Biochemical Characterization of the Catalytic Domain of Human Matrix Metalloproteinase 19

EVIDENCE FOR A ROLE AS A POTENT BASEMENT MEMBRANE DEGRADING ENZYME*

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From the ‡School of Biological Sciences, University of East Anglia, Norwich, NR4 7TJ, United Kingdom, the ¶Departamento de Bioquímica y Biología Molecular, Universidad de Oviedo, 33006 Oviedo, Spain, and the †Celltech Chirotechnology, 216 Bath Road, Slough SL1 4EN, United Kingdom

We have recently cloned MMP-19, a novel matrix metalloproteinase, which, due to unique structural features, was proposed to represent the first member of a new MMP subfamily (Pendás, A. M., Knäuper, V., Puente, X. S., Llano, E., Mattei, M. G., Apte, S., Murphy, G., and López-Otin, C. (1997) J. Biol. Chem. 272, 4281–4286). A recombinant COOH-terminal deletion mutant of MMP-19 (proΔ260–508MMP-19), comprising the propeptide and the catalytic domain, was expressed in Escherichia coli, refolded, and purified. Interestingly, we found that proΔ260–508MMP-19 has the tendency to autoactivate, whereby the Lys97-Tyr98 peptide bond is hydrolyzed, resulting in free catalytic domain. Mutation of two residues (Glu88 → Pro and Pro90 → Val) within the propeptide latency motif did not prevent autoactivation but the autoylation rate was somewhat reduced. Analysis of the substrate specificity revealed that the catalytic domain of MMP-19 was able to hydrolyze the general MMP substrate Mcapro-Leu-Gly-Dpa-Ala-Arg-NH2 and, with higher efficiency, the stromelysin substrate Mcapro-Leu-Ala-Nva-Dpa-Ala-Arg-NH2. Kinetic analysis of the interactions of the catalytic domain of MMP-19 with the natural MMP inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), showed strong inhibition using TIMP-2, TIMP-3, and TIMP-4, while TIMP-1 was less efficient. We also demonstrated that synthetic hydroxamic acid-based compounds efficiently inhibited the enzyme. The catalytic domain of MMP-19 was able to hydrolyze the basement membrane components type IV collagen, laminin, and nidogen, as well as the large te nascin-C isoform, fibronectin, and type I gelatin, suggesting that MMP-19 is a potent proteinase capable of hydrolyzing a broad range of extracellular matrix components. Neither the catalytic domain nor the full-length MMP-19 was able to degrade triple-helical collagen. Finally, and in contrast to studies with other MMPs, MMP-19 catalytic domain was not able to activate any of the latent MMPs tested in vitro.

The human matrix metalloproteinases (MMPs)† are a group of homologous zinc-dependent endopeptidases that degrade the different macromolecular components of the extracellular matrix. They have been implicated in the remodeling of connective tissues during such diverse processes as normal mammalian development and growth, wound healing, cartilage degradation during arthritis, and cancer metastasis (1–3). At present 18 members of the human MMP family have been cloned, and they have been classified into different subfamilies according to their substrate specificity and cellular location. This classification comprises the collag enases, gelatinases, stromelysins, and membrane-type MMPs (MT-MMPs). We have recently cloned a new member of the matrix metalloproteinase family, MMP-19, which showed the typical domain organization of soluble members of the MMP family, namely a signal sequence, a propeptide domain with the cysteine residue essential for maintaining latency, a catalytic domain with the typical zinc binding motif, a linker region, and a COOH-terminal fragment with sequence similarity to hemopexin (4). However, the enzyme lacks various structural features distinctive of the diverse MMP subfamilies, e.g., the fibronectin-like repeats of gelatinases or the Asp, Tyr, and Gly residues near the active site of collagenases, but possesses a unique insertion of five Glu residues within the linker region, an unusual latency motif in the propeptide domain ( . . . PROCGLED . . . ) and an additional Cys residue in the catalytic domain, when compared with other MMPs. In addition, the MMP-19 gene is the first MMP gene found to be located on chromosome locus 12q14 and initial data on its genomic organization has revealed a unique intron/exon distribution. MMP-19 may therefore represent the first member of a new subfamily of MMPs (4), whose role in vivo remains to be investigated. However, recently, MMP-19 mRNA was found to be constitutively expressed in arthritic (RA) and traumatic synovial membranes, which may imply the involvement of MMP-19 in this tissue during normal ECM remodeling processes (5).

Northern blot analysis of polyadenylated RNA from various normal human tissues revealed strong expression of MMP-19 in placenta, ovary, lung, pancreas, spleen, and intestine, whereas expression in brain and leukocytes was undetectable (4). Since adult cells under non-pathological conditions do not frequently produce MMPs, it is possible that MMP-19 participates in normal ECM turnover or in activation of secreted and membrane-bound proteins such as growth factors and protein-tissue inhibitor of metalloproteinase; ECM, extracellular matrix; RA, rheumatoid arthritis; Mca, (7-methoxycoumarin-4-yl)-acetyl; Cha, 3-cy clohexylalanyl; Nva, norvalyl; Dpa, N-3-(2,4-dinitrophenyl)-L-2,3-dia minoprooprol; Dnp, 2,4-dinitrophenyl; APMA, p-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MT, membrane-type; MES, 4-morpholinooethanesulfonic acid; CAPS, 3-cyclohexylamino propanesulfonic acid.

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§ The abbreviations used are: MMP, matrix metalloproteinase; TIMP, 3-(2,4-dinitrophenyl)-L-2,3-diaminoprooprol; Dpa, N-3-(2,4-dinitrophenyl)-L-2,3-diaminoprooprol; Dnp, 2,4-dinitrophenyl; APMA, p-aminophenylmercuric acetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; MT, membrane-type; MES, 4-morpholinooethanesulfonic acid; CAPS, 3-cyclohexylamino propanesulfonic acid.
ases (4). More recently, Sedlacke and co-workers (6) reported that enhanced anti-MMP-19 autoantibody titers appear to be frequent among patients suffering from RA, and that MMP-19 was detected using an anti-peptide antibody on the surface of lymphatic cells such as activated peripheral blood mononuclear cells, T<sub>g</sub>1 lymphocytes, and Jurkat T lymphoma cells. Furthermore, a distinct expression of MMP-19 was observed, associated with the smooth muscle cells in the tunica media of synovial blood vessels of an RA patient, as well as in normal skin and uterine ligaments (7). In contrast, in capillaries of acutely inflamed RA synovium strong MMP-19 expression was also detected in the endothelial layer (8). The same authors report an elevated MMP-19 mRNA expression of smooth muscle cells in vitro after stimulation with 12-O-tetradecanoylphorbol-13-acetate, epidermal growth factor, and basic fibroblast growth factor, whereby proliferating smooth muscle cells exhibited higher levels of MMP-19 mRNA than resting cells. MMP-19 protein and mRNA was detected in vitro in endothelial cells from various tissues, e.g. umbilical artery, skin, and fat tissue. These data support the hypothesis that MMP-19 participates in angiogenic processes and lymphocyte extravasation during arthritic diseases and therefore may be involved in the invasion of the inflamed synovial pannus into the joint space and thus in the destruction of joint tissues. Here we describe the expression, refolding, and enzymatic characterization of the catalytic domain of MMP-19.

**EXPERIMENTAL PROCEDURES**

*Expression, Refolding, and Purification of Pro<sup>260–508</sup>MMP-19—* An expression vector for pro<sup>260–508</sup>MMP-19 was generated by PCR using the following primers: 5′-GGCCGCTGACAATCTGACCTGACGAGCAGGCTGACTGCAAGCT-3′ (A) and 5′-AACGGATCTCTACACCTGCATGTTCCAGTTCC-3′ (B) using the full-length MMP-19 CDNA in pSP64 as a template, thereby introducing a PetI site at the 5′ end and a stop codon following Ser<sub>259</sub> flanked by a unique A and 5′-GGCGCCTGCAGACTACCTGTCACAATAT-3′ recognition sequence for the pRSET B expression vector (Invitrogen), respectively, in combination with primers A and B from above. Subcloning, expression, refolding, and purification were performed as described for the wild type pro<sup>260–508</sup>MMP-19. A protein containing alterations in both, propeptide and catalytic domain, was produced, thereby altering E88FP/P90V and C168S by ligation of the PetI and NcoI fragment from pro<sup>260–508</sup>MMP-19/E88F/P90V into the pRSET B vector containing the mutation for C168S in the catalytic domain, previously cleaved with the same restriction enzymes. Expression and refolding were performed as described above. All expression vectors were sequenced using the dye-terminator cycle-sequencing method and confirmed the correct sequence for each construct.

*Expression and Purification of Full-length MMP-19—* Human MMP-19 was purified from culture medium conditioned by N90 mouse myeloma cells that had been transfected with MMP-19 cDNA, essentially as described previously (10). The expression vector, transfection method, and culture conditions were as described previously (11, 12).

*Expression and Purification of Recombinant TIMPs—* Recombinant forms of human TIMP-1, -2, and -3 were generated as described previously (10). The expression vector, transfection method, and culture conditions were as described previously (11, 12).

**Activity Assays, Active Site Titration, and Kinetic Analysis of Inhibitor Binding—** Enzymatic activity was determined after activation of wild type or mutated pro<sup>260–508</sup>MMP-19 with 1 mM APMA for 30 min at 37 °C (16). Routine assays were performed at 37 °C using 1 mM enzyme and the synthetic quenched fluorescent peptide (7-methoxycoumarin-4-y-lacetol-Pro-Leu-Ala-Nva-[3-2, 4-dinitrophenyl]-t-r, 2,3-di-aminomproinophenyl]-Ala-Arg-NHz<sub>2</sub> 328 μM, 394 nM) at different concentration of 1 μM in a bioreactor (20, 21) at pH 7.6, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 0.05% (v/v) Brj 35, 0.01% NaN<sub>3</sub>. The concentration of active enzyme was evaluated using the fluorescent assay by titration against a standard TIMP-2 solution of known concentration (12). Determination of the substrate specificity of the catalytic domain of MMP-19 was carried out using the same assay with different quenched fluorescent synthetic peptide substrates at concentrations of 1 μM, which fulfilled the requirements of [S] ≪ K<sub>m</sub>. All peptide substrates were kindly provided by Dr. Graham Knight (University of Cambridge, Cambridge, United Kingdom). The pseudo-first-order rate constants (k<sup>i</sup>) for the formation of the EI complex of 1 nM active wild type pro<sup>260–508</sup>MMP-19 with TIMP-1, -2, -3, and -4, and the Δ<sub>128–194</sub> TIMP-2 mutants S2E, Y36G, and A70K were determined by analysis of the progress curves of McaPLA<sub>Na</sub>PPaARNH<sub>2</sub>, hydrolysis (12). The dependence of k<sup>i</sup> on TIMP concentration was evaluated using various amounts of TIMP (5–20 nM). In addition, the apparent K<sup>i</sup> values for TIMP inhibition were determined using 1 nM enzyme incubated with a range of inhibitor concentrations for 24 h at 37 °C to reach equilibrium before assayed as described above.

*Human Matrix K<sup>+</sup> Values for the inhibition of active wild type pro<sup>260–508</sup>MMP-19 with the synthetic hydroxamic acid-based inhibitors CT-1746, Ro31-9790, and BB-94 were determined by incubation of 1 nM enzyme with a range of inhibitor concentrations for 24 h at 37 °C before assayed.

**pH Dependence of Pro<sup>260–508</sup>MMP-19 Activity—** The pH dependence of the activity of pro<sup>260–508</sup>MMP-19 was determined at 37 °C, after complete activation with 1 mM APMA for 30 min, using the synthetic fluorescent substrate McaPLA<sub>Na</sub>PPaARNH<sub>2</sub> in a buffer of 20 mM MES, 20 mM Tris, 20 mM CAPS, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 0.05% (v/v) Brj 35. The pH was adjusted with HCl in steps of 0.5 before every measurement, covering a range from pH 5 to pH 11.

**Cleavage of Extracellular Matrix Components—** A wide variety of extracellular matrix molecules such as collagen type I, gelatin type I, collagen type IV, laminin, nidogen, fibronectin, tenasin-C (small and large isoforms), fibrin, and fibrinogen were incubated in time-course experiments at 37 °C (if not stated otherwise) with active Δ<sub>128–194</sub>MMP-19 prior to analysis by SDS-PAGE. Type I collagen was prepared from rat skin, as described previously (17). Tenasin-C small and large isoforms were obtained from baby hamster kidney cells transfected with tenasin-C cDNA constructs (18). Mouse laminin, human fibronectin and human fibrinogen were purchased from Sigma. Collagen type IV and nidogen were generous gifts from Klaus Kühn and Rupert Timpl, respectively. Fibrin was generated from fibrinogen by clotting with thrombin prior to incubation with active Δ<sub>128–194</sub>MMP-19 (19). Monoclonal antibodies to fibronectin domains were purchased from Dako (Carpinteria, CA). Technogenetics (Slough, UK) recombinant stromelysin-1 (MMP-3) and gelatinase A (MMP-2), which contain the proteinase domain, were prepared as described previously and activated by trypsin and APMA, respectively, (20, 21). The enzyme/substrate ratio (w/w) used in these experiments was 1/10. Furthermore, 14C-labeled gelatin and casein were used in an assay described by Cawston and Barrett (17) to quantify the specific catalytic activity of wild type Δ<sub>128–194</sub>MMP-19 hydrolyzing these molecules. Gelatin and casein zymography was performed...
activation of other matrix metalloproteinases by active
\Delta_{260-508}MMP-19. The proenzymes of human collagenase-1 (MMP-1),
gelatinase A (MMP-2), stromelysin-1 (MMP-3), neutrophil collagenase
(MMP-8), gelatinase B (MMP-9), collagenase-3 (MMP-13), and MT1-
MMP (MMP-14) were incubated for 24 h with active \Delta_{260-508}MMP-19 at
a 1:1 (w/w) ratio of enzyme versus substrate at 37 °C in 50 mM Tris/HCl,
\pH 7.6, 150 mM NaCl, 10 mM CaCl₂, 0.05% (v/v) Brij 35. Aliquots of the
reaction mixture were removed at the time intervals indicated and
diluted to a final enzyme concentration of 25 pM, and assayed for activity
in the fluorometer using the synthetic quenched fluorescent peptide
substrate (7-methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-[3-(2-
4-dinitrophenyl)-1,2,3-diaminopropionyl]-Ala-Arg-NH₂
(λₐ = 328 nm, λₑ = 393 nm) at a concentration of 1 μM. Molecular mass
changes were monitored by SDS-PAGE and silver staining.

NH₂-terminal Sequence Determination—Proteins were purified by
reverse phase high performance liquid chromatography using a C₁₈
column followed by automated Edman degradation using a PE Bioso-
systems 492 Procise protein sequencer operated according to manufactur-
er’s instructions.

**RESULTS AND DISCUSSION**

Human MMP-19 is a novel member of the matrix metallo-
proteinase family, cloned recently from a human liver cDNA
library (4). Consequently, biochemical analysis of the activa-
tion mechanism, substrate specificity, and inhibition profile of
MMP-19 is of vital importance in order to understand its
possible function in vivo and to design specific inhibitors as poten-
tial new therapeutic agents. Since purification of full-length
MMP-19 expressed in mammalian and bacterial expression
systems resulted in low yields, autoproteolytic activation, and
partial fragmentation, we decided to use the COOH-terminal
truncated form of MMP-19, \pro_{Δ_{260-508}}MMP-19 (numbering
starts at Met¹, GenBank™/EBI accession number X92521), for
this study and analyzed its enzymatic properties.

Refolding, Purification, and Activation of \proΔ_{260-508}MMP-19,
and Assessment of Mutations within the Pro and Catalytic Domains—
In order to further characterize the catalytic activity
of MMP-19, we expressed and refolded the COOH-terminal
truncated form of MMP-19, \pro_{Δ_{260-508}}MMP-19, comprising
the pro and catalytic domains, and analyzed the protein bio-
chemically in detail. After solubilization and refolding,
\pro_{Δ_{260-508}}MMP-19 was purified using nickel-nitriotriacetic
acid-agarose and the eluted proenzyme displayed the expected
mass of 30 kDa when analyzed by SDS-PAGE (Fig. 1A).

Surprisingly, following dialysis to remove the imidazole, all the
enzyme autoactivated, resulting in the generation of active
enzyme with a mass of 20 kDa under reducing conditions (Fig.
1B). NH₂-terminal amino acid sequence determination of the
active catalytic domain confirmed that the Lys⁹⁷-Tyr⁹⁸ peptide
bond was hydrolyzed during autoproteolytic activation (Fig. 2).

Similar autoactivation was observed when we attempted to
purify full-length MMP-19 from the culture medium of stable
transfected NS0 cells (data not shown). In order to define the
residues in the proenzyme that make \proMMP-19 prone to
autoactivation, we performed site-directed mutagenesis exper-
timents by targeting the latency motif PRCGLE²⁶⁰DP⁹⁰ of
\proMMP-19 and the residue Cys⁶⁶ in the catalytic domain,
which are structural elements different from other MMPs. The
mutants \proΔ_{260-508}MMP-19(C166S), \proΔ_{260-508}MMP-19(E88P/P90V), and
\proΔ_{260-508}MMP-19(C166S/E88P/P90V) were expressed, solubilized,
refolded, and purified in the same way as described for the wild type \proΔ_{260-508}MMP-19. All
mutants are of the same size as wild type when analyzed by
SDS-PAGE and activate spontaneously during refolding and
purification (Fig. 1 and data not shown). However, the tenden-
cy to undergo activation is slowed by approximately
50% for the two mutants \proΔ_{260-508}MMP-19(E88P/P90V) and
\proΔ_{260-508}MMP-19(C166S/E88P/P90V), confirming previ-
ously published data, where the effects of single mutations
within the latency motif of transin (rat stromelysin) upon
activation were reported (23). In both of these \proΔ_{260-508}MMP-19
mutants, the alterations reestablish the sequence PRCGYPD,
conserved within the propeptide of MMPs. Thus, our results
suggest that the tendency of \proΔ_{260-508}MMP-19 and full-length
MMP-19 to undergo autoactivation can be ascribed partially
to its unique latency motif. Cys⁶⁶ within the catalytic domain
seems not to influence activation and its function
remains unclear. However, we observed low amounts of
dimers of active recombinant wild type \proΔ_{260-508}MMP-19 by
casein zymography and SDS-PAGE, whereas the mutants
containing C166S did not form any dimers under the same
conditions (data not shown). There is no in vivo evidence, how-
ever, that the residue Cys⁶⁶ facilitates dimerization or linkage to
other proteins.

If not stated otherwise, we used the active wild type catalytic
domain of MMP-19 (active \proΔ_{260-508}MMP-19) for further bio-
chemical characterization.

**Kinetic Analysis of Cleavage of Quenched Fluorescent Sub-
strates and TIMP Binding—**In order to assess the substrate
specificity of active \proΔ_{260-508}MMP-19 further, seven synthetic
quenched fluorescent peptide substrates were employed (Table
1). Only the general MMP substrate Mca-Pro-Leu-Gly-Dpa-Ala-
Arg-NH₂ and the stromelysin substrate Mca-Pro-Leu-Ala-Nva-
Dpa-Ala-Arg-NH₂ were hydrolyzed efficiently, while other
substrates were resistant to hydrolysis, thus confirming our
preliminary data (4). The \(k_{cat}/K_m\) values obtained for these two
substrates are in agreement with our earlier results. Therefore,
the catalytic domain of MMP-19 has a substrate specificity
similar to that for the stromelysin subfamily of MMPs since it
preferably hydrolyzed Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-
NH₂ which was designed as a substrate to study stromelysin
activity. However, stromelysin-1 (MMP-3) hydrolyzes both sub-
strates, Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂ and Mca-Pro-
Leu-Ala-Nva-Dpa-Ala-Arg-NH₂, more efficiently than active
\proΔ_{260-508}MMP-19 (20).

Furthermore, we followed \proΔ_{260-508}MMP-19 activity over a
wide pH range (pH 5.0–11.0) using a constant enzyme concen-
tration of 1 nM and Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH₂ as
substrate in the quenched fluorescent assay (Fig. 3). The activity,
measured in steps of 0.5, showed low \(k_{cat}/K_m\) values at pH
5.0 and a sudden increase toward pH 7.0, where the highest

![Fig. 1. SDS-PAGE analysis of purified recombinant pro\( \Delta_{260-508} \)MMP-19 produced by bacterial cells. Panel A, lane 1, molecular size standard; lane 2, purified pro\( \Delta_{260-508} \)MMP-19, processing from the latent to the active form. Panel B, lane 1, molecular size standard; lane 2, active \( \Delta_{260-508} \)MMP-19. Molecular mass markers are indicated on the left. The left and active forms of the mutants pro\( \Delta_{260-508} \)MMP-19(C166S), pro\( \Delta_{260-508} \)MMP-19(E88P/P90V), and pro\( \Delta_{260-508} \)MMP-19(C166S/E88P/P90V) display the same masses as wild type pro\( \Delta_{260-508} \)MMP-19 (data not shown).](http://www.jbc.org/)

**Human MMP-19 Catalytic Domain**

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value was obtained, followed by a gradual decrease toward pH 10.5, where no activity was measurable. Thus, MMP-19 is active over a wide pH range and displays maximum activity at pH 6.0 (24). In contrast, the pH 7.0 in our assay. A comparison of the pH profiles of MMP-19 and MMP-3 reveals that the latter seems to prefer a more acidic environment, reaching maximum activity at pH 6.0 (24).

The apparent $K_i$ values for the inhibition of active $\Delta_{260-508}$MMP-19 with the various TIMPs were determined using a constant enzyme concentration of 1 nM in the quenched fluorescent assay at 37 °C with Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ as substrate (Table II). Under these conditions, only the $K_i$ value for TIMP-1 was determined with accuracy, since its value (57.6 nM) is well above the employed enzyme concentration and represents the weakest value ascertained for full-length TIMPs. In contrast, the $K_i$ values for TIMP-2, TIMP-3, and TIMP-4 were all in the range of 4–5 μM and are only rough estimates of the true values, because we were not able to determine them using enzyme concentrations below $K_{\text{app}}$ due to the lack of assay sensitivity. However, the $K_i$ values in the picomolar range indicate very strong enzyme-inhibitor interactions. The same restrictions apply to the $K_{\text{app}}$ for the interactions of COOH-terminal truncated forms of enzyme and inhibitor, active $\Delta_{260-508}$MMP-19 and $\Delta_{128-194}$TIMP-2, which also result in values within the picomolar range and are, thus, not accurate. In addition, we investigated the inhibitory potential of mutants of $\Delta_{128-194}$TIMP-2, containing single amino acid changes, with the aim to determine the sites in the NH$_2$-terminal, inhibitory region of TIMP-2, which are important for interactions with MMP-19 (Table II). From the three mutants used in this study, Y36G, A70K and S2E, only A70K (745 pM) was 1.22 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ for TIMP-1, and 3.049 $\times$ 10$^4$ M$^{-1}$ s$^{-1}$ for TIMP-3. In comparison, the association of TIMP-2 and

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**TABLE I**

Substrate specificity of active $\Delta_{260-508}$MMP-19. $k_{\text{cat}}/K_{\text{m}}$ values for various synthetic quenched fluorescent substrates at 37 °C.

| Substrate | $k_{\text{cat}}/K_{\text{m}}$ ($M^{-1} s^{-1}$) |
|-----------|------------------------------------------|
| 1. Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH$_2$ | 1.93 $\times$ 10$^4$ |
| 2. Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ | 3.09 $\times$ 10$^4$ |
| 3. Mca-Pro-Glu-Gly-Leu-Arg-Dpa-NH$_2$ | Not cleaved |
| 4. Mca-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH$_2$ | Not cleaved |
| 5. Mca-Thr-Glu-Gly-Glu-Ala-Arg-Gly-Ser-Dpa-NH$_2$ | Not cleaved |
| 6. Mca-Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Ser-Arg-Lys(Dpn)-NH$_2$ | Not cleaved |
| 7. Mca-His-Tyr-Gly-Lys-Leu-Pro-Gln-Lys-Ser-His-Gly-Arg-Lys(Dpn)-D-Arg-OH | Not cleaved |

**TABLE II**

Kinetic analysis of the inhibition of MMP-19 catalytic domain by various TIMPs

| Inhibitor | $k_{\text{on}}$ ($\times$ 10$^4$) | $K_{\text{app}}$ (pM) |
|-----------|-------------------------------|---------------------|
| TIMP-1    | 1.22                          | 57,600 ($\pm$ 6,995) |
| TIMP-3    | 3.049                         | $<5$                |
| TIMP-4    | 13.0 ($\pm$ 2.5)              | $<5$                |
| TIMP-2    | 41.8 ($\pm$ 3.5)              | $<5$                |
| $\Delta_{128-194}$TIMP-2 | 10.2 ($\pm$ 1.9) | 14 ($\pm$ 7) |
| $\Delta_{128-194}$TIMP-2 (Y36G) | 13.8 ($\pm$ 0.49) | 24.2 ($\pm$ 9) |
| $\Delta_{128-194}$TIMP-2 (A70K) | 16.0 ($\pm$ 2.7) | 745.5 ($\pm$ 69) |
| $\Delta_{128-194}$TIMP-2 (S2E) | 1.05 ($\pm$ 0.34) | 61,570 ($\pm$ 4337) |

Further more, the association rate constant of enzyme/inhibitor complex. All values were measured at 37 °C.

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**FIG. 2.** NH$_2$-terminal sequence determination of pro$\Delta_{260-508}$MMP-19 in pRSET B and active $\Delta_{260-508}$MMP-19. The NH$_2$ terminus of active $\Delta_{260-508}$MMP-19 is indicated with an arrow. The numbering of the amino acid sequence starts with Met$^1$.

**FIG. 3.** Determination of the pH dependence of $\Delta_{260-508}$MMP-19 activity in the fluorescent assay $k_{\text{cat}}/K_{\text{m}}$ values were obtained at 37 °C using 1 nM enzyme and the synthetic quenched fluorescent substrate Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ at 1 μM.
TIMP-4 with the enzyme were considerably faster (4.18 \times 10^5 and 1.3 \times 10^3 \text{ m}^{-1} \text{ s}^{-1}, respectively). TIMP-4 shows similar features in the inhibition of the catalytic domain of MMP-19 as TIMP-2, the strongest known inhibitor for MMP-19 (4), which is in agreement with the high homology between these two inhibitors (26). Interestingly, the interaction of the catalytic domain of MMP-19 with TIMP-2 was found to be about 4 times faster than the one observed with the COOH-terminal deletion mutant \( \Delta_{128-194} \text{TIMP-2} \) (4.18 \times 10^5 \text{ m}^{-1} \text{ s}^{-1} and 1.02 \times 10^5 \text{ m}^{-1} \text{ s}^{-1}, respectively), suggesting an influence of the COOH terminus of TIMP-2 on the association with active \( \Delta_{260-508} \text{MMP-19} \), which is in agreement with our previous data on the domain interactions involved in the binding of TIMPs to MMPs and suggestions from recent crystallographic analysis of the MT1-MMP/TIMP-2 complex (12, 25, 27). However, the small differences in \( K_{\text{app}} \) observed between TIMP-2 and \( \Delta_{128-194} \text{TIMP-2} \) (both picomolar) confirm that the NH2-terminal domain of TIMP-2 alone is able to form stable complexes with the enzyme and, thus, is sufficient for the inhibition of MMP-19 (Table II). These results are confirmed by data on the interactions of \( \Delta_{128-194} \text{TIMP-2} \) with MMP-3 (28).

The \( \Delta_{128-194} \text{TIMP-2} \) mutants Y36G and A70K do not show a significant change of association if compared with the wild type \( \Delta_{128-194} \text{TIMP-2} \). However, the mutation S2E slows down the association of active \( \Delta_{260-508} \text{MMP-19} \) and inhibitor with 1 order of magnitude (1.05 \times 10^5 \text{ m}^{-1} \text{ s}^{-1}), and results in a 10,000-fold increase in \( K_{\text{app}} \) (61.57 nM), therefore weakening the inhibitory properties considerably. In case of A70K, merely the \( K_{\text{app}} \) was increased about 50 times when compared with the wild type \( \Delta_{128-194} \text{TIMP-2} \), whereas the association rate constant was unchanged. From our data we can deduce that both mutations, A70K and S2E, result in increased enzyme-inhibitor complex dissociation, suggesting that the exchange of these amino acids compromise vital enzyme-inhibitor interactions. We therefore conclude that for the formation of the enzyme-inhibitor complex, similar to other MMPs, \( \Delta_{260-508} \text{MMP-19} \) interacts with the so-called “ridge” region in TIMP-2 (Cys1-Cys5 and Ser48-Cys37) (15, 27, 29–32).

Kinetic Analysis of Hydroxamate Inhibitor Binding—The involvement of MMPs in the breakdown and remodeling of the connective tissue under pathological conditions such as arthritis and cancer makes them attractive targets for the development of specific inhibitors for therapeutic intervention. In recent years, hydroxamic acid-based peptide inhibitors were developed, which react with 1:1 stoichiometry with MMPs, as revealed by x-ray crystallography (33, 34). The apparent \( K_{\text{app}} \) values for the inhibition of active \( \Delta_{260-508} \text{MMP-19} \) with the inhibitors BB-94 (British Biotech), CT-1746 (Celltech), and Ro31-9790 (Hoffmann-La Roche) were determined (Table III). Only the \( K_{\text{app}} \) for Ro30-9790 was determined with accuracy (1.22 nM), since its value is above the employed enzyme concentration, whereas the values obtained for the other two inhibitors were both in the low picomolar range and therefore must be regarded as rough estimates of the real values. Interestingly, the \( K_{\text{app}} \) value for the inhibition of stromelysin-1 (MMP-3) with Ro31-9790 (119 nM) is about 100 times higher than our value determined for \( \Delta_{260-508} \text{MMP-19} \). In addition, the other two synthetic compounds tested seem also to inhibit \( \Delta_{260-508} \text{MMP-19} \) more efficiently than stromelysin-1, suggesting that the architectures of the active sites of these two enzymes, responsible for inhibitor interactions, are different (Table III).

Hydrolysis of Extracellular Matrix Components—A large variety of extracellular matrix components, purified from different connective tissues, were incubated with the catalytic domain of MMP-19 in time-course experiments prior to SDS-PAGE analysis to study its substrate specificity and to evaluate its possible function in vivo (Figs. 4–8). These experiments were also performed using stromelysin-1 as a comparison, since active \( \Delta_{260-508} \text{MMP-19} \) exhibits similar specificity toward the synthetic fluorescent peptide substrate Mca-Pro-Leu-Ala-Nva-Arg-GL-A (34). As expected, collagen type I was resistant to hydrolysis by the catalytic domain of MMP-19 (Fig. 4A). However, full-length MMP-19 purified from the medium of stable transfected NS0 cells was also unable to cleave triple-helical collagen. Furthermore, catalytic domain and full-length MMP-19 exhibit similar activity versus gelatin (data not shown). Our data show that the influence of the COOH terminus on MMP-19 substrate specificity is negligible for the substrates studied, that the enzyme is non-collagenolytic, and thus the catalytic domain

| Inhibitor          | \( K_{\text{app}} \) (nM) | \( K_{\text{app}} \) (nM) |
|--------------------|--------------------------|--------------------------|
| BB-94              | 0.06                     | 2.0                      |
| Ro31-9790          | 1.2                      | 119                      |
| CT-1746            | 0.05                     | 10.9                     |
represents a good model to study macromolecular substrate hydrolysis.

The catalytic domain of MMP-19 was able to degrade gelatin efficiently (Fig. 4B). However, at equivalent substrate/enzyme ratios (w/w), stromelysin-1 (MMP-3) and gelatinase A (MMP-2) display a considerably higher activity against this macromolecule, if analyzed by SDS-PAGE (Fig. 4B). Although Δ260–508MMP-19 degraded gelatin, hydrolysis is not sufficient to generate a definable zone of lysis using gelatin zymography presumably due to the large mass of the generated fragments. Thus, it is not possible to use gelatin zymography to detect MMP-19 activity (Fig. 4C). In contrast, casein is hydrolyzed efficiently and Δ260–508MMP-19 activity can therefore be monitored using casein zymograms (Fig. 4D). In comparison to Δ260–508MMP-19, stromelysin-1 hydrolyzes gelatin and can therefore be analyzed by gelatin zymography. In addition, the specific activity of MMP-19 catalytic domain was assessed using 14C-labeled gelatin and casein (17). In this assay, the enzyme was able to hydrolyze gelatin with a specific activity of 78.45 units/μmol and casein with 8491 units/μmol. In comparison, the specific activities for the hydrolysis of gelatin by stromelysin-1 and gelatinase A are 2 to 4 orders of magnitude higher than observed for Δ260–508MMP-19, respectively (20, 21). On the other hand, specific activities of Δ260–508MMP-19 and stromelysin-1 for the hydrolysis of casein are similar (20).

The two different isoforms of human tenascin-C were incubated with the MMP-19 catalytic domain (Fig. 5) (35). Analysis of the reaction products revealed that the small isoform was resistant (data not shown), as expected (18), while the large tenascin-C isoform was cleaved into two high molecular mass fragments displaying molecular masses of 190 and 123 kDa. The cleavage pattern is identical to the one generated by stromelysin-1 under the same conditions, but MMP-19 seems to be more effective in processing the large isoform of tenasin-C. In addition, it was demonstrated that MMP-19 catalytic domain is able to hydrolyze the large isoform to the same sized products as gelatinase A (18).

Fibronectin was degraded by MMP-19 catalytic domain in a
with its expression in the tunica media, the enzyme may have a role during angiogenesis (7). To further investigate the ability of MMP-19 to hydrolyze ECM components, \( \Delta_{260-508} \text{MMP-19} \) was incubated with fibrinogen and fibrin, both molecules with a distinct role during new blood vessel formation (Fig. 7D). The cleavage pattern showed complete hydrolysis of the fibrinogen \( \alpha \alpha \) and B\( \beta \) chains after 1 h and loss of the \( \gamma \) chain after 5 h of incubation. After 24 h, fragments of sizes between 35 and 42 kDa seem to represent the final cleavage products. Fibrin was hydrolyzed by MMP-19 catalytic domain in a similar fashion to fibrinogen, resulting in three major cleavage products between 35 and 42 kDa and a double band at 72 kDa, which appeared to be derived from the \( \gamma \) dimers (19). In comparison, hydrolysis of fibrinogen and fibrin by stromelysin-1 for 24 h resulted in only two distinct fragments of approximately 42 and 37 kDa, whereas, in case of fibrin, a doublet at 42 kDa was obtained (Fig. 7D).

**Activation of Other Pro-matrix Metalloproteinases—**MMPS have been implicated in the activation and hydrolysis of secreted or membrane-bound proteinases (e.g. other MMPS) and precursors of growth factors (40, 41). Thus, in order to determine the possible role of MMP-19 upon activation of other MMPS, we incubated various pro-MMPS (MMP-1, -2, -3, -8, -9, -13, and -14) with active \( \Delta_{260-508} \text{MMP-19} \). However, only human progelatinase B (MMP-9) was processed by the enzyme in a time-dependent fashion generating a final form of \( M_r 82,000 \) after 24 h of incubation when analyzed by SDS-PAGE (Fig. 8). The MMP-19 catalytic domain was not able to process any of the other MMPs tested (MMP-1, -2, -3, -13, and -14; data not shown). The processed form of human gelatinase B does not show hydrolytic activity against the synthetic quenched fluorescent substrate Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH\(_2\). NH\(_2\)-terminal sequencing revealed that \( \Delta_{260-508} \text{MMP-19} \) cleaves the Lys\(^{73}\)-Ala\(^{74}\) bond upstream of the ...PRCGVPD... sequence within the propeptide region of MMP-9, leaving its latency motif intact. This cleavage may induce conformational changes in the propeptide exposing the final activation site (Arg\(^{97}\)-Phe\(^{98}\)) to be hydrolyzed by a second proteolysis, which cannot be performed by MMP-19 (42).

Relatively high levels of MMP-19 expression were detected by Northern blot analysis in a wide variety of normal tissues (4). This pattern of expression is unusual for MMPS, which are not constantly produced by adult cells but are mostly induced during physiological conditions associated with extensive connective tissue remodeling, such as wound healing, uterine postpartum involution, or mammary gland involution. Therefore, MMP-19 may be involved in normal ECM remodeling processes. Interestingly, MT1-MMP (MMP-14) shows an expression pattern similar to that of MMP-19 and is regarded as major player in surface activation of other MMPS and ECM remodeling (43–46).

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Biochemical Characterization of the Catalytic Domain of Human Matrix Metalloproteinase 19: EVIDENCE FOR A ROLE AS A POTENT BASEMENT MEMBRANE DEGRADING ENZYME

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