Human membrane type-4 matrix metalloproteinase (MT4-MMP) is encoded by a novel major transcript: isolation of complementary DNA clones for human and mouse *mt4-mmp* transcripts

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Abstract Five distinct membrane-type matrix metalloproteinases (MT-MMP) have been reported by cDNA cloning. However, the *mt4-mmp* gene product (MMP-17) has not been identified yet in spite of the cDNA isolation [Puente et al. (1996), Cancer Res. 56, 944–949]. In this study, we re-examined the transcripts for human *mt4-mmp* by 5′ RACE and identified two types of transcripts. The minor one corresponded to the cDNA reported by Puente et al. and failed to express protein, and the other is the major transcript that has an extended open reading frame and expressed 67 and 71 kDa translation products. Thus, functional *mt4-mmp* has been identified for the first time.

Key words: Matrix metalloproteinase; Membrane-type enzyme; MT4-MMP

1. Introduction

Matrix metalloproteinases (MMPs), also called 'Matrixin', are zinc metalloendopeptidases that play critical roles in tissue formation, maintenance and remodeling of the extracellular matrix [1–3]. In addition to the soluble-type MMPs, we and others recently identified a new subgroup of MMPs, membrane-type MMPs (MT-MMPs), that are anchored to the plasma membrane through the transmembrane domain present at the C-terminus [4].

Complementary DNA sequences for five human MT-MMPs have been reported [5–9]. MT1, MT2, MT3 and MT5-MMPs (MMP-14, 15, 16 and 24, respectively) are closely related to each other in their amino acid sequences (70–80% homology) and share an activity to induce pro-gelatinase A (proMMP-2) activation by introducing a cleavage in the propeptide [10–14]. On the other hand, MT4-MMP (MMP-17) reported by Puente et al. [8] is unique compared to the other four MT-MMPs in that the expected translation product does not show obvious homology (less than 40%). In addition, MT4-MMP is different from all other MMPs in that it lacks a signal peptide for secretion. The translation product from the reported cDNA sequence is expected to start from the middle of the propeptide sequence (Fig. 1A), though the product has not been detected yet. Thus, there is no information about the mechanism of secretion and the biochemical nature of MT4-MMP.

In this study, we re-examined the transcripts from the human *mt4-mmp* (*mt4-mmp(h)*) gene by the 5′ RACE method and identified a new major transcript that can encode a gene product in addition to the one previously identified by Puente et al.

2. Materials and methods

2.1. Cells and culture

A human acute monocytic leukemia cell line, THP-1 (ATCC 455303), and a human breast carcinoma cell line, T-47D (ATCC 45528), were cultured in RPMI 1640 supplemented with 10% fetal calf serum (FCS) under a 5% CO_2 atmosphere. For transfection experiments, COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. Cells were transfected with plasmids using FuGene 6 (Roche Molecular Biochemicals, Basel, Switzerland) according to the manufacturer's instructions.

2.2. Cloning and sequencing

An oligo(dT)-primed cDNA library was constructed from mouse 17-day embryo brain poly(A)$^+$ RNA using lamdaZAP II (Stratagene) as a vector. The cDNA library was screened with a ^{32}P-labeled human *mt4-mmp* cDNA (233–1899 nt of Puente's sequence) [8] under standard conditions. Positive phage plaques were isolated and subjected to in vivo excision according to the manufacturer's protocol.

cDNA clones of *mt4-mmp(h)* were obtained by screening a human brain cDNA library (Clontech) using the same probe. But none of them have enough length to encode a signal peptide.

2.3. 5′ RACE method

The RNA-ligase-mediated 5′ RACE was performed according to the method by Chen [15]. Poly(A)$^+$ RNA (0.2 μg) extracted from the human monocytic leukemia cell line THP-1 was transcribed into cDNA using Superscript II (Gibco BRL) with a gene-specific primer (5′-GGTTCCTCTTGTTCCACTTGG-3′). A single-stranded oligonucleotide adapter (5′-GTAGGAATTCGGGTTGTAGGGAGGTCGACATTGCC-3′) was ligated to the cDNAs using T4 RNA ligase. The first round of polymerase chain reaction (PCR) employs the gene-specific primer [16] and an adapter primer (5′-GGCAATGTCGACCTCCCTACAAC-3′), which is complementary to the 3′ portion of the adapter. In the second round, nested PCR is performed with another gene-specific primer (5′-GGAGCTGTCTAAGGCCATCACA-3′) and an adapter primer (5′-CTCCCTACAACCCGAATTCCTAC-3′), which is complementary to the 5′ portion of the adapter. The PCR reactions were performed using Taq DNA polymerase (LA Taq with GC buffer, TaKaRa Co. Ltd.). The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA).

2.4. Reverse-transcription polymerase chain reaction (RT-PCR)

First-strand cDNA was synthesized from 3 μg of total RNA using 0.3 μg of random primers (Gibco BRL, Gaithersburg, MD, USA) and 200 U of Superscript II RNase H$^-$ reverse transcriptase (RTase) (Gibco BRL, Gaithersburg, MD, USA). After removing random

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primers, 1 μl of the RT product was used as a template for PCR. Primer sequences are indicated in the figure legends. To measure the amount of template, the same PCR products but with an internal deletion were used as competitors (Fig. 3).

2.5. Immunoblotting

Cells were collected 36 h after transfection, washed three times with PBS, and lysed in the SDS-PAGE loading buffer. Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane (Hybond-ECL, Amersham, Buckinghamshire, UK). After blocking with 10% fat free dry milk in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl), the membrane was probed with anti-FLAG M2 monoclonal antibodies (Eastman Kodak, New Haven, CT, USA). The final concentration of M2 antibody was 5 μg/ml in 1% BSA/TBS (with 1 mM CaCl) for 16 h at room temperature. After washing with TBS, the membrane was incubated with anti-mouse IgG conjugated with alkaline phosphatase (Sigma Co. Ltd., St. Louis, MO, USA) diluted to 1:10 000 in 1% BSA/TBS at room temperature for 1 h.

3. Results

3.1. Analysis of the human mt4-mmp transcript

We adopted the 5′ RACE method to analyze the transcripts for *mt4-mmp(h)*, since we thought there might be a heterogeneity in the 5′ region of the transcripts and some of them may contain a coding region for a signal peptide like other MMP genes. The RNA sample was prepared from the human monocytic leukemia cell line THP-1 that expresses *mt4-mmp(h)* at high levels and the primer for reverse transcription was designed from the coding sequence for the catalytic domain (Fig. 1B). The amplified 5′ RACE products were subcloned into a plasmid vector and nucleotide sequences of 50 isolates were analyzed. Three of them completely matched Puente's sequence, however, the other 47 isolates were different from Puente's sequence at the 5′ region (Fig. 1B). The new sequence has an open reading frame that starts from the ATG codon and it is continuous to the reported open reading frame for MT4-MMP(h) [8]. The predicted N-terminal sequence contains a hydrophobic stretch that can act as a signal peptide showing similarity to the other reported MMP genes [17]. Thus, two types of transcripts for human *mt4-mmp(h)* were identified in the THP-1 cells. The minor transcript corresponds to the previously reported sequence ('Puente-type') and the new transcript is the major one that is expected to encode functional proMT4-MMP (DDBJ/EMBL/GenBank database: AB021225).

To confirm that mRNA species similar to the new *mt4-mmp* transcript in human also exist in mouse, *mt4-mmp(m)* cDNA was screened from a mouse embryo brain cDNA library. The mouse cDNA (DDBJ/EMBL/GenBank database: AB021224) shows more than 90% conservation along with the newly identified human sequence (Fig. 1B). At the 5′ region, sequence conservation of the mouse cDNA was only found with the human major transcript but not with the 'Puente type'. No mouse transcripts that correspond to the 'Puente type' were found at least in the 16 cDNA clones analyzed.

3.2. Analysis of the two types of mt4-mmp(h) transcripts

The 5′ region (1–137 nt) unique to the 'Puente-type' transcript contains a repetitive Alu sequence [17] which exists usually in an intron and spliced out from mature transcripts. A consensus sequence for the splice acceptor is also found immediately upstream of 136 nt of the reported sequence (Fig. 1B). Analysis of the genomic DNA fragment containing

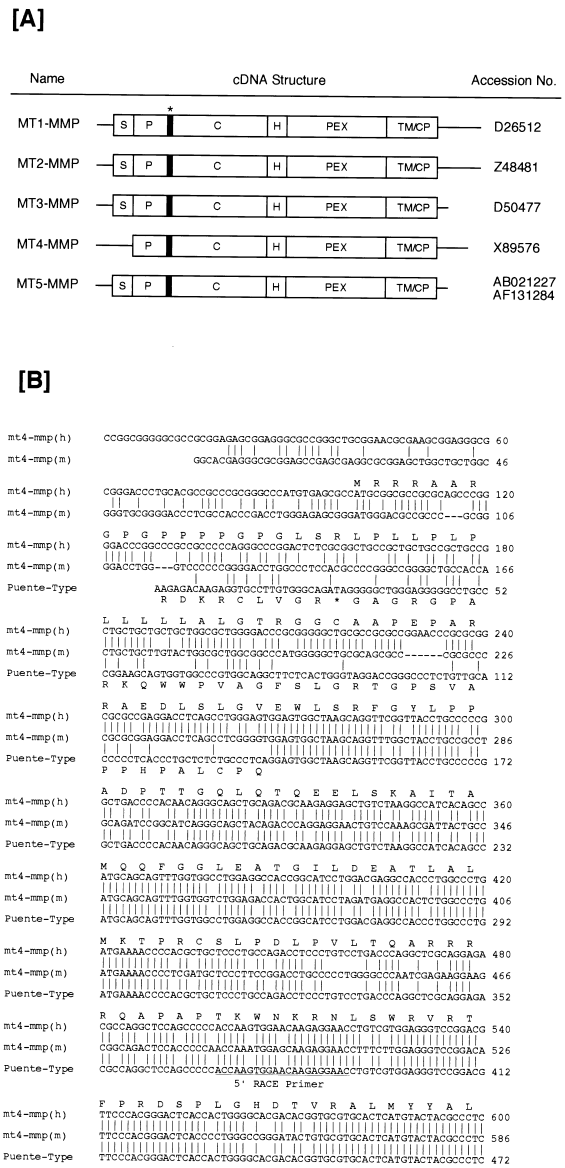

Fig. 1. Analysis of the 5′ region of human and mouse *mt4-mmp* cDNAs. A: Schematic illustration of MT-MMPs. The cDNAs for four human MT-MMPs and their open reading frames (ORFs) in relation to the domain structures are illustrated. A newly identified major transcript for MT4-MMP(h) is also presented at the bottom. The initiation codon for 'Puente-type' MT4-MMP can be seen only in the propeptide domain. S, signal peptide; P, propeptide domain; *, furin motif; C, catalytic domain; H, hinge; PEX, hemopexin-like domain; TM/CP, transmembrane and cytoplasmic domain. Accession numbers for the genes are also listed. B: Sequence analysis of the 5′ region of human *mt4-mmp* transcripts by 5′ RACE. The *mt4-mmp* transcripts in THP-1 cells were analyzed by the 5′ RACE method as described in Section 2. The 5′ region of the major transcript is aligned with *mt4-mmp(m)* together with the alignment with the 'Puente-type' *mt4-mmp(h)* sequence. Translated amino acids are indicated along with the sequences. Identical nucleotides are indicated by vertical lines. Initiation codons for *mt4-mmp(h)* and *mt4-mmp(m)* are indicated by bold letters. The primer sequence for the 5′ RACE is underlined.

this region revealed that the 5′ region of the 'Puente-type' transcript (1–136 nt) matched the intron sequence that is contiguous to the second exon of the major transcript (data not shown).

Expression of the two types of human transcripts was ex-

amined by RT-PCR using various human tumor cell lines. The 5′ primer was specific to either one of the transcripts and the 3′ primer was common to both. A set of primers that amplifies the common region of both transcripts was also used. Transcripts for *mt4-mmp(h)* were detected in nine of the 18 tumor cell lines by the common primers and the major transcript was detected in all of these cell lines (Fig. 2). However, 'Puente-type' transcript was detected in four of the nine cell lines and it was negligible in the other five cell lines. It is noteworthy that *mt4-mmp* is expressed at high levels in three of the four monocytic leukemia cell lines. A human breast carcinoma cell line, ZR-75-1, from which cDNA for the 'Puente-type' transcript was originally obtained, also expressed both types of the transcripts.

The amount of the two types of transcripts in breast carcinoma T-47D cells was measured using specific competitors for each primer set (Fig. 3). The major transcript was 4.5 pg per 100 ng of total RNA and that of the 'Puente-type' transcript was about 1/12 of the major transcript. Thus, the result is equivalent to the 5′ RACE analysis of THP-1 cells.

3.3. Detection of translation products

To analyze which transcripts direct expression of MT4-MMP(h), cDNA fragments were subcloned into a mammalian expression vector and transfected into COS-1 cells. For detection of the products, they were expressed as fusion proteins having a FLAG epitope. The FLAG tag was inserted downstream of the putative furin motif. Lysates of the cells transfected with the plasmids were analyzed by Western blotting using a specific anti-FLAG antibody M2 (Fig. 4). The antibody detected a 67 kDa product for MT4-MMP(m). For MT4-MMP(h), 67 and 71 kDa bands were detected only in the cells that express the major human transcript but not in those expressing the 'Puente-type' transcript. Both transfected genes were confirmed to be transcribed at the same levels (data not shown). Thus, we concluded that only the major transcript encodes the gene product (MT4-MMP(h)).

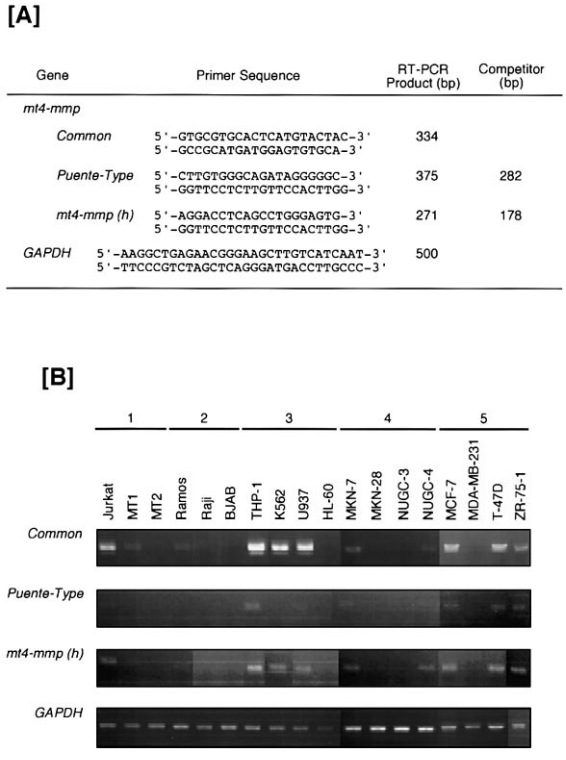

Fig. 2. Expression of two types of *mt4-mmp(h)* transcripts in tumor cell lines. A: Primers to detect *mt4-mmp(h)* transcripts, the sizes of the RT-PCR product and specific competitors are summarized. Common primers are designed from the common sequences between two types of the transcripts. The 5′ primer for 'Puente-type' and major transcripts was derived from respective 5′ unique sequences, and the 3′ primer is common between the two. B: Expression of two types of transcripts was analyzed by RT-PCR. Total RNA was extracted from the cells indicated. Cell types are 1, T-cell leukemia; 2, B-cell leukemia; 3, monocytic leukemia; 4, gastric cancer; 5, breast cancer. RT-PCR products were analyzed by agarose gel electrophoresis.

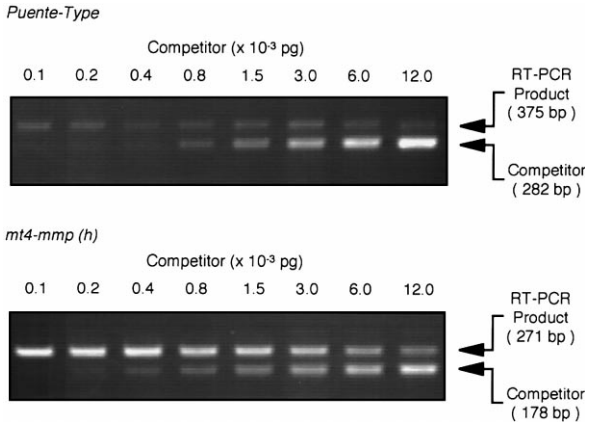

Fig. 3. Relative ratio of the two types of transcripts. A RNA sample extracted from T-47D cells was analyzed by competitive PCR. The RT-PCR reaction was carried out as described in Section 2 except that specific competitors (Fig. 2A) were added at the concentrations indicated.

4. Discussion

A new major *mt4-mmp(h)* transcript was identified in ad-

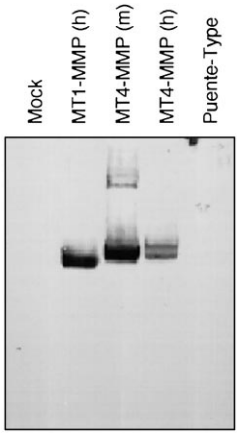

Fig. 4. Detection of translation products. Both types of candidates were expressed in COS-1 cells using a eukaryotic expression vector pSG5 (Stratagene). For detection, a FLAG epitope was incorporated into the expected products downstream of the furin motif. Cells were transiently transfected with the expression plasmids as indicated and lysates were prepared and analyzed by Western blotting using an anti-FLAG monoclonal antibody M2. FLAG-tagged MT1-MMP(h) (63 kDa) was used as a positive control.

dition to the previously reported 'Puente-type' transcript. The 'Puente-type' transcript could not direct expression of the gene product but the new transcript expressed 67 and 71 kDa products. Presumably the 71 kDa product corresponds to proMT4-MMP and the 67 kDa one to the processed version at the furin motif. A mouse *mt4-mmp* cDNA that corresponds to the human new transcript was also obtained and directed expression of a 67 kDa protein. Thus, functional cDNAs encoding human and mouse MT4-MMPs were identified for the first time. From the cDNA sequences, human and mouse MT4-MMP are composed of 605 and 587 amino acids, respectively.

Both types of transcripts are expressed in several human tumor cell lines, but the 'Puente-type' transcript was detected only in the cells that express the major transcript. Though the two types of *mt4-mmp(h)* transcripts are surely expressed in the cells, cDNA for the major transcript has not been obtained from the conventional cDNA libraries as Puente described [8]. The sequence of the major transcript obtained by 5′ RACE revealed that the 5′ region unique to the major transcript is rich in GC content (85%). This would explain that cDNA clones containing this region could be rare in the cDNA libraries.

It is not clear how the 'Puente-type' transcript is generated. The most plausible possibility is alternative splicing at the first intron, because the 'Puente type' retains the first intron sequence at its 5′ end. In the previous report by Puente et al. [8], a weak band of 7.5 kb was detected in addition to the major 2.7 kb band by Northern blotting. This minor transcript may correspond to the 'Puente type'. Indeed our preliminary estimation of the first intron by PCR is about 5 kb.

Mt4-mmp was originally isolated from a human breast carcinoma cell line. Both transcripts were detected in three of the four breast carcinoma cell lines. In contrast, expression of the gene in gastric carcinoma cell lines was low. High levels of expression were also found in three of the four monocytic leukemia cell lines. Expression of MT4-MMP in tumor cells may contribute to their malignant phenotypes by degrading the surrounding extracellular matrix or causing processing of the cell surface molecules.

In conclusion, human and mouse *mt4-mmp* genes and their products were identified. Our results provide a sure basis for further characterization of MT4-MMP.

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