa, Leu-Leu-Val-Tyr and Ala-Ala-Pro-Phe are substrates for chymotrypsin, and Ala-Ala-Ala is the substrate for elastase.*

<sup>a</sup>No cleavage activity was detected with these substrates at a concentration of 200 μM.

As appropriate, leupeptin was included in the medium at 100 μg/ml. Media were removed, and cells were rinsed with 1× phosphate-buffered saline (PBS) and collected by centrifugation following trypsinization. After washing one more time with 1× PBS, cell pellets were frozen in dry ice. The frozen cell pellets were either stored at −80 °C for later extraction or immediately extracted as described below. Cells were thawed on ice, extracted by suspension in buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM sodium fluoride, 10 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1% Triton X-100. Extracts were clarified by centrifugation for 15 min at 12,000 × g in a microcentrifuge, and the protein concentration was determined by BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as standard. About 2 mg of protein of extracts was immunoprecipitated using anti-c-Met antibody and Pansorbin (Calbiochem, La Jolla, CA). The protein-antibody-Pansorbin immunocomplex was collected by centrifugation, washed twice with extraction buffer, and then dissociated by boiling in SDS-sample buffer. Pansorbin was removed by centrifugation, the supernatant fractions were subjected to 8% SDS-PAGE, and the proteins were detected by Western immunoblot using anti-phosphotyrosin antibody. The same immunoblots were then stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 82.5 mM Tris-HCl (pH 7.6) for 30 min at 50 °C and reprobed with anti-c-Met antibody.

**RESULTS**

**Matriptase Selectively Cleaves Peptide after an Arg or Lys Residue**—In our previous study, the trypsin-like activity of matriptase was suggested by the observations that 1) an Arg residue positioned at the bottom of a substrate binding pocket of the serine protease domain of matriptase and 2) matriptase is able to cleave various synthetic substrates containing Arg or Lys as P1 sites (6). In the current study, to investigate in detail the substrate specificity of matriptase, we measured the K_{m} and V_{max} of matriptase for a variety of protease substrate peptides. Table I shows that the most reactive peptide substrates for matriptase are N-t-Boc-Glu-Ala-Arg-AMC, with a K_{m} of 4.89 μM, and N-t-Boc-Bz-Glu-Ala-Arg-AMC, with a K_{m} of 3.81 μM. N-t-Boc-Gln-Ala-Arg-AMC and N-t-Boc-Bz-Glu-Ala-Arg-AMC were reported to be good substrates for bovine trypsin and human factor XIa, respectively (12). No released fluorescence was detected from the substrates for chymotrypsin or elastase (Table I, peptide substrates 7, 8, and 9). Matriptase...
Matriptase Is Able to Activate HGF/SF—HGF/SF is secreted as an inactive, single chain precursor by stromal cells, and it is activated by proteolytic conversion to the two-chain form factor by cleavage at Arg or Lys for their activation. We, therefore, set out to examine whether matriptase can activate three such substances, HGF/SF, plasminogen, and pro-uPA, in addition to characterizing the synthetic substrates for matriptase.

Matriptase Activates HGF and Pro-uPA

Fig. 1. Matriptase converts single-chain HGF into smaller fragments, which can be recognized by anti-α-chain HGF and β-chain HGF antibodies. A, protein staining. Shown are the silver-stained protein patterns of HGF incubated overnight without (–) or with (+) matriptase. B. Western immunoblot. HGF incubated overnight without (–) or with (+) matriptase was immunoblotted with anti-βHGF (β chain) or anti-α-HGF (α chain).

appears to prefer to bind to peptides containing small side-chain amino acids, such as Ala and Gly, at P2 site (Table I, peptide substrates 1–5). Peptides containing P2 Ala are better substrates for matriptase than peptides containing P2 Gly (compare peptides 1 and 2 with peptides 3–5). The binding affinity of matriptase to the former is about 30-fold higher than that to the latter. Interestingly, a change from Gln to Glu at the P3 site significant reduces the Vmax (compare peptide 1 with 2) without causing a significant change to the Km.

Considering its trypsin-like activity and its presentation on the surfaces of epithelial cells and cancer cells, matriptase could serve as a cell surface activator for other secreted proteases and growth factors. Particularly relevant substrates could include those factors that are produced by stromal cells, that function on epithelial cells, and that require proteolytic cleavage at Arg or Lys for their activation. We, therefore, set out to examine whether matriptase can activate three such substances, HGF/SF, plasminogen, and pro-uPA, in addition to characterizing the synthetic substrates for matriptase.

Matriptase Converts Single-Chain HGF into Smaller Fragments, which Can Be Recognized by Anti-α-Chain HGF and β-Chain HGF Antibodies. A, protein staining. Shown are the silver-stained protein patterns of HGF incubated overnight without (–) or with (+) matriptase. B, Western immunoblot. HGF incubated overnight without (–) or with (+) matriptase was immunoblotted with anti-βHGF (β chain) or anti-α-HGF (α chain).
absence of HGF (No HGF). The tyrosine-phosphorylated c-Met in the cells treated with uncleaved HGF is about one-fifth of that in the cells treated with matriptase-cleaved HGF. Again, consistent with a limited degree of contamination of cleaved HGF in our latent HGF preparation. Phosphotyrosine detected in c-Met of cells incubated with untreated HGF appears to be caused by the residual active HGF contamination in the preparation. Leupeptin did not affect the total c-Met expression, the c-Met phosphorylation, nor the total pattern of tyrosine phosphorylation (data not shown).

Plasminogen shares high homology with HGF, and its activation also requires a cleavage at Arg. Therefore, it seemed likely that plasminogen would be a substrate of matriptase as well. However, matriptase failed to cleave plasminogen. As shown in Fig. 4, plasminogen remained as a 94-kDa single-chain form, even in the presence of matriptase at a concentration 8-fold higher than that required for cleavage of HGF.

Matriptase Could Function as an Initiator of Matrix-degrading Protease Cascade—Plasmin has long been regarded as the enzyme that converts pro-uPA to active uPA. However, the level of active uPA is not reduced in the urine of mice bearing a targeted disruption of the plasminogen gene (17), suggesting the existence of plasmin-independent pro-uPA activation. Plasma kallikrein (18), trypsin-like proteases from human ovarian tumors (19), a T cell-associated serine protease (20), cathepsins B and L (21, 22), nerve growth factor (23), human mast cell tryptase (24), and prostate-specific antigen (25) have also been reported to activate pro-uPA. However, the relevance of these studies of pro-uPA activation in vitro is uncertain for understanding roles of these enzymes in vivo. During the preparation of this manuscript, activation of pro-uPA was reported by the recombinant serine protease domain of matriptase/MT-SP1 (26). This observation is consistent with our studies using the purified, 70-kDa active matriptase containing both CUB and LDL domains. Fig. 5A showed that, after incubation with matriptase, the 55-kDa single-chain pro-uPA was converted into smaller fragments. One of these cleavage products clearly appeared on the protein gel as the 33-kDa molecule, which resembles the size of the active uPA protease (Fig. 5A). The cleaved product exhibited enzymatic activity toward the fluorescent peptide substrate, N-t-Boc-Leu-Gly-Arg-AMC, for uPA (Fig. 5B, compare the closed circles with closed triangles). This
Matriptase Activates HGF and Pro-uPA

Fig. 4. Plasminogen is not a substrate for matriptase. Shown are the silver-stained protein patterns of plasminogen incubated overnight without (0) or with increasing amount of matriptase. The highest amount of matriptase used is 8-fold of the lowest amount of matriptase that cleaves HGF.

A

![Pro-uPA is activated by matriptase cleavage. A, single-chain pro-uPA is converted into two-chain form uPA by matriptase. Pro-uPA was incubated overnight with active matriptase in the absence (0) or presence of increasing amount of matriptase. The cleaved products were analyzed by electrophoresis followed by silver-staining. B, matriptase cleavage of pro-uPA generates an active protease. Pro-uPA was either incubated for 30 min with matriptase (closed circles) or incubated 1 min with plasmin (opened circles) prior to the assay. Plasmin (opened triangles), uPA (closed triangles), and matriptase (closed squares) all exhibit low activity.](image)

activity was not derived from matriptase, because matriptase alone only exhibited negligible background activity (Fig. 5B, closed squares). The same amounts of pro-uPA produced a similar activity after cleavage by plasmin (Fig. 5B, opened circles). These results suggest that matriptase itself is able to activate pro-uPA and that the CUB domains and LDL receptor domains of matriptase do not interfere with its activation activity.

DISCUSSION

By using the 70-kDa, active matriptase isolated from human milk, we report in this study that matriptase cleaves and converts HGF into a biologically functional factor that can induce c-Met activation and stimulate epithelial cell scattering. In addition, we also noted that matriptase can activate pro-uPA but not plasminogen. These results further support our working hypothesis that matriptase is an upstream regulator of cellular migration and extracellular matrix degradation. Most significantly, these results reveal a novel mechanism in the control of tissue remodeling that involves an upstream epithelial membrane activator and downstream stromal effectors.

Tissue remodeling is a process observed both in physiological and pathologic processes. Two essential changes occur during these processes: 1) an epithelial-mesenchymal transition transforms relatively rigid epithelial cells to the more mobile migratory mesenchymal cells; 2) extracellular matrix degradation opens pathways for the migrating cells. HGF/SF is a potent inducer of epithelial-mesenchymal transition (27); engagement of HGF to its epithelial receptor c-Met triggers various intracellular signaling pathways. HGF is secreted as an inactive precursor by stromal cells, and it is proteolytically activated in the extracellular environment (13). Therefore, activation of HGF/SF needs to occur in the close vicinity of the epithelial cells. uPA and the zinc-dependent metalloproteinases have been proposed to be responsible for the majority of proteolysis of pericellular proteins (28). However, both systems are largely synthesized by the stromal cells and require indirect mechanisms for their recruitment and activation on the surfaces of epithelial cells. Thus, an epithelial-derived protease, like matriptase, could provide a missing link in this process.

Matriptase appears to have selectivity for its macromolecular substrates. In our experiments, matriptase did not cleave plasminogen, despite the high sequence homology between plasminogen and HGF. This selectivity was also reported by others utilizing the serine protease domain of matriptase (26). In the same report, it was also shown that matriptase/MT-SP1 has selectivity for a basic residue at the P3 or P4 site. The sequence at the activation cleavage site of HGF and plasminogen is P4-(Lys)-P3-(Gln)-P2-(Gly)-P1-(Arg) and P4-(Cys)-P3-(Pro)-P2-(Leu)-P1-(Arg), respectively. Thus, the lack of a P3 or P4 basic residue in plasminogen might contribute to its lack of cleavage by matriptase. Alternatively, there might be conformational differences between HGF and plasminogen. The kringle domains of plasminogen contain a Lys-binding site that serves to mediate its localization to fibrin and to cellular surfaces. Plasminogen circulates in the blood in a globular and closed conformation; upon binding to the surface, it shifts to an extended and opened conformation. This conformation change promotes its recognition by its activator and its rapid conversion to plasmin. The kringle domains on HGF/SF also contribute to its binding to c-Met. However, cleavage activation of HGF/SF does not depend on its binding to c-Met. It is possible that the single chain form HGF/SF exhibits a more open conformation than does plasminogen and that matriptase can distinguish this subtle structural difference.

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