Characterization of matrix metalloproteinase-26, a novel metalloproteinase widely expressed in cancer cells of epithelial origin

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Identification of expanding roles for matrix metalloproteinases (MMPs) in complex regulatory processes of tissue remodelling has stimulated the search for genes encoding proteinases with unique functions, regulation and expression patterns. By using a novel cloning strategy, we identified three previously unknown human MMPs, i.e. MMP-21, MMP-26 and MMP-28, in comprehensive gene libraries. The present study is focused on the gene and the protein of a novel MMP, MMP-26. Our findings show that MMP-26 is specifically expressed in cancer cells of epithelial origin, including carcinomas of lung, prostate and breast. Several unique structural and regulatory features, including an unusual 'cysteine-switch' motif, discriminate broad-spectrum MMP-26 from most other MMPs. MMP-26 efficiently cleaves fibrinogen and extracellular matrix proteins, including fibronectin, vitronectin and denatured collagen. Protein sequence, minimal modular domain structure, exon–intron mapping and computer modelling demonstrate similarity between MMP-26 and MMP-7 (matrilysin). However, substrate specificity and transcriptional regulation, as well as the functional role of MMP-26 and MMP-7 in cancer, are likely to be distinct. Despite these differences, matrilysin-2 may be a suitable trivial name for MMP-26. Our observations suggest an important specific function for MMP-26 in tumour progression and angiogenesis, and confirm and extend the recent findings of other authors [Park, Ni, Gerkema, Liu, Belozerov and Sang (2000) J. Biol. Chem. **275**, 20540–20544; Uría and López-Otín (2000) Cancer Res. **60**, 4745–4751; de Coignac, Elson, Delneste, Magistrelli, Jeannin, Aubry, Berthier, Schmitt, Bonnefoy and Gauchat (2000) Eur. J. Biochem. **267**, 3323–3329].

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INTRODUCTION

Remodelling of the extracellular matrix is an essential characteristic of normal and pathological conditions [1–4]. Complexity of cell–matrix interactions implies a plethora of proteolytic enzymes capable of directly cleaving extracellular matrix proteins. Matrix metalloproteinases (MMPs) are a comprehensive family of zinc metalloenzymes that are involved in the breakdown of extracellular matrix proteins [3]. To date, approx. 20 human MMPs have been cloned and partially characterized. They are classified into four main subfamilies according to their substrate specificity, amino acid sequence and cellular location [4,5]. These subfamilies comprise the collagenases, gelatinases, stromelysins and membrane-type MMPs (MT-MMPs), although some enzymes, such as macrophage metalloelastase (MMP-12) [6] and MMP-19 [7] cannot be convincingly classified within any group. Synthesized as inactive precursors, MMPs undergo proteolytic activation in order to exhibit enzymic activity. Once activated, MMPs can be inhibited by ubiquitous tissue inhibitors of metalloproteinases (TIMPs), such as TIMP-1, TIMP-2, TIMP-3 and TIMP-4 [8,9]. Activation that may occur intracellularly and extracellularly requires the cleavage of a prodomain located downstream of a signal peptide [3]. A unique free cysteine residue in the prodomain can interact with the zinc ion of the catalytic domain to maintain the enzyme in its inactive state [10]. In addition to calcium binding sites, the catalytic domain contains a conserved HEXGHXXGXXHS/T zinc-binding motif (where single-letter amino-acid notation has been used). A linker region, referred to as the hinge, separates the catalytic domain from the C-terminal haemopexin-like domain that consists of four repeats similar to haemopexin and vitronectin [11,12]. Both matrilysin (MMP-7) and MMP-23 are missing the C-terminal domain. Although this domain is not critical for enzyme activity, it seems to be essential for regulating activity and substrate specificity of at least several known MMPs [1,3,13–15]. In addition to this modular domain structure, some MMPs display additional features, such as fibronectin type II repeats in gelatinases (MMP-2 and MMP-9), a membrane-spanning hydrophobic region and cytoplasmic tail in MT-MMPs (excluding MT4- and MT6-MMPs), a vitronectin-like insert in XMMP (from *Xenopus*), a hydrophobic N-terminal signal-anchor in MMP-23 [16] and a proprotein convertase recognition motif, RXRXKR, in some MMPs, including MT-MMPs and stromelysin-3 [1,17].

Although structure–function relationships of MMPs are far from being understood in detail, these enzymes have been shown to be strongly associated with cancer cell invasion, metastasis and tumour neovascularization [4,18–20]. Evidence is emerging that MMPs are involved in inflammation, arthritis and certain

Abbreviations used: AP, activator protein; DMEM, Dulbecco's modified Eagle's medium; Dpa, 3(2,4-dinitrophenyl)diaminopropionic acid; Dnp, dinitrophenyl; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; Mca, 7-methoxycoumarin; MMP, matrix metalloproteinase; MT-MMPs, membrane-type MMPs; MTC, multiple tissue cDNA; Nva, norvaline; PEA3, polyoma virus enhancer A-binding protein-3; RACE, rapid amplification of cDNA ends; RT-PCR, reverse-transcriptase PCR; TIMPs, tissue inhibitors of metalloproteinases.

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The nucleotide sequence data for MMP-26 and MMP-28 appear in the GenBank® Nucleotide Sequence Database under the accession numbers AF291664, AF291665 and AF315683.

cardiovascular and neurodegenerative diseases [2,21–23], and that the role of MMPs is not limited to the proteolytic cleavage of the extracellular matrix [22,24–27]. In addition to pericellular proteolysis, some MMPs may be essential in the processing of cytokines, growth factors, and hormone and cell adhesion receptors, and are therefore critical in apoptosis, morphogenesis, proliferation and development [22,28]. Identification of expanding roles for MMPs in complex regulatory and remodelling processes has stimulated the search for genes coding for proteinases with unique functions, regulation and expression patterns [7,29–40].

In the present study we report a molecular cloning strategy that facilitated the identification of three novel MMP genes. We refer to these proteinases as MMP-21, MMP-26 and MMP-28. A similar strategy may facilitate identification of additional genes in a supergene family like the MMPs. This paper is focused on the biochemical and structural data which extend our knowledge of MMP-26, an unusual broad-spectrum protease expressed in cancer cells of epithelial origin.

All alignments, models (in PDB format) and VRML files for visualization of surface properties discussed in the present paper are available at the bioinformatics server of the Burnham Institute at http://bioinformatics.burnham.org under the link 'Additional material'.

EXPERIMENTAL

Materials

Restriction endonucleases, T4 DNA ligase and other reagents for molecular cloning were purchased from New England Biolabs (Beverly, MA, U.S.A.). AdvanTaq™ Plus DNA Polymerase for PCR was obtained from ClonTech Laboratories (Palo Alto, CA, U.S.A.). Collagen (types I, II, III and IV), fibronectin, vitronectin, fibrinogen, laminin V, TIMP-1, TIMP-2 and GM6001 (also known as Ilomastat) were obtained from Chemicon International (Temecula, CA, U.S.A.). α_1-Antitrypsin and α_2-macroglobulin were purchased from Calbiochem (San Diego, CA, U.S.A.). The fluorogenic peptide substrates were obtained from Bachem (King of Prussia, PA, U.S.A.). The catalytic domain of MT1-MMP was purified from *Escherichia coli* cells and refolded as previously described [41]. Other chemicals and reagents were purchased from Sigma (St. Louis, MO, U.S.A.).

Molecular cloning

Known MMP sequences were depleted from the first-strand mixed cDNA library representing the human multiple tissue cDNA (MTC) panel I, the human fetal MTC panel and the human tumour MTC panel (ClonTech Laboratories) by using the following 19 biotinylated probes: U1, 5′-AGGTGGACCA-ACAATTTCAGAGAG-3′; U2, 5′-GCAAGTGGGGCTTCT-GCCCTGACC-3′; U3, 5′-CAATGGACAAAGGATACAAC-AGG-3′; U4, 5′-CTGGACGGATGGTAGCAGTCTAGG-3′; U5, 5′-TGGACCAACACCTCCGCAAATTAC-3′; U6, 5′-AGCGACAAGAAGTGGGGCTTCTGC-3′; U7, 5′-TGGAC-AGAAGATGCATCAGGCACC-3′; U8, 5′-ACCTGGACTA-TCGGGGATGACCAG-3′; U9, 5′-TTCTGGACTACACAT-TCAGGAGGC-3′; U10, 5′-ACCTGGACAAGTAGTTCCA-AAGGC-3′; U11, 5′-TCTGGACTGAGGGGACCTACCGT-G-3′; U12, 5′-GAAGTGGACTATGGGAACGAATGG-3′; U13, 5′-TACAGCTGGAAGAAAGGCGTGTGG-3′; U14, 5′-TGGACTGTCAGGAATGAGGATCTG-3′; U15, 5′-CTTCT-CCAGCACTGACCTGCATGG-3′; U16, 5′-GAGCCATGG-ACACTAGGAAATCC-3′; U17, 5′-ACCTTCCGCTCCTCG-GATGCCCAC-3′; U18, 5′-CGCTAGGAAATGCCAACCAT-GAC-3′; and U19, 5′-GACCTGGACTTTTGGGTCAAAA-GG-3′. The following sequences released by GenBank®, NM_002421, PO8253, NM_002422, PO9237, JO5556, NM_004994, NM_002425, NM_005940, NM_002426, NM_002427, NM_002429, NM_004771, AJ005256, 4826833, 4505310, 5174582, AB021225, 5729928 and AAG17007, were used to design these unique U1–U19 primers for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-11, MMP-12, MMP-13, MMP-19, MMP-20, MMP-23, MT1-MMP, MT2-MMP, MT3-MMP, MT4-MMP, MT5-MMP and MT6-MMP respectively. For these purposes, the first-strand cDNAs from 24 tissues (0.6 ng each in a total volume of 72 μl) were denatured for 5 min at 100 °C, diluted with 6 × SSC/1 × Denhardt's hybridization buffer [where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate, and Denhardt's buffer is 0.02 % Ficoll 400, 0.02 % polyvinylpyrrolidone and 0.02 % BSA] and allowed to hybridize with the U1–U19 mixture (1 pmol of each probe) for 1 h at 56 °C. The hybrids were removed from the library by using Dynabeads Streptavidin M-280 magnetic beads (Dynal, Lake Success, NY, U.S.A.). Subsequently, the biotinylated P3 probe 5′-GCNCAYGARAWTGGNCAYNC-NYTNGGNYT-3′ (1 pmol) was allowed to hybridize to the depleted library. The resulting hybrids were bound with the M-280 beads. After heating the beads at 100 °C, the free cDNAs were amplified by touch-down PCR using AdvanTag Plus DNA Polymerase (ClonTech Laboratories) and the P1 5′-GGNGW-NRCNCAYTTYGAY-3′ forward primer (0.2 μM) and the P2 5′-YTGNAYNCCNNNNAYRTCRTC-3′ reverse primer (0.2 μM). The PCR products were separated by 2 % agarose gel electrophoresis. The DNA bands with a size of 200–240 bp were extracted and cloned into the pCRII vector (Invitrogen, Carlsbad, CA, U.S.A.). Approx. 600 white colonies were analysed by PCR using a mixture of the U1–U19 forward primers (0.1 μM each) and the P2 reverse primer (0.4 μM). Out of the colonies 29 failed to provide any amplifiable 160–230 bp fragment. *Eco*R1 restriction analysis of these 29 plasmids confirmed the existence of the 210–230 bp DNA insert in six clones. These DNA inserts were then sequenced to verify whether they coded for novel MMPs. One insert corresponded to the cDNA fragment coding for MMP-26. Two other inserts encoded human MMP-28 and MMP-21. The MMP-26 cDNA gene was further amplified from Lung Marathon-Ready™ cDNA (ClonTech Laboratories) by using the 5′- and 3′-rapid amplification of cDNA ends (RACE) protocols. Finally, the full-length MMP-26 cDNA from three independent clones was sequenced to exclude any errors in the gene sequence.

Tissue specificity of novel MMP genes

Expression of MMP-26 in various tissues was analysed by PCR amplification of the human fetal MTC panel, the human MTC panel I and the human tumour MTC panel by employing the 5′-TCCATCGGAATGGGACAGACC-3′ and 5′-GCCCACTGC-CAGAAAGAAACC-3′ primers specific to the sequence of MMP-26 that gave rise to a 289 bp fragment.

Chromosomal mapping

The chromosomal location of the MMP-26 gene in the genome was examined by screening the G3 83 human radiation hybrid panel (Research Genetics, Huntsville, AL, U.S.A.). The forward and reverse primers used for PCR amplification were the unique sequences 5′-TCCATCGGAATGGGACAGACC-3′ and 5′-GCCCACTGCCAGAAAGAAACC-3′, respectively, derived from the MMP-26 gene sequence.

Expression of the catalytic domain of MMP-26 in *E. coli*

A 498 bp fragment of the MMP-26 gene, corresponding to the catalytic domain, was amplified with 5′-ACATATGACCTC-CATCTCGCCAG-3′ and 5′-TGAATTCTTATCCATACAAA-TGCTGG-3′ primers. PCR products were cloned into the pCRII plasmid and then subcloned with *Nde*I and *Eco*RI into the pFLAG-ATS (Sigma) expression vector under the regulatory elements of the *tac*-promoter. Resulting constructs were transformed into BL21 (DE3) competent cells and grown in 2YT medium (16 g/l tryptone, 10 g/l yeast extract and 5 g/l NaCl) supplemented with 50 μg/ml ampicillin. The expression of MMP-26 was induced by 2.5 mM isopropyl β-D-thiogalactoside (IPTG). After incubation for 5 h at 37 °C, *E. coli* cells were collected by centrifugation and lysed. Inclusion bodies containing the denatured catalytic domain of MMP-26 were isolated from cell lysates by centrifugation.

Inclusion bodies were solubilized in 20 mM Tris (pH 8.0), containing 8 M urea and 10 mM dithiothreitol (DTT), and were subjected to ion-exchange chromatography on a 1 cm × 5 cm Mono Q column (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Bound material was eluted with a linear NaCl gradient (0–500 mM). The eluted fractions were analysed for the presence of MMP-26 using SDS/PAGE and gelatin zymography. To refold the catalytic domain of MMP-26, the combined fractions containing the purified protein were dialysed at room temperature against 4 M urea in 50 mM Tricine buffer (pH 7.5), containing 1 mM DTT for 2 h and then overnight against buffer A [50 mM Hepes (pH 7.5), 200 mM NaCl, 10 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.01 % Brij 35] at 4 °C. The refolded catalytic domain of MMP-26 was used immediately in enzyme assays. In addition, the purified catalytic domain was used to raise anti-(MMP-26) antibodies in rabbits.

Enzyme assays

Enzymic activity of the purified catalytic domain of MMP-26 was measured using the following quenched fluorogenic peptide substrates: Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [where Mca is 7-methoxycoumarin, and Dpa is 3(2,4-dinitrophenyl) diaminopropionic acid], Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2, Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2 (where Dnp is dinitrophenyl, and Nva is norvaline) and Mca-Arg-Pro-Lys-Pro-Val-Ala-Nva-Trp-Arg-Lys(Dnp) -NH_2, All fluorimetric measurements were made with an fMax fluorescence microplate reader (Molecular Devices, Sunnivale, CA, U.S.A.) (λ_{exc} = 328 nm and λ_{emm} = 405 nm) in 96-well plates at 37 °C. The reaction mixture contained 2 μM substrate and the refolded MMP-26 catalytic domain (200 ng) or the catalytic domain of MT1-MMP (200 ng) in buffer A (100 μl).

Hydrolysis of macromolecular substrates

Cleavage of native and denatured collagens (types I, II, III and IV), fibronectin, vitronectin, fibrinogen, laminin V, α_1-antitrypsin and α_2-macroglobulin by the MMP-26 catalytic domain or the MT1-MMP catalytic domain was followed by reducing SDS/PAGE. All assays were performed in buffer A overnight at 37 °C, except native collagens, which were assayed at 25 °C. The reaction mixture contained 1 μg of the individual protein substrate and either 100 ng of MMP-26 or 20 ng of MT1-MMP. The reactions were stopped with 20 mM EDTA. The samples were mixed with an equal volume of 2× SDS/PAGE sample buffer [125 mM Tris/HCl (pH 6.8), 4 % (v/v) SDS, 0.005 % Bromophenol Blue and 20 % (v/v) glycerol] containing 20 mM DTT, boiled for 5 min at 100 °C, and resolved by SDS/PAGE (4–20 % polyacrylamide) (Novex, San Diego, CA, U.S.A.).

In addition, α1-antitrypsin, fibrinogen, fibronectin and vitronectin (5 μg of each) were digested by the MMP-26 catalytic domain (100 ng) in buffer A at 37 °C overnight. Subsequently, the digestion products were transferred on to a PVDF membrane, and stained with Coomassie Brilliant Blue R250. After destaining, the major stained bands were excised and subjected to N-terminal protein microsequencing in order to identify the cleavage site.

Cell transfection

Human MMP-26 cDNA was cloned into the mammalian expression vector pcDNA.3-neo (Invitrogen). MCF7 breast carcinoma cells (A.T.C.C., Rockville, MD, U.S.A.) were stably transfected using LIPOFECTAMINE™ (Gibco BRL, Rockville, MD, U.S.A.) according to the manufacturer's instructions. Several cell clones resistant to 0.6–0.8 mg/ml G418 (Sigma) were further analysed by immunofluorescence. The control cell line was generated as a pool of non-cloned antibiotic-resistant cells after transfection with the original plasmid. Transfected cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (v/v) fetal calf serum and 0.25 mg/ml G418.

Immunofluorescence

Eight-well glass chambers (LabTek II; Nalge Nunc International, Naperville, IL, U.S.A.) were pre-coated overnight at 4 °C with type I collagen (Vitrogen 100; Cohesion, Palo Alto, CA, U.S.A.) in PBS (50 μg/ml). Wells were washed with PBS (pH 7.4) and then serum-free DMEM. MCF7 cells were plated in complete medium at 2×10^4 cells/chamber. Following incubation for 16–18 h, cells were washed with PBS, fixed for 20 min with an ice-cold 1:1 mixture of methanol/acetone and blocked with PBS containing 20 % (v/v) goat serum for 30 min. Next, cells were stained with control or rabbit MMP-26 antiserum diluted 1:5 with PBS containing 10 % (v/v) goat serum. Following incubation for 1 h, cells were extensively washed with PBS and then incubated for 30 min with 10 μg/ml of the secondary goat anti-rabbit IgG conjugated with Oregon Green (Molecular Probes, Eugene, OR, U.S.A.). After rinsing in PBS, cells were embedded into SlowFade Light Antifade solution (Molecular Probes) and studied under an inverted epifluorescent microscope (Nikon TE300) equipped with a Spot-RT cooled colour CCD camera. Digital recorded images were processed with Adobe Photoshop software (Adobe, San José, CA, U.S.A.).

RESULTS

Molecular cloning strategy

To facilitate the isolation of previously uncharacterized MMP genes we modified the routine cloning strategy. Briefly, our approach involved the following steps: (1) depletion of known MMP genes from a comprehensive multi-tissue first-strand gene library by using unique biotin-labelled primers immobilized on streptavidin-Dynabeads; (2) isolation of the uncharacterized genes using a biotin-labelled degenerate primer immobilized on streptavidin-Dynabeads; (3) amplification of the resulting cDNA pool by PCR; and (4) cloning of the PCR products followed by analysis of the individual colonies. This allowed us to identify three previously unknown sequences of human MMPs, i.e. MMP-26 (deposited in the GenBank® database with accession numbers AF291665 and AF291664, for the cDNA gene and the genomic

gene respectively), MMP-21 and MMP-28 (accession number AF315683). The strategy described in detail in our previous publication [42], and the protein sequences of the identified MMPs, which were uncharacterized at that time, are shown in Figure 1.

The first human gene identified in our work was highly similar in sequence to the XMMP/MMP-21 gene earlier identified in *Xenopus* [43]. The available 1038 bp fragment of human MMP-21 encodes a partial sequence of the putative catalytic domain followed by the complete sequence of the putative hinge and haemopexin-like domains. The catalytic domain of MMP-21 has the HEIGHVLGLPHT zinc-binding site. The known amino acid sequence of human MMP-21 is 73% identical to the corresponding region of *Xenopus* XMMP/MMP-21 [43]. These findings suggest that the MMP-21 found in *Xenopus* is also expressed in human tissues.

While this study was in progress, three other groups simultaneously and independently identified the structure of the second novel gene, i.e. MMP-26 ('endometase') [29,33,37]. The third novel gene has been tentatively named MMP-28 [42]. Recently, the existence and the structure of MMP-28 have been independently reported by another group [31]. These authors used the name 'epilysin' to emphasize the expression of MMP-28 in epithelium.

We subsequently used human kidney, lung and testis Marathon-Ready™ cDNA gene libraries in RACE experiments in order to extend the length of the novel genes. Successive 3′- and 5′-RACE experiments allowed us to obtain fragments that were long enough to contain the entire coding information for MMP-26. In the present study, we focus on the characterization of the gene and the protein of MMP-26.

MMP-26 peptide sequence

Computer analysis of the 998 bp cDNA sequence obtained from the human kidney cDNA library (Figure 2, top panel; deposited in GenBank® with accession number AF291664) revealed an open reading frame that coded for a 261-amino-acid preproproteinase with a predicted molecular mass of 29.6 kDa. MMP-26 does not have the C-terminal haemopexin-like domain. In the structure of MMP-26, a signal peptide containing 17 hydrophobic residues follows the initiation methionine residue, suggesting that this proteinase is secreted [33]. Downstream of the signal peptide, a prodomain containing a putative unique unpaired Cys^{82} is followed by the catalytic domain, which contains the highly conserved HEIGHSLGLQHS zinc-binding motif. Further downstream, a stretch of seven amino acids separates the zinc-binding motif from Met^{226}. The position of this methionine residue, an essential component of the 'methionine turn', is strictly conserved in MMPs [44]. These structural features indicate that MMP-26 belongs to the MMP family of enzymes. In its minimal modular domain design, MMP-26 is most closely related to MMP-7, the shortest member of the MMP family.

There is a unique feature that distinguishes MMP-26 from all known MMPs. In MMP-26, the highly conserved PRCGXXD 'cysteine-switch' motif involved in the latency of the MMP precursors is replaced by PH^{81}CGVPD. The presence of this highly unusual His^{81} in MMP-26 has been independently confirmed by other authors [29,33,37]. All other MMPs have an arginine residue at this position of the cysteine-switch motif.

A single unpaired cysteine residue in the cysteine-switch motif is present in all known MMPs except MMP-23. Other cysteine residues, located primarily in the fibronectin-type II repeats of MMP-2 and MMP-9 and the haemopexin-like C-terminal region of MMPs, are paired [3]. In contrast, the sequence of pro-MMP-26 exhibits three cysteine residues: a critical unpaired Cys^{82} of the PHCGVPD cysteine-switch motif, Cys^{97} in the enzyme's catalytic domain and Cys^{256} at the C-terminal part of the enzyme's polypeptide chain. It is probable that Cys^{97} and Cys^{256} form a disulphide bridge, thereby positioning the C-terminal region in proximity to the N-terminal part of the mature enzyme, and making the structure of MMP-26 more rigid relative to that of most other MMPs.

Evolution of MMP-26

Pairwise comparisons between MMP-26 and other MMPs showed that identities ranged from 19% to approx. 45%. Specifically, the MMP-26 protein sequence including the prodomain and the catalytic domain was compared with the corresponding sequences of the five most similar human MMPs, MMP-7, MMP-12, MMP-13, MMP-19 and MMP-28, and were found to be 39, 47, 41, 42 and 38% identical respectively (Figure 2, bottom panel). MMP-26 cannot be classified as gelatinase, collagenase or stromelysin as the protease does not share the specific motifs of these groups. The existence of three residues (Tyr^{214}, Asp^{235} and Gly^{237} in MMP-13 numbering) in the catalytic domain is a fundamental determinant of collagenase specificity [3]. The equivalent residues in MMP-26 at these positions are Tyr^{200}, Asn^{221} and Ser^{223}, making it unlikely that MMP-26 is a collagenase. Similarly, stromelysins are characterized by an insertion of a 9-amino-acid motif XPPVPTXXV in the C-terminal part of their catalytic domain. This insertion is missing in MMP-26. Accordingly, MMP-26 cannot be classified as a stromelysin. By size, MMP-26 is related to matrilysin (MMP-7), the shortest member of the MMP family. However, sequence similarity between MMP-26 and MMP-7 is relatively low. The presence of a Thr^{207} residue upstream of the zinc-binding motif in MMP-26 is characteristic of all known mammalian MMP-7 enzymes (Figures 1 and 2). This feature has been postulated as a structural signature of matrilysins [45]. However, the sequence of the HEIGHSLGLQHS zinc-binding motif of MMP-26 has two residue positions (underlined), which strongly discriminate this motif from the HELGHSLGMQHS motif of human MMP-7. These structural features and a relatively low overall sequence similarity with MMP-7 do not allow us to unambiguously identify MMP-26 as a classical matrilysin.

The deduced amino acid sequence for MMP-26 lacks the fibronectin-like and transmembrane domains that are present in gelatinases and membrane-anchored MT-MMPs respectively. MMP-26 has a region that demonstrates certain similarity with the furin-cleavage motif. Instead of the consensus RXKR sequence, MMP-26 bears the RCKWNK sequence downstream of the cysteine-switch motif. A more detailed examination of the partial sequence of MMP-26, along with those of known MMPs, is shown in Figure 3. It is likely that during evolution, MMP-19 and MMP-26 lost the furin-cleavage motif that existed in the ancestral MMPs.

Computer analysis using the CLUSTAL X and Laser Gene computer programs (DNASTAR, Madison, WI, U.S.A.) failed to group MMP-26 and MMP-7 within a single evolutionary cluster (results not shown). Similarly, a recently published dendrogram of the catalytic domains of human MMPs aligned with the CLUSTAL W computer program demonstrated the close evolutionary relationships between MMP-26 and MMP-12 [31]. In view of all these considerations, it is possible that the similarity between MMP-7 and MMP-26 could result from convergent, but not divergent, evolution. This speculation may point to an important function of MMP-26, an enzyme that has

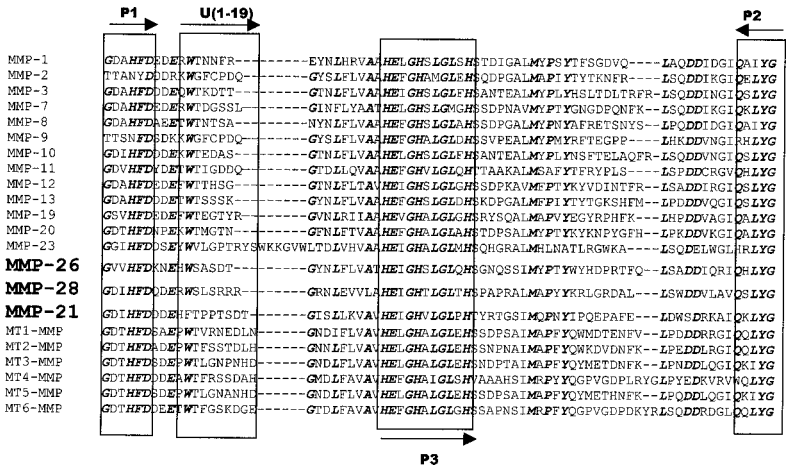


Figure 1 Amino acid sequence alignment of the catalytic domain of human MMPs

Regions corresponding to the nucleotide sequences of the unique U1–U19 probes, and the degenerate P1, P2 and P3 oligonucleotides used for molecular cloning of MMP-21, MMP-26 and MMP-28 are boxed. Conserved amino acids are in bold. Biotin-labelled U1–U19 probes corresponding to the sequence of the nineteen known MMPs were used for depleting the first-strand gene library. The biotin-labelled P3 probe was employed for isolating the residual pool of MMPs. Primers P1 and P2 were used in the PCR amplification of this pool to give rise to the 200–240 bp fragments. After the cloning of the 200–240 bp fragments, the U1–U19 and P2 primers were utilized in PCR amplification to screen each of the 600 individual *E. coli* colonies and to discard the positive clones. Restriction analysis and sequencing of the residual 29 negative colonies identified individual clones which coded for the sequences of the catalytic domains of MMP-21 [43], MMP-26 [29,33,37] and MMP-28 [31,42].

evolved to acquire some structural characteristics and the modular design of MMP-7.

Chromosomal location and genomic structure of the MMP-26 gene

In order to examine the chromosomal location of the MMP-26 gene in the human genome, a linkage analysis based on a PCR strategy was employed to screen a panel of hybrid somatic cell lines containing a single human chromosome in a hamster background (results not shown). The results were compared with previously defined markers to obtain the linkage data. According to linkage mapping, the MMP-26 gene is located in the 11p15.3 loci, and is therefore distant from the 11q21–q23 cluster of at least eight MMP genes (MMP-1, MMP-3, MMP-7, MMP-8, MMP-10, MMP-12, MMP-13 and MMP-20) [46].

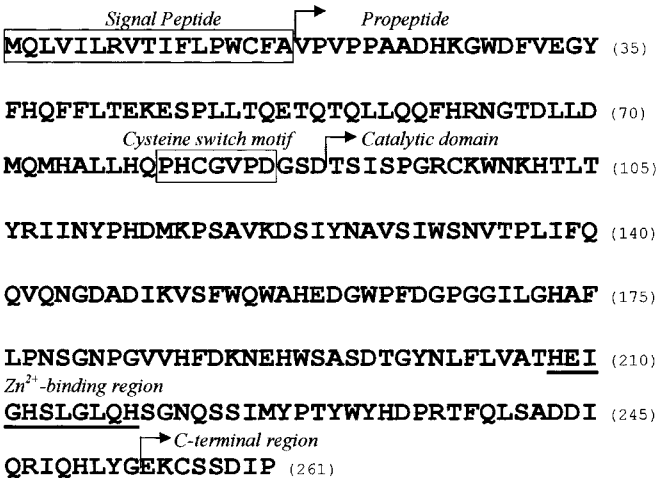
Structural organization of the MMP-26 gene

During the preparation of this paper, a nucleotide sequence (accession number AC020597.4) was released by GenBank®. This sequence represents a 186999 bp working draft of 22 contigs of *Homo sapiens* chromosome 11 clone RP11-390G21 and includes the genomic sequence of MMP-26. We mapped the exon–intron junctions and determined an exon–intron map of the MMP-26 gene by comparing the cDNA and genomic sequences (Figure 3). The chromosomal gene of MMP-26 consists of six exons. Exon–intron boundaries and the sizes of introns and exons are shown in Table 1. The exon–intron boundaries conform to the GT/AT rule for splice sites. All exons, except exon 1, were present within the genomic clone RP11-390G21. Hence, the size of intron 1 is still unknown. The splice sites are at positions conserved among most MMP genes. The exon–intron structure of MMP-26 is strikingly similar to that of MMP-7, thus indicating close relationships between these two MMPs. So far, no alternative splicing of MMP-26 has been identified.

Expression of the MMP-26 gene in cells and tissues

Expression of MMP-26 was analysed by PCR amplification of the first-strand cDNA panels and by reverse-transcriptase PCR (RT-PCR) of the mRNA pool isolated from cells and tissues. To control for the amount of cDNA, a parallel PCR reaction with glyceraldehyde-3-phosphate dehydrogenase-specific primers was carried out. Tissues such as heart, liver, spleen, placenta, lung and pancreas were clearly negative. The MMP-26 mRNA was identified in fetal kidney, brain, thymus and skeletal muscle (Figure 4), and adult peripheral blood leukocytes, prostate, small intestine, spleen, testis and descending and transverse colon (results not shown). The highest level of MMP-26 expression was found in adult kidney. Based on these findings, it appears that MMP-26 exhibits a relatively restricted expression pattern in normal tissues. Our findings disagree with a recent report demonstrating relatively high expression of the MMP-26 mRNA in placenta [29,37]. Another recent report stated the expression of MMP-26 in the endometrium of the uterus and in certain endometrial tumours [33]. The finding of MMP-26 expression in the adenovirus-transformed kidney cell line HEK-293 [29] correlates well with high levels of MMP-26 in kidney.

To evaluate whether MMP-26 is specifically associated with different types of cancer, we analysed the expression of the MMP-26 gene in tumour cell lines of diverse tissue origin. First, we evaluated the first-strand cDNA tumour panel (ClonTech Laboratories) that included two unrelated lung carcinomas, two unrelated colon adenocarcinomas, a breast carcinoma, a prostatic adenocarcinoma, an ovarian carcinoma and a pancreatic adenocarcinoma. There were no positive signals specific for MMP-26 in this panel (results not shown). We also performed PCR amplification of cDNAs generated by reverse-transcription of mRNA isolated from tumour cell lines including breast, lung and epidermoid carcinomas, prostate adenocarcinoma, lung fibroblastoma, fibrosarcoma and glioma. MMP-26 gene expression was confirmed for LNCap prostate adenocarcinoma, A549 lung carcinoma and MRC5 lung fibroblasts. Relatively low expression



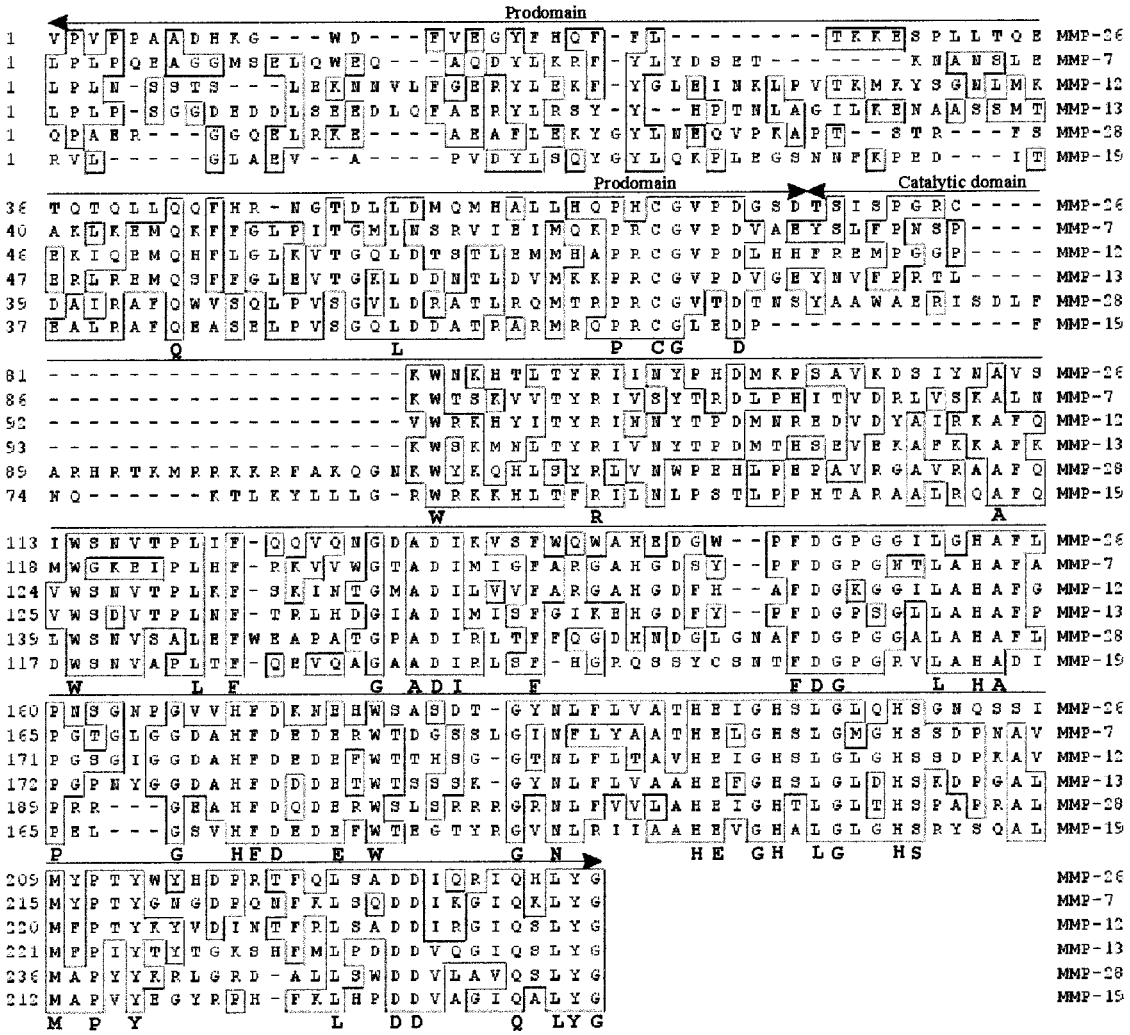


Figure 2 Amino acid sequence (top panel), domain structure and multiple sequence alignment of MMP-26 and the five most similar human MMP zymogens (bottom panel)

Top panel: sequences of a signal peptide and a cysteine-switch motif are boxed. The zinc-binding motif is underlined. There are three potential N-glycosylation sites at Asn[64], Asn[133] and Asn[222] in the sequence of MMP-26. The sequence of the signal peptide has been recently published by other authors [33]. Bottom panel: peptide sequences of human MMPs, including MMP-7, MMP-12, MMP-13, MMP-19 and MMP-28, were retrieved from the GenBank® database and were aligned with the MMP-26 peptide sequence. Identical amino acid residues are boxed. Identical amino acid residues in all six MMPs are indicated below the sequences. MMP-26 domains are indicated above the sequence. Unique His[81] and Thr[94] residues distinguish MMP-26 and MMP-28 from other MMPs respectively [31,42]. An unusually long insert of 22 amino acids between the cysteine-switch motif and the furin-cleavage motif distinguishes MMP-28 from other MMPs [31,42]. Since the C-terminal haemopexin-like domain is missing in both MMP-7 and MMP-26, the sequence of this domain in MMP-12, MMP-13, MMP-19 and MMP-28 has not been included in the alignment.

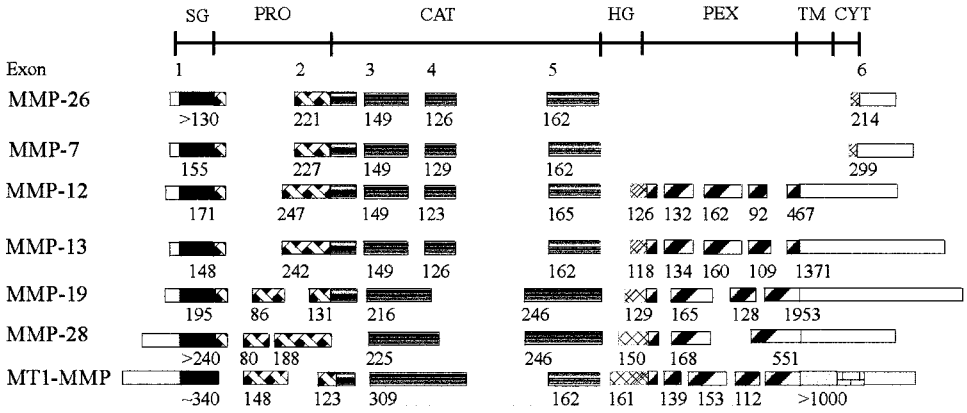


Figure 3 Comparison of exon–intron structure of human MMPs

The exons (numbered from the 5′-end of the gene) in MMP-26, MMP-7, MMP-12, MMP-13, MMP-19, MMP-28 and MT1-MMP are shown as boxes, with their sizes in nucleotides given below. White boxes indicate non-coding regions. The size of the first intron is unknown since the corresponding sequence is missing in the genomic clone RP11-390G21. The domain structure of MMPs is shown in the upper part of the Figure (SG, signal peptide; PRO, prodomain; CAT, catalytic domain; HG, hinge; PEX, haemopexin domain; TM, transmembrane domain; CYT, cytoplasmic tail).

Table 1 Exon–intron structure of the genomic gene of MMP-26

The sequence of clone RP11-390G21 (a 186 999 bp working draft of 22 contigs of *H. sapiens* chromosome 11) released by the GenBank® database with accession number AC020597.4 was used to identify introns and exons of the genomic gene of MMP-26. Intron and exon sequences are in lowercase and uppercase respectively. Donor and acceptor sites are in boldface. Amino acids in parentheses are encoded by codons positioned at exon–intron junctions.

Exon position	Splice donor	Splice acceptor	Intron size (bp)
	Met Gln Leu Val ...	Asp Phe Val Glu[33]	
1*–117	*AGGGTTTGGC*ATG CAG CTC GTC ...	GAC TTT GTT GAG**gt**aggtgaa	1337
	Gly Tyr Phe His ...	Leu Thr Tyr (Arg[107])	
1454–1675	tctgattc**ag**GGC TAT TTC CAT ...	CTA ACT TAC AG**gt**gcttgtta	729
	(Arg) Ile Ile Asn Tyr ...	Trp Gln Trp (Ala[157])	
2404–2553	tcctca**ag**G ATT ATC AAT TAC ...	TGG CAG TGG G**gt**aagaaatt	623
	(Ala) His Glu Asp ...	Ser Asp Thr (Gly[199])	
3176–3302	tcgtccac**ag**CC CAT GAA GAT ...	TCA GAC ACT G**gt**aaatgcct	467
	(Gly) Tyr Asn Leu ...	His Leu Tyr (Gly[253])	
3769–3931	aatttttc**ag**GA TAT AAT CTG ...	CAT TTG TAT G**gt**ctgtgctg	89
	(Gly) Glu Lys Cys Ser	Ser Asp Ile Pro[261] *stop*	
4020–4234	gtttccat**ag**GA GAA AAA TGT TCA	TCT GAC ATA CCT TAA-(178 bp)-*TTAAAGAGT*†	

* The size of intron 1 is not yet known since exon 1 was not present within the genomic clone RP11-390G21.

† Polyadenylation site of the mature mRNA. A 178 bp sequence separates the stop codon from the TTA triplet of the polyadenylation site.

of MMP-26 was identified in MCF7 breast carcinoma cells (Figure 4). These results correlate well with published observations [29,33,37]. Our findings and the data of other authors convincingly demonstrate that the MMP-26 gene is specifically expressed in carcinomas of endometrium, lung and prostate. Generally, expression of the MMP-26 gene in tumours of epithelial origin suggests a significant physiological role for MMP-26 in epithelial tumours. Additional studies are needed to identify the clinical significance of the expression of the MMP-26 gene, and to elucidate the functions of MMP-26 in tumours.

Promoter region and the putative transcription binding sites

The coding part of the MMP-26 gene is located 587 bp downstream of a stop codon of a putative protein encoded by the Alu-like sequence. There are four potential mRNA polyadenylation sites located approx. 150–200 bp downstream of the stop codon of this putative protein. Most probably, the promoter and the transcription factor-binding motifs of MMP-26 are located within this 587 bp genomic region. The sequence of this region demonstrates a putative recognition site for several regulatory proteins. There is a canonical TATA-box at the position −60 relative to the translation start. The putative binding sites of the activator protein (AP)-1, AP-4 and hepatocyte nuclear factor-3 transcription factors are located at positions −86, −144 and −435 respectively. The positions of the TATA-box and the AP-1 site of MMP-26 are similar to those found in the promoter regions of most other MMPs (Figure 5). Many MMP promoters share another element that can be observed in conjunction with the AP-1 binding site. This binding element, designated as polyoma virus enhancer A-binding protein-3 (PEA3), is located upstream of the AP-1 element in many MMPs, including MMP-7, MMP-12, MMP-13 and MMP-19. However, the PEA3 consensus sequence 5′-(C/G)AGGAAG(T/C)-3′ [47] was not found in the non-coding region of MMP-26. These results provide the basis for future characterization of the regulation of MMP-26 gene expression in cancer cells.

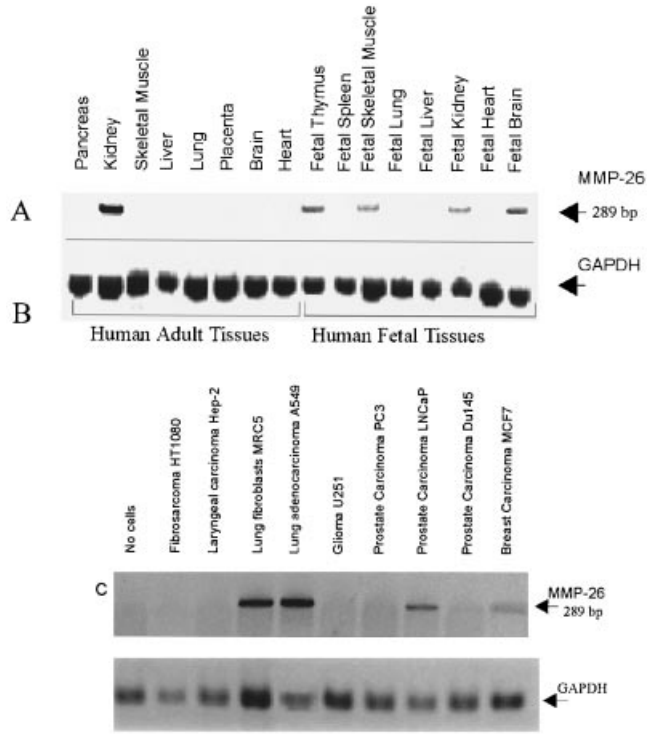


Figure 4 Expression of MMP-26 in various tissues and tumour cell lines

Expression was evaluated by PCR amplification of the MTC panels isolated from adult and fetal tissues (ClonTech Laboratories) (**A** and **B**) and by RT-PCR of the mRNA pool isolated from tumour cell lines (**C**). The unique primers derived from the sequence of the MMP-26 gene were used in a PCR reaction to generate a 289 bp fragment. The PCR reaction was analysed using 2% agarose gels. Amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (**B**) was used to ensure that equal amounts of cDNA were loaded in each reaction.

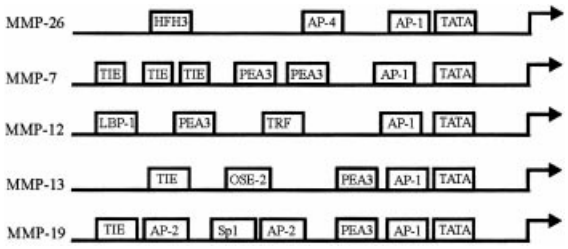


Figure 5 Regulatory elements of the promoter regions of MMPs

Putative transcription factor binding sites are indicated within boxes. TATA, TATA-box; TIE, TGF-β inhibitory element; Sp1, Sp-1 binding site; OSE-2, osteoblast-specific element-2; TRF, octamer binding protein; LBP-1, leader binding protein; HFH3, hepatocyte nuclear factor-3. The transcription start point is indicated with a bent arrow.

Production of recombinant MMP-26 in bacterial cells

As a necessary step to elucidate whether the isolated cDNA genes encode a enzyme with proteolytic activity, a partial cDNA coding for the catalytic domain of MMP-26 was subcloned into the modified pFLAG-ATS expression vector under the control of the regulatory elements of the *tac*-promoter. The resulting plasmid was transformed into *E. coli* BL21 (DE3) cells. Transformed cells were induced with IPTG. Cell extracts were prepared from the induced bacteria and analysed by SDS/PAGE. The insoluble fraction of the *E. coli* extracts contained the MMP-26 catalytic domain, with the expected molecular mass of 18 kDa. The domain was solubilized in 8 M urea and further purified by ion-exchange chromatography on a Mono Q column. This allowed us to isolate the homogeneous catalytic domain of MMP-26 (Figure 6). Microsequencing confirmed the expected N-terminal sequence of the catalytic domain, with an extra methionine residue coded for by the initiation codon (MT^{90}SISXG). Gelatinolytic activity was verified by analysing the protein samples in gelatin gels. The gelatinolytic activity of the MMP-26 catalytic domain was similar to that of the catalytic domain of MT1-MMP, but at least several hundred-fold lower relative to the activity of gelatinases, such as MMP-2 and MMP-9 (results not shown).

To facilitate the isolation and purification of the catalytic domain, the partial cDNA sequence was recloned into the pET2a(+) plasmid (Novagen, Madison, WI, U.S.A.). *E. coli* cells transformed with the recombinant plasmid expressed high levels of the His-tagged catalytic domain of MMP-26. The C-

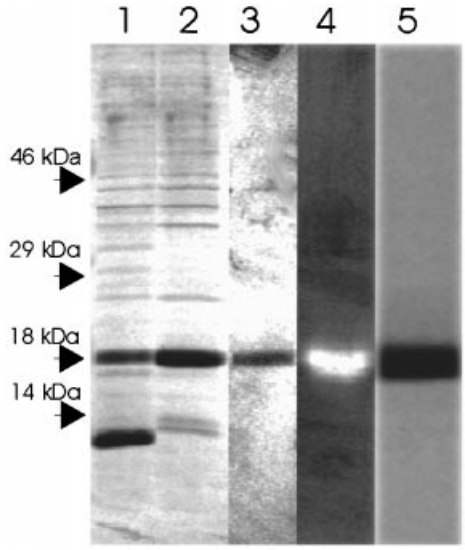


Figure 6 Expression of MMP-26 in *E. coli*

Total cell lysate (lane 1), inclusion bodies (lane 2) and the purified catalytic domain of MMP-26 (lanes 3–5) were analysed by SDS/PAGE (lanes 1–3), gelatin zymography in gels containing 0.1% gelatin (lane 4) and Western blotting with anti-(MMP-26) rabbit serum (lane 5). Positions of molecular mass markers are shown on the left.

Table 2 Cleavage of the fluorogenic peptide substrates by MMP-26 and MT1-MMP

The reaction mixture contained 2 μM of the respective fluorogenic substrate and either the catalytic domain of MMP-26 (200 ng; approx. 11 pmol) or MT1-MMP (200 ng; approx. 10 pmol) in buffer A (100 μl). Assays were performed at 37 °C in wells of a 96-well plate. Fluorimetric measurements were made with an fMax fluorescence microplate reader (Molecular Devices) (λ_{ex} = 328 nm, λ_{emm} = 405 nm).

	Specific activity (arbitrary units)	
Peptide substrate	MMP-26	MT1-MMP
Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2	100	1273
Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2	35	833
Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2	25	426
Mca-Arg-Pro-Lys-Pro-Val-Ala-Nva-Trp-Arg-Lys(Dnp)-NH_2	16	24

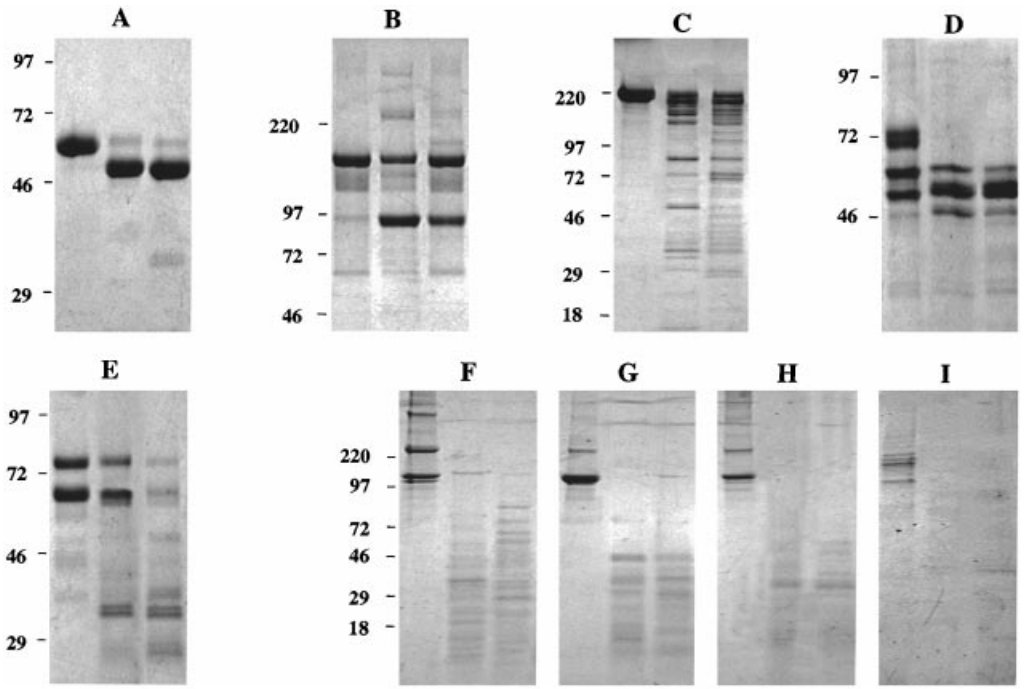


Figure 7 Degradation of high-molecular-mass substrates by MMP-26

(**A**) α1-antitrypsin, (**B**) α2-macroglobulin, (**C**) fibronectin, (**D**) fibrinogen, (**E**) vitronectin, (**F**) denatured type I collagen, (**G**) denatured type II collagen, (**H**) denatured type III collagen, (**I**) denatured type IV collagen (1 μg of each) were incubated with buffer alone (left-hand lane), with 100 ng of MMP-26 (right-hand lane) or with 20 ng of MT1-MMP (middle lane) for 16 h at 37 °C, except for collagen samples that were incubated at 25 °C. The samples were resolved by reducing SDS/PAGE (4–20% polyacrylamide). Positions of molecular mass markers are shown in kDa on the left.

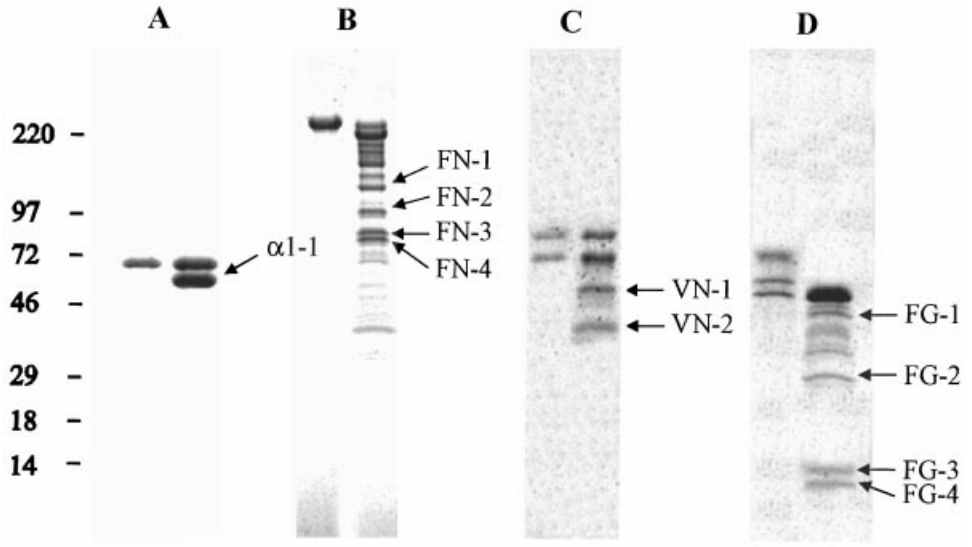


Figure 8 Sequence analysis of the cleavage sites

(**A**) α1-antitrypsin (α1), (**B**) fibronectin (FN), (**C**) vitronectin (VN) and (**D**) fibrinogen (FG) (5 μg of each) were incubated with buffer alone (left-hand lane) or with 100 ng of MMP-26 (right-hand lane) for 16 h at 37 °C. The reaction products were separated by reducing SDS/PAGE (4–20% polyacrylamide) and were transferred on to a membrane support. The marked proteolytic fragments were excised and subjected to N-terminal protein microsequencing. The identified N-terminal sequences were as follows: α1-1, E^{25}DPQGDA; FN-1, V^{45}SQSKPG; FN-2, V^{45}SQSKPG; FN-3, V^{45}SQSKPG; FN4, V^{45}SQSKPG; VN-1, I^{135}DSRPET; VN-2, I^{135}DSRPET; FG-1, M^{104}IDAAT; FG-2, A^{20}DSGEG; FG-3, L^{433}VTSKGD; and FG-4, L^{442}RTGKE. Positions of molecular mass markers are shown in kDa on the left.

Table 3 Cleavage specificity of MMP-26

Proteins (5 μg of each) were incubated overnight at 37 °C with the catalytic domain of MMP-26 (100 ng) in buffer A. The cleavage products were separated by SDS/PAGE and transferred on to a membrane, and their N-terminal sequences (*italicized*) were identified by microsequencing. The cleavage bond is indicated by a star symbol.

Protein	Cleavage sequence
Fibronectin	Pro-Val-Ala^{44} *Val^{45}-*Ser-Gln-Ser*
Vitronectin	Pro-Glu-Gly^{134} *Ile^{135}-*Asp-Ser-Arg*
Fibrinogen, γ-A chain	Lys-Pro-Asn^{103}*Met^{104}-*Ile-Asp-Ala*
Fibrinogen, α-E chain	Thr-Glu-Lys^{433}*Leu^{434}-*Val-Thr-Ser*
Fibrinogen, α-E chain	Asp-Lys-Glu^{441}*Leu^{442}-*Arg-Thr-Gly*
Consensus cleavage site	Pro-Glu-Xaa *Leu- Xaa-Thr-Xaa

terminal His-tagged domain was solubilized in 8 M urea and isolated by metal-affinity chromatography on a nickel column (results not shown). Unexpectedly, the tagged construct was completely inactive. All our attempts to refold the tagged domain failed. It cannot be excluded that the highly positively-charged tag affected the structure of the relatively short catalytic domain, thereby prohibiting its proper folding.

Substrate specificity of MMP-26

The purified MMP-26 catalytic domain was refolded as described in the Experimental section, and the substrate-cleavage specificity of this enzyme was further analysed. The cleavage data points to a wide substrate specificity of MMP-26. Purified and refolded MMP-26 was capable of cleaving the fluorogenic substrates, such as Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2, Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(Dnp)-NH_2 and Mca-Arg-Pro-Lys-Pro-Val-Ala-Nva-Trp-Arg-Lys(Dnp)-NH_2 (Table 2). The specific activity of MMP-26 against these peptide substrates was low compared with that of the MT1-MMP catalytic domain. The catalytic activity of MMP-26 was efficiently inhibited by TIMP-2, TIMP-1 and GM6001, a hydroxamate class inhibitor (results not shown).

To further elaborate these findings, we hydrolysed native and denatured protein substrates including human α1-antitrypsin, α2-macroglobulin, laminin V, fibronectin, vitronectin, fibrinogen, denatured collagens (gelatins; types I, II, III and IV) and native collagens (types I, II, III and IV) with the catalytic domain of MMP-26 or MT1-MMP. The samples were subsequently analysed by SDS/PAGE (Figure 7). MMP-26 exhibits wide substrate specificity in cleaving extracellular matrix and basement membrane proteins, which is indicative of its important functional role in cancer. Denatured type I, type II, type III and type IV collagen were susceptible to MMP-26. MMP-26 efficiently cleaved α1-antitrypsin (molecular mass of 63 kDa) generating its major 58 kDa proteolytic fragment. Both the 63 kDa and the 58 kDa bands demonstrated the same N-terminal sequence, EDPQGDAAQ, suggesting that the cleavage occurred within the C-terminal portion of the α1-antitrypsin molecule by removing a 5–6 kDa C-terminal fragment (Figures 7 and 8). Both MMP-26 and MT1-MMP cleaved fibrinogen, fibronectin and vitronectin. MMP-26 was comparable with MT1-MMP in cleaving the last two extracellular matrix substrates. MMP-26 was incapable of cleaving native collagen (types I, II, III and IV), tenascin C and laminin V (results not shown).

To generate the amounts of cleavage products sufficient for sequence analysis, the experiments employing α1-antitrypsin, fibronectin, vitronectin and fibrinogen were scaled up. The proteolytic fragments were separated by SDS/PAGE, transferred on to a membrane and subjected to N-terminal microsequencing (Figure 8 and Table 3).

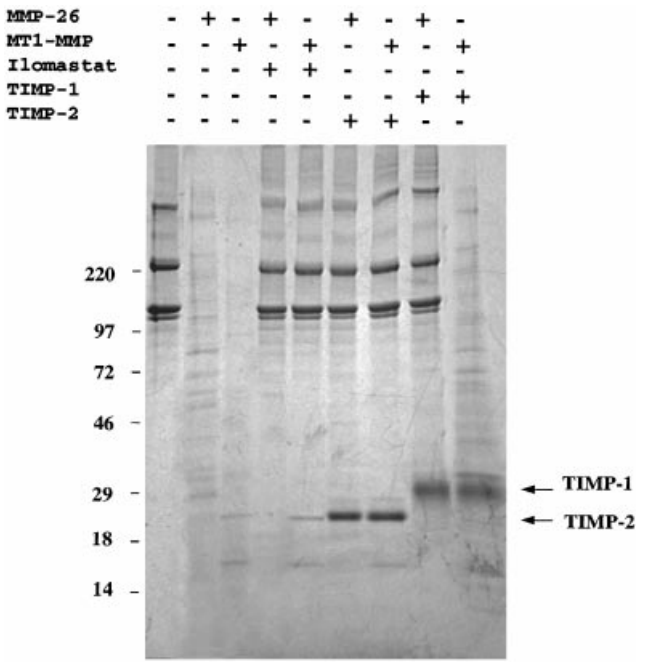


Figure 9 MMP-26 is sensitive to TIMP-1, TIMP-2 and a hydroxamate inhibitor, GM6001

Denatured collagen (gelatin) type I (1 μg) in 100 μl of buffer A was cleaved with 100 ng of the catalytic domain of MMP-26 or 100 ng of the catalytic domain of MT1-MMP alone or with 10 μM GM6001, 300 ng of TIMP-2 (1:3 molar ratio) or 800 ng of TIMP-1 (1:6 molar ratio). After incubation for 18 h at 37 °C, the protein samples were resolved by reducing SDS/PAGE (4–20% polyacrylamide) and stained with Coomassie Brilliant Blue R-250. Positions of molecular mass markers are shown in kDa on the left.

Analysis of the N-terminal sequences of the proteolytic fragments generated by MMP-26 cleavage demonstrated the wide substrate specificity of the enzyme (Table 3). No consensus cleavage site could be drawn from these cleavage data. However, there is one specific feature of all identified cleavage sites. This is the presence of a hydrophobic residue (alanine, isoleucine, methionine or leucine) in the P_1' position of the substrate. Residues at both the P_1 and P_2 positions have less effect on the specificity of MMP-26 (Tables 2 and 3). These findings allow us to suggest that the MMP-26 enzyme could significantly degrade the extracellular matrix. TIMP-2, TIMP-1 and GM6001 inhibited the cleavage of type I collagen (gelatin) by MMP-26. The cleavage of gelatin by MT1-MMP was highly resistant to the inhibition by TIMP-1. These data are in agreement with earlier observations [4], and confirm that TIMP-1 is a poor inhibitor of MT1-MMP (Figure 9).

Expression of MMP-26 in stably transfected breast carcinoma cells

The MMP-26 coding sequence was re-cloned into the pCDNA3-neo expression vector under the control of the cytomegalovirus promoter. This recombinant vector was stably transfected into MCF7 breast carcinoma cells. Multiple stably transfected clones resistant to G418 were selected, for the subsequent analysis. Transfection experiments employing the control pcDNA3-neo plasmid bearing the full-length MT1-MMP cDNA insert were

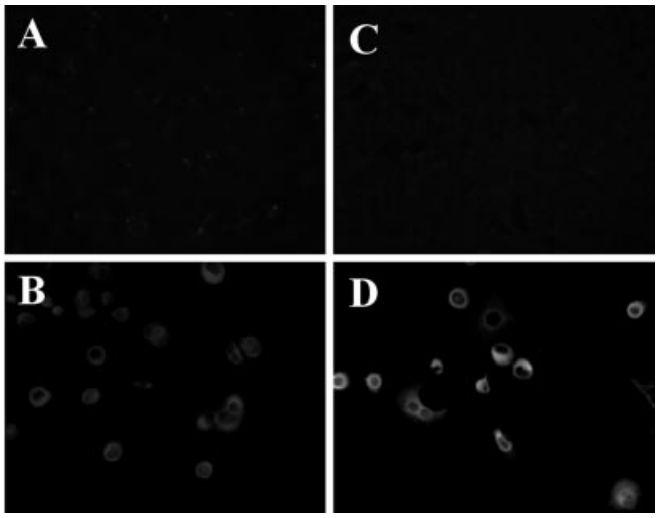


Figure 10 Immunofluorescence of MCF7 breast carcinoma cells stably transfected with MMP-26

MCF7 cells transfected with the original pcDNA3-neo plasmid (**A** and **B**) or with the pCDNA3.neo plasmid bearing the MMP-26 cDNA insert (**C** and **D**) were fixed with methanol/acetone and stained with normal rabbit serum (**A** and **C**) or rabbit serum raised against MMP-26 (**B** and **D**) followed by staining with goat anti-rabbit IgG conjugated with Oregon Green. Cell images were observed and recorded with a TE300 Nikon fluorescent microscope equipped with a 40× objective and a real-time camera. Magnification ×240.

carried out in parallel to confirm the efficiency of cell transfection. These control experiments confirmed high levels of MT1-MMP expression in the resulting stable clones (results not shown). Unexpectedly, activity assays with fluorogenic peptide substrates, gelatin zymography and Western blotting failed to unambiguously identify any significant immunological reactivity and proteolytic activity of MMP-26 in the medium conditioned by MMP-26 transfected cells. Furthermore, we failed to detect any expression of MMP-26 tagged with a FLAG tag (DYKDDDDK) in the medium conditioned by transiently transfected COS-7 cells (results not shown).

To test whether MMP-26 was expressed intracellularly in stably transfected MCF7 cells, several clones resistant to G418 were selected and stained with anti-serum against the catalytic domain of MMP-26. These fluorescence studies identified an intracellular pool of MMP-26, thereby confirming that the enzyme was preferentially accumulated within the cells (Figure 10). Recent reports by two other groups independently confirmed the poor secretion of soluble MMP-26 by transfected cells, and identified the main fraction of MMP-26 inside the cells [29,37]. It cannot be excluded that certain unusual mechanisms may be implicated in the processing, trafficking and secretion of MMP-26 by the cells. The insufficiency of these mechanisms may at least partially explain poor expression and secretion of MMP-26 in COS-7 cells and MCF7 breast carcinoma cells.

DISCUSSION

MMPs are promising targets for pharmacological intervention in numerous pathological conditions, including cancer, and neurodegenerative and cardiovascular diseases. There has been a significant interest in identifying novel members of the MMP family [7,29–40,42,43].

In our search for novel MMP genes, we modified the traditional cloning strategy. Our molecular cloning strategy greatly facilitated the identification of three novel MMP genes with low abundance in human gene libraries, thereby confirming the efficiency of our experimental approach.

One of the three newly isolated genes encoded MMP-26, a unique enzyme distinguished by the PHCGVPD cysteine-switch motif. The latency of zymogens of all known MMPs is maintained by co-ordination of the active site zinc ion by an unpaired cysteine sulphydryl group within a PRCG(V/N)PD conserved sequence motif of the propeptide domain. MMP-26 differs from all MMPs characterized so far by the presence of His^{81} in this motif. His^{81} functionally substitutes for arginine, the amino acid residue that is positively charged in the physiological pH range. In contrast, histidine is only positively charged at pH values below 7. This intrinsic feature of histidine may affect the stability and, probably, the activation mechanisms of the MMP-26 zymogen. Relative to other MMPs, activation of MMP-26 is probably more sensitive to changes in pH.

The three-dimensional structures of several MMPs are known, thereby providing templates for the identification of structural determinants of MMP-26 that are likely to control its substrate specificity and the proenzyme state [11,12,44,48–51]. The most significant difference among MMP catalytic domains is the size of the S_1' specificity pocket. The existence of Arg^{195} in MMP-1 limits the size of the S_1' pocket. In contrast, leucine in the corresponding position increases the size of the predominantly hydrophobic S_1' pocket in MMP-3, MMP-8 and MT1-MMP, since the side chain of leucine is orientated away from the catalytic zinc. The tyrosine residue in the corresponding position of MMP-7 limits the size of the S_1' pocket, restricting the specificity of this protease to aliphatic and aromatic residues in the P_1' position of the substrate. Since MMP-26 has Leu^{204} in the corresponding site, the enzyme should demonstrate a wide substrate specificity and a preference for hydrophobic residues at the P_1' position. Our experimental results, where alanine, isoleucine, methionine and leucine were found at the P_1' position of the substrate, confirm this hypothesis.

To further elaborate on the structure of MMP-26, we performed surface modelling of MMP-7 and MMP-26. Modelling was performed using an automated modelling server at http://bioinformatics.burnham.org, which uses a profile–profile alignment FFAS [52] and a modelling program MODELLER [53]. Structures were further refined using minimization subroutines from the SYBYL modelling package (www.tripos.com). Modelling of MMP-26, employing the available X-ray structures of MMP-7 (PDB code 1mmr) and MMP-3 (PDB code 1slm) as templates, allowed us to identify a substantial difference in the regions involving the active-site groove and substrate binding pocket of the enzymes (Figures 11 and 12). A model of the catalytic domain of MMP-26 was built on the matrylisin (MMP-7) template and compared with the matrilysin experimental structure. The surface features and the charge distribution of both models, calculated by the program Delphi [54] and visualized using the GRASS server [55], are presented in Figure 11. Relative to MMP-7, the active-site groove of MMP-26 is narrow and less negatively charged. An additional loop in MMP-26 partially blocks the substrate-binding site at the region that is distinct from the S_1' pocket. This predicted structural difference between MMP-7 and MMP-26 strongly suggests that the substrate binding and cleavage specificity of MMP-26 should be distinct compared with those of most other MMPs. Importantly, the surface of the MMP-7 molecule opposite to the active site is highly positively charged, thereby indicating that this enzyme is likely to exist as a soluble monomer. In contrast, the surface of MMP-26 is highly hydrophobic, suggesting that this protease is more likely to form homo- and/or hetero-molecular complexes, than to exist as a

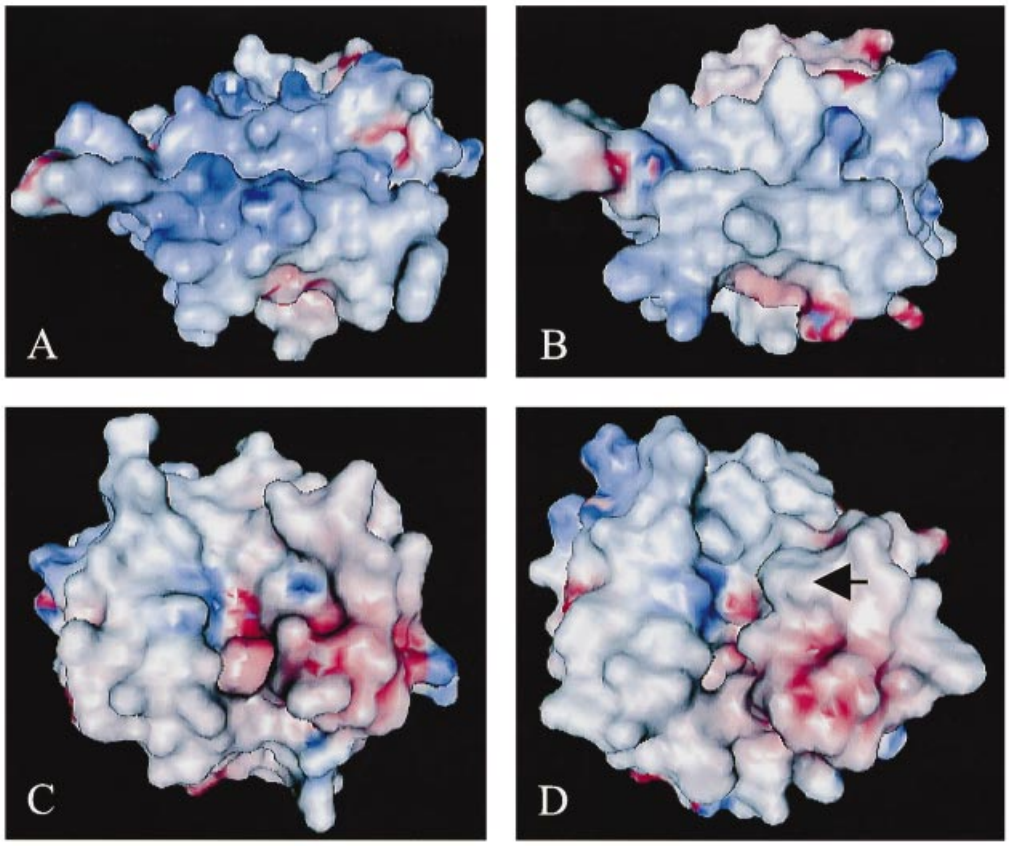


Figure 11 Three-dimensional views of MMP-7 (A and C) and MMP-26 (B and D)

(**A** and **B**) The interaction surface opposite to the active-site groove. (**C** and **D**) Comparison of the active-site groove. Negative and positive charges are represented by red and blue respectively. In MMP-7, the interaction surface (**A**) is highly positively charged, whereas in MMP-26 the same region (**B**) is hydrophobic, indicating that MMP-26 will have a tendency to form complexes. The active-site groove of MMP-7 (**C**) is relatively wide and negatively charged inside. The predicted active-site groove of MMP-26 (**D**) is relatively narrow and less charged. An additional loop (indicated by the arrow) partially blocks the substrate pocket of MMP-26. Surface modelling and electrostatic calculations were performed using the GRASP and DELPHI computer programs [54,55].

soluble monomeric protein. It cannot be ruled out that a putative complex formation is involved in trafficking and secretion of MMP-26. This unusual surface hydrophobicity may explain inefficient secretion of MMP-26 by transfected cells.

Furthermore, detailed modelling of the region involving the cysteine-switch motif and the active-site catalytic zinc allowed us to identify effects of His^{81} on the MMP-26 fold. Models of the structures of the proenzymes of MMP-7 and MMP-26 were built using the stromelysin (MMP-3) structure as a template. The detailed position of the active-site zinc ligands (His^{208}, His^{212} and His^{218} in MMP-26 numbering) and amino acid residues stabilizing the prodomain are illustrated in Figure 12, with the rest of the protein shown as a cartoon representation prepared using the Molscript program [56]. In the MMP-7 structure (Figure 12A), the active site is blocked by a beta strand from the prodomain. The position of this beta strand is stabilized by the ion pair involving Arg^{81} and Asp^{86}, and is surrounded by a hydrophobic cluster composed of Phe^{40} and Phe^{61}. All these residues are highly conserved in MMPs, with the exception of MMP-26 ($Arg^{81} \rightarrow His$). Figure 12(B) illustrates the important structural differences between MMP-26 and MMP-7. Substitutions existing in the MMP-26 prodomain result in a partially unfolded beta strand. Since the histidine side chain is short, the $Arg^{81} \rightarrow His$ substitution precludes the formation of both the ion pair with Asp^{86} and the hydrophobic cluster involving the phenylalanine residues. Simple minimization did not improve the packing of the MMP-26 prodomain. Thus we conclude that the prodomain of MMP-26 must undergo larger rearrangements to stabilize the cysteine-switch relative to the catalytic zinc. Limitations of the current algorithms do not allow us to precisely model the rearrangements involved in the stabilization of MMP-26. However, it could be concluded that the prodomain structure of MMP-26 is likely to be significantly different relative to that of other MMPs, especially at high pH when His^{81} is not protonated.

In agreement with the observations of other authors [37], MMP-26 was able to degrade a number of protein substrates, such as fibronectin, fibrinogen, vitronectin, α1-antitrypsin, α2-macroglobulin and denatured collagen (types I–IV; gelatins). In contrast, tenascin C and laminin V, as well as native collagen (types I–IV), were resistant to proteolysis by MMP-26. Relative to MT1-MMP, MMP-26 was most effective at cleaving fibronectin and vitronectin. It is remarkable that MMP-26 may cleave fibrinogen in a very similar manner to that of MT1-MMP [21]. The fact that MMP-26 may be active in fibrinolysis opens up the possibility that MMP-26, similarly to MT1-MMP, may participate in processes such as neovascularization and angiogenesis, which involve fibrin formation and degradation.

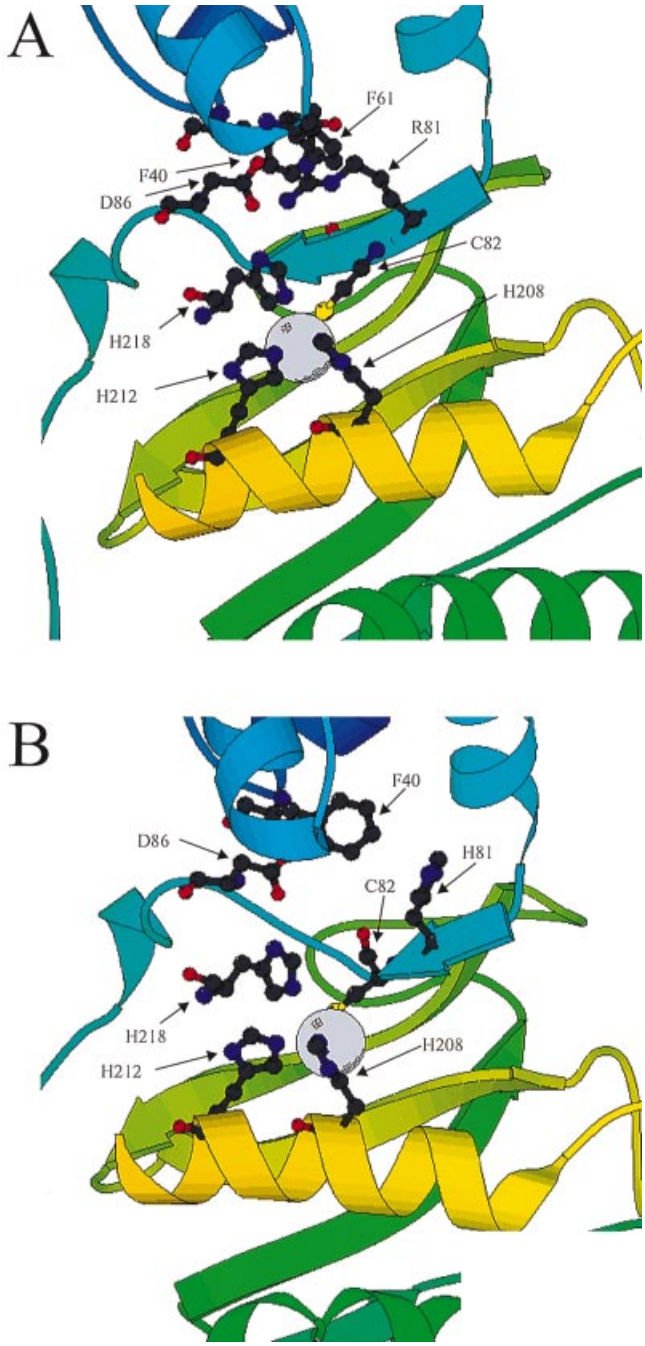


Figure 12 Modelling of the cysteine-switch motif interactions with the active-site zinc in MMP-7 (A) and MMP-26 (B)

(**A**) The prodomain (coloured in shades of blue) blocks the active site with a strand inserted in the active-site groove and Cys^{82} of the cysteine-switch motif co-ordinating the catalytic zinc ion. The conserved Arg^{81} from the cysteine-switch motif points away from the active site and forms an ion pair with the Asp^{86} residue. This ion pair is further stabilized by a pair of highly conserved phenylalanine residues (Phe^{40} and Phe^{61}), thereby forming the core of the prodomain. These interactions support the maintenance of the proenzyme state of MMP-27. (**B**) Substitutions existing in the prodomain and, especially, His^{81} in the cysteine-switch motif of MMP-26 destabilize the folding of the region involving the prodomain and the catalytic zinc. His^{81} cannot reach Asp^{86}. The phenylalanine residues (Phe^{40} and Phe^{61}) are incapable of stabilizing the core of the prodomain making the structure wide open and unstable. Phe^{61} is not visible, since it moves away from the cluster. Since Cys^{82} is incapable of stably chelating the catalytic zinc, MMP-26 would fail to maintain the proenzyme state. Carbon, black; nitrogen, dark blue; oxygen, red; sulphur, yellow.

Unlike other MMPs, which are primarily expressed *in vivo* by stromal cells, expression of MMP-7 is largely restricted to epithelium and the tumour cells themselves [57–59]. Since MMP-7 and MMP-26 are similar, expression of MMP-26 in epithelial tumours is intriguing, since it may indicate the existence of an important function for MMP-26. We speculate that MMP-26 is likely to be involved in the massive destruction of necrotic tissue in oxygen-deprived tumours. Regulatory elements and transcription factors involved in specific regulation of MMP-26 are not known. The availability of the MMP-26 cDNA, including the 5′-untranslated region, and the recombinant protein will allow further biochemical characterization of this novel, potent, proteinase, and the study of its regulation and biological function in normal and pathological conditions.

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