Purification and Characterization of a Complex Containing Matriptase and a Kunitz-type Serine Protease Inhibitor from Human Milk*

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Matriptase, a trypsin-like serine protease with two potential regulatory modules (low density lipoprotein receptor and complement C1r/s domains), was initially purified from T-47D breast cancer cells. Given its plasma membrane localization, extracellular matrix-degrading activity, and expression by breast cancer cells, this protease may be involved in multiple aspects of breast tumor progression, including cancer invasion. In breast cancer cells, matriptase was detected mainly as an uncomplexed form; however, low levels of matriptase were detected in complexes. In striking contrast, only the complexed matriptase was detected in human milk. The complexed matriptase has now been purified. Amino acid sequences obtained from the matriptase-associated proteins reveal that they are fragments of a Kunitz-type serine protease inhibitor that was previously reported to be an inhibitor of the hepatocyte growth factor activator. In addition, matriptase and its complexes were detected in milk-derived, SV40 T-antigen-immortalized mammary luminal epithelial cell lines, but not in human foreskin fibroblasts or in HT-1080 fibrosarcoma cells. These results suggest that the milk-derived matriptase complexes are likely to be produced by the epithelial components of the lactating mammary gland in vivo and that the activity and function of matriptase may be differentially regulated by its cognate inhibitor, comparing breast cancer with the lactating mammary gland.

Matriptase is a trypsin-like serine protease with two regulatory modules: two tandem repeats of the complement C1r/s domain and four tandem repeats of the low density lipoprotein receptor domain (1). Matriptase was initially identified from T-47D human breast cancer cells as a major gelatinolytic activity on a gelatin zymogram, with a migration rate between those of gelatinase A (72 kDa; MMP-2) and gelatinase B (92 kDa; MMP-9) (2); it has been proposed to play a role in breast cancer invasion (3). The primary cleavage specificity of matriptase was identified to be arginine and lysine residues, similar to the majority of serine proteases, including trypsin and plasmin. In addition, matriptase, as does trypsin, exhibits broad spectrum cleavage activity, and such activity is likely to contribute to its gelatinolytic activity on a gelatin zymogram.

HAI-1 (hepatocyte growth factor activator inhibitor-1) (4) is a Kunitz-type serine protease inhibitor that is able to inhibit the hepatocyte growth factor (HGF) activator, a blood coagulation factor XII-like serine protease (5). The mature form of this protease inhibitor has 478 amino acid residues, with a calculated molecular mass of 53,319 Da. A putative transmembrane domain is located at its carboxyl terminus. HAI-1 contains two Kunitz domains (domain I spans residues 246–306, and domain II spans residues 371–431) separated by a low density lipoprotein receptor domain (residues 315–360). The presumed P1 residue of the active-site cleft is likely to be arginine 260 in HAI-1 and lysine 385 in domain II by alignment with bovine pancreatic trypsin inhibitor (aprotinin) and with other Kunitz-type inhibitors (6–8). Thus, HAI-1 has specificity against trypsin-type proteases. Although the HGF activator is exclusively expressed by liver cells, HAI-1 was originally purified from the conditioned medium of carcinoma cells as a 40-kDa fragment doublet, rather than the proposed mature, membrane-bound 53-kDa form (4).

The protein inhibitors of serine proteases can be classified into at least 10 families, according to various schemes (9). Among them, serpins such as maspin (10) and Kunitz-type inhibitors such as urinary trypsin inhibitor (11) have been previously implicated in suppression of cancer invasion. The Kunitz-type inhibitors form very tight, but reversible complexes with their target serine proteases. The reactive sites of these inhibitors are rigid and can simulate optimal protease substrates (12). The interaction between a serine protease and a Kunitz-type inhibitor depends on complementary large surface areas of contact between the protease and inhibitor. The inhibitory activity of the recovered Kunitz-type inhibitor from protease complexes can always be reconstituted. The Kunitz-type inhibitors may be cleaved by cognate proteases, but such cleavage is not essential for their inhibitory activity.

In this study, to begin to investigate the role of matriptase under physiological conditions such as differentiation and lactation, we studied its expression in human milk. We found that milk-derived matriptase strongly interacts with fragments of HAI-1 to form complexes.

MATERIALS AND METHODS

Cell Lines and Culture Conditions—Four milk-derived, immortalized mammary luminal epithelial cell lines (MTSV-1.1B, MTSV-1.7, MRSV-4.1, and MRSV-4.2) were a gift from Dr. J. Taylor-Papadimitriou (Im-

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peripheral Cancer Research Fund, London) (13) and were maintained in modified Iscove’s minimal essential medium (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 10 μg/ml bovine insulin, 5 μg/ml hydrocortisone (Sigma), and antibiotics. Human foreskin fibroblasts and the fibroaoma cell line HT-1080 (from American Type Culture Collection) were maintained in modified Iscove’s minimal essential medium supplemented with 10% fetal calf serum. To collect cell-conditioned medium, monolayers of these cells at confluency were washed twice with phosphate-buffered saline and were cultured for 2 days in the absence of the serum in modified Iscove’s minimal essential medium supplemented with insulin/transferrin/ selenium (Biofluids, Inc., Rockville, MD).

Identification and Partial Isolation of Matriptase-related Proteases from Human Milk—To isolate matriptase-related proteases, 1.5 liters of frozen human milk from the Georgetown University Medical Center Milk Bank were thawed and centrifuged to remove the milk fat and insoluble debris. Ammonium sulfate powder was added to the milk with continuous mixing to 40% saturation and allowed to precipitate in a cold room for at least 2 h. Protein precipitates were obtained by centrifugation at 10,000 × g for 20 min. The pellets were saved, and the supernatant was further precipitated by addition of ammonium sulfate powder to 60% saturation. The protein pellets were dissolved in water and then dialyzed against 20 mM Tris-HCl, pH 8.0, for DEAE chromatography or against 10 mM phosphate buffer, pH 6.0, for CM chromatography. Insoluble debris was cleared by centrifugation, and the supernatant was divided into five batches; each batch was loaded onto a DEAE-Sepharose FF column (2.5 × 20 cm; Amersham Pharmacie Biotech) equilibrated with 20 mM Tris-HCl, pH 8.0. The column was washed with 10 column volumes of the equilibration buffer. Bound material was eluted with a linear gradient of 0–1 M NaCl in DEAE equilibration buffer with a total volume of 500 ml. Fractions (14 ml) were collected and assessed by immunoblotting using mAb 21-9. To perform CM chromatography, the 95-kDa fraction from DEAE chromatography or the precipitate derived directly from ammonium sulfate precipitation was dialyzed against 10 mM phosphate buffer, pH 6.0. Insoluble debris was cleared by centrifugation, and the supernatant was loaded onto a CM-Sepharose FF column (2.5 × 20 cm; Amersham Pharmacie Biotech) equilibrated with 10 mM phosphate buffer, pH 6.0. The column was washed with 10 column volumes of the equilibration buffer. Bound material was eluted with a linear gradient of 0–0.5 M NaCl in 10 mM phosphate buffer, pH 6.0, with a total volume of 500 ml. Fractions (14 ml) were assessed by immunoblotting using mAb 21-9.

Immunooaffinity Chromatography—Preparation of an immunooaffinity column by coupling mAb 21-9 to Sepharose 4B (5 mg of IgG/ml of beads) was performed using CNBr-activated Sepharose 4B as described previously (3). Partially purified 95-kDa matriptase complex from DEAE or CM chromatography was loaded onto a 1-ml column at a flow rate of 7 ml/h. The column was washed with 1% Triton X-100 in phosphate-buffered saline. Bound protease was then eluted using 0.1 M glycine HCl, pH 2.4. Fractions were immediately neutralized using 2 M NaCl in 10 mM phosphate buffer, pH 6.0, with a total volume of 500 ml. Fractions (14 ml) were assessed by immunoblotting using mAb 21-9.

Hybridoma Fusion—Two 6-week-old female BALB/c mice were immunized with matriptase complexes (10 μg/dose) at intervals of 2 weeks. Complete Freund’s adjuvant was used for the initial immunizations, whereas incomplete adjuvant was used for boosts. Three days after the second boost, antisera was collected from the tail vein, and the immunoresponse was determined by immunoblotting. The final boost was conducted with the matriptase complex in the absence of adjuvant by tail vein injection. The splenocytes were collected and fused with mouse myeloma cells (SP2 or NS1) by polyethylene glycol methodology, and the successful hybridoma cells were selected by HAT medium (14).

Matriptase and a Kunitz-type Serine Protease Inhibitor

Matriptase-related Proteases in Human Milk—In our previous study (3), matriptase was observed to exist either in a major uncomplexed form or in two minor complexed forms with apparent molecular masses of 110 and 95 kDa. The matriptase-binding protein(s) was not identified. To identify these binding protein(s), we now have examined the matriptase complexes found in human milk. Our hypothesis has been that the binding protein is a protease inhibitor and that its expression may be associated with a specific physiological status such as differentiation or lactation. In human milk, two immunoreactive bands of 95 and 110 kDa in size (Fig. 1, A and B) were detected, after boiling in the absence of reducing agents, to a smaller immunoreactive band. This band corresponds in molecular weight to the previously described complex of the matriptase from breast cancer (Fig. 1C). Thus, matriptase appears to be a component of the 95-kDa complex, both in breast cancer cells and in milk. Although most of the matriptase detected in breast cancer cells is uncomplexed; the opposite is true in milk.

Most of the minor 110-kDa immunoreactive polypeptide in milk was precipitated by a 40% saturation of ammonium sul-
Protein had a reduced rate of migration after boiling; however, the PAGE and subjected to Western blotting using mAb 21-9. The 110-kDa B 40–60% pool (matriptase. Two bands of 95 and 110 kDa were detected as indicated; two pools by addition of ammonium sulfate: a 0–40% pool (A). matriptase mAb 21-9. milk of 110- and 95-kDa proteins immunoreactive to anti-matriptase after boiling. In addition to matriptase, 40- and 25-kDa bands after boiling (lane 6). These results suggest that both 40- and 25-kDa bands are components of the 95-kDa matriptase complex. In our biochemical approach, a 95-kDa complex, a smaller complex with an apparent size of 85 kDa was also obtained (lane 1); this 85-kDa matriptase complex could also be converted to uncomplexed matriptase and a 25-kDa band after boiling (lane 2). Molecular mass markers are indicated. BP-40 and BP-25, 40- and 25-kDa binding proteins, respectively.

**Fig. 1.** Identification and partial purification from human milk of 110- and 95-kDa proteins immunoreactive to anti-matriptase mAb 21-9. Human milk proteins were fractionated into two pools by addition of ammonium sulfate: a 0–40% pool (A) and a 40–60% pool (B). Both fractions were further purified by DEAE chromatography. The DEAE fractions were examined by immunoblot analysis using mAb 21-9, which is directed against cancer cell-derived matriptase. Two bands of 95 and 110 kDa were detected as indicated; uncomplexed matriptase was not detected. In C, both pooled 110-kDa (lanes 1 and 2) and 95-kDa (lanes 3 and 4) fractions were incubated in 1× SDS sample buffer in the absence of reducing agents at room temperature (+ boiling) or at 95 °C (+ boiling) for 5 min prior to SDS-PAGE and subjected to Western blotting using mAb 21-9. The 110-kDa protein had a reduced rate of migration after boiling; however, the 95-kDa protein was converted to uncomplexed matriptase after boiling.

**Fig. 2.** Immunoaffinity purification of matriptase complexes. The partially purified matriptase complex from ion-exchange chromatography (see Fig. 1) was loaded onto a mAb 21-9-Sepharose column. The bound proteins were eluted with glycine buffer, pH 2.4, and neutralized by addition of 2 M Trizma. The eluted proteins were incubated in 1× SDS sample buffer in the absence of reducing agents at room temperature (– boiling) or at 95 °C (+ boiling) for 5 min. The samples were resolved by SDS-PAGE and stained by colloidal Coomassie. In some batches of purification, as described in the accompanying paper (1), the 95-kDa matriptase complex was obtained as the major band; this 95-kDa complex was capable of being converted to uncomplexed matriptase and a 40-kDa doublet after boiling. In some other batches, in addition to the 95-kDa complex, a smaller complex with an apparent size of 85 kDa was also obtained (lane 1); this 85-kDa matriptase complex could also be converted to uncomplexed matriptase and a 25-kDa band after boiling (lane 2). Molecular mass markers are indicated. BP-40 and BP-25, 40- and 25-kDa binding proteins, respectively.

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**Purification of Matriptase Complexes from Human Milk—**

The milk-derived 110-kDa matriptase complex had a reduced rate of migration on an SDS-polyacrylamide gel after boiling (Fig. 1C). These results suggest that this milk-derived 110-kDa immunoreactive polypeptide is not likely to be a protease complex. Alternatively, this milk-derived 110-kDa immunoreactive polypeptide is likely to be a serpin complex, which is known to be stable in SDS.

In contrast, the 110-kDa species from breast cancer cells was converted by boiling into matriptase and another unidentified species (3). This milk-derived 110-kDa species was thus distinct from the 110-kDa matriptase complex previously isolated from T-47D breast cancer cells.

**Purification of Matriptase Complexes from Human Milk—**

The milk-derived 110-kDa matriptase complex has been isolated using an anti-matriptase mAb 21-9 immunoaffinity column. This highly purified 95-kDa matriptase complex can be converted to matriptase, after boiling, in conjunction with the appearance of a protein doublet with an apparent molecular mass of 40 kDa (1). In some batches of milk, another protease complex doublet, with an apparent molecular mass of 85 kDa, was also observed, in addition to the 95-kDa complex (Fig. 2, lane 1). Both 95- and 85-kDa matriptase complexes released matriptase after boiling. In addition to matriptase, 40- and 25-kDa protein bands were observed (Fig. 2, lane 2).

Biochemical and immunological approaches have been taken to prove that the 40- and 25-kDa bands are components of matriptase complexes. In our biochemical approach, a 95-kDa matriptase complex preparation, which also contains low levels of uncomplexed matriptase, was subjected to nonboiling/boiling diagonal gel electrophoresis. In this gel electrophoresis system, proteins whose migration rate on an SDS-polyacrylamide gel are not changed by boiling will be seen on the diagonal line. Proteins whose configuration is changed by boiling, resulting in a lower migration rate, will be seen beyond the diagonal line. The sample was first resolved by SDS-PAGE, and a strip of gel was sliced off. The sliced gel strip was boiled in 1× SDS sample buffer in the absence of reducing agents, placed on a second SDS-polyacrylamide gel, and electrophoresed (Fig. 3). In the case of the 95-kDa matriptase complex, both the 40-kDa protein doublet and matriptase were observed below the diagonal line and on the same electrophoretic path (Fig. 3). This result thus confirms that matriptase and the 40-kDa doublet are components of the 95-kDa matriptase complex. On the other hand, uncomplexed matriptase was seen on the diagonal line (Fig. 3).

In our immunological approach, a panel of mAbs was obtained using matriptase complexes as immunogens (Fig. 4). A new anti-matriptase mAb (M92) recognized both 95- and 85-kDa complexes under nonboiling conditions (Fig. 4A, lane 5); this mAb recognized uncomplexed matriptase, but not the 40- and 25-kDa bands, after boiling (lane 6). These results suggest that matriptase is a component of these two complexes. In contrast to mAb M92, mAb M19 recognized both 40- and 25-kDa bands, but not matriptase, under boiling conditions (Fig. 4A, lane 4). This mAb also recognized both 95- and 85-kDa complexes under nonboiling conditions (Fig. 4A, lane 3). These results suggest that both 40- and 25-kDa bands are components of these matriptase complexes.

A third antibody type, mAb M58, was also selected. This mAb selectively recognized only the 95-kDa matriptase complex (not the 85-kDa complex) under nonboiling conditions (Fig. 4A, lane 1). Furthermore, mAb M58 recognized only the 40-kDa band (not the 25-kDa band) after boiling (Fig. 4A, lane 2). Both 40- and 25-kDa bands are fragments of a Kunitz-type serine protease inhibitor; the 25-kDa band is a carboxyl-terminal degraded product of the 40-kDa band (see below). Thus, the epitope that is recognized by mAb M58 is likely to reside on the
amino terminus of the 40-kDa band. These results, combined with the results in Fig. 2, suggest that the 95-kDa matriptase complex is composed of matriptase and the 40-kDa component; the 85-kDa matriptase complex is composed of matriptase and the 25-kDa component. In Fig. 4B, we summarize the structures of both 95- and 85-kDa matriptase complexes and their interactions with these mAbs.

The Binding Proteins of Matriptase Are Fragments of a Kunitz-type Serine Protease Inhibitor—When the amino-terminal sequences of the 40- and 25-kDa binding proteins were determined, the sequence of the 40-kDa binding protein (GPP-PAPPGPLPG) was found to be identical to the amino-terminal sequence of a Kunitz-type serine protease inhibitor (4), which was previously identified as HAI-1 (4). The amino acid residues (TQGFGGSGS) obtained from the N terminus of the 25-kDa binding protein mAbs (M58 and M19) are presented here. mAb M92 recognized both 95- and 85-kDa matriptase complexes under nonboiling conditions (lane 1). Another mAb, M58, recognized only the 95-kDa matriptase complex (not the 85-kDa complex) under nonboiling conditions (lane 2), but not with the 40- and 25-kDa bands after boiling. Anti-binding protein mAb M19 detected a band with an apparent size of 55 kDa in the cell lysate of HAI-1-transfected COS-7 cells (Fig. 6). Anti-binding protein mAb M19 detected a band with an apparent size of 55 kDa in the cell lysate of HAI-1-transfected COS-7 cells (Fig. 6, lane 2) and in the 2 M KCl-washed membrane fraction of T-47D human breast cancer cells (lane 4), but not in the COS-7 cells (lane 3) or in matriptase-transfected COS-7 cells (lane 1). The immunoreactivity of the anti-binding protein mAb for HAI-1 provides a second line of evidence that the binding protein of matriptase is HAI-1. Because the size of the immunoreactive 55-kDa band is close to the calculated molecular mass (53,319 Da) of mature

**Fig. 3. Diagonal gel electrophoresis of the 95-kDa matriptase complex: evidence that this complex corresponds to uncomplexed matriptase in association with its 40-kDa binding protein doublet.** The 95-kDa matriptase complex from human milk was subjected to diagonal gel electrophoresis. In the first dimension (D), the 95-kDa matriptase complex, without boiling treatment, was resolved by SDS-PAGE. Then a gel strip was sliced out, boiled in 1× SDS sample buffer in the absence of reducing agents for 5 min, and electrophoresed on a second SDS-polyacrylamide gel. The proteins were stained by colloidal Coomassie. After this procedure, the 95-kDa matriptase complex disappeared from the diagonal line and was converted to matriptase and a 40-kDa binding protein doublet (BP-40). The uncomplexed matriptase was observed on the diagonal line, as expected, suggesting that its migration rate was not changed by boiling.

**Fig. 4. Structural characterization of matriptase complexes by monoclonal antibodies that are directed against matriptase and its binding protein.** A, a panel of mAbs was produced using the milk-derived matriptase complexes as immunogens. These mAbs were characterized by immunoblot analysis using the preparation containing both 95- and 85-kDa matriptase complexes described in the legend to Fig. 2. The matriptase preparation was dissolved in 1× SDS sample buffer in the absence of reducing agents and incubated at room temperature (lanes 1, 3, and 5; −Boiling) or at 95 °C (lanes 2, 4, and 6; +Boiling) for 5 min. Among these mAbs, an anti-matriptase mAb (M92) and two anti-binding protein mAbs (M58 and M19) are presented here. MAb M92 recognized both 95- and 85-kDa matriptase complexes under nonboiling conditions (lane 2) and interacted with the dissociated matriptase after boiling (lane 6), but not with the 40- and 25-kDa bands after boiling. Anti-binding protein mAb M19 recognized both 95- and 85-kDa matriptase complexes under nonboiling conditions (lane 3) and both 40- and 25-kDa bands after boiling (lane 4). Another mAb, M58, recognized only the 95-kDa matriptase complex (not the 85-kDa complex) under nonboiling conditions (lane 1); this mAb also detected the 40-kDa band, but not the 25-kDa band or the dissociated matriptase (lane 2). B, shown is a summary of the structures of matriptase-containing complexes and mAbs that are directed against these complexes and their subunits. BP-40 and BP-25, 40- and 25-kDa binding proteins, respectively.

**Fig. 5. Amino acid sequence comparison of binding protein and HAI-1.** The deduced amino acid sequence of human HAI-1 was obtained from Ref. 4. Twelve-amino-acid (GPP-PAPPGPLPG) and seven-amino-acid (TQGFGGGS) sequences of the amino terminus obtained from the 40-kDa binding protein doublet and the 25-kDa binding protein, respectively, and were identical to amino acids 36–47 and 154–160 of HAI-1. The two stretches of larger band of the 40-kDa binding protein doublet were compared with the results in Fig. 2, suggest that the 95-kDa matriptase complex was not changed by boiling. The tryptic digests were examined by MALDI-MS. Twelve unique peptides from the tryptic digests were matched with the HAI-1 sequences by searching the entire HAI-1 sequence for observed MALDI-MS masses (Fig. 5). These 12 peptides cover 87 residues that span positions 135–310. These results indicate that the binding proteins of matriptase are fragments of HAI-1.

We further examined the immunoreactivity of an anti-binding protein mAb with HAI-1 that was expressed by HAI-1-transfected COS-7 cells (Fig. 6). Anti-binding protein mAb M19 detected a band with an apparent size of 55 kDa in the cell lysate of HAI-1-transfected COS-7 cells (Fig. 6, lane 2) and in the 2 M KCl-washed membrane fraction of T-47D human breast cancer cells (lane 4), but not in the COS-7 cells (lane 3) or in matriptase-transfected COS-7 cells (lane 1). The immunoreactivity of the anti-binding protein mAb for HAI-1 provides a second line of evidence that the binding protein of matriptase is HAI-1. Because the size of the immunoreactive 55-kDa band is close to the calculated molecular mass (53,319 Da) of mature HAI-1. The deduced amino acid sequence of human HAI-1 was obtained from Ref. 4. Twelve-amino-acid (GPP-PAPPGPLPG) and seven-amino-acid (TQGFGGGS) sequences of the amino terminus obtained from the 40-kDa binding protein doublet and the 25-kDa binding protein, respectively, and were identical to amino acids 36–47 and 154–160 of HAI-1. The two stretches of larger band of the 40-kDa binding protein doublet were compared with the results in Fig. 2, suggest that the 95-kDa matriptase complex was not changed by boiling. The tryptic digests were examined by MALDI-MS. Twelve unique peptides from the tryptic digests were matched with the HAI-1 sequences by searching the entire HAI-1 sequence for observed MALDI-MS masses (Fig. 5). These 12 peptides cover 87 residues that span positions 135–310. These results indicate that the binding proteins of matriptase are fragments of HAI-1.

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and the 2 M KCl-washed membrane fraction of T-47D human breast cancer cells (3). The HAI-1 cDNA fragment that was generated by reverse transcriptase-polymerase chain reaction and that contains the entire coding region was inserted into the expression vector pcDNA3.1 and transfected into COS-7 cells. Cell lysates from HAI-1-transfected COS-7 cells (lane 2), COS-7 cells (lane 3), and matriptase-transfected COS-7 cells (lane 1), and the 2 M KCl-washed membrane fraction of T-47D human breast cancer cells (lane 4) were subjected to Western blot analysis using anti-binding protein mAb M19.

mammary luminal epithelial cells (Fig. 7, lanes 1–3) suggest that the protease and its binding protein are produced by the epithelial components of the lactating mammary gland. In contrast to milk, the immortalized mammary luminal epithelial cells expressed detectable uncomplexed matriptase and a 110-kDa complex. This 110-kDa complex species was not detected in milk, but was detected in T-47D breast cancer cells (3).

**DISCUSSION**

Although matriptase has been previously purified from breast cancer cells (3), we now report on its expression in the normal lactating mammary gland and in immortalized mammary luminal epithelial cells. Expression of matriptase in the lactating mammary gland is, however, characterized by its strong association with HAI-1 fragments to form complexes that were previously identified as minor forms of matriptase in cultured breast cancer cells. Expression of matriptase in milk in its complexed form with a protease inhibitor suggests that the activity of matriptase is highly regulated in the lactating mammary gland.

Because the P1 residues of both Kunitz domains of HAI-1 are likely to be arginine 260 and lysine 385, HAI-1 is likely to be an inhibitor directed against trypsin-like serine proteases. The trypsin-like activity of matriptase was previously confirmed by its cleavage activity against various synthetic substrates with arginine and lysine as P1 sites and by Asp positioned at the bottom of its substrate specificity pocket. Thus, HAI-1 is not only a matriptase-binding protein, but also a potential inhibitor of matriptase. However, because HAI-1 is a Kunitz-type protease inhibitor that exhibits its inhibitory activity in a competitive and reversible manner, residual activity of matriptase was consistently observed in association with matriptase/HAI-1 complexes. This residual activity was detected both as gelatinolytic activity and cleavage activity against synthetic substrates. This residual activity may also contribute to the cleavage of a 40-kDa fragment of HAI-1 at Arg-153 in the process of purification, resulting in the 25-kDa fragment of HAI-1 and the 85-kDa matriptase complex in some purified batches.

Although HAI-1 was originally identified as an inhibitor of the HGF activator, it is possible that matriptase is an *in vivo* target protease of this Kunitz-type inhibitor, at least in the mammary gland. Both matriptase and HAI-1 are colocalized on the surfaces of T-47D cells, they are coexpressed by four different immortalized mammary luminal epithelial cell lines; and they are copurified from human milk as complexes. Activation of HGF requires cleavage at an arginine residue as the P1 site by a protease such as the HGF activator or urokinase-type plasminogen activator (17); the putative P1 arginine residue in Kunitz domain I of HAI-1 makes the HGF activator a possible target. However, the HGF activator, a serine protease, is produced mainly in the liver and circulates in the blood as an inactive zymogen (5). HAI-1 is expressed mainly in the kidney, pancreas, prostate, small intestine, colon, fetal lung, and fetal kidney; but negligible expression of HAI-1 was observed in adult and fetal liver (4). Furthermore, HAI-1 activity is detected in some lung and stomach carcinoma cell lines, but it is negligible in hepatoma cell lines and in immortalized cells from human fetal liver. Therefore, it is not clear that HAI-1 is an *in vivo* inhibitor for the HGF activator, particularly since HAI-1 is expressed at negligible levels in the lung and liver, where highly regulated activity of HGF is thought to play a crucial role in repair following injury (18, 19).

In summary, in this study, we have provided evidence that the previously described breast cancer cell-derived matriptase is also produced by the lactating mammary gland. In milk, however, the protease tightly associates with a 40-kDa fragment of a Kunitz-type protease inhibitor, forming a 95-kDa protease complex. The formation of a protease complex appears to be selective for lactating mammary epithelial cells; increased levels of uncomplexed protease are associated with epithelial...
immortalization and, in particular, with breast tumorigenesis. Study of the activity of this plasma membrane-associated protease and its potential regulation by complexation with the Kunitz-type inhibitor could provide insight into how cancer cells utilize a physiological protease for multiple malignant processes such as invasion and metastasis.

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