Inhibition of MMP-2 gelatinolysis by targeting exodomain–substrate interactions

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MMP-2 (matrix metalloproteinase 2) contains a CBD (collagen-binding domain), which is essential for positioning gelatin substrate molecules relative to the catalytic site for cleavage. Deletion of the CBD or disruption of CBD-mediated gelatin binding inhibits gelatinolysis by MMP-2. To identify CBD-binding sites on type I collagen and collagen peptides with the capacity to compete CBD binding of gelatin and thereby inhibit gelatinolysis by MMP-2, we screened a one-bead one-peptide combinatorial peptide library with recombinant CBD as bait. Analyses of sequences from the CBD-binding peptides pointed to residues 715–721 in human $\alpha 1(I)$ collagen chain as a binding site for CBD. A peptide (P713) including this collagen segment was synthesized for analyses. In SPR (surface plasmon resonance) assays, the CBD and MMP-2^{E404A}, a catalytically inactive MMP-2 mutant, both bound immobilized P713 in a concentration-dependent manner, but not a scrambled control peptide.

Furthermore, P713 competed gelatin binding by the CBD and MMP-2^{E404A}. In control assays, neither of the non-collagen binding alkylated CBD or MMP-2 with deletion of CBD (MMP-2 Δ CBD) bound P713. Consistent with the exodomain functions of the CBD, P713 inhibited $\sim 90\%$ of the MMP-2 gelatin cleavage, but less than 20% of the MMP-2 activity on a peptide substrate (NFF-1) which does not require the CBD for cleavage. Confirming the specificity of the inhibition, P713 did not alter MMP-2 Δ CBD or MMP-8 activities. These experiments identified a CBD-binding site on type I collagen and demonstrated that a corresponding synthetic peptide can inhibit hydrolysis of type I and IV collagens by competing CBD-mediated gelatin binding to MMP-2.

Key words: collagen, collagen-binding domain (CBD), exodomain, gelatinolysis, matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase inhibitor.

INTRODUCTION

The MMP (matrix metalloproteinase) family of endopeptidases has at least 23 members that are capable of cleaving many macromolecules of the extracellular matrix [1]. Potentially equally significantly, recent studies have shown that MMPs have important roles in processing and shedding of growth factors and membrane molecules [2]. By virtue of these properties, MMPs are essential to normal physiological remodelling events as well as chronic inflammatory diseases and metastasis in cancer [3]. The functions of MMPs rely on a characteristic structure, which includes a propeptide domain containing a cysteine residue which interacts with a catalytic site zinc to maintain enzyme latency [4,5], a catalytic domain with a zinc-binding motif that is essential for the proteolytic activity [6], and a C-terminal haemopexin-like domain which is essential to substrate binding [7]. An additional unique CBD (collagen-binding domain) formed by three fibronectin type II-like modules is present only in MMP-2 and MMP-9 [8,9]. MMPs are inhibited specifically by TIMPs (tissue inhibitors of metalloproteinases) that bind the catalytic site directly, but also have additional functions in activation of MMP-2 [10,11].

Exodomains are essential for optimal MMP function. Murphy et al. [12] demonstrated that deletion of the C-termini of MMP-1 and MMP-3 abrogated the capacity of the enzymes to cleave native type I collagen. Importantly, hybrid enzymes in which the C-termini were exchanged between the two enzymes did not retain the capacity to cleave native type I collagen, pointing to essential structural specificities and the requirement for the C-terminal

exodomains. Other substrates, such as gelatin and casein, were cleaved in the absence of the C-terminus, suggesting that other parts of the enzymes were utilized and sufficient for cleavage of those molecules [12]. The CBDs in MMP-2 and -9 are the primary sites of interaction with multiple collagens and gelatin [9,13,14] and also mediate binding to elastin [9,15,16]. Importantly, as observed for the C-termini in MMP-1 and -3, the CBDs in MMP-2 and -9 are required for cleavage of collagen and elastin. CBD-deletion mutants of MMP-2 had a $\sim 90\%$ reduction in gelatin hydrolysis [17] and elastin was not cleaved by MMP-2 and -9 after deletion of the CBDs [16]. To verify that the loss of activity did not result from structural perturbations following deletion of the ~ 20 kDa internal CBD, we demonstrated that a soluble CBD competed for and reduced gelatinolysis of intact MMP-2 and -9 [18]. Of note, the CBD was required for hydrolysis of larger collagen α -chains, but not short collagen-like peptides [18]. Together, these results have emphasized the essential contributions of exodomains to MMP specificities and activities.

The lack of success in clinical trials that used active-site-specific MMP inhibitors such as hydroxamic acid derivatives [19,20] have prompted reconsideration of strategies for more enzyme-specific MMP inhibition by targeting the exodomain–substrate interactions as reviewed by Overall and Lopez-Otin [21]. In the present experiments, we tested the hypothesis that a CBD-binding-site-specific peptide from type I collagen would have the capacity to block MMP-2 interactions with gelatin substrate molecules and, in turn, inhibit their cleavage. By screening a random peptide library, we identified a CBD-binding peptide with

Abbreviations used: AP, alkaline phosphatase; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; CBx, CNBr fragment x; CBD, collagen-binding domain; AlkCBD, alkylated CBD; Dnp, 2,4-dinitrophenyl; Mca, (7-methoxycoumarin-4-yl)acetyl; MMP, matrix metalloproteinase; RFU, relative fluorescent units; RU, response units.

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high identity with a short segment of the $\alpha 1(I)$ collagen chain which, as a synthetic peptide, specifically blocked interactions of both isolated CBD and full-length MMP-2 with gelatin, and inhibited gelatinolysis by MMP-2. Together, our binding and enzyme activity experiments demonstrated that blocking the CBD–substrate interactions provides a strategy for specific inhibition of MMP-2.

MATERIALS AND METHODS

Expression and purification of recombinant proteins

The CBD from human MMP-2 was expressed in *Escherichia coli* transformed with the expression vector pGYMX containing the CBD coding region as detailed previously [9]. Constitutively active MMP-2 without the prodomain was expressed using the pRSETA expression vector (Invitrogen) after ligation with coding cDNA amplified from the MMP-2 plasmid p186.2 (provided by Dr Ivan Collier, Dermatology Department, Washington University, St. Louis, MO, U.S.A.) as described in [22,23]. To obtain MMP-2 with intact ligand binding but abrogated catalytic activities (MMP-2^{E404A}), Glu⁴⁰⁴ in the MMP-2 active site was replaced with alanine using PCR-based site-directed mutagenesis as detailed previously [24]. For studies of MMP-2 interactions with gelatin in the absence of CBD, a second MMP-2 with deletion of the CBD (MMP-2 Δ CBD) was constructed in pRSETA from the plasmid Psp65-MMP2 Δ CBD (provided by Dr Gillian Murphy, Cancer Research UK, Cambridge Research Institute, Cambridge, U.K.) and expressed in *E. coli* as described previously [24].

All recombinant proteins were expressed in inclusion bodies and required solubilization with 8 M urea, 0.1 M NaH₂PO₄ and 0.01 M Tris/HCl, pH 8.0. Refolding and purification procedures varied between the recombinant proteins and were optimized as detailed previously [18,23,24]. Typically, since all recombinant proteins contained a His₆ tag, the first step in protein purification included Ni²⁺-affinity chromatography under denaturing conditions. The CBD was purified further over a gelatin–Sepharose affinity column (GE Healthcare). The identities of the proteins were verified by their masses analysed by migration in SDS/PAGE, by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS at the University of Texas Health Science Center at San Antonio (UTHSCSA) Laboratory for Mass Spectrometry, and by reaction with appropriate antibodies in Western blot analyses [25]. The presence and loss of enzymatic activities for MMP-2 and MMP-2^{E404A} respectively were confirmed by gelatin substrate zymography and assays using fluorescently labelled gelatin or peptide substrates. The proteins were dialysed against 50 mM Tris/HCl and 150 mM NaCl, pH 7.4 and stored at -80°C until analysis. Recombinant proteins generated by these procedures were all functionally folded and displayed the properties predicted from their native counterparts [9,18,23].

Protein modifications

Biotinylation of recombinant CBD and recombinant MMP-2

For use in screening the peptide library, the CBD was biotinylated. After dialysis against 0.1 M NaHCO₃, pH 8.5, 3 ml of 200–300 $\mu\text{g}/\text{ml}$ recombinant CBD was incubated with 300 μg of sulfo-NHS (*N*-hydroxysuccinimido)-LC (long-chain)-biotin (Sigma–Aldrich) 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 and 150 mM NaCl, pH 7.4, and the protein biotinylation was verified by slight increases in masses relative to control proteins on Coomassie Blue-stained SDS/PAGE minislab gels after electrophoretic separation and by reaction with AP (alkaline phosphatase)–

conjugated streptavidin (Pierce) in plate assays using PNPP (*p*-nitrophenyl phosphate disodium) as substrate. Protein binding assays verified that the biotinylated CBD retained its collagen-binding properties.

Alkylation

To generate collagen-binding-deficient CBD control protein with disrupted disulfide bonds [9], the CBD was equilibrated with 8 M urea, 65 mM DTT (dithiothreitol), 2 mM EDTA and 0.5 M Tris/HCl, pH 8.0, overnight at 4°C . The reduced CBD was then incubated for 1 h at 50°C and subsequently reacted with 130 mM iodoacetic acid (Sigma–Aldrich) for 30 min at 22°C . The alkylated CBD (AlkCBD) was dialysed thoroughly against 50 mM Tris/HCl and 150 mM NaCl, pH 7.4, and was stored at -80°C until further analysis.

Screening of bead-conjugated peptide library

Synthesis of peptide library

A one-peptide one-bead library was synthesized using the approach of Lam et al. [26]. Using the split synthesis approach with 19 reaction vessels on an Advanced Chem Tech Multiple Peptide Synthesizer, the octapeptide library was synthesized incorporating all natural amino acids except cysteine at the UTHSCSA Protein Core Facility. The combinatorial library method allows the identification of binding peptides to a variety of macromolecular targets [27]. The composition and randomness of the peptide library was confirmed by amino acid analysis of representative beads.

Screening of the bead library

For screening, we used 0.5 ml of peptide library beads (3×10^6 random octapeptide beads). Beads were washed with water, equilibrated with PBS/Tween [8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl and 0.1 % (v/v) Tween 20], and then reacted with 3 ml of biotinylated CBD probe at a concentration of 200 nM at 22°C for 2 h. To detect beads with surface-bound CBD, the beads were washed thoroughly with PBS/Tween and incubated with AP-conjugated streptavidin at a 1:30 000 dilution for 1 h at 22°C . Excess unbound AP was removed by washes with PBS/Tween and then with TBS (Tris-buffered saline: 2.5 mM Tris/HCl and 13.7 mM NaCl) before the colour reaction which used 0.165 mg/ml BCIP (5-bromo-4-chloroindol-3-yl phosphate) (Sigma–Aldrich) in 0.1 M Tris/HCl, 0.1 M NaCl and 2.34 mM MgCl₂, pH 9.5, at 22°C for 1 h. Positive beads yielded a dark-blue colour (Figure 1A) and were transferred to microtubes with a fine-tip pipette. The peptide sequences for the beads were determined by automated Edman protein sequencing in the UTHSCSA Protein Core Facility.

Sequence analysis, and peptide design and synthesis

To detect consensus sequences and motifs, sequences of CBD-binding peptides were aligned and analysed with gene-analysis software allowing for gapped alignments. All peptides were searched for identity with known binding site motifs or other biologically significant sites and domains by protein motif searches on the ProSite database, and aligned with the human $\alpha 1(I)$ collagen chain (Swiss-Prot accession number P02452) to identify possible CBD-binding sites on collagen.

No consensus or other known binding motifs were identified. However, since one peptide had 71 % identity with a segment of the human $\alpha 1(I)$ pro-collagen sequence, a collagen-like peptide (P713) corresponding to the identified segment and a control peptide (Pscr) with identical amino acids, but with scrambled

order and no apparent similarity with P713, were designed for functional studies. The peptides included amino acids corresponding to one gap of one amino acid in the alignment, an N-terminal glycine residue for structural flexibility preceding a cysteine residue required for immobilization of peptides on to sensor chips in subsequent SPR analyses. Furthermore, to match post-translational modifications of collagen, each of two proline residues at X' positions of G-X-X' repeats were replaced with hydroxyproline. The resulting 12-mer peptides were synthesized at the UTHSCSA Protein Core Facility (see Figure 1C and the Results section) by sequential addition of Fmoc (fluoren-9-ylmethoxycarbonyl)-protected amino acids in a Multiple Peptide Synthesizer Model 396 MPS (Advanced Chemtech).

Peptide–protein binding assays

Surface plasmon resonance

SPR analyses were carried out at the UTHSCSA Surface Plasmon Resonance Core Laboratory using a Biacore 3000 SPR instrument with CM5 sensor chips (GE Healthcare). Each CM5 chip contains separate flow cells, on to which we immobilized P713 or Pscr. One uncoated cell served as a negative control. Briefly, for coating peptides, CM5 chips were activated by injection of 15 μ l of 0.2 M *N*-ethyl-*N*⁹-(dimethylaminopropyl)carbodi-imide and 0.05 M *N*-hydroxysuccinimide for 6 min. Then the surfaces were modified by PDEA [2-(2-pyridinyldithio)ethaneamine] thiol-coupling reagent hydrochloride (GE Healthcare). Peptides in acetate buffer, pH 4.0, were then immobilized in the flow cells at $\sim$ 500 RU (response units), followed by blocking with 30 μ l of cysteine (6 mg/ml). In peptide–protein binding assays, recombinant CBD, AlkCBD, MMP-2^{E404A} or MMP-2 Δ CBD (30 or 50 μ l each; concentration from 0.5 to 40 μ M) in Biacore buffer (10 mM Hepes, 150 mM NaCl and 0.005 % surfactant P20, pH 7.4) were passed over the coated surfaces. Interactions of proteins (analytes) with immobilized peptide ligand were expressed on RU against time sensorgrams. The surfaces were regenerated by injection of 6 M guanidinium chloride between analyses of peptide interactions with different proteins.

Competitive binding assays by SPR

Denatured type I collagen (gelatin) was immobilized on CM5 chips activated by 0.2 M *N*-ethyl-*N*⁹-(dimethylaminopropyl)carbodi-imide and *N*-hydroxysuccinimide (4600 RU) as described above and blocked with 1 M ethanolamine. After immobilization, a concentration range of CBD (0.5–40 μ M) and MMP-2^{E404A} (0.5–28 μ M) were passed over the immobilized gelatin to define an appropriate concentration for the competition within the linear range of the binding to gelatin. On this basis, and because both the CBD and MMP-2^{E404A} bound to gelatin in a concentration-dependent and saturable manner (results not shown), competition assays used 1 μ M CBD or MMP-2^{E404A} alone (control) or mixed with a concentration range of competing peptides P713 or Pscr (4–500 μ M). The capacity of the peptides to inhibit binding of the CBD and MMP-2^{E404A} to gelatin was determined by the reduction in binding RU in the presence of peptide competitors and expressed relative (%) to the binding of the proteins in the absence of competing peptides. The percentage inhibition was plotted against the concentration of competing peptide.

Enzyme activity assays

The inhibitory effects of the synthetic peptides on gelatinolytic activities of MMP-2 were measured by changes in cleavage of fluorescently labelled porcine type I gelatin and or human

placenta type IV collagen substrates (D-12054 and D-12052; Molecular Probes) as detailed previously [18]. Total reaction volumes of 200 μ l in 96-well microplates contained 75 nM MMP-2 or 530 nM MMP-2 Δ CBD added alone or simultaneously with 6.25–100 μ M synthetic peptides (P713 or Pscr), and DQ-gelatin (0.5 μ g/well) or DQ-collagen IV (2 μ g/well) in reaction buffer (50 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl₂ and 2 mM NaN₃, pH 7.6). All activity assays were performed at 22 °C in a SpectraMAX Gemini XS fluorescent plate reader (Molecular Devices). The increase in fluorescence emitted in the reaction is proportional to the amount of substrate cleaved. Results were expressed in RFU (relative fluorescent units) or the rate of cleavage (RFU $\cdot$ s⁻¹).

In addition to DQ-gelatin, we measured P713 inhibition of the cleavage of a short 11-amino-acid collagen-like peptide substrate (NFF-1) [Mca-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Lys(Dnp)-Gly, where Mca is (7-methoxycoumarin-4-yl)acetyl and Dnp is 2,4-dinitrophenyl] (provided by Dr Gregg B. Fields, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, FL, U.S.A.) by MMP-2 and MMP-2 Δ CBD [18,28]. Typical reactions contained 5 μ M NFF-1 in reaction volumes of 200 μ l of assay buffer (50 mM Tris/HCl, pH 7.0, 200 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂ and 0.05 % Brij-35), 150 nM MMP-2 or MMP-2 Δ CBD and 0 and 6.25–100 μ M P713 inhibitor. The reaction temperature was 22 °C, and enzyme activities were measured with λ_{ex} at 325 nm and λ_{em} at 393 nm at 30 min and expressed as for DQ-gelatin.

To assess the specificity of the peptides, we tested their inhibition of human neutrophil collagenase (MMP-8), which does not contain a CBD. MMP-8 (Calbiochem) was activated by incubation with 1 mM APMA (4-aminophenylmercuric acetate) at 37 °C for 2 h. Activated MMP-8 (3.5 nM) was incubated with 5 μ M peptide substrate (peptide 3163v) [Mca-Pro-Leu-Gly-Leu-A₂pr(Dnp)-Ala-Arg-NH₂, where A₂pr is 2,3-diaminopropionic acid] (Peptides International) in the presence of P713 (0 and 6.25–100 μ M) in 200 μ l volumes of assay buffer (50 mM Tris/HCl, pH 7.0, 200 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂ and 0.05 % Brij-35). The reactions were carried out at 22 °C for 30 min, and enzyme activities were measured with λ_{ex} at 325 nm and λ_{em} at 393 nm and expressed as the rate of cleavage (RFU $\times$ 10⁻³ $\cdot$ s⁻¹).

RESULTS

Identification of a CBD-binding segment in type I collagen

To identify a potential binding site for the MMP-2 CBD on type I collagen, we used a recombinant CBD as bait to screen a bead-conjugated octapeptide combinatorial library. Before the screening, we verified that biotinylation did not alter recombinant CBD binding to native or denatured type I collagen (results not shown) [9]. Among $\sim$ 3 million beads that were screened, 50 beads demonstrated varying degrees of reaction after substrate development indicating binding of the CBD (Figure 1A). After isolation from non-staining beads, the 20 most homogeneous and darkest staining beads were submitted for amino acid sequencing (Figure 1B).

Alignment of the 20 isolated peptide sequences did not reveal a unique consensus sequence, and database searches did not identify a known binding site motif. However, alignment of peptides with the human α 1(I) pro-collagen chain protein sequence showed either no homology or low homology with a very big gap located within CNBr fragments CB7 or CB8. One peptide had 71 % identity with the Pro⁷¹⁵–Gln⁷²¹ region in CB3 (corresponding to Pro⁵³⁷–Gln⁵⁴³ in the mature α 1(I) collagen chain) when allowing for one gap at position 716 in a potentially non-contiguous binding

Figure 1 Identification of MMP-2 CBD-binding peptides from a random peptide library

(A) A one-bead one-peptide combinatorial peptide library was screened for members binding specifically to the CBD from MMP-2. Biotinylated CBD-binding beads were readily identified with AP-conjugated streptavidin and BCIP substrate and isolated under a light microscope ($\times 40$ magnification). (B) The amino acid sequences were determined by Edman sequencing (*, either residue possible; +, unresolved residue identity); (—), gap in alignment relative to Swiss-Prot $\alpha 1(I)$ collagen amino acid sequence. (C) Among all analysed peptides, only one had a 71% sequence identity with the $\alpha 1(I)$ collagen chain. The collagen sequence Gly⁷¹³–Ala⁷²³ with an N-terminal cysteine residue was synthesized as peptide P713 ([†], proline in the X' positions of G-X-X' triplets were replaced with hydroxyproline, HYP). A synthetic scrambled control peptide, Pscr, had the same amino acid composition as P713, but arranged in a random order. Amino acid numbers presented correspond to the $\alpha 1(I)$ collagen sequence (Swiss-Prot accession number P02452).

motif (Figure 1C). This high level homology with the $\alpha 1(I)$ pro-collagen chain warranted further investigation on the role of this collagen segment relative to its potential interactions with the CBD and MMP-2 and its role in gelatinolysis by MMP-2. Consequently, we synthesized a slightly longer 12-amino-acid peptide (P713) corresponding to the Gly⁷¹³–Ala⁷²³ sequence of the type I collagen $\alpha 1$ chain accommodating a one-amino acid gap, SPR experiments and post-translational modifications as detailed in the Materials and methods section and presented in Figure 1(C). This peptide (P713) had excellent ligand-binding properties in subsequent functional experiments. It is noteworthy that other investigators found no binding with the CBD for phage-display peptides of less than six residues in length, but good binding for 15-mer peptides [29]. A scrambled control peptide (Pscr) contained the same amino acids as P713, but arranged in random order (Figure 1C).

P713 interacts specifically with the CBD and MMP-2^{E404A}

SPR assays were used to analyse the interactions of immobilized synthetic peptides with the recombinant CBD, MMP-2^{E404A}, MMP-2 Δ CBD and AlkCBD (Figure 2). When injected at a 2 μ M concentration, a classical CBD–peptide association phase, followed by dissociation of the CBD from the peptide was detected in the flow cells containing immobilized P713 (Figure 2A). However, the CBD did not bind the immobilized scrambled control peptide, Pscr (Figure 2A). Importantly, and consistent with our previous observations for CBD–gelatin interactions [9], the non-collagen-binding negative control, AlkCBD, did not bind P713 when added at concentrations of up to 10 μ M (Figure 2B). This indicates that the mechanism of CBD binding to P713 reflects the interaction of the CBD with Type I collagen α -chains. Since

the CBD accounts for binding of its parental enzyme, MMP-2, to the collagen substrate [9], we subsequently analysed the peptide interactions with an active-site mutant of MMP-2, MMP-2^{E404A}, which is catalytically inactive, but retains collagen-binding properties [30], and a second modification of MMP-2 in which the CBD has been deleted, MMP-2 Δ CBD [24]. As observed in experiments with the CBD, MMP-2^{E404A} injected at 2 μ M also bound specifically to P713, but not to Pscr (Figure 2C). In contrast, MMP-2 Δ CBD at 4 μ M did not bind any of the peptides (Figure 2D), verifying that the binding between MMP-2 and P713 involves the CBD, but not other exodomains of MMP-2. That the lack of peptide binding by MMP-2 Δ CBD did not result from structural perturbations introduced by the deletion of the ~ 20 kDa CBD was verified by intact binding of the same modified enzyme to fibronectin, an interaction which is known to occur through the C-terminal domain of MMP-2 (results not shown) [7].

To verify that the interactions between peptides and proteins were specific and saturable and to evaluate the affinity of interaction, we used a concentration range of the CBD and MMP-2^{E404A} as analytes in SPR. Results showed that the binding of the CBD (Figure 3A and 3B) and MMP-2^{E404A} (Figures 3C and 3D) to the synthetic collagen-like P713 were concentration-dependent. The apparent K_d values were 5.4 μ M for the CBD and 10.2 μ M for MMP-2^{E404A}. Consistently, there was no binding of the CBD (Figure 3B) or MMP-2^{E404A} (Figure 3D) to Pscr even at up to 40 μ M CBD and 28 μ M MMP-2^{E404A}.

CBD-binding P713 competes interactions of MMP-2^{E404A} with gelatin

Having demonstrated that the synthetic collagen-like peptide P713 bound specifically to both the CBD and MMP-2, we

Figure 2 CBD mediates interactions of MMP-2 with collagen-like peptide P713

In SPR binding assays, CM5 chips were coated with a collagen-like peptide, P713, or a scrambled control peptide, Pscr. Subsequently, 30 or 50 μ l aliquots of purified recombinant CBD (A), AlkCBD (B), MMP-2^{E404A} (C) or MMP-2 Δ CBD (D) (mobile-phase analytes) were passed over the immobilized peptide surfaces at 2 or 4 μ M for 6 or 10 min in Biacore buffer. Biacore sensorgrams showed specific binding measured in RU of CBD and MMP-2^{E404A} to P713, but not to Pscr. The negative-control proteins, AlkCBD and mutated MMP-2 with deletion of CBD (MMP-2 Δ CBD) bound neither P713 nor Pscr.

investigated the capacity of P713 to compete the CBD and MMP-2 interactions with gelatin. Such an inhibition predictably should occur if the binding sites of gelatin and P713 on the CBD of MMP-2 were identical or closely positioned, thereby causing steric binding interference. Confirming this assumption, P713 inhibited the binding of the CBD as well as MMP-2^{E404A} to gelatin in a concentration-dependent manner in competitive binding experiments (Figures 4A and 4B). Results showed that P713 could inhibit the binding of the CBD to gelatin by 80 % and the binding of MMP-2^{E404A} to gelatin by 90 % at 500 μ M. Half-maximal inhibition (IC_{50}) of MMP-2^{E404A} binding to gelatin was achieved with $\sim$ 40 μ M P713, whereas 500 μ M Pscr inhibited less than 15 % of CBD or MMP-2^{E404A} binding to gelatin (Figures 4A and 4B). Thus these experiments demonstrated that P713 reduced MMP-2 interactions with gelatin by competing access for this substrate to the CBD.

P713 inhibits MMP-2 activities by blocking substrate access to the CBD exodomain

Previous studies have shown that CBD-mediated positioning of gelatin is required for cleavage of gelatin by MMP-2 [17,18]. Having determined that P713 inhibited MMP-2 binding to gelatin, we then tested whether the specific P713 binding of the CBD resulted in the inhibition of the gelatinolytic activities of MMP-2. Indeed, enzyme activity assays demonstrated that P713 significantly inhibited MMP-2 cleavage of gelatin in a dose-dependent manner with an IC_{50} of $\sim$ 30 μ M (Figure 5A). In contrast, Pscr

inhibited the MMP-2 catalytic activities by <10 % at peptide concentrations of 100 μ M (Figure 5A). Since type IV collagen is another major substrate of MMP-2 [31], we investigated whether P713 had the capacity to also inhibit the type IV collagenase activities of MMP-2. Results showed that P713 significantly inhibited the cleavage of this substrate in a concentration-dependent manner with an IC_{50} of $\sim$ 10 μ M, whereas the negative control Pscr had no effect on MMP-2 hydrolysis of type IV collagen (Figure 5B).

To verify that P713 exerted its inhibitory effect on MMP-2 only for substrates that require interactions with the CBD for positioning relative to the catalytic site, an 11-amino-acid-long fluorescent peptide substrate, NFF-1, was included in the competitive MMP-2 activity assays. We have shown previously that cleavage of NFF-1 by MMP-2 does not require CBD-mediated substrate binding for degradation to occur [18]. The present experiments demonstrated that cleavage of NFF-1 by MMP-2 was not inhibited in the presence of 50 μ M P713 and by only 18 % with 100 μ M P713 (Figure 5A), a concentration which reduced gelatin cleavage by more than 90 % (Figure 5A). These experiments validated the hypothesis that blocking access of the substrate molecule to the CBD abrogates their cleavage by MMP-2.

P713 does not inhibit enzymatic activities of MMP-2 Δ CBD and MMP-8

To eliminate the possibility that P713 inhibited gelatin cleavage by direct interaction with the catalytic domain of MMP-2, we

Figure 3 Interactions between the CBD and MMP-2^{E404A} and the collagen-like binding site peptide P713 are concentration-dependent

To determine concentration-dependence of the CBD and MMP-2^{E404A} interactions with P713, CM5 chips were coated with P713 or Pscr and binding of concentration ranges of the CBD (0.5–40 μ M) or MMP-2^{E404A} (1–28 μ M) was monitored in SPR experiments. Plotting the binding (RU) of the CBD (**A** and **B**) and MMP-2^{E404A} (**C** and **D**) to P713 and Pscr peptide surfaces as a function of protein concentration revealed that both proteins had concentration-dependent binding interactions with P713, but no binding to Pscr (**B** and **D**).

analysed the P713 effects on the CBD deletion mutant of MMP-2, MMP-2 Δ CBD, in enzymatic assays. Of note, other investigators demonstrated previously that deletion of the CBD from MMP-2 reduced the activity on gelatin by $\sim 90\%$, but had little effect on cleavage of short peptide substrates [17]. Consistent with that observation, we found unchanged cleavage of the 11-mer peptide substrate NFF-1 by MMP-2 Δ CBD even in the presence of up to 100 μ M P713 (Table 1). Likewise, we detected little or no additional inhibition of gelatin degradation by MMP-2 Δ CBD at this concentration range of P713 (Table 1). Collectively, these experiments excluded the possibility that P713 exerted its inhibitory effect directly on the catalytic site of MMP-2.

To ensure that the observed CBD-mediated P713 inhibitory effects on MMP-2 did not apply to other MMPs without a CBD, we measured the rate of cleavage of a heptamer peptide substrate by neutrophil collagenase, MMP-8, in the presence of P713. Results showed that P713 inhibited only $\sim 15\%$ of the hydrolysis at P713 concentrations up to 100 μ M (Table 1). This is significantly less than the $> 90\%$ inhibitory effect of P713 on MMP-2 (Figure 5).

Overall, in the present study, we identified a segment on the $\alpha 1(I)$ collagen chain that represents a new binding site for MMP-2. A synthetic peptide (P713) corresponding to this MMP-2-binding site on collagen blocked CBD-mediated MMP-2 interactions with gelatin and inhibited gelatinolysis by a mode of action that involved the CBD, but was independent of the MMP-2 catalytic

site. Contrary to the effects on MMP-2, P713 had no activity on MMP-8.

DISCUSSION

Emerging from the recognition that MMP inhibitors generally lack specificity for individual MMPs, hence causing undesired side effects in clinical trials, alternative approaches for developing MMP-specific inhibitors have been sought. Novel strategies such as blocking enzyme–substrate interactions were reviewed by Overall and Lopez-Otin [21]. In this context, we and other investigators have demonstrated that disruption of interactions between the CBD in MMP-2 and gelatin reduces gelatinolysis by the enzyme [17,18,32]. That the collagen interactions for MMPs-2 and -9 reside in the CBD domain, which is not found in other MMPs, provides the opportunity to identify specific unique inhibitors of substrate binding in two MMPs that would not modify the functions of MMPs without the CBD. An example of alternative inhibitors are long-chain unsaturated fatty acids. However, although these preferentially bind CBD, they inhibit MMP-2 cleavage of both native and denatured type I gelatin, as well as an octapeptide substrate pointing to interactions with both the CBD and the active site [33].

Three fibronectin type 2-like modules that each possess an accessible hydrophobic surface form the CBD in MMP-2 [30,34,35]. Rather than uniting in a contiguous binding motif, the

Figure 4 Competitive inhibition of the CBD and MMP-2^{E404A} binding to gelatin by P713

In competitive binding assays, CM5 chips were coated with denatured type I collagen (gelatin). Subsequently, 1 μM CBD or MMP-2^{E404A} alone, or mixed with a concentration range of competing P713 or Pscr peptides (4–500 μM), were passed over the gelatin surfaces. The maximal response at each concentration of competitive peptides were recorded by Biacore sensorgrams and expressed by percentage inhibition relative to the control as described in the Material and methods section. (A) and (B) present the inhibition of the CBD and MMP-2^{E404A} binding as a function of competing P713 concentration respectively. Pscr did not alter protein interactions. That P713 had the capacity to inhibit the CBD and MMP-2^{E404A} binding to gelatin indicated that P713 corresponded to an essential CBD-binding site on the α1(I) collagen chain or blocked access for this and additional binding sites on gelatin to the collagen-binding sites on the CBD.

hydrophobic pockets are oriented outwards in a conformation that has been described as a 'three-pronged fishhook', thereby forming three putative ligand-binding sites in the CBD [30]. Although not yet fully solved, the location of collagen binding to these site(s) on the CBD is generally recognized [36,37]. In comparison, little is known about MMP-2/CBD-binding sites on collagen. Our earlier investigations found that the CBD interacts specifically with telopeptide segments of native type I collagen and also with three CNBr fragments (CB2, CB7 and CB8) [9]. Our observation that peptic removal of the telopeptides strongly reduced the binding affinity of the CBD to native type I collagen demonstrated that unwinding of the collagen triple helix exposed cryptic binding sites, such as those represented in the three tested CNBr collagen fragments. On this basis, our hypothesis was that sequences from collagen that mimic binding motif(s) for the CBD could specifically compete the CBD-dependent binding of MMP-2 to collagen and thereby inhibit the catalytic activities of this MMP.

Other investigators have screened phage display decameric peptide libraries with MMP-2 and MMP-9 as baits [38]. One identified decameric synthetic peptide with the sequence CGYGRFSPPC had the capacity to inhibit the catalytic activity of MMP-9 as well as cancer cell migration [39]. Subsequently,

Figure 5 P713 inhibits cleavage by MMP-2 of type I gelatin and type IV collagen, but has no effect on a peptide substrate

The capacity of P713 to inhibit enzymatic activities of MMP-2 was measured in enzyme activity assays. MMP-2 (75 nM) was added alone or with a concentration range of either P713 or Pscr peptides (0, 3–100 μM) to DQ-gelatin (0.5 μg/well) and a 12-amino-acid peptide substrate (NFF-1; 5 μM) (A) or to type IV DQ-collagen (B). The cleavage reactions were performed at 22 °C, and MMP-2 degradation of the substrates were measured after 30 min by changes in the fluorescent intensity (RFU) with λ_{ex} at 495 nm and λ_{em} at 515 nm for DQ-gelatin and DQ-collagen and with λ_{ex} at 325 nm and λ_{em} at 393 nm for NFF-1. The MMP-2 activity was expressed as a function of the P713 concentration as a percentage of the MMP-2 activity without P713 (100%). Results verified that P713 inhibits gelatinolysis and type IV collagenase activities by MMP-2, but not CBD-independent cleavage of short collagen-like substrate molecules. Data points are means $\pm$ S.D. of duplicate measurement for two to four experiments.

Table 1 Inhibition of MMP-2ΔCBD and MMP-8 activities in the presence of P713

The effects of peptide inhibitor P713 (0–100 μM) on cleavage of the fluorescent peptide substrates NFF-1 and DQ-gelatin by recombinant MMP-2ΔCBD and 3163v by MMP-8 respectively were analysed as detailed in the Materials and methods section.

Concentration of P713 (μM) ...	Rate of cleavage (RFU $\times 10^{-3} \cdot s^{-1}$)					
	0	6.25	12.5	25	50	100
MMP-2ΔCBD on NFF-1	2899	3045	3100	3092	3110	3069
MMP-2ΔCBD on DQ-gelatin	59	60	52	57	61	68
MMP-8 on peptide 3163v	918	909	901	884	851	788

Trexler et al. [29] used the CBD as bait for screening a phage-displayed peptide library. Two isolated and subsequently synthesized 15-mer peptides bound the CBD and were used for structural studies of their interactions with the CBD. Surprisingly, these peptides did not have collagen-like sequences, and the investigators did not report any MMP inhibitory properties [29].

Interestingly, we previously isolated a phage clone from a phage display peptide library using the CBD from MMP-2 as bait. While

the cloned phage bound well to the CBD, the corresponding synthetic peptide did not bind the CBD bait protein (X. Xu, Y. Wang, Z. Chen and B. Steffensen unpublished work). One should be aware that unique conformational presentations of peptides on phages may be critical to their functions and may be difficult to replicate in synthetic peptides [38]. For example, when displayed on phage, CGYGRFSPPC [39] may have a cyclic disulfide-bonded conformation. Likewise, when cysteine residues were replaced by serine residues in the CTTHWGFTLC peptide, the resulting STTHWGFTLS peptide had a 10-fold lower MMP-2 inhibitory activity compared with the cyclic peptide [38].

Recognizing the lack of success in isolating collagen-like peptide sequences from phage display peptide libraries by ourselves and others, in the present study we elected to screen a one-bead one-peptide combinatorial library using the CBD as bait [27]. Each bead is coated with a single peptide, which, under the experimental conditions, enabled us to screen $\sim 3 \times 10^6$ random peptide motifs. This was sufficient to examine all possible combinations of pentapeptide CBD-binding motifs and large proportions of longer motifs. Among 20 CBD-binding beads that we isolated and analysed, we did not identify a unique shared consensus sequence or a known binding site motif. However, alignments of the isolated peptides with the amino acid sequence of the $\alpha 1(I)$ chain from human type I collagen revealed a number of matches of differing levels of homology. These sites were distributed throughout the sequence, and included sites in CB fragments 2, 3, 4, 6, 7 and 8 [40]. Most sites were characterized by one or more gaps of different lengths and low level identities in the range 12–25%. However, one peptide demonstrated a 71% identity with residues 715–721 of the $\alpha 1(I)$ collagen chain (Figure 1). This collagen segment in CB3 was selected for further in-depth analyses. Of note, we did not report a CBD-binding site in CB3 in our earlier studies because this CB fragment was not available for study at the time [9]. Yet, as we reviewed the distribution of all putative binding sites, we did find lower homology sites in CB2, CB7 and CB8 that we found previously to contain binding sites for the CBD [9].

Combinatorial libraries have been used previously to identify compounds which can disrupt MMP-2 interactions with the $\alpha V\beta 3$ integrin [41] suggesting that a short peptide, such as P713, could have the capacity to block collagen interactions with the CBD. SPR analyses demonstrated that the synthetic collagen-like P713 bound both the isolated CBD and full-length MMP-2. Furthermore, competitive binding assays demonstrated that P713 reduced MMP-2 binding to gelatin in a concentration-dependent manner. These results verified the specificity of interaction and showed that P713 and gelatin interacted with the same or sterically closely positioned binding site residues on the CBD. Importantly, subsequent experiments revealed that P713 had inhibitory effects on the gelatinolytic activity of MMP-2. The IC_{50} of P713 for inhibition of MMP-2 binding to gelatin was $40 \mu M$, which was close to the concentration of P713 at which we achieved 50% inhibition ($IC_{50} = 30 \mu M$) of the MMP-2 gelatinolytic activity. In comparison, a scrambled Pscr without CBD-binding properties also did not inhibit the gelatinolysis by MMP-2. These results clearly implied that P713 inhibited MMP-2 by interfering with the binding of the enzyme to its substrate, gelatin.

To verify further that P713 did not act directly on the catalytic site, we investigated the inhibitory effects of P713 on MMP-2 hydrolysis of a short collagen-like peptide substrate, NFF-1, because cleavage of this substrate does not require positioning by the CBD [18]. The observation that P713 at $100 \mu M$ reduced the hydrolysis of NFF-1 by <20%, but the collagen α -chains of gelatin by >90% excluded an inhibitory mechanisms involving P713 binding in the active site of the enzyme. This assumption was substantiated further by the lack of P713 effects on NFF-1

and gelatin cleavage by a CBD deletion mutant of MMP-2 (MMP-2 Δ CBD). To ensure that CBD-targeted inhibition of MMP-2 by P713 did not apply non-specifically to MMPs without a CBD, such as the collagenases, our experiments demonstrated that MMP-8 cleavage of a peptide substrate was inhibited <15% with a P713 concentration of $100 \mu M$.

In addition to MMP-2, MMP-9 is the only MMP which contains a CBD consisting of three fibronectin type II-like modules. We recently demonstrated that the CBDs of MMP-2 and -9 have very similar substrate-binding properties, compete for substrate binding and competitively inhibit gelatinolytic activities of MMP-2 and -9 [24]. Although identified by its specific interaction with the CBD from MMP-2, P713 also bound the CBD from MMP-9 and inhibited the gelatinolytic activity of MMP-9 (results not shown). While this observation confirmed our conceptual approach to inhibit MMPs via disruption of exosite-mediated substrate binding, it also indicated that P713 inhibition via the CBD applied to both CBD-containing MMPs. The inhibitory effect of P713 is lower than the optimized, but non-specific, active site hydroxamic acid inhibitor batimastat [20]. Further efforts are needed to identify the precise binding site residues on the CBD that are critical for collagen binding in MMP-2 so that a rational structurally based approach may be applied to chemically modify and develop inhibitors with optimal CBD-binding and MMP-2 inhibitory properties.

In summary, in the present study we have identified a collagen peptide, which can specifically bind the CBD of MMP-2. The binding of the peptide to CBD blocks positioning of substrate molecules relative to the catalytic site and thereby inhibits MMP-2 activities.

We gratefully acknowledge Dr Gillian Murphy, Cancer Research UK, Cambridge Research Institute, Cambridge, U.K., for providing us with MMP-2 Δ CBD plasmids and Dr Gregg B. Fields, Department of Chemistry and Biochemistry, Florida Atlantic University, Boca Raton, FL, U.S.A., for providing the MMP substrate NFF-1. Dr Eileen Lafer and Patricia Schwarz, at the UTHSCSA Center for Macromolecular Interactions and Dr Susan Weintraub at the UTHSCSA Mass Spectrometry Laboratory provided valuable guidance in the experiments and data interpretation. This work was supported by NIH (National Institutes of Health) grants DE12818, DE14236 and DE016312, and the San Antonio Area Foundation.

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Received 3 May 2007/17 May 2007; accepted 21 May 2007

Published as BJ Immediate Publication 21 May 2007, doi:10.1042/BJ20070591