Identification and Characterization of Human MT5-MMP, a New Membrane-bound Activator of Progelatinase A Overexpressed in Brain Tumors

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Abstract

A cDNA encoding a new member of the membrane-type (MT) matrix metalloproteinase (MMP) family has been identified and cloned from a human brain cDNA library. The isolated cDNA encodes a polypeptide of 645 amino acids that displays a similar domain organization as other MMPs, including a predominant with the activation locus, a zinc-binding site, and a hemopexin domain. The deduced amino acid sequence contains a COOH-terminal extension, rich in hydrophobic residues and similar in size to the equivalent domains identified in MT-MMPs. Immunofluorescence and Western blot analysis of COS-7 cells transfected with the isolated cDNA revealed that the encoded protein is localized in the plasma membrane. On the basis of these features, this novel human MMP has been called MT5-MMP because it represents the fifth member of the MT-MMP subfamily of MMPs. Fluorescent in situ hybridization experiments showed that the human MT5-MMP gene (MMP-24) maps to 20q11.2, a region frequently amplified in tumors from diverse sources. Northern blot analysis demonstrated that MT5-MMP is predominantly expressed in brain, kidney, pancreas, and lung. In addition, MT5-MMP transcripts were detected at high levels compared to normal brain tissue in a series of brain tumors, including astrocytomas and glioblastomas. The catalytic domain of MT5-MMP, produced in Escherichia coli as a fusion protein with glutathione S-transferase, exhibits a potent proteolytic activity against progelatinase A, leading to the generation of the M62,000 active form of this enzyme. These data suggest that MT5-MMP may contribute to the activation of progelatinase A in tumor tissues, in which it is overexpressed, thereby facilitating tumor progression.

Introduction

The MMPs, also known as matrixins, form a family of zinc-dependent endopeptidases that degrade the different protein components of the extracellular matrix and basement membranes. These enzymes have been implicated in the connective tissue remodeling occurring in normal processes, such as embryonic development, bone growth, or wound healing (1). In addition, abnormal expression of these enzymes may contribute to a variety of pathological processes, including atherosclerosis, pulmonary emphysema, rheumatoid arthritis, and tumor invasion and metastasis (2). To date, 17 distinct human MMPs have been characterized at the amino acid sequence level (1–4). According to their primary structures, substrate specificity, and cellular localization, these human MMPs can be classified into at least four main subfamilies: the collagenases, gelatinases, stromelysins, and MT-MMPs.

The MT-MMP subfamily is the most recently described subclass of MMPs and is composed of four members that have been identified using the reverse transcriptase-PCR technique and degenerate primers corresponding to conserved regions of MMP genes (5–8). MT-MMPs are type I membrane proteins with a single membrane-spanning domain and a short cytoplasmic tail located after the hemopexin domain, which are characteristic of most MMPs. In addition, these enzymes contain a conserved sequence of basic amino acids between the propeptide and catalytic domains that has been implicated in the intracellular activation of these membrane proteases by furin or furin-like enzymes (9), although extracellular mechanisms for MT-MMPs activation have been also proposed (10). MT-MMPs have raised additional interest for their role as cell surface activators of progelatinase A, which has a propeptide that is not generally susceptible to the serine proteinase-mediated process of activation occurring in other pro-MMPs. Because gelatinase A is an important enzyme for basement membrane invasion due to its ability to degrade type IV collagen, its activation, mediated by MT-MMPs on the tumor cell surface, is thought to play a critical role in the invasive phenotype of tumor cells (11, 12). Furthermore, although MT-MMPs were first characterized by virtue of their ability to activate progelatinase A, we have recently provided evidence that their activating role may be also extended to other MMP family members, such as procollagenase-3, which is efficiently activated by MT1-MMP (13). This has led us to propose that these three enzymes could form a proteolytic cascade operating in those physiological and pathological conditions, including tumor processes, in which MT1-MMP, gelatinase A, and collagenase-3 are coexpressed (14). In addition to this role of MT-MMPs as membrane-bound activators of other MMPs, several studies have shown that these membrane proteases can also degrade a number of extracellular matrix proteins, such as gelatin, fibronectin, vitronectin, fibrillar collagens, or aggrecan (15). Furthermore, Hiraoka et al. (16) have recently provided evidence that MT1-MMP has the ability to regulate neovascularization processes by acting as a pericellular fibrinolysin. These enzymatic activities extend the number of biological functions in which MT-MMPs could be involved and suggest that their proposed ability to favor the invasive potential of tumor cells may not necessarily be the result of progelatinase A activation at the cell surface.

Identification of new members of the MT subclass of MMPs would be important for a better understanding of the properties of these membrane proteinases, the biological functions of which appear to be distinct from previously described family members. In this work, we...
report the molecular cloning of a novel human MT-MMP that has been called MT5-MMP and show that it is localized in the plasma membrane. We also report the chromosomal location of the MT5-MMP gene (MMP-24) and analyze its expression in normal and tumor tissues. Finally, we describe the expression of the gene in Escherichia coli and perform an analysis of the enzymatic activity of the recombinant MT5-MMP, including its characterization as a progelatinase A activator.

Materials and Methods

Materials. A human brain cDNA library constructed in Agt11 and two Northern blots containing polyadenylated RNAs from different human tissues were from Clontech (Palo Alto, CA). A human PAC was provided by the Human Genome Mapping Resource Center (Cambridgeshire, United Kingdom). A human BAC library was provided by Dr. P. J. de Jong (Roswell Park Cancer Institute, Buffalo, NY). Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim (Mannheim, Germany). Synthetic oligonucleotides were prepared in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with [32P]dCTP (300 Ci/mmole) purchased from Amersham International (Buckinghamshire, United Kingdom) using a commercial random-primer kit from the same company.

Probe Preparation and Screening of a Human Brain cDNA Library. A computer search of the GenBank database of human ESTs for entries with similarity to previously described MMPs led us to identify a sequence (accession no. AA324134) derived from a cerebellum cDNA clone, showing significant similarity with the hemepxin domain of MMPs. To obtain this DNA fragment, we performed PCR amplification of a panel of cDNAs (Quick Screen; Clontech) with two specific primers, 5'-TTCAACACAGTGCGCTCTTC-3' and 5'-GCCAGGCTGTTGGGA-3', derived from the AA324134 sequence. The PCR was carried out in a GeneAmp 2400 PCR system from Perkin-Elmer/Cetus (Norwalk, CT) for 40 cycles of denaturation (94°C, 15 s), annealing (57°C, 15 s), and extension (72°C, 30 s). The 245-bp PCR product, amplified from human brain cDNA was sequenced and found to be virtually identical (99% identities) to the EST sequence. This cDNA fragment was then radiolabeled and used to screen a human brain cDNA library according to standard procedures.

5'-Extension of Isolated cDNAs. The 5' ends of cloned cDNAs were extended by successive cycles of RACE using RNA from human brain and kidney and the Marathon cDNA amplification kit (Clontech), essentially as described by the manufacturer. Each cycle of RACE allowed the extension of 60–100 bp of cDNA toward the 5' end. After cloning and sequencing the amplified products, we synthesized new specific oligonucleotides and used them for the next RACE experiment. Finally, the complete cDNA was obtained by PCR amplification using the Expand Long PCR kit (Boehringer Mannheim). The PCR was performed for 35 cycles of denaturation (94°C, 15 s), annealing (64°C, 15 s), and extension (68°C, 2 min) with primers 5'-ATGGCTATCTGCTTCCTATGACC-3' and 5'-GGCACCTATTTGGAGAT-3'. Following gel purification, the amplification product was cloned and sequenced by the dideoxy chain termination method, using the Sequenase Version 2.0 kit (United States Biochemical, Clevelend, OH). All nucleotides 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.

Chromosomal Mapping. Fluorescent in situ hybridization mapping of genomic DNA clones for MT5-MMP was performed as described previously (4). Briefly, DNA from isolated PAC and BAC clones were obtained with the standard alkaline lysis method using Qiaegen columns (Qiaegen, Chatsworth, CA) and nick-translated with biotin-16-DUTP. Then, labeled probes were hybridized to normal male metaphase chromosomes obtained from phytohemagglutinin-stimulated cultured lymphocytes and detected using two avidin-biotinylated peroxidase conjugates (Vector Laboratories, Burlingame, CA) and observed in a BioRad confocal laser microscope. COS-7 extracts were also obtained for Western blot analysis of the MT5-MMP-HA protein.

Preparation of Cell Membrane Fractions and Western Blot Analysis. COS-7 cells were transiently transfected with the pCEN-MT5-MMP-HA plasmid as described previously. Cells were rinsed in PBS and scraped from the plates. Membrane fractions were prepared essentially following the procedure described by Strongin et al. (11). Extracts were separated by SDS-PAGE and analyzed by Western blotting with an anti-HA monoclonal antibody and detected with an ECL chemiluminescent kit (Amersham). 

In Vitro Transcription and Translation. cDNA insert from plasmid pCEN-MT5-MMP-HA was released by HindIII and cloned in pcDNA3 (Invitrogen). One μg of this plasmid was then transfected and translated using the coupled reticulocyte TNT T7 Kit (Promega) in the presence of 35S-methionine (Amersham), following the manufacturer’s instructions. Protein translation...
products were analyzed by SDS-PAGE, followed by overnight autoradiography.

Results

Cloning and Characterization of a Human Brain cDNA Encoding a New MT-MMP. As part of our studies directed toward identifying new members of the MMP family produced by normal and tumor tissues (4, 8, 17–19), we screened the GenBank database of ESTs looking for sequences similar to previously described MMPs. This analysis led us to the finding of a 262-bp EST cloned from a fetal brain cDNA library that, when translated, generated an open reading frame with significant sequence similarity to the hemopexin domain, which is characteristic of MMPs. A cDNA containing part of this EST was PCR-amplified from total λ-phage DNA prepared from a human brain cDNA library and used as a probe to screen this library. Upon screening of ~1 × 10^6 plaque-forming units, six positive clones, named 1.1–1.6, were identified and characterized. DNA was isolated from these positive clones, and their nucleotide sequence was determined by standard procedures. A comparative analysis of the sequence obtained for the largest clone (1.3) with those corresponding to other MMPs, suggested that it was incomplete at the 5' end. To extend this sequence, we performed 5'-RACE experiments using a specific oligonucleotide deduced from the end of the 1.3 clone and RNA from human brain as a template. Successive 5'-RACE experiments performed in similar conditions finally led us to obtain a fragment long enough to contain most of the entire coding information for the identified MMP. However, after several 5'-RACE experiments using RNA from different human tissues, we were unable to extend the 5' sequence beyond the region coding for the putative propeptide of this MMP. To overcome this problem, likely due to the extremely high GC content of this region, we performed a genomic approach to try to complete the nucleotide sequence encoding this protease. To isolate these genomic clones, two genomic libraries (PAC and BAC) were screened with the isolated cDNA cloned as described above. A total of five positive PAC clones and 13 BAC clones were identified on the basis of their positive hybridization with the probe. Southern blot analysis of DNA isolated from these clones, followed by nucleotide sequencing of selected bands, revealed that most of them contained sequences close to the 5' end of the gene. However, only a series of restriction fragments generated from BAC clones hybridized with the most 5' cDNA probe obtained by RACE experiments. Then, a 1-kb BAC EcoRI/PstI genomic fragment positive for the 5'-RACE probe was subcloned and sequenced. Nucleotide sequence analysis of this DNA fragment revealed the presence of a region encoding a typical signal sequence as well as an in-frame ATG start nucleotide codifying for the first methionine. Computer analysis of the obtained sequence (Fig. 1; EMBL database accession no. AF131284) revealed an open reading frame coding for a protein of 645 amino acids with a predicted molecular mass of 73.2 kDa. This sequence contains two potential sites of N-glycosylation (Asn-Tyr-Thr and Asn-Lys-Thr at positions 174 and 627, respectively). Further analysis of the identified amino acid sequence revealed a significant similarity with other human MMPs, the maximum percentage of identities (63%) being with MT3-MMP. It should be also mentioned that a murine nucleotide sequence that is ~94% identical to the human cDNA sequence reported herein has been recently released to the GenBank (accession no. AJ010262). According to this high percentage of identities, it is likely that the murine protein encoded by this cDNA sequence is the homologue of the human enzyme identified here.

The deduced amino acid sequence shown in Fig. 1 displays a number of features that are characteristic of MMPs. Thus, it contains a signal sequence, a prodomain with a Cys residue essential for maintaining the latency of these enzymes, a catalytic domain of ~170 residues, including the consensus sequence HEXGlixirGXXHS involved in zinc binding, and a fragment of ~200 amino acids with sequence similarity to hemopexin. In addition, this novel sequence contains three insertions characteristic of the MT subclass of MMPs (Fig. 1). The first one (IS-1, nine amino acids) is located between the propeptide and catalytic domains and ends in a RXKR furin activation consensus sequence. The second insertion (IS-2) is 8 amino acids long and is located in the catalytic domain of all MT-MMPs, with the exception of MT4-MMP. Finally, the identified sequence contains a COOH-terminal extension rich in hydrophobic residues and similar in size to the equivalent domains present in the different MT-MMPs characterized to date. On the basis of these structural characteristics, we suggest that the isolated brain cDNA codes for a novel human MT-MMP, which we propose calling MT5-MMP. Furthermore, following the nomenclature system proposed for vertebrate MMPs, we propose designating this novel MT-MMP MMP-24 because MMP-23 corresponds to the last family member first cloned by Gururajan et al. (3) and, subsequently, by Velasco et al. (4).

Membrane Localization of MT5-MMP. To provide further support on the subcellular distribution of MT5-MMP, we transfected COS-7 cells with pCEP-MT5-MMP-HA, a construct containing the HA epitope in the COOH terminus of MT5-MMP. Transfected cells were then analyzed by immunofluorescence with a mouse monoclonal antibody (12CA5) specific for this viral epitope. As shown in Fig. 2, a clear fluorescent pattern surrounding the cell was visualized in a serial optical section obtained by the confocal microscope. This observation provides strong evidence that the human MT5-MMP is a membrane-bound MMP, fitting the requirement for a cell surface activator of progelatinase A. To further verify the nature of the MT5-MMP recombinant protein, we analyzed lysates from COS-7 cells transfected with the MT5-MMP-HA by SDS-PAGE, followed by Western blotting detection with anti-HA monoclonal antibody. A band of the expected molecular weight (M, 64,000) was detected in the membrane-enriched fractions but not in the soluble fraction, reinforcing the above results proposing its membrane localization (Fig. 2). It is remarkable that the electrophoretic mobility of the protein detected in membrane extracts of COS-7-transfected cells was very similar to that obtained by SDS-PAGE analysis of the protein product generated in an in vitro transcription and translation assay of MT5-MMP cDNA (Fig. 2).

Chromosomal Mapping of the Human MT5-MMP Gene. The isolation and chromosomal localization of genomic clones encoding MT5-MMP would contribute to the further delineation of its structural and evolutionary relationship to other members of the MMP gene family, especially to the remaining MT-MMPs. Toward this end, DNA isolated from PAC 198F17 was used for fluorescence in situ hybridization experiments on human metaphase spreads. As shown in Fig. 3, fluorescent signals corresponding to biotinylated MT5-MMP clones were located on chromosome 20, and no other chromosome site was labeled above background. After DAPI banding of 50 metaphases showing hybridization in both chromosomes 20, the MT5-MMP fluorescent signal was assigned to the centromeric region of the long arm of chromosome 20, in the q11.2 region. The gene encoding gelatinase B (MMP-9) has been also located in the long arm of chromosome 20 (20). However, this location is distinct from that reported for all MT-MMP genes, which have been mapped to chromosomes 14q11 (MT1-MMP/MMP14), 16q13 (MT2-MMP/MMP15), 8q21 (MT3-MMP/MMP16), and 12q24 (MT4-MMP/MMP17; Refs. 21 and 22).

Production of Recombinant MT5-MMP in Escherichia coli and Analysis of its Activity on the Processing of Progelatinase A. Because MT5-MMP has a number of structural features characteristic of
MT-MMPs, we next examined whether it has also the ability to induce activation of progelatinase A, a key event in the progression of various human carcinomas (5, 11, 12). As a preliminary step to elucidate this question, we expressed the cloned cDNA in E. coli. For this purpose, an 815-bp fragment encoding part of the prodomain and the entire catalytic domain of human MT5-MMP was PCR-amplified and cloned into the expression vector pGEX 3X. The resulting plasmid (pGEX-3X MT5) was transformed into E. coli BL21(DE3), and the transformed bacteria were induced to produce the recombinant protein by treatment with isopropyl-1-thio-D-galactopyranoside. Protein extracts were prepared from the induced bacteria and analyzed by SDS-PAGE. As shown in Fig. 4, the bacteria transformed with the recombinant plasmid contained a fusion protein of 44,600, which was not present in the control extracts. The fusion protein containing MT5-MMP was purified by affinity chromatography in a glutathione-Sepharose 4B column, and as shown in Fig. 4, a single band of the expected size was detected by SDS-PAGE analysis. It is also remarkable that GST-MT5-MMP showed autoproteolytic activity, despite having a prodomain with the cysteine switch involved in maintaining the latency of the proenzyme. Similar results have also been obtained with a GST-MT1-MMP fusion protein enclosing the same structural domains as GST-MT5-MMP, and are likely due to the disturbing effect of the NH2-terminal heterologous GST sequence on the switch function of the prodomain of these enzymes (23).

Fig. 1. A, comparison of the amino acid sequence of MT5-MMP (EMBL database accession no. AF131284) with other human MT-MMPs. The amino acid sequences of human MT-MMPs were extracted from the SwissProt data base and the multiple alignment was performed with the PILEUP program of the GCG package. Conserved residues in all five human MT-MMPs are shaded. B, domain organization of MT5-MMP. Insertions that are characteristic of MT-MMPs are indicated (IS-1, IS-2, and transmembrane and cytoplasmic domains).

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MT5-MMP fusion protein was then used to determine its ability to activate progelatinase A (MMP-2) and progelatinase B (MMP-9; Mr 72,000 and 92,000, respectively; Fig. 4) secreted to the medium by the human fibrosarcoma HT1080 cell line. To do that, we incubated conditioned medium from HT1080 cells with the GST-MT5-MMP fusion protein and analyzed by gelatin zymography. As can be seen in Fig. 4, these analyses revealed that recombinant human MT5-MMP exhibits a significant proteolytic activity against progelatinase A, giving rise to a Mr 62,000 band corresponding to the active gelatinase A. In contrast, similarly to previous studies with the remaining MT-MMPs, no activation effect of MT5-MMP on progelatinase B was observed (Fig. 4). Finally, we examined the possibility that the progelatinase A is efficiently activated by MT5-MMP, suggest that this novel membrane-bound enzyme may contribute to the generation of the gelatinolytic activity that is necessary to facilitate tumor progression.

Discussion

The localization of proteases at the cellular surface appears to be a general strategy essential for the focusing of a variety of degradative processes, including tumor invasion (24). Over the past few years, an
increasing number of proteases belonging to different families and acting at the cell surface have been identified (5–8, 24). In this work, we describe the finding of a new human membrane-bound proteinase of the MMP family, which we have called MT5-MMP. The approach followed to identify MT5-MMP was first based on a computer search of the EST database, looking for sequences with similarity to previously characterized MMPs. A sequence, presumably encoding part of the hemopexin domain of a new MMP, was identified, PCR-amplified from human brain cDNA, and used to screen a brain cDNA library. After screening of this library, further 5′-RACE experiments, and analysis of PAC and BAC genomic libraries, a cDNA coding for this MMP was finally identified and characterized. Structural analysis of the identified amino acid sequence shows that it contains a series of protein domains characteristic of MMPs, including a prodomain, a catalytic domain, a hinge region, and a COOH-terminal hemopexin domain organized in four recognizable repeats. In addition, this sequence contains three insertions that are characteristic of members belonging to the MT subfamily of MMPs, including the COOH-terminal extension rich in hydrophobic residues, and involved in the membrane anchoring of these proteases (5–8). According to these structural data, it seems clear that the identified sequence is a member of the MT-MMP subfamily. Consistent with this, immunofluorescence analysis of COS-7 cells transfected with an MT5-MMP expression vector revealed the plasma membrane localization of this enzyme, reinforcing its classification as the fifth member of the MT-MMP subfamily. Nevertheless, the structure of MT5-MMP is unique among members of this family due to the presence of a series of repeated sequences in the regions corresponding to the signal sequence and propeptide domains of this protein. These trinucleotide repeats (CCG, CTG, and GCG) generate stretches of eight Pro residues and six Leu residues in the signal sequence and six Ala residues in the propeptide domain, and they are the cause by which the NH2-terminal region of MT5-MMP is significantly longer than that corresponding to the remaining MT-MMPs. Because these nucleotide repeats are usually polymorphic in the human genome due to their high genetic instability (25), it will be of interest to examine the possibility that any of the identified repeats could be also polymorphic, giving rise to MT5-MMP protein variants that could differ in structural and/or functional properties.

Here, we have also provided functional evidence that MT5-MMP is an enzymatically active member of this subfamily of membrane-bound MMPs, as assessed by examining its ability to act as a progelatinase A activator. In fact, the catalytic domain of MT5-MMP produced in E. coli as a fusion with GST, is able to activate progelatinase A to its M, 62,000 active final form. The finding that different MT-MMPs share the ability to activate this gelatinase may reflect on evolutionary adaptation to cleave similar substrates in different tissues. Consistent with this proposal, the pattern of MT5-MMP expression in human tissues is distinct from the remaining MT-MMPs. Thus, here, we have shown that this gene is abundantly expressed in brain, kidney, pancreas, and lung. None of the remaining MT-MMPs shows a similar pattern of expression (5–8). In fact, MT1-MMP is widely expressed in normal tissues but it is not detected at significantly levels in brain. MT2-MMP is also undetectable in brain, whereas MT3-MMP and MT4-MMP, which are expressed in brain, are not detected in kidney or pancreas, which are also major sources of MT5-MMP expression. On this basis, it is tempting to speculate that this novel membrane proteinase could play some specific role in any of the

![Fig. 4. SDS-PAGE analysis of GST-MT5-MMP produced in E. coli and activation of progelatinase A.](Image)

![Fig. 5. Northern blot analysis of MT5-MMP expression in normal and tumor tissues. About 2 µg of polyadenylated RNA from the indicated normal tissues (A) and 10 µg of total RNA from different brain tumors (B) were analyzed by hybridization with the full-length cDNA isolated for human MT5-MMP. The positions of RNA size markers are shown. Filters were subsequently hybridized with a human actin probe to ascertain the differences in RNA loading among the different samples (data not shown).](Image)
matrix-remodeling processes occurring in these tissues in which its levels are significantly higher when compared with those corresponding to the remaining MT-MMPs. Similarly, MT5-MMP may act as a membrane-bound progelatinase A activator in those tumors in which it is overexpressed, thus contributing to the facilitation of tumor invasion and metastasis. Interestingly, a survey of a series of brain tumors for their ability to produce MT5-MMP has revealed that this gene is significantly overexpressed in a number of astrocytomas, anaplastic astrocytomas, and glioblastomas. In contrast, all analyzed meningiomas showed very low or undetectable levels of MT5-MMP RNA transcripts. Further clinical studies, now in progress, will try to evaluate the possibility that MT5-MMP expression may have a critical role in brain tumor progression, as already shown for other MT-MMP family members overproduced in different human tumors such as breast carcinomas, lung carcinomas, or papillary thyroid carcinomas (5, 26). In an attempt to provide further insights into potential associations of MT5-MMP with tumor processes, we have also established in this work the chromosomal location of the gene encoding this proteinase. According to our results, the MT5-MMP gene (MMP-24) is located at chromosome 20q11, a unique position among all MT-MMP genes mapped to date (21, 22). The fact that all members of the MT-MMP subfamily map to distinct chromosomes indicates that besides duplication of their putative common ancestor, transposition events to different chromosomes have played a major role in the evolutionary diversification of this gene family, as opposed to the MMPs from other family members. J. Biol. Chem., 272: 17124–17131, 1997.


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