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

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Hyun I. Park‡, Benjamin E. Turk§, Ferry E. Gerkema‡, Lewis C. Cantley§, and Qing-Xiang Amy Sang‡¶

From the ‡Department of Chemistry and Biochemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306-4390 and the §Department of Medicine, Harvard Medical School, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

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–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 was preferred at P1. The overall specificity was similar to that of other MMPs with the exception 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 only a 40-fold difference in K_i^{app} values against MMP-26 compared with a 250-fold difference against neutrophil collagenase, indicating that MMP-26 is less stereoselective for its inhibitors. MMP-26 autodigested 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 (PHC^{82}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 ~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 α_1-proteinase inhibitor and His^{140}–Val^{141} in insulin-like growth factor-binding protein-1, probably 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 (30,000/molar second) with MMP-26. This report proposes a working model for the future studies of pro-MMP-26 activation, the design of inhibitors, and the identification of optimal physiological and pathological substrates of MMP-26 *in vivo*.

Matrix metalloproteinases (MMPs)[1] share a conservative metal binding sequence of HE*X*GH*XX*G*XX*HS 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

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¶ To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, Florida State University, Chemistry Research Bldg. (DLC), Rm. 203, Tallahassee, FL 32306-4390. Tel.: 850-644-8683; Fax: 850-644-8281; E-mail: qxsang@chem.fsu.edu; Website: www.chem.fsu.edu/editors/sang/sang.html.

[1] The abbreviations used are: MMP, matrix metalloproteinase; α_1-PI, α_1-protease inhibitor; Brij-35, polyoxyethylene lauryl ether; IGFBP-1, insulin-like growth factor binding protein-1; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ECM, extracellular matrix; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Dnp, 2,4-dinitrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl; Nva, non-valine.

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-β-cyclohexylalanyl-Gly-Nva-His-Ala-Dpa-NH_2, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2, 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-NH_2 and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 were purchased from Bachem. Hydroxamic acid derivatives of amino acids, buffers, cysteine, α_1-protease inhibitor (α_1-PI), and 1,10-phenanthroline were purchased from Sigma. Metal salts, Brij-35, sodium dodecyl sulfate, dithioerythreitol, and 2-mercaptoethanol were purchased from Fisher. Peptide libraries were synthesized at the Tufts University Core Facility (Boston, MA) as described previously (12).

Preparation of Partially Active MMP-26—MMP-26 was expressed in the form of inclusion bodies from transformed *E. coli* cells as described previously (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 ~5 mg/ml. The protein solution was diluted to ~100 μg/ml in 8 M urea and 10 mM dithiothreitol for 1 h, dialyzed in 4 M urea, 1 mM dithiothreitol, 50 mM HEPES, or Tricine, pH 7.5, for at least 1 h and then folded by dialysis in buffer containing 50 mM HEPES or Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, 20 μM $ZnSO_4$, 0.01% Brij-35, pH 7.5, for 16 h. To enhance the activity of MMP-26, the folded enzyme was dialyzed twice for 24 h at 4 °C in the folding buffer without Zn^{2+} ion. The total enzyme concentration was measured by UV absorption using $\epsilon_{280} = 57130\ M^{-1}\ cm^{-1}$, which was calculated by Genetics Computer Group software.

Peptide Library Methods—The methods were performed as described previously (12). 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 $CaCl_2$ at 37 °C until 5–10% 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 were normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicated 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.

The specificity of the unprimed side was determined by libraries with the sequence MAXXXXXLRGAARE(K-biotin) for the P3 site and MAXXPXXLRGGGEE(K-biotin) for other sites, where X represents a degenerate position, K-biotin is ε-(biotinamidohexanoyl)lysine, and the amino 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 of 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 $CaCl_2$, 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-NH_2, and 5–10 different inhibitor concentrations. The substrate stock solutions were prepared in Me_2SO and then further diluted to 50% Me_2SO in water. The final Me_2SO concentration in the assays was 1%. The inhibitors were dissolved in Me_2SO to 5 or 2 mM and diluted with methanol with the exception of 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 57,130 $M^{-1}\ cm^{-1}$. 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 ~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).

IGFBP-1 and 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 2× SDS-PAGE sample buffer containing 2% SDS, 100 mM dithiothreitol, 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 h, 1day, 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 α_1-PI were determined by measuring the mass of the cleavage products with a Bruker protein time-of-flight mass spectrometer. The reaction mixture was mixed with an equivalent volume of α-cyano-4-hydroxycinamic acid (4.5 mg/ml in 50% CH_3CN, 0.05% trifluoroacetic acid) matrix solution containing synthetic peptide calibrants. Because the high salt concentration increased the noise in the mass spectra, the digestion reaction was performed with 10 mM HEPES buffer containing 5 mM $CaCl_2$ 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 Fig. 1. The residues preferred at each site from P4–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 with the P1′ site, a lack of a preference for a basic residue (Arg or Lys) 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, although small residues such as serine were preferred. The preference of MMP-26 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 Fig. 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 *versus* 4

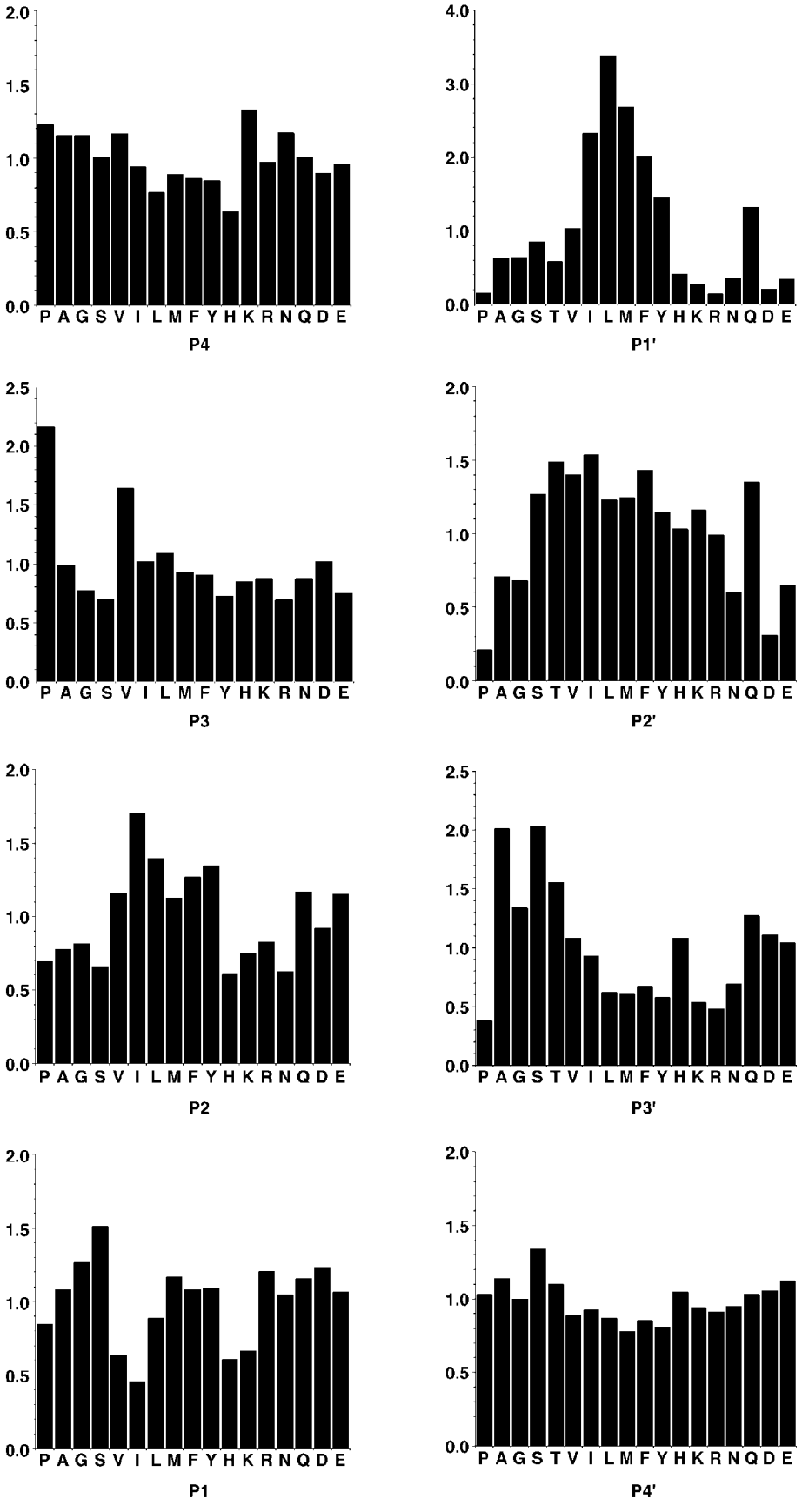

FIG. 1. **Cleavage site specificity of MMP-26 (endometase).** The *figures* on the *right* represent the relative distribution of amino acid residues at positions COOH terminus (P1′–P4′) to the MMP-26 cleavage site determined by sequencing the cleavage fragments of a random dodecamer (Ac-*XXXXXXXXXXXX*). 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 because of poor yield during sequencing. The *figures* on the *left* represent specificity of positions amino terminus to the MMP-26 cleavage site. For the P3 position, data shown were obtained using the library MA*XXXXX*LRGAARE(K-biotin). For all other positions, the P3 proline library MG*XX*P*XX*LRGGGEE(K-biotin) was used. Glutamine and threonine were omitted in some cycles because of high background on the sequencer. Data were normalized as for the primed sites.

nM). There was a 40-fold difference between the K_i^{app} values of the pair of stereoisomers with MMP-26 (60 *versus* 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 collagenases MMP-1 and MMP-8 (both are 1 μM) (25).

TABLE I
Cleavage site motifs for MMP-26[a] compared with those of six other MMPs[b]

Enzyme	Cleavage position							
	P4	P3	P2	P1	P1′	P2′	P3′	P4′
MMP-26	Lup (1.3)	Pro (2.2) Val (1.6)	Ile (1.7) Leu (1.4) Tyr (1.3)	Ser (1.5)	Leu (3.4) Met (2.7) Ile (2.3) Phe (2.0) Tyr (1.5) Gln (1.3)	Ile (1.5) Iwe (1.5) Phe (1.4) Gln (1.4)	Ser (2.0) Ada (2.0) Thr (1.6) Gly (1.3)	Ser (1.3)
MMP-1	Val	Pro	Met	Ser	Met	Met	Ala	
MMP-2	Ile	Pro	Val	Ser	Leu	Arg	Ser	
MMP-3	Lys	Pro	Phe	Ser	Met	Met	Met	
MMP-7	Val	Pro	Leu	Ser	Leu	Val	Met	
MMP-9	Val	Pro	Leu	Ser	Leu	Arg	Ser	
MMP-14	Ile	Pro	Glu	Ser	Leu	Arg	Met	
MMP	Val	Pro	Leu	Ser	Leu	Arg	Ala	
Consensus[c]		Val	Tyr		Met Ile	Ile		

[a] Quantities were determined from sequencing data as described for Fig. 1, and values ≥1.3 are listed. All primed sites were obtained using the library Ac-*XXXXXXXXXXXX*. MA*XXXXX*LRGAARE(K-biotin) and MG*XXPXX*LRGGGEE(K-biotin) were used to produce the data at the unprimed sites.
[b] Data from Turk *et al.* (12). A series of consensus peptides/optimal cleavage site motifs were selected and listed for each MMP.
[c] Data summarized from Turk *et al.* (12). These listed residues were selected among amino acids that appeared at least in 5 of the 6 MMPs with values ≥1.3.

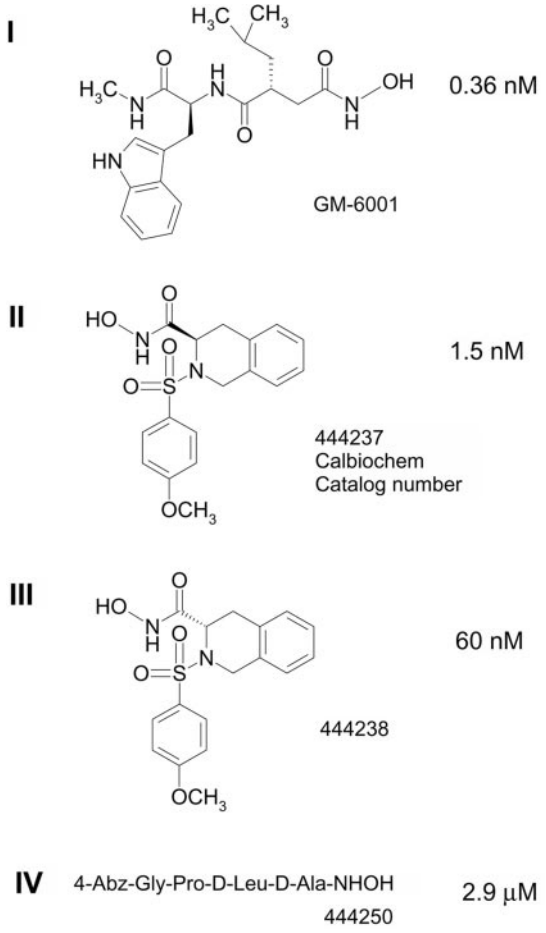

FIG. 2. **The structures of MMP inhibitors and their inhibitor dissociation constants with MMP-26.** The apparent inhibition constants (K_i^{app} values) were determined by Morrison's equation for tight binding inhibitors (compounds *I*, *II*, and *III*) (19), and the inhibition constant (K_i value) 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.

Autocleavage Sites of Recombinant MMP-26—Dialysis of the folded pro-form of MMP-26 results in an increase in activity because of autolysis of the prodomain. 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 (Fig. 3). The band near 30 kDa was confirmed to be pro-MMP-26 by amino-terminal sequencing (Fig. 3, *lane 2*) (14). Several bands below 30 kDa appeared after the dialysis, three of which were located between 20 and 25 kDa (Fig. 3, *lane 3*). One or more of the three cleavage products may be active forms of MMP-26 and was analyzed by amino-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). The cleavage at either site does not remove the cysteine switch sequence PH**C**[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 (Fig. 4) using the least efficient substrate tested as described under "Experimental Procedures." The titration analysis revealed the concentration of active MMP-26 to be ~5% of the total enzyme concentration (21 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 (Fig. 5). The specificity constants (k_{cat}/K_m) 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 α_1-PI and IGFBP-1—MMP-26 cleaved α_1-PI near the COOH terminus to produce a COOH-terminal fragment of approximately 5 kDa (Fig. 6, *lanes 6* and *7*). This fragment was detected by silver staining of a 15% SDS-PAGE gel run under optimized conditions to identify proteins of molecular masses <10 kDa as described previously (31). A 24-h

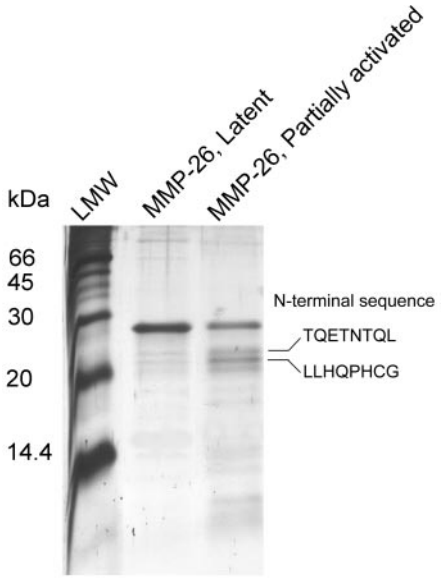

FIG. 3. **Autolysis of MMP-26 during dialysis.** *Lanes 1–3* were low molecular weight markers and the folded MMP-26 before and after dialysis at 4 °C for 24 h, respectively. The cleavage sites of MMP-26 that formed the two major bands around 20 kDa were revealed to be The^{51}–Gln^{52} and Ala^{75}–Leu^{76} by amino-terminal sequencing.

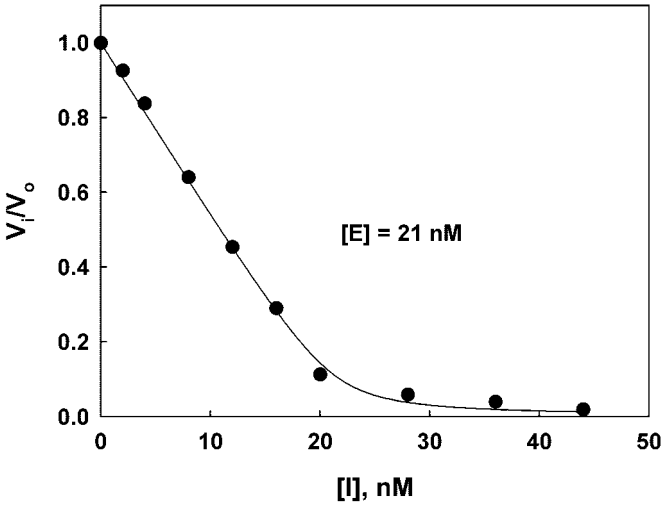

FIG. 4. **Determination of the active MMP-26 concentration by titration of MMP-26 with inhibitor I.** Total MMP-26 concentration was estimated to be 400 nM by molar absorptivity. The estimated active concentration was 21 nM by fitting the titration data into Morrison's equation (19). The assays were performed as described under "Experimental Procedures" with 1 μM of the substrate.

incubation of α_1-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 α_1-PI and MMP-26 mixture (Fig. 7*B*) exhibited two new peaks located at 4260 and 4774, which were not observed in the spectrum of α_1-PI alone (Fig. 7*A*). Based on molecular mass analysis, the cleavage sites resulting in these fragments should be Phe^{352}–Leu^{353} (~4774 Da) and Pro^{357}–Met^{358} (~4260 Da) near the COOH terminus of α_1-PI.

A comparison of *lanes 2* and *7* in Fig. 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 1 or 2 days (*lanes 4* and *5*, respectively). The amino-terminal sequence of this band was determined to be Val-The-Asn-Ile-Lys-Lys-Trp-Lys, demonstrating that it arises

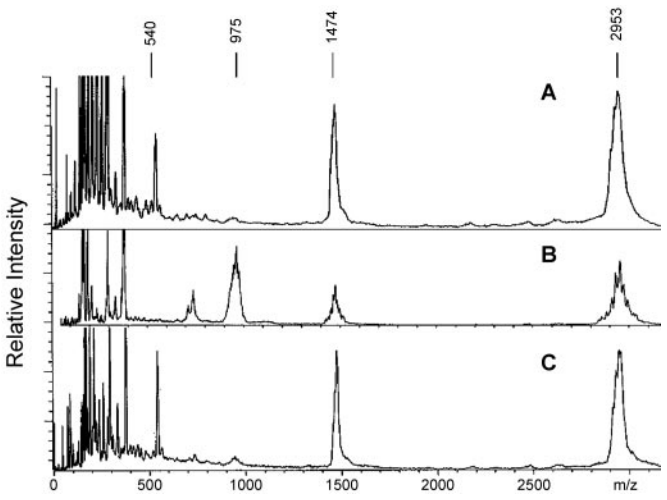

FIG. 5. **An example of the determination of fluorogenic peptide cleavage sites by MALDI TOF mass spectrometry.** 80 μM peptide substrate III (Table II), Dnp-Pro-Leu-Gly-Leu-Trp-Ala-(D)-Arg-OH) was incubated overnight with 5 nM MMP-9 (human neutrophil gelatinase) (*A*), alone (*B*), and with 20 nM endometase (*C*), pH 7.5, and 10 mM HEPES containing 5 mM $CaCl_2$ at room temperature. The two peaks observed at *m/z* 1474 and 2953 were internal synthetic peptide mass calibrants. The peaks at *m/z* 975 and 542 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.

TABLE II
Peptide substrates of MMP-26[a]

Fluorogenic substrate cleavage sites[b] P3 P2 P1 P1′ P2′ P3′ P4′	k_{cat}/K_m $s^{-1}\,M^{-1}$
Dnp-Pro-Leu-Gly–Met-Trp-Ser-Arg-OH (I)	9.4×10^3
Dnp-Pro-Leu-Ala–Tyr-Trp-Ala-Arg-OH (II)	3.5×10^3
Dnp-Pro-Leu-Gly-Leu-Trp-Ala-(D)Arg-OH (III)	4.9×10^3
Mca-Pro-Cha-Gly–Nva-His-Ala-Dpa-NH_2 (IV)	1.7×10^4
Mca-Pro-Leu-Ala–Nva-Dpa-Ala-Arg-NH_2 (V)	3.0×10^4
Mca-Pro-Leu-Gly–Leu-Dpa-Ala-Arg-NH_2 (VI)	2.2×10^4

[a] All of the assays were performed in pH 7.5 buffer containing 50 mM HEPES, 0.2 M NaCl, 0.01 M CaCl2, 0.01% Brij-35 at 25°C. The range of substrate concentrations used were 1μM, 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 under "Experimental procedures" and Fig. 5.

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 is preferred at P3, and serine is preferred 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, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-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*.

The inhibition constants of four synthetic inhibitors with MMP-26 were comparable to those with gelatinases and collagenases, the enzymes for which the inhibitors were designed. This corroborates the findings that the substrate specificity of

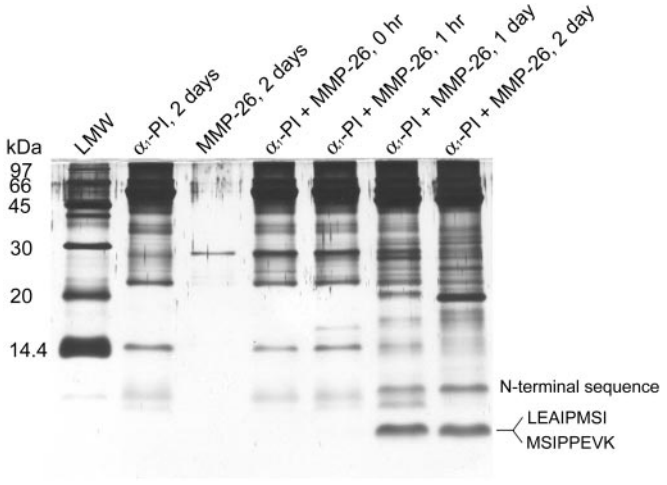

FIG. 6. **Cleavage of human α_1-PI by MMP-26.** After incubation of an α_1-PI (900 μg/ml) and MMP-26 (13 μg/ml) mixture for 1 day (*lane 6*) and 2 days (*lane 7*) at room temperature, the COOH-terminal cleavage products were detected by silver staining a 15% SDS-PAGE gel. Samples containing α_1-PI were overloaded to detect the bands of around 4.5 kDa in *lanes 6* and *7*, which might be 4.8- and 4.2-kDa fragments produced by MMP-26 proteolysis of α_1-PI. The two amino-terminal sequences were deduced from the mass spectrometry results shown in Fig. 6 compared with the primary structure of human α_1-PI.

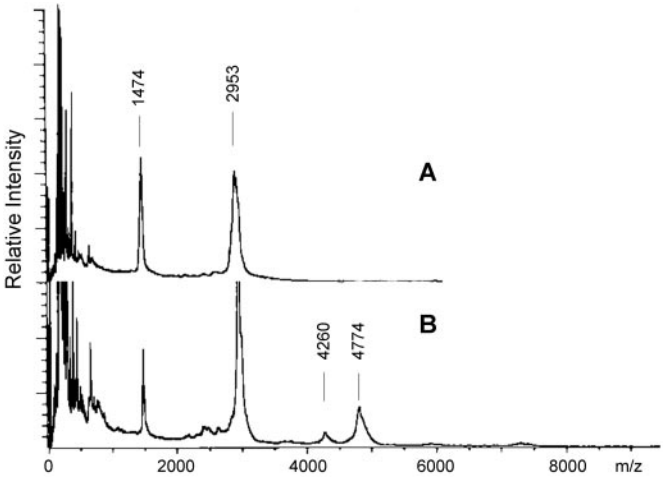

FIG. 7. **Cleavage sites of α_1-PI by MMP-26 determined by MALDI TOF mass spectrometry.** α_1-PI alone (*A*) and with MMP-26 (*B*) were incubated for 1 day in 10 mM HEPES buffer at pH 7.5 containing 5 mM $CaCl_2$. The peaks at *m/z* 1474 and 2953 were two internal calibrants. The two peaks observed at *m/z* 4260 and 4774 were produced from α_1-PI cleavage by MMP-26 at the sites Pro^{357}–Met^{358} and Phe^{352}–Leu^{353}.

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^{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^{app} values of the stereoisomers with MMP-26, indicating that MMP-26 is less stereoselective for its inhibitors. Inhibitor IV was more selective for MMP-1 and MMP-8 (IC_{50} = 1 μM against both enzymes) than MMP-9 (IC_{50} = 30 μM) and MMP-3 (IC_{50} = 150 μM) (25). This inhibitor has an IC_{50} value of 3.4 μM 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 residue predictions by the peptide libraries, although 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 probably suboptimal for cleavage by MMP-26. The folding topology of the protein may be a contributing factor to the enzyme-substrate interactions. Although the protein cleavage site may not be the optimal sequence, the peptide chain might assume a conformation that is easily accessible to a protease active site; for example, an exposed loop is found in the bait region of α_2-macroglobulin (33), and the reactive loop is found in the bait region of α_1-PI (34). Alternatively, the 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 α_1-PI (14, 17). It has been reported that the cleavage of the reactive loop residues around 350–365 in α_1-PI by MMP-1 and MMP-3 inactivates the inhibitor (34–36). The digestion of α_1-PI by MMP-26 generates two major peaks

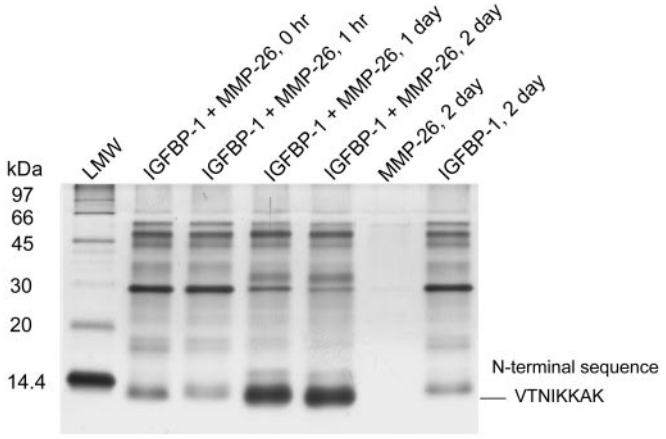

FIG. 8. **Cleavage of IGFBP-1 by MMP-26.** IGFBP-1 (80 μg/ml) was incubated with MMP-26 (13 μg/ml) for 0 h (*lane 2*), 1 h (*lane 3*), 1 day (*lane 4*), and 2 days (*lane 5*). The dense band below 14.4 kDa observed after 1 day (*lane 4*) was the product of IGFBP-1 cleavage by MMP-26 at the His^{140}–Val^{141} site.

TABLE III
Protein sequences hydrolyzed by MMP-26

Proteins	Cleavage sites[a]
α_1-PI[a]	GAMF-LEAI
	EAIP-MSIP
MMP-26 (autolysis)[b]	QMHA-LLHQ
	SPLL-TQET
MMP-26 (autolysis)[c]	QLLQ-QFHR
IGFBP-1[b]	KALH-VTNI
Fibronectin[d]	SPVA-VSQS
Vitronectin[d]	KPEG-IDSR
Fibrinogen[d]	SKPN-MIDA
	HTEK-LVTS
	GDKE-LRTG

[a] A line is inserted in the cleavage site.
[b] Data from this study.
[c] Data from Marchenko *et al.* (41).
[d] Data from Marchenko *et al.* (17).

that originate from the cleavage at two sites near the COOH-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 α_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 α_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, IGFBPs, and IGFBP proteases are involved in the regulation of somatic growth and cellular proliferation. The level of free insulin-like growth factor in a system is modulated by rates of insulin-like growth factor 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 insulin-like growth factor concentration in the surrounding medium (32). MMP-26 cleaves the His^{140}–Val^{141} bond in IGFBP-1 as does MMP-11. 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′, with the exception 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-NH_2. This peptide appears to be very close to optimal sequences determined by the peptide library studies where there is a selected residue at essentially every position (see Fig. 1 and Table I) with the exception 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, and Ile are preferred at P1′ (Fig. 1). This finding is consistent with the protein cleavage site data shown in Table III in which 7 of the 11 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 3.0×10^4 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 1.7×10^4 M^{-1} s^{-1} and 2.2×10^4 M^{-1} s^{-1}, respectively (Table II). The worst substrate of MMP-26 in Table II is peptide II with a specificity constant ~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 (3.97×10^5 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**H**^{81}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**R**CGVPDV) of the prodomain and the active site zinc atom in the catalytic domain. The 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.* (41) have challenged the cysteine-switch model. Their report showed that the activating cleavage site of pro-MMP-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 P**H**^{81}CGVPDGSD cysteine-switch motif of pro-MMP-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, the cleavage at these two sites also does not remove the cysteine switch sequence (PH**C**^{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 ~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 pro-MMP-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 the future design of MMP-26 inhibitors, studies of pro-MMP-26 activation, and identification of optimal physiological and pathological substrates of MMP-26 *in vivo*.

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ENZYME CATALYSIS AND REGULATION:

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

Hyun I. Park, Benjamin E. Turk, Ferry E. Gerkema, Lewis C. Cantley and Qing-Xiang Amy Sang

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