Identification and Characterization of a Novel Human Matrix Metalloproteinase with Unique Structural Characteristics, Chromosomal Location, and Tissue Distribution*

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We have cloned a novel member of the matrix metalloproteinase (MMP) family of proteins from a human liver cDNA library. The isolated cDNA contains an open reading frame coding for a polypeptide of 508 amino acids, which has been tentatively called MMP-19. This protein exhibits the domain structure characteristic of previously described MMPs, including a signal sequence, a prodomain with the cysteine residue essential for maintaining the latency of these enzymes, an activation locus with the zinc-binding site, and a COOH-terminal fragment with sequence similarity to hemopexin. However, it lacks a series of structural features distinctive of the diverse MMP subclasses, including the Asp, Tyr, and Gly residues located close to the zinc-binding site in collagenases, the fibronectin-like domain of gelatinases, the transmembrane domain of membrane-type (MT) MMPs, and the furin-activation sequence common to stromelysin-3 and MT-MMPs. In addition, the 9-residue insertion rich in hydrophobic amino acids present at the hinge region in stromelysins is replaced in MMP-19 by a longer insertion very rich in acidic residues. On the basis of these structural characteristics, we propose that MMP-19 does not belong to any of the previously defined MMP subclasses and may represent the first member of a new MMP subfamily. Chromosomal location of the MMP-19 gene revealed that it maps to chromosome 12q14, which is also a unique location for the MMP-19 gene. Northern blot analysis of polyadenylated RNAs isolated from a variety of human tissues revealed that MMP-19 is mainly expressed in placenta, lung, pancreas, ovary, spleen, and intestine, suggesting that it may play a specialized role in these tissues.

MMP-19 proteolytic activity was abolished by TIMP-2 and EDTA, thus providing additional evidence that the isolated cDNA codes for an authentic MMP. Northern blot analysis of polyadenylated RNAs isolated from a variety of human tissues revealed that MMP-19 is mainly expressed in placenta, lung, pancreas, ovary, spleen, and intestine, suggesting that it may play a specialized role in these tissues.

The human matrix metalloproteinases (MMPs)1 are a group of structurally related endopeptidases that degrade the different macromolecular components of the extracellular matrix and basement membranes at a neutral pH. These enzymes have been implicated in the remodeling of connective tissues occurring in normal and pathological processes (1–4). At present, the family of human MMPs is composed of 14 members that can be classified into four different families: collagenases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs), although there are some enzymes like macrophage metalloelastase (5) and stromelysin-3 (6) that do not belong to these groupings. The collagenases (fibroblast collagenase, neutrophil collagenase, and collagenase-3) cleave the native helix of fibrillar collagens at a single peptide bond, generating fragments of about three-fourths and one-fourth the size of the intact molecule (7–10). The gelatinases (72- and 92-kDa type IV collagenases) recognize and degrade basement membranes and denatured collagens and may act synergistically with collagenases in the degradation of fibrillar collagens (11, 12). The stromelysins (stromelysins-1 and -2 and matrilysin) exhibit a broad substrate specificity and have the ability to degrade many extracellular proteins, including proteoglycans, laminin, and fibronectin (13–15). Finally, the MT-MMP subclass is composed of at least four members mainly characterized by the occurrence of a putative transmembrane domain in their amino acid sequences and whose proposed role is the proteolytic activation of other MMPs like 72-kDa gelatinase and collagenase-3 (16–22).

Because of the importance of these enzymes in both normal and pathological processes, over the last years we have been interested in examining the possibility that additional yet un-
characterized members of the MMP family could be produced by human tissues (9, 21). In this work, we describe the molecular cloning and complete nucleotide sequence of a cDNA coding for a novel member of this family of proteolytic enzymes, which has been tentatively called MMP-19. We also report the expression of the gene in Escherichia coli and the preliminary enzymatic characterization of the recombinant enzyme. Finally, we report the chromosomal location of the MMP-19 gene in the human genome and analyze its expression in human tissues showing that it is mainly expressed in placenta, lung, pancreas, ovary, spleen, and intestine, which suggests that this novel MMP may play some specialized role in these tissues. On the basis of its structural characteristics, chromosomal location, and expression pattern in human tissues, we propose that MMP-19 may represent the first member of a new MMP subfamily.

EXPERIMENTAL PROCEDURES

Materials—A human liver cDNA library constructed in λgt11 and two Northern blots containing polyadenylated RNAs from different human tissues were purchased from Clontech (Palo Alto, CA). Oligonucleotide primers were synthesized in an Applied Biosystems (Pomona, City, CA) model 381A DNA synthesizer. Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim (Mannheim, Germany). Double-stranded DNA probes were radiolabeled with [32P]dCTP (3000 Ci/mmol) from Amersham Corp. (Amer- sham, UK) using a commercial random priming kit purchased from Pharmacia Biotech Inc. (Uppsala, Sweden).

Screening of a Human Liver cDNA Library—Searching the GenBank™ data base of human expressed sequence tags (ESTs) for sequences with homology to MMPs, we identified a sequence (R56524; deposited by R. K. Wilson, Merck EST project) that, when translated, showed a significant similarity to amino acid sequences previously determined for human MMPs. This DNA fragment was obtained by PCR amplification of cDNAs from commercially available libraries (Quick Screen, Clontech) as follows. Total γ-phage DNA from the different human cDNA libraries was screened for the presence of the EST using two specific primers 5'-CCAGTCTCAGGTCAGCTG-3' and 5'-AAAGGAGGCGGCAGTGACGCGC (primer 2) derived from the R56624 sequence. The PCR reaction was carried out in a GeneAmp 2400 PCR system from Perkin-ElmerCetus for 40 cycles of denaturation (94°C, 15 s), annealing (62°C, 15 s), and extension (72°C, 30 s). The PCR products were phosphorylated with T4 polynucleotide kinase and cloned into an M13mp19 cut pUC18 vector. The cloned cDNA was sequenced and was found to be closely related (96% identities) to the R56224 sequence. This cDNA was then excised from the vector, radio-labeled, and used to screen a human liver cDNA library according to standard procedures (29). Following plaque purification, the cloned insert was digested with EcoRI, and the resulting fragments were subcloned into the EcoRI site of pEMBL19.

Nucleotide Sequence Analysis—Selected DNA fragments were inserted in the polylinker region of phage vector M13mp19 (24) and sequenced by the dideoxy chain termination method (25), using either M13 universal primer or cDNA specific primers and the Sequenase Version 2.0 kit (U. S. Biochemicals, Cleveland, OH). All nucleotide sequences were identified in both strands. Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group (26).

Chromosomal Location—Total DNA from a panel of 24 humanchromosomal somatic cell hybrids containing a single human chromosome in a mouse or hamster cell line background (kindly provided by the Human Genome Mapping Resource Center, Cambridge, UK) was PCR-screened for the presence of the genomic sequence flanked by the above described primers 1 and 2. Amplification conditions were identical to those previously described for generation of the DNA probe used in the screening of the human liver cDNA library.

Fluorescent in Situ Hybridization—A high density grided human P1 artificial chromosome (PAC) genomic library (kindly supplied by the Human Genome Mapping Resource Center, Cambridge, UK) was screened by PCR hybridization with the full-length MMP-19 cDNA as probe. Six independent clones were identified enclosing the MMP-19 gene as demonstrated by PCR and Southern blot analysis. DNA from one of the PAC clones was further employed for fluorescent in situ hybridization (FISH) mapping. PAC DNA was obtained with the standard alkaline lysis method using QIAGEN columns (QIAGEN Inc, Chatsworth, CA). 2 μg of the PAC DNA was nick translated with biotin-16-dUTP, whereas the centromeric probe D12Z1 was labeled by nick translation with digoxi-genin-11-dUTP (Boehringer Mannheim). Both probes were hybridized to normal male metaphase chromosomes obtained from phytohemag-glutinin-stimulated cultured lymphocytes (27). Digoxigenated and bi- otinylated probes were subsequently detected using a biotinylated antibody and two avidin-fluorescein layers, respectively (28). Chromosomes were dyed-mine-2-phenylindole dihydrochloride (DAPI)-labeled, and images were captured in a Zeiss axiophot fluores- cent microscope equipped with a CCD camera (Photometrics).

RNA Analysis by Northern Blot Hybridization—Northern blots contain- ing 2 μg of poly(A)+ RNA of different human tissue specimens were prehybridized at 42°C for 3 h in 50% formamide, 5 × saline/sodium/ phosphate/EDTA (1 × = 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 10 × Denhardt’s solution, 2% SDS, and 100 μg/ml of denatured herring sperm DNA and then hybridized with radiolabeled MMP-19 full-length cDNA for 20 h under the same conditions. Filters were washed with 0.1 × SSC, 0.1% SDS for 2 h at 50°C and exposed to autoradiography. RNA integrity and equal loading was assessed by hybridization with an actin probe.

Construction of Expression Vectors for MMP-19 and Expression in E. Coli—The 1600-bp fragment of the MMP-19 cDNA generated by KpnI and EcoRI cleavage was ligated in frame into the pRESET E. coli expression vector (Invitrogen) previously cleaved with the above restriction enzymes. The recombinant vector into E. coli BL21(DE3)pLyS0 competent E. coli cells and grown on agar plates containing chloramphenicol and ampicillin. Single colonies were used to inoculate 2-ml cultures in 2YT medium supplemented with 33 μg/ml chloramphenicol and 50 μg/ml ampicillin. 500 μl of the corresponding culture was used to inoculate 200 ml of 2YT medium containing the above antibiotics. After culture reached an A600 of 0.6, expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) (0.5 mM final concentration) followed by further incubation for 5–20 h at 30°C.

Refolding of the Recombinant proMMP-19—The full-length MMP-19 was obtained in inclusion bodies and was solubilized using 20 mM Tris buffer, pH 8.0, containing 6 M urea and 5 mM DTT. The solubilized protein was purified by gel filtration chromatography using S-200 (Pharmacia Biotech Inc.), which had been equilibrated in 50 mM HEPES/NaOH, pH 7.4, 25 mM NaCl, 0.5 mM EDTA (DAPI)-banded, and images were captured in a Zeiss axiophot fluores- cent microscope equipped with a CCD camera (Photometrics).

RESULTS AND DISCUSSION

Molecular Cloning and Nucleotide Sequencing of a cDNA Encoding Human MMP-19—As a previous step to identify new MMPs that could be produced by human tissues, we performed an extensive analysis of the GenBank™ data base of ESTs looking for sequences with homology to human MMPs. This search led to the identification of a short DNA fragment that, when translated, generated an open reading frame with significant sequence similarity to a region of the propeptide domain found in all MMPs characterized to date. A cDNA containing part of the EST was obtained by PCR amplification of total P1-phage DNA prepared from different human cDNA libraries. Positive PCR amplification results were obtained when DNA prepared from liver and placenta cDNA libraries were used as template. The amplified DNA fragment (about 250 bp), whose identity was confirmed by nucleotide sequenc- ing analysis, was cloned and used as a probe to screen a human liver cDNA library. Upon screening of approximately 1 × 106

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plaque forming units, 7 positive clones were identified and characterized. One of them, named 4.1, had an insert of 1.8 kilobases, which could be large enough to contain the complete coding information for an archetypal MMP. The corresponding insert was subcloned in M13 and its complete nucleotide sequence was determined in both strands. Computer analysis of the obtained sequence (Fig. 1) revealed an open reading frame 1527-bp long, starting with an ATG codon at position 102 and ending with a TGA codon at position 1628. Assuming that translation starts at this first ATG, the identified open reading frame codes for a protein of 508 amino acids with a predicted molecular weight of 57,356.

Pairwise comparisons for sequence similarities between the identified amino acid sequence and those determined for other human MMPs showed that the percentage of identities ranged from 35% with MT1-MMP to 28% with neutrophil collagenase. Despite this overall limited sequence identity, the deduced amino acid sequence from the human cDNA isolated in this work, contains a number of structural features characteristic of MMPs (Figs. 1 and 2). Thus, the presence of a stretch of hydrophobic amino acids close to the initial methionine strongly suggests the presence of the signal peptide, which is also present in the remaining MMPs and which targets these proteinases to the secretory pathway. In addition, the identified open reading frame also contains a prodomain region with the activation locus including the essential cysteine residue (at position 85), a catalytic domain of about 160 residues including the consensus sequence HEXXXXXHS (at positions 212–223) involved in the coordination of the zinc atom at the active site, and finally, a COOH-terminal fragment of about 200 amino acids with sequence similarity to hemopexin and found in all family members with the exception of matrilysin. Taken together, these structural comparisons strongly suggest that the identified open reading frame codes for a putative new member of the MMP family of proteins that we propose to call MMP-19 tentatively, MMP-18 corresponding to a novel collagenase recently identified in *Xenopus laevis* (31).

Then, and in order to try to classify MMP-19 in one of the established MMP subclasses, we performed a more detailed comparison of its amino acid sequence with the remaining MMPs characterized to date (Fig. 2). Thus, according to structural comparisons between MMPs, it has been previously described that, close to the zinc-binding site of these enzymes, there are three residues (Tyr-214, Asp-235, and Gly-237 in collagenase-3 numbering) that are conserved in all collagenases characterized to date and that have been proposed as fundamental determinants of collagenase specificity (32). The equivalent residues in MMP-19 at these positions are Val-204, Tyr-225, and Gln-227, thus making it unlikely that MMP-19 is a collagenase (Fig. 2A). Similarly, stromelysins are characterized by the occurrence in the COOH-terminal region of their catalytic domain of an insertion of nine amino acids, most of them being hydrophobic. The introduction of these residues in the equivalent region of collagenases results in complete loss of the collagenolytic activity of the chimeric enzymes (33). A comparative examination of this hinge region in the sequence of MMP-19 shows the presence of a longer insertion (16 amino acids) and whose sequence, rich in acidic residues, completely deviates from that of stromelysins, which is mainly hydrophobic (Fig. 2B). Furthermore, the deduced amino acid sequence reading frame also contains a prodomain region with the activation locus including the essential cysteine residue (at position 85), a catalytic domain of about 160 residues including the consensus sequence HEXGHXXEHHX (at positions 212–223) involved in the coordination of the zinc atom at the active site, and finally, a COOH-terminal fragment of about 200 amino acids with sequence similarity to hemopexin and found in all family members with the exception of matrilysin. Taken together, these structural comparisons strongly suggest that the identified open reading frame codes for a putative new member of the MMP family of proteins that we propose to call MMP-19 tentatively, MMP-18 corresponding to a novel collagenase recently identified in *Xenopus laevis* (31).
for MMP-19 lacks the fibronectin-like domain present in all gelatinases as well as the COOH-terminal extension rich in hydrophobic residues characteristic of MT-MMPs. Finally, MMP-19 lacks the 8–11 residue insertion located between the propeptide and the catalytic domain of MT-MMPs and stromelysin-3, which contains the furin activation consensus sequence R-X-R-X-R, mediating their intracellular activation (16–21, 34, 35). It is also noteworthy that MMP-19 does not contain in its catalytic domain the N-glycosylation site (N-Y-S/T), which is absolutely conserved in collagenses, stromelysins-1 and -2, macrophage metalloelastases, 92-kDa type IV collagenses, and MT1-, 2-, and 3-MMPs, and whose effective glycosylation has been demonstrated for several of these MMPs. By contrast, MMP-19 contains two potential sites of N-glycosylation in the hemopexin-domain (N-I-S and N-T-T at positions 464–466 and 485–487), which appear to be unique for this protein. According to all these structural comparisons, it seems that MMP-19 does not belong to any of the previously described subclasses of MMPs and, thus, may represent the first member of a novel MMP subfamily.

Chromosomal Location of the Human MMP-19 Gene—The above structural data show that MMP-19 exhibits the typical domain organization of other MMPs and especially that characteristic of collagenses and stromelysins, which are codified by genes clustered in the long arm of chromosome 11 (36, 37). However, it also seems clear that MMP-19 has diverged considerably from them, at least in terms of absence of specific residues assumed to be important for the respective function of either collagenses and stromelysins. One possibility to explain this observation may be that the gene encoding MMP-19 is located on a different chromosome. In order to examine this hypothesis, studies were undertaken to determine the chromosomal location of the MMP-19 gene in the human genome. To this purpose, a PCR-based strategy was carried out to screen a panel of somatic cell hybrid lines containing a single human chromosome in a mouse or hamster background. The sequence tagged site specific for the MMP-19 gene was based in the same set of primers (primers 1 and 2) originally used to amplify the cDNA encoding part of this protein from human liver and placenta cDNA libraries. As can be seen in Fig. 3, positive amplification results were only obtained in the hybrid containing the autosomal number 12 although the resulting PCR-amplified product differed in 300 bp with respect to the size of the cDNA, presumably due to the insertion of the putative intron 2 of the MMP-19 gene in the amplified fragment. Since no amplification products were observed in the hybrids containing the remaining human chromosomes, we can conclude that the MMP-19 gene maps to chromosome 12. In order to localize more precisely the MMP-19 gene within chromosome 12, double color FISH experiments were performed using a digoxigenated centromeric probe from chromosome 12 as an anchor marker (red color) and a biotinylated large genomic PAC clone enclosing the MMP-19 gene (yellow color). In complete agreement with the human-rodent somatic hybrid studies, yellow fluorescent signals were located on chromosome 12, and no other chromosome site was labeled above background (Fig. 4). After DAPI-banding of 60 metaphases showing hybridization in both chromosomes 12, the yellow MMP-19 fluorescent signal was assigned to the q14 region of the long arm of the chromosome 12. This position differs from those reported for the other human MMP genes (36–41), thus providing additional support to the proposal that MMP-19 may be the first representative of a novel subfamily of MMPs.

Production of Active Recombinant MMP-19 in Bacterial Cells—To elucidate whether the isolated MMP-19 cDNA codes for a biologically active proteinase, we expressed the cloned cDNA in a bacterial system. The complete cDNA coding for human MMP-19 (proMMP19) was subcloned into the expression vector pRSETA, and the resulting plasmid was transformed into E. coli BL21(DE3)/pLysS. Transformed bacteria were induced with IPTG, and extracts were then prepared from the induced bacteria and analyzed by SDS-PAGE. According to the obtained results, insoluble fraction of the bacteria transformed with the recombinant plasmid contained a protein of the expected size that was not present in the control extracts (data not shown). Then, in order to examine the enzymatic activity and TIMP inhibition properties of proMMP-19, the solubilized protein was purified by size-exclusion chromatography in Sephacryl S-200 in the presence of 4 M urea and 5 M DTT, and the resulting fractions were analyzed by SDS-PAGE (Fig. 5). The purified proMMP-19 was refolded as described under “Experimental Procedures,” and its degrading activity against specific substrates for MMPs was examined. Refolded proMMP-19 was activated by trypsin treatment in a time-dependent manner, and full activation was achieved after 50 min of incubation at 37 °C. As shown in Fig. 6, the recombinant proMMP-19 displayed a clear proteolytic activity on the synthetic peptide McaPLGLDApARNH₂ after activation with trypsin. Similar results were obtained by using purified proMMP-19, a C-terminal deletion mutant of proMMP-19, lacking the hemopexin domain (Fig. 5 and data not shown). By contrast, control experiments performed without activation of

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**Fig. 3. Chromosomal location of MMP-19 in the human genome.** 100 ng of total DNA from the 24 monochromosomal somatic cell lines was PCR amplified with primers 5'-CCAGTCTAGGGTCAAGCTGAG-TG (primer 1) and 5'-AAGGAAGAGCCGATGTCAGCCG (primer 2) as described under “Experimental Procedures.” pBR322 digested with HaeIII (Marker V, Boehringer Mannheim) was used as a size marker.

**Fig. 4. Chromosomal location of the human MMP-19 gene by FISH.** The red hybridization signal corresponding to the centromeric probe of chromosome 12 and the yellow signal corresponding to the MMP-19 PAC clone are detected simultaneously in chromosome 12. Metaphase cells were counterstained in blue with DAPI.
hydrolyzed two of these substrates (McaPLANvaDpaARNH₂, synthetic quenched fluorescent peptide substrates. MMP-19 apperent activity was detected against McaPChaGNvaHAD-paNH₂. These data indicate that MMP-19 is more closely re-
folded proMMP-19 was activated with trypsin for the indicated times, and its proteolytic activity was assayed against the fluorescent sub-
strate McaPLGLDpaARNH₂. Molecular mass markers are indicated on the left.

proMMP-19 revealed that the latent protein displayed no ac-
activity. In addition, the enzymatic activity of trypsin-activated proMMP-19 was extensively abolished by TIMP-2, a natural inhibitor of matrix metalloproteinases, as well as by EDTA (data not shown). Taken together, these preliminary functional analyses indicate that the cloned cDNA codes for an authentic matrix metalloproteinase with the general substrate specificity and sensitivity to inhibitors characteristic of this family of proteolytic enzymes. To further assess the substrate specificity of MMP-19, we then determined the \( k_{cat}/K_m \) values for three synthetic quenched fluorescent peptide substrates. MMP-19 hydrolyzed two of these substrates (McaPLANvaDpaARNH₂ and McaPLGLDpaARNH₂) with distinct \( k_{cat}/K_m \) (1.96 \( \times \) 10⁴ \( m^{-1} \) s⁻¹ and 1.32 \( \times \) 10⁵ \( m^{-1} \) s⁻¹, respectively). By contrast, no apparent activity was detected against McaPChaGNvaHAD-paNH₂. These data indicate that MMP-19 is more closely re-
lated in terms of enzymatic activity to members of the stromelysin subfamily of MMPs since it preferably hydrolyzed McaPLANvaDpaARNH₂, which was designed as a stromelysin substrate (42), and while it showed no activity against McaPChaGNvaHADpaNH₂, which is a good collagenase sub-
strate (10). Further studies, now in progress, will be required to define the specific substrates that could be targeted by this novel member of the MMP family.

Expression Analysis of MMP-19 in Human Tissues—In order to analyze the expression of the MMP-19 gene in human tis-

cyes, colon, small intestine, ovary, testis, prostate, thymus, spleen, pancreas, kidney, skeletal muscle, liver, lung, placenta, brain, and heart) were indetectable by Northern blot analysis. The finding of MMP-19 expression in all these normal tissues is somewhat unusual since these proteo-
lytic enzymes are not frequently produced by adult cells under normal quiescent conditions. By contrast, expression of most MMPs is strongly induced in those physiological conditions involving extensive connective tissue remodeling, like during bone growth and resorption, or wound healing, or in a series of hormonally controlled processes specific to reproduction such as uterine post-partum involution or mammary gland involution after lactation or ovulation (1–4). Therefore, the detection of relatively high levels of MMP-19 expression in a wide variety of normal adult tissues suggests that this enzyme could play a role in matrix remodeling processes taking place in all of them. However, it should also be considered that MMPs may participate in biological processes other than the direct degradation of the diverse components of the extracellular matrix. In fact, MMPs are also able to catalyze hydrolysis of precursor proteins leading to activation of secreted proteinases or membrane-bound precursors of cytokines and growth factors (43–45). In addition, several reports have described their degrading activity on a variety of substrates including serum-amyloid A (46), insulin-like growth factor binding proteins (47, 48), proteinase inhibitors (49–52), or more recently interleukin-1β (53). Consequently, MMP-19 may therefore function in some of these processes in the diverse tissues in which it is produced. In this regard, it is interesting to note that the pattern of expression of MMP-19 is somewhat similar to that found for MT1-MMP, which is also widely expressed in normal human tissues with the exception of brain and leukocytes (19, 20). Although there are some differences in the tissue distribution and relative levels of expression between these two MMPs, the parallelism in MMP-19 and MT1-MMP expression in some normal tissues
could be important in functional terms if we consider that MT1-MMP appears to play a key role as a cell surface activator of other MMPs under physiological and pathological conditions (16–18, 22). Furthermore, and although MT1-MMP was first identified and characterized by virtue of its ability to activate progelatinase A (MMP-2), we have recently demonstrated that its activating role may be also extended to other MMP family members like collagenase-3, thereby generating extracellular collagenolytic activity and potentiating extracellular matrix degradation (22). On this basis, MMP-19 may be also a target of MT1-MMP mediated activation in those normal tissues in which both MMPs appear to be coordinately expressed. The availability of recombinant MMP-19 will be very helpful to examine these questions as well as to study the potential role of this novel MMP in protein degradative processes associated to pathological conditions including cancer invasion and metastasis.

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