Functional basis for the overlap in ligand interactions and substrate specificities of matrix metalloproteinases-9 and -2

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The MMPs (matrix metalloproteinases) MMP-9 and -2 each possess a unique CBD (collagen-binding domain) containing three fibronectin type II-like modules. The present experiments investigated whether the contributions to ligand interactions and enzymatic activities by the CBD of MMP-9 (CBD-9) corresponded to those of CBD in MMP-2 (CBD-2). The interactions of recombinant CBD-9 with a series of collagen types and extracellular matrix molecules were characterized by protein–protein binding assays. CBD-9 bound native and denatured type I, II, III, IV and V collagen, as well as Matrigel and laminin, with apparent K_d values of $(0.1–6.8) \times 10^{-7}$ M, which were similar to the K_d values for CBD-2 $[(0.2–3.7) \times 10^{-7}$ M]. However, CBD-9 bound neither native nor denatured type VI collagen. We also generated two modified MMPs, MMP-9^{E402A} and MMP-2^{E404A}, by site-specific mutations in the active sites to obtain enzymes with intact li-

gand binding, but abrogated catalytic properties. In subsequent competitive binding assays, CBD-9 and MMP-9^{E402A} inhibited the interactions of MMP-2^{E404A} and, conversely, CBD-2 and MMP-2^{E404A} competed with MMP-9^{E402A} binding to native and denatured type I collagens, pointing to shared binding sites. Importantly, the capacity of CBD-9 to disrupt the MMP-9 and MMP-2 binding of collagen translated to inhibition of the gelatinolytic activity of the enzymes. Collectively, these results emphasize the essential contribution of CBD-9 to MMP-9 substrate binding and gelatinolysis, and demonstrate that the CBDs of MMP-9 and MMP-2 bind the same or closely positioned sites on type I collagen.

Key words: collagen, gelatinolysis, matrix metalloproteinase (MMP).

INTRODUCTION

The MMP (matrix metalloproteinase) family of endopeptidases is composed of at least 23 members that collectively are capable not only of cleaving most major macromolecules of the extracellular matrix [1,2], but also play key roles in activation of growth factors and chemokines [3,4]. MMPs play important roles in normal tissue homeostasis [5], and their activity is up-regulated in pathologies such as arthritis [6] and periodontal disease [7]. The domain structure is remarkably similar between the MMPs [8,9]. Three common domains include: (1) the catalytic domain (≈ 170 amino acids), which contains a zinc-binding active-site motif and is essential for the proteolytic activity of MMPs [10]; (2) the prodomain (≈ 80 amino acids), from which a conserved cysteine interacts with the catalytic zinc to maintain the latency of pro-MMPs [11]; and (3) the hemopexin-like domain, which plays a functional role in substrate binding [12] and in the interaction with the TIMPs (tissue inhibitors of metalloproteinases). However, only the two gelatinases MMP-9 and -2 have each of the three 58-amino-acid fibronectin-like type II modules inserted in their catalytic domains. These modules form CBDs (collagen-binding domains), which interact specifically with collagens and other extracellular matrix molecules and are critical for the positioning of substrates for cleavage [13–16].

In addition to similar domain structures, MMP-9 and -2 have very similar substrate specificities, targeting collagen types IV, V, VII and X, elastin, fibrin and fibrinogen, fibronectin, interleukin-1 and the $\alpha 1$ -proteinase inhibitor [1–3]. Among the features that distinguish MMP-9 and -2, MMP-2 is synthesized constitutively

by mesenchymal cells such as fibroblasts, macrophages, endothelial and epithelial cells, and is activated by membrane-type MMPs (MT-MMPs) [17,18], whereas MMP-9 is produced mainly by inflammatory cells, such as monocytes/macrophages, neutrophils and eosinophils, and is activated by other MMPs, such as MMP-3 [19]. The expression of MMP-9 is highly regulated compared with that of MMP-2.

Extensive characterization of the CBD-2 ligand-binding interactions have demonstrated that the CBD is responsible for virtually all the collagen-binding properties of MMP-2 [14,20], and binds several native and denatured collagen types, as well as elastin and heparin [1,14]. Recently, we showed that the binding of gelatin via the CBD-2 is required for degradation of denatured type I collagen α -chains, but not short collagen peptide substrates [15]. Furthermore, competitive disruption of MMP-2 cleavage of native type I collagen by soluble CBD inhibited cancer cell migration on collagen [21]. Thus the CBD-mediated binding to enzyme substrates and other extracellular matrix molecules is essential for both proper function of MMP-2 and cell migration.

As found in MMP-2, the CBD-9 in MMP-9 is essential for enzyme function, and deletion of the domain abrogated hydrolysis of type I, V and XI collagens and elastin [22,23]. On this basis, the present studies were designed to characterize in more detail the ligand-binding properties of the CBD-9 and to investigate the basis for the overlap in substrate specificity between MMP-9 and -2, as this might involve the CBDs. The experiments demonstrated that CBD-9 and -2 contribute similar ligand-binding properties to their respective parental enzymes. Competitive protein binding assays pointed to shared or closely positioned binding

Abbreviations used: AlkCBD, alkylated rCBD (recombinant collagen-binding domain); AP, alkaline phosphatase; CBD, collagen-binding domain; CBD-9 and -2, CBDs of MMP-9 and -2 respectively; DTT, dithiothreitol; MMP, matrix metalloproteinase; PNPP, *p*-nitrophenyl phosphate disodium salt; RFU, relative fluorescence units; TIMP, tissue inhibitor of metalloproteinase; λ_{em} , emission wavelength; λ_{ex} , excitation wavelength.

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sites on type I collagen for the CBDs of MMP-9 and -2. Indeed, competition between the two CBDs for substrate binding translated to the capacity of either of the CBDs to inhibit gelatinolytic activities by the other gelatinase via occupation of shared substrate-binding sites.

MATERIALS AND METHODS

Extracellular matrix proteins

Acid-soluble native type I collagen was prepared from rat tail tendons as described by Piez, but with modifications [14]. To obtain gelatin, native type I collagen was heat-denatured at 56 °C for 30 min. Bovine type II and human type III and VI collagen were from Biodesign (Saco, ME, U.S.A.), and human type V collagen, murine type IV collagen, Matrigel and laminin were from BD Biosciences (Franklin Lakes, NJ, U.S.A.).

Expression and purification of recombinant proteins

The recombinant (r)CBD from MMP-9 (CBD-9; Val²¹⁷–Glu³⁹¹) was expressed in *Escherichia coli* Le392. The gene sequence encoding CBD-9 was amplified by PCR from the vector pCEP-4MMP9 (kindly provided by Dr M. Seiki, Institute of Medical Science, University of Tokyo, Japan) with primers containing NheI and PstI sites for directional ligation into the expression vector pGYMX [24,25], which introduces an N-terminal His₆ tag (forward primer, 5'-atcgctagcGTGGTTCCAACCTCGGTTTGG-3'; reverse primer, 5'-aactgcagTCATTATTGGTCCGGGCA-GAAGCC-3', where the lowercase letters correspond to the restriction sites added to enable ligation of the PCR-amplified gene sequence to the expression vector). The rCBD-9 was expressed and purified as described in detail for rCBD-2 [26]. In brief, rCBD-9 was expressed as inclusion bodies that were denatured and purified under non-reducing conditions with 8 M urea in 0.1 M NaH₂PO₄/0.01 M Tris/HCl, pH 8.0. Protein was refolded by gradual dialysis against 0.1 M sodium borate, pH 10, and then against 50 mM Tris/HCl/150 mM NaCl, pH 7.4, prior to purification by Ni³⁺- and gelatin–Sephacrose (Amersham Biosciences) affinity chromatography.

The rCBD-2, rMMP-2 and rMMP-9 proteins were expressed and purified as described previously [15,21]. To optimize the enzyme activities of MMP-9 and MMP-2 and to avoid confounding effects of partial enzyme activation, both MMP-9 and MMP-2 were expressed without their prodomains in constitutively active forms. To obtain MMP-2 without CBD (MMP-2ΔCBD), the coding gene sequence was amplified by PCR (forward primer, 5'-ccgctcgagTACAACCTTCTTCCTCGCAAG-3'; reverse primer, 5'-cgggaattcCCTGTGGGAGCCAGGGCC-3') from the plasmid Psp65-MMP2ΔCBD (kindly provided by Professor G. Murphy, Cambridge University, Cambridge, U.K.) and ligated into the XhoI or EcoRI sites of the expression vector pRSETA (Invitrogen, San Diego, CA, U.S.A.), which expresses recombinant proteins with a His₆ tag. Proteins expressed in inclusion bodies in *E. coli* BL21(DE3) pLysS were denatured with 8 M urea in 0.1 M NaH₂PO₄/0.01 M Tris/HCl, pH 8.0, purified by Ni³⁺-affinity chromatography under denaturing conditions and then refolded by gradual dialysis against 0.1 M sodium borate, pH 10, or by adding denatured protein drop by drop into 50 mM Hepes/0.2 M NaCl/1 M NDSB201 [3(1-pyridinio)-1-propane sulphonate], pH 7.5, at a volume ratio of 1:20 to 1:50 with magnetic stirring. The refolded proteins were then dialysed against 50 mM Tris/HCl/150 mM NaCl, pH 7.4.

To obtain rMMP-9 and rMMP-2 with intact ligand-binding properties but without catalytic activities, the active site Glu⁴⁰² of proMMP-9 and Glu⁴⁰⁴ of proMMP-2 were substituted for

alanine residues to generate MMP-9^{E402A} and MMP-2^{E404A} [27]. Point mutations were introduced into the coding DNA by overlap-extension PCR using the following primer pairs: forward, 5'-GTGGCGGCGCATGCGTTCGGCCACGCG-3', and reverse, 5'-CGCGTGGCCGAACGCATGCGCCGCCAC-3', for MMP-9^{E402A}; and forward, 5'-GTGGCAGCCACGCGTTTGGCCACGCC-3', and reverse, 5'-GGCGTGGCCAAACGCGTGGGCTGCCAC-3', for MMP-2^{E404A}. Previously described expression constructs for MMP-9 and MMP-2 in pRSETA [21] served as templates in PCR reactions that included 5 µl of 10 × reaction buffer, 100 ng of template DNA, 125 ng of each primer, 25 µM dNTP mixture, and 2.5 units Pfu Turbo DNA polymerase. The cycles included 95 °C for 2 min, then 95 °C for 30 s, 55 °C for 1 min and 72 °C for 10 min for 12 cycles. Subsequently, 1 µl of the DpnI restriction enzyme was added for 1 h at 37 °C to digest the parental DNA. *E. coli* BL21 (DE3) competent cells were transformed with 1 µl of DNA and the mutated enzymes were expressed and purified as described above for MMP2ΔCBD. All new expression constructs and induced mutations were verified by double-stranded DNA sequencing and analysis with restriction enzymes (results not shown).

Chemical protein modifications

Biotinylation of proteins

After dialysis against 0.1 M NaHCO₃, 3 ml of 200–300 µg/ml rCBD-9, rCBD-2, rMMP-9^{E402A} or rMMP-2^{E404A} were incubated with 300 µg of Sulfo-NHS-LC-Biotin (Sigma–Aldrich Corp., St Louis, MO, U.S.A.) for 20 min at 22 °C, and then for 2 h at 4 °C. Free biotin was removed by dialysis against 50 mM Tris/HCl/150 mM NaCl, pH 7.4, and the biotinylation of the proteins was verified by slight increases in masses relative to control proteins on Coomassie-Blue-stained gels after separation by SDS/PAGE and by reaction with AP (alkaline phosphatase)-conjugated streptavidin (Pierce, Rockford, IL, U.S.A.) in plate assays using PNPP (*p*-nitrophenyl phosphate disodium) as substrate. Protein binding assays verified that all biotinylated proteins retained their collagen binding, but not their catalytic activities.

Alkylation of rCBD

The ligand binding of rCBD requires intact disulphide bonds [14]. To generate binding-deficient control protein with disrupted disulphide bonds, the rCBD-9 or -2 were equilibrated with 2 mM EDTA/0.5 M Tris/HCl, pH 8, containing 8 M urea and 65 mM DTT (dithiothreitol) overnight at 4 °C. The reduced rCBDs were incubated for 1 h at 50 °C and then with 130 mM iodoacetic acid for 30 min at 22 °C. The AlkCBDs (alkylated rCBDs) were thoroughly dialysed against 50 mM Tris/HCl/150 mM NaCl, pH 7.4.

Protein–protein binding experiments

CBD-9 interactions with extracellular matrix molecules

Equal amounts (0.5 µg/well) of either native or denatured type I, II, III, IV, V or VI collagens, Matrigel or laminin in 0.1 M NaHCO₃/Na₂CO₃, pH 9.6, were coated in 96-well plates overnight at 4 °C. BSA (2.5 %) was used to block non-specific binding sites for 1 h at 22 °C. Serially diluted (from 3000 to 0.3 nM, and 0 nM), biotinylated rCBD-9 in PBS was added for 1 h at 22 °C. After washes with PBS/0.5 % (v/v) Tween 20 to remove unbound protein, bound CBD-9 was detected by AP-conjugated streptavidin at a 1:10 000-fold dilution (Pierce) and with 1 mg/ml PNPP substrate (Sigma), and A₄₀₅ was measured with an Opsys MR plate reader (Dynex, Chantilly, VA, U.S.A.). Apparent K_d

values were calculated from binding curves using a four-parameter, non-linear curve-fitting algorithm (Sigma Plot, SPSS Corp., Chicago, IL, U.S.A.). All experiments were performed in duplicate and repeated two to four times.

Competitive protein binding experiments

The assays used the principle that binding of biotinylated proteins to coated collagen could be detected by AP-conjugated streptavidin, whereas a non-labelled protein competing for identical or closely positioned binding sites would remain undetected. If effective, the competing protein would reduce the binding of the biotinylated protein as a function of the molar ratio of the two proteins. Wells were coated with 0.1 μ g of denatured type I collagen or 0.5 μ g of native type I collagen overnight at 4°C. The concentrations of the biotinylated proteins, which resulted in 50 % of maximum binding, were determined and used in the subsequent assays. Biotinylated rCBD-9 and rCBD-2 were used at 160 and 70 nM respectively for native collagen binding experiments, and at 60 and 45 nM respectively in the gelatin binding experiments. Biotinylated rMMP-9^{E402A} and rMMP-2^{E404A} were used at 40 and 25 nM for the native type I collagen binding, and at 11 nM and 12 nM for the gelatin binding assays respectively. In the competitive binding experiments, biotinylated proteins were added alone (control) or simultaneously with a range of molar ratios of non-labelled competitor proteins (typically 0.5–16-fold) to wells coated with immobilized native or denatured (gelatin) type I collagen. The capacity of the biotinylated protein to bind in the presence of the competing protein was measured with AP-conjugated streptavidin and PNPP as described above, and expressed as a function of the molar ratio of the competing:biotinylated test protein. All experiments were performed in triplicate, and repeated at least twice.

Enzyme activity assays

Assays with fluorescent substrates

The gelatinolytic activities of MMP-9 and -2 were measured by the cleavage of the fluorescence-labelled porcine gelatin substrate (DQ gelatin from Molecular Probes, Eugene, OR, U.S.A.) as detailed previously [15]. In brief, gelatinase assays were carried out in 96-well microplates in total reaction volumes of 200 μ l with reaction buffer [50 mM Tris/HCl (pH 7.6)/150 mM NaCl/5 mM CaCl₂/2 mM NaN₃ containing 2 μ g of DQ gelatin substrate and 0–10 μ g of MMP-2 or -9]. Reactions were performed at 22°C, and the changes in fluorescence intensity were expressed in RFU (relative fluorescent units) as measured with λ_{ex} (excitation wavelength) at 495 nm and λ_{em} (emission wavelength) at 515 nm in a SpectraMAX Gemini XS fluorescent plate reader (Molecular Devices, Sunnyvale, CA, U.S.A.).

Enzymography

For zymographic analyses, 150 μ g/ml heat-denatured acid-soluble type I gelatin was co-polymerized with SDS/10%-PAGE gels. Purified MMP-2 and MMP-9, and MMP-2^{E404A} and MMP-9^{E402A} were separated under non-reducing conditions. After incubation and processing of gels as described previously [14], enzyme activities appear as translucent bands on a Coomassie-Blue-stained background. Positive controls included conditioned medium from ROS 17/2.8 cell cultures, which contains high amounts of MMP-2.

Competitive enzyme activity assays

In competitive enzyme activity assays, MMP-9 or -2 was added to enzyme assay buffer [50 mM Tris/HCl (pH 7.6)/150 mM

Figure 1 SDS/PAGE and enzymography analyses of purified recombinant proteins

(A) Samples (400 ng/lane) of rMMP-2 and -9 (MMP-2 and MMP-9), active site mutants MMP-2^{E404A} and MMP-9^{E402A}, MMP-2 with deletion of CBD (MMP-2 Δ CBD) and CBD-2 and -9 were reduced with DTT and separated on 12 % (w/v) cross-linked polyacrylamide mini-slab gels and stained with Coomassie Brilliant Blue R-250. The migration relative to protein standards (M_r) showed high protein purity and predicted molecular masses for each recombinant protein. The positions of protein standards (in kDa) are indicated in the leftmost lane of the gel. (B) The activities of rMMP-2 and -9 and the catalytically inactive mutants (10 ng of protein per lane) were then analysed using SDS/10 %-PAGE gels containing 150 μ g/ml heat-denatured gelatin. MMP activities in conditioned medium from ROS 17/2.8 cells (ROS) served as positive controls. The positions and M_r of the latent (66 000), intermediary (62 000), and active (59 000) forms of MMP-2 from ROS cells are indicated.

NaCl/5 mM CaCl₂/2 mM NaN₃] at final concentrations of 300 or 150 nM respectively, either alone or simultaneously with a concentration range (typically 0.5–8-fold of the molar ratio) of competitor proteins. The fluorescent gelatin substrate (DQ gelatin; Molecular Probes) was then added to the well at 0.5 μ g/well to yield final reaction volumes of 200 μ l. Substrate cleavage in the presence of the competing protein was measured at 22°C with λ_{ex} at 495 nm and λ_{em} at 515 nm, as described above. The enzyme activity was expressed in RFU, and the effect of the competitor was expressed in terms of the percentage of non-competed control as a function of the competitor protein/MMP-9 or -2 molar ratio.

RESULTS

Expression and purification of recombinant proteins from *E. coli*

The identities of the new recombinant proteins rCBD-9, rMMP-2^{E404A}, rMMP-9^{E402A} and rMMP-2 Δ CBD were confirmed by their predicted masses (20 947, 66 518, 70 795 and 47 052 Da respectively) and migration on SDS/PAGE analysis (Figure 1A), and by Western blotting (results not shown). Furthermore, analysis of purified rMMP-9^{E402A} and rMMP-2^{E404A} by enzymography demonstrated that enzymatic activities were abolished for these mutated MMPs up to at least 200 ng/lane, whereas wild-type rMMP-9 and rMMP-2 efficiently digested gelatin at as low as 1 ng/lane (Figure 1B). The loss of enzymatic activity of MMP-9^{E402A} and MMP-2^{E404A} was confirmed further in enzyme activity assays with the fluorescent DQ gelatin as substrate (results not shown).

CBD-9 interacts with several collagen types and other extracellular matrix molecules

Previous investigations have shown that the CBD from MMP-9 binds type I collagen [16] and is essential for hydrolysis of

Figure 2 CBD-9 interactions with native and denatured forms of several collagen types, Matrigel and laminin

Equal amounts (0.5 µg/ml) of native or heat-denatured type I, II, III, IV, V and VI collagen, Matrigel or laminin were coated in 96-well microwell plates as detailed in the Materials and methods section. Serially diluted biotinylated CBD-9 (from 3000 to 0.3 nM, and 0 nM) were added to the wells and incubated for 1 h at 22 °C. After thorough rinses, bound CBD-9 was detected using AP-conjugated streptavidin and 1 mg/ml PNPP as substrate, and quantified at 405 nm. Data points represent the means ± S.D. (bars) for two to four experiments performed in duplicate. Curve fitting was achieved using a four-parameter algorithm.

Table 1 Apparent K_d values for CBD-9 interactions with native and denatured extracellular matrix proteins

Proteins were heat-denatured for 30 min at 56 °C; the apparent K_d values (M) were determined using a four-parameter, non-linear curve-fitting algorithm. See Figure 2 for the various binding curves.

Matrix protein	Apparent K_d (M)	
	Native state	Denatured state
Collagen type		
I	1.2×10^{-7}	1.4×10^{-8}
II	5.0×10^{-8}	8.0×10^{-9}
III	1.0×10^{-7}	1.5×10^{-8}
IV	2.4×10^{-7}	2.0×10^{-8}
V	7.0×10^{-8}	1.0×10^{-8}
VI	No binding	No binding
Matrigel	1.2×10^{-7}	2.5×10^{-8}
Laminin	3.5×10^{-7}	

type V and XI collagens [22]. However, contrary to CBD-2, where the interactions with a series of collagen types and other extracellular matrix proteins have been thoroughly characterized [14,26], little is known about CBD-9 and MMP-9 interactions with other potential ligands. The present results showed that CBD-9 binds specifically to native and denatured types I, II, III, IV and V collagen, as well as Matrigel and laminin (Figure 2) with apparent K_d values of $0.1\text{--}6.8 \times 10^{-7}$ M (Table 1). However, CBD-9 did not bind type VI collagen. The binding of CBD-9 to denatured forms of the collagens was generally 5–10-fold stronger than that to the native forms, as judged by the apparent K_d values.

MMP-9 and -2 compete for substrate binding via the CBD domains

Because the ligand-binding properties of CBD-9 corresponded to those reported for CBD-2 [26], we predicted that MMP-9 and MMP-2 through their CBDs might: (a) bind same or closely positioned sites on type I collagen or other extracellular matrix proteins; and (b) compete for cleavage of substrate molecules that require positioning by the CBDs for cleavage. To investigate these scenarios, we used native and denatured type I collagen (gelatin) as ligands and the mutated, catalytically inactive MMP-9^{E402A} and MMP-2^{E404A} to eliminate confounding effects from enzyme autolysis and ligand cleavage in the assays.

MMP-9^{E402A} and MMP-2^{E404A} both inhibited binding to gelatin of their identical, but biotin-labelled, counterparts (Figure 3 and Table 2). Control experiments showed that labelled and unlabelled proteins had similar binding to coated ligands when bound proteins were detected using specific antibodies that reacted equally with biotinylated and non-labelled proteins (results not shown). Subsequent competitive binding assays comparing MMP-9 and MMP-2 gelatin interactions showed that non-labelled MMP-2^{E404A} inhibited gelatin binding of MMP-9^{E402A} (Figure 3A). Likewise, non-labelled MMP-9^{E402A} could inhibit binding of MMP-2^{E404A} to gelatin in a concentration-dependent manner (Figure 3B). The results from these competitive ligand binding experiments and those showing that CBD-9 bind the same ligands as CBD-2 are consistent with the concept that the CBDs in both MMP-9 and MMP-2 provide the enzymes with gelatin-binding properties. Adding to those results our observation that non-labelled MMP-2 with deletion of the CBD (MMP-2ΔCBD) did not block the binding to gelatin of either MMP-9^{E402A} or MMP-2^{E404A} (Figures 3A and 3B), it appears that MMP-9 and MMP-2 bind the same or closely positioned site(s) on gelatin via the CBDs.

Figure 3 Competition between MMP-9 and MMP-2 for binding to gelatin

After coating gelatin (0.1 μ g/well) in microwell plates overnight at 4 °C and blocking non-specific binding sites with 10 mg/ml BSA, catalytically inactive but gelatin-binding, biotinylated MMP-9^{E402A} (A) or MMP-2^{E404A} (B) (test protein) was added to the wells alone or with competing proteins consisting of non-labelled MMP-9^{E402A}, MMP-2^{E404A} or MMP-2 Δ CBD as a negative control at a range of competing protein/test protein molar ratios from 16:1 to 0.5:1. After incubation for 1 h at 22 °C, unbound proteins were removed by thorough washes and bound biotinylated MMP-9^{E402A} and MMP-2^{E404A} were quantified at 405 nm after reaction with AP-conjugated streptavidin and PNPP substrate, as detailed in the Materials and methods section. Data points shown are the means $\pm$ S.D. (bars) for three independent experiments performed in triplicate.

To investigate further the overlapping ligand-binding properties of CBD-9 and CBD-2 and their parental MMPs, we extended the competitive binding assays as shown in Table 2. Soluble CBD-2 had the capacity to inhibit gelatin binding of both MMP-9^{E402A} and CBD-9 and, likewise, CBD-9 could compete the binding of both CBD-2 and MMP-2^{E404A} to gelatin. Of considerable interest, both CBD-9 and CBD-2 had less competitive effects and required substantially greater molar concentrations to achieve the same level of inhibition as the parental full-length MMPs in competition experiments analysing MMP binding to gelatin (Table 2). Importantly, the non-gelatin binding negative controls, AlkCBD-9 and AlkCBD-2, did not inhibit the binding of either CBDs or MMPs to gelatin. This observation confirmed the specificity of the competitive assays (Table 2).

In additional competitive binding experiments, we analysed the relative binding of MMPs and CBDs to native type I collagen, which is also a ligand and a substrate of MMP-9 and -2. Results from these assays corresponded to those obtained in the competitive gelatin binding assays, indicating that CBD-9 and CBD-2 also bind to the same or closely positioned sites on native type I collagen (Table 3).

Collectively, these experiments showed that CBD-9 and CBD-2 contribute very similar ligand-binding properties to MMP-9 and MMP-2 respectively, and bind identical or closely positioned sites on type I collagen.

CBD-9 inhibits gelatinolytic activity of MMP-2 and MMP-9

We have recently demonstrated that CBD-2 is required for the gelatinolytic activity of MMP-2, and that soluble CBD-2 can compete the gelatinolytic activity of MMP-2 [15]. Since the preceding experiments showed that CBD-9 and CBD-2 are characterized by similar ligand interactions, we investigated whether the MMP-9 enzymatic activity also required substrate binding via CBD-9. The results demonstrated that CBD-9 inhibited gelatinolysis by MMP-9 in a concentration-dependent manner (Figure 4A), verifying that CBD-9 is required for the activity by MMP-9. CBD-9 also inhibited gelatin degradation by MMP-2 (Figure 4C) and, conversely, CBD-2 inhibited the gelatinolytic activity of

Table 2 Competition between MMP-9, MMP-2, CBD9 and CBD2 for binding to denatured type I collagen

Data are shown as the means $\pm$ S.D. from duplicate measurements verified in 3-5 separate experiments.

Test protein	Competitive protein	Ratio . . .	Binding of test protein at different competitive protein/test protein molar ratios			
			1:1	2:1	4:1	8:1
MMP-9 ^{E402A}	MMP-9 ^{E402A}		63.2 $\pm$ 1.0*	41.8 $\pm$ 6.7	31.2 $\pm$ 7.3	20.9 $\pm$ 1.3
	MMP-2 ^{E404A}		80.4 $\pm$ 1.8	65.7 $\pm$ 1.5	52.4 $\pm$ 2.1	40.6 $\pm$ 2.1
	CBD-9		93.0 $\pm$ 5.8	83.5 $\pm$ 3.9	72.4 $\pm$ 1.2	45.1 $\pm$ 1.8
	CBD-2		101.5 $\pm$ 4.0	95.9 $\pm$ 3.9	93.9 $\pm$ 6.1	77.3 $\pm$ 6.7
	AlkCBD-9		96.9 $\pm$ 1.6	96.5 $\pm$ 4.9	98.7 $\pm$ 3.3	97.5 $\pm$ 2.7
MMP-2 ^{E404A}	MMP-9 ^{E402A}		53.2 $\pm$ 2.7	32.3 $\pm$ 2.9	19.3 $\pm$ 3.3	9.8 $\pm$ 2.0
	MMP-2 ^{E404A}		73.2 $\pm$ 3.4	49.7 $\pm$ 4.0	32.2 $\pm$ 1.6	23.0 $\pm$ 1.3
	CBD-9		94.8 $\pm$ 1.0	82.6 $\pm$ 3.4	65.8 $\pm$ 5.3	39.2 $\pm$ 6.0
	CBD-2		100.4 $\pm$ 2.5	91.6 $\pm$ 1.9	81.2 $\pm$ 4.0	65.1 $\pm$ 5.0
	AlkCBD-2		99.2 $\pm$ 2.1	104.1 $\pm$ 1.4	106.0 $\pm$ 1.2	102.9 $\pm$ 1.2
CBD-9	CBD-9		34.9 $\pm$ 1.2	18.7 $\pm$ 1.9	11.3 $\pm$ 1.2	7.3 $\pm$ 1.4
	CBD-2		40.6 $\pm$ 8.0	23.0 $\pm$ 4.0	13.7 $\pm$ 2.1	9.1 $\pm$ 1.6
	AlkCBD-9		115.4 $\pm$ 3.2	116.3 $\pm$ 4.2	116.4 $\pm$ 1.1	113.7 $\pm$ 3.7
CBD-2	CBD-9		50.0 $\pm$ 3.2	32.9 $\pm$ 3.6	21.8 $\pm$ 3.6	14.4 $\pm$ 2.8
	CBD-2		45.0 $\pm$ 4.9	28.2 $\pm$ 2.9	15.8 $\pm$ 3.0	11.4 $\pm$ 1.6
	AlkCBD-2		102.4 $\pm$ 4.6	100.3 $\pm$ 3.4	99.1 $\pm$ 6.9	99.1 $\pm$ 6.9

* The percentage binding of test protein in the presence of a molar range of competing protein relative to non-competed control is shown.

Table 3 Competition between MMP-9, MMP-2, CBD9 and CBD2 for binding to native type I collagen

Date are presented are means ± S.D. from duplicate measurements verified in three to five separate experiments.

		Binding of test protein at different competitive protein/test protein molar ratios				
Test protein	Competitive protein	Ratio . . .	1:1	2:1	4:1	8:1
MMP-g ^{E402A}	MMP-g ^{E402A}		66.0 ± 6.7*	48.5 ± 7.2	28.5 ± 8.6	24.9 ± 2.8
	MMP-2 ^{E404A}		73.5 ± 4.1	68.0 ± 8.3	71.7 ± 3.8	51.0 ± 4.1
	CBD-9		79.8 ± 5.4	77.1 ± 5.1	69.6 ± 3.5	38.1 ± 5.7
	CBD-2		83.7 ± 6.0	75.6 ± 3.8	76.7 ± 9.8	72.9 ± 1.9
	AlkCBD-9		109.7 ± 2.6	112.3 ± 2.0	104.7 ± 2.0	105.2 ± 8.8
MMP-2 ^{E404A}	MMP-g ^{E402A}		44.0 ± 5.4	28.2 ± 4.2	21.8 ± 1.5	17.2 ± 1.3
	MMP-2 ^{E404A}		62.7 ± 1.8	49.1 ± 1.8	35.5 ± 1.1	24.1 ± 1.2
	CBD-9		90.2 ± 5.1	79.9 ± 3.0	60.8 ± 2.1	34.9 ± 1.9
	CBD-2		93.6 ± 3.0	84.0 ± 6.6	73.9 ± 5.4	43.7 ± 1.8
	AlkCBD-2		103.5 ± 6.9	110.9 ± 3.6	104.2 ± 0.3	105.8 ± 5.7
CBD-9	CBD-9		32.7 ± 3.1	19.4 ± 1.9	13.6 ± 1.7	10.4 ± 1.5
	CBD-2		37.6 ± 2.7	25.7 ± 1.1	17.5 ± 1.9	13.3 ± 1.6
	AlkCBD-9		102.7 ± 2.9	105.3 ± 8.6	105.1 ± 8.4	107.9 ± 6.1
CBD-2	CBD-9		51.9 ± 1.7	35.7 ± 5.5	24.6 ± 3.0	17.3 ± 1.8
	CBD-2		47.8 ± 5.5	31.6 ± 3.1	20.5 ± 3.5	13.7 ± 2.7
	AlkCBD-2		92.3 ± 6.1	90.2 ± 2.0	93.0 ± 1.3	100.7 ± 3.3

* The percentage binding of test protein in the presence of a molar range of competing protein relative to non-competed control is shown.

Figure 4 Competition between MMP-9 and MMP-2 for CBD-binding sites on gelatin reduces gelatinolytic activities

The gelatin-degrading activities of MMP-9 (A and B) and MMP-2 (C and D) were competed with CBD-2 and -9, and the catalytically inactive but gelatin-binding enzyme mutants (MMP-g^{E402A} and MMP-2^{E404A}). Non-collagen-binding AlkCBDs (AlkCBD-2 and -9) served as negative controls. Experiments using competing proteins derived from MMP-9 and MMP-2 are shown in (A, C) and (B, D) respectively. In enzyme activity assay with DQ gelatin as substrate (0.5 μg/well), active MMP-9 and MMP-2 were used at final concentrations of 300 and 150 nM respectively, either alone or in the presence of competing proteins added at a range of molar concentrations in an 8–0.5-fold ratio relative to the active enzymes. Substrate cleavage by the MMPs was monitored at 22 °C for 30 min with λ_{ex} 495 nm and λ_{em} 515 nm. Data points are means of duplicate measurements from two to four experiments, and recorded within the linear ranges of the assays.

MMP-9 (Figure 4B). These results supported further our working hypothesis that CBD-9 and CBD-2 target identical or closely positioned substrate binding sites.

Translating the data on competition for substrate-binding sites to effects on substrate cleavage, our results showed that MMP-9^{E402A} inhibited MMP-2 gelatinolytic activities (Figure 4C), and that MMP-2^{E404A} inhibited MMP-9 (Figure 4B). This implied

that not only the substrate specificities [1] but also the mechanism of function of MMP-9 and MMP-2 at a minimum are very similar, and probably identical. Extending our observation that the full-length enzymes had greater ligand-binding affinities than the isolated CBDs (Tables 2 and 3), the inhibition of enzyme activities by full-length MMPs (MMP-9^{E402A} and MMP-2^{E404A}) were also more efficient than the CBDs when used at the same enzyme-to-competitor molar ratios in the assays (Figure 4).

DISCUSSION

Data from our laboratory and other investigators have shown that disruption of the CBD-2-mediated interactions of MMP-2 with gelatin by DMSO inhibits more than 50 % of the gelatinolytic activity of the enzyme [15,28]. Surprisingly, this compound was reported to have less of an inhibitory effect on MMP-9 [16], suggesting that the CBD-9 might contribute different functional properties to MMP-9 compared with CBD-2 in MMP-2. No detailed characterization of the ligand-binding properties has been presented for CBD-9, and we have previously found significant differences in ligand interactions between the collagen-binding domains of MMP-2 and fibronectin [21,26], although both, like the CBD-9, are formed by fibronectin type II modules. Therefore we designed the present series of experiments to characterize the contributions of CBD-9 to MMP-9 in the context of the functions of CBD-2 in MMP-2. Collectively, our results have shown that CBD-9 binds a series of collagen types and other extracellular matrix molecules with specificities and affinities that are very similar to those observed for CBD-2 [14,26]. Moreover, we present evidence that overlapping ligand interactions of MMP-9 and MMP-2 are based on CBD-mediated interactions with identical or closely positioned binding sites on type I collagen. Indeed, the two enzymes reciprocally compete for substrate binding and cleavage. These observations confirm the functional requirement for CBD-9 in positioning substrate molecules for cleavage by MMP-9, as shown previously for MMP-2 [15].

The binding of CBD-9 to denatured forms of the collagen ligands was consistently 5–10-fold stronger than the binding to native forms of the same collagens, as expressed by the apparent *K_d* values. This binding pattern corresponded to that observed in

previous studies of the CBD-2 ligand interactions, and suggests that denaturation of collagen triple helices exposes cryptic binding sites [14]. Other investigators have found weak binding of full-length, latent MMP-9 to native type IV collagen by surface plasmon resonance experiments, and only the $\alpha 2(\text{IV})$ chain was pulled down from solubilized pericellular membranes by latent MMP-9 coupled to AffiGel-10 resin [29]. Interestingly, Morgunova et al. [27] found that Phe³⁷ from the prodomain inserts into the hydrophobic pocket of the third fibronectin module in latent MMP-2. If such intermolecular interactions were also to occur in latent MMP-9, then the weak binding to native type IV collagen could be accounted for by reduced access for binding of the rigid triple-helical type IV molecule to the CBD by either the coupled AffiGel resin or by steric hindrance resulting from prodomain–CBD interactions. Of note, the competitive binding experiments presented here used MMP-2 and MMP-9 that were expressed without the prodomains and rendered catalytically inactive by active-site glutamine-to-alanine mutations.

Since the ligand-binding properties of CBD-9 mirrored those of CBD-2, and MMP-9 and -2 have overlapping substrate specificities [1–3], we developed the working hypothesis that CBD-9 and CBD-2 mediate competition between MMP-9 and MMP-2 for binding to identical substrate sites and, in turn, hydrolysis of the same substrates. To first test this hypothesis in competitive ligand binding assays, we generated the mutated MMP-9^{E402A} and MMP-2^{E404A} to eliminate any confounding effects from enzyme autolysis and substrate cleavage. The mutations of the glutamate residues to alanines at positions 402 or 404 in the active sites of MMP-9 or MMP-2 respectively completely abolished enzymatic activities (Figure 1B), as also reported by others [27]. Importantly, these mutations do not introduce significant structural changes [27], and our control studies showed that binding to native and denatured type I collagen remained intact. In our experiments, MMP-9 and MMP-2 competed for binding to native and denatured type I collagen. To validate our experimental approach, we found that neither the non-collagen binding, CBD-deletion mutant of MMP-2 (MMP-2 Δ CBD) nor AlkCBD-9 or AlkCBD-2 were able to compete the binding of the functional MMP-9 or MMP-2 to denatured or native type I collagen.

Extending our previous reports showing that CBD is required for the gelatinolytic activity of MMP-2 [15], we tested the possibility that the competition between CBD-9 and CBD-2 for ligand binding translated to effects on the enzyme activities. Indeed, both CBD-9 and -2 had the capacity to inhibit gelatinolysis by MMP-9 and MMP-2. This observation verified that CBD-9 is a required exosite for gelatin degradation by MMP-9. Importantly, the chemically modified negative control CBDs, AlkCBD-9 and AlkCBD-2, did not inhibit the MMP-9 activities.

Prior studies of CBD-deletion mutants of MMP-2 [28] and MMP-9 [22] detected an approx. 90 % reduction in gelatinolysis as a result of the domain deletion. Although a variant of MMP-2 consisting of the isolated recombinant 19 kDa catalytic domain also cleaved gelatin, this occurred at a much lower rate compared with full-length MMP-2 [30], and may be ascribed to hydrolysis of short α -chain fragments that do not require positioning by CBD for cleavage [15]. Interestingly, in our present experiments, the catalytically inactive mutants MMP-9^{E402A} and MMP-2^{E404A} had stronger inhibitory effects on both intact MMP-9 and MMP-2 ligand binding and gelatinolysis compared with either CBD-9 or CBD-2 when added at the same molar ratios. This observation is indicative of as yet unresolved intermolecular interactions between the CBD and other parts of the enzymes which contribute to substrate binding, and is consistent with recent results implicating the hemopexin-like domain as well as the CBD in collagenolysis by MMP-2 [31].

The biological reasons for the redundancy in function of MMP-9 and MMP-2 are uncertain. In the absence of specific inhibitors to discriminate the biological functions of the two gelatinases [32], the effects of gene knock-outs have been examined. Genetic deficiencies of either MMP-9 or MMP-2 significantly reduced smooth muscle cell migration in response to wounding, and also impaired cell invasion through gelatin [33]. However, neither the loss of MMP-9 nor MMP-2 alone completely inhibited smooth muscle cell migration, suggesting that the two MMPs contribute distinct properties to the cells and that neither can fully compensate for the lack of the other enzyme [33]. MMP-9 contributes importantly to the migration of epithelial cells during wound healing [34], and we recently demonstrated that MMP-2 is important to migration of cancer cells on collagen [21]. In the context of earlier key observations on the contributions of MMP-1 to cell migration [35], we consider it most likely that several co-ordinated MMPs are involved in cell migration in a manner that is both substrate- and cell-type-dependent.

Both MMP-2^{-/-} and MMP-9^{-/-} mice develop without apparent phenotypic abnormalities [36,37], or only with a subtle delay in their growth [38], suggesting that MMP-2 and MMP-9 functions *in vivo* are synergistically co-ordinated [39]. In addition to the functions of MMPs in matrix remodelling, previous publications have revealed the involvement of MMP-2 in growth factor and chemokine processing and shedding [4,40]. These functions greatly expand the biological significance of MMPs. A lack of the proper execution of those biological functions could explain the aberrant accumulation of inflammatory cells in lungs of allergen-challenged MMP-9^{-/-} and MMP-2/9^{-/-} mice [41], or the attenuation of macular degeneration in MMP-2/9^{-/-} over single gene-deficient mice [39]. Alternatively, our observation of substrate overlap and competition might reflect a potential for synergism, rather than competition, between MMP-9 and MMP-2. For example, MMP-2 is constitutively expressed by multiple cell types in the extracellular matrix [2,7]. In comparison, a major source of MMP-9 are inflammatory cells, such as polymorphonuclear leucocytes, which may be targeted to and release MMP-9 at times and sites of unique needs, including acute and chronic arthritic and periodontal inflammation [7,42]. Thus combined activities of MMP-9 and MMP-2 may provide an additive response to increased local needs for their activities. In neoplastic diseases such as breast carcinomas, the mRNA for MMP-2 has been found to be more abundant in stromal fibroblasts and endothelial cells than in carcinoma cells. In comparison, MMP-9 was expressed at high levels in carcinoma cells but at considerably lower levels in stromal fibroblasts and endothelial cells [43]. The different distribution of the two gelatinases in the carcinomas and surrounding tissues may point to synergism between tumour and stromal cells directed at the disruption of basement membranes and tumour expansion by means of MMP-9 and -2 activities.

Collectively, our studies have demonstrated that rCBD-9 of MMP-9 binds not only denatured type I collagen, but the native and denatured forms of a series of collagen types as well as Matrigel and laminin, with affinities corresponding to those of CBD-2 from MMP-2. The capacity of MMP-9 and MMP-2 to compete for substrate binding and degradation indicate that the two enzymes interact with identical or closely positioned substrate sites, and as in MMP-2, the CBD is also required for the gelatinolytic activities of MMP-9.

This work was supported by NIH grants DE12818, DE14236 and DE016312, and the San Antonio Area Foundation. We gratefully acknowledge Dr Gregg B. Fields, (Florida Atlantic University, Boca Raton, FL, U.S.A.) for providing the MMP substrate NFF-1 and Dr M. Seiki (Institute of Medical Science, University of Tokyo, Japan) and Professor

G. Murphy (Department of Oncology, University of Cambridge, Cambridge Institute for Medical Research, U.K.) providing us with MMP-9 and MMP2 Δ CBD plasmids.

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