Matriptase-2, a Membrane-bound Mosaic Serine Proteinase Predominantly Expressed in Human Liver and Showing Degrading Activity against Extracellular Matrix Proteins*

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Glória Velasco‡, Santiago Cal§§, Víctor Quesada, Luis M. Sánchez§, and Carlos López-Otín¶

From the Departamento de Bioquímica y Biología Molecular, Instituto Universitario de Oncología, Universidad de Oviedo, 33006 Oviedo, Spain

We have identified and cloned a fetal liver cDNA encoding a new serine proteinase that has been called matriptase-2. This protein exhibits a domain organization similar to other members of an emerging family of membrane-bound serine proteinases known as type II transmembrane serine proteinases. Matriptase-2 contains a short cytoplasmic domain, a type II transmembrane sequence, a central region with several modular structural domains including two CUB (complement C1r/C1s, complement factor C1r, urchin embryonic growth factor, bone morphogenetic protein) domains and three low density lipoprotein receptor class A (LDLr) domains used for assaying serine proteinases and endog-

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† These authors contributed equally to this manuscript.

‡ These authors contributed equally to this manuscript.

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To whom correspondence should be addressed: Departamento de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad de Oviedo, 33006 Oviedo, Spain. Tel.: 34-985-104201; Fax: 34-985-103564; E-mail: CLO@correo.uniovi.es.

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This article has been withdrawn by the authors upon request from the Journal. In Fig. 6, a duplicated image of the lane corresponding to untreated fibrinogen was used in the PMSF-inactivated Matriptase-2 lane. In Fig. 8, actin panels were reused from previous publications of the group using the same commercial membranes, and the actin panel for the Northern blot containing spleen, thymus, prostate, testis, ovary, intestine, colon, and leukocyte samples was rotated 180 degrees. The liver-specific expression of Matriptase-2/TMPRSS6 shown in Fig. 8 can be compared to independent global RNA-seq studies (GTEx portal, https://www.gtexportal.org/home/gene/TMPRSS6). The authors assert that all of the results reported in this article are valid.

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1 The abbreviations used are: MMP, matrix metalloproteinase; LDLR, low density lipoprotein receptor; TTSS, type II transmembrane serine proteinase; uPA, urokinase-type plasminogen activator; HAT, human airway trypsin-like protease; contig, group of overlapping clones; HA, hemagglutinin; PBS, phosphate-buffered saline; GST, glutathione S-transferase; N-t-Boc, N-t-tert-butoxy-carbonyl; AMC, 7-aminomethylcoumarin.

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in vitro activator of pro-atrial natriuretic peptide, a cardiac hormone essential for the regulation of blood pressure (16, 17). HAT, originally isolated from the sputum of patients with chronic airway diseases, may be involved in the host defense system on the mucous membrane (18). Recently, a HAT-related protease isolated from rat tissues has been found to cleave pro-γ-melanotropin at the adrenal cortex, stimulating the mitogenic actions of this peptide (19). Spinesin, predominantly expressed at synapses, may play specific roles in neural function (20). Finally, insertion of β-satellite repeats into the gene encoding TMPRSS3 causes a form of autosomal recessive deafness, suggesting a role for this protease in the development or maintenance of the inner ear or in the turnover of the protein contents of the perilymph and endolymph (21).

The expression of virtually all TTSPs characterized to date is widely deregulated during the development and progression of tumor processes. Thus, matriptase-MT-SP1 was originally identified in breast cancer cells and is highly expressed in breast, prostate and colorectal cancers (22–25). Inhibition of this protease abolishes both primary tumor growth and metastasis in a murine model of prostate cancer (24, 26), whereas stabilization of active matriptase through glycosylation by N-acetylglucosaminyltransferase V is associated with the pro-metastatic effects of this enzyme (27). Hepsin is overexpressed in ovarian and prostate carcinomas (28–31), and its expression correlates inversely with measures of patient prognosis (32). Epithilisin is also overexpressed in prostate carcinomas, and a mutated form of this protease has been found in a case of aggressive disease (33–35). TMPRSS3/TADG-12 is expressed in ovarian cancer (36), and TMPRSS4 is overexpressed in pancreatic cancer (37). Finally, the matriptase precursor (DESC1) was identified as a consequence of its differential expression in squamous cell carcinoma (38).

These recent findings have led to the search for interactions modulated by these mediators of proteases and their regulation, and as part of our study we have examined the possibility of a large family of membrane proteases dually encoded in human tissues, with the discovery of a novel human member, designated matriptase-2. We describe the molecular cloning of a complete nucleotide sequence of a DNA coding for this protein and report an analysis of its expression in human tissues. We also report the production of recombinant matriptase-2 in Escherichia coli and perform an analysis of its enzymatic activity against synthetic and endogenous substrates. Finally, we demonstrate that matriptase-2 is bound to the cell membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—A human fetal liver cDNA library constructed in ADR2 and Northern blots containing polyadenylated RNAs from different adult and fetal human tissues were from CLONTECH (Palo Alto, CA). Chemicals, reagents, and synthetic and macromolecular substrates for proteases, including fibronectin, fibrinogen, laminin, type I collagen, and type I gelatin, were obtained from Sigma. Single-chain uPA was purchased from OncoGene Research Products (Boston, MA). Recombinant gelatinases A and B were kindly provided by Dr. G. Murphy (University of East Anglia, Norwich, UK). Recombinant collagenase-3 (MMP-13) was produced as described previously (38). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. Synthetic oligonucleotides were prepared in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with [α-32P]dCTP (3000 Ci/mmol) purchased from Amersham Biosciences using a random-priming kit from the same company.

**Bioinformatic Screening of the Human Genome and cDNA Cloning**—The BLAST program was used to search public (www.ncbi.nlm.nih.gov) and private (www.celera.com) human genome data bases, looking for regions with sequence similarity to previously described TTSPs. After identification of a DNA contig in chromosome 22 encoding a region similar to the catalytic domain of matriptase-MT-SP1, we analyzed in this contig the possible presence of regions encoding the remaining domains characteristic of TTSPs. This approach allowed us to identify DNA regions potentially encoding a full-length sequence for a novel member of this family of serine proteases. The nucleotide sequence of a cDNA coding for this protein and report an analysis of its expression in human tissues. We also report the production of recombinant matriptase-2 in Escherichia coli and perform an analysis of its enzymatic activity against synthetic and endogenous substrates. Finally, we demonstrate that matriptase-2 is bound to the cell membrane.
with the different inhibitors. Cleavage of type I collagen, type I gelatin, type I laminin, fibronectin, and fibrinogen by recombinant matriptase-2 was followed by SDS-PAGE. All of the assays were performed in the above described assay buffer for 4–12 h at 37°C. The experiments using type I collagen as a substrate were also performed at 28°C. The enzymatic activity ratio (w/w) used in these experiments was 1/100. Finally, to examine the activation of other proteinases by matriptase-2, the proenzymes of human uPA, plasmin, gelatinase A, and gelatinase B were incubated with active matriptase-2 at a 1:100 w/w enzyme/substrate ratio. The incubations were performed in assay buffer without MeSO for 4–12 h at 37°C. The processing of the different precursor proteins was assessed by SDS-PAGE.

Homology Modeling—A three-dimensional model of the catalytic domain of matriptase-2 was calculated using Swiss-Model, a semi-automated modeling server (40), and analyzed with the Swiss-Pdb Viewer. Briefly, the amino acid sequence of the predicted catalytic domain of matriptase-2 was compared with the sequences of the macromolecules deposited in the Protein Data Bank to identify suitable templates. After ranking the possible templates by sequence similarity to matriptase-2, the resulting models was verified automatically with WhatCheck and manually with Swiss-Pdb Viewer. Electrostatic analyses of the model were performed with MolMol (41). We added hydrogens and utilized a protein dielectric constant of 3. Partial atomic charges were taken from the Amber94 force field. The figures were modeled with MolMol and rendered with Megavop and POV-Ray (www povray.org).

Northern Blot Analysis—Nylon filters containing 2 μg of poly(A)+ RNA of a wide variety of human tissues were prehybridized at 42°C for 3 h in 50% formamide, 5× SSPE (1× SSPE = 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 10× Denhardt’s solution, 2% SDS, 100 μg/ml of denatured herring sperm DNA and then hybridized with radiolabeled matriptase-2-specific probe 2.2 kb long, containing the nucleotides 220–2420 of the isolated cDNA. Hybridization was performed for 20 h under the same conditions used for prehybridization. The filters were washed with 0.1× SSC, 0.1% SDS, 5 × 10-3 M NaCl, and 1 × 10-3 M NaH2PO4, 100°C, and autoradiography. RNA integrity and hybridization with an actin probe confirmed that the cloned human cDNA encoded a novel membrane-bound serine proteinase with a type II transmembrane domain. To generate a cDNA clone for this gene, we performed PCR using a panel of human cDNA libraries, and specific oligonucleotides derived from the identified genomic sequence. A fragment of the expected size (~2.5 kb) containing in-frame initiator and stop codons was amplified from a cDNA library prepared from human fetal liver. After cloning and sequencing the PCR-amplified product, we confirmed by conceptual translation that the generated sequence was distinct from that reported for all previously identified human TTSPs (Fig. 1A). Computer analysis of the full-length cDNA sequence revealed that it codes for a protein of 802 amino acids, with a calculated molecular weight of 88,901 and showing significant sequence similarity to all other human membrane serine proteinases belonging to the TTSP family. Further analysis of the predicted sequence indicated that the minimum percentage of identities (35%) was with matriptase/MT-SP1 and spinesin. The percentage of identities with other members of the TTSP family, such as TMPRSS2, TMPRSS3, TMPRSS4, DESC1, corin, MSPL, and HAT, was of about 30%.

An alignment of the deduced amino acid sequence from the isolated liver cDNA confirmed that the identified protein possesses all domains characteristic of TTSPs (Fig. 1B). Thus, close to the initiator methionine residue there is a hydrophobic domain spanning from positions 44–66 that is not preceded by a recognizable signal sequence. Computer analysis using the TMHMM (transmembrane helices Markov model) program (available at www.cbs.dtu.dk) revealed that this domain is predicted to act as a type II membrane anchor sequence. The transmembrane domain is followed by a stem region containing two CUB domains (the first one substantially degenerate with respect to the consensus CUB) and three LDLR repeats. This stem region is very similar to that present in matriptase, which contains two CUB and four LDLR repeats. Finally, there is a catalytic domain located at the C-terminal region of the identified protein and showing about 45% identities with the equivalent domain of matriptase. This catalytic domain also contains all structural hallmarks of functional serine proteinases (Fig. 2). In fact, an alignment of this sequence with that of other members of this class of proteolytic enzymes allows identification of a prodomain region ending in a conserved Arg-Ile-Val-Gly-Gly motif that is highly conserved in serine proteinases and that contains the Arg-Ile and that is cleaved for protease activation (Fig. 2). The alignment also allows identification of the activation site as that present at position 573 within the Arg-Ile of Asp-Ser-Gly-Gly. The His and Asp residues of the catalytic activity should be those present in the enzyme, respectively. The sequence alignment with the side chains of other members of this family reveals a high level of conservation of the side chains of Cys593, Cys609, Cys724, Cys738, and Cys749–Cys778, a fourth cysteine located in the transmembrane domain spanning from positions 773–791. The putative catalytic domain contains the six conserved cysteine residues of TTSP family members. The formation of disulfide bonds between Cys579–Cys698 of the catalytic domain and Cys559 located at the prodomain. The formation of this predicted disulfide bond observed in many serine proteinases including matriptase would suggest that the active catalytic domain of the identified protein should still remain located at the cell surface even after cleavage at the activation site. Finally, analysis of consensus motifs present in the identified sequence revealed the presence of a potential phosphorylation site in the cytoplasmic tail (Ser-Lys-Arg at position 34) that may participate in the recruitment of intracellular proteins potentially involved in activation of signal transduction pathways. This analysis also revealed six potential sites of N-glycosylation (Asn-Xaa-Thr or Asn-Xaa-Ser) at positions 127, 175, 329, 424, 444, and 509. All of these structural features are also conserved in the amino acid sequence deduced from a mouse cDNA isolated as part of a large scale cDNA sequencing project (42). The protein encoded by this mouse cDNA (accession numbers AK004939 and BAB23684) likely corresponds to the mouse ortholog of the human serine proteinase identified in this work (Fig. 2). The percentages of identities between the human and mouse enzymes were 85.1% in nucleotides and 84.3% in amino acids.

In summary, and according to this structural analysis, we can conclude that the cloned human cDNA encodes a novel membrane serine proteinase that, according to its significant sequence similarity and overall domain organization with matriptase, we propose to call matriptase-2.

Membrane Localization of Matriptase-2—To provide experimental support to the proposal that matriptase-2 is a membrane-bound protease, we transfected COS-7 cells with pcDNA3-matriptase-2-HA, a construct containing the HA epitope at the C-terminal region of the enzyme. Transfected...
cells were then analyzed by immunofluorescence with a mouse monoclonal antibody (12CA5) specific for this viral epitope. As shown in Fig. 3A, a fluorescent pattern surrounding the cell membrane was clearly visualized in transfected cells expressing matriptase-2. Furthermore, we performed SDS-PAGE analysis of lysates from COS-7 cells transfected with the matriptase-2-HA construct, followed by Western blotting detection with anti-HA monoclonal antibody. As can be seen in Fig. 3B, matriptase-2 was detected in the membrane-enriched fractions but not in the soluble fraction. Taken together, these
results provide strong experimental evidence that matriptase-2 is a membrane-anchored protein.

Production of Recombinant Matriptase-2 in E. coli and Analysis of Its Enzymatic Properties—To analyze the enzymatic activity of matriptase-2, we first expressed the catalytic domain of this mosaic membrane-bound protein in bacterial cells. To this purpose, a cDNA coding for the catalytic region was subcloned into the expression vector pGEX-3X, and the resulting plasmid was transformed into the E. coli bacterial strain BL21(DE3)pLysS. After induction of transformed bacteria with isopropyl-1-thio-/beta/-D-galactopyranoside, a protein band of the expected size (52 kDa) was detected by SDS-PAGE analysis of bacterial protein extracts (Fig. 4). This recombinant fusion protein was purified using glutathione-Sepharose chromatography as described previously (43) (Fig. 4). The soluble GST-matriptase-2 fusion protein eluted from the affinity column was directly used for enzymatic analysis. We did not release the catalytic domain of matriptase-2 with Factor Xa, because any putative trace of this factor that could remain after the purification process would interfere with assays to analyze the enzymatic activity of a protein such as matriptase-2, which belongs to the same class of proteolytic enzymes as Factor Xa. However, and similar to the case of a fusion protein containing the catalytic domain of matriptase (26), the GST-matriptase-2 fusion protein was apparently autoactivated after incubation at 37 °C in the course of the different activity assays. Thus, SDS-PAGE analysis of the recombinant fusion protein incubated for 12 h at 37 °C showed the presence of an additional band of 26 kDa that likely corresponds to the catalytic domain of matriptase-2 after proteolytic release of the GST-moiety (data not shown). To analyze the substrate specificity of this recombinant matriptase-2, a series of synthetic quenched fluorescent peptides commonly used for assaying serine proteinases were used. As can be seen in Fig. 5A, the peptides N-t-Boc-Gln-Ala-Arg-AMC and N-t-Boc-Gln-Gly-Arg-AMC were hydrolyzed by matriptase-2 (12.1 and 18.3 nM AMC/min, respectively). Other peptides, including N-t-Boc-Ala-Phe-Lys-AMC, N-t-Boc-Ala-Pro-Ala-AMC, and N-t-Boc-Val-Leu-Lys-AMC, were not signif-

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**Fig. 1—continued**
significantly hydrolyzed by the enzyme. The catalytic activity of the recombinant matriptase-2 was further characterized using N-t-Boc-Gln-Gly-Arg-AMC as a substrate, yielding $K_m = 1.49$. The hydrolytic activity of matriptase-2 against this substrate was substantially abolished by a number of inhibitors of serine proteinases (phenylmethylsulfonyl fluoride, 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin, and aprotinin) but not by EDTA or E-64, inhibitors of metallo- and cysteine-proteinases, respectively (Fig. 5B). Tosyl-L-phenylalanine chloromethyl ketone was also a poor inhibitor of matriptase-2 (Fig. 5A).

**FIG. 2.** Amino acid sequence comparisons of the catalytic domains of matriptase-2 and matriptase. The amino acid sequence of human matriptase-2 identified in this work is shown in **bold**. The sequences for human and mouse matriptase were extracted from the SwissProt data base, whereas the sequence BAB23684 corresponding to the putative mouse matriptase-2 was deduced from a cDNA sequence reported in (42). The multiple alignment was performed with the PILEUP program of the GCG package. Gaps are indicated by **dots**. Common residues to all sequences are **shaded**. The **numbering** corresponds to the sequence of human matriptase-2.

**FIG. 3.** Membrane localization of matriptase-2. A, immunofluorescent detection of matriptase-2 with a monoclonal anti-HA antibody. Fluorescence was localized to the surface of cells transiently transfected with pcDNA3-matriptase-2-HA. B, Western blot analysis from COS-7 cells transfected with the same pcDNA3-matriptase-2-HA vector. The matriptase-2 band was detected in the total extracts (lane 1) and in the plasma membrane fractions (lane 3) but not in the soluble fraction (lane 2). The sizes of the molecular weight markers are shown to the left.

**FIG. 4.** Production of recombinant matriptase-2 in E. coli. 5 μl of bacterial extracts transformed with pGEX-3X (lane 2) or pGEX-3X-mat2 (lane 3) and purified GST-matriptase-2 (lane 4) were analyzed by SDS-PAGE. The sizes of the molecular weight markers (MWM) (lane 1) are shown to the left.

**FIG. 5.** Analysis of enzymatic activity of matriptase-2 on synthetic peptides. A, synthetic fluorescent peptides N-t-Boc-Gln-Ala-Arg-AMC, N-t-Boc-Gln-Gly-Arg-AMC, N-t-Boc-Ala-Phe-Lys-AMC, N-t-Boc-Ala-Pro-Ala-AMC, and N-t-Boc-Val-Leu-Lys-AMC (100 μM) were incubated with 5 μl of matriptase-2 in 50 mM Tris-HCl, 20 mM NaCl, 2.5% Me2SO, pH 8.0, for 4 h at 37 °C, in a final volume of 200 μl. The fluorometric measurements were made at $\lambda_{ex} = 360$ nm and $\lambda_{em} = 460$ nm. B, for inhibition assays, synthetic peptide N-t-Boc-Gln-Gly-Arg-AMC was incubated with matriptase-2 in the presence or absence of 2 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 mM EDTA, 10 mM E-64, 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 0.2 mM tosyl-L-phenylalanine chloromethyl ketone (TPCK), 80 μM leupeptin, 0.2 μM aprotinin, and fluorescence was measured as above.
The substrate specificity of the matriptase-2 catalytic domain is consistent with structural features of this protein. In fact, it is well established that the S1 specificity of proteinases is largely determined by the residues amino acids N-terminal to the active site Ser residue. This position is occupied by an Asp residue in matriptase and matriptase-2, giving rise to a cleavage specificity for substrates with Asp at the P1 position. The presence of a polar Gln residue (position 802) very close to the catalytic Ser is also consistent with specificity for substrates with Arg/Lys at the P1 position. The amino acid sequence similarity between matriptase-2 and human matriptase-2 compared with that corresponding to human matriptase. As can be seen, there is a significant degree of similarity between them around their catalytic site, both showing a deep negatively charged S1 pocket and a similar overall charge distribution (Fig. 7A). The occurrence of this deep S1 pocket in both enzymes is consistent with a somewhat relaxed specificity for the catalytic activity of matriptases when compared with other serine proteinases. An additional structural feature shared by matriptase and matriptase-2 may be deduced from the analysis of the loops surrounding the active site cleft.
Fig. 7. Homology model of the catalytic domain of matriptase-2. A, molecular surface of the catalytic domains of matriptase and matriptase-2 modeled with the peptide Arg-Ser-Ala-Met-Phe in the canonical conformation. The arrow indicates the clash of the P2’ Phe with matriptase-2. Electrostatic potentials lower than −1.8 V are in red, those higher than 1.8 V are in blue, and those that are neutral are in white. Intermediate values are interpolated. B, ribbon representations of the catalytic domains of matriptase-2, and thrombin, showing the 60 loop. The residues Asp60(A) and Asp60(B) and the corresponding matriptase-2, and thrombin, showing the 60 loop. The residues Glu60(A) and Asp60(B) are also shown. These residues are proposed to stabilize the conformation of the 60 loop in both proteins.

DISCUSSION

Over the last years, there has been an increasing interest in the characterization of proteolytic processes localized at the cell surface (47, 48). Most of the membrane-associated proteolytic systems have been metalloproteinases, but very recently, new families of membrane-bound serine proteinases known as TTSPs have received considerable attention because of their potential role in multiple normal and pathological conditions, including cancer. Immunofluorescence and Western blot analysis of COS-7 cells transfected with the isolated cDNA confirmed that matriptase-2 is anchored to the cell surface. In addition, functional analysis of the recombinant catalytic domain of matriptase-2 produced in E. coli provided additional evidence that the isolated cDNA codes for a catalytically active serine proteinase. In fact, the purified recombinant protein exhibits a significant proteolytic activity against fluorogenic substrates used for assaysing the enzymatic properties of this class of proteinases. In addition, this degrading activity was abolished by inhibitors of serine proteinases but not by inhibitors of any other class of proteolytic enzymes. The substrate specificity of matriptase-2 against synthetic peptides is similar to that of matriptase, with N-t-Boc-Gln-Gly-Arg-AMC and N-t-Boc-Gln-Ala-Arg-AMC being the preferred substrates for matriptase-2 and matriptase, respectively (Ref. 49 and this work). Matriptase-2 also shares with matriptase the ability to degrade extracellular matrix components, suggesting that this novel protease may participate in some of the matrix-degrading processes occurring in both normal and pathological conditions, including cancer pro-
Interestingly, several TTSP genes lie on the long arm of human chromosome 11; spinepsin, TMPRSS4, and MSPL genes are clustered in 11q23, whereas the gene for matriptase is located at 11q25. Similarly, three TTSP genes are located on chromosome 21, TMPRSS2 and TMPRSS3 genes are located at 21q22, and the gene for enteropeptidase is located at 21p11. Likewise, three TTSP genes are located at chromosome 4, HAT and DESC1 are located at 4q13, and corin is located at 4p12. Finally, the hepsin gene maps to 19q13 in a region containing several genes encoding serine proteinases (55). It is worthwhile mentioning that the region containing the matriptase-2 gene is frequently altered in several human tumors, such as insulinomas, ependymomas, and breast and colorectal carcinomas (56–59). Genetic lesions in the 22q13 region have been also linked to diverse diseases including schizophrenia susceptibility (60). It will be of future interest to examine the possibility that the matriptase-2 gene could be a direct target of some of these genetic abnormalities.

Finally, in this work, as a step to try to define the physiological role of matriptase-2, we have examined the distribution of this protein in human tissues. Similar to the case of most TTSPs, matriptase-2 expression in normal tissues is very restricted, being only detected in significant amounts in fetal and adult liver. This might explain the role for this enzyme in some of the pathologies occurring in this tissue during development or in adult life as proposed for other proteases (61, 62). These observations suggest that matriptase-2 in liver may also play a role in physiological processes. Furthermore, the above mentioned structural differences between both proteases could serve to guide the search for specific inhibitors of each enzyme (52). The functional role of the CUB and LDLR repeats of matriptase (44), although the catalytic domain of matriptase (4), and only contain one or two modular repeats in their respective domains that can strongly influence the substrate specificity and catalytic activity of TTSPs. Accordingly, further studies with full-length matriptase-2 produced in eukaryotic expression systems will be required to provide additional information about the nature of the diverse macromolecular structures presumably targeted by this enzyme.

To further characterize the structure of the catalytic domain of matriptase-2, we performed a homology model for this novel enzyme. The recombinant protein shows a low specific activity and lacks the ancillary noncatalytic domains that can strongly influence the substrate specificity and catalytic activity of TTSPs. Furthermore, the above mentioned structural differences between both proteases could also be consistent with distinct catalytic properties for this novel enzyme. Finally, the identification of a putative murine ortholog of human matriptase-2 raises the possibility of generating mice deficient in this gene, as recently described for matriptase (63). These mutant mice could contribute to clarify the role of matriptase-2 in physiological processes.

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