Identification of Structural Elements Important for Matrix Metalloproteinase Type V Collagenolytic Activity as Revealed by Chimeric Enzymes. Role of Fibronectin-like Domain and Active Site.*

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Running Title: Elements Important for Type V Collagenolytic Activity

* This work was supported by National Institutes of Health Grant AR-41843 and American Cancer Society Grant GR CB-146.

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Abstract

Digestion of type V collagen by the gelatinases is an important step in tumor cell metastasis as this collagen maintains the integrity of the extracellular matrix that must be breached during this pathological process. However, the structural elements that provide the gelatinases with this unique proteolytic activity among matrix metalloproteinases had not been thoroughly defined. To identify these elements, we examined the substrate specificity of chimeric enzymes containing domains of gelatinase B and fibroblast collagenase. We have found that the addition of the fibronectin-like domain of gelatinase B to fibroblast collagenase is sufficient to endow the enzyme with the ability to cleave type V collagen. In addition, the substitution of the catalytic zinc-binding active site region of fibroblast collagenase with that of gelatinase B increased the catalytic efficiency of the enzyme three to four fold. This observation led to the identification of amino acid residues, Leu 397, Ala 406, Asp 410, and Pro 415, in this region of gelatinase B that are important for its efficient catalysis as determined by substituting these amino acids with the corresponding residues from fibroblast collagenase. Leu 397 and Ala 406 are important for the general proteolytic activity of the enzyme, while Asp 410 and Pro 415 specifically enhance its ability to cleave type V collagen and gelatin, respectively. These data provide fundamental information about the structural elements that distinguish the gelatinases from other matrix metalloproteinases in terms of substrate specificity and catalytic efficiency.
Introduction

Matrix metalloproteinases (MMP)\(^1\) are a constantly growing family of calcium- and zinc-dependent endoproteinases. These enzymes are secreted as catalytically latent species that are processed to their activated forms \textit{in vivo} by other proteinases. MMPs function in the remodeling of the extracellular matrix by degrading its component proteins, such as collagen and fibronectin. Several studies have implicated MMP involvement in both normal physiological and pathological processes such as wound healing (1, 2), skeletal growth and remodeling (3, 4), cancer (5-10), arthritis (11-14), and periodontal disease (15, 16).

MMPs can be sub-divided according to substrate specificity. The collagenases (MMPs-1, 8, and 13) are well known for their ability to cleave fibrillar collagens I-III. The stromelysins (MMPs-3, 10, and 11) digest a wide variety of extracellular matrix proteins including fibronectin, laminin, elastin and collagens IX and X. The gelatinases, A (MMP-2) and B (MMP-9), digest denatured collagen (gelatin) with relatively high efficiency and cleave fibrillar collagens V and XI, among other proteins. Lastly, membrane-type (MT) MMPs (MMPs-14-17) are known for their ability to activate gelatinase A at the cell surface, which may be an important step for inducing tumor cell invasion (17).

The morbidity and mortality of cancer is directly related to the ability of tumor cells to metastasize. There is evidence that tumor cell invasion is facilitated by the actions of degradative enzymes, particularly the gelatinases, that are capable of digesting the components of the basement membrane and stromal matrix barriers (reviewed in (18)). Type V collagen maintains the integrity of the extracellular matrix by acting as a molecular anchor, linking the...
basement membrane to the stromal matrix (19). Thus, the degradation of this molecule by the
gelatinases is an important step in tumor cell invasion. Undoubtedly, studying the mechanism by
which these enzymes interact with and degrade type V collagen will increase our understanding
of metastasis and perhaps lead to strategies to inhibit this lethal pathological process.

It is known that the ability of MMPs to recognize particular substrates depends on the
presence of certain domains and particular amino acids within the enzyme structure. Almost
every MMP possesses three common domains: an amino-terminal pro-domain that is partially or
completely removed during the processing and activation of the enzyme, a catalytic domain that
contains the catalytic zinc-binding active site region (AS), and a carboxy-terminal domain that
consists of a proline-rich hinge region followed by four hemopexin-like repeats. The ability of
the collagenases to bind and cleave type I collagen, but not casein or gelatin, is dependent on the
presence of their carboxy-terminal domains (20-23). However, this domain does not appear to be
important for the substrate specificity of the gelatinases (24-26) or the stromelysins (21, 22, 27).
The gelatinases are the only MMPs that contain three contiguous fibronectin type 2 homology
units, collectively known as the fibronectin-like domain, that are inserted into their catalytic
domains. Several reports have indicated that this domain plays a role in the various proteolytic
activities of these enzymes. It has been shown that the presence of this domain greatly increases
their gelatinolytic and elastinolytic activities (26, 28, 29). In addition, we have recently shown
that the presence of the fibronectin-like domain in gelatinase B is critical for its ability to bind
and digest collagen types V and XI, but not a small peptide substrate (29). However, it was not
known whether the addition of this domain to an MMP is sufficient to confer type V
collagenolytic activity. In fact, there are MMPs, such as the collagenases and the stromelysins,
that can bind collagen via their carboxy-terminal domains (22) but are unable to cleave type V
collagen (30-33). This raised the possibility that determinants of type V collagenolytic activity may also be located in other domains of the gelatinases. In this report, we have achieved a definitive understanding of the structural elements that are required for type V collagenolytic activity by studying the substrate specificity of chimeric enzymes containing domains of gelatinase B and fibroblast collagenase (FC). We were able, for the first time, to demonstrate that the insertion of the fibronectin-like domain into the catalytic domain of an MMP (FC) is sufficient to endow the enzyme with the ability to cleave type V collagen. In addition, we were able to identify amino acid residues in the AS of gelatinase B (Gel.B/AS) that dictate and differentiate its type V collagenolytic, gelatinolytic, and peptidolytic activities.
Experimental Procedures

**Plasmid Construction for Chimeric Enzymes** — The full length FC cDNA was cloned into the pET12C (Novagen) expression vector via engineered *Bam*HI and *Nhe*I sites to produce pET/FC. The pET/FC+Fib expression vector was designed to produce FC with the fibronectin-like domain of gelatinase B, amino acids 216-394, inserted into its catalytic domain (Fig. 1). This vector was created by amplifying the region of the gelatinase B cDNA encoding the fibronectin-like domain by anchor PCR using pNG 4.1 (34) as a template. The 5′ primer, 5′-GGGGATGCTCATTTTGATGAACATGAAAGGTGGACCAACAATTTCACAGAGTACACGTCGTGGTTCCAACACTCGG-3′, contained nucleotides, shown in bold, complementary to the 5′ end of the region of the gelatinase B cDNA encoding the fibronectin-like domain, amino acids 216-221. The 5′ portion of the primer contained a *Msl*I site and encoded amino acids 193-211 of the catalytic domain of FC. The 3′ primer, 5′-GGAGAGTCCAAGAGAATGGCCAAGCACATGCGCCGCCACGCAGAA-3′, contained nucleotides, shown in bold, complementary to the 5′ end of the region of the gelatinase B cDNA encoding the AS, amino acids 396-402. Amino acids 215-219 of FC are identical to residues 398-402 in gelatinase B. The underlined nucleic acid represents a substitution to replace Leu 397 in gelatinase B with the corresponding Arg 214 in FC. The 5′ portion of the primer contained a *Bst*XI site and encoded amino acids 220-227 of the FC AS. The 0.6 kb PCR product was digested with *Bst*XI and *Msl*I and ligated with 5 kb *Sal*I/*Bst*XI and 0.6 kb *Msl*I/*Sal*I fragments from pET/FC to produce the final expression vector.

The pET/FC+Fib+Gel.B/AS expression vector was designed to produce the pET/FC+Fib chimera with the additional substitution of the AS of FC, amino acids 212-254, for the Gel.B/AS,
amino acids 395-437 (Fig. 1). This vector was constructed by amplifying the region of the gelatinase B cDNA in pNG 4.1 encoding the fibronectin-like domain and AS, amino acids 216-437, using anchor PCR. The same 5′ primer used for the construction of pET/FC+Fib was used in this reaction. The 3′ primer, 5′-TTGGGAACGTCCATATATGGCTTGATCCATTCACTCTTTT-3′, contained nucleotides, shown in bold, complementary to the 3′ end of the region of the gelatinase B cDNA encoding the AS, amino acids 433-437. The 5′ portion of the primer encoded FC amino acids 255-264 of the hinge region and contained a XcmI site. The resulting 0.7 kb PCR product was digested with MslI and XcmI and ligated with 0.6 kb MslI/SalI and 4.9 kb XcmI/SalI fragments from pET/FC to produce the final expression vector.

The pET/FC+Gel.B/AS expression vector was designed to produce FC with its AS substituted with the Gel.B/AS (Fig. 1). This vector was created by using anchor PCR with pET/FC+Fib+Gel.B/AS as the template. The 5′ primer, 5′-CGTCGACGGGATCCCACAGCTTT-3′, bound 5′ to a SalI site in the extreme 5′ end of the cDNA. The 3′ primer, 5′-CGCCGCCACGAGGAACAAAGTTGTACTCTGTGAAATTGTG-3′, contained nucleotides, shown in bold, complementary to the region of the FC+Fib+Gel.B/AS cDNA encoding FC amino acids 205-211 of the catalytic domain, which is immediately 5′ to the region encoding the fibronectin-like domain. The 5′ portion of the primer was complementary to the 5′ end of the region encoding the Gel.B/AS, amino acids 395-400, which contained a BssSI site. This 0.7 kb PCR product was digested with BssSI and SalI and ligated to 0.8 kb BssSI/DraIII and 4.5 kb SalI/DraIII fragments produced from pET/FC+Fib+Gel.B/AS. This effectively deleted the region of the cDNA encoding the fibronectin-like domain from pET/FC+Fib+Gel.B/AS to produce pET/FC+Gel.B/AS.
The pET/FC+Fib+Gel.B/AS-COOH expression vector was designed to produce the FC+Fib+Gel.B/AS chimera without the carboxy-terminal domain of FC (Fig. 1). This construct was produced from the pET28/Δ3′NG expression vector. pET28/Δ3′NG contains the gelatinase B cDNA corresponding to amino acids 1-444 inserted into pET28C (Novagen). An engineered stop codon immediately following the codon for Gly 444 enables this vector to produce a carboxy-terminal truncated form of gelatinase B. A 5.5 kb XbaI/SacII fragment was produced from pET28/Δ3′NG that contained nucleotides encoding a portion of the fibronectin-like domain and the Gel.B/AS. This fragment was ligated with a 1.2 kb NdeI/SacII fragment from pET/FC+Fib+Gel.B/AS, encoding the remaining portion of the fibronectin-like domain from gelatinase B and the catalytic and pro-domains of FC, to produce pET/FC+Fib+Gel.B/AS-COOH.

All of the PCR derived DNA for these constructs was found to be free of mutations by dideoxy sequencing. The plasmids encoding the chimeric enzymes were transformed either into Escherichia coli BL21(DE3) or BL21(DE3)pLysS cells. With the exception of cells transformed with pET/FC+Fib+Gel.B/AS-COOH, BL21(DE3) transformed cells were maintained in 50 μg/ml carbenicillin while BL21(DE3)pLysS transformed cells were maintained in 50 μg/ml carbenicillin plus 34 μg/ml chloramphenicol. The pET/FC+Fib+Gel.B/AS-COOH vector was transformed into BL21(DE3) cells that were maintained in 30 μg/ml kanamycin.

Site-Directed Mutagenesis of Gelatinase B AS Residues—PCR was used to create the L397R, A406S, D410S, and P415I point mutations in the gelatinase B cDNA. The following mutagenic primers were used (substituted nucleotides underlined): L397R, 5’-GGATAACAGTTTGTTCGCGGTG-3’; A406S, 5’-TGTTTCTCTCTTGCGCGCATGAGTTCCGCCACTCGCTGG-3’; D410S, 5’-
GTTCGGCCACGCGCTGGGCTTATCTCCTTATCATTCC-3’; P415I, 5’-
CATGAGCGCTTCATCCTGAGGAATG-3’. The expected mutations were confirmed and all PCR derived DNA was found to be free of secondary mutations by dideoxy sequencing. Plasmids encoding the mutant enzymes were introduced into *E. coli* BL21(DE3) cells that were maintained in 50 µg/ml carbenicillin.

*Protein Expression and Purification*—Transformed cells were grown to mid-log phase and induced with the addition of 100-120 mg/L isopropyl-β-D-thiogalactopyranoside. Cells were then allowed to grow 4-19 h before being pelleted at 6,370 X g for 10 min. The supernatant was decanted and the pellets stored at -20 °C for further purification. Latent gelatinase B, point mutants, FC+Fib, FC+Fib+Gel.B/AS, and FC+Fib+Gel.B/AS-COOH were purified using a gelatin Agarose affinity column essentially as described previously (34).

Activated FC and FC+Gel.B/AS were purified by a zinc-chelating column. FC containing bacterial pellets were resuspended in buffer containing 50 mM Tris, pH 7.5, 5 mM CaCl₂, 0.5 M NaCl, 0.05% (w/v) Brij-30, and 2 mM phenylmethylsulfonyl fluoride. The resulting suspension was then sonicated three times, 30 sec each time, at 30% duty cycle and centrifuged at > 20,000 X g for 30 min. To activate the enzymes, *p*-aminophenylmercuric acetate (APMA) was added to the supernatant at a final concentration of 1 mM and the resulting mixture was incubated 4-5 h at 37 °C. During the incubation period, precipitant formed that was removed by centrifugation at > 20,000 X g for 30 min. The supernatant was then dialyzed against buffer containing 25 mM Na₂B₄O₇ (Borax), pH 8.0, 5 mM CaCl₂, 1 M NaCl, and 10 mM imidazole (dialysis buffer) and then loaded onto a 5 ml iminodiacetic acid Sepharose column (Pharmacia) that had been charged with 50 mM ZnCl₂ and equilibrated with dialysis buffer. The column was then washed with dialysis buffer until the *A*₂₈₀ of the effluent was approximately
zero. The protein was then eluted with a 10-33 mM gradient of imidazole in dialysis buffer.
Selected fractions were combined and concentrated. FC+Gel.B/AS was purified in essentially the same manner except that the dialysis buffer contained 0.5 M NaCl and a 10-40 mM imidazole gradient was used to elute the enzyme. All of the purified enzymes were finally dialyzed against buffer containing 50 mM Tris, pH 7.5, 5 mM CaCl$_2$, 150 mM NaCl, and 2 µM ZnCl$_2$ and stored at -80 °C for further use.

*Activation of Enzymes*—Gelatinase B and point mutants were activated either by 1 mM APMA for 12-15 h at 37 °C or 16 units/μl stromelysin-1 for 2 h at 37 °C. One unit of stromelysin-1 degrades 1 nM/s of (7-methoxycoumarin-4-yl) acetyl (Mca)-Pro-Leu-Gly-Leu-[3-(2′,4′-dinitrophenyl)-L-2,3-diaminopropionyl](Dpa)Ala-Arg-NH$_2$ (BACHEM Bioscience Inc., King of Prussia, PA) at 23 °C. Pro-stromelysin-1 was activated by incubation at 52 °C for 1 h. Stromelysin-1, at concentrations used in the enzyme assays, had no proteolytic activity towards any of the substrates. FC+Fib, FC+Fib+Gel.B/AS, and FC+Fib+Gel.B/AS-COOH were activated by 1 mM APMA for 1 h at 37 °C.

*Amino-terminal sequencing* — Prior to the loading of samples, 8% SDS-polyacrylamide electrophoresis (PAGE) gels were electrophoresed for 20 min. The buffer in the upper reservoir contained either 1 mM glutathione or mercaptoacetic acid. When glutathione was used, the upper buffer was removed and replaced with buffer containing no glutathione prior to the loading of samples. Samples were heated for 15 min at 60 °C under reducing conditions before loading onto the gels. After electrophoresis, proteins were transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 80 min at 120 mA in 10 mM 3-(cyclohexylamino)propanesulfonic acid, pH 11, and 10% (v/v) methanol. Protein bands were
visualized by Coomassie Blue staining and sequenced at Midwest Analytical Inc. (St. Louis, MO).

**Determination of $k_{\text{cat}}/K_m$ values for the fluorogenic peptide substrate, Mca-PLGL(Dpa)AR-NH$_2$**—Enzymes were mixed with Mca-PLGL(Dpa)AR-NH$_2$ in assay buffer that was composed of 50 mM Tris, pH 7.5, 5 mM CaCl$_2$, 150 mM NaCl, and 2 µM ZnCl$_2$. The concentrations of Mca-PLGL(Dpa)AR-NH$_2$ used fulfilled the condition of $K_m >> [S]$. The initial rate of substrate hydrolysis was determined by measuring the increase in fluorescence intensity ($\lambda_{ex}$ 328 nm, $\lambda_{em}$ 393 nm) as a function of time using an Aminco Bowman luminescence spectrophotometer (SLM Instruments, Inc., Urbana, IL) at 23 °C. Assays were performed in duplicate and the results averaged. The $k_{\text{cat}}/K_m$ values were calculated from the following equation:

$$k_{\text{cat}}/K_m \equiv \frac{v_o}{[E]_T[S]}$$

Concentrations of active enzymes, $[E]_T$, were found by active-site titration with the amino-terminal, 14-kDa, inhibitory domain of recombinant tissue inhibitor of metalloproteinase-2 (a kind gift from Dr. Harold Tschesche, Lerstuhl für Biochemie, Universität Bielefeld, Bielefeld, Germany) as described previously (29). This method was used to determine the concentration of enzyme used in every assay in this report. Concentrations of active enzymes found by titration correlated with those determined by the Bradford dye-binding technique (standard Bio-Rad Laboratories assay) using bovine serum albumin (BSA) as a standard.

**Analysis of Gelatinolytic Activity**—The gelatinolytic activity of enzymes was determined using $^{14}$C-labeled gelatin essentially as described previously (35). The specific activity was calculated by dividing the amount of gelatin degraded per hour (mg or µg) by the nmoles of enzyme used in the reaction. Each assay was conducted in duplicate and the results averaged.
Analysis of Type V Collagenolytic Activity—Various concentrations of enzymes were added to reaction mixtures containing 0.1 \( \mu \text{g/\mu l} \) pepsin-solubilized human placental type V collagen (a kind gift from Dr. Jerome Seyer, VAMC, Norfolk, VA) and 0.8 \( \mu \text{g/\mu l} \) BSA in assay buffer that consisted of 50 mM Tris, pH 7.5, 5 mM CaCl\(_2\), 150 mM NaCl, and 2 \( \mu \text{M ZnCl}_2 \). A control reaction was also conducted that contained no enzymes. Reaction mixtures were incubated for 18 h at 30 °C before being quenched by the addition of (ethylenedinitrilo)tetraacetic acid (EDTA) to final concentration of 10 mM. The specific activities were determined by preparing reaction mixtures as described above and incubating them for 2 h at 30 °C. Reactions were performed in duplicate and a control reaction was also conducted that contained no enzymes. All reaction mixtures were then boiled for 5 min and electrophoresed on 6% SDS-PAGE gels under nonreducing conditions. The amount of collagen degraded was determined by subtracting the integrated intensities of the remaining intact collagen chains after incubation with enzyme from that of the control reaction. The integrated intensities of the protein bands were determined using an Alpha Imager 2000 Documentation and Analysis System (Alpha Innotech Corp., San Leandro, CA). Specific activities were calculated by dividing the amount of collagen cleaved (ng) in 2 h by the pmoles of enzyme used in the reaction. The relationship between the concentration of enzyme and the amount of collagen degraded was linear at the enzyme levels used.

Molecular Modeling—Amino acid substitutions in the crystal structure of neutrophil collagenase (NC) (36) were made using Insight II software (Molecular Simulations Inc., San Diego, CA). Substitutions were made and the energy of the resulting structure was minimized by the Biopolymer and Discover modules, respectively.
Results

Type V Collagenolytic Activity of Chimeric Enzymes—One of the most distinctive characteristics of the gelatinases is their ability to cleave type V collagen. Our previous experiments indicated that the type V collagenolytic activity of gelatinase B is determined, at least in part, by its fibronectin-like domain (29). To assess whether the fibronectin-like domain and the Gel.B/AS are sufficient to provide an MMP with type V collagenolytic activity, the proteolytic properties of an FC molecule containing these motifs were examined. The chimera, FC+Fib+Gel.B/AS, consisted of full length FC with its AS (amino acids 212-254) replaced by the Gel.B/AS (amino acids 395-437) and the fibronectin-like domain of gelatinase B (amino acids 216-394) inserted into its catalytic domain as it is in the gelatinases (Fig. 1).

The chimeric enzyme, latent gelatinase B, and latent FC were expressed in E. coli and purified as described in “Experimental Procedures”. Wild type FC was activated during the purification procedure by APMA and recovered as a 42-kDa species (Fig. 2, lane 1) as expected (37). FC+Fib+Gel.B/AS (Fig. 2, lane 5) was recovered as a latent enzyme of 68-kDa, which is consistent with the addition of the fibronectin-like domain to the FC molecule. This enzyme was processed by APMA to an active 62-kDa species (Fig. 2, lane 6). The activity of these enzymes toward type V collagen was then assessed using APMA-activated gelatinase B as a standard. As shown in Fig. 3, FC, at concentrations up to 5.4 µM (lanes 2 and 3), was not able to cleave type V collagen as previously observed (30). However, at the same
concentration, FC+Fib+Gel.B/AS (Fig. 3, lane 9) was able to degrade 66% of the type V collagen. This indicated that the presence of the fibronectin-like domain and Gel.B/AS is sufficient to endow FC with type V collagenolytic activity.

To examine the individual contributions of the fibronectin-like domain and the Gel.B/AS to this endowed activity, these motifs were individually inserted into the FC molecule as shown in Fig. 1. FC+Fib was composed of FC with the fibronectin-like domain of gelatinase B inserted into its catalytic domain immediately amino-terminal to the AS as it is in the gelatinases. FC+Gel.B/AS consisted of FC with its AS replaced with the Gel.B/AS. These chimeras were also expressed in *E. coli* as latent enzymes. FC+Gel.B/AS, like wild type FC, was activated during the purification procedure by APMA and recovered as a 42-kDa species (Fig. 2, lane 2). FC+Fib (Fig. 2, lane 3) was recovered as a latent enzyme of 68-kDa and processed by APMA to an active 62-kDa species (Fig. 2, lane 4). As shown in Fig. 3, FC+Gel.B/AS, at concentrations of up to 5.4 µM (lanes 4 and 5), was not able to cleave type V collagen, indicating that the substitution of the Gel.B/AS for that of FC is not sufficient to confer type V collagenolytic activity to the enzyme. However, FC+Fib, at the same concentration, was able to digest 57% of the collagen (Fig. 3, lane 7). This demonstrates that the presence of the fibronectin-like domain alone is sufficient to endow FC with type V collagenolytic activity. The specific activities of the chimeras for the collagen substrate were obtained by assaying the enzymes for a short period of time. As shown in Table I, the specific activity of FC+Fib+Gel.B/AS towards type V collagen was 5.3 (ng/h)/pmole. This value is about four times higher than that obtained for FC+Fib (1.4 (ng/h)/pmole). These data indicate that although the Gel.B/AS in itself is not sufficient to furnish FC with type V collagenolytic activity, it substantially contributes to the endowed collagenolytic activity of FC+Fib+Gel.B/AS. Despite the high degree of amino acid conservation in the AS of
MMPs, there are several residues that distinguish the gelatinases from FC (Fig. 4). This suggests that these residues contribute to the efficiency of type V collagen degradation by the gelatinases. It has been shown that the carboxy-terminal domain of FC is able to bind collagen (22). Therefore, the possible contribution of this domain to the type V collagenolytic activity of these chimeras was examined by deleting it from FC+Fib+Gel.B/AS. The truncated chimera, FC+Fib+Gel.B/AS-COOH (Fig. 1), was expressed in *E. coli* and recovered as a 45-kDa latent species (Fig. 2, lane 7) that was processed to an active 39-kDa species (Fig. 2, lane 8) by APMA. The *M*ₐ values for the FC+Fib+Gel.B/AS-COOH species were consistent with the removal of the carboxy-terminal domain from FC+Fib+Gel.B/AS. As shown in Fig. 3, FC+Fib+Gel.B/AS-COOH (*lanes* 10 and 11), like its parental chimera, was able to cleave type V collagen. However, its specific activity against this substrate was 1.7 times higher than that of FC+Fib+Gel.B/AS (Table I). This indicated that the type V collagenolytic activity of FC+Fib+Gel.B/AS was not dependent on the carboxy-terminal domain of FC. In fact, it appears that the presence of this domain had an inhibitory effect on this activity, probably by competing with the fibronectin-like domain for binding to the collagen. This data provides further conclusive evidence that the presence of the fibronectin-like domain is sufficient to endow an MMP with type V collagenolytic activity.

It has been shown that the specific activity of FC depends on the amino-terminus of the active species generated upon treatment with different reagents (38, 39). Treatment of latent FC with APMA generates a mixture of active species with amino-termini of V¹⁰¹LTEG and L¹⁰²TEGN (38). However, activation of the enzyme by plasmin generates an active species having L⁸⁸KVMK as the amino-terminus. It has been demonstrated that the specific activity of the enzyme having the amino-termini of V¹⁰¹LTEG or L¹⁰²TEGN is three times higher than that
with the amino-terminus of L\textsuperscript{84}KVMK (38). To exclude the possibility that the differences in type V collagenolytic activity of the chimeras were due to the generation of different amino-termini upon activation, the amino-termini of the APMA-activated enzymes were determined. Amino-terminal sequence analyses of the active species of FC and FC+Gel.B/AS, enzymes lacking type V collagenolytic activity, yielded a mixture of V\textsuperscript{101}LTEG and L\textsuperscript{102}TEGN sequences, identical to that reported for native FC (38). This indicated that the substitution of the Gel.B/AS for that of FC had no effect on autocatalytic cleavage within the pro-domain. All the amino-terminal sequences of the active species of the chimeras containing the fibronectin-like domain were L\textsuperscript{84}KVMK, which were not processed further by longer activation periods (data not shown). These data indicated that the differences in specific activity of the chimeras containing the fibronectin-like domain were not due to the generation of different amino-termini upon activation. Interestingly, in contrast to APMA-activated FC and FC+Gel.B/AS but similar to plasmin-activated FC and APMA-activated gelatinase B, these chimeras retained the PRCGVPD sequence in the pro-domain containing the conserved cysteine residue that is involved in the “cysteine switch” mechanism of activation (40) (41). These results suggest that in addition to providing the enzyme with type V collagenolytic activity, the inclusion of the fibronectin-like domain in FC has influenced the autocatalytic processing of the pro-domain.

Activity of Chimeric Enzymes Towards Gelatin and a Peptide Substrate, Mca-PLGL(Dpa)AR-NH\textsubscript{2}—The effect of the addition of the fibronectin-like domain and Gel.B/AS on the general proteolytic activities of FC was assessed by determining the catalytic efficiencies of the chimeras against gelatin and the fluorogenic peptide substrate, Mca-PLGL(Dpa)AR-NH\textsubscript{2}. As demonstrated in Table I, addition of the fibronectin-like domain to FC, FC+Fib, decreased its catalytic efficiency ($k_{cat}/K_m$) towards the peptide substrate by four times. The $k_{cat}/K_m$ values of
FC and FC+Fib for this substrate were 4.3 and 1.1 mM⁻¹s⁻¹, respectively. As mentioned previously, it was found that the amino-termini of activated FC and FC+Fib were Val¹⁰¹/Leu¹⁰² and Leu⁸⁴, respectively. According to Suzuki and coworkers (38), the specific activity of FC having amino-termini of Val¹⁰¹/Leu¹⁰² is three fold higher than that having the amino-terminus of Leu⁸⁴. Therefore, the decreased catalytic efficiency of FC+Fib towards the peptide substrate of FC+Fib compared to FC could be due, at least in part, to the generation of a different amino-terminus that renders the enzyme less active. However, substitution of the AS of FC with the Gel.B/AS increased the catalytic efficiency of the enzyme towards this substrate by about four times. The increase in catalytic efficiency caused by replacing the AS of FC with the Gel.B/AS was also demonstrated by the greater $k_{cat}/K_m$ value of FC+Fib+Gel.B/AS compared to FC+Fib (Table I). The $k_{cat}/K_m$ values of FC+Fib+Gel.B/AS and FC+Fib for the peptide substrate were 2.5 and 1.1 mM⁻¹s⁻¹, respectively. The removal of the carboxy-terminal domain from FC+Fib+Gel.B/AS had little effect on its catalytic efficiency towards the peptide substrate (Table I). These data suggest that amino acid residues in the Gel.B/AS are responsible for the observed increases in catalytic efficiency of the enzymes for this substrate.

The addition of the fibronectin-like domain to FC, FC+Fib, increased the specific activity of the enzyme towards gelatin by more than four times (Table I). This is consistent with several previously published reports indicating that the presence of the fibronectin-like domain in the gelatinases substantially increases their capacity to degrade gelatin (26, 28, 29, 42). As seen with the peptide substrate, the replacement of the AS of FC with the Gel.B/AS, FC+Gel.B/AS, caused an increase in the specific activity of the enzyme towards gelatin of more than two fold. Interestingly, the insertion of the fibronectin-like domain and the replacement of the AS of FC with the Gel.B/AS, FC+Fib+Gel.B/AS, had a synergistic effect on the gelatinolytic activity of
the enzyme. The specific activity of FC+Fib+Gel.B/AS towards gelatin was thirteen times higher than that of FC. These data show that the substitution of the AS of FC with that of gelatinase B resulted in chimeric enzymes with greater catalytic efficiencies towards all the substrates examined here compared to the parental enzyme (FC or FC+Fib). These observations suggest that the AS of gelatinase B contains unique amino acid residues that, although may not play a role in substrate specificity, contribute to the enhanced proteolytic activity of the enzyme.

Identification of critical amino acid residues in the AS of gelatinase B involved in catalysis—To identify specific amino acid residues in the Gel.B/AS that may be important for enhancing its proteolytic activity, a partial amino acid sequence alignment from this region of FC, neutrophil collagenase (NC), collagenase 3, and the gelatinases from various species was made and is shown in Fig. 4. This alignment revealed four amino acids in gelatinase B that might be responsible for the enhanced proteolytic activity of the enzyme: Leu 397, Ala 406, Asp 410, and Pro 415. These residues are conserved in all the species variants of gelatinase B but substituted in FC. The significance of these residues to the enzymatic activity of gelatinase B was assessed by individually replacing them with the corresponding residues from FC.

The gelatinase B mutants were expressed in *E. coli* and purified as latent enzymes as described previously (34). The wild type and the mutant enzymes were activated and processed by stromelysin-1 to 42-kDa species within 2 h (data not shown, (43)). This indicated that all of the mutant enzymes were properly folded and catalytically competent. As seen in Table II, the effects of the L397R and A406S mutations on the enzymatic activity of gelatinase B were quite similar. Both mutations resulted in a ~30% decrease in the catalytic efficiency of the enzyme towards the peptide substrate. However, the specific activities of these mutants towards gelatin and type V collagen were reduced by significantly greater extents (59-79%). The replacement of
Leu 397 by Arg reduced the specific activity of the enzyme towards gelatin and type V collagen by 52% and 64%, respectively. This effect was more pronounced for the A406S mutation as it decreased the specific activity of the enzyme towards gelatin and type V collagen by almost 80%. Interestingly, replacement of Asp 410 with Ser increased the catalytic efficiency of gelatinase B towards the peptide substrate by almost two fold and its specific activity towards gelatin by 26%. However, this mutation reduced the specific activity of the enzyme towards type V collagen by 62%. This suggests that Asp 410 is specifically important for the type V collagenolytic activity of the enzyme. The P415I mutation had little effect on the enzyme’s activity towards the peptide substrate and type V collagen but decreased its specific activity for gelatin by 51%. This indicated that a Pro at position 415 is specifically important for the gelatinolytic activity of gelatinase B. The fact that the effects of these mutations on the activities of gelatinase B were specific suggests that the observed decreases in catalytic efficiencies were not due to a conformational change associated with the mutation.
Discussion

We have recently shown that the fibronectin-like domain of gelatinase B is required for its ability to bind and degrade type V collagen (29). To investigate whether this domain in itself is sufficient to endow an MMP with type V collagenolytic activity or other structural elements are also involved, we generated a series of chimeric enzymes containing domains of FC and gelatinase B and studied their substrate specificities. By all indications, these enzymes were properly folded and catalytically competent. All of the chimeras were of the expected $M_r$, capable of autocatalytic processing, and showed a shift in electrophoretic mobility in the presence versus the absence of a reducing agent (data not shown) that is indicative of disulfide bond formation in the proteins. These studies clearly demonstrate, for the first time, that the presence of the fibronectin-like domain in an MMP is sufficient to grant the enzyme type V collagenolytic activity. This is based on the observation that the insertion of the fibronectin-like domain from gelatinase B into FC resulted in a chimeric enzyme, FC+Fib, with the ability to cleave type V collagen. Further replacement of the AS of FC with the Gel.B/AS, FC+Fib+Gel.B/AS, increased the type V collagenolytic activity of the enzyme by about four times. However, this substitution alone, FC+Gel.B/AS, was not enough to confer type V collagenolytic activity to FC. This indicates that although the presence of the Gel.B/AS is not sufficient to provide FC with the ability to cleave type V collagen, it is required for the efficient utilization of this substrate.

The presence of the fibronectin-like domain in FC not only endowed the enzyme with type V collagenolytic activity, it gave FC other characteristics similar to that of gelatinase B. The inclusion of the fibronectin-like domain in the FC molecule increased its gelatinolytic
activity. In addition, interestingly, treatment of the pro-forms of the chimeras containing this domain with APMA led to the formation of active species that, unlike FC but similar to gelatinase B, retained the conserved PRCGVPD sequence in the pro-domain that is involved in maintaining catalytic latency (40, 41, 44, 45). These observations suggest that the presence of the fibronectin-like domain also affects the autocatalytic processing of the pro-domain. This is consistent with the crystal structure of latent gelatinase A indicating that the pro-peptide interacts with the fibronectin-like domain via hydrogen bonding and a salt bridge (46). This interaction may affect the availability of cleavage sites within the pro-domain during activation.

The fibronectin-like domain of the gelatinases consists of three contiguous fibronectin-like type II homology units. However, the function of each of the units with regards to substrate binding and catalysis is still not thoroughly understood. The individual fibronectin type II homology units from gelatinase A have been studied with regards to stability (47) and gelatin binding affinity (48). Although significant differences in the stabilities of individual units were found (47), it appeared that there is interaction between units resulting in a fixed arrangement of the three units. In fact, when various combinations of these units were expressed as β-galactosidase fusion proteins (48), it was shown that they act cooperatively with regards to gelatin-binding. However, according to the recently published X-ray structure of gelatinase A (46), the fibronectin type II homology units do not form a collective binding domain as once thought. Instead, they turn away from each other to take the shape of a “three-pronged fishhook”. The basic structure of each unit comprises a pair of β sheets that are connected by an alpha helix. The β sheets form a hydrophobic pocket that is probably where substrates bind. It appears that binding and positioning of the substrate to the active site of gelatinase A occurs via the third unit. The first and second units may function to correctly position the third with respect
to the active site. The crystal structure of gelatinase B is not available, however, this
arrangement of units would seem counterproductive for this enzyme considering that it was
found that its second unit had a much greater gelatin-binding affinity than the other two (49).
Substrate binding by the second unit would compete with productive substrate binding through
the third unit and severely decrease the catalytic efficiency of the enzyme. Further experiments
are needed to provide information about the role of each of the fibronectin type II homology
units in the gelatinolytic and collagenolytic activities of the gelatinases.

As mentioned above, FC+Gel.B/AS, an FC chimera in which the Gel.B/AS was
substituted for that of FC, did not exhibit type V collagenolytic activity. However, this
substitution resulted in chimeric enzymes with significantly greater catalytic efficiencies towards
all the substrates tested compared to the parental enzyme (FC or FC+Fib). This suggested that
unique amino acids in the AS of gelatinase B are important for enhancing the catalytic efficiency
of the enzyme towards these substrates. An amino acid sequence comparison of the AS of FC
and gelatinase B revealed that Arg 214, Ser 223, Ser 227, and Ile 232 in FC have been replaced
by Leu 397, Ala 406, Asp 410, and Pro 415 in gelatinase B, respectively (Fig. 4). This suggested
that these substitutions are responsible for augmenting the proteolytic activity of gelatinase B.
This was confirmed by the observation that the individual substitutions of Leu 397, Ala 406, Asp
410, and Pro 415 in wild type gelatinase B with the corresponding residues in FC, in most cases,
substantially decreased the catalytic efficiency of the enzyme towards these substrates. Our
mutational analysis indicates that Leu 397 and Ala 406 are important for the general catalytic
activity of the enzyme, while Asp 410 and Pro 415 specifically contribute to enhancing its ability
to cleave type V collagen and gelatin, respectively. These amino acids are conserved in all the
species variants of the gelatinases except for the homologous Asp for Glu substitution in
gelatinase A (Fig. 4). In addition, several of these residues are also conserved in NC and collagenase 3 that also have significantly greater activities towards the peptide substrate and gelatin compared to FC (50) (Fig. 4). Therefore, it is likely that these conserved amino acids function not only to increase the proteolytic activity of the gelatinases but of other MMPs as well.

Although the X-ray structure of gelatinase B is not available, we were able to gain further insight into the roles of these particular amino acids in the proteolytic activity of the enzyme by using molecular modeling of the crystal structure of NC (36) (Fig. 5). NC shares relatively high amino acid sequence identity with gelatinase B in the active site region and also contains Leu and Pro residues at positions 213 and 231 that correspond to Leu 397 and Pro 415 in gelatinase B. In order to make a model of the AS of gelatinase B that contained all four of the amino acids that were found to be important for the activity of the enzyme, a molecular modeling program was used to substitute Ser 222 and Ala 226 in the structure of NC for Ala and Asp residues, respectively (Fig. 5). Leu 397 and Ala 406 were found to be important for the general catalytic activity of gelatinase B. This is based on the observation that replacement of Leu 397 and Ala 406 with Arg and Ser residues, respectively, significantly reduced the ability of the enzyme to cleave all three substrates tested here. Leu 397 is situated at the beginning of the first alpha helix in the AS (Fig. 5). It is generally accepted that the amino acid in this position defines the depth and character of the $S_{1}´$ pocket of MMPs. It has been noted that MMPs, such as NC (36, 51) and stromelysin-1 (52), that contain a Leu at this position have a deep and hydrophobic $S_{1}´$ pocket. However, FC contains a longer Arg in this position that actually points towards the substrate to provide a shallower and less hydrophobic pocket (53). On the basis of these observations, it would be logical to surmise that MMPs with a deeper $S_{1}´$ pocket would be able to
hydrolyze a wider variety of peptide bonds with higher efficiency because of their ability to accommodate more sizable amino acids at the P₁⁻ position. In fact, studies with synthetic peptide substrates (54, 55) have shown that enzymes (gelatinases and NC) with a Leu in the S₁⁻ pocket tend to accommodate more bulky hydrophobic amino acids at the P₁⁻ position than FC. Consistent with this observation is our finding that the type V collagenolytic activity of the L397R mutant was significantly lower than that of the wild type enzyme. Niyibizi et al. (56) have shown that the cleavage of type V collagen by gelatinase B generates peptide fragments having large hydrophobic amino acids, Val and Leu, at the P₁⁻ positions. Therefore, it is reasonable to assume that the replacement of Leu 397 with an Arg reduces the ability of the enzyme to accept these amino acids in the S₁⁻ pocket. Being able to accommodate a wider variety of amino acids at the P₁⁻ position would also facilitate the degradation of a substrate like gelatin in which all the peptide bonds are accessible to the enzyme and numerous sites are cleaved. This is supported by the observation that the substitution of Leu 397 with an Arg also resulted in lower gelatinolytic activity. Ala 406 is located near the end of the first alpha helix in the AS (Fig. 5). The decrease in general catalytic activity caused by the substitution of this residue for a slightly longer Ser suggests that an Ala in this position enhances proteolysis because of its small size.

Site-directed mutagenesis revealed that Asp 410 is specifically important for the type V collagenolytic activity of gelatinase B. Asp 410 is located very close to the catalytic zinc ion (Fig. 5) and may come in close contact with P₂ and P₃ residues of substrate. It is possible that Asp 410 interacted with positively charged residues in type V collagen to provide better contact between enzyme and substrate. It was also noted that the D410S mutant had an approximately two fold higher catalytic efficiency towards the peptide substrate than the wild type enzyme.
Having an uncharged Ser at this position apparently provides for a better interaction with the Leu at the P$_2$ position in the peptide than the negatively charged Asp residue.

The substitution of Pro 415 for an Ile exclusively reduced the ability of gelatinase B to cleave gelatin. Pro 415 (Fig. 5) is located in the middle of a tight turn at the bottom of the AS. Proline residues are often found in flexible regions of proteins, suggesting that Pro 415 may play a role in movement within the AS during the binding and hydrolysis of gelatin that is beneficial to catalysis.

In conclusion, our data has provided a more comprehensive understanding of the structural elements that contribute to the substrate specificity and catalytic efficiency of MMPs. We have shown that the addition of the fibronectin-like domain to an MMP is sufficient to confer type V collagenolytic activity. In addition, our mutational analysis has given insight on the roles of conserved amino acids in the AS of the gelatinases for their important gelatinolytic and type V collagenolytic activities.
Acknowledgments—The authors thank Drs. Harold Tschesche (Lerstuhl für Biochemie, Universität Bielefeld, Bielefeld, Germany) and Jerome Seyer (VAMC, Norfolk, VA) for providing the inhibitory domain of recombinant tissue inhibitor of metalloproteinase-2 and the human placental type V collagen, respectively.
Footnotes

1The abbreviations used are: MMP, matrix metalloproteinase; MT, membrane-type; AS, catalytic zinc-binding active site region; FC, fibroblast collagenase; Gel.B/AS, gelatinase B catalytic zinc-binding active site region; APMA, p-aminophenylmercuric acetate; Mca, (7-methoxycoumarin-4-yl) acetyl; Dpa, [3-(2’,4’-dinitrophenyl)-L-2,3-diaminopropionyl]; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; EDTA, (ethylenedinitrilo)tetraacetic acid; NC, neutrophil collagenase.
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Figure Legends

FIG. 1. **Domain structures of chimeric enzymes.** A, domain structures of gelatinase B, FC, and chimeric enzymes. Construction of the expression vectors for the chimeric enzymes is described under “Experimental Procedures”. B, amino acid sequences near the junctions of domains from gelatinase B and FC in chimeric enzymes. Amino acids from gelatinase B are shown in bold.

FIG. 2. **SDS-PAGE analysis of purified chimeric enzymes.** Purified FC+Fib, FC+Fib+Gel.B/AS, and FC+Fib+Gel.B/AS-COOH were activated by 1 mM APMA for 1 h at 37 °C. FC and FC+Gel.B/AS were activated by 1 mM APMA during purification as described under “Experimental Procedures”. Samples were boiled for 5 min and electrophoresed on a 10% SDS-PAGE gel under reducing conditions. Protein bands were visualized by Coomassie Blue staining. *Lane 1*, activated FC; *lane 2*, activated FC+Gel.B/AS; *lane 3*, latent FC+Fib; *lane 4*, activated FC+Fib; *lane 5*, latent FC+Fib+Gel.B/AS; *lane 6*, activated FC+Fib+Gel.B/AS; *lane 7*, latent FC+Fib+Gel.B/AS-COOH; *lane 8*, activated FC+Fib+Gel.B/AS-COOH. Positions of molecular mass standards are shown to the left of the gel.

FIG. 3. **Analysis of Type V collagenolytic activity by chimeric proteins.** Various concentrations of enzymes were mixed with 1 µg/µl of pepsin-solubilized human placental type V collagen and 0.8 µg/µl BSA in assay buffer that consisted of 50 mM Tris, pH 7.5, 5 mM CaCl$_2$, 150 mM NaCl, and 2 µM ZnCl$_2$. A control reaction containing no enzyme was also
included. Reaction mixtures were incubated for 18 h at 30 °C before being quenched by 10 mM EDTA. Reactions were then boiled for 5 min and electrophoresed on a 6% SDS-PAGE gel under nonreducing conditions. Protein bands were visualized by Coomassie Blue staining. *Lane* 1, type V collagen alone; *lane* 2, FC, 2.7 µM; *lane* 3, FC, 5.4 µM; *lane* 4, FC+Gel.B/AS, 2.7 µM; *lane* 5, FC+Gel.B/AS, 5.4 µM; *lane* 6, FC+Fib, 2.7 µM; *lane* 7, FC+Fib, 5.4 µM; *lane* 8, FC+Fib+Gel.B/AS, 2.7 µM; *lane* 9, FC+Fib+Gel.B/AS, 5.4 µM; *lane* 10, FC+Fib+Gel.B/AS-COOH, 1 µM; *lane* 11, FC+Fib+Gel.B/AS-COOH, 2 µM; *lane* 12, gelatinase B, 22 nM; *lane* 13, gelatinase B, 43 nM. Positions of molecular mass standards are shown to the left of the gel.

FIG. 4. **Sequence alignment of amino acids in the AS of FC and gelatinases from various species.** Residues that distinguish the gelatinases from FC are shown in bold.

FIG. 5. **Location of conserved amino acids in the AS of gelatinase B.** This figure was modeled after the X-ray structure of NC (36). To make a model that highlighted all four of the amino acids that were found to be important for the activity of gelatinase B, a molecular modeling program (see “Experimental Procedures”) was used to substitute Ser 222 and Ala 226 in the structure of NC for Ala and Asp residues, respectively. Leu 397, green; Ala 406, white; Asp 410, yellow; Pro 231, orange.
Table I

*Kinetic parameters of chimeric enzymes*

Experimental details are described under “Experimental Procedures”. All enzymes were activated by APMA. Values are listed ± standard error (S.E). ND, not determined.

| Enzyme                      | Mca-PLGL(Dpa)AR-NH₂ | Gelatin     | Type V Collagen |
|-----------------------------|---------------------|-------------|-----------------|
|                             | $k_{cat}/K_m$, mM⁻¹s⁻¹ | (µg/h)/nmole | (ng/h)/pmole    |
| FC                          | 4.3 ± 0.6           | 272 ± 9     | ND              |
| FC+Gel.B/AS                 | 16 ± 3              | 692 ± 25    | ND              |
| FC+Fib                      | 1.1 ± 0.1           | 1,217 ± 50  | 1.4 ± 0         |
| FC+Fib+Gel.B/AS             | 2.5 ± 0             | 3,440 ± 283 | 5.3 ± 0.6       |
| FC+Fib+Gel.B/AS-COOH        | 2.3 ± 0.1           | 2,130 ± 88  | 9.2 ± 0.3       |
| gelatinase B                | 216 ± 16            | 90,526 ± 8,506 | 549 ± 32       |
| gelatinase B                |                     |             |                 |
Table II  

*Kinetic parameters of gelatinase B mutants*

Experimental details are described under “Experimental Procedures”. All enzymes were activated by stromelysin-1. Values are listed as a percentage of the value for wild type gelatinase B ± standard error (S.E.). Absolute values are listed in parentheses.

| Enzyme    | $k_{cat}/K_m$, mM$^{-1}$s$^{-1}$ | Specific Activity, (mg/h)/nmole | Specific Activity, (ng/h)/pmole |
|-----------|---------------------------------|---------------------------------|---------------------------------|
| gelatinase B | 100 ± 10, (184)             | 100 ± 3, (101)          | 100 ± 3, (521)                  |
| L397R     | 68 ± 2                         | 48 ± 3                     | 36 ± 1                          |
| A406S     | 71 ± 9                         | 21 ± 0                     | 24 ± 0                          |
| D410S     | 180 ± 11                       | 126 ± 13                   | 38 ± 3                          |
| P415I     | 93 ± 5                         | 49 ± 4                     | 93 ± 3                          |
B

FC+Gel.B/AS

NH₂..........E²⁰⁹ Y²¹⁰ N²¹¹ L³⁹⁵ F³⁹⁶ L³⁹⁷ ..........D⁴³⁵ V⁴³⁶ N⁴³⁷ G²⁵⁵ I²⁵⁶ Q²⁵⁷ ..........COOH

FC+Fib

NH₂..........E²⁰⁹ Y²¹⁰ N²¹¹ V²¹⁶ V²¹⁷ V²¹⁸ ..........A⁴⁰⁰ H⁴⁰¹ E⁴⁰² L²²⁰ G²²¹ H²²² ..........COOH

FC+Fib+Gel.B/AS

NH₂..........E²⁰⁹ Y²¹⁰ N²¹¹ V²¹⁶ V²¹⁷ V²¹⁸ ..........D⁴³⁵ V⁴³⁶ N⁴³⁷ G²⁵⁵ I²⁵⁶ Q²⁵⁷ ..........COOH

FC+Fib+Gel.B/AS-COOH

NH₂..........E²⁰⁹ Y²¹⁰ N²¹¹ V²¹⁶ V²¹⁷ V²¹⁸ ..........N⁴³⁷ G⁴³⁸ R⁴³⁹ H⁴⁴⁰ L⁴⁴¹ Y⁴⁴² G⁴⁴₄ COOH
| Enzyme Type                     | Sequence             |
|--------------------------------|----------------------|
| human FC                       | RVAHELGHSLGLSHSTDIGAL|
| human NC                       | LVAAHEFGHSLGLASHSDPGAL|
| human collagenase 3            | LVAAHEFGHSLGLDHSKDPGAL|
| human gelatinase B             | LVAAHEFGHALGLDHSSPEAL|
| mouse gelatinase B             | LVAAHEFGHALGLDHSSPEAL|
| rat gelatinase B               | LVAAHEFGHALGLDHSSPEAL|
| bovine gelatinase B            | LVAAHEFGHALGLDHTSPEAL|
| human gelatinase A             | LVAAHEFGHAMGLEHSQDPGAL|
| chicken gelatinase A           | LVAAHEFGHAMGLEHSEDPGAL|
| mouse gelatinase A             | LVAAHEFGHAMGLEHSDPGAL|
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*J. Biol. Chem.* published online May 22, 2000

Access the most updated version of this article at doi: [10.1074/jbc.M003936200](http://dx.doi.org/10.1074/jbc.M003936200)

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