The Structural Basis for the Elastolytic Activity of the 92-kDa and 72-kDa Gelatinases

ROLE OF THE FIBRONECTIN TYPE II-LIKE REPEATS*

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J. Michael Shipley‡, Glenn A. R. Doyle‡, Catherine J. Fliszar§, Qi-Zhuang Ye†, Linda L. Johson, Steven D. Shapiro†, Howard G. Welgus§, and Robert M. Senior**

From the Respiratory and Critical Care Division and the Division of Dermatology, Departments of Medicine and Cell Biology and Physiology, Washington University School of Medicine at Jewish Hospital, St. Louis, Missouri 63110 and the Department of Biochemistry, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105

Several matrix metalloproteinases, including the 92-kDa and 72-kDa gelatinases, macrophage metalloelastase (MME), and matrilysin degrade insoluble elastin. Because elastolytically active MME and matrilysin consist only of a catalytic domain (CD), we speculated that the homologous CDs of the 92-kDa and 72-kDa gelatinases would confer their elastolytic activities. In contrast to the MME CD, the 92 and 72 CDs expressed in Escherichia coli (lacking the internal fibronectin type II-like repeats) had no elastase activity, although both were gelatinolytic and cleaved a thiopeptolide substrate at rates comparable to the full-length gelatinases. To test the role of the fibronectin type II-like repeats in elastolytic activity, we expressed the 92-kDa gelatinase CD with its fibronectin type II-like repeats (92 CD/FN) in yeast. 92 CD/FN degraded insoluble elastin with activity comparable to full-length 92-kDa gelatinase. 92 and 72 CDs lacking the fibronectin type II-like repeats did not bind elastin, whereas the parent enzymes and 92 CD/FN did bind elastin. Furthermore, recombinant 92-kDa fibronectin type II-like repeats inhibited binding of the 92-kDa gelatinase to elastin. We conclude that the 92- and 72-kDa gelatinases require the fibronectin type II-like repeats for elastase activity.

Elastin is an extracellular matrix protein composed of highly cross-linked, hydrophobic tropoelastin monomers which provides resilience to elastic fibers. The hydrophobicity and extensive cross-linking of tropoelastin monomers result in an insoluble elastic fiber which is highly resistant to proteolysis (1). Thus, under normal physiologic conditions, elastin undergoes minimal turnover (2). However, certain pathologic situations, including pulmonary emphysema (3) and abdominal aortic aneurysm (4), are characterized by proteolytic destruction of elastin fibers. The involvement of serine proteases in such pathologies has long been suspected. More recently, participation of cysteine proteinases (5) and matrix metalloproteinases (MMPs, 6–8) in these diseases has been proposed.

The MMPs comprise a gene family that collectively is capable of degrading all components of extracellular matrix in physiologic and pathologic states (9, 10). As presently recognized, this family consists of fibroblast (11), neutrophil (12), and breast carcinoma-derived (13) collagenases, three stromelysins, 92-kDa and 72-kDa gelatinases, macrophage metalloelastase (MME, MMP-12), matrilysin, and a recently described 66-kDa membrane-type metalloproteinase (14). These enzymes are organized into homologous structural domains, with some differences in domain composition and number. All members share a zinc domain, containing the conserved PRGCGPD motif involved in enzyme latency, and a zinc-binding CD. Most members also contain a hemopexin-like domain at their C terminus, the exception being matrilysin, which lacks this domain completely. Unique to the 72-kDa and 92-kDa gelatinases is an additional domain composed of three fibronectin type II-like repeats inserted in tandem within the zinc-binding CD. The 92-kDa gelatinase also contains an a2(V) collagen-like domain not found in any of the other family members.

The issue of substrate specificity has received considerable attention recently in MMP biology. The determinants which confer substrate specificity to these enzymes appear to be localized within discrete structural domains. For example, the ability of the collagenases to degrade triple-helical collagen requires the presence of the C-terminal hemopexin-like domain (15–17). In contrast, the stromelysins degrade a variety of substrates in a manner which is independent of the C-terminal hemopexin-like domain (16, 18–20). Likewise, the C-terminal domain of the 92-kDa and 72-kDa gelatinases is not required for these enzymes to degrade gelatin (21, 22). However, the fibronectin type II-like repeats within the CDs of the gelatinases confer high affinity binding of these enzymes to gelatin (23–26).

Four members of the MMP family have the capacity to degrade insoluble elastin. These are the 92-kDa and 72-kDa gelatinases (27, 28), macrophage metalloelastase (7, 8), and matrilysin (28). In their fully processed active forms, matrilysin and MME consist only of a zinc-binding CD. Because the zinc-binding CDs of other MMPs such as collagenase and...
Elastolytic Activity of 92-kDa and 72-kDa Gelatinases

Strategies cleaved by the parent enzymes. Inclusion of the fibronectin type II-like repeats within the CD of the 92-kDa gelatinase fully restored its elastolytic activity. We conclude that the structural determinants required for elastase cleavage by the gelatinases are distinct from those of matrilysin or MME.

MATERIALS AND METHODS

Reagents

Bovine ligament elastin, obtained from Elastin Products, Owensville, MO, was radiolabeled with [3H]iodoacetylborohydride (DuPont NEN) to a specific activity of ~1000 cpm/ng (29). Isopropl-1-thio-β-galactoside, heparin, agarose, heparin agarose, 5-5′dithio-bis-(2-nitrobenzolic acid) (DTNB), trypsin, soybean trypsin inhibitor, and Luria broth (LB) were obtained from Sigma. Acc-Pro-Leu-Gly-S-Leu-Leu-Gly-OEt (thiopentide) was obtained from Bachem Biosience, King of Prussia, PA. 72-kDa gelatinase, recombinantly expressed in vaccinia virus and free of TIMP-2 (30), was kindly provided by W. Stetler-Stevenson, NCI, National Institutes of Health, Bethesda, MD. Bovine dermal type I collagen (Vitrogen 100) was from CELLTRIX, Palo Alto, CA. The Pichia pastoris expression system was obtained from Invitrogen. Truncated stromelysin-1 was prepared by expression of the CD in Escherichia coli as described previously (20). TIMP-1 was from Synergen (Boulder, CO). The BCA reagent for determining protein concentration was from Pierce.

Expression, Purification, and Activation of the Full-length 92-kDa Gelatinase

92-kDa gelatinase was expressed in E1A- and 92-kDa gelatinase-transfected fibroblasts and purified to homogeneity as described previously (31). In this system, the 92-kDa enzyme is obtained free of TIMP-1. The 92-kDa progelatinase was activated with a truncated version of stromelysin-1 as previously reported (32). Full conversion of the 92-kDa gelatinase to its active form was assessed by SDS-PAGE. Full catalytic activity was established by assays against gelatin and elastin as compared to previous batches of the enzyme.

Activation of the 72-kDa Gelatinase

The 72-kDa progelatinase was activated with 1 mM aminophenylmercuric acetate at 37°C for 2 h (33). Complete conversion of the 72-kDa proenzyme to its active form was assessed by SDS-PAGE.

Construction of the CDs of the 92-kDa Gelatinase, 72-kDa Gelatinase, and MME

92 CD—The fibronectin type I-like repeats of the 92-kDa gelatinase, which split the CD, were deleted by recombinant PCR (34). The region of the CD 5′ to the fibronectin type I-like repeats was amplified with the primers 5'-CTGTCGCACTGAGCGGACATCAATTTGAG-3' and 5'-CGAGGAAACACTGATCAGGCTCCCTGGAGG-3'. The introduction of the NcoI site in the 5′ primer created an additional Met-Gly dipeptide at the N terminus in order to start translation at this point within the cDNA. The region 3′ to the fibronectin type I-like repeats was amplified with the primers 5'-CCCTGGGCGACGGCGGCTTGATA - CAGTGTGGTCTGAG-3' and 5'-CAACTCTAGGATCAGGGCTCCG-3'. A stop codon was placed in the 3′ primer after residue 443, thereby eliminating the collagen type V and hemopexin-like domains. After gel purification of both PCR products, the products were annealed to each other by virtue of the complementary design of the internal PCR primers and amplified using the outside primers. The final PCR product was digested with NcoI and XhoI and subcloned into the pET-14b vector (Novagen, Madison, WI) for expression in E. coli. The resulting fragment was subcloned into the pHL-S1 yeast expression vector. The 92 CD contained residues 107–216 of the parent molecule fused to residues 391–443. Constructs were confirmed by DNA sequencing.

The resulting 92 CD construct in pET-14b was transformed into the E. coli BL21(DE3) strain (Novagen) for expression. Colonies were grown in 10 ml of LB media containing 50 μg/ml ampicillin to log phase and induced with 0.4 mM isopropyl-1-thio-β-galactoside for 4 h. After centrifugation, the pellet was resuspended in 2.5 ml of 50 mM Tris, pH 7.5, 10 mM CaCl2, 30 mM NaCl and sonicated 5 × 15 s on ice. 8 mM deionized urea in the same buffer was added to a final concentration of 2 M urea and the extract was rocked gently at 4°C overnight, prior to centrifugation at 12,000 × g for 10 min in a Sorval SS34 rotor. The sample was dialyzed successively against 4 M, 2 M, and 1 M urea in the same buffer containing 20 μM ZnCl2 and 0.05% Brij and finally against urea-free buffer (50 mM Tris, pH 7.5, 10 mM CaCl2, 30 mM NaCl, 0.05% Brij). Initial conversion of the fibronectin type II-like repeats to the active form was assessed by SDS-PAGE. The region of the NcoI site in the 5′ primer creates an additional Met-Gly dipeptide at the amino terminus in order to start translation at this point within the cDNA. The 3′ primer replaces Gly392 with a stop codon, eliminating the carboxyl-terminal hemopexin-like domain. The resulting protein contains residues 93–265 of the parent enzyme. This protein was expressed and purified as described for the 92 CD except that the zinc chelate chromatography step was replaced by chromatography over a 1-mL heparin-agarose column. The protein was expressed in a buffer containing 50 mM Tris, pH 7.5, 10 mM CaCl2, 1 mM NaCl, 0.05% Brij and dialyzed against the same buffer containing 30 mM NaCl.

Expression of the CD of the 92-kDa Gelatinase Containing the Fibronectin Type II-like Repeats (92 CD/FN) and the Fibronectin Type II-like Repeats Alone in Yeast

PCR was used to generate the CD of the 92-kDa gelatinase containing the fibronectin type II-like repeats as well as the repeats themselves. The 92 CD/FN (amino acids 18–444) was amplified using the forward primer 5'-CACGAAATTCTCGTGCCCCACAG-3' and reverse primer 5'-CACGAAATTCTCGTGCCCCACAG-3'. Both primers contained EcoRI sites for subcloning. The reverse primer incorporated a conversion of Pro465 to Ala. The fibronectin type II-like repeats alone (amino acids 217–391) were amplified with the forward primer 5'-CTCGCGTGGAGTTCAACCCATGGATGAG-3' and the reverse primer 5'-CAACTCTAGGGATCAGGGCAGAA-3'. Both primers incorporated XhoI sites for subcloning, and the reverse primer incorporated the conversion of Gly392 to a stop codon. The PCR products were subcloned into the pHL-S1 yeast expression vector. The 92 CD/FN contains an additional Arg-Glu dipeptide at its N terminus after processing of the yeast acid phosphatase signal sequence, and the fibronectin type II-like repeats contain an additional Arg residue.

Expression constructs were transformed into P. pastoris strain GS115 by the method described by the manufacturer. Colonies were screened for high level protein expression and secretion by Western analysis upon induction with methanol. Briefly, yeast donors were grown to a high density in minimal glycerol medium for 2 days, then shifted to culture in 1/5 volume of inducing minimal methanol complex media, containing 0.5–5% methanol, and allowed to grow for 3 days. The conditioned medium was collected, equilibrated to gelatin column buffer (10 mM Tris, pH 7.5, 5 mM CaCl2, 150 mM NaCl), and 92-kDa gelatinase proteins were purified by affinity chromatography over a gelatin-agarose column (Sigma). Gelatin-agarose columns were loaded with sample, washed with 10 volumes of column buffer followed by 20 volumes of high salt column buffer (10 mM Tris, pH 7.5, 5 mM CaCl2, 1 mM NaCl). Bound protein was eluted with high salt buffer containing 10% dimethyl sulfoxide (v/v). Eluted fractions were analyzed for protein by Western blot. Fractions containing the appropriate proteins were pooled and dialyzed to completion.

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against elastin assay buffer (50 mM Tris, pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.02% Brij) containing 20 μM ZnCl₂. The 92 CD/FN was activated using truncated stromelysin as described previously (32).

Assays

Protein—Protein concentrations were determined using the BCA method.

Elastase Activity—Elastase activity was determined by quantifying the solubilization of insoluble ³H-labeled elastin (29). Reactions were carried out at 37 °C in 50 mM Tris HCl, pH 7.5, 10 mM CaCl₂, 150 mM NaCl, 0.02% Brij, in a final volume of 100 μl with an excess of elastin (~70 μg). Following centrifugation, radioactivity released into the supernatant was collected and counted. Buffer blanks were subtracted to determine net values. Initial velocities were calculated in ranges which were linear over time and enzyme concentration. When assaying 92-kDa gelatinase and 92 CD/FN, blanks also contained stromelysin-1 to correct for elastin-degrading functions that are not inherently obvious by inspection of the primary sequences.

Comparison of the CD Mutants—To determine whether the CD mutants purified and reconstituted from E. coli were enzymatically competent and elastolytic, three substrates were used. First, we employed a previously described thiopetolide substrate assay (35). Fig. 3 demonstrates that recombinantly expressed 92 CD, 72 CD, and MME CD are at least as active as the thiopetolide activity in control reaction mixtures in which the enzyme was not exposed to insoluble elastin. Binding to elastin was expressed as the percent reduction in thiopetolide activity produced by incubation with elastin. The fibronectin type II-like repeats of the 92-kDa gelatinase (6.6 × 10⁻⁶ τ, 13-fold excess over enzyme) were included in some experiments. In these experiments, the repeats were preincubated with elastin for 10 min at room temperature prior to the addition of enzyme.

RESULTS

Comparison of the CDs of Elastolytic and Nonelastolytic MMPs—An alignment of the amino acid residues of the MME CD, the gelatinase CDs (lacking the fibronectin type II-like repeats), and the CD of collagenase is shown in Fig. 1. The CDs of the elastolytic MMPs (MME and the gelatinases) share no more homology with each other (49–60%) than they do with fibroblast collagenase (53–55%), a nonelastolytic MMP. Consequently, regions within the CDs that might be involved in elastin-degrading functions are not inherently obvious by inspection of these primary sequences.

Expression and Purification of Recombinant Proteins—The purified 92 CD, 72 CD, and MME CD proteins expressed in E. coli are shown in Fig. 2. Purification as described under "Materials and Methods" yielded a protein band of Mᵋ ~ 20,000 for each construct.

Enzymatic Activity of CD Mutants—To determine whether the CD mutants purified and reconstituted from E. coli were enzymatically competent and elastolytic, three substrates were used. First, we employed a previously described thiopetolide substrate assay (35). Fig. 3 demonstrates that recombinantly expressed 92 CD, 72 CD, and MME CD are at least as active as the thiopetolide substrate as the full-length enzymes.

A second substrate tested was gelatin prepared from type I collagen. Although the fibronectin type II-like repeats of the gelatinases have been implicated in gelatin binding, it has been shown that removal of these repeats from the 72-kDa gelatinase results in an enzyme with reduced, but detectable, gelatinolytic activity (24). Similarly, the 92-kDa gelatinase actively degrades gelatin in the presence of 10% dimethyl sulfoxide, which disrupts the binding of the fibronectin type II-like domain to gelatin (23). However, in each of these studies, the gelatinase constructs contained intact carboxyl-terminal domains. Therefore, we assessed whether the 92 CD and 72 CD, which lack both the fibronectin type II-like domains and the carboxyl-terminal domains, were capable of degrading gelatin. Fig. 4A demonstrates that both CDs were readily capable of digesting gelatin, although the activity of the 92 CD appeared...
greater than that of the 72 CD. The gelatinolytic activity was specific to the gelatinase CDs, as it was completely inhibited by TIMP-1 (data not shown). As reported for the 72 CD relative to the full-length 72-kDa gelatinase (25), the 92 CD exhibits a reduced capacity to degrade gelatin relative to the full-length activated 92-kDa gelatinase. However, inclusion of the fibronectin type II-like repeats into the 92 CD restored full gelatinolytic activity (data not shown). In fact, this construct is more active against gelatin than the full-length 92-kDa gelatinase.

Finally, to determine whether the CDs of the gelatinases were elastolytic, the CDs were tested for their ability to degrade insoluble elastin. Although the native gelatinases and the MME CD were elastolytic, both the 92 CD and 72 CD were completely inactive against insoluble elastin (Fig. 5). Even very high concentrations of the gelatinase CDs (in excess of 100 μg/ml) produced only barely detectable activity against elastin (data not shown).
elastolytic activity from expressing clones was inhibitable by TIMP-1 (data not shown).

Binding of the Gelatinases to Elastin Occurs through the Fibronectin Type II-like Repeats—To determine whether the lack of elastase activity of the 92 CD and 72 CD proteins related to elastin binding, we compared the elastin binding potential of these constructs with that of the full-length parent proteins (Fig. 6). As shown in Fig. 6A, the full-length 92-kDa and 72-kDa gelatinases, as well as the MME CD, demonstrated substantial binding to insoluble elastin. In contrast, the 92 CD and 72 CD showed virtually no binding to insoluble elastin. Interstitial collagenase, a MMP devoid of elastase activity, also did not bind to elastin in this assay. This suggested that binding of the gelatinases, but not of MME, to elastin might require the fibronectin type II-like or carboxyl-terminal domains. Steffensen et al. (36) recently demonstrated that the 72-kDa gelatinase fibronectin type II-like domains bind to elastin. Indeed, inclusion of the fibronectin type II-like repeats in the 92 CD (92 CD/FN) restores binding to elastin. To further investigate the role of the fibronectin type II-like repeats in elastin binding, we expressed the tandem repeats of the 92-kDa gelatinase in a yeast system. Fig. 6B shows that exogenously added recombinant fibronectin type II-like repeats inhibited binding of the full-length 92-kDa gelatinase to elastin, while having little effect on the binding of the MME CD. We conclude that the fibronectin type II-like repeats of the 92-kDa gelatinase participate in the binding of this enzyme to insoluble elastin, and that MME and the gelatinases represent two distinct classes of elastolytic MMPs.

**DISCUSSION**

The property of degrading insoluble elastin is restricted to select members of the MMP family. Previous studies have demonstrated that interstitial collagenases and stromelysins have virtually no elastolytic activity, whereas the 92-kDa and 72-kDa gelatinases, MME, and matrilysin are elastolytic (7, 8, 27, 28). However, the overall amino acid homology among the CDs of the elastolytic MMPs does not distinguish them from the nonelastolytic members of the family (Fig. 1). In fact, the elastolytic MMPs are as similar to fibroblast collagenase, a nonelastolytic enzyme, as they are to each other. Consequently, regions of these enzymes which may be involved in elastin...
binding and degradation are not readily apparent by inspection.

Because MME and matrilysin in their activated forms are functional elastases consisting only of the typical catalytic zinc-binding domain, we speculated that the CDs of the 92-kDa and 72-kDa gelatinases would be functional against elastin. Accordingly, we expressed constructs in E. coli encoding the CDs of the 92-kDa gelatinase, the 72-kDa gelatinase, and MME. The gelatinase CDs lacking the fibronectin type II-like repeats which split the CD in the native enzymes were devoid of elastase activity although they did display catalytic activity against both a synthetic thiopeptilide substrate and gelatin, indicating that they were enzymatically active. The MME CD expressed in the same system was enzymatically active, showing that this activity can be reconstituted in an E. coli expression system. Restoration of the fibronectin type II-like repeats into the CD of the 92-kDa gelatinase restored elastin-binding and elastin-degrading activity. This finding indicated that the fibronectin type II-like repeats in the 92-kDa gelatinase are necessary for the elastolytic activity of this enzyme. It also revealed that the carboxyl-terminal type V collagen-like and hemopexin-like domains of the native enzyme are not required for elastase activity. These carboxyl-terminal domains are also not required for gelatin degradation by either of the gelatinases (21, 22).

It may be argued that the CDs of the gelatinases contain the elements necessary for elastin binding and degradation, and that removal of the fibronectin type II-like domain results in a steric alteration which prohibits these elements from acting in concert. We believe this scenario is unlikely for the following reasons. First, the CDs retain activity on other substrates. More importantly, exogenous fibronectin type II-like domains strongly inhibit binding of the full-length enzyme to elastin. These data, coupled with the observation of Steffensen et al. (36) that the fibronectin type II-like repeats of the 72-kDa gelatinase bind directly to elastin, strongly suggest that the gelatinases bind elastin through the fibronectin type II-like domain.

The requirement of the fibronectin type II-like domains for elastase activity was unexpected, but there is precedence for the participation of these repeats in matrix binding. Several investigators have shown that these repeats confer high affinity binding to type IV collagen and type I gelatin (23-26). However, there is some question as to whether gelatin binding through the fibronectin type II-like domain is rate-limiting for catalysis. A 72-kDa gelatinase mutant lacking the fibronectin type II-like repeats had only 10% the gelatinolytic activity of the native enzyme, suggesting that this was a rate-limiting event (24). Likewise, the 72 CD also has a reduced ability to degrade gelatin relative to the native enzyme (25). This result is in contrast to the data of Collier et al. (23) regarding the 92-kDa gelatinase (23). These investigators demonstrated that Me$_2$SO concentrations which inhibit $>$90% of the binding of the recombinant fibronectin type II-like domain to gelatin inhibit only $\sim$20% of its gelatinolytic activity, suggesting that binding of the 92-kDa gelatinase to gelatin through the fibronectin type II-like repeats is not rate-limiting for catalysis. We found that the 92 CD has only 20-30% the gelatinolytic activity of the full-length gelatinase (Fig. 4B). However, restoration of the fibronectin type II-like repeats resulted in gelatinase specific activity which was greater than that of the full-length 92-kDa gelatinase (data not shown). These data suggest that gelatin binding through the fibronectin type II-like domain is rate-limiting for catalysis.

With respect to elastin, the ability of the fibronectin type II-like repeats to play a role in elastase activity received sup-

port from a recent study showing that the fibronectin type II-like repeats of the 72-kDa gelatinase themselves bind elastin with high affinity (36). In this report, we present evidence indicating that the fibronectin type II-like repeats of the 92-kDa gelatinase bind elastin. First, the CD of this enzyme lacking these repeats does not bind to elastin. Second, the CD containing the repeats both binds to and degrades elastin. Third, exogenous recombinant 92-kDa gelatinase fibronectin type II-like repeats inhibit the binding of the 92-kDa gelatinase to elastin. Collier et al. (23) demonstrated that the second fibronectin type II-like repeat of the 92-kDa gelatinase is responsible for most of the elastin binding and speculated that the other repeats may be involved in binding to other matrix substrates. We are currently investigating the role of the individual repeats of the 92-kDa gelatinase in elastin binding.

The necessity of the fibronectin type II-like repeats of the gelatinases for elastolytic activity indicates that the gelatinases differ from MME and matrilysin in their mechanism of elastolysis. Moreover, the inability of exogenous fibronectin type II-like repeats to inhibit binding of the MME CD to elastin while they do inhibit binding of the 92-kDa gelatinase suggests that the gelatinases and MME bind to different sites on the elastin molecule. Interestingly, the 92-kDa gelatinase and MME have different cleavage site preferences within insoluble elastin. The binding of the gelatinases to elastin through the fibronectin type II-like repeats is an attractive model since the repeats interrupt the CD immediately adjacent to the active site, thereby potentially bringing the active site into close proximity with the substrate.

In conclusion, these studies reveal unexpected complexity in the domain requirements of MMPs having elastolytic activity. There appear to be two classes of elastolytic MMPs: 1) macrophage metalloelastase and matrilysin that require only the CD for elastin binding and degradation, and 2) the gelatinases which require the fibronectin type II-like repeats for elastin binding. Thus, structure/function relationships that apply to one elastolytic MMP cannot be assumed to apply to other elastolytic MMPs.

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