Unconventional Activation Mechanisms of MMP-26, a Human Matrix Metalloproteinase with a Unique PHCGXXD Cysteine-switch Motif*

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ProMMP-26 has the unique Pro-His81-Cys-Gly-Xaa-Xaa-Asp cysteine-switch motif that discriminates this protease from all other matrix metalloproteinases (MMPs) known so far. The conserved, free cysteine residue of the conventional PRCXXPD sequence interacts with the zinc ion of the catalytic domain and provides the fourth coordination site for the catalytic zinc, thereby preventing latent proMMPs from becoming active. MMPs become functionally active when proteolytic cleavage releases the prodomain and the PRCXXPD sequence and exposes the zinc atom. Here, we report that the Pro-His81-Cys-Gly-Xaa-Xaa-Asp motif is not functional in proMMP-26 and consequently is not involved in the activation mechanisms. Organomercurial treatment failed to activate proMMP-26. The autolytic Lys-Lys-Gln59-Phe-His cleavage upstream of the Pro-His81-Cys-Gly-Xaa-Xaa-Asp motif induced the proteolytic activity of recombinant proMMP-26 whereas any further cleavage inactivated the enzyme. The His81→Arg mutation restored the conventional cysteine-switch sequence in the prodomain but failed to induce the cysteine-switch activation mechanism. These data and computer modeling studies allowed us to hypothesize that the presence of His81 significantly modified the fold of proMMP-26, abolished the functionality of the cysteine-switch motif, and stimulated an alternative intramolecular activation pathway of the proenzyme.

Members of the matrix metalloproteinase (MMP) family of enzymes share a number of structural and functional features (1). To date, about 25 human MMPs have been cloned and partially characterized (2, 3). MMPs are found in most primary and metastatic tumors where they are involved in the degradation of tissue barriers and the processing of cytokines, growth factors, hormones, and cell receptors, thereby being essential for tumor neovascularization, metastasis, and invasion (4–7). Evidence is emerging that MMPs are involved in inflammation, arthritis, and certain cardiovascular and neurodegenerative diseases (8–12).

The catalytic domain of all known MMPs contains an active site zinc that binds to three conserved histidines in the HEXX-HXXGXXH/S/T/C_M zinc-binding motif (1, 13). With the exception of MMP-23 (14, 15), the prodomain of all known MMPs has a highly conserved free cysteine residue within the consensus PRCXXPD sequence motif (13). This motif regularly called the cysteine-switch (16) has been proposed to prevent latent enzymes from becoming inappropriately active by binding to the catalytic zinc atom. The cysteine residue of the PRCXXPD motif can interact with the zinc ion of the catalytic domain and provide the fourth coordination site for the catalytic zinc ion. The cysteine residue may act in concert with other residues to maintain the conformation of the prodomain and shield the enzyme’s active site from substrates. MMPs become functionally active when proteolytic cleavage releases the prodomain, exposing the zinc atom and freeing the active site to interact with substrate (1).

Recently, we and other authors identified the human genes coding for novel matrix metalloproteinase, MMP-26 (endometase; matrilysin-2) (2, 17–19). Remarkably, proMMP-26 exhibited the unique PHCGVPD cysteine-switch motif. The presence of His81 but not Arg in this motif distinguishes proMMP-26 from all other proMMPs known so far. Our earlier studies demonstrated relatively close relations of MMP-26 with MMP-7 (matrilysin) (2). It is likely that MMP-7 and MMP-26 are both apically secreted by tumor cells in vivo (20). In contrast, most other soluble MMPs in tumors are made by stromal cells, not carcinoma cells (5). Expression of MMP-26 is largely restricted to carcinomas of epithelial origin (21).

Computer modeling of MMP-26 led us to a hypothesis that the prodomain of MMP-26 must undergo significant rearrangements to stabilize the cysteine-switch relative to the catalytic zinc (2). Conversely, the prodomain fold of MMP-26 with the non-functioning cysteine-switch is likely to be distinct from that of other MMPs leading to the suggestion that this protease employs the alternative mechanisms to maintain its activation or the proenzyme state.

Our findings reported here suggest that the unique PHCGXXD sequence is not involved in the latency of proMMP-26 and that the activated species of MMP-26 retains the cysteine-switch motif.

EXPERIMENTAL PROCEDURES

Materials—All reagents for molecular cloning except AdvanTaq™ Plus DNA polymerase used in PCR (CLONTECH, Palo Alto, CA) were purchased from New England Biolabs (Beverly, MA). The individual catalytic domain of MT2-MMP, MT3-MMP, MT4-MMP, and a hydroxamate inhibitor (GM6001) were from Chemicon (Temecula, CA). The individual catalytic domain of MT1-MMP was prepared as described earlier (2). The fluorescence peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (where Mca is 7-methoxy coumarin and Dpa is 3-(2,4-dinitrophenyl)aminopropionic acid) was from Bachem (King of Prussia, PA). Other chemicals were from Sigma. TIMP-2-free proMMP-2 was isolated from conditioned medium of p2AHT2A72 cells derived from HT-1080 fibrosarcoma cell line sequentially transfected with E1A and MMP-2 cDNA (22).

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Construction of the Mutants—Cloning of the full-length proMMP-26 cDNA was described previously (3). The fragments of the proMMP-26 cDNA encoding the wild type catalytic domain (catMMP-26 WT) and the proenzyme lacking the signal peptide were amplified by PCR and recloned for the subsequent expression in *Escherichia coli* into the pET21a (+) vector (Novagen, Madison, WI). PCR mutagenesis was used to replace His\(^{81}\) of the putative Pro-His\(^{81}\)-Cys-Gly-Val-Pro-Asp "cysteine switch" motif with the Arg residue in the sequence of proMMP-26 as well as to construct the C-termi nally truncated catMMP-26-3HLYGEKSSDIP mutant and the mutants with the modified C-terminal region such as catMMP-26-LYGKN, catMMP-26-LYGKNSRP7GCRSRR, and catMMP-26-LYGLEHIIIIIIIIIIII (modified amino acid residue positions are italicized). All constructs were sequenced to exclude PCR errors.

Expression of MMP-26 in *E. coli*—The wild type and mutant MMP-26 were expressed in *E. coli* BL21 (DE3) cells on induction with 2.5 mM isopropyl-β-D-galactopyranoside as insoluble inclusions. After incubation for 3–5 h at 37 °C, *E. coli* cells were collected by centrifugation and resuspended in 20 mM Tris-HCl buffer, pH 7.6, followed by sonication of the suspension and centrifugation at 14,000 × g. The pellet (insoluble inclusions) containing denatured MMP-26 was additionally purified by using a Bug Buster HT protein extraction reagent (Novagen). Purified inclusion bodies were solubilized in 20 mM Tris-HCl, pH 8.0, containing 8 M urea and 10 mM DTT, and subjected to ion-exchange chromatography on a 1 × 5-cm Mono Q column (Amersham, Biosciences). The bound material was eluted with a linear 0–500 mM NaCl gradient. The eluted fractions were analyzed for the presence of MMP-26 by SDS-PAGE and gelatin zymography. To refold the MMP-26, the fractions containing the purified material were combined, 2-fold diluted in Buffer A (50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM CaCl\(_2\), 50 μM ZnCl\(_2\), 0.01% Brij 35) containing 1 mM DTT, and then dialyzed overnight against Buffer A without DTT at 4 °C. The refolded MMP-26 samples were immediately used in our assays.

Enzyme Assays—The enzymatic activity was measured using quenched fluorescent peptide substrate. Fluorescence measurements were made on a Max fluorescence microplate reader (Molecular Devices, Sunnyvale, CA) by following excitation/emission at 328/405 nm in 96-well plates at 37 °C. The reaction mixture (100 μl) in Buffer A contained 2 μM substrate and refolded MMP-26 (100 ng).

N-terminal Protein Sequencing—The activation products of proMMP26 were subjected to SDS-PAGE, transferred on to an Immobilon membrane (Millipore, Bedford, MA), and stained with Coomassie. After destaining, the stained bands were excised and subjected to the N-terminal protein sequencing.

Activation of proMMP-26—Pro-MMP-26 and proMMP-2 (100 ng each in Buffer A) were treated for 4 h at ambient temperature with 2 mM 4-aminophenylmercuric acetate (APMA). Alternatively, proMMP26 (100 ng in Buffer A) was processed with N-tosyl-l-phenylalanin chloromethyl ketone-treated bovine trypsin (5 ng, the enzyme-substrate ratio ~1:20) for 0.1–24 h at ambient temperature. The reaction was stopped by adding 1 mM phenylmethylsulfonyl fluoride. Additionally, proMMP26 (100 ng in Buffer A) was incubated at 37 °C for 16 h at the approximately 1:3 enzyme-substrate ratio with the individual catalytic domain of each MT1-MMP, MT2-MMP, MT3-MMP, and MT4-MMP or the activated MMP-2 enzyme. The digested samples were mixed with an equal volume of 2 × SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.005% bromphenol blue, and 20% glycerol) containing 20 mM DTT, boiled for 5 min, and examined by SDS-PAGE (4–20% polyacrylamide) (Novex, San Diego, CA) and by gelatin zymography in 0.1% gelatin, 15% polyacrylamide gels. Regularly, the gels were stained with Coomassie or, where indicated, with Silver Xpress stain (Novex). Purified proMMP2 (23) was used as control in our activation studies. Protein concentration was measured with Coomassie protein assay reagent (Pierce).

RESULTS AND DISCUSSION

Computer Modeling of MMP-26—Modeling of the region involving the cysteine-switch motif and the active site zinc ion allows us to speculate that the proMMP-26 prodomain fold is distinct from that of other MMPs. Thus, in the structure of related MMP-7, a β-strand from the prodomain blocks the active site (Fig. 1). The position of this β-strand is stabilized by the ion pair involving Arg\(^{81}\) and Asp\(^{86}\) and is surrounded by a hydrophobic cluster composed of Phe\(^{80}\) and Phe\(^{81}\). All these residues are conserved in MMPs with the exception of MMP-26 (Arg\(^{81}\) → His\(^{81}\)). Substitutions existing in the MMP-26 prodomain result...
in a partially unfolded β-strand. Because the histidine side chain is short, the Arg<sup>81</sup> → His<sup>81</sup> substitution precludes the formation of both the ion pair with the Asp<sup>86</sup> and the hydrophobic cluster involving the phenylalanine residues. Simple minimization does not improve the packing of the MMP-26 prodomain suggesting that the prodomain must undergo larger rearrangements to maintain the proenzyme state. Limitations of the current algorithms do not allow us to model the rearrangements involved in the stabilization of MMP-26. However, it could be concluded that the prodomain fold of MMP-26 is likely to be different relative to that of other MMPs, especially at high pH when His<sup>81</sup> is not protonated.

### FIG. 2. Expression and mutagenesis of MMP-26

A, expression of catMMP-26-WT and proMMP-26-WT in E. coli. Insoluble inclusions, purified catMMP-26-WT, and proMMP-26 were analyzed by SDS-PAGE (right panel) and gelatin zymography in gels containing 0.1% gelatin (left panel). Positions of molecular mass markers are shown on the right. B, MT1-MMP degrades but does not activate proMMP-26-WT. ProMMP-26 alone was analyzed by gelatin zymography (lane 1) and by SDS-PAGE (lane 4; silver-stained gel). An aliquot of proMMP-26 (100 ng) was mixed with MT1-MMP (30 ng) and immediately analyzed by gelatin zymography (lane 2) or incubated for 16 h and then analyzed by gelatin zymography (lane 3) and SDS-PAGE (lane 5, silver-stained gel). C, schematic representation of the wild type and mutant constructs of proMMP-26 and catMMP-26. Modified residues are italicized. Arrow indicates the autocleavage site. The specific activity of the solubilized, purified, and refolded constructs against the fluorescence substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub> are shown in arbitrary units/mg of protein. The presence and absence of gelatinolytic activity in zymography depicted by + and −, respectively. The N-terminal methionine is in parentheses. D, APMA does not activate proMMP-26. APMA activates MMP-26 and mutant proMMP-26-Arg were both treated with APMA and analyzed by SDS-PAGE (left panel). Right panel, proMMP-2 was activated by APMA or MT1-MMP and analyzed by SDS-PAGE. A band marked by arrowhead has the N terminus of the prodomain (<sup>16</sup>MVPVPP). E, autocatalytic activation of proMMP-26. The samples were analyzed by SDS-PAGE (lanes 1 and 2) and by gelatin zymography (lanes 3–5). ProMMP-26-WT (lanes 2 and 5, proenzyme alone) was autocatalytically activated at 1 mg/ml in Buffer A for 3 h at 4 °C (lanes 1 and 3). Further, the gelatinolytic band that corresponded to proMMP-26 (lane 5) was excised, extracted from the gel, and re-run on gelatin gel (lane 4). The protein bands marked as A, B, C, and D (lane 1) were transferred onto a membrane, and their N-terminal protein sequence was determined. The N-terminal sequence was <sup>16</sup>MVPVPP, <sup>60</sup>QFHRN, <sup>60</sup>QFHRN, and <sup>60</sup>QFHRN, respectively. Note that the bands in lane 1 are highly similar to those observed in lanes 3 and 4. Positions of molecular mass markers are shown on the right.
Consequently, analysis of proMMP-26 and identification of the mechanisms involved in its activation are of interest. These studies may facilitate rational manipulation of this protease in a clinically advantageous manner and understanding of its function in vivo. Because the yield of MMP-26 expressed in mammalian systems was low, we employed a bacterial expression system to generate the full-length MMP-26 zymogen and the truncated species of MMP-26 representing its individual catalytic domain (catMMP-26).

Expression in E. coli—To characterize the catalytic activity and the activation mechanisms of MMP-26, we expressed, solubilized, purified, and refolded both the wild type proMMP-26-WT and catMMP-26-WT. Following induction with isopropyl-β-D-thiogalactoside, both forms of MMP-26 were found highly expressed in E. coli as insoluble inclusions. On solubilization in 8 M urea both proMMP-26-WT and catMMP-26-WT could be purified under denaturing conditions to give a major band at the molecular mass of 28 and 19 kDa when analyzed by SDS-PAGE under non-reducing conditions, respectively (Fig. 2A). Following transfer to a membrane, the main MMP-26 forms were analyzed by the N-terminal sequencing. The N-terminal sequencing further confirmed that the 28-kDa band corresponded to proMMP-26-WT (Met-Val18-Pro-Val-Pro-Pro-Ala) whereas the 19-kDa form represented the catMMP-26-WT species (Met-Thr90-Ser-Ile-Ser-Xaa-Gly) (Fig. 2A). As expected, both forms exhibited one extra initiation codon coded for a methionine residue. Both proMMP-26-WT and catMMP-26-WT exhibited gelatinolytic activity in gelatin zymography (Fig. 2A).

Activation of ProMMP-26 and Mutagenesis of the Cysteine-switch Motif—Generally, activation of latent zymogens of MMPs requires cleavage of the N-terminal propeptid by an external protease or by intramolecular autocatalytic mechanisms induced by organomercurial compounds (24). These compounds modify the thiol group of the unique free cysteine residue of the PRCXXPD cysteine-switch sequence. The treatment of the refolded proMMP-26-WT with organomercurials such as APMA, which are known to result in the activation of other members of the MMP family or proteases including trypsin, MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MMP-2, and catMMP-26-WT failed to induce the activation of proMMP-26-WT. Thus, the individual catalytic domain of MT1-MMP degraded proMMP-26-WT without generating the stable proteolytic fragments that might correspond to the active MMP-26 enzyme (Fig. 2B). The cleavage profile of proMMP-26-WT treated with other MT-MMPs was highly similar. The treatment of proMMP-26-WT with individual MT-MMPs did not enhance the proteolytic activity against the fluorescence peptide substrates (data not shown). In turn, proMMP-2 used as control in our studies was activated either by APMA or MT1-MMP and readily converted into the known activated species of MMP-2 (Fig. 2D). The catMMP-26-WT generated multiple cleavage products of proMMP-26-WT (data not shown) suggesting a possibility for an autocatalytic intramolecular pathway.

To identify a functional significance of the highly unusual His81 (underlined) of the PHCGVPD cysteine-switch, we performed site-directed mutagenesis of proMMP-26-WT to restore the conventional PRCGVPD motif in the mutant (proMMP-26-Arg) prodomain. This motif is conserved in all proMMPs except MMP-26. The mutant was expressed in E. coli as insoluble inclusions, solubilized, purified, and refolded as described for proMMP-26-WT. However, the pro-MMP-26-Arg mutant also resisted activation by any tested MMPs and was significantly more sensitive to trypsin suggesting that the mutation destabilized the mutant construct (data not shown).

Remarkably, the treatment of either proMMP-26-WT or proMMP-26-Arg with APMA also failed to induce limited proteolysis and activation of the zymogen (Fig. 2D). No increase in the proteolytic activity of proMMP-26-WT or proMMP-26-Arg was observed following APMA treatment. To further confirm that APMA did not induce activation of proMMP-26-WT, we identified the N-terminal peptide sequence of the APMA-treated samples. This sequence (18MVPVPP) confirmed the
presence of the zymogen with the intact N terminus. Thus, the restoration of the conventional cysteine-switch motif failed to induce the cysteine-switch activation mechanism of mutant proMMP-26.

Autoactivation of ProMMP-26—Conversion of proMMP-26 into the active enzyme was most efficiently accomplished by concentration-dependent autoproteolysis. Thus, self-activation of the refolded proMMP-26-WT at 0.1 mg/ml was accomplished in about 7–10 days in Buffer A at 4 °C. At 1 mg/ml proMMP-26-WT is fully autolytically processed in several hours (Fig. 2, C and E). Autoactivation was completely inhibited by 1 μM GM6001, a hydroxamate inhibitor of MMPs. SDS-PAGE of self-activated proMMP-26-WT confirmed the presence of the forms with the molecular mass in the range of 18–29 kDa. The expected molecular mass of the enzyme of MMP-26 cleaved downstream of the cysteine-switch motif is about 19 kDa. The C and D species with molecular masses of 19 and 17 kDa, respectively, were most active in gelatin zymography (Fig. 2E).

Importantly, the species of MMP-26 with the molecular mass below 18 kDa exhibited no gelatinolytic activity, thereby representing inactive degradation by-products. Self-activation of proMMP-26 correlated to an increase in the proteolytic activity of the samples. The specific activity of self-activated proMMP-26 was lower than but still comparable with that of catMMP-26-WT (Fig. 2C). This activity was fully suppressed by 1 μM GM6001 or 100 ng/ml TIMP-2.

To identify the N terminus of active MMP-26, we subjected the gelatinolytically active forms to the N-terminal sequencing. The proenzyme had the expected 13MVPVPP sequence whereas the cleavage products demonstrated the same N-terminal peptide sequence (6(Q)HFRN) localized upstream of the cysteine-switch motif. These findings suggest the presence of the cysteine-switch motif in all proteolytically active forms of MMP-26. Further, the difference in the molecular mass of the activation products indicated the C-terminal processing of autolytically activated proMMP-26-WT.

To support our data, we fully denatured proMMP-26-WT by SDS followed by protease renaturation and self-proteolysis. For these purposes, proMMP-26-WT was separated in gelatin gel, and the gelatinolytically active proMMP-26 band was excised from the gel, minced, and again denatured by resuspending the sample in 2× SDS-sample buffer. Further, the sample was subjected to gelatin zymography. The observed gelatinolytically active activation products were highly similar to those that resulted from self-activation at 4 °C. Hence, these observations strongly support our hypothesis that the active forms of MMP-26 preserve the cysteine-switch sequence and that the next shorter activation product unavoidably loses the activity. These findings agree with our computer modeling studies and with the resistance of proMMP-26 to organomercurials and suggest that the PHCGVPD cysteine-switch motif is not functional in proMMP-26 and that the cysteine residue is not involved in the maintenance of the proenzyme state. It is likely that similarly to bacterial metalloproteinases (e.g. prothrombin), which do not exhibit the cysteine-switch motif, proMMP-26 can naturally mature via an autocatalytic intramolecular pathway (25). Furthermore, it is tempting to hypothesize that if the fold of proMMP-26 is relatively similar to that of other MMPs, the four histidine residues existing in proMMP-26 (the three from the active site domain and the one from the cysteine-switch motif) may all directly interact with the zinc atom similarly to those observed in the Zn$^{2+}$ finger motifs.

The C-terminal Portion Is Essential for the Folding of MMP-26—Our data suggest the autolytic cleavage of the C-terminal HLYGEKCSSDIP sequence of MMP-26 and a possible functional role of the Cys$^{256}$ residue. We speculated that the C-terminal portion of MMP-26 is important for the folding of the enzyme. To address this question we constructed the following catMMP-26 mutants with the truncated or modified C-terminal region, catMMP-26-HLYGEKCSSDIP, -LYGKN, -LYGKNSRPGTCSR, and -LYGLEHHHHHHH (modified residues are italicized) (Fig. 2C). The constructs were expressed in E. coli as insoluble insolutions, solubilized, purified, and refolded as described for catMMP-26-WT (Fig. 3). Following refolding, only the catMMP-26-LYGKN and -LYGKNSRPGTCSR mutants demonstrated the proteolytic activity. The activity level of these mutants was similar to that of catMMP-26-WT whereas both other mutants (catMMP-26-HLYGEKCSSDIP and -LYGLEHHHHHHH) were completely inactive both in gelatin zymography and activity assay (Fig. 3). These findings suggest that the C-terminal portion including the LYG sequence, which is highly conserved among all MMPs, affects the folding rather than the activity of the catMMP-26 constructs. The peptide sequence downstream the conserved LYG is less critical for the folding: the presence of the Cys$^{256}$ is not essential; however, the existence of the positively charged Lys$^{258}$ facilitates the folding of MMP-26. The strong positive charge in the C-terminal LYGRKNSRKKK peptide sequence of MMP-7 (GenBank™ accession number P09237) supports our suggestions. Presumably, the C-terminal region acts as an intramolecular chaperone in vivo. However, if the folding is completed, this sequence may be truncated (autolytically or by an external protease) without significantly affecting the catalytic efficiency of the resulting construct.

In conclusion, we suggest that proMMP-26 could be autolytically activated by the mechanisms, which do not involve the cysteine-switch. Prior studies have shown that the disruption of the Cys-Zn$^{2+}$ coordination alone is not sufficient to activate MMP-3 (stromelysin 1) (29) and that time-dependent conformational changes (probably involving the Arg$^{31}$ to Asp$^{36}$ salt bridge; numbering is given according to the MMP-26 peptide sequence) are needed prior to the expression of proteolytic activity (30). The functionality of the cysteine-switch motif in tumor necrosis factor-γ converting enzyme (TACE) or disintegrin and metalloproteinase (ADAM) family members is posing a question regarding its role in the folding of the latent zymogens (31). Further, Kotra et al. (32) presented evidence for the critical importance of a protonation event at the coordinated thiolate as a prerequisite for the departure of the propeptide from the active site of the proMMPs. Modeling of the MMP-26 structure and wet chemistry experiments suggest that both the presence of the His$^{81}$ but not the Arg$^{83}$ and the folding of the prodomain of MMP-26 eliminated the cysteine-switch mechanism in this proteinase, which evolved to adapt a distinct activation pathway. To this end, the catalytic domain of MMP-26 is 45% identical to that of MMP-7 whereas the identity of the prodomain of MMP-26 to that of MMP-7 is hardly seen and is significantly below 20%. It is tempting to hypothesize that the Arg → His substitution existing in the unique Pro-His$^{81}$-Cys-Gly-Xaa-Xaa-Asp cysteine-switch motif of proMMP-26 abolished the ability of the Cys$^{85}$ to interact with the zinc ion of catalytic domain and that the cysteine-switch motif is not involved in the maintenance of the proenzyme state of MMP-26.

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