Membrane type (MT) matrix metalloproteinases (MMPs) are recently recognized members of the family of Zn\(^{2+}\)- and Ca\(^{2+}\)-dependent MMPs. To investigate the proteolytic capabilities of human MT4-MMP (i.e. MMP-17), we have cloned DNA encoding its catalytic domain (CD) from a breast carcinoma cDNA library. Human membrane type 4 MMP CD (MT4-MMPCD) protein, expressed as inclusion bodies in *Escherichia coli*, was purified to homogeneity and refolded in the presence of Zn\(^{2+}\) and Ca\(^{2+}\). While MT4-MMPCD cleaved synthetic MMP substrates Ac-PLG-[2-mercapto-4-methylpentanoyl]-LG-OEt and Mca-PLGL-AR-NH\(_2\) with modest efficiency, it catalyzed with much higher efficiency the hydrolysis of a pro-tumor necrosis factor-α converting enzyme synthetic substrate, Mca-PLAQAV-Dpa-RSSSR-NH\(_2\). Catalytic efficiency with the pro-tumor necrosis factor-α converting enzyme substrate was maximal at pH 7.4 and was modulated by three ionizable enzyme groups (pK\(_{a2}\) = 6.2, pK\(_{a2}\) = 8.3, and pK\(_{a1}\) = 10.6). MT4-MMPCD cleaved gelatin but was inactive toward type I collagen, type IV collagen, fibronectin, and laminin. Like all known MT-MMPs, MT4-MMPCD was also able to activate 72-kDa progelatinase A to its 68-kDa zymogen at pH 7.4 and was modulated by three ionizable enzyme groups (pK\(_{a2}\) = 6.2, pK\(_{a2}\) = 8.3, and pK\(_{a1}\) = 10.6). MT4-MMPCD cleaved gelatin but was inactive toward type I collagen, type IV collagen, fibronectin, and laminin. Like all known MT-MMPs, MT4-MMPCD was also able to activate 72-kDa progelatinase A to its 68-kDa form. EDTA, 1,10-phenanthroline, reference hydroxamic acid MMP inhibitors, tissue inhibitor of metalloproteinases-1, and tissue inhibitor of metalloproteinases-2 all potently blocked MT4-MMPCD enzymatic activity. MT4-MMP is, therefore, a competent Zn\(^{2+}\)-dependent MMP with unique specificity among synthetic substrates and the capability to both degrade gelatin and activate progelatinase A.

Matrix metalloproteinases are members of a family of homologous Zn\(^{2+}\)-dependent endopeptidases that participate in physiological and pathological processes involving tissue remodeling or cell migration (1, 2). MMP\(^{1}\) gene expression is highly regulated in different cell types in response to various physiological stimuli (3), and dysregulated MMP activity is implicated in the development of many diseases involving matrix remodeling, including cancer (4, 5), arthritis (6), and cardiovascular disease (7). With nearly 20 MMPs identified and more likely to be discovered, delineation of the functional capabilities of these proteases becomes crucial as MMP inhibitors are developed as therapeutic agents.

Five human MT-MMPs (MT1-, MT2-, MT3-, MT4-, and MT5-MMP) have been cloned (8–12). A common functional role proposed for MT1-, MT2-, MT3-, and MT5-MMP is the proteolytic activation of the 72-kDa progelatinase A (pro-MMP-2) (8, 10, 12, 13). However, the nature of the complex formed between the MT-MMP and progelatinase A, the identity of the MT-MMP domain(s) involved (15–17), and whether a TIMP participates (17) have been debated. A MT1-MMP-TIMP-2-progelatinase A complex was reportedly required for processing to give activated gelatinase A (17). However, transmembrane domain deletion mutants of MT1-MMP can proteolytically activate progelatinase A in vitro in the absence of TIMP (15, 16). A similar function for MT4-MMP has not yet been defined.

Human MT4-MMP (i.e. MMP-17) was recently cloned from a breast carcinoma cDNA library (11) using primers that were directed to the conserved propeptide region and catalytic Zn\(^{2+}\)-binding site of MMPs (1, 2). In addition to being present in breast cancers, MT4-MMP mRNA was found highly expressed in brain, colon, ovary, and testis tissues as well as in leukocytes (11). The deduced amino acid sequence of MT4-MMP contained regions homologous to all of the domains conserved in MMPs, but two invariant aspartic acid residues that ligate structural Ca\(^{2+}\) ions in related MMPs were replaced by Tyr-137 and Asn-141 in MT4-MMP (11). Although antibodies to MT4-MMP detected a ~70-kDa protein in human brain extracts, the catalytic competence of this putative protease was not evaluated (11).

The expression and characterization of MMP catalytic domains has facilitated structure-function studies of these proteases (15, 16, 18–22). For example, three-dimensional structures of the human MMP-3CD (stromelysin-1) in complex with small molecule inhibitors and TIMP-1 have been solved by x-ray crystallography and NMR methods (23–26). Furthermore, MMP CDs designed without a propeptide domain obviate activation steps and simplify their use.

In our continuing studies of MMPs, we set out to characterize the catalytic capabilities of MT4-MMP. We cloned the MT4-MMPCD DNA from a breast carcinoma cDNA library, purified the bacterially expressed protein, and defined its catalytic competence with synthetic peptide substrates and extracellular matrix components. Consistent with the expectation that MT4-

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1 The abbreviations used are: MMP, matrix metalloproteinase; MT, membrane type; MT1-, MT2-, MT3, MT4-, and MT5-MMPCD, human membrane type 1–5 matrix metalloproteinase catalytic domain(s), respectively; TIMP, tissue inhibitor of metalloproteinases; CD, catalytic domain; TACE, pro-tumor necrosis factor-α converting enzyme; MES, 2-(N-morpholino)ethanesulfonic acid; ECM, extracellular matrix; FP1, (7-methoxyoumarin-4-y)acetyl-PLGL-[3-(2, 4-dinitrophenyl)-L-2,3-diaminopropionyl]-AR-NH₂; FP2, (7-methoxyoumarin-4-y)acetyl-

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PLAQAV-[8-(2, 4-dinitrophenyl)-L-2,3-diaminopropionyl]-RSSRR-NH₂; TPL or thiopeptolide, Ac-PLG-[2-mercaptop-4-methylpentanoyl]-LG-OEt; PCR, polymerase chain reaction; APMA, p-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; bp, base pair.
MMP is a member of the Zn$^{2+}$-dependent MMP family, we show that the MT4-MMPCD can activate progelatinase A; it can cleave synthetic substrates and the ECM component gelatin, and is inhibited by metal ion chelators, reference MMP inhibitors, and TIMPs.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primers were synthesized by Genosys and PAGE-purified. cDNA libraries were obtained from CLONTECH, while breast cancer cell lines were from the American Type Culture Collection. The TRI-REAGENT kit, leupeptin, pepstatin, 5,5'-dithiobis(2-nitrobenzoic acid), mouse basement membrane type IV collagen, bovine plasma fibrinogen, murine basement membrane laminin, and Brij 35 were obtained from Sigma. Other detergents were obtained from Calbiochem.

Rat tail type I collagen (Collaborative Biomedical Products) was obtained from Becton Dickinson. Superdex 75 resin was from Amersham Pharmacia Biotech; ZnCl$_2$ (99.999%) and CaCl$_2$ (99.999%) were from Johnson Matthey; and pGEMEX-1, Escherichia coli, BL21(DE3)/pLysS cells, and restriction enzymes were from Promega. Other enzymes, ultrapure urea, phenylmethylsulfonyl fluoride, isopropyl b-D-thiogalactopyranoside, and the SuperScript reverse transcriptase-PCR kit were from Life Technologies. Pfu DNA polymerase was a product of Stratagene, and Superbroth was from Digene. Synthetic peptide substrates (Thr-Pro-Arg-NH$_2$ (FP1) (27) and (7-methoxycoumarin-4-carboxamido)tetrapeptide RSSSR-NH$_2$ (FP2) (28, 29) as well as a thioester substrate Ac-PLG-[2,3-diaminopropionyl]-$\beta$-thiogalactopyranoside, cells were harvested by centrifugation, washed with 50 mM Tris-HCl buffer (pH 7.4), and then lysed by suspension in buffer A containing 0.1% Triton X-100, 10 mM EDTA, 0.1 mg of DNase I, 0.1 ml phenylmethylsulfonyl fluoride, and 1 $\mu$g/ml leupeptin and pepstatin. The inclusion body pellet resulting from centrifugation of this mixture (10,000 g, 20 min) was washed four times by suspension in buffer A containing 10 mM EDTA plus the protease inhibitors followed by centrifugation. After the washed inclusion body pellet was solubilized by vortexing in buffer A containing 8% urea, this mixture was centrifuged (10,000 g, 20 min), and the supernatant fluid was concentrated to ~3 ml by ultrafiltration. This soluble protein fraction was applied to a column containing Superdex 75 resin equilibrated with buffer A plus 8 M urea, this mixture was centrifuged (10,000 g, 20 min), and the supernatant fluid was concentrated to ~3 ml by ultrafiltration. This soluble protein fraction was applied to a column containing Superdex 75 resin equilibrated with buffer A plus 8 M urea, the protein content of eluted fractions was estimated using the Bradford reagent (Bio-Rad) and bovine serum albumin as the standard. The proteolytic activity was typically assessed in a fluorimetric kinetic assay (see below) by diluting an aliquot of each fraction 20-fold into buffer A containing 0.005% Brij 35, 50 mM CaCl$_2$, 1–5 $\mu$M ZnCl$_2$, and 30 $\mu$M FP2. Fractions containing the MT4-MMPCD were pooled, and the protein content of eluted fractions was estimated using the Bradford reagent (Bio-Rad). The Bradford reagent was prepared in Me$_2$SO. Assay mixtures contained buffer B plus 5 mM CaCl$_2$, 0.005% Brij 35, and 50 mM edetic acid (EDTA) plus the protease inhibitors followed by centrifugation. The pH dependence of $k_{\text{cat}}/K_{\text{m}}$ was determined from the relationship $F_1 = F_0 + F_{\text{max}}(1 - e^{-k_{\text{cat}}/K_{\text{m}}t})$ where $F_1$ is the fluorescence at time $t$, $F_0$ is the initial fluorescence, $F_{\text{max}}$ is the total change in fluorescence, and $k_{\text{cat}}/K_{\text{m}}$ is the first order rate constant for enzyme-catalyzed substrate hydrolysis. For MT4-MMPCD-catalyzed complete hydrolysis of FP2 was studied at 25 °C using a three-component, constant ionic strength buffer system (32) consisting of 100 mM MES (pH 6.2), 50 mM Tris (pH 8.1), and 50 mM ethanolamine (pH 9.6) buffers containing 0.005% Brij 35 and 10 mM CaCl$_2$. The FP2 concentration used (0.7 mM) was well below its $K_{\text{m}}$ of 20 $\mu$M.

**Inhibition of the MT4-MMPCD by Metal Ion Chelators, Reference MMP Inhibitors, and TIMPs**—Reference MMP inhibitors were synthesized in the Parke-Davis Chemistry Department. Stock solutions were prepared in Me$_2$SO. Assay mixtures contained buffer B plus 5 mM CaCl$_2$, 0.005% Brij 35, and 50 mM edetic acid (EDTA) plus the protease inhibitors followed by centrifugation. In addition to running vehicle controls, inhibitors were evaluated at various concentrations from 1 to 1000 $\mu$M (EDTA and 1,10-phenanthroline), 1–200 $\mu$M (reference MMP inhibitors), or 0.003–500 $\mu$M (TIMPs). Refolded MT4-MMPCD (2–10 mM) was used to initiate reactions, and the initial rates of product formation were assessed at 25 °C. Rates without enzyme were negligible. The percentage of control activity was calculated using Equation 3.

**Expression and Purification of the MT4-MMPCD**—The MT4-MMPCD was expressed at 37 °C in E. coli BL21(DE3)/pLysS cells transformed with pGEMEX-1/MT4-MMPCD. Four hours after induction with 1 mM isopropyl b-D-thiogalactopyranoside, cells were harvested by centrifugation, washed with 50 mM Tris- HCl buffer (pH 7.4) (buffer A), and then lysed by suspension in buffer A containing 0.1% Triton X-100, 10 mM EDTA, 0.1 mg of DNase I, 0.1 ml phenylmethylsulfonyl fluoride, and 1 $\mu$g/ml leupeptin and pepstatin. The inclusion body pellet resulting from centrifugation of this mixture (10,000 g, 20 min) was washed four times by suspension in buffer A containing 10 mM EDTA plus the protease inhibitors followed by centrifugation. After the
% control activity = (inhibited rate/control rate) × 100  

(eq. 3)

IC_{50} values were calculated using a least-squares algorithm by fitting the percent of control activity versus inhibitor concentration data to Equation 4 for a sigmoid inhibition pattern as follows,

% control activity = 100/[1 + (I/IC_{50})^{nH}]

(eq. 4)

where [I] is the inhibitor concentration, IC_{50} is the concentration of inhibitor where the reaction rate is 50% inhibited relative to the control, and nH is the slope of the curve at its inflection point.

Cleavage of ECM Proteins by the MT4-MMPCD—Incubations (30 μl) were carried out at 25 °C for 16 h in 100 mM Tris-HCl buffer (pH 7.4) containing 0.005% Brij 35 and 0.5 mM CaCl₂. Gelatin was prepared by denaturing a 1 mg/ml solution of type I collagen for 20 min at 95 °C. Each reaction contained 5 μg of ECM protein test substrate (type I collagen, type IV collagen, gelatin, fibronectin, or laminin) and either 0 or 50 nM MT4-MMPCD. The reaction mixtures were subjected to SDS-PAGE analysis carried out under nonreducing conditions using Tris/glycine 8−16% polyacrylamide gradient gels (Novex).

Activation of Human Progelatinase A by Purified MT4-MMPCD and MT1-MMPCD—Conditioned cell culture medium from genetically modified human HT-1080 cells was used as described previously (17) (a source of 72 kDa progelatinase A. Incubations were carried out for 2 h at 37 °C in mixtures that contained 50 mM Tris-HCl buffer (pH 7.4), 5 mM CaCl₂, 0.01% Brij 35, 150 mM NaCl, a 5-fold dilution of progelatinase A-containing conditioned culture medium, and 0 or 100 nM MT4-MMPCD or MT1-MMPCD. As a positive control, progelatinase A was activated by incubation with 1 mM APMA for 30 min at 37 °C. The activation of progelatinase A in the samples was assessed by zymography carried out according to the manufacturer’s protocols using a 10% SDS-polyacrylamide gel containing 0.1% gelatin (Novex).

RESULTS

PCR Amplification of the MT4-MMPCD from a Breast Carcinoma cDNA Library—Using primers 1 and 2, we amplified a 540-bp MT4-MMPCD DNA fragment from a human breast carcinoma cDNA library (data not shown). The sequence of the MT4-MMPCD coding region was determined and found to be in disagreement with the published sequence (11) at five positions (Fig. 1), including three deletions in a G/C-rich region and two single nucleotide conflicts. As highlighted in Fig. 1b, these differences would cause several changes in the amino acid sequence of the encoded protein. In particular, two invariant aspartic acid residues, Asp-137 and Asp-141, which ligate structural Ca²⁺ ions in related MMPs, Gly-118, was Ala-118 in the previously reported sequence of the MT4-MMPCD (top line) with the corresponding region of MT4-MMP as published (bottom line) (11). Asp-137 and Asp-141 (numbering based on the full-length enzyme), two putative Ca²⁺ ligands in the MT4-MMPCD, are underlined. The sequence of the MT4-MMPCD agrees completely with the recently revised sequence in GenBank™ for MT4-MMP (g364294).

Expression and Purification of the MT4-MMPCD—The MT4-MMPCD protein was expressed in E. coli and purified from the inclusion body pellet was solubilized with 8 M urea; and the soluble protein was purified in one size exclusion chromatography run. SDS-PAGE analysis showed that the pure MT4-MMPCD migrated as a single protein band (Fig. 2). About 70 mg of purified MT4-MMPCD protein was obtained from a 1-liter fermentation (30 g of cell paste).

MT4-MMPCD Refolding—When MT4-MMPCD was rapidly refolded by dilution into assay mixtures containing Ca²⁺, Zn²⁺, and substrate, maximal enzymatic activity was observed at a ratio of approximately 1 Zn²⁺ ion added per enzyme. Higher levels of Zn²⁺ reduced the catalytic activity obtained. With a 4-fold molar excess of added Zn²⁺, the catalytic activity was only 60% of that obtained with 1 mol eq of added Zn²⁺, while at a 20-fold molar excess of Zn²⁺ only 10% of the maximal activity remained (data not shown). On the other hand, when the MT4-
MMPCD was first refolded with optimal levels of Ca\(^{2+}\) (50 mM) and Zn\(^{2+}\) (1:1), the enzymatic activity of the soluble enzyme was not inhibited by up to a 450-fold molar excess of Zn\(^{2+}\) (data not shown). With regard to the effect of Ca\(^{2+}\) under rapid refolding conditions, MT4-MMPCD activity increased hyperbolically with increasing Ca\(^{2+}\) concentrations. At the optimal 1:1 ratio of Zn\(^{2+}\) added/enzyme, the activation \(K_a\) for Ca\(^{2+}\) was 10 ± 1 mM (data not shown).

We also assessed the ability of various detergents to assist in the refolding of the MT4-MMPCD under optimal Zn\(^{2+}\) and Ca\(^{2+}\) conditions. When no detergent was used, no enzymatic activity was recovered. Low levels of Brij 35 and Tween 20 gave high activity, whereas \(\beta\)-octyl glucoside was less effective. The highest enzymatic activity was obtained with 0.005% Brij 35.

Because the purified MT4-MMPCD is stored in the presence of 8 mM urea, we probed the effect of urea on the enzymatic activity of the refolded enzyme. Urea lowered the activity of the MT4-MMPCD in a linear manner such that in 1.64 mM urea only 43% of the activity detected in 0.04 mM urea remains (data not shown). Nevertheless, extrapolation of the data indicated that the catalytic activity of MT4-MMPCD in 0.04 mM urea (i.e. the residual urea concentration in a typical activity assay) was 99% of that expected with no urea.

Finally, in preparation for screening of the MT4-MMPCD with inhibitor test compounds, we evaluated the effect of the inhibitor vehicle, Me\(_2\)SO, on enzyme activity. At up to 2% Me\(_2\)SO, the activity was >90% of the control level, but it decreased linearly with increasing Me\(_2\)SO such that in 5% Me\(_2\)SO only 65% of the activity without Me\(_2\)SO remained (data not shown).

**Determination of Steady State Kinetic Constants of the MT4-MMPCD**—The cleavage of a number of synthetic substrates as catalyzed by the MT4-MMPCD was quantified at pH 7.4 and 25 °C. When hydrolysis of FP1 or FP2 is monitored, there is strong quenching of the product’s fluorescent signal by substrate. The amount of quenching is proportional to the substrate concentration up to about 40 \(\mu\)M substrate (Ref. 31 and data not shown). Our estimates of \(K_m\) are derived from hyperbolic fits of quench-corrected initial velocities versus substrate concentration for rates measured at substrate concentrations up to 40 \(\mu\)M. Under our typical assay conditions, \(K_m = 8 \pm 1 \mu\)M for FP1 (data not shown), whereas \(K_m = 26 \pm 2 \mu\)M for FP2 (Fig. 3). The thioester substrate, TPL, had a \(K_m = 650 \mu\)M (data not shown). The MT4-MMPCD did not catalyze the cleavage of any of the following peptides (tested at 5, 20, and 50 \(\mu\)M): acetyl-LAQAVSK-dimethoxycoumarin-CONH\(_2\); 7-methoxycoumarin-4-yl)acetyl-LAQA-4-yl]-LAQAVRSK-dimethoxycoumarin-CONH\(_2\); 7-methoxycoumarin-4-yl)acetyl-LAQAVSRK-(2, 4-dinitrophenyl)-NH\(_2\); and 4-(4-dimethylaminophenylazo)benzoyl-LAQAVRSSKR-5-[2-aminoethyl]-amino]-naphthalene-1-sulfonic acid.

Steady state kinetic constants for cleavage of TPL, FP1, and FP2 by the MT4-MMPCD are summarized in Table I. The catalytic efficiency (\(k_{cat}/K_m\)) values were determined in the complete substrate hydrolysis experiments under conditions of \([S] \ll K_m\). The total extent of cleavage of these substrates was >98% as determined by liquid chromatography/electrospray ionization-mass spectrometric analyses of the reaction mixtures at completion (data not shown). As can be seen in Table I, MT4-MMPCD cleaves with highest catalytic efficiency FP2 (\(k_{cat}/K_m = 16,000 \text{ } \text{m}^{-1} \text{ } \text{s}^{-1}\)) at 25 °C. Reaction of the MT4-MMPCD with the commonly used MMP substrates FP1 (\(k_{cat}/K_m = 5,800 \text{ } \text{m}^{-1} \text{ } \text{s}^{-1}\)) or TPL (\(k_{cat}/K_m = 4,600 \text{ } \text{m}^{-1} \text{ } \text{s}^{-1}\)) was much less catalytically efficient than cleavage of the TACE substrate.

When the pH dependence of \(k_{cat}/K_m\) for cleavage of FP2 by the MT4-MMPCD was studied, the catalytic efficiency was found to be maximal at pH 7.4. The pH-activity profile (Fig. 4) is fairly complex with a shoulder of low activity at high pH. The data did not fit an equation describing a bell-shaped (i.e. two-proton ionization) model, and we were, therefore, fit to Equation 2, which describes a three-proton ionization model (see Scheme 1). The derived ionizations were \(pK_{a1} = 6.2 \pm 0.1\), \(pK_{a2} = 8.3 \pm 0.1\), and \(pK_{a3} = 10.6 \pm 0.3\). Also derived from the fit are catalytic efficiency values for the two routes to product of \((k_{cat}/K_m)_{1} = 5,400 \pm 800 \text{ } \text{m}^{-1} \text{ } \text{s}^{-1}\) and \((k_{cat}/K_m)_{2} = 17,900 \pm 800 \text{ } \text{m}^{-1} \text{ } \text{s}^{-1}\).

**Cleavage of ECM Protein Components by the MT4-MMPCD**—As shown in Fig. 5, when incubated with extracellular matrix proteins, the purified MT4-MMPCD cleaved gelatin to form smaller protein fragments but was completely inactive toward type I collagen, type IV collagen, fibronectin, and laminin.

**Activation of Progelatinase A by the MT4-MMPCD and MT1-MMPCD**—As assessed by gelatin zymography (Fig. 6), the mercurial compound, APMA, fully activated progelatinase A from the 72-kDa pro form (lane 1) to the mature 66-kDa form (lane 2). Consistent with previous findings with transmembrane deletion mutants of MT1-MMP (15), the MT1-MMPCD was also able to partially proteolytically activate progelatinase A to the 68-kDa intermediate form but not to the mature 66-kDa form (Fig. 6, lane 4). Like MT1-MMPCD, the MT4-MMPCD was able to partially proteolytically activate progelatinase A to the 68-kDa form (Fig. 6, lane 3). A very small amount of 66-kDa gelatinase A was present in both the MT1- and MT4-MMPCD-treated progelatinase A samples.

**Inhibition of the MT4-MMPCD**—Table II lists IC\(_{50}\) values
determined for inhibition of the MT4-MMPCD by small molecule inhibitors. The metal ion chelators EDTA and 1,10-phenanthroline inhibited the MT4-MMPCD with IC50 values of 5.6 ± 0.1 μM and 6.7 ± 0.1 μM, respectively (Fig. 7). All of the reference MMP inhibitors tested were potent, nanomolar inhibitors of the MT4-MMPCD (Fig. 8).

Table II gives IC50 values determined for U24522, CGS 27023A, marimastat, batimastat, and galardin.

As indicated in Table III, both TIMP-1 and TIMP-2 were potent inhibitors of the MT4-MMPCD. In parallel determinations, the catalytic domain of stromelysin-1 was inhibited by TIMP-1 with a similar potency. TIMP-1 was a very weak inhibitor of the MT1-MMPCD. TIMP-1 was a very weak inhibitor of the MT1-MMPCD. TIMP-2 was also a potent inhibitor of both the stromelysin-1 CD and the MT1-MMPCD.

DISCUSSION

MMPs are being discovered at an ever-increasing rate. When the cloning and sequencing of the human MT4-MMP gene was reported, the catalytic activity of the putative protease was not assessed (11). The amino acid sequence deduced at that time for MT4-MMP unexpectedly lacked two invariant aspartic acid residues that ligate structural Ca2+ ions in related MMPs (2, 25). To clarify the sequence of MT4-MMP and investigate its enzymic activity, we have cloned, expressed, and purified the

MT4-MMPCD.

MT4-MMP was first cloned from a breast carcinoma cDNA library (11) using degenerate oligonucleotide primers directed to the consensus propeptide and catalytic Zn2+-binding regions of MMPs (2). In our work, specific primers based on the published sequence of MT4-MMP (11) were used to clone the catalytic domain that spans residues Gln-42 to Val-216 but contains

a Y. Wang, unpublished result.
Proteolytic Activities of the MT4-MMPCD

The catalytic activity of the refolded MT4-MMPCD (20 nM) with 30 μM FP2 was measured in the presence of various levels of EDTA (○) and 1,10-phenanthroline (▲). Data shown are the average from two separate titrations with each chelator. For inhibition of the MT4-MMPCD by EDTA, the IC$_{50}$ = 5.6 ± 0.1 μM, while 1,10-phenanthroline inhibited this catalytic domain with IC$_{50}$ = 6.7 ± 0.1 μM.

**Fig. 7. Inhibition of the MT4-MMPCD by metal ion chelators.** The catalytic activity of the refolded MT4-MMPCD (20 nM) with 30 μM FP2 was measured in the presence of various levels of EDTA (○) and 1,10-phenanthroline (▲). Data shown are the average from two separate titrations with each chelator. For inhibition of the MT4-MMPCD by EDTA, the IC$_{50}$ = 5.6 ± 0.1 μM, while 1,10-phenanthroline inhibited this catalytic domain with IC$_{50}$ = 6.7 ± 0.1 μM.

**Fig. 8. Inhibition of the MT4-MMPCD by reference MMP inhibitors.** Assay conditions were as described for the data in Fig. 7, and experiments were carried out twice for each inhibitor with each substrate. The data shown are from one experiment using FP2. Average IC$_{50}$ values determined with FP2 and TPL as substrate are listed in Table II. The refolded MT4-MMPCD displayed amidolytic and thioesterolytic activity with several synthetic substrates. The commonly used MMP substrates TPL (a thioester) and FP1 (a quenched fluorogenic peptide) were cleaved with rather low catalytic efficiency, which hindered initial kinetic characterization of the MT4-MMPCD. In a search for alternative substrates, we found that the MT4-MMPCD cleaved with much higher catalytic efficiency FP2, a substrate that was designed for use with TACE (28, 29). Whereas TACE cleaves FP2 between Ala-5 and Val-6, liquid chromatography/electrospray ionization-mass spectrometric analyses indicated that the MT4-MMPCD cleaves exclusively between Ala-3 and Gln-4 (data not shown). Whether MT4-MMP might be able to cleave pro-TNF-α to liberate mature TNF-α in vivo or in vitro remains to be shown, but localization of this MT-MMP on the cell surface where it can potentially interact with membrane-bound substrates such as pro-TNF-α makes this a definite possibility. Work is under way to isolate a clone that will express full-length, membrane-bound MT4-MMP so that this question can be answered definitively in a cell-based assay system. Interestingly, MT4-MMP is the only MT-MMP described so far that is expressed in leukocytes (11). Such cell-specific expression may influence the role of MT4-MMP and its interactions with substrates in vivo.

When the MT4-MMPCD was tested for its proteolytic activity toward extracellular matrix components, it was found to cleave gelatin (i.e. denatured type I collagen) but was inactive toward cleavage of the ECM proteins type I collagen, type IV collagen, fibronectin, and laminin. Although the minimal MT4-MMPCD used in these studies exhibited proteolytic activity with unique selectivity toward certain ECM components, an assessment of the substrate specificity of a full-length MT4-MMP protein that includes its natural C-terminal domains is warranted.

**Table III**

Inhibition of purified catalytic domains of human MT4-MMP, MT1-MMP, and stromelysin-1 by purified recombinant human TIMP-1 and TIMP-2

| MMP                  | TIMP-1 | TIMP-2 |
|----------------------|--------|--------|
| MT4-MMPCD            | 28     | 9.7    |
| MT1-MMPCD            | 340    | 6.9    |
| Stromelysin-1 CD     | 14     | 19     |

The catalytic activity of the refolded MT4-MMPCD clone was amplified at several positions with the published gene sequence. Two putative Ca$^{2+}$ ligands, Asp-137 and Asp-141, absent from the published sequence, were restored in our clone, and several other amino acid residues were changed. When we amplified and sequenced an internal fragment of MT4-MMP from various other human tissue cDNA sources, each sequence we determined agreed with that of our MT4-MMPCD clone. Indeed, while our work was in progress the DNA sequence archived in GenBank™ for MT4-MMP was revised to a sequence that agrees with all of our determined DNA sequences.

MMP CDs have been routinely solubilized from inclusion bodies using denaturants and then refolded in the presence of Ca$^{2+}$ and Zn$^{2+}$ by dilution of the denaturant. The active protease is then purified by column chromatography (18, 19, 21, 22). In contrast, the MT4-MMPCD was solubilized, purified, and stored in buffers all containing 8 M urea. The MT4-MMPCD protein was expressed as inclusion bodies in E. coli, but was denatured using 8 M urea, and was purified to homogeneity in a single size exclusion chromatography run.

MMP CDs typically bind both a structural and a catalytic Zn$^{2+}$ ion (24). However, in our initial studies when the MT4-MMPCD was rapidly refolded by dilution into an assay mixture containing substrate, Zn$^{2+}$, and Ca$^{2+}$, Zn$^{2+}$ levels in excess of the enzyme concentration greatly reduced the activity. On the other hand, exposure of the refolded MT4-MMPCD to excess Zn$^{2+}$ did not lower activity. Since other MMP CDs are generally unaffected by slight excesses of Zn$^{2+}$, this observation was critical for us to enhance recovery of MT4-MMPCD activity. Rapidly refolding the protein by dilution into buffers containing excess Zn$^{2+}$ may lead to formation of some improperly folded protein that is either inactive or potently inhibited by Zn$^{2+}$. A similar inhibitory effect of Zn$^{2+}$, but at much higher molar excesses, was previously observed with the gelatinase A catalytic domain (18). The molecular basis for the excess Zn$^{2+}$-mediated loss of catalytic activity in these MMP CDs is unknown.

The refolded MT4-MMPCD displayed amidolytic and thioesterolytic activity with several synthetic substrates. The commonly used MMP substrates TPL (a thioester) and FP1 (a quenched fluorogenic peptide) were cleaved with rather low catalytic efficiency, which hindered initial kinetic characterization of the MT4-MMPCD. In a search for alternative substrates, we found that the MT4-MMPCD cleaved with much higher catalytic efficiency FP2, a substrate that was designed for use with TACE (28, 29). Whereas TACE cleaves FP2 between Ala-5 and Val-6, liquid chromatography/electrospray ionization-mass spectrometric analyses indicated that the MT4-MMPCD cleaves exclusively between Ala-3 and Gln-4 (data not shown). Whether MT4-MMP might be able to cleave pro-TNF-α to liberate mature TNF-α in vivo or in vitro remains to be shown, but localization of this MT-MMP on the cell surface where it can potentially interact with membrane-bound substrates such as pro-TNF-α makes this a definite possibility. Work is under way to isolate a clone that will express full-length, membrane-bound MT4-MMP so that this question can be answered definitively in a cell-based assay system. Interestingly, MT4-MMP is the only MT-MMP described so far that is expressed in leukocytes (11). Such cell-specific expression may influence the role of MT4-MMP and its interactions with substrates in vivo.

When the MT4-MMPCD was tested for its proteolytic activity toward extracellular matrix components, it was found to cleave gelatin (i.e. denatured type I collagen) but was inactive toward cleavage of the ECM proteins type I collagen, type IV collagen, fibronectin, and laminin. Although the minimal MT4-MMPCD used in these studies exhibited proteolytic activity with unique selectivity toward certain ECM components, an assessment of the substrate specificity of a full-length MT4-MMP protein that includes its natural C-terminal domains is warranted.
Like the other four known MT-MMPs that share the ability to proteolytically cleave and thereby activate gelatinase A, the MT4-MMPCD was also able to activate gelatinase A to its intermediate 68-kDa form. However, in our experiments the total extent of activation to the 68-kDa and conversion to the 66-kDa forms of gelatinase A was low. This outcome may reflect the fact that the MT1- and MT4-MMPCDs used in our studies contain only a minimal catalytic domain with no hemopexin-like or transmembrane domains that could be important for highly efficient, complete activation. Nevertheless, it is clear that the MT4-MMPCD has the ability to proteolytically activate gelatinase A.

The pH-activity profile for the MT4-MMPCD catalytic efficiency with FP2 resembles that reported for human stromelysin-1 (33, 34) in that it is best fit to a model with three proton ionizations and two active enzyme forms that can lead to product. Since FP2 is not expected to ionize in the pH range studied, we conclude that these ionizations arise from groups on the free enzyme. The $k_{cat}/K_m$ value was maximal at the physiological pH of 7.4. Although the functional groups in stromelysin-1 that ionize at two pH extremes are not well defined, the third (middle) $pK_a$ of 6.2 for that enzyme has been assigned by site-directed mutagenesis as due to deprotonation of His-224 (34); the analogous residue in MT4-MMP is Gln-187 (see Fig. 1). While it is unlikely that Gln-187 is the group in the MT4-MMPCD that ionizes with the middle $pK_a$ = 8.3, the identities of the functional groups involved in the different ionizations in MT4-MMPCD are under investigation.

Metal ion chelators and reference MMP inhibitors are sensitive probes of MMP structure and function. That the MT4-MMPCD catalytic activity was strongly inhibited by EDTA and $K_m$-dependent MMPs. No selectivity of the inhibition of the MT4-MMPCD with low nano-molar IC$_{50}$ values. Nonetheless, the substrate specificity results presented here should facilitate characterization of the enzymatic activity of the full-length MT4-MMP protein. In the meantime, the MT4-MMPCD will be useful in functional studies of this enzyme. Determination of the three-dimensional structure of this enzyme by NMR or x-ray crystallography is also now feasible.

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