Peptide Substrate Specificities and Protein Cleavage Sites of Human
Endometase/Matriplysin-2/Matrix Metalloproteinase-26†

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**Abbreviations**

$\alpha_1$-PI, $\alpha_1$-protease inhibitor, $\alpha_1$-antitrypsin, $\alpha_1$-antiproteinase; Brij-35, polyoxyethylene lauryl ether; cha, $\beta$-cyclohexylalanyl; Dnp, 2,4-dinitrophenyl; Dpa, N-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; DTT, dithiothreitol; ECM, extracellular matrix; EST, expressed sequence tag; HEPES, $N$-2-hydroxyethylpiperazine-$N'$-2-ethane sulfonate; Mca, (7-methoxycoumarin-4-yl)acetyl; 2-ME, 2-mercaptoethanol; IC$_{50}$, inhibitor concentration at 50% enzyme activity; IGFBP-1, insulin-like growth factor-binding protein-1; $K_i$, inhibitor dissociation constant; MALDI TOF MS, Matrix Assisted Laser Desorption Ionization, Time of Flight Mass Spectrometry; MMP, matrix metalloproteinase; MMP-1, interstitial collagenase; MMP-2, gelatinase A; MMP-3, stromelysin-1; MMP-7, matrilysin; MMP-8, neutrophil collagenase; MMP-9, gelatinase B; MMP-11, stromelysin-3; MMP-12, metalloelastase; MMP-14 (MT1-MMP), membrane-type 1 matrix metalloproteinase; MMP-26, endometase, matrilysin-2; Nva, nor-valine; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TACE, tumor necrosis factor-$\alpha$ (TNF-$\alpha$) converting enzyme; TIMP, tissue inhibitor of metalloproteinases; Tricine, $N$-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
SUMMARY

Human endometase/matrilysin-2/matrix metalloproteinase-26 (MMP-26) is a novel epithelial and cancer specific metalloproteinase. Peptide libraries were used to profile the substrate specificity of MMP-26 from the P4 to P4′ sites. The optimal cleavage motifs for MMP-26 were Lys-Pro-Ile/Leu-Ser (P1)-Leu/Met (P1′)-Ile/Thr-Ser/Ala-Ser. The strongest preference was observed at the P1′ and P2 sites where hydrophobic residues were favored. Proline was preferred at P3 and Serine at P1. The overall specificity was similar to that of other MMPs except that more flexibility was observed at P1, P2′, and P3′. Accordingly, synthetic inhibitors of gelatinases and collagenases inhibited MMP-26 with similar efficacy. A pair of stereoisomers had a only 40-fold difference in $K_{\text{app}}$ values against MMP-26 compared to a 250-fold difference against neutrophil collagenase, indicating that MMP-26 is less stereo-selective for its inhibitors. MMP-26 auto-digested itself during the folding process; two of the major autolytic sites were Leu$_{49}$-Thr$_{50}$ and Ala$_{75}$–Leu$_{76}$, which still left the cysteine switch sequence (PH$_{C82}$GVPD) intact. This suggests that Cys$_{82}$ may not play a role in the latency of the zymogen. Interestingly, inhibitor titration studies revealed that only approximately 5% of the total MMP-26 molecules was catalytically active, indicating that the thiol groups of Cys$_{82}$ in the active molecules may be dissociated or removed from the active site zinc ions. MMP-26 cleaved Phe$_{352}$–Leu$_{353}$ and Pro$_{357}$–Met$_{358}$ in the reactive loop of alpha 1 proteinase inhibitor and His$_{140}$–Val$_{141}$ in insulin-like growth factor binding protein-1, likely rendering these substrates inactive. Among the fluorescent peptide substrates analyzed, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ displayed the highest specificity constant (30000 / Molar second) with MMP-26. This report proposes a working model for the future studies of proMMP-26 activation, the design of inhibitors, and the identification of optimal physiological and pathological substrates of MMP-26 in vivo.
INTRODUCTION

Matrix metalloproteinases (MMPs) share a conservative metal binding sequence of HEXGHXXGXXHS and a turn containing methionine (1). Evidence suggests that MMPs may play important roles in extracellular matrix (ECM) remodeling in physiological processes (2,3). Excessive breakdown of the ECM by MMPs is observed in pathological conditions, including periodontitis, rheumatoid arthritis, and osteoarthritis. MMPs also participate in tumor cell invasion and metastasis by degrading the basement membrane and other ECM components and allowing the cancer cells to gain access to blood and lymphatic vessels (4). Analyses of a large number of peptide and protein substrates and more recent work with phage display and synthetic peptide libraries have led to the identification of consensus cleavage site motifs for a number of different MMPs (5–13). The substrate specificities of MMPs are quite similar to each other, showing strong preferences for hydrophobic residues at P1’. Although distinct MMPs often prefer the same type of amino acid residues at corresponding positions surrounding the cleavage site, differences in the orders of preference for specific residues at each position may more precisely determine MMP specificity for substrates.

Endometase (matrilysin-2/MMP-26) is the smallest member of the MMP family, with a molecular mass of 28 kDa (14–17). Sequence homology calculations identified metalloelastase (MMP-12) and stromelysin-1 (MMP-3) as the closest relatives. Nevertheless, the specificity constant profile of peptide substrates with MMP-26 was quite different from that with MMP-12 and MMP-3 (14). According to protein substrate studies in vitro, MMP-26 might process matrix proteins, such as fibronectin, vitronectin, fibrinogen, type IV collagen, gelatinase B (MMP-9), and gelatin (14–17).
MMP-26 has been found to be highly expressed in several cancer cell lines. A significant level of expression in normal tissues was found only in the uterus and placenta. The limited occurrence of MMP-26 in normal tissues suggests that the production of this enzyme may be strictly regulated during specific events, such as implantation, and that MMP-26 could be a target enzyme for the treatment of cancer and other pathological conditions.

The biological function and substrate specificity of MMP-26 are not yet fully understood. According to the protein substrate studies in vitro, it may participate in ECM degradation. In this study, we take a step forward toward understanding the biochemical properties and functions of MMP-26 by identifying the cleavage sites of protein and peptide substrates, characterizing the substrate specificities of MMP-26, and measuring the potencies of synthetic inhibitors.

**EXPERIMENTAL PROCEDURES**

Materials—Dnp-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH, Dnp-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-OH, Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH₂, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂, insulin-like growth factor binding protein-1 (IGFBP-1), and MMP-specific synthetic inhibitors were purchased from Calbiochem, and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH₂ and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH₂ were purchased from Bachem. Hydroxamic acid derivatives of amino acids, buffers, cysteine, α₁-protease inhibitor (α₁-PI), and 1,10 phenanthroline were purchased from Sigma Chemical Co. Metal salts, Brij-35, sodium dodecyl sulfate, dithioerythreitol, and 2-mercaptoethanol were purchased from Fisher Biotech. Peptide libraries were synthesized at the Tufts University Core Facility (Boston, MA) as described (12).

*Preparation of partially active MMP-26*— MMP-26 was expressed in the form of inclusion bodies from transformed *E. Coli* cells as described before (14). The inclusion bodies...
were isolated and purified using B-PER™ bacterial protein extraction reagent according to the manufacturer’s instructions. The insoluble protein was dissolved in 8 M urea to about 5 mg/ml. The protein solution was diluted to ~100 µg/ml in 8 M urea and 10 mM DTT for 1 hour, dialyzed in 4 M urea, 1 mM DTT, 50 mM HEPES or Tricine, at pH 7.5 for at least 1 hour, then folded by dialysis in buffer containing 50 mM HEPES or Tricine, 0.2 M NaCl, 10 mM CaCl₂, 20 µM ZnSO₄, 0.01% Brij-35, pH 7.5 for 16 hours. To enhance the activity of MMP-26, the folded enzyme was dialyzed twice for 24 hours at 4°C in the folding buffer without Zn²⁺ ion. The total enzyme concentration was measured by UV absorption using ε₂₈₀ = 57130 M⁻¹ cm⁻¹, which was calculated by Genetics Computer Group software.

**Peptide library methods**—The methods were performed as described previously (12). In brief, to determine the specificity for the primed positions (18), an amino-terminally acetylated dodecamer peptide mixture (1 mM) consisting of a roughly equimolar mixture of the 19 naturally occurring L-amino acids, excluding cysteine, at each site was incubated with MMP-26 in 50 mM HEPES, pH 7.4, 200 mM NaCl, 5 mM CaCl₂ at 37°C until 5 to 10% of the peptides were digested. An aliquot (10 µL) of the mixture was subjected to automated amino-terminal peptide sequencing. The data in each sequencing cycle was normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicates the average value. Undigested peptides and the amino-terminal fragments of digested peptides are amino-terminally blocked and therefore do not contribute to the sequenced pool.

Specificity of the unprimed side was determined by libraries with the sequence MAXXXXXXLRAERE(K-biotin) for the P3 site and MAXXPPXXLRGGEE(K-biotin) for other sites, where X represents a degenerate position, K-biotin is ε-(biotinamidohexanoyl)lysine, and the N terminus is unblocked. Libraries were partially digested with MMP-26 as described
above, quenched with EDTA (10 mM) and treated in batch with 400 µL avidin agarose resin (Sigma). The mixture was transferred to a column, which was washed with 25 mM ammonium bicarbonate. The unbound fraction was evaporated to dryness under reduced pressure, suspended in water, and sequenced. Data were normalized as described above.

**Kinetic assays**—Assays of fluorescent peptide substrates were performed by following the procedures reported in the literature (14, 29). For substrates containing the tryptophan residue, the fluorescence was observed at an excitation wavelength of 280 nm and emission wavelength of 360 nm, and for substrates containing 3-methoxycoumarin, fluorescence was measured at an excitation wavelength of 328 nm and emission wavelength of 393 nm. All of the kinetic experiments were conducted in 50 mM HEPES buffer containing 10 mM CaCl₂, 0.2 M NaCl, and 0.01% Brij-35. To assess inhibition potency for tight-binding inhibitors, the apparent inhibitor dissociation constants (\(K_{i}^{app}\) values) were calculated by fitting the data to Morrison’s equation (19); the inhibitor dissociation constants (\(K_{i}\) values) were determined by Dixon’s plot (20) for less potent inhibitors. The inhibition assays were performed with a peptide substrate (1 µM), Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂, and 5 to 10 different inhibitor concentrations. The substrate stock solutions were prepared in DMSO, and then further diluted to 50% DMSO in water. The final DMSO concentration in the assays was 1%. The inhibitors were dissolved in DMSO to 5 mM or 2 mM and diluted with methanol except for inhibitor IV (Calbiochem catalogue number: 444250), which was dissolved in assay buffer. The final methanol concentration in the inhibition assays was 5% (V/V). The specificity constants (\(k_{cat}/K_{m}\) values) were determined by the equation, \(v = (k_{cat}/K_{m})[E][S]\), which is modified from the Michaelis-Menten equation when \([S] \ll K_{m}\).
The enzyme became a mixture of several states after partial activation by dialysis. The total concentration of 400 nM MMP-26 was measured by absorption at 280 nm and calculated using a molar extinction coefficient of 57130 M\(^{-1}\) cm\(^{-1}\). The enzyme was titrated with MMP inhibitor I (GM-6001) to determine the concentration of catalytically active MMP-26. The titration analysis revealed the concentration of active MMP-26 to be 21 nM, which was approximately 5% of the total protein concentration after dialysis. For an accurate titration, the concentration of an enzyme is required to be at least 100-fold more than the inhibition constant of the titrant (21). To avoid the depletion of substrate by a high MMP-26 concentration, a less specific substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH\(_2\), designed for MMP-3 (22), was used for detection of the initial rate. The cleavage of this substrate by MMP-26 was the slowest among peptide substrates studied in our laboratory (14).

*Insulin-like growth factor binding protein-1 (IGFBP-1) and alpha 1 proteinase inhibitor (α\(_1\)-PI) digestion by MMP-26*—IGFBP-1, α\(_1\)-PI, and MMP-26 solutions were diluted or dissolved in 50 mM HEPES buffer at pH 7.5 containing 10 mM CaCl\(_2\), 0.2 M NaCl, and 0.01% Brij-35. IGFBP-1 (4 µg) and MMP-26 (0.63 µg), in a total volume of 50 µL, were incubated for 2 days at room temperature. Each day, 10 µL of reaction mixture was taken, and the reaction was stopped by boiling for 5 min after 2X SDS PAGE sample buffer containing 2% SDS, 100 mM DTT, and 50 mM EDTA was added. The cleaved products were separated by a 12% acrylamide gel and detected by silver staining. For cleavage of α\(_1\)-PI, 90 µg of α\(_1\)-PI were incubated with 1.3 µg of MMP-26 in a total volume of 100 µL. The samples were collected after 1 hr, 1 day, and 2 days. The cleaved products were separated by a 15% SDS PAGE and detected by silver staining.
Determination of cleavage products by Matrix Assisted Laser Desorption Ionization, Time of Flight Mass Spectrometry (MALDI-TOF MS) — The cleavage sites of fluorogenic peptide substrates and α₁-PI were determined by measuring the mass of the cleavage products with a Brucker Protein Time of Flight (TOF) mass spectrometer. The reaction mixture was mixed with an equivalent volume of α-cyano-4-hydroxycinamic acid (4.5 mg/mL in 50% CH₃CN, 0.05% TFA) matrix solution containing synthetic peptide calibrants. Since the high salt concentration increased the noise in the mass spectra, the digestion reaction was performed with 10 mM HEPES buffer containing 5 mM CaCl₂ overnight at room temperature. For fluorogenic substrates, MMP-9 was used as a positive control.

RESULTS

Substrate specificities of MMP-26 — The substrate specificity of MMP-26 was investigated using a recently described peptide library method (12). Data are shown in figure 1. The residues preferred at each site from P₄ to P₄' are summarized in table I. The strongest selectivity was seen at the P₁' site, where large hydrophobic residues were preferred. Small residues, alanine and serine, were preferred at the P₃' site. Although P₂' and P₄' displayed indistinct specificity compared to the P₁' site, lack of a preference for a basic residue (R or K) at the P₂' site was unique to MMP-26 (Table I). Among the unprimed positions, the P₃ site showed the highest selectivity preferring proline and valine. The P₁ site was not as selective as the P₃ site, though small residues, such as serine, were preferred. MMP-26’s preference for proline at P₃, hydrophobic residues at P₂ and P₁' sites and serine at P₁ is similar to that of other MMPs (5–13).
Inhibition of MMP-26 by synthetic inhibitors—Inhibition constants for several inhibitors designed for collagenases and gelatinases were measured with MMP-26, and these values are shown in figure 2. Among the four inhibitors tested, inhibitor I (23) was the most potent for MMP-26 with a $K_{i}^{app}$ of 0.36 nM. Inhibitor II inhibited MMP-26 with a $K_{i}^{app}$ of 1.5 nM, which is similar to the inhibition constant with neutrophil collagenase (MMP-8) (4 nM) (24). Inhibitor III is a less potent stereoisomer of inhibitor II, and MMP-8 discriminates between the two with a 250-fold difference in their inhibition constants (1000 nM vs. 4 nM). There was a 40-fold difference between the $K_{i}^{app}$ values of the pair of stereoisomers with MMP-26 (60 nM vs. 1.5 nM). Inhibitor IV inhibited MMP-26 with a $K_{i}^{app}$ of 2.9 µM and an IC$_{50}$ value of 3.4 µM. This IC$_{50}$ value is similar to the IC$_{50}$ values with interstitial collagenase (MMP-1) and MMP-8 (both are 1 µM) (25).

Autocleavage sites of recombinant MMP-26—Dialysis of the folded pro-form of MMP-26 results in an increase in activity due to autolysis of the pro-domain. MMP-26 was collected after two 24-h dialyses with fresh buffer at 4°C (further dialysis or incubation gradually reduced the activity). Partially activated MMP-26 was compared with the zymogen form on a silver-stained polyacrylamide gel (Figure 3). The band near 30 kDa was confirmed to be pro-MMP-26 by N-terminal sequencing (Figure 3, Lane 2) (14). Several bands below 30 kDa appeared after the dialysis, three of which were located between 20 and 25 kDa (Figure 3, Lane 3). One or more of the three cleavage products may be active forms of MMP-26 and were analyzed by N-terminal sequencing. Only the top two bands were successfully sequenced. The top band resulted from cleavage of a peptide bond between Leu$_{49}$ and Thr$_{50}$, and the band below it was a product of cleavage between Ala$_{75}$ and Leu$_{76}$ (sequence based on ref. 14). Cleavage at either site does not remove the cysteine switch sequence PHC$_{82}$GVPDGSD.
Cleavage of fluorogenic substrates by MMP-26—Initial screening of a number of fluorogenic peptide substrates revealed that gelatinase and collagenase peptide substrates were most efficiently cleaved by MMP-26 (14,17). Therefore, we chose peptide substrates designed for gelatinases or collagenases for further study, three of which contained Trp and two of which contained 7-methoxy coumarin as the fluorogenic group, respectively (26–30). The active MMP-26 concentration was determined by active site titration with inhibitor I (Figure 4) using the least efficient substrate tested as described in the Experimental Procedures. The titration analysis revealed the concentration of active MMP-26 to be approximately 5% of the total enzyme concentration (21 nM of 400 nM). The cleavage sites of the six fluorogenic peptide substrates were determined by identifying the mass of the products by mass spectrometry. Mass spectra of the cleavage products revealed that the cleavage sites of the substrates by MMP-26 and MMP-9 were identical as shown in the example of peptide III (Figure 5). The specificity constants (kcat/Km) of these six peptide substrates with MMP-26 were measured and calculated as shown in table II. MMP-26 hydrolyzed peptide V with the highest specificity constant (3.0 × 10^4 M^{-1} s^{-1}), which is still 10-fold lower than the specificity constant with MMP-2 (3.97 ×10^5 M^{-1} s^{-1}) (26).

Cleavage site of α1-PI and IGFBP-1—MMP-26 cleaved α1-PI near the C-terminus to produce a C-terminal fragment of around 5 kDa (Figure 6, Lane 6 and Lane 7). This fragment was detected by silver staining of a 15% SDS PAGE gel run under optimized conditions to identify proteins of molecular weights less than 10 kDa as previously described (31). A 24-hour incubation of α1-PI with MMP-26 at room temperature led to the formation of a fragment below 14.4 kDa (Lane 6), which was not cleaved any further after 2 days of incubation (Lane 7). The mass spectrum of the α1-PI and MMP-26 mixture (Figure 7, B) exhibited two new peaks located
at 4260 and 4774 which were not observed in the spectrum of $\alpha_1$-PI alone (Figure 7, A). Based on molecular mass analysis, the cleavage sites resulting in these fragments should be Phe$^{352}$–Leu$^{353}$ ($\sim$4774 Da) and Pro$^{357}$–Met$^{358}$ ($\sim$4260 Da), near the C-terminus of $\alpha_1$-PI.

Comparison of lane 7 and lane 2 in figure 8 indicated that there was no detectable proteolysis of IGFBP-1 without MMP-26. The dark band around 30 kDa (IGFBP-1) disappeared, and a band below 14.4 kDa appeared when IGFBP-1 was incubated with MMP-26 for one day or two days (Lane 4 and 5, respectively). The N-terminal sequence of this band was determined to be Val-The-Asn-Ile-Lys-Lys-Trp-Lys, demonstrating that it arises from cleavage at the same site (His$^{140}$–Val$^{141}$) as stromelysin-3 (MMP-11), which produces an inactive 9 kDa fragment (32).

**DISCUSSION**

The results obtained from peptide library studies indicate that MMP-26 substrate specificities are similar to those of other MMPs, where hydrophobic residues are preferred at P1′ and P2, proline at P3, and serine at P1. The optimal cleavage motifs/consensus peptide sequences for MMP-26 were Lys-Pro-Ile/Leu-Ser (P1)-Leu/Met (P1′)-Ile/Thr-Ser/Ala-Ser (Table I), which are not identical to those of MMP-1, -2, -3, -7, -9, and -14 (12). Based on this sequence specificity knowledge, new fluorescence resonance energy transfer substrates more specific for MMP-26 will be designed and developed. These data may provide critical information applicable to the design of new MMP-26 specific inhibitors and to the identification of novel physiological and pathological substrates of MMP-26 *in vivo*.

Inhibition constants of four synthetic inhibitors with MMP-26 were comparable to those with gelatinases and collagens, the enzymes for which the inhibitors were designed. This
corroborates the findings that the substrate specificity of MMP-26 is quite close to that of other MMPs. Inhibitor I/GM6001 was the most potent inhibitor of MMP-26 tested with a $K_{i}^{\text{app}}$ of 0.36 nM. GM6001 also potently inhibits MMP-2 ($K_{i} = 0.5$ nM) and MMP-8 ($K_{i} = 0.1$ nM), but is less effective against MMP-3 ($K_{i} = 27$ nM) (23). Inhibitor III is a less potent stereoisomer of inhibitor II, and MMP-8 discriminates between the two with a 250-fold difference in their inhibition constants. There was only 40-fold difference between the $K_{i}^{\text{app}}$ values of the stereoisomers with MMP-26, indicating MMP-26 is less stereo-selective for its inhibitors. Inhibitor IV was more selective for MMP-1 and MMP-8 (IC$_{50} = 1 \mu$M against both enzymes) than MMP-9 (IC$_{50} = 30 \mu$M) and MMP-3 (IC$_{50} = 150 \mu$M) (25). This inhibitor has an IC$_{50}$ value of 3.4 $\mu$M with MMP-26, similar as that with MMP-1 and MMP-8.

A survey of known protein cleavage sites determined in vitro for MMP-26 is summarized in table III. The survey indicates that hydrophobic residues are preferred at P1$'$ and appear in almost all of the substrates. Residues occurring at other positions that agree with the consensus from the peptide libraries include proline (3 times) at P3, hydrophobic residues (6 times) at P2, and Ser, Ala, and Thr (4 times) at P3$. Residues at the other positions seem random and do not coincide with residues predictions by the peptide libraries, though the libraries do indicate less stringent selectivity at these positions. Accordingly, no individual protein cleavage site precisely matches the consensus motif determined by the peptide library studies, suggesting that the cleavage sites in these protein substrates are likely to be suboptimal for cleavage by MMP-26. The folding topology of the protein may be a contributing factor to the enzyme-substrate interactions. Though the protein cleavage site may not be the optimal sequence, the peptide chain might assume a conformation easily accessible to a protease active site, for example an exposed loop is found in the bait region of $\alpha_{2}$-macroglobulin (33) and the reactive loop of $\alpha_{1}$-PI (34).
Alternatively, cleavage of a suboptimal site may be promoted by recruitment to the enzyme via a substrate-binding exosite. In addition, the presence of unfavorable residues around the cleavage site may slow down the rate of digestion by a protease, regulating the degradation process.

MMP-26 has been shown to digest several components of the extracellular matrix, such as fibronectin, collagens, fibrinogen, and vitronectin, but not any of several plasma proteins tested, with the exception of $\alpha_1$-PI (14,17). It has been reported that the cleavage of the reactive loop residues around 350–365 in $\alpha_1$-PI by MMP-1 and MMP-3 inactivates the inhibitor (34-36). Digestion of $\alpha_1$-PI by MMP-26 generates two major peaks that originate from cleavage at two sites near the C-terminal region, Phe$^{352}$–Leu$^{353}$ (~4774 Da) and Pro$^{357}$–Met$^{358}$ (~4260 Da). These are the same cleavage sites for MMP-1 (35). In addition, MMP-3 cleaves the Pro$^{357}$–Met$^{358}$ bond (34). MMP-11 cleaves the Ala$^{350}$–Met$^{351}$ bond (36), a site distinct from those of MMP-26 and MMP-1. Interestingly, direct evidence showed that $\alpha_1$-PI was a critical substrate for MMP-9 in vivo in a mouse model of the autoimmune disease bullous pemphigoid (37). Thus, MMP-26 may inactivate $\alpha_1$-PI like the other MMPs to promote serine proteinase activity, enhancing extracellular matrix degradation in cancers or other pathological processes.

The insulin-like growth factors (IGFs), insulin-like growth factor binding proteins (IGFBPs), and IGFBP proteases are involved in the regulation of somatic growth and cellular proliferation. The level of free IGF in a system is modulated by rates of IGF production and clearance and the degree of binding to IGFBPs (38). IGFBP-1 inhibits IGF-I-induced proliferation of the MCF-7 human breast adenocarcinoma (32). Through their inactivation of IGFBP-1, MMPs were able to promote cell growth and survival by the increase of the effective IGF concentration in the surrounding media (32). MMP-26 cleaves the His$^{140}$–Val$^{141}$ bond in
IGFBP-1 as MMP-11 does. Therefore, the cleavage of IGFBP-1 by MMP-26 to produce the 9 kDa inactive form may sustain the survival of cancer cells, increasing the chance of metastasis.

The cleavage sites in the fluorogenic substrates seem in good agreement with the motifs determined by the peptide library approach. Although the six commercial fluorogenic peptide substrates tested were not designed for the specificity of MMP-26, some of them resemble closely to the consensus sequences of peptide substrates for MMP-26 determined by the peptide library studies—proline at P3, a hydrophobic residue at P2, P1′ and P2′, and small residues at P3′—except that serine is preferred at P1 and P4′, Lys is preferred at P4, but a basic residue is not preferred at P2′. The best substrate tested for MMP-26 was peptide V, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂. This peptide appears to be very close to optimal sequences determined by the peptide library studies; there is a selected residue at essentially every position (see Figure 1 and Table I), except that the peptide libraries do not have Nva at P1′.

The cleavage sites in the protein substrates tested do not match exactly the optimal motifs identified by the peptide library approach; however, upon close examination of the protein cleavage site data presented in Table III, it seems that the amino acid residues at P1 and P4′ are less selective, this is in good agreement with the peptide library data. Furthermore, P1′ is more selective and Leu, Met, Ile are preferred at P1′ (Figure 1). This is consistent with the protein cleavage site data shown in Table III, in which seven of the eleven residues (64%) at P1′ are these residues. Moreover, two Lys residues are found at the P4 and two Ser residues are found at P4′ of the protein cleavage sites, which is also unique to MMP-26 according to the library data.

The relative rates of cleavage in the six fluorogenic substrates also correspond to the peptide library data relatively well. The best substrate is peptide V with a specificity constant of $3.0 \times 10^4$ M⁻¹ s⁻¹. In addition to peptide V, peptides IV and VI are also relatively good substrates.
for MMP-26 with specificity constants of $1.7 \times 10^4$ M$^{-1}$ s$^{-1}$ and $2.2 \times 10^4$ M$^{-1}$ s$^{-1}$, respectively (Table II). The worst substrate of MMP-26 in Table II is peptide II, with a specificity constant approximately 10 times slower than peptide V. Neither Ala at P1 nor Tyr at P1' in the peptide II is preferred. On the other hand, the rate of cleavage of peptide V, the best peptide of MMP-26 in Table II, is 10 times slower than the rate of substrate cleavage by MMP-2 ($3.97 \times 10^5$ M$^{-1}$ s$^{-1}$) (26). The slower rate of peptide and protein digestion by MMP-26 suggests that this enzyme is not the most powerful MMP catalytically or the optimal substrates for MMP-26 have not been identified.

It is also possible that a manageable rate of MMP-26 catalysis may be required in biological processes such as normal implantation where tight control of substrate degradation is highly desirable. In the latter scenario, the function of MMP-26 may not be limited to the direct degradation of ECM; MMP-26 may play a more critical role in controlling the activities of growth factors or proteases that mediate such processes. Consequently, biologically significant substrates of MMP-26 may be growth factor-binding proteins, receptors, zymogens, and enzyme inhibitors.

MMP-26 is not only unique in terms of its tissue and cell specific expression as reported by us and others (14-17), but also because of its unique cysteine switch sequence (P$^8$H$^8$CGVPDGSD), and thus, its unique pathway of proenzyme activation. Many members of the MMP family follow the classic cysteine-switch activation model (39, 40). The inactivity of a pro-MMP is generally attributable to a complex between the sulfhydryl group of a cysteine residue in the cysteine switch sequence (P$^C$RGVPDV) of the prodomain and the active site zinc atom in the catalytic domain. Activation of a pro-MMP can be achieved proteolytically by hydrolysis of the propeptide on the carboxyl-terminal side of the cysteine switch residue near the
border between the propeptide and catalytic domains. This proteolytic step may be catalyzed by another proteinase or it may be an autolytic step (39, 40). However, Marchenko et al. have challenged the cysteine-switch model (41). Their report showed that the activating cleavage site of proMMP-26 occurs at Gln\(^{59}\)-Gln\(^{60}\), leaving the putative cysteine switch sequence intact. It was suggested that the Arg to His substitution existing in the unique PH\(^{81}\)CGVPDGSD cysteine-switch motif of proMMP-26 abolishes the ability of Cys\(^{82}\) to interact with the zinc ion of the catalytic domain (41).

We have identified two of the major autolytic sites in MMP-26 to be Leu\(^{49}\)-Thr\(^{50}\) and Ala\(^{75}\)–Leu\(^{76}\). Although different from the Gln\(^{59}\)-Gln\(^{60}\) site, cleavage at these two sites also does not remove the cysteine switch sequence (PH\(^{82}\)GVPD) from the enzyme, suggesting that Cys\(^{82}\) may not play a role in the latency of the zymogen, which is consistent with the hypothesis proposed by Marchenko et al. (41). Alternatively, the thiol group of Cys\(^{82}\) could be transiently dissociated from the zinc ion at the active site, allowing a water molecule to bind to the zinc ion and the enzyme to exhibit catalytic activity. Our inhibitor titration data demonstrated that approximately 5% of the total enzyme molecules was active. This observation may support the concept that the thiol groups of Cys\(^{82}\) in the active enzyme molecules are dissociated or removed from the active site zinc ions, and the thiol groups of the Cys\(^{82}\) in remaining 95% of the total enzyme molecules are still coordinated with the zinc ions at the active sites, forming a steady-state equilibrium between the active enzyme molecules and the zymogen molecules. However, this hypothesis and the detailed activation mechanisms of proMMP-26 remain to be thoroughly investigated (42). In summary, this work provides new knowledge on the MMP-26 substrate specificity to build a working model for future design of MMP-26 inhibitors, studies of
proMMP-26 activation, and identification of optimal physiological and pathological substrates of MMP-26 in vivo.

Acknowledgements

We thank Ms. Margaret Seavy at the Bioanalytical Facility for protein N-terminal sequencing and Ms. Sara C. Monroe for her editorial assistance with the manuscript preparation at the Florida State University. We appreciate Dr. Jian Ni at the Human Genome Sciences Inc for his previous collaboration on the human MMP-26 project.

REFERENCES


**Figure Legends**

**Figure 1.** Cleavage site specificity of MMP-26 (endometase). The figures on the right present the relative distribution of amino acid residues at positions C-terminal (P1′ to P4′) to the MMP-26 cleavage site determined by sequencing the cleavage fragments of a random dodecamer (Ac-XXXXXXXXXXXXXXX). Data are normalized so that a value of 1 corresponds to the average quantity per amino acid in a given sequencing cycle and would indicate no selectivity. Tryptophan was not included in the analysis due to poor yield during sequencing. The figures on the left present specificity of positions N-terminal to the MMP-26 cleavage site. For the P3 position, data shown were obtained using the library MAXXXXXLRGAARE(K-biotin). For all other positions, the P3 proline library MGXXPXXLRGGGEE(K-biotin) was used. Glutamine and threonine were omitted in some cycles due to high background on the sequencer. Data were normalized as for the primed sites.

**Figure 2.** The structures of MMP inhibitors and their inhibitor dissociation constants with MMP-26. The apparent inhibition constants ($K_i^{app}$ values) were determined by Morrison’s equation for tight-binding inhibitors (compounds I, II, III) (19), and the inhibition constant ($K_i$ value) was determined by Dixon’s plot for a less potent inhibitor (compound IV) (20). The values were 0.36, 1.5, 60, and 2900 nM for compounds I, II, III, and IV, respectively.

**Figure 3.** Autolysis of MMP-26 during dialysis. Lane 1, Lane 2, and Lane 3 were low molecular weight marker and the folded MMP-26 before and after dialysis at 4°C for 24-h, respectively. The cleavage sites of MMP-26 that formed the two major bands around 20 kDa were revealed to be The$^{51}$–Gln$^{52}$ and Ala$^{75}$–Leu$^{76}$ by N-terminal sequencing.
**Figure 4.** Determination of the active MMP-26 concentration by titration of MMP-26 with inhibitor I. Total MMP-26 concentration was estimated to be 400 nM by molar absorptivity. The estimated active concentration was 21 nM by fitting the titration data into Morrison’s equation (19). The assays were performed as described in the *Experimental Procedures* with 1 µM of the substrate.

**Figure 5.** An example of the determination of fluorogenic peptide cleavage sites by MALDI TOF mass spectrometry. 80 µM of peptide substrate III (Table II, Dnp-Pro-Leu-Gly-Leu-Trp-Ala-(D)-Arg-OH) was incubated overnight with 5 nM of MMP-9 (human neutrophil gelatinase) (A), alone (B), and with 20 nM of endometase (C) in pH 7.5, 10 mM HEPES containing 5 mM CaCl$_2$ at room temperature. The two peaks observed at 1474 and 2953 m/z were internal synthetic peptide mass calibrants. The peaks at 975 and 542 m/z were the substrate and the cleaved peptide fragment, Leu-Trp-Ala-(D)-Arg-OH produced by cleavage of the Gly-Leu peptide bond by MMP-9 and endometase, respectively.

**Figure 6.** Cleavage of human $\alpha_1$-PI by MMP-26. After incubation of an $\alpha_1$-PI (900 µg/mL) and MMP-26 (13 µg/mL) mixture for one day (Lane 6) and two days (Lane 7) at room temperature, the C-terminal cleavage products were detected by silver staining a 15% SDS PAGE gel. Samples containing $\alpha_1$-PI were overloaded to detect the bands around 4.5 kDa in Lane 6 and 7, which might be 4.8 and 4.2 kDa fragments produced by MMP-26 proteolysis of $\alpha_1$-PI. The two N-terminal sequences were deduced from the mass spectrometry results shown in figure 6 compared with the primary structure of human $\alpha_1$-PI.
**Figure 7.** Cleavage sites of $\alpha_1$-PI by MMP-26 determined by MALDI TOF mass spectrometry. $\alpha_1$-PI alone (A) and with MMP-26 (B) were incubated for one day in 10 mM HEPES buffer at pH 7.5 containing 5 mM CaCl$_2$. The peaks at 1474 and 2953 m/z were two internal calibrants. The two peaks observed at 4260 and 4774 m/z were produced from $\alpha_1$-PI cleavage by MMP-26 at the sites Pro$^{357}$–Met$^{358}$ and Phe$^{352}$–Leu$^{353}$.

**Figure 8.** Cleavage of IGFBP-1 by MMP-26. IGFBP-1 (80 $\mu$g/mL) was incubated with MMP-26 (13 $\mu$g/mL) for 0 hr (Lane 2), 1 hr (Lane 3), 1 day (Lane 4), and 2 days (Lane 5). The dense band below 14.4 kDa observed after one day (Lane 4) was the product of IGFBP-1 cleavage by MMP-26 at the His$^{140}$–Val$^{141}$ site.
| Enzyme | Cleavage Position |
|--------|-----------------
|        | P4 | P3 | P2 | P1 | P1′ | P2′ | P3′ | P4′ |
| MMP-26 | K (1.3) | P (2.2) | I (1.7) | S (1.5) | L (3.4) | I (1.5) | S (2.0) | S (1.3) |
|        | V (1.6) | L (1.4) | Y (1.3) | M (2.7) | I (2.3) | F (1.4) | Q (1.4) | T (1.6) |
| MMP-1  | V   | P   | M   | S   | S   | M   | M   | A   |
| MMP-2  | I   | P   | V   | S   | L   | R   | S   |
| MMP-3  | K   | P   | F   | S   | M   | M   | M   |
| MMP-7  | V   | P   | L   | S   | L   | V   | M   |
| MMP-9  | V   | P   | L   | S   | L   | R   | S   |
| MMP-14 | I   | P   | E   | S   | L   | R   | M   |
| MMP Consensus | V | P | L | S | L | R | A |
|        | V | Y | M | I |   |   |   |

aQuantities were determined from sequencing data as described for Figure 1, and values ≥ 1.3 are listed. All primed sites were obtained using the library Ac-XXXXXXXXXXXXX. MAXXXXXXLRLGAAARE(K-biotin) and MGXXPXXLRRGGE(K-biotin) were used to produce the data at the unprimed sites.

bData from (12). A series of consensus peptides/optimal cleavage-site motifs were selected and listed for each MMP.

cData summarized from (12). These listed residues were selected among amino acids that appeared at least in five of the six MMPs with values ≥ 1.3.
Table II. Peptide substrates of MMP-26a

Fluorogenic substrate cleavage sitesb

<table>
<thead>
<tr>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P1′</th>
<th>P2′</th>
<th>P3′</th>
<th>P4′</th>
<th>k_{cat}/K_m (s^{-1} M^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dnp-Pro-Leu-Gly--Met-Trp-Ser-Arg-OH (I)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>9.4 \times 10^3</td>
</tr>
<tr>
<td>Dnp-Pro-Leu-Ala--Tyr-Trp-Ala-Arg-OH (II)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.5 \times 10^3</td>
</tr>
<tr>
<td>Dnp-Pro-Leu-Gly--Leu-Trp-Ala--(D)Arg-OH (III)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.9 \times 10^3</td>
</tr>
<tr>
<td>Mca-Pro-Cha-Gly--Nva-His-Ala-Dpa-NH_2 (IV)</td>
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<tr>
<td>Mca-Pro-Leu-Ala--Nva-Dpa-Ala-Arg-NH_2 (V)</td>
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<tr>
<td>Mca-Pro-Leu-Gly--Leu-Dpa-Ala-Arg-NH_2 (VI)</td>
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<td></td>
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<td>2.2 \times 10^4</td>
</tr>
</tbody>
</table>

a All of the assays were performed in pH 7.5 buffer containing 50 mM HEPES, 0.2 M NaCl, 0.01 M CaCl_2, 0.01% Brij-35 at 25°C. The range of substrate concentrations used were 1 to 4 \mu M, and the active MMP-26 concentration used was 2 nM for the substrates containing the Mca group and 10 nM for the substrates containing the Trp residue.

b The cleavage sites of the substrates were determined by mass spectrometry as described in Experimental procedures and Figure 5.
Table III. Protein Sequences Hydrolyzed by MMP-26

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Cleavage sites&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
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<tr>
<td>α&lt;sub&gt;1&lt;/sub&gt;-PI&lt;sup&gt;b&lt;/sup&gt;</td>
<td>GAMF–LEAI</td>
</tr>
<tr>
<td></td>
<td>EAIP–MSIP</td>
</tr>
<tr>
<td>MMP-26 (autolysis)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>QMHA–LLHQ</td>
</tr>
<tr>
<td></td>
<td>SPLL–TQET</td>
</tr>
<tr>
<td>MMP-26 (autolysis)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>QLLQ–QFHR</td>
</tr>
<tr>
<td>IGFBP-1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>KALH–VTNI</td>
</tr>
<tr>
<td>Fibronectin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SPVA–VSQS</td>
</tr>
<tr>
<td>Vitronectin&lt;sup&gt;d&lt;/sup&gt;</td>
<td>KPEG–IDSR</td>
</tr>
<tr>
<td>Fibrinogen&lt;sup&gt;d&lt;/sup&gt;</td>
<td>SKPN–MIDA</td>
</tr>
<tr>
<td></td>
<td>HTEK–LVTS</td>
</tr>
<tr>
<td></td>
<td>GDKE–LRTG</td>
</tr>
</tbody>
</table>

<sup>a</sup>A line is inserted in the cleavage site.
<sup>b</sup>Data from this study.
<sup>c</sup>Data from Marchenko et al. (41).
<sup>d</sup>Data from Marchenko et al. (17).
Park et al., Figure 1
Park et al., Figure 2
Park et al., Figure 3
$[E] = 21 \text{ nM}$

Park et al., Figure 4
Park et al., Figure 5
Park et al., Figure 6
Park et al., Figure 8
Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26

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