

Membrane Type 4 Matrix Metalloproteinase (MMP17) Has Tumor Necrosis Factor- α Convertase Activity but Does Not Activate Pro-MMP2*

Received for publication, January 21, 2000

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Membrane type 4 matrix metalloproteinase (MT4-MMP) shows the least sequence homology to the other MT-MMPs, suggesting a distinct function for this protein. We have isolated a complete cDNA corresponding to the mouse homologue which includes the signal peptide and a complete pro-domain, features that were lacking from the human form originally isolated. Mouse MT4-MMP (mMT4-MMP) expressed in COS-7 cells is located at the cell surface but does not show ability to activate pro-MMP2. The pro-catalytic domain was expressed in *Escherichia coli* as insoluble inclusions and active enzyme recovered after refolding. Activity of the isolated catalytic domain against synthetic peptides commonly used for MMP enzyme assays could be inhibited by TIMP1, -2, and -3. The recombinant mMT4-MMP catalytic domain was also unable to activate pro-MMP2 and was very poor at hydrolyzing components of the extracellular matrix with the exception of fibrinogen and fibrin. mMT4-MMP was able to hydrolyze efficiently a peptide consisting of the pro-tumor necrosis factor α (TNF α) cleavage site, a glutathione S-transferase-pro-TNF α fusion protein, and was found to shed pro-TNF α when co-transfected in COS-7 cells. MT4-MMP was detected by Western blot in monocyte/macrophage cell lines which in combination with its fibrinolytic and TNF α -converting activity suggests a role in inflammation.

Matrix metalloproteinases (MMPs)¹ are a large family of structurally related zinc-dependent endopeptidases that are essential in tissue remodeling under physiological and patho-

logical conditions. These include morphogenesis, angiogenesis, tissue repair, arthritis, tumor invasion, and migration of inflammatory leukocytes (1–5)

A subset of the MMP family, the membrane type MMPs (MT-MMPs) have more recently been identified (6, 7). To date six such enzymes have been described, and MT1-, -2-, and -3-MMP have been shown to have a wide range of activities against extracellular matrix proteins (8–13). The activity of MT-MMPs has been implicated in cell invasion of the ECM (4). Possibly more importantly, they are also activators of pro-MMP2 and pro-MMP13 which has been correlated with the invasiveness of tumor and other cells (7, 9, 14, 15). Like other MMPs, the activity of MT-MMPs can be modulated by the tissue inhibitors of MMPs or TIMPs; however, TIMP1 was found to be a poor inhibitor of MT1-, -2-, and -5-MMP (9, 10, 16). TIMP2 and MT1-MMP can also associate to form a pro-MMP2 receptor at the cell surface, leaving the C terminus of TIMP2 free to bind pro-MMP2. This allows efficient activation of pro-MMP2 by adjacent TIMP2-free MT1-MMP and may be a common mechanism for pro-MMP2 activation by MT-MMPs (17).

The fourth member of the MT-MMP family, MT4-MMP, has the least degree of sequence identity to the other family members (7, 18). The human cDNA (hMT4-MMP) originally isolated from a human breast carcinoma cell line was found to lack the signal sequence and part of the pro-domain. Northern blot analysis of the tissue mRNA distribution showed hMT4-MMP to be localized in brain, colon, ovary, and testis. More interesting was the high level of mRNA found in leukocytes, which suggested a possible novel function for MT4-MMP (18). More recent investigations of the distribution of MT4-MMP mRNA in a variety of cell lines has shown MT4-MMP to be expressed in many transformed and non-transformed cell types (19).

Here we describe the isolation of a mouse cDNA homologue to MT4-MMP (mMT4-MMP) which includes the complete signal sequence and pro-peptide. This has allowed us to express transiently mMT4-MMP in mammalian cells and express the pro-catalytic domain as insoluble inclusions in *Escherichia coli*, from which active enzyme was recovered after refolding. Characterization of the catalytic activity of either the isolated catalytic domain alone or the entire enzyme transiently expressed in mammalian cells has shown it to be quite distinct in specificity from MT1-, -2-, -3-, and -5-MMP.

EXPERIMENTAL PROCEDURES

Materials and General Procedures—All chemicals and reagents were obtained from Sigma or BDH (Poole, UK) and were of the highest quality available unless otherwise specified. Fibrinogen was obtained from Calbiochem. Fibronectin, tenascin long and short forms, and type I gelatin were prepared and purified as described by d'Ortho *et al.* (20).

* This work was supported by European Union Fourth Framework Initiatives ERBB104CT96-0464 and BMH4CT96-0017, the Medical Research Council, the Arthritis Research Campaign, and the Wellcome Trust. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ010731.

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¹ The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane type MMP; ECM, extracellular matrix; TIMP, tissue inhibitor of MMP; TNF α , tumor necrosis factor α ; TACE, TNF α -converting enzyme; GST, glutathione S-transferase; Mca, (7-methoxycoumarin-4-yl)acetyl; Dnp, 2,4-dinitrophenol; Dansyl, 5-(dimethylamino)naphthalene-1-sulfonyl; HA, hemagglutinin; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; APMA, P-amino-phenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide.

Thrombin and α_2 -macroglobulin were obtained from Sigma. Type IV collagen and type I laminin were purchased from Collaborative Research (UK). Recombinant MT1-MMP catalytic domain, pro-MMP2, pro-MMP9, pro-MMP13, and mouse TACE catalytic domain were purified from conditioned medium from transfected cell lines as published previously (9, 21, 22). GST-pro-TNF α was expressed and purified as described previously (20). Monoclonal antibody to human TNF α were obtained from Chemicon (Harrow, UK). ELISA for human TNF α was purchased from Cambridge Biosciences (Cambridge, UK).

Expression of the Human MT4-MMP Catalytic Domain in *E. coli* as Insoluble Inclusions and the Production of Specific Sheep Polyclonal Antibodies—The catalytic domain of human MT4-MMP was subcloned by PCR from the human cDNA originally isolated by Puentes *et al.* (18) using primers 5'-AAAAGAATTCAACCGTACAGCTG-3' and 5'-AAAA-CATGGCCAGGCTCCAGCC-3'. The PCR product was ligated into pET23d (Novagen) using *NcoI/EcoRI* sites and sequenced. Human MT4-MMP catalytic domain was expressed in *E. coli* BL21 (DE3) pLysS at 30 °C on the addition of 0.5 mM isopropyl-1-thio- β -D-galactopyranoside at an $A_{600\text{ nm}}$ of 0.2–0.3 for 3 h. The insoluble inclusions were prepared as described previously (23) and were solubilized in 8 M urea, 50 mM Tris-HCl, 5 mM EDTA, 100 mM dithiothreitol, pH 8.0, prior to addition to a DEAE-Sepharose column equilibrated with 6 M urea, 25 mM Tris-HCl, pH 7.5. Partially pure MT4-MMP catalytic domain (estimated >65%) was then eluted with the equilibration buffer containing 1 M NaCl using a linear gradient from 0 to 1 M NaCl. Fractions were analyzed by reducing SDS-PAGE, and those containing MT4-MMP catalytic domain were then combined and dialyzed against PBS. The antigen was injected into sheep and IgG purified from sera. Specific antibody was purified against the antigen originally used by dot blotting as described by Stanton *et al.* (24). Purified antibody was checked for cross-reactivity against other MT-MMPs by Western blot by loading 5 μ g of protein per well. The antibody was not found to cross-react with the catalytic domain MT1-, -2-, or -3-MMP.

Cloning of the mMT4-MMP cDNA—A mouse brain cDNA library constructed in λ gt11 was from CLONTECH (Palo Alto, CA). Restriction endonucleases and other reagents used for molecular cloning were from Roche Molecular Biochemicals. Synthetic oligonucleotides were prepared in an Applied Biosystems (Foster City, CA) model 392A DNA synthesizer. Double-stranded DNA probes were radiolabeled with [32 P]dCTP (3000 Ci/mmol) purchased from Amersham Pharmacia Biotech using a commercial random-priming kit from the same company. A cDNA fragment (1.6 kilobase pairs) encoding human MT4-MMP was radiolabeled and used to screen a mouse brain cDNA library according to standard procedures. Hybridization to the radiolabeled probe was carried out for 18 h in 6 \times SSC (1 \times = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5 \times Denhardt's (1 \times = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll), 0.1% SDS, and 100 μ g/ml denatured herring sperm DNA at 55 °C. The membranes were washed twice for 1 h at 55 °C in 2 \times SSC, 0.1% SDS and exposed to autoradiography. After plaque purification, cloned inserts were excised by *EcoRI* digestion, and the resulting fragments were subcloned into the *EcoRI* site of pUC18. DNA fragments selected for nucleotide sequencing were inserted in the polylinker region of phage vector M13mp19 and sequenced by the dideoxy chain termination method using either M13 universal primer or cDNA-specific primers and the Sequenase version 2.0 kit (United States Biochemical Corp.). All nucleotides were identified in both strands. Sequence ambiguities were solved by substituting dITP for dGTP in the sequencing reactions. Computer analysis of DNA and protein sequences was performed with the software package of the University of Wisconsin Genetics Computer Group.

Construction of Eukaryotic Expression Vectors for MT4-MMP-HA and Immunolocalization—A cDNA fragment encoding the first 527 residues of mouse MT4-MMP was PCR-amplified and cloned between the *HindIII* (blunt-ended) and *BamHI* sites of pcDNA3-KZ-HA (a gift from Dr. Piero Crespo, Instituto Investigaciones Biomédicas, Madrid, Spain). Then, a second cDNA fragment encoding residues 528–578 was PCR-amplified and cloned between the *BglIII* and *EcoRI* sites of this plasmid. The final construct contained the hemagglutinin (HA) epitope of human influenza virus in frame to the MT4-MMP, located between residues 527 and 528 of the murine polypeptide, at the end of the hemopexin domain and immediately before the C-terminal hydrophobic extension of MT4-MMP. Thus, the resulting MT4-MMP protein was HA-tagged in an inner part of the molecule, avoiding problems derived from putative proteolytic processing of the protein either at N- or C-terminal ends. COS-7 cells were transfected with 1 μ g of plasmid DNA, using Fugene 6 reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. 48 h after transfection, cells were fixed for 10 min in cold 4% paraformaldehyde in PBS for 10 min,

washed in PBS, and incubated for 10 min in 0.2% Triton X-100 in PBS. Fluorescent detection was performed by incubating the slides with monoclonal antibody 12CA5 (Roche Molecular Biochemicals) against HA (diluted 1:100) followed by another incubation with goat anti-mouse fluoresceinated antibody (diluted 1:50). After washing in PBS, slides were mounted with vectashield (Vector, Burlingame, CA) and observed in a Bio-Rad confocal laser microscope. COS-7 extracts were also obtained for Western blot analysis of the MT4-MMP-HA protein.

Preparation of Cell Membrane Fractions and Western Blot Analysis—COS-7 cells were transiently transfected with the pcDNA3-MT4-MMP-HA plasmid as described under "Experimental Procedures." Cells were rinsed in PBS and scraped from the plates. Membrane fractions were prepared essentially following the procedure described by Strongin *et al.* (25). 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 Pharmacia Biotech).

Transient Expression of mMT4-MMP in CHO and COS-7 Cells for the Activation of Exogenous Pro-MMP2 and Pro-MMP13 and Processing of Pro-TNF α —CHO and COS cells were transiently transfected for 48 h with MT4-MMP or MT1-MMP using Ca(PO $_4$) or LipofectAMINE (Life Technologies, Inc.) in a 24-well plate using the appropriate vector controls. Total cell lysates of transiently transfected cells were prepared by washing the cells with PBS prior to the addition of cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM CaCl $_2$, 0.02% NaN $_3$ (v/v), 1% Triton X-100 (v/v), pH 8.0, with 0.1 mM phenylmethylsulfonyl fluoride, 5 μ g ml $^{-1}$ E64, and 5 μ g ml $^{-1}$ pepstatin) and scraping. Total cell lysates were analyzed by Western blot using the anti-hMT4-MMP sheep polyclonal antibody. Activation of pro-MMP2 or pro-MMP13 by CHO or COS-7 cells transiently expressing MT4-MMP was followed by the addition of pro-MMP2 or pro-MMP13 to the culture media 24 h after transfection and then analyzed by gelatin zymography (26) and Western blotting, respectively. For the processing of pro-TNF α by transiently expressed MT4-MMP, human pro-TNF α in psfCAV (a kind gift from J. Arribas, Laboratori de Recerca Oncologica, Barcelona, Spain) was co-transfected with mMT4-MMP in pcDNA3.0 into COS-7 cells in 6-well plates with LipofectAMINE (Life Technologies, Inc.) using 1 μ g of each DNA. Where expression of TNF α alone was monitored, the cells were co-transfected with empty pcDNA3.0 vector. Shedding was inhibited by the addition of either 10 μ M CT-1746 or 200 nM TIMP-1. The conditioned medium and cell lysates were then harvested 24 h post-transfection. Human TNF α was detected in cell lysates using a monoclonal antibody, and MT4-MMP was detected using the affinity purified sheep anti-human polyclonal by Western blotting. Shedding of soluble human TNF α was quantitated using ELISA (Cambridge Biosciences, UK).

Culture Conditions of Leukocytes—Rat alveolar macrophages were isolated by bronchial lavage of the lungs of freshly sacrificed rats using phosphate-buffered saline (PBS). Macrophages were then cultured as described previously (27). The cells were stimulated for 24 h in the presence of 2 μ g ml $^{-1}$ LPS or 20 ng ml $^{-1}$ PMA before total cell lysates were prepared using cell lysis buffer. Macrophages were also cultured for 24 h before stimulation with 20 ng ml $^{-1}$ PMA and then cultured for a further 24 h before harvesting. MT4-MMP in total cell lysates was detected by Western blot using the anti-hMT4-MMP sheep polyclonal antibody. Jurkat cells were cultured in the presence or absence of 10 ng ml $^{-1}$ PMA for up to 8 h. U937 and THP-1 cells were stimulated with 5 ng ml $^{-1}$ PMA for up to 72 h. Total cell lysates of Jurkat, U937, and THP-1 cells were prepared and probed for the presence of MT4-MMP in the same manner as for the rat alveolar macrophages.

Expression, Refolding, and Purification of mMT4-MMP Pro-catalytic Domain in *E. coli*—The pro-catalytic domain of mMT4-MMP was subcloned by PCR from the pcDNA3 MT4-MMP-HA construct using primers 5'-GCGCCTCGAGGGGAGGACCTCAGCCTCGGGGTGGAGTGG-C-3' and 5'-AAAAGATCTTACTCTCGGGCTCTGGGGTATCCAGC-TGGGC-3'. The PCR product was then ligated into pRSETB using *XhoI/BglIII* sites, which introduces a N-terminal His $_6$ tag, and sequenced. The pro-mMT4-MMP catalytic domain was expressed from pRSETB (Invitrogen) in *E. coli* BL21 (DE3) pLysS at 37 °C. mMT4-MMP pro-catalytic domain was purified as described under "Experimental Procedures," except that the DEAE-Sepharose equilibration and elution buffers included 5 mM dithiothreitol. mMT4-MMP pro-catalytic domain was then refolded by dropwise addition to 50 mM Tris-SO $_4$, 250 mM (NH $_4$) $_2$ SO $_4$, 10 mM CaSO $_4$, 0.1 mM ZnSO $_4$, 0.05% Brij (v/v), pH 8.0, to a final concentration of 0.1 mg ml $^{-1}$ and left overnight at 4 °C. The mMT4-MMP pro-catalytic domain was then further purified on Ni $^{2+}$ -nitrilotriacetic acid-agarose pre-equilibrated with 25 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl $_2$, pH 7.5, and eluted in the

equilibration buffer containing 150 mM imidazole. Fractions containing mMT4-MMP pro-catalytic domain were immediately dialyzed against 25 mM Tris-HCl, 10 mM CaCl₂, 10% (v/v) glycerol, pH 7.5, and stored at -80 °C.

mMT4-MMP pro-catalytic domain was normally activated with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin for 15 min in assay buffer at a concentration of 10 μ g ml⁻¹ at 20 °C followed by the inhibition of the trypsin with 0.1 mM phenylmethylsulfonyl fluoride. Alternatively, mMT4-MMP was activated for 30 min at 20 °C in the presence of 1 mM APMA. Activated protein was routinely stored at -20 °C. Samples were transferred onto polyvinylidene difluoride membrane and stained with Coomassie Blue before N-terminal sequence analysis was performed on a PE Biosystems 494 Procise protein sequencer, using standard gas phase cycles for sequencing polyvinylidene difluoride-blotted protein.

Activity against Peptide Substrates, Inhibitors, and TIMPs—Hydrolysis of the following quenched fluorescent peptides were examined: Mca-PLGL-Dpa-AR-NH₂, Mca-GTEGEARGS-Dpa-NH₂, Mca-PLA-Nva-Dpa-AR-NH₂, Mca-APKVE-Nva-Dpa-R-NH₂, Mca-PEGLR-Dpa-NH₂, Mca-PEGL-Dpa-R-NH₂, Mca-P-Cha-G-Nva-HA-Dpa-NH₂, Mca-TEGEARGS-Dpa-NH₂, Mca-SPLAQAVRSSSRK(Dnp)-NH₂, Mca-SSMAQTLTLRSSSK(Dnp)-NH₂, Mca-KLDKSFNS-Nle-K(Dnp)-NH₂ (where Nva is Norvalyl and Nle is 3-cyclohexylalanyl) (kindly provided by C. G. Knight, University of Cambridge, UK). All fluorimetric measurements were made with an LS50B Perkin-Elmer spectrofluorimeter ($\lambda_{\text{exc}} = 328$ nm, $\lambda_{\text{em}} = 393$ nm) equipped with thermostable cuvette holders (25 °C) in assay buffer (2.5 ml of 50 mM Tris-SO₄, 250 mM (NH₄)₂SO₄, 10 mM CaCl₂, 10% glycerol (v/v), 0.05% Brij v/v, pH 7.5) keeping the concentration of Me₂SO constant (2% v/v). Kinetic analyses were achieved with the software package GraFit 3.0 (Erithacus Software Ltd, Staines, UK). Initial velocities were monitored with substrate concentrations between 0.25 and 2 μ M, which were estimated to be well below the Michaelis constant ($S \ll K_m$). k_{cat}/K_m was calculated as described by Amour *et al.* (22). The enzyme concentration E_0 was determined as described below. Analysis of the cleavage products of Mca-SPLAQAVRSSSRK(Dnp)-NH₂ and Mca-SSMAQTLTLRSSSK(Dnp)-NH₂ and comparison with those of TACE were performed as described by Amour *et al.* (22). Reaction mixtures between mMT4-MMP (3 nM) and inhibitors were preincubated for 30 min at 25 °C with BB-94 (British Biotech Pharmaceuticals (Oxford, UK)), Ro31-9790 (Roche Molecular Biochemicals), CT-1746, CT-572, and CT-1869 (Celltech, Slough, UK) at concentrations ranging from 0 to 100 nM and TIMPs from 0 to 30 nM. TIMPs were prepared as described previously (21, 28, 29). The steady-state velocities V_S were measured with Mca-PLGL-Dpa-AR-NH₂ (1 μ M). Apparent inhibition constants K_I^{app} (equal to $K_I(1 + S/K_m)$) and E_0 were estimated by least squares fit to Equation 1 for competitive tight-binding inhibition (30),

$$V_S = (V_0/2E_0) \times \{E_0 - I_0 - K_I^{\text{app}} + ((E_0 - I_0 - K_I^{\text{app}})^2 + 4E_0K_I^{\text{app}})^{1/2}\} \quad (\text{Eq. 1})$$

where I_0 and V_0 are the total concentration of inhibitor and velocity in absence of inhibitor, respectively. Enzyme concentration was approximated using this equation by averaging the calculated value obtained from fits to the inhibition curves of at least three hydroxamate inhibitors whose K_I^{app} values were calculated to be in the low nM range. Enzyme concentrations determined in this manner were typically in close agreement between hydroxamate inhibitors and TIMP2 and -3. The association rate constants (k_{on}) at 25 °C of mMT4-MMP with TIMP1, -2, and -3 were estimated as described previously (28).

Hydrolysis of Macromolecular Substrates—Cleavage of type I collagen, type I gelatin, fibronectin, laminin-I, tenascin, COMP, fibrinogen, fibrin, and α_2 -macroglobulin by mMT4-MMP catalytic domains were followed by reducing SDS-PAGE. All assays were performed in assay buffer overnight (16 h) in the presence of 1% Sigma mixture of inhibitors (containing AEBSF, pepstatin A, E64, bestatin, leupeptin, and aprotinin) at 20 °C. Where activity was seen, the time dependence of cleavage was followed by stopping the reaction by boiling in reducing SDS-PAGE buffer. Time-dependent cleavage of GST-pro-TNF α by mMT4-MMP, MT1-MMP catalytic domain or TACE catalytic domain was followed by reducing SDS-PAGE. Cleavage of GST-pro-TNF α was also examined by Western blotting using a polyclonal antibody to TNF α . Processing of pro-MMP2 and pro-MMP9 was followed by incubation with various molar ratios of mMT4-MMP or MT1-MMP catalytic domain:pro-MMP2 or 9 for 24 h and visualized by gelatin zymography.

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GAATTCGGCGGGAGCGGAGCGGAGCGGAGCTGGCTGCTGGCGGGTGGGGGACCTCGCCACCCGACCTGGG 75
AGAGCGGGATGGGACGCCGCCCGGGGACCTGGTCCCCCGGGGACCTGGCCCTCCACGCCCGCCGGGGGGC 150
  M G R R R P R G P P G S P R G G F P P R P G P G L
TGCCACCAGTGTCTGTACTGGCGCTGGCGGCCATGGGGCTGCGCCAGCCGCCCGCCCGGGGGAGGAC 225
  P L L L V L A L A A H G G C A A P A P R G E D L
TCAGCCTCGGGTGGAGTGGCTAAGCAGGTTGGCTACCTGCGCCTGCGAGATCCGGCATCAGGGCAGCTACA 300
  S L G V E W L S R F G Y L P P A D P A S G Q L Q T
CCGAGGAGACTGTCAAAGCGATTACTGCGCATGCGAGCATTGGTGTCTGGAGACCATGGATCTGATGAT 375
  Q E E L S K A I T A M Q Q F G G L E T T G I L D E
AGGCCACTCTGGCCCTGATGAAACCCCTCGATGCTCCCTTCGGGACCTGCCCTGGGGCCCAATCGAAGA 450
  A T L A L M K T P R C S L P D L P P G A Q S R R K
AGCGGACTCCACCCCAACCAATGAGGACGAGGAGCATCTTCTGGAGGCTCGGACATCTCCACGGGACT 525
  R Q C T R Q T P T K W S K R N L S W R V R T F P P R D S
CACCCCTGGCGGGATACTGTGCGTGCCTCATGTACTAGCCCTCAAAGTCTGGATGACATCACACCTTGA 600
  P L G R D T V R A L M Y Y A L K V W S D I T P L N
ACTTCCAGAGTAGCGGGCAACGGCGGCGCATCCAGATCGACTTCCAGAGGCGACACAGATGACGGCTAC 675
  F H E V A G N A A D I Q I D F S K A D H N D G Y P
CCTTCGATGGCCCTGGTGGCAGCGTGGCCCGCAGCATTCTTCCCTGGTGACACCAACAGCGGGGACACCT 750
  F D G P G G T V A H A H A F P P G D H H T A G D T H F
TTGATGAGATGAGCCATGGACCTTCGTTCTCAGATGCCACGGGATGACCTGTTTCAGTGGCGTCCATG 825
  D D D E F P W T F R S S D A H G M D L F A V A V H E
AGTTGGTCATGCCATGGTCTGAGCCATGTTGCCGCCCAAGCTCATCATGATGACCGGTACTCCAGGGCC 900
  F G H A I G L S H V A A P S S I M Q P Y Y Q G P V
TGGTGACCCCTAGCCTTGGACTTCCCTATGAGACAGGGTGGCTGTCTGGCAGTTAGCGTGTGGGGAT 975
  G D P L R Y G L P Y E D R V R V W Q L Y G V R E S
CCGTGTCCTACTGCCAGCTGGATACCCAGAGCCGAGGACCCCTCTGCCAGAGCCGCCCAACATC 1050
  V S P T A Q L D T P E P E E P P L L P E P P N N R
GGTCTAGCCTCCGCCGAGAGGAGCTGCTCAGCGTGCCTGCCACTGCCACTTGTATGCTGTGGCCAGATT 1125
  S S T P P Q K D V P H R C T A H F D A V A Q I R G
CGGAGCATTCTTTTCAAAGGCAAGTATTCTGGAGGCTGACCCGGGACCGACACTGGTGTCTGCGAGCCGG 1200
  E A F F F K G K Y F W R L T R D R H L V S L Q P A
CTCAATGCATCGCTCTGGCGGGCTGCCGCTGCACCTGGACSTGGACGCTGTGATGAGCTACCAGT 1275
  Q M H R F W R G L P L H L D A S V Y E R T S D
ACCACAAGATTCTCTCAAAGGACAGACTACTGGTGTAAAGGACAAACAGTAAAGGAAAGGATCCCGG 1350
  H K I V F F K G D R Y W F K D N N W E E G Y P R
GACCTGTCTCCGACTCAGCCTCCCGCAGGTGGCATGATGCTGTCTTCTCCTGGGCCCAATGACAGGACT 1425
  P V S D F S L P P G G I D A V F S W A H N D R T Y
ATTTCTTAAAGGACAGCTGTACTGGCGCTATGATGACACACAGCGGCATGGACCTTGGCTACCTGCCA 1500
  F F K D Q L Y W R Y D D H T R R M D P G Y P A Q G
GACCCCTGGGAGGAGTGTCCCGCAGATGTTGGATGATGCCATCGGCTGGTGTGATGGTGCATCTTCTT 1575
  P L W R G V P S M L D D A M C R S D R G S Y F F R
GAGGCGAGGAGTCTGGAAAGTGTGGATGCGGAGCTGGAAGCAGCCCGGGTAPCCAGATCTACAGCCCG 1650
  G Q E Y W K V L D G E L E A A P P Y Q P G S T A R D
ACTGGCTGGTATGCGGTGAGCCGCTGGCGGATGCGGAGGATGAGGGCTGGACCCCGGGCGGAGTGGGGCC 1725
  W L V C G E P L A D A E D V G P G P Q G R S G A Q
AAGATGGCTGGCAGTATGTTCTGCGACTTCCAGCGCACAGGTTGGCATTGCTGCTCTGCTGCTGACT 1800
  D G L A V G S C S T S D A H R L A L P S L L L L T P
CACTGTGTGGGGCTGTGAACCTCAGTCTCTGCCAAGGCATCCTGAGGGCAGTGTAGCCCTTGGCATCA 1875
  L L W G L *
GCCAAGGGGAGCAGGACACACTGGCCAGTACTCAGCAGGACTGTGCTCCAAGTCTCCGGTCCCTCGCTCT 1950
  CTTCTCTCTTCTTGAACCCAGGGGCTCCGGAATTC 1987

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FIG. 1. Nucleotide sequence of murine MT4-MMP cDNA isolated from mouse brain. The deduced amino acid sequence is shown below the nucleotide sequence. Structural features of the MT4-MMP sequence are indicated.

RESULTS

Cloning of Mouse mMT4-MMP—To isolate cDNA clones encoding the murine homologue of human MT4-MMP, a mouse brain cDNA library was screened with the cDNA coding for the human enzyme (18). Upon screening of approximately 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 (1.4) with those corresponding to other MMPs, including human MT4-MMP, suggested that it could encode a full-length cDNA for mouse MT4-MMP. Computer analysis of the sequence obtained (Fig. 1 and GenBank™ accession number AJ010731) revealed an open reading frame coding for a protein of 578 amino acids with a predicted molecular mass of 64.3 kDa. This sequence contains two potential sites of N-glycosylation (Asn-Leu-Ser and Asn-Arg-Ser at positions 136 and 322, respectively). Further analysis of the identified amino acid sequence revealed a significant similarity with other MMPs, the maximum percentage of identities (89%) being with human MT4-MMP (Fig. 2). While this manuscript was under review an identical mouse sequence was reported by Kajita *et al.* (38) as well as an equivalent form of a human sequence.

The deduced amino acid sequence is shown in Fig. 1 displays

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mMT4 1 MGRRRPGRGSGRPGFPPRPGLP.....PLLLVLAARHGCAA..PAPRGEGLSLGVEWLSRFGYL 61
hMT4 1 MRRAA....ARGPFPFPPGFLSRLPLPLPLLLLLALGTRGCAAEFARRAEDLSLGVWLSRFGYL 65

mMT4 62 PPADPASGQLQTQEELSKAITAMQQGGLETTGILDEATLALMKTFRCSLPDLPFGAQRKRKQTPPPFK 131
hMT4 66 PPADPTGQLQTQEELSKAITAMQQGGLEATGILDEATLALMKTFRCSLPDLVLTQARRRQAPAPFK 135

mMT4 132 WSKRNLNWRVRFPPRDSPLGRDTVRALMYALKVWSDITPLNFHEVAGNADIQIDFSKADHNDGYFFDG 201
hMT4 136 WNKRNLNWRVRFPPRDSPLGHDTVRALMYALKVWSDIAPLNFHEVAGSTADIQIDFSKADHNDGYFFDG 205

mMT4 202 PGGTVAHAFFPGOHTAGDTHFDDEPWFTRSSDAHGMDLFAVAVHEFGHAIGLSHVAAPSSIMQPYQG 271
hMT4 206 PGGTVAHAFFPGHHHTAGDTHFDDEAWTFTRSSDAHGMDLFAVAVHEFGHAIGLSHVAHAHSIMRPHYQG 275

mMT4 272 PVGDPLRYGLPYEDKRVKVLQYGVRESVSPAQDTPPEEPEPLLEPPNNRSTPPKQDVPHRCTAHD 341
hMT4 276 PVGDPLRYGLPYEDKRVKVLQYGVRESVSPAQ.....PEEPLLEPPDNRSSAPPKQDVPHRCTHFD 340

mMT4 342 AVAQRGEAFFFGKGYFWRLTRDRHLVSLQPAQMHRFWGLPLHLSDVDAVYERTSDHKIVFFKGDYRW 411
hMT4 341 AVAQRGEAFFFGKGYFWRLTRDRHLVSLQPAQMHRFWGLPLHLSDVDAVYERTSDHKIVFFKGDYRW 410

mMT4 412 FKDNVVEEGYFRVSDSFLPPGGIDAVFSWAHNDRTYFFKDQLYRWYDDHTRRMDPGYPAQSLWRGVPS 481
hMT4 411 FKDNVVEEGYFRVSDSFLPPGGIDAAFSAHNDRTYFFKDQLYRWYDDHTRRMDPGYPAQSLWRGVPS 480

mMT4 482 MLDDAMRWDGASVYFRGQYKWLVDGELVAPGYQSTARDWLVCGEPLAD...AEVGV.....PG 540
hMT4 481 TLDDAMRWDGASVYFRGQYKWLVDGELVAPGYQSTARDWLVCQSDGSGAAGVDAEGRAPPPG 550

mMT4 541 PQGRSGAQDGLAVCSCTSDAHR.....LALPSLLLLTPLLWGL..... 578
hMT4 551 QHDQSRSEDGYEVCSCTSGASSPPGAPGLVAATMLLLPLPSGALWTAQAQLTL 606

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FIG. 2. Sequence comparison of murine and human MT4-MMPs. Sequences were extracted from the Swiss-Prot data base, and the alignment was performed with the program PILEUP of the GCG package.

a number of features 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 about 170 residues including the consensus sequence HEXGHXXGXXH involved in zinc binding, and a fragment of approximately 200 amino acids with sequence similarity to hemopexin. In addition, the deduced amino acid sequence contains a 9-residue insertion between the propeptide and the catalytic domain, which is a common feature of MT-MMPs. This amino acid sequence ends with the consensus motif RXKR, which appears to be essential in the activation of these proteinases by furin. Finally, the identified sequence contains a C-terminal extension rich in hydrophobic residues and similar in size to that found in human MT4-MMP. Interestingly, this C-terminal region of both mouse and human MT4-MMP is shorter than the equivalent domains present in all the remaining MT-MMPs characterized to date and lacks the putative cytoplasmic tail also present in members of the MT-MMP subfamily. Based on all these structural characteristics, it is very likely that the murine protein encoded by the isolated mouse brain cDNA is the homologue of the human MT4-MMP (MMP-17).

Transient Expression and Localization of MT4-MMP-HA in CHO and COS Cells—To provide further support on the subcellular distribution of MT4-MMP, COS-7 cells were transfected with pcDNA3-MT4-MMP-HA, a construct containing the HA epitope in the C-terminal end of the hemopexin domain of murine MT4-MMP. The HA epitope was inserted to aid detection, whereas using immunofluorescence as the sheep anti-human polyclonal proved only to be suitable to Western blot detection of MT4-MMP. Transfected cells were then analyzed by immunofluorescence with a mouse monoclonal antibody (12CA5) specific for this viral epitope. As shown in Fig. 3A, a clear fluorescent pattern surrounding the cell was visualized in a serial optical section obtained using confocal microscopy. This observation strongly suggests that mouse MT4-MMP is a membrane-bound MMP. To verify this observation further, lysates from COS-7 cells transfected with the MT4-MMP-HA were analyzed by SDS-PAGE followed by Western blotting detection with anti-HA monoclonal antibody. A band of the expected molecular mass (64 kDa) was detected in the membrane-en-

riched fractions, but not in the soluble fraction, confirming its membrane localization (Fig. 3B). Transiently transfected CHO and COS-7 cells were also found to express a 64-kDa protein detectable by Western blot using a sheep polyclonal antibody raised against the human MT4-MMP catalytic domain. Untransfected CHO cells were also found to express a 54-kDa unidentified protein on occasion which also cross-reacted with the affinity purified anti-human MT4-MMP antibody, whereas untransfected COS-7 cells did not (Fig. 4A). The size of the MT4-MMP detected in transiently transfected cells corresponds to that expected of the pro-enzyme. Treatment with peptide *N*-glycosidase F did not result in a reduction of mass of the MT4-MMP detected suggesting the 64-kDa form is not *N*-glycosylated (results not shown). Equally the addition of the furin inhibitor decanoyl-RVKR-chloromethylketone at concentrations up to 25 μ M did not result in the appearance of larger species of MT4-MMP (results not shown). The addition of recombinant pro-MMP2 to the culture medium of MT4-MMP transiently transfected CHO cells did not result in activation of pro-MMP2 as revealed by gelatin zymography of the conditioned medium after 24 h, whereas CHO cells transfected with MT1-MMP did activate pro-MMP2 (Fig. 4B). COS-7 cells transiently transfected with MT4-MMP also did not activate pro-MMP2 or pro-MMP13 (data not shown).

Detection of MT4-MMP in Cultured Leukocytes—In order to examine the distribution of MT4-MMP expression, different cell lines and primary cells were probed by Western blot using the antibody that we developed. Since leukocytes were shown to express high levels of MT4-MMP mRNA, detectable by Northern blot (18), we analyzed cell lysates of stimulated and unstimulated Jurkat (T-cell), U937 (monocyte), THP-1 (monocyte), and primary rat alveolar macrophages. MT4-MMP was not detectable in total cell lysates of Jurkat either stimulated or unstimulated by 10 ng ml⁻¹ PMA for up to 8 h. MT4-MMP was not detected in unstimulated U937 cells but was found to be present after stimulation with PMA for 72 h (Fig. 5A), by which time the cells had become adherent and changed morphology. In THP-1 cells, MT4-MMP could be detected, and levels of expression were not modulated by PMA (Fig. 5A). MT4-MMP was also detected in unstimulated rat alveolar macrophages, and MT4-MMP levels were visibly increased in cells treated with LPS and PMA (Fig. 5A). Typically, only a 64-kDa form of MT4-MMP was detected in all cases corresponding to the mass of the pro-form. However, macrophages cultured for 24 h prior to stimulation with PMA for a further 24 h resulted in the detection of both pro- and active forms of MT4-MMP (Fig. 5A). These results indicate that MT4-MMP is expressed by monocytes and macrophages, and the formation of matrix by these cells regulates activation of MT4-MMP.

Expression, Refolding, Purification, and Activation of Pro-mMT4-MMP Catalytic Domain—Pro-mMT4-MMP catalytic domain was efficiently expressed in *E. coli* on induction with isopropyl-1-thio- β -D-galactopyranoside and accumulated as insoluble inclusions bodies in the cytoplasm, as seen with other MMPs expressed using similar systems (9, 10, 23). After solubilization with urea under reducing conditions, pro-mMT4-MMP catalytic domain was partially purified under denaturing conditions on DEAE-Sepharose to remove *E. coli* DNA and cell wall components, factors known to decrease refolding yields (29). At this stage it was found that approximately 25–40 mg of protein (of which >80% is pro-mMT4-MMP catalytic domain) could be obtained from 1 liter of culture. After refolding, soluble pro-enzyme was purified using the N-terminal His₆ tag on Ni²⁺-nitrilotriacetic acid-agarose, resulting in two species close in molecular mass seen on reducing SDS-PAGE and by Western blot (Fig. 6). N-terminal sequencing of this material

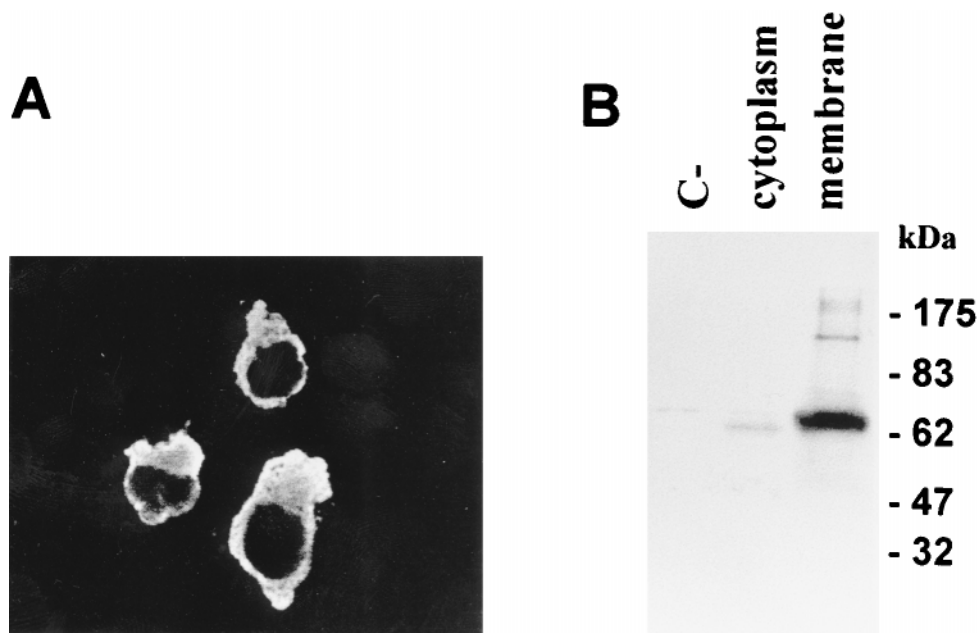


FIG. 3. *A*, immunofluorescent detection of MT4-MMP-HA in transiently transfected COS-7 cells with a monoclonal anti-HA antibody. Fluorescence was observed under a confocal laser microscope and localized to the surface of the COS-7 cells. *B*, Western blot analysis from COS-7 cells transiently transfected with the same MT4-MMP-HA vector. The 64-kDa MT4-MMP band was detected predominantly in the membrane fraction. *C*, control.

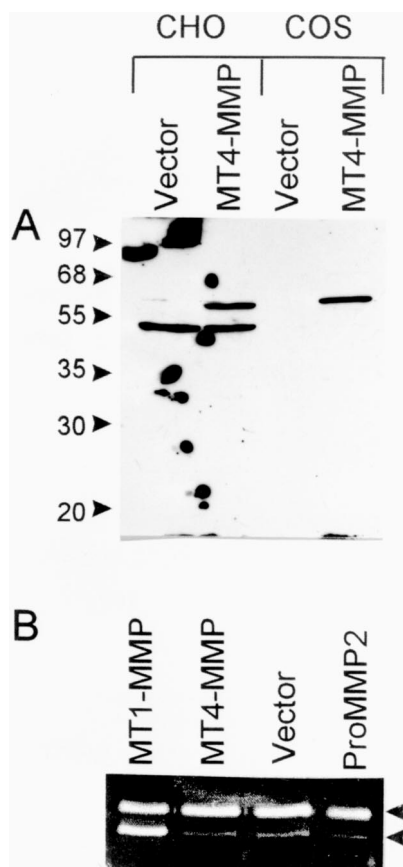


FIG. 4. Western blots of cell lysates of MT4-MMP transiently transfected CHO and COS cells and activation of pro-MMP2. *A*, Western blot of cell lysates probed with an anti-hMT4-MMP polyclonal sheep antibody. Each lane contained 100 μ g of total protein. *B*, gelatin zymogram of the conditioned medium of CHO cells transiently transfected with mMT4-MMP and hMT1-MMP containing exogenous recombinant pro-MMP2. Arrowheads, top to bottom, indicate the pro- and active forms of MMP2, respectively.

did not produce a unique sequence for the larger form and indicated the His₆ tag was degraded. The smaller form also did not produce a unique sequence but did have a main sequence of LQTQEEL indicating degradation within the pro-region around residue 71 of the mouse enzyme (Fig. 2). The Ni²⁺-agarose-purified material activated to a species of 25 kDa in the presence of trypsin, the expected mass for the active enzyme. As found with MT1- and -2-MMP (9, 10) activation by trypsin was extremely rapid, with no discernible intermediates being seen (Fig. 7A). N-terminal sequencing revealed that trypsin was processing mMT4 at amino acid 125, immediately after the proposed furin site RRKR, generating an N terminus of QTTPPTK. Interestingly, although at a slower rate, pro-mMT4-MMP catalytic domain could also be activated by APMA to a slightly larger form of 27 kDa (Figs. 6 and 7B), not observed with MT1- or -2-MMP over a similar time scale (9, 10).² The APMA-activated mMT4-MMPs N terminus was found to be at residue 119, giving a sequence of QSRRKRQTP, and precedes the furin site. The size discrepancy between the trypsin- and APMA-activated forms indicates that trypsin also cleaves at the C terminus of mMT4-MMP. This is most likely to occur between Arg-296 and Glu-297 (see Fig. 2), and MT1-MMP is also thought to be processed at a similar site (9).

Activity of mMT4-MMP Catalytic Domain against Peptides and Inhibitors—The mMT4-MMP catalytic domain activated by trypsin hydrolyzed a wide variety of peptide substrates (Table I). Activity against peptide substrates was routinely monitored at 25 °C as the enzyme showed poor stability at higher temperatures. Loss of activity at higher temperatures (*i.e.* 37 °C) was found to be irreversible, probably due to denaturation, as the protein was not observed to have undergone degradation on subsequent analysis by SDS-PAGE. Interestingly, mMT4-MMP was not only able to cleave the substrates commonly hydrolyzed by other MMPs (*i.e.* Mca-PLGL-Dpa-AR-NH₂ and Mca-PLA-Nva-Dpa-AR-NH₂) but also those corresponding to the pro-TNF α cleavage site, substrates for TACE

² W. R. English, X. S. Puente, J. M. P. Freije, V. Knäuper, A. Amour, A. Merryweather, C. López-Otín, and G. Murphy, unpublished observations.

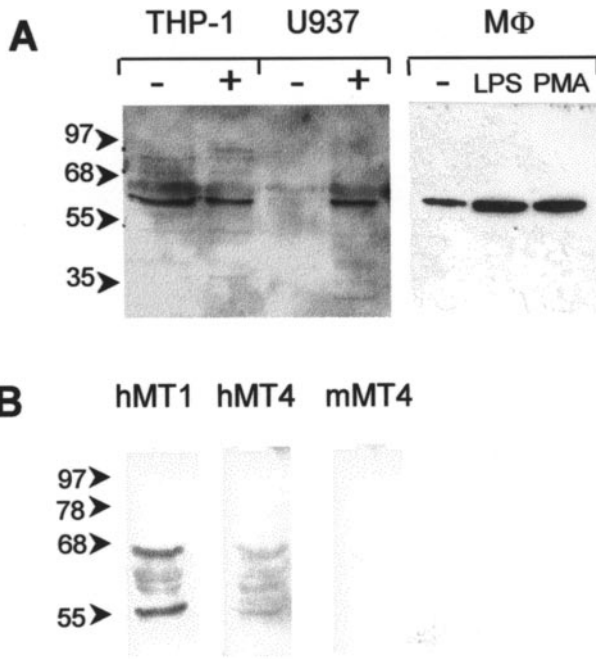


FIG. 5. Expression of MT4-MMP in leukocytes. Total cell lysates corresponding to approximately 2×10^5 cells were loaded per lane on a 10% reducing SDS-PAGE gel. MT4-MMP was subsequently detected by Western blot using an anti-hMT4-MMP sheep polyclonal antibody. *A*, THP-1 and U937 cells were cultured in the presence (+) or absence (-) of 5 ng ml^{-1} PMA for 72 h. Rat alveolar macrophages (MΦ) were stimulated with either $2 \mu\text{g ml}^{-1}$ LPS or 20 ng ml^{-1} PMA for 24 h. *B*, rat alveolar macrophages stimulated with 20 ng ml^{-1} for 24 h PMA after prior culturing for 24 h. The anti-hMT4-MMP IgG was pre-treated with either MT1-MMP catalytic domain, hMT4-MMP catalytic domain, or mMT4-MMP catalytic domain to remove cross-reacting IgG.

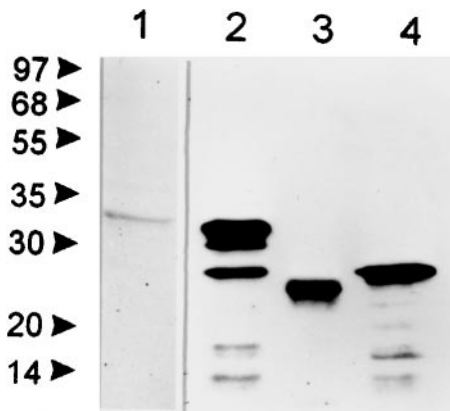


FIG. 6. SDS-PAGE of proMT4-MMP catalytic domain after refolding and purification. Lane 1, Coomassie Blue-stained 10% reducing SDS-PAGE gel of purified proMT4-MMP catalytic domain. Lanes 2 and 3, Western blot of 10% reducing SDS-PAGE gel of different purified forms of proMT4-MMP catalytic domain visualized using a sheep anti-human polyclonal antibody. Lane 2, pro-MT4-MMP catalytic domain. Lane 3, treatment with $10 \mu\text{g ml}^{-1}$ trypsin for 15 min at ambient temperature. Lane 4, treatment with 1 mM APMA for 1 h at 20 °C.

(22). The human peptide was hydrolyzed the most efficiently (Mca-SPLAQAVRSSSRK(Dnp)-NH₂) of all the peptides examined, although the murine peptide (Mca-SSMAQTLTLRS-SSK(Dnp)-NH₂) was actually hydrolyzed more rapidly by mMT4-MMP at 25 °C than by the mTACE catalytic domain at 37 °C with a k_{cat}/K_m of $6 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$ compared with $3.5 \times 10^3 \text{ s}^{-1} \text{ M}^{-1}$, respectively (22). mMT4-MMP was only observed to produce two products on cleavage of the human and murine pro-TNF α peptides on analysis by reverse phase-high performance liquid chromatography, indicating cleavage at a unique

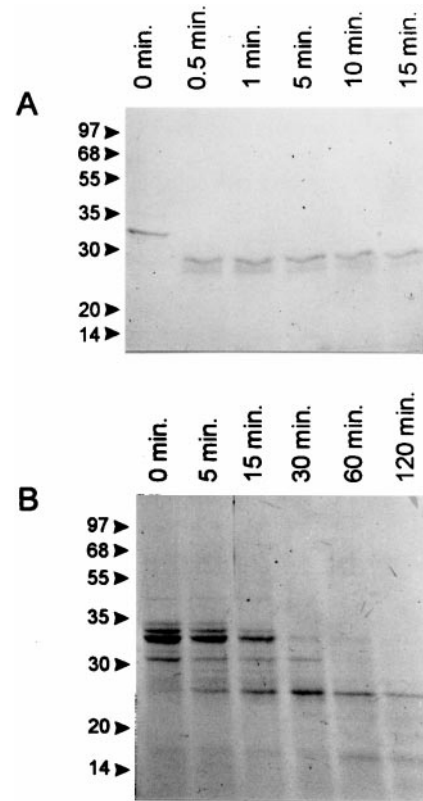


FIG. 7. Coomassie Blue-stained 10% reducing SDS-PAGE of the time of activation of pro-T4-MMP catalytic domain by trypsin and APMA. The enzyme was either incubated with $10 \mu\text{g ml}^{-1}$ trypsin (*A*) or 1 mM APMA (*B*) at 20 °C for the times shown, and the reaction was stopped as described under “Experimental Procedures.”

TABLE I
 k_{cat}/K_m for MT4-MMP catalytic domain versus quenched fluorescent peptide substrates

All assays were conducted at 25 °C, and errors were typically less than 5% of the calculated value.

Peptide	Substrate for/based on	k_{cat}/K_m $\text{M}^{-1} \text{ s}^{-1}$
Mca-PLGL-Dpa-AR-NH ₂ (QF24)	Most MMPs	2.4×10^4
Mca-PLA-Nva-Dpa-AR-NH ₂ (QF35)	MMP3	3.0×10^4
Mca-APKPVE-Nva-Dpa-R-NH ₂ (QF38)	MMP3	1.1×10^4
Mca-PEGLR-Dpa-NH ₂ (QF39)	α_2 -Macroglobulin bait region	0.82×10^4
Mca-PEGL-Dpa-R-NH ₂ (QF40)	α_2 -Macroglobulin bait region	0.6×10^4
Mca-P-Cha-G-Nva-HA-Dpa-NH ₂ (QF41)	MMP1/MMP9	2.0×10^4
Mca-SPLAQAVRSSSRK(Dnp)-NH ₂ (QF45)	Human pro-TNF α cleavage site	3.5×10^4
Mca-SSMAQTLTLRS-SSK(Dnp)-NH ₂ (QF48)	Mouse pro-TNF α cleavage site	0.6×10^4

site. Electrospray mass spectrometry analysis of the products indicated that mMT4-MMP cleaves the peptides at SPLA ↓ QAVRSSSRK and SSMAQT ↓ LTLRS-SSK for the human and murine, respectively (the arrow indicates the peptide bond hydrolyzed). Neither of these sites are those reported for soluble TNF α , where the expected N termini are VRSS and LRSS for the human and murine forms, and are generated by TACE (22). Wang *et al.* (41) have also described the cleavage of

TABLE II

K_I^{app} (10^{-9} M) for MT4-MMP catalytic domain versus right-hand side hydroxamate peptide analogue inhibitors at and comparison with other MMPs at 25 °C and TACE at 37 °C

Inhibitor	MMP1	MMP2	MMP3	MMP9	MMP14	MMP17	TACE ^a
CT-572	150	0.02	5.92	ND ^b	0.66	0.85	ND
CT-1746	122	0.04	10.9	0.42	1.1	4.7	126
BB-94	1.5	0.26	2.0	0.47	3.1	0.3	0.54
Ro31-9790	4.2	1.8	119	6.0	3.3	2.3	21
CT-1847	2.9	1.55	90	5.2	6.0	3.1	ND

^a Values for TACE were from Amour *et al.* (22). Errors were typically less than 10% of calculated values.

^b ND, not determined.

a similar human based pro-TNF α peptide at the same site SPLA \downarrow QAVRSSSRK by the catalytic domain of hMT4-MMP. MT1-, -2-, and -3-MMP did not show any detectable ability to hydrolyze the human pro-TNF α peptide, using up 20 nM of enzyme over a period of 5 h (data not shown). The mMT4-MMP catalytic domain was also unable to hydrolyze a peptide (Mca-KLDSKFS-Nle-K(Dnp)-NH₂) corresponding to the ectodomain cleavage site of L-selectin. Overall the specificity of MT4-MMP is different from most MMPs and TACE, suggesting a more defined role.

mMT4-MMP was found to be inhibited by a range of hydroxamate peptide analogue inhibitors that bind to the primed side of the active site in MMPs (32). All of the inhibitors tested had affinities for mMT4-MMP in the nM range, and none showed any significant specificity for mMT4-MMP (Table II). TACE and MMP1 do not tolerate the chlorophenyl propyl group at the P₁' position of CT-1746 as well as the isobutyl group of BB-94, unlike MT4-MMP, indicating MT4-MMP has a deeper S₁' pocket more in common with MMP2 and MT1-MMP.

The association constants of TIMP2 and 3 with mMT4-MMP were found to be similar and of the same order of magnitude as those for MT1- and -2-MMP (Table III). Unlike MT1-MMP, TIMP1 was found to associate with mMT4-MMP although approximately 10–20-fold slower than with TIMP2 or 3, as reported for MT2-MMP (Table III). Due to the instability of mMT4-MMP over extended periods (typically a loss of 30% after 24 h was observed), apparent K_I values for the binding of TIMPs to mMT4-MMP were calculated after complex formation at high concentrations (μ M range) for 1 h followed by dilution to give a final enzyme concentration of 3 nM. Although this method does not give a truly accurate K_I^{app} , it does allow comparative estimations to be made. K_I^{app} values for TIMP2 and mMT4-MMP was found to be 400 pM (Table IV) which is close to that calculated for MT1-MMP at 200 pM using similar conditions and enzyme concentration. TIMP3 was found to be a marginally better inhibitor of mMT4-MMP with K_I^{app} values approximated to 190 pM. As k_{on} was found to be very close for TIMP2 and TIMP3, differences in K_I^{app} result from differences in k_{off} , the dissociation constant. However, although TIMP1 was found to have the slowest association rate with MT4-MMP, its K_I^{app} value was found to be the lowest at ≤ 70 pM and effectively titrated the enzyme.

Processing of Pro-MMP2 and Pro-MMP9 by mMT4-MMP Catalytic Domain—The catalytic domains of MT1-, -2-, -3-, and -5-MMP have all been shown to be able to activate pro-MMP2 but not pro-MMP9 (7, 9–11), hence the potential of mMT4-MMP to activate pro-MMP2 was also investigated. mMT4-MMP catalytic domain was incubated with pro-MMP2 at 20 °C for approximately 24 h at various molar ratios, and the activation was followed by zymography. mMT4-MMP was found to be an extremely poor activator of pro-MMP2 on direct comparison with MT1-MMP, requiring a 200 molar excess of mMT4-MMP over pro-MMP2 for any indication of processing. MT1-MMP was found to fully activate pro-MMP2 under these conditions, although it also required a molar excess of enzyme at 20 °C. As

TABLE III

Association rate constants (k_{on}) of the onset of inhibition of MT1-, -2-, and -4-, MMP and TACE catalytic domain by TIMP1, -2, and -3 ($\times 10^6$ M⁻¹ s⁻¹)

Enzyme	TIMP1	TIMP2	TIMP3
MT1-MMP (at 37 °C) ^a	0.00005	4.2	3.1
MT2-MMP (at 25 °C) ^a	0.25	1.6	2.0
MT4-MMP (at 25 °C)	0.17	4.7	4.0
TACE at (37 °C) ^b	NC ^c	NC	1.0

^a Values for MT1 and 2-MMP were taken from Butler *et al.* (10).

^b TIMP1 and -2 were found to be poor inhibitors of TACE, and k_{on} was not calculated (20).

^c NC designates values not calculated. Errors were typically 10% of calculated values.

TABLE IV

Inhibition constants (K_I^{app}) for TIMPs with MT1-MMP, MT4-MMP, and TACE catalytic domains

TIMP2 inhibition refers to an ^oIC25. NI denotes no inhibition observed with μ M concentrations of TIMP. NC designates values not calculated. Errors were typically 5–10% of calculated values.

Enzyme	TIMP1	TIMP2	TIMP3
MT1-MMP ^a	NI	2×10^{-10}	NC
MT4-MMP	0.7×10^{-10} M	3.8×10^{-10}	1.9×10^{-10} M
TACE ^b	NI	1.3×10^{-7}	1.8×10^{-10} M

^a Values for MT1-MMP were taken from Butler *et al.* (10).

^b Values for TACE were from Amour *et al.* (22).

mMT4-MMP catalytic domain was found to be able to hydrolyze a wide variety of peptide substrates including those designed for MMP3, an activator of pro-MMP9 (Table I), the ability of mMT4-MMP to activate pro-MMP9 was also investigated in a similar manner. The results obtained showed MT4-MMP is also unable to activate pro-MMP9.

Hydrolysis of Macromolecular Substrates by the Catalytic Domain of MT4-MMP—MT1-, -2-, and -3-MMP have all been shown to have the ability to degrade a large number of ECM components suggesting a direct role for MT-MMPs in tissue remodeling events that are not necessarily dependent on their role as activators of MMP2 (4). The ability of mMT4-MMP catalytic domain to hydrolyze components of the ECM was found to be much more limited than reported for MT1-MMP. Substrates tested that were not hydrolyzed included type I collagen, type IV collagen, laminin-I, and tenascin long and short forms. As triple helix activity of MMPs has been shown to be dependent on the presence of the hemopexin domain the catalytic domain of mMT4-MMP would only be expected to have the potential of telopeptidase activity against type I collagen, although this was not observed. The inability of mMT4-MMP to cleave laminin or type IV collagen would suggest it may not play a direct role in cell invasion of the basement membrane. Although mMT4-MMP did show very marginal activity against fibronectin, close examination suggested the activity of mMT4-MMP was not significant. mMT4-MMP catalytic domain did cleave α_2 -macroglobulin (data not shown),

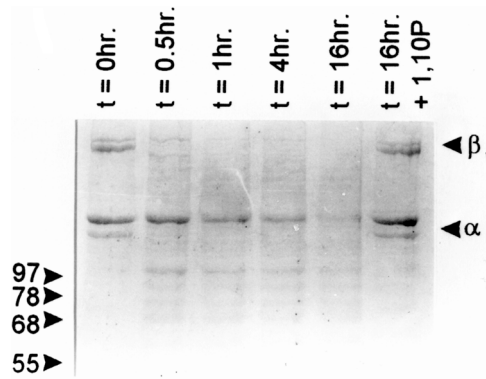


FIG. 8. Coomassie Blue-stained 7% reducing SDS-PAGE gel of the degradation of type I gelatin by MT4-MMP catalytic domain. 15 μ g of gelatin incubated with 0.6 μ g of active MT4-MMP catalytic domain per lane at 20 $^{\circ}$ C for the times indicated. The control consisted of incubation in the presence of 1,10-phenanthroline for 16 h.

type I gelatin, fibrinogen, and fibrin. mMT4-MMP activity toward type I gelatin was not particularly potent and showed activity predominantly toward the β chain (Fig. 8). mMT4-MMP showed its most potent activity toward fibrinogen and fibrin. Hydrolysis of fibrinogen led to complete degradation of the A α chain occurring within 30 min and almost complete degradation of the B β chain occurring within 24 h (Fig. 9A). MT1-MMP has also been reported to cleave the A α and B β chains (33), and direct comparison with MT4-MMP catalytic domain showed MT1-MMP catalytic domain was 2–3-fold more effective, particularly toward the B β chain (Fig. 9B). Degradation of the A α and B β chain by the MT4-MMP catalytic domain also prevented clotting by thrombin (data not shown). MT4-MMP did not show activity toward the γ chain of fibrinogen also in common with MT1-MMP (Fig. 9). Degradation of fibrin by MT4 and MT1-MMP catalytic domains showed both were capable of degrading the β chain within 30 min (Fig. 10). MT1-MMP was also found to show activity toward the α chain after 1–3 h not seen with mMT4-MMP. The catalytic domain of mMT4-MMP was also found to produce fragments of discrete size at 46 and 34 kDa within 30 min (Fig. 10A).

mMT4-MMP was found to show potent activity toward a GST-pro-TNF α fusion protein (Fig. 11). In comparison with mTACE, mMT4-MMP was the more active, reducing the majority of the fusion protein to the expected 17-kDa form of TNF α within 30 min, as compared with TACE which required 3 h under identical conditions using 0.4 μ M of each enzyme. MT1-MMP has also been reported to process the GST-pro-TNF α fusion protein but not uniquely at the site that generates mature TNF α (20). Comparison of the rate of degradation of GST-pro-TNF α by MT1-MMP and mMT4-MMP catalytic domains at 0.4 μ M showed MT1-MMP to be approximately 2-fold faster (data not shown).

To investigate whether MT4-MMP could shed native membrane-bound pro-TNF α , human pro-TNF α was co-transfected into COS-7 cells with either an empty vector or a vector containing MT4-MMP. It has been previously shown that the disappearance of the membrane-bound pro-form of TNF α can be an unreliable method of detecting shedding as internalization and subsequent degradation of TNF α also occurs in addition to ectodomain proteolysis giving misleading results (44). Hence an ELISA specific for human TNF α was used to detect the abundance of the shed soluble form. The results show that MT4-MMP co-transfected with pro-TNF α leads to an approximate 2-fold increase in shedding over background levels. Equally this could be inhibited to a significant amount, although not entirely, by the hydroxamate inhibitor CT-1746 and TIMP1 (Fig. 12). Expression of MT4-MMP was verified by

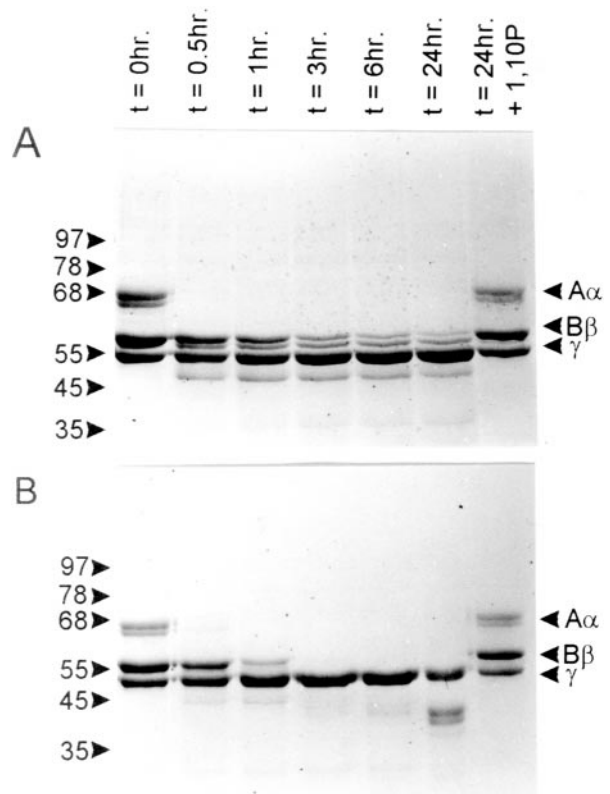


FIG. 9. Coomassie Blue-stained 7.5% reducing SDS-PAGE gel of the degradation of fibrinogen by MT4-MMP (A) or MT1-MMP (B). 20 μ g of fibrinogen were incubated with equal amounts of enzyme (0.3 μ g) as determined by active site titration, at 20 $^{\circ}$ C for the times indicated. Control consisted of incubation in the presence of 1,10-phenanthroline for 24 h.

Western blotting with the affinity purified sheep anti-human MT4-MMP polyclonal antibody (data not shown).

DISCUSSION

Here we describe the isolation of a mouse cDNA with a high degree of sequence similarity to the human MT4-MMP cDNA, which we have designated mouse MT4-MMP. The mouse homologue has proven more useful for *in vivo* and *in vitro* characterization of activity as this cDNA has a complete signal sequence and pro-domain. This has also allowed us to resolve the recent ambiguities in data recently published on the activity of the recombinant human MT4-MMP catalytic domain with respect to pro-MMP2 activation (40, 41).

MT4-MMP-HA transiently transfected into COS-7 cells shows it to be localized at or near the plasma membrane. This conforms with the recent data of Itoh *et al.* (39) showing that both human and mouse MT4-MMP are glycosylphosphatidylinositol-anchored proteins. Western blots of cell extracts from transiently transfected CHO or COS-7 cells showed the enzyme had the mass expected of the pro-form, including an HA tag, of 64 kDa. Equally the pro-form of MT4-MMP detected in monocyte/macrophage cell lines was normally the 63-kDa pro-form, with the exception of macrophages cultured for 24 h prior to stimulation with PMA where a species of MT4-MMP could be detected corresponding to the size expected of the active form. Although MT4-MMP has potential glycosylation sites, it does not appear to undergo such modification.

Expression of the pro-catalytic form of mMT4-MMP as insoluble inclusions and subsequent refolding have allowed further insights into possible roles for the enzyme. The mMT4-MMP catalytic domain not only shows the least sequence homology with MT1-, -2-, -3-, and -5-MMP (7, 14) but also very little

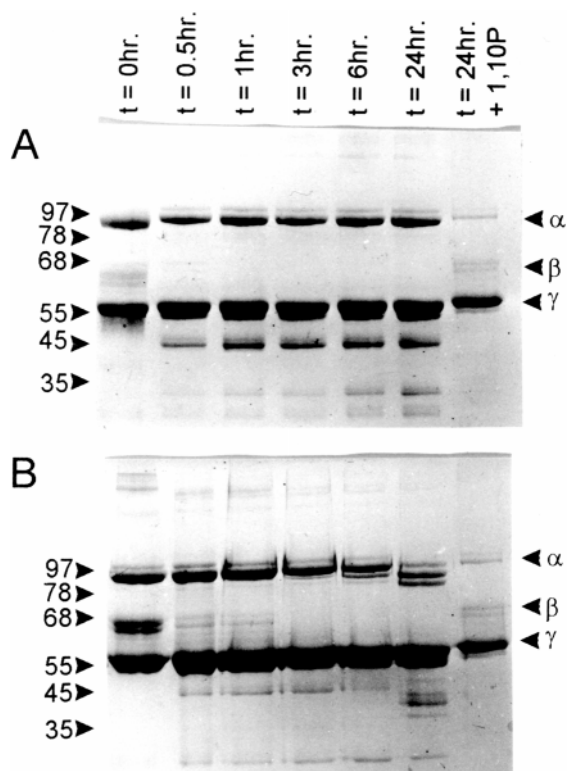


FIG. 10. Coomassie Blue-stained 7.5% reducing SDS-PAGE gel of the degradation of fibrin by MT4 and MT1-MMP. 1 mg of fibrin was incubated with 3 μ g of MT4 (A) or MT1-MMP (B) at 20 $^{\circ}$ C for the times shown. The reaction was stopped by the addition of SDS-PAGE loading buffer and immediately boiling for 5 min. A volume corresponding to approximately 25 μ g of fibrin was then loaded per lane. Control tube consisted of the 24-h reaction in the presence of 1,10-phenanthroline.

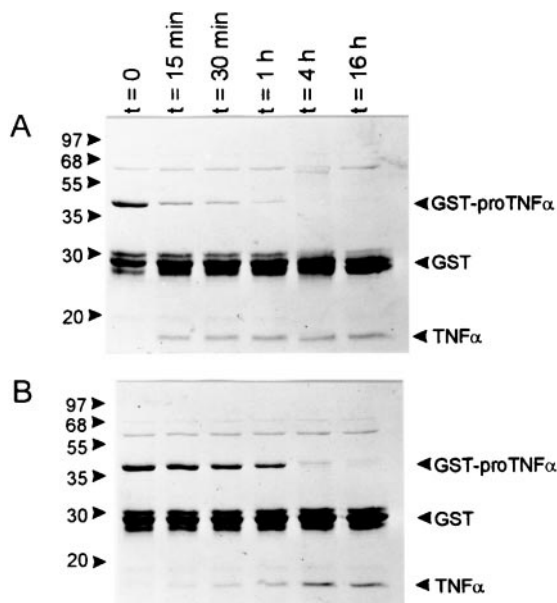


FIG. 11. Coomassie Blue-stained 10% reducing SDS-PAGE gel of the degradation of GST-pro-TNF α by MT4-MMP and TACE. 10 μ g of GST-pro-TNF α (estimate) incubated with 0.4 μ M of each enzyme as determined by active site titration at 20 $^{\circ}$ C for the times indicated. The identity of the fusion protein and soluble TNF α was confirmed by Western blotting and probing with an anti-human rabbit polyclonal antibody (data not shown).

similarity at a functional level. In the first instance we have found MT4-MMP is virtually unable to process pro-MMP2 to its active form, in contrast with MT1-, -2-, -3-, and -5-MMP, which

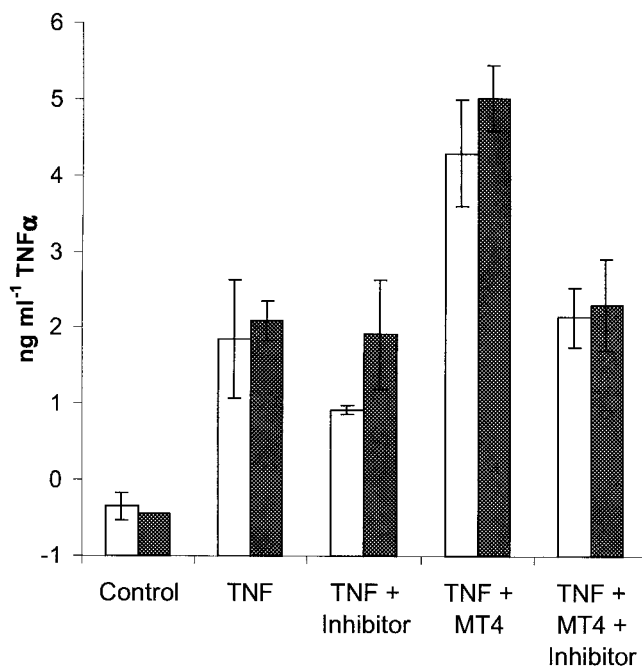


FIG. 12. ELISA of conditioned medium for hTNF α of COS-7 cells transiently co-transfected with pro-TNF α and a control vector or MT4-MMP cDNA. The conditioned medium was removed 24 h after transfection. White bars are each the result of four separate transfections, where 10 μ M CT-1746 was the inhibitor used. The dark bars represent an independent experiment, again where each bar is the result of four separate transfections, but where TIMP1 at 200 nM was used as the inhibitor. Error bars represent the standard deviation.

are all efficient activators of pro-MMP2. In this respect our data support the findings of Kolkenbrock *et al.* (40) who found no pro-MMP2 activation, rather than Wang *et al.* (41), who showed that the human MT4-MMP catalytic domain activated pro-MMP2, albeit inefficiently. One difference between the pro-MMP2 activating MT-MMPs and MT4-MMP and other MMPs is the presence of the second insertion in the catalytic domain of MT1-, -2-, -3-, and -5-MMP termed the "MT Loop" (4, 7, 14). This eight-amino acid insertion may prove to be a key structural requirement for pro-MMP2 activation. This hypothesis is supported by the recent isolation of a sixth member of the MT-MMP family that is homologous to MT4-MMP and has also been shown to be poor at activating pro-MMP2 in comparison with MT1-MMP (13). In the second instance MT4-MMP is also very poor at hydrolyzing components of the ECM in comparison with other MT-MMPs (4) and is in agreement in this respect with the lack of activity reported for the human enzyme (40, 41). A third distinction is that MT4-MMP is most potently inhibited by TIMP1 rather than TIMP2 or 3 like the pro-MMP2-activating MT-MMPs. These three key differences between MT4-MMP and the other family members are likely to preclude it from playing a major role in tissue remodeling or cell invasion and metastasis in the same manner prescribed for the other MT-MMPs, either through activating pro-MMP2 or directly as an enzyme in its own right. However, it is clearly expressed by a large number of carcinoma cell lines derived from different tissues as well as leukemic cells (18, 19).

It is known that MMPs, and most likely MMP3 and MT-MMPs, are responsible for the break down of the fibrin cap of atherosclerotic plaques and the non-serine protease-linked invasion of fibrin by endothelial cells during neovascularization (33, 34). We have found that the mMT4-MMP catalytic domain shows significant ability to degrade fibrinogen at the A α and B β but not γ chain in common with MT1-MMP. mMT4-MMP also showed as potent activity toward the fibrin β chain as

MT1-MMP. Hiraoka *et al.* (33) proposed that MT-MMPs could be important in regulating pericellular fibrinolysis, promoting cell migration. Their efficacy is dependent on their cell membrane localization and efficient furin activation mechanism. However, mMT4-MMP differs from MT1-MMP which degrades the α and β chains, leading to the breakdown of the fibrin gel (33), and instead produces fragments of a discrete size. The exact role of these fragments is not as yet known, but it is possible that they may modulate interactions with integrins such as $\alpha_{IIb}\beta_3$, $\alpha_V\beta_3$, or $\alpha_M\beta_2$ (35, 36). As we have identified MT4-MMP as being expressed at the protein level in monocytes and macrophages, it would be well placed to modulate such interactions.

Zinc-dependent membrane-bound endopeptidases have been implicated in the shedding of growth factors, cytokines, and their receptors from the cell surface, with TACE (ADAM17) being the most thoroughly characterized in this respect. TACE, in addition to being shown to be responsible for the phorbol ester-induced shedding of TNF α , has been implicated in the shedding of L-selectin, transforming growth factor- α , interleukin-6, and TNF receptor II (42). Both a peptide corresponding to the ectodomain cleavage site of pro-TNF α and a GST-pro-TNF α fusion protein were found to be very good substrates for MT4-MMP and on a comparative basis was more potent than mTACE. Both the peptide and GST fusion protein containing the cleavage site of pro-TNF α required for the generation of soluble TNF α were cleaved at a unique site, the N termini generated were not those expected of the soluble TNF α produced *in vivo* on stimulated shedding or TACE *in vitro* (22). MMP1 and MMP9 have also been reported to hydrolyze the human pro-TNF α peptide at the site cleaved by mMT4-MMP but also cleave at the same site as TACE (37). In this respect MT4-MMP also shows different substrate specificity from MMP1 and MMP9 which cleave at two sites, SPLA \downarrow QA \downarrow VRSSSRK, and is also different from ADAM9 (Meltrin- γ) which also cleaves at two sites SPLA \downarrow QAVRS \downarrow SSRK (37). Co-transfection of MT4-MMP and pro-TNF α into COS-7 cells led to a significant increase in shedding of TNF α into the conditioned medium that was inhibited by a hydroxamic acid inhibitor of MMPs and TIMP1. This result is interesting for two reasons. First, this shows that the lack of activation of pro-MMP2 or pro-MMP13 by cells transfected with MT4-MMP is not due to the absence of catalytically active MT4-MMP, lending weight to the argument that activation of pro-MMPs is not the role of MT4-MMP. Second and more importantly, it suggests that MT4-MMP may have a role in the regulation of cell surface proteins. The physiological importance of MT4-MMP as a TNF α -converting enzyme has yet to be investigated although its role as a glycosylphosphatidylinositol-anchored enzyme (38) means that it is unlikely to be involved in the rapidly induced shedding stimulated by phorbol esters associated with TACE (43). It is more probable that MT4-MMP would be involved in physiological shedding seen in the low levels of background shedding observed in unstimulated cells. The differing TIMP inhibition profiles of TACE, MT1-MMP, and MT4-MMP will also be of future use in aiding the identification of which metalloshedase is involved in ectodomain cleavage events.

In conclusion, MT4-MMP appears to be distinct in function from the other MT-MMPs described to date. Its high expression in leukocytes in combination with its potential in ectodomain shedding events and ability to degrade fibrinogen and fibrin may indicate a role in inflammatory processes, although its true role remains to be ascertained.

Acknowledgments—We thank the following for their assistance and advice: Anastasia Sobolewski for the preparation of the rat alveolar

macrophages, Rosalind Hembry for help with antiserum preparation, Mike Butler for the hMT4-MMP catalytic domain expression vector, and Mike Hutton for the TIMP preparation. We give special thanks to Graham Knight and Emily Hargreaves of the Department of Biochemistry, University of Cambridge, UK, for the fluorescent peptide substrates and electrospray mass analysis, and Bryan Smith of Celltech, Slough, UK, for the N-terminal sequence analysis.

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