Isolation of a mouse MT2-MMP gene from a lung cDNA library and identification of its product

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Abstract We have isolated a new MT-MMP related gene of 3.3 kb from a mouse lung cDNA library using a human *MT1-MMP* cDNA as a probe. The deduced protein sequence shows 87% homology to human MT2-MMP and 52, 50 and 29% to MT1-MMP, MT3-MMP and MT4-MMP, respectively. Thus the gene is thought to be a mouse homologue of human *MT2-MMP*. A monoclonal antibody raised against a synthetic peptide recognized mouse MT2-MMP as a 70 kDa protein. Like MT1- and MT3-MMPs, mouse MT2-MMP caused activation of pro-gelatinase A upon co-transfection into COS-1 cells.

Key words: Matrix metalloproteinase; Membrane type matrix metalloproteinase; MT2-MMP; Gelatinase A activation

1. Introduction

Matrix metalloproteinases (MMPs) are zinc-binding endopeptidases that degrade macromolecules of extracellular matrix (ECM). Recently four membrane-type matrix metalloproteinases (MT-MMPs) have been identified using the RT-PCR technique and primers corresponding to the conserved regions of MMP genes [1–6]. They contain a transmembrane domain and a short cytoplasmic tail downstream of the hemopexin-like domain. Pro-MT-MMPs like prostromelysin-3 contain a conserved sequence of four basic amino acid residues between propeptide and catalytic domains that is a potential cleavage site of preprotein convertase, furin [1]. Indeed, furin was demonstrated to cleave this sequence in prostromelysin-3 and proMT1-MMP resulting in enzyme activation [7–9]. Thus activation of MT-MMPs seems to be regulated differently from other MMPs [10].

MT1-MMP induces autocatalytic activation of pro-gelatinase A by introducing a cleavage into the propeptide domain [8,9,11,12] at the reported N-terminus of the intermediate form [13] and also degrades ECM components [8,14]. Other MT-MMPs remain to be characterized biochemically although MT3-MMP was shown to also activate pro-gelatinase A [4]. Characterization of mouse MT-MMP genes is an important step in future analysis of the biological functions of these enzymes by generating knock-out and transgenic mice. Here we report isolation of a new mouse *MT-MMP* gene that eventually turned out to be the mouse homologue of human *MT2-MMP*. The gene product was identified using a monoclonal antibody raised against a synthetic peptide and its ability to activate progelatinase A was also demonstrated for the first time.

2. Materials and methods

2.1. Screening of a cDNA library and molecular cloning

A λgt10 cDNA library of mouse lung (Clontech, Palo Alto, CA) was screened with a human *MT1-MMP* cDNA (accession number D26512) fragment (1–1485 bp) as a probe [2]. Phage plaques were transferred to nitrocellulose filters, hybridized with the ^{32}P-labeled probe at 60°C in Rapid Hybridization buffer (Amersham) and washed with 0.5×SSC, 0.1% SDS at 50°C. DNA inserts of the purified phages were excised with *Eco*RI and subcloned into a pBluescript vector. Nucleotide sequence was determined using an automated sequencer (Applied Biosystems model 373A DNA sequencer).

2.2. Cell culture and transfection

Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum and 2 mM glutamine under a 5% CO_2 atmosphere. Transfection of the cells has been carried out using a calcium phosphate method as described previously [15]. For expression of the cloned gene, an eukaryotic expression vector, pSG5 (Stratagene, La Jolla, CA), that has a SV40 early gene promoter was used. Lysates of the COS-1 cells transfected with MT-MMP plasmids were prepared and examined by Western blotting as described previously [1].

2.3. Monoclonal antibodies and Western blotting

Mouse monoclonal antibodies were raised against MT-MMP derived synthetic peptides. Monoclonal antibodies 113-15E [1] and 117-4E3 [4] were against C^{319}DGNFDTVAMLRGEMA^{333} and F^{166}EEVPYSELENGKRD^{181} peptides of human MT1-MMP [1] and anti-MT3-MMP [4] respectively. For mouse MT2-MMP, T^{282}DNFQLPEDDLRGI^{295} peptide was synthesized and used for immunization of mice to obtain a monoclonal antibody 162-4E3. Western blot analysis of the COS-1 cell lysates was performed as described [1].

2.4. Gelatin zymography

Samples were mixed with the SDS sample buffer in the absence of a reducing agent, incubated for 2 0min at 37°C and separated on a 10% polyacrylamide gel containing 0.1% gelatin. After electrophoresis, gels were soaked in 2.5% Triton X-100 for 1 h and incubated in 50 mM Tris-HCl (pH 7.6) containing 150 mM NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3 at 37°C for 24 h. The gels were stained with 0.1% Coomassie brilliant blue R250, and the location of gelatinolytic activity was detected as clear bands on the background of uniform staining [1].

3. Results and discussion

To isolate a mouse *MT1-MMP* and its related genes we screened a mouse lung cDNA library with the human *MT1-MMP* cDNA fragment as a probe and obtained eight positive

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                                                                CGGACTCCCCTTTCTCTGACTCTGGAGC          28 nt

ATGGGCAGCGACCGGAGCGCACTCGGACGTCCAGGCTGCACTGGCAGCTGCCTCAGCAGCCGAGCTTCGCTGCTCCCGCTGCTACTGGTG          118
MetGlySerAspArgSerAlaLeuGlyArgProGlyCysThrGlySerCysLeuSerSerArgAlaSerLeuLeuProLeuLeuLeuVal  30 aa

CTTCTGGACTGTCTGGGCCATGGTACAGCGTCTAAAGACGCCGAAGTGTACGCCGCGGAGAACTGGCTGCGGCTCTATGGCTACCTACCC          208
LeuLeuAspCysLeuGlyHisGlyThrAlaSerLysAspAlaGluValTyrAlaAlaGluAsnTrpLeuArgLeuTyrGlyTyrLeuPro  60

CAGCCCAGCCGCCACATGTCCACCATGCGCTCTGCCCAGATCCTGGCCTCCGCCCTTGCCGAGATGCAGAGTTTCTATGGGATCCCTGTC          298
GlnProSerArgHisMetSerThrMetArgSerAlaGlnIleLeuAlaSerAlaLeuAlaGluMetGlnSerPheTyrGlyIleProVal  90

ACGGGTGTGCTTGATGAAGAGACGAAAACGTGGATGAAGCGGCCCCGATGTGGGGTTCCTGATCAGTTTGGGGTACATGTGAAAGCCAAC          388
ThrGlyValLeuAspGluGluThrLysThrTrpMetLysArgProArgCysGlyValProAspGlnPheGlyValHisValLysAlaAsn  120

CTGCGTCGACGGCGGAAACGTTACACCCTGACAGGAAAGGCATGGAACAATTACCACCTGACCTTCAGCATCCAGAACTACACTGAGAAG          478
LeuArgArgArgArgLysArgTyrThrLeuThrGlyLysAlaTrpAsnAsnTyrHisLeuThrPheSerIleGlnAsnTyrThrGluLys  150

CTGGGCTGGTACAACTCCATGGAGGCGGTGCGCAGGGCTTTCCAAGTGTGGGAGCAGGTCACACCATTGGTCTTCCAGGAAGTATCCTAT          568
LeuGlyTrpTyrAsnSerMetGluAlaValArgArgAlaPheGlnValTrpGluGlnValThrProLeuValPheGlnGluValSerTyr  180

GATGACATTCGGCTACGAAGGCGAGCGGAGGCTGACATCATGGTACTCTTTGCCTCTGGCTTCCATGGCGACAGCTCACCGTTTGATGGC          658
AspAspIleArgLeuArgArgArgAlaGluAlaAspIleMetValLeuPheAlaSerGlyPheHisGlyAspSerSerProPheAspGly  210

GTGGGTGGCTTTCTGGCCCACGCTTATTTCCCCGGCCCTGGTCTGGGTGGGGACACCCATTTCGACGCAGATGAACCCTGGACCTTCTCC          748
ValGlyGlyPheLeuAlaHisAlaTyrPheProGlyProGlyLeuGlyGlyAspThrHisPheAspAlaAspGluProTrpThrPheSer  240

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SerThrAspLeuHisGlyIleSerLeuPheLeuValAlaValHisGluLeuGlyHisAlaLeuGlyLeuGluHisSerSerAsnProSer  270

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AlaIleMetAlaProPheTyrGlnTrpMetAspThrAspAsnPheGlnLeuProGluAspAspLeuArgGlyIleGlnGlnLeuTyrGly  300

TCCCCAGATGGTAAGCCACAGCCCACCCGGCCTCTCCCCACTGTAAGGCCCCGGCGGCCAGGACGGCCAGATCACCAGCCACCTCGGCCT         1018
SerProAspGlyLysProGlnProThrArgProLeuProThrValArgProArgArgProGlyArgProAspHisGlnProProArgPro  330

CCCCAGCCACCACATCCAGGTGGGAAGCCAGAAAGGCCCCCCAAACCAGGGCCCCCACCCCAGCCCCGAGCCACAGAGAGGCCTGACCAG         1108
ProGlnProProHisProGlyGlyLysProGluArgProProLysProGlyProProProGlnProArgAlaThrGluArgProAspGln  360

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TyrGlyProAsnIleCysAspGlyAsnPheAspThrValAlaValLeuArgGlyGluMetPheValPheLysGlyArgTrpPheTrpArg  390

GTCAGGCACAACCGCGTTCTAGACAACTACCCCATGCCAATTGGCCACTTCTGGCGCGGTCTGCCGGGGAACATCAGTGCTGCCTACGAG         1288
ValArgHisAsnArgValLeuAspAsnTyrProMetProIleGlyHisPheTrpArgGlyLeuProGlyAsnIleSerAlaAlaTyrGlu  420

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AGGTACTGGCGCTTCAACGAGGAGACACAGCATGGAGACCCTGGCTACCCCAAGCCCATCAGTGTCTGGCAGGGGATCCCCACCTCTCCC         1558
ArgTyrTrpArgPheAsnGluGluThrGlnHisGlyAspProGlyTyrProIleSerValTrpGlnGlyIleProTyrSerProPro  510

AAAGGGGCCTTCCTCAGCAACGATGCAGCCTACACCTACTTCTACAAGGGCACCAAGTACTGGAAATTCAACAACGAACGCCTACGGATG         1648
LysGlyAlaPheLeuSerAsnAspAlaAlaTyrThrTyrPheTyrLysGlyThrLysTyrTrpLysPheAsnAsnGluArgLeuArgMet  540

GAACCCGGCCACCCCAAATCCATCCTGCGGGACTTCATGGGCTGCCAGGAGCACGTGGAGCCCCGATCGCGATGGCCCGATGTGGCTCGT         1738
GluProGlyHisProLysSerIleLeuArgAspPheMetGlyCysGlnGluHisValGluProArgSerArgTrpProAspValAlaArg  570

CCACCCTTCAACCCCAACGGGGGTGCCGAGCCTGAGGCAGATGGTGACAGCAAGGAAGAGAACGCGGGTGACAAGGATGAGGGCAGCCGC         1828
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GCCTTTGCTCTGGTGCAGATGCAGCGCAAGGGCGCCCCTCGCATGCTGCTCTACTGCAAGCGCTCACTGCAGGAGTGGGTGTGATCAGGT         2008
AlaPheAlaLeuValGlnMetGlnArgLysGlyAlaProArgMetLeuLeuTyrCysLysArgSerLeuGlnGluTrpVal  657

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CTTTGGTTCCGCTTTCCTGACTCGGGCCAGGAGATGCTTTGAGATCTCCCGGAGTGTAGTACCGCCCCAGGCAGGAACGAAGGGACAGAG         2278
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GTTTGCCTTGAAGGAGCCCACCTCGGCTCTGCCCTCCTGAATTTGCCTTAGGGAGAGTATATGCTCCCCCCTGGGGGGGCACCCTGATAA         2458
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CAGCACTCTAGATGTCCCCTCTGCAGAACCTGAGAGTCAGCAAGGGGTGGGGCGTCCACCCCCCCCACTCCCCATCGTTTAGTTCTGTAGA         3268
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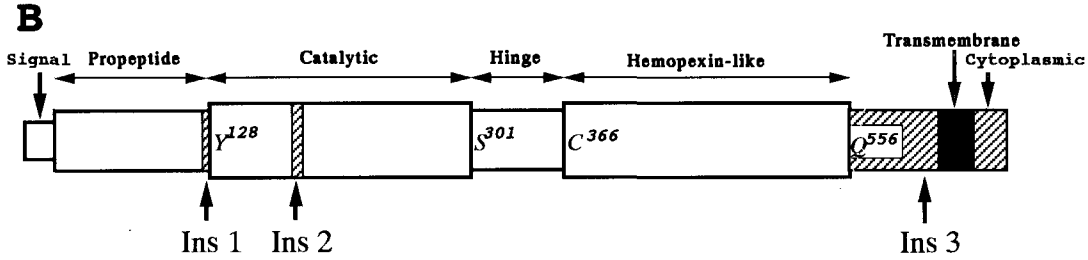

Fig. 1. The nucleotide and predicted protein sequences of a new mouse MMP gene. A: The nucleotide sequence of 3.3 kb (accession number D86332) coding for the mouse MT2-MMP gene is presented. The sequence contains an open reading frame that starts at the ATG codon and terminates at the TGA codon. A conserved PRCGVPD sequence in the propeptide domain which is part of the cysteine switch mechanism important in enzyme activation is boxed. A potential furin-sensitive site, RXKR, is underlined with a dotted line. An eight amino acid residues insertion in the catalytic domain is underlined with arrows. Zinc binding site is single underlined. The predicted transmembrane domain at the C terminus is double underlined. B: Schematic illustration of the domain structure of mouse MT2-MMP. Domains are indicated above the illustration. Insertions unique to MT-MMPs are indicated by the shaded boxes (Ins 1, 2 and 3).
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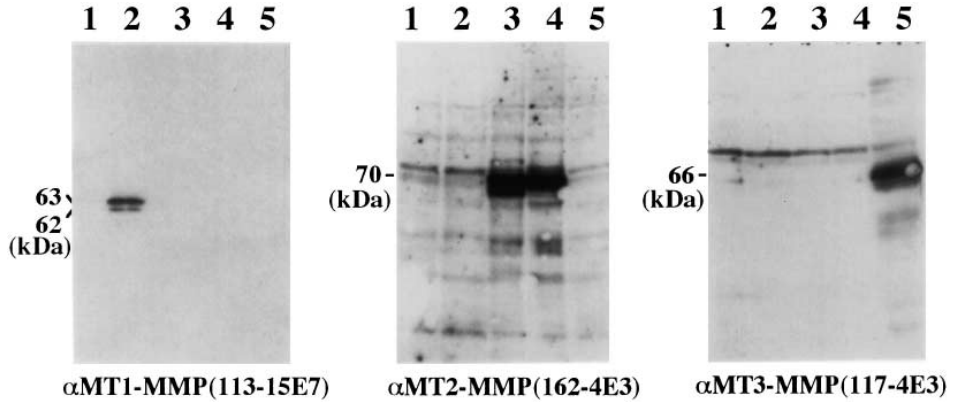

Fig. 2. Detection of human and mouse MT2-MMP by Western blotting. COS-1 cells were transiently transfected with human MT1-, MT2-, MT3-MMP and mouse MT2-MMP plasmids as follows: lane 1, no DNA; lane 2, human *MT1-MMP*; lane 3, human *MT2-MMP*; lane 4, mouse *MT2-MMP*; lane 5, human *MT3-MMP*. Cell lysates were prepared and examined by Western blotting using mAbs as described in Section 2.

clones. Sequence analysis revealed that six of them corresponded to the mouse *MT1-MMP* gene [3] and the gene was used for the analysis of *MT1-MMP* expression during mouse embryogenesis as reported previously [16]. The nucleotide sequences of the other two clones were different from mouse *MT1-MMP* showing 62% homology to the probe sequence while mouse *MT1-MMP* showed 88% homology. Since these clones did not contain the entire open reading frame (ORF), we used one of the fragments for further screening of the library under high stringency conditions to obtain a cDNA clone that contains an intact ORF for MMP.

A new cDNA clone consisting of 3339 bp and containing an ORF coding for a protein of 657 amino acids was obtained (Fig. 1). The predicted protein has a modular domain structure similar to MT-MMPs including a transmembrane domain and a cytoplasmic tail at the C-terminus (Ins 3). MT1-MMP, MT2-MMP and MT3-MMPs have two unique insertions (Ins 1 and 2) compared to other MMPs [4]. Ins 1 is between propeptide and catalytic domains and Ins 2 is in the catalytic domain. Both of these insertions were also found in the predicted protein. Comparison of the amino acid sequence of the newly isolated mouse MT-MMP to human MT-MMPs showed the highest homology to human MT2-MMP (87%) with homology of 52% for MT1-MMP, 50% for MT3-MMP and 29% for MT4-MMP. Accordingly, only human *MT2-MMP* cDNA clones were isolated when a human placenta cDNA library was screened under a high stringency condition with this mouse cDNA as a probe (data not shown). Thus the new gene is thought to be the mouse homologue of *MT2-MMP*.

To identify the product of the gene, a monoclonal antibody (anti-MT2-MMP, 162-4E3) was raised against a synthetic peptide corresponding to the insertion (Ins 2) in mouse MT2-MMP. Human and mouse MT-MMPs were expressed in COS-1 cells by transient transfection of the corresponding expression plasmids. Western blot analysis of the lysates demonstrated that the mAb 162-4E3 recognized a 70 kDa protein only in the lysates of cells transfected with the human and mouse MT2-MMP plasmids and did not react with human MT1-MMP and MT3-MMP (Fig. 2). On the other hand, MT1-MMP and MT3-MMP were recognized as 63 and 66 kDa proteins by mAbs 113-15E [1] and 117-4E3 [4], respectively.

MT1-MMP was first identified as a pro-gelatinase A activator [1] and, later, MT3-MMP was shown to have a similar activity although it was less efficient [4]. Thus we have examined whether human and mouse MT2-MMPs can activate pro-gelatinase A. Human and mouse MT2-MMPs were expressed in COS-1 cells together with pro-gelatinase A by transient co-transfection. The culture medium was collected and analyzed by gelatin zymography. Pro-gelatinase A was de-

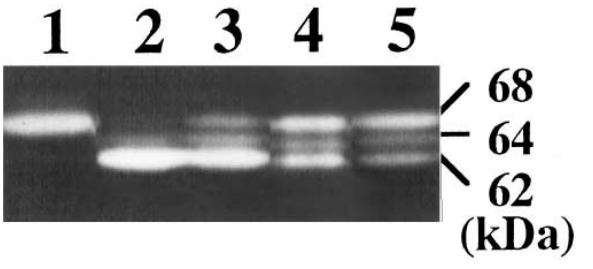

Fig. 3. Activation of progelatinase A by human and mouse MT2-MMPs. COS-1 cells were co-transfected with the expression plasmids for pro-gelatinase A and one of the human MT1-, MT2-, MT3-MMP or mouse MT2-MMPs. 24 h after transfection the cells were transferred to serum free DMEM and cultured further for 24 h. The culture medium was collected and analyzed by gelatin zymography. Lanes correspond to those in Fig. 2 except all MT-MMP plasmids were co-transfected with gelatinase A.

tected as a 68 kDa band when it was expressed alone (Fig. 3). Co-expression of MT1-MMP and MT3-MMP generated a processed gelatinase A corresponding to intermediate (64 kDa) and fully activated (62 kDa) forms. Expression of human and mouse MT2-MMPs also induced processing of pro-gelatinase A (Fig. 3). Thus, although no function of human MT2-MMP has been described yet, we demonstrated that both human and mouse MT2-MMP have the ability to activate pro-gelatinase A when co-expressed in COS-1 cells. The substrates of MT2-MMP and MT3-MMP in ECM have not been determined yet either. It is possible to speculate that they share some common target proteins while the expression of the genes is regulated differently.

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