Identification and Characterization of Human Endometase (Matrix Metalloproteinase-26) from Endometrial Tumor*

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Hyun I. Park†, Jian Niš, Ferry E. Gerkema‡, Ding Liu§, Vladimir E. Belozerov,‡ and Qing-Xiang Amy Sang¶†

From the ‡Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306-4390 and §Human Genome Sciences, Inc., Rockville, Maryland 20850-3338

We report the discovery, cloning, and characterization of a novel human matrix metalloproteinase 26 (MMP-26) (matrixin) gene, endometase, an endometrial tumor-derived metalloproteinase. Among more than three million expressed sequence tags sequenced, the endometase gene was only obtained from human endometrial tumor cDNA library. Endometase mRNA was expressed specifically in human uterus, not in other tissues/cells tested, e.g. testis, heart, brain, lungs, liver, thymus, and melanoma G361. Endometase protein has a signal peptide, a propeptide domain, and a catalytic domain with a unique "cysteine switch" propeptide sequence, PRCG(V/N)PD, and a zinc-binding motif, VAT-HEIGHSLGLQH. Endometase is 43, 41, and 39% identical to human metalloelastase, stromelysin, collagenase-3, and matrilysin, respectively. The zymogen was expressed and isolated from Escherichia coli as inclusion bodies with a molecular mass of 28 kDa. The identity and homogeneity of the recombinant protein was confirmed by protein N-terminal sequencing, silver stain, and immunoblot analyses. The pro-enzyme was partially activated during the folding process. Endometase selectively cleaved type I gelatin and α1-proteinase inhibitor; however, it did not digest collagens, laminin, elastin, β-casein, plasminogen, soybean trypsin inhibitor, or Bowman-Birk inhibitor. It hydrolyzed peptide substrates of matrixins and tumor necrosis factor-α converting enzyme. Endometase may selectively cleave extracellular matrix proteins, inactivate serpins, and process cytokines.

Matrix metalloproteinases (MMPs)§ (also known as matrixins) are a family of highly homologous zinc metalloenzymes that digest extracellular matrix proteins and process pro-growth factors and cytokines; their activities are inhibited by tissue inhibitors of metalloproteinases (1). Matrixins may play many important physiological and pathological functions, including reproduction, angiogenesis, development, morphogenesis, tissue remodeling, arthritis, cardiovascular diseases, neurologic diseases, and cancer progression and metastasis (1–4). Knowledge about the structure-function relationship of the matrixins has been growing rapidly during past decade. New members of the matrixin family have been discovered continuously (1). So far, at least 22 MMPs have been reported; the most recent two are MT5-MMP/ MMP-24 (5, 6) and MT6-MMP/MMP-25 (7, 8). All the matrixins have a propeptide domain containing the highly conserved cysteine switch sequence PRG(V/N)PD and a catalytic domain with a zinc-binding motif, HEXHXHXXGXXH (1).

We report a novel member of the matrixin family, endometase (MMP-26), which was cloned from human endometrial tumor cDNA library. Its mRNA was detected only in human uterus tissue among all the human tissues tested. Endometase has a pro-domain with a unique cysteine switch sequence, PHCGVPDGSD, and a catalytic domain with the common zinc-binding motif. This new MMP has a different substrate profile from other closely similar MMPs. Endometase selectively cleaves rat tail tendon type I (RTTI) gelatin and human plasma α1-proteinase inhibitor (α1-PI). Our data show that endometase is an authentic new member of the MMP family with unique biochemical characteristics and substrate specificities. Endometase may be tissue-specific and may have unique biological and pathological functions.

EXPERIMENTAL PROCEDURES

Materials—Human α1-antitrypsin (α1-PI) and most chemicals and reagents were purchased from Sigma. Human matrilysin, stromelysin, and recombinant catalytic domain of collagenase-3 (cd-collagenase-3) were kindly provided by Drs. Harold E. van Wart of Roche Bioscience, L. Jack Windsor of Indiana University, and Harald Tschesche of the University of Bielefeld, respectively. Recombinant rabbit catalytic domain of metalloelastase (cd-metalloelastase) was kindly provided by Dr. C. Bruun Schiødt, OsteoPro A/S.

Molecular Cloning of Endometase Full-length cDNA Sequence—A data base of more than three million expressed sequence tags (ESTs) was obtained from over 750 different cDNA libraries. This data base has been generated through the combined efforts of Human Genome Science Inc. and the Institute for Genomic Research using high throughput automated DNA sequence analysis of randomly selected human cDNA clones (9). Sequence homology comparisons of each EST were performed against the GenBank™ data base using the Basic Local Alignment Search Tool (BLAST) and BLASTN (BLAST for nucleic acid) algorithms (10). A specific homology and motif search using the known amino acid sequence of matrilysin against this human EST data base revealed several ESTs that had translated sequences with >38% identity to that of matrilysin. One clone, HETBW05, from human endometrial tumor cDNA library encoding an intact N-terminal signal peptide and the
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Fig. 1. Nucleotide and deduced protein sequence of the full-length cDNA clone encoding human endometase (GenBankTM accession number AF248646). The predicted signal peptide, the propeptide domain, and the catalytic domain are separated by an arrow-head. The highly conserved cysteine switch sequence and the zinc-binding motif for MMPs are underlined. The unique histidine residue in the catalytic domain. Represent ones that are highly conserved in the propeptide domain and the zinc-binding motif for MMPs are boldface. Underlined sequences were selected as antigens.

Northern Blot Analysis—Five Northern blot membranes containing 2 µg of poly(A)+ RNAs per lane from human multiple tissues, human amniotic fluid, and 250 rabbits were incubated with 32P-labeled HETBW05 cDNA probe and the human β-actin cDNA positive control probe. Northern blotting was performed with the ExpressHyB™ hybridization solution (PT1190–1) from CLONTECH following the manufacturer's manual.

Expression of Pro-endometase in Escherichia coli Cells and Refolding of the Denatured Protein—The full-length open reading frame of the cDNA clone encoding human endometase (GenBank™ accession number AF248646) was confirmed.

The full-length protein was selected for further investigation. This EST was sequenced on both strands to the 3'-end, and its homology to matrixins was confirmed.

The predicted signal peptide, the propeptide domain, and the catalytic domain are separated by an arrow-head. Underlined sequences were selected as antigens. Three potential N-glycosylation sites are both italic and underlined. A segment of the nucleic acid sequence (nucleotides 1–36, underlined) of the noncoding region may be of vector origin based on the VecScreen program.

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Fig. 2. Multiple sequence alignment of human endometase and five most similar human MMPs. This alignment was determined using the Genetics Computer Group Wisconsin Package, Version 9.1, Madison, WI, 1997) program PILEUP with a default gap weight of 8 and a gap length weight of 2 based on the full protein sequences, including the signal peptides. Boldface amino acid residues represent ones that are highly conserved in the propeptide domain and the catalytic domain. Underlined sequences were selected as antigens to make antibodies. The five most similar human MMPs are macrophage metalloelastase (MMP-12, GenBank™ accession number P39900) (19), stromelysin 1 (MMP-3, accession number NP_002413.1) (20), collagenase 3 (MMP-13, accession number NP_002418.1) (21), membrane-type 1 matrix metalloproteinase (MMP-20) (22) (accession number NP_004762.1), and matrixin 4 (MMP-7, accession number P09237) (23).

Immunological and Biochemical Characterization of Endometase—Anti-human endometase peptide polyclonal antibodies were developed in rabbits as described (11). Polyclonal antibody against endometase peptide starting from residue 188 (pAb-E188) was directed against a peptide just a few residues upstream the zinc binding site,188DKNE-2054105 of the prepro-enzyme. Using BLAST search method at the National Center for Biotechnology Information web site against all of the sequences in the data banks, no peptide with 45% level of identity was found. Thus, the antibody against this peptide should be very specific. Western blot analysis and SDS-PAGE experiments characterizing different refolded endometase samples were performed as described previously (11, 12). Protein substrates were digested by enolase.

0.05% Brij-35, pH 7.5, for 16 h. Several other folding conditions were also tested.
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TABLE I

|               | Endometase | Metalloelastase | Stromelysin | Collagenase-3 | Enamelysin |
|---------------|------------|----------------|-------------|---------------|------------|
| Metalloelastase| 43 (48)    | 53 (62)        | 51 (61)     | 46 (56)       | 48 (55)    |
| Stromelysin   | 41 (48)    | 48 (55)        | 46 (55)     | 46 (56)       | 48 (55)    |
| Collagenase-3 | 41 (49)    | 44 (52)        | 50 (56)     | 48 (56)       | 48 (55)    |
| Enamelysin    | 39 (46)    | 48 (57)        | 50 (56)     | 48 (56)       | 48 (55)    |
| Matrilysin    | 39 (46)    | 48 (57)        | 50 (56)     | 48 (56)       | 48 (55)    |

FIG. 3. Northern blot analysis of endometase gene expression in eight different human tissues. A Northern blot membrane containing 2 µg of poly(A)⁺ RNAs per lane from human multiple tissues was obtained from CLONTECH. Two µg of 2.0-kb human β-actin poly(A)⁺ RNAs per lane was a positive control. The membrane was probed with both the 3²P-labeled HETBW05 cDNA probe and the human β-actin cDNA positive control probe. Northern blotting was performed with the ExpressHyb™ hybridization solution (PT1190–1) from CLONTECH following the manufacturer’s manual. The endometase RNA is a single 1.05-kb band, and the β-actin RNA is a single 2.0-kb band.

mometase and the products were detected by SDS-PAGE gel with Coomassie Blue staining (12–14). Pro-endometase and α,-PI digestion products were also analyzed by protein N-terminal sequencing (12–14).

Simthetic Fluorogenic Peptide Substrate Cleavage Assays—The following substrates were purchased from BACHEM Bioscience Inc.: substrate 1, Mca-PLGLDpaAR-NH₂ (15); substrate 2, Mca-PKPLADpaAR-NH₂; substrate 3, Mca-RPKPYANvaWMK2,4-dinitrophenyl)-NH₂ (16); substrate 4, Mca-RPKPVENvaWRK2,4-dinitrophenyl)-NH₂ (16); and substrate 5, Mca-PLAQAVDpaRSSSRR-NH₂ (17), where Nva represents norvaline. The first four are MMP substrates, and the last one is a substrate of the tumor necrosis factor-α converting enzyme (TACE). Substrates were prepared as 50–500 µM stock solution in 1:1 dimethyl sulfoxide (Me₂SO) and water. Fluorescent assays were performed at λ excitation = 328 nm and λ emission = 393 nm using a Perkin-Elmer LS 50B luminescence spectrometer equipped with a constant-temperature water bath. The relationship between fluorescence units and nmol of product produced was determined from the fluorescence value obtained when all the substrate was hydrolyzed. Assays for obtaining kinetic parameters were performed at 25 °C in 10 mM CaCl₂, 0.2 mM NaCl, and 0.05% Brij-35 in 50 mM HEPES, pH 7.5, over a substrate concentration range of 1–4 µM and an enzyme concentration range of 0.06–50 nM under steady-state conditions. Stock solutions of MMPs were diluted to 1–500 nM HEPES buffer containing 10 mM CaCl₂, 0.2 mM NaCl, and 0.05% Brij-35 or 50 mM Tricine buffer with the same constituents. A typical assay was carried out by incubating 186 µl of buffer solution and 4 µl of substrate solution in an assay cuvette at least for 15 min at 25 °C and then adding 10 µl of enzyme solution to the assay cuvette. Initial hydrolysis rates were monitored for 10–30 min.

RESULTS

Molecular Cloning of Endometase and Homology of Endometase to Other MMPs—By searching EST data bases, we found one clone, HETBW05, from a human endometrial tumor cDNA library, that encodes a MMP homologue with an intact N-terminal signal peptide. This clone was not found in any other cDNA libraries derived from other human tissues/cells. The protein expressed from this clone was selected for further investigation, and it was named endometase (for endometrial tumor-derived matrix metalloproteinase).

The cDNA nucleotide and deduced protein sequences of the full-length clone encoding human endometase are shown in Fig. 1. Those sequences were submitted to GenBank™ with accession number AF248646. The signal peptide sequence was predicted according to its homology to other MMPs and using the PSORT computer program. The activation cleavage site, between the propeptide domain and the catalytic domain was predicted according to its homology to other MMPs (18). Endometase has a unique histidine residue instead of an arginine residue in the cysteine switch sequence (PHCQVPDGS). All other MMPs have an arginine residue at that position. In addition, endometase has the MMP zinc-binding consensus sequence in the catalytic domain. The endometase protein sequence was scanned against the GenBank™ data bases, and all of the sequences with significant relatedness to the new sequence were identified. The endometase protein sequence was compared with those of five most similar human MMPs, macrophage metalloelastase (MMP-12, GenBank™ accession number P39900) (19), stromelysin 1 (MMP-3, accession number NP_002413.1) (20), collagenase 3 (MMP-13, accession number...
endometase was incubated with \(4\) \(\mu\)g of \(\alpha\)-PI alone was incubated in 27 \(\mu\)l of assay buffer for 0 and 3 days (lanes 2 and 3, respectively), at 37 °C; 320 \(\mu\)g of endometase was incubated with 4 \(\mu\)g/ml gelatin in 27 \(\mu\)l of assay buffer for 0 and 3 days (lanes 4 and 5, respectively); and 320 \(\mu\)g of \(\alpha\)-PI by endometase. The specificity of pAb-E188 was verified because it did not recognize human stromelysin, collagenase-1, collagenase-3, matrilysin, MT1-MMP, gelatinase A, or gelatinase B (data not shown). Protein N-terminal sequencing further confirmed that we have isolated the pro-endometase with one extra initiation codon coded methionine residue, MVPVPAAADH.

**Hydrolysis of Protein Substrates by Endometase—Heat-denatured RTTI collagen (gelatin) was cleaved slowly by endometase compared with that by matrilysin (Fig. 4). 275 ng of endometase and matrilysin was incubated with 4 mg/ml gelatin for 0, 1, 2, and 3 days at 37 °C in 100 \(\mu\)l of assay buffer containing 50 mM HEPES, 0.2 mM NaCl, 10 mM CaCl\(_2\), and 0.05% Brij-35, pH 7.5. Endometase cleaved RTTI gelatin and generated three-quarter, one-quarter, and other fragments of \(\alpha\)-I and \(\alpha\)-II subunits. However, endometase did not cleave RTTI collagen, mouse type IV collagen, laminin, bovine elastin, \(\beta\)-casein, or human milk lactoferrin, demonstrating that the endometase cleavage of type I gelatin is specific. Human plasma \(\alpha\)-PI (63 kDa) was cleaved by endometase and generated a visible band at about 58 kDa (Fig. 5). Both the 63- and the 58-kDa protein bands have the same sequence, EDPGQDAAQQ, as determined by the N-terminal sequencing technique, suggesting that \(\alpha\)-PI was cleaved near the C terminus by removing a 5-kDa fragment. This sequence represents the N-terminal sequence of the mature \(\alpha\)-I PI protein (24). However, endometase did not cleave other two serine proteinase inhibitors (serpins) tested, soybean trypsin inhibitor and trypsin-chymotrypsin inhibitor (Bowman-Birk inhibitor). Moreover, endometase did not digest any of the other human plasma proteins tested (albumin, \(\alpha\)-1-acid glycoprotein, apo-transferrin, small intestine, colon, peripheral blood leukocytes, and peripheral blood leukocytes. 2 kb of human \(\beta\)-actin poly(A)\(^+\) RNA per lane was a positive control for all the Northern blot membranes. The membrane was probed with both \(^{32}\)P-labeled HETBW05 cDNA probe and the human \(\beta\)-actin cDNA positive control probe. Except for 2-kb \(\beta\)-actin bands, no detectable band was found in blots 1–4. Endometase mRNA was expressed only by human uterus as a 1.03-kb band (Fig. 3) in blot 5; it was not expressed in any other human tissues or human cancer cells tested. Because endometrium is the mucous membrane lining of the uterus, these data demonstrate that endometase may be uterus-specific.

**Biochemical and Immunological Analysis of Endometase Protein Samples—**The pro-endometase inclusion bodies were isolated and purified using B-PER\textsuperscript{TM} bacterial protein extraction and purification reagent. Lysozyme was used for preparation of the inclusion bodies. This method allowed us to obtain highly homogenous endometase from the inclusion bodies as demonstrated by SDS-PAGE silver stain gel and immunoblot analysis using our anti-catalytic domain peptide antibody (pAb-E188) (data not shown). The purified recombinant pro-endometase was partially activated containing both latent (28 kDa) and active (19 kDa) species during the folding process. The specificity of pAb-E188 was verified because it did not recognize human stromelysin, collagenase-1, collagenase-3, matrilysin, MT1-MMP, gelatinase A, or gelatinase B (data not shown). Protein N-terminal sequencing further confirmed that we have isolated the pro-endometase with one extra initiation codon coded methionine residue, MVPVPAAADH.

**Hydrolysis of synthetic peptide substrates of matrixins (nos. 1–4) and that of tumor necrosis factor-\(\alpha\) converting enzyme (no. 5) by endometase, \(\text{cd-metalloelastase}, \text{stromelysin, cd-collagenase-3, and matrilysin}\)**

Assays were performed at 25 °C in 50 mM HEPES, 0.2 mM NaCl, 10 mM CaCl\(_2\), 0.05% Brij-35, pH 7.5. [S] = 1–4 \(\mu\)M, [endometase] = 19–48 nM, [cd-metalloelastase] = 0.06–1.2 nM, [stromelysin] = 0.7–7 nM, [cd-collagenase-3] = 25–50 nM, and [matrilysin] = 0.2–1 nM.

| Fluorogenic peptide substrate | Endometase | Metalloelastase | Stromelysin | Collagenase-3 | Matrilysin |
|------------------------------|------------|----------------|-------------|--------------|-----------|
| 1. Mca-PLGLDpa-AR-NH\(_2\)   | 1.85 \(\times\) 10\(^2\) | 6.10 \(\times\) 10\(^2\) | 6.50 \(\times\) 10\(^2\) | 2.52 \(\times\) 10\(^2\) | 1.31 \(\times\) 10\(^2\) |
| 2. Mca-PKRLALDpa-AR-NH\(_2\) | 3.43 \(\times\) 10\(^2\) | 9.88 \(\times\) 10\(^2\) | 4.33 \(\times\) 10\(^2\) | 6.83 \(\times\) 10\(^3\) | 3.29 \(\times\) 10\(^3\) |
| 3. Mca-RPKFPAVNa-WMK(Dnp)-NH\(_2\) | 1.67 \(\times\) 10\(^2\) | 2.43 \(\times\) 10\(^2\) | 2.07 \(\times\) 10\(^2\) | 4.08 \(\times\) 10\(^2\) | 2.83 \(\times\) 10\(^2\) |
| 4. Mca-RPKPVEVNa-WRK(Dnp)-NH\(_2\) | 1.60 \(\times\) 10\(^2\) | 8.33 \(\times\) 10\(^3\) | 8.02 \(\times\) 10\(^4\) | <10 | 5.62 \(\times\) 10\(^2\) |
| 5. Mca-PLAQAVDpa-RSSSR-NH\(_2\) | 1.85 \(\times\) 10\(^2\) | 1.30 \(\times\) 10\(^4\) | 1.20 \(\times\) 10\(^5\) | <10 | 3.42 \(\times\) 10\(^3\) |
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and plasminogen), further showing that the hydrolysis of α1-PI by endometase is specific.

Hydrolysis of Fluorogenic Synthetic Peptide Substrates by Endometase—Endometase hydrolyzed four synthetic MMP peptide substrates and one TACE substrate (Table II). It hydrolyzed the common MMP substrate, Mca-PLGDP-Arg-NH$_2$ (15), with a $k_{cat}/K_m$ value of 1850 m$^{-1}$ s$^{-1}$, which is similar to those of stromelysin and the cd-collagenase-3 but is much lower than that of matrilysin and cd-metalloelastase. Endometase also digested the other three MMP substrates. Interestingly, it has the ability to hydrolyze the TACE substrate with a $k_{cat}/K_m$ value of 185 m$^{-1}$ s$^{-1}$, which is much lower than that of cd-metalloelastase (13,000 m$^{-1}$ s$^{-1}$).

DISCUSSION

We have cloned and identified a novel member of the MMP family, endometase/MMP-26. Its prepro-enzyme has 261 amino acid residues, making it the smallest member of the matrixin family (Figs. 1 and 2 and Table I). Endometase has a unique cysteine switch sequence, P$^2$H$^G$V$^P$DP$^G$DS, and a zinc-binding consensus sequence in the catalytic domain, HEGHXHXXGXXH. It also has three potential N-glycosylation sites. One is at residue Asn$^{64}$ of the propeptide domain; the other two are at the residues Asn$^{193}$ and Asn$^{221}$, distributed within the catalytic domain. The possibility of N-glycosylation of endometase in human and mammalian systems remains to be investigated. This unique enzyme was only cloned from a human endometrial tumor cDNA library, and its mRNA was only detected in human uterus, not in the other tissues and cancer cell lines tested, indicating a possible tissue-specific distribution and function of endometase.

Endometase has a unique protein substrate specificity. Endometase cleaved gelatin and generated three-quarter, one-quarter, and other fragments of α1(I) and α2(I) subunits (Fig. 4), indicating that it may have gelatin cleavage sites identical or similar to those of human interstitial collagenase (MMP-1) and frog collagenase-4 (MMP-18) (13). Although the catalytic domain of endometase was found to be 49% identical to that of human collagenase-3, it did not dissolve type I collagen, possibly due to the presence of a hemopexin-like domain. Endometase also did not cleave type IV collagen, laminin, elastin, ß-casein, and lactoferrin, demonstrating that the endometase cleavage of type I gelatin was specific. Although the catalytic domain of endometase was found to be 54, 51, and 46% identical to those of metalloelastase, stromelysin, and matrilysin, respectively, endometase did not hydrolyze type IV collagen, elastin, laminin, plasminogen, or ß-casein, further illustrating that endometase may have its unique substrates.

Endometase selectively hydrolyzed human plasma α1-PI (Fig. 5). It did not cleave two other serpins, soybean trypsin inhibitor and Bowman-Birk inhibitor. Moreover, endometase did not digest any other human plasma proteins tested, e.g. albumin, α2-acid glycoprotein, transferrin, and plasminogen, further showing that the hydrolysis of α1-PI by endometase was specific. Our results suggested that the α1-PI protein was cleaved by endometase near the C terminus by removing a 5-kDa fragment. Interestingly, α1-PI protein was cleaved by MMP-1 at two sites near the C terminus, Phe$^{375}$-Leu$^{376}$ and Pro$^{357}$-Met$^{358}$ of the mature α1-PI protein, which indicated that a fragment of about 4.5 kDa was removed from the C terminus (25). Stromelysin-3 also inactivated α1-PI by cleaving Ala$^{350}$-Met$^{351}$ within the reactive site loop in breast cancer (26). Therefore, one of the potential functions of endometase would be selective inactivation of serpin, thus promoting serine proteinase activity and enhancing extracellular matrix degradation in endometrial cancer.

The peptide substrate specificity of endometase was tested and compared with four more similar MMPs, cd-metalloelastase, stromelysin, cd-collagenase-3, and matrilysin (Table II). Endometase hydrolyzed four synthetic MMP peptide substrates and one TACE substrate. Endometase shares some of the same substrates as other MMPs; however, it has its own unique catalytic specificities and efficiencies. In addition, endometase hydrolyzed a TACE substrate. Interestingly, matrixin has been reported to release tumor necrosis factor-α in a herniated disc resorption model (27). The possibility of processing pro-tumor necrosis factor-α by endometase warrants further investigation. Moreover, our preliminary studies showed that endometase was inhibited by tissue inhibitors of metalloproteinases-1 and -2, further confirming that endometase is an authentic matrixin. Our data suggest that endometase may be uterus-specific and may have unique biochemical characteristics and physiological and pathological functions.

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2 F. Yu and Q.-X. A. Sang, unpublished results.