Peptide Ligands for the Fibronectin Type II Modules of Matrix Metalloproteinase 2 (MMP-2)∗

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The interaction of matrix metalloproteinase 2 (MMP-2) with gelatin is mediated by three repeats homologous to fibronectin type II (FN2) modules, which are inserted in the catalytic domain in proximity of the active site. We screened a random 15-mer phage display library to identify peptides that interact with the FN2 modules of MMP-2. Interestingly, the selected peptides are not gelatin-like and do not share a common, obvious sequence motif. However, they contain a high proportion of aromatic residues. The interactions of two peptides, WHWHR0IRPLQLAAGR and THSHQWRRHQPFPAPT, with constructs comprising the in-tandem first and second and third FN2 modules of MMP-2 (Col-12 and Col-23, respectively) were characterized by NMR. Both peptides interact with Col-12 and Col-23 with apparent association constants in the mM−1 range. Peptide binding results in perturbation of signals from residues located in the gelatin-binding pocket and flexible parts of the molecule. Although the former finding suggests that the gelatin-binding site is involved in the contact, the interpretation of the latter is less straightforward and may well reflect both the direct and indirect effects of the interaction.

Matrix metalloproteinase 2 (MMP-2,† gelatinase A), and the closely related MMP-9 (gelatinase B) are unique among the metalloproteinases in that three gelatin-binding fibronectin type II (FN2) modules (Col-1, Col-2, and Col-3) are inserted in their catalytic domain in the vicinity of the active site (1). The solution conformation of each FN2 repeat from human MMP-2 has been characterized via NMR spectroscopy (2–4). Moreover, the x-ray crystallographic structure of the intact human pro-MMP-2 has been reported (5).

In the second FN2 module from each MMP-2 and MMP-9, residues that are important for the interaction with gelatin have been identified via site-directed mutagenesis (6, 7). Additionally, the ligand binding surfaces of all three modules of MMP-2 have been mapped from 1H and 15N NMR perturbations induced by (PPG)6 and the longer chain analog, (PPG)12, synthetic peptide mimics of gelatin (2–4). In line with the crystallographic evidence, which shows that the FN2 modules in MMP-2 point away from each other (5), our NMR studies of the interaction between Col domains and (PPG)6 and (PPG)12 have shown that consecutive Col modules contain distinct ligand-binding sites in which affinities for these ligands are virtually identical to those of the individual domains (3, 4, 8).

Although the affinity of the MMP-2 Col domains for collagenous ligands appears by now to be well established, less is known regarding the specificity of the interaction. In our previous studies we found that the peptide PIHKFPGDV, which corresponds to segment 33–42 of the pro-MMP-2, interacts with the three Col domains of MMP-2 in a manner that mimics the interaction with the collagen-like (PPG)6 and (PPG)12 peptides (3, 4). Preference for binding to Col-3 was indicated, consistent with the x-ray crystallographic structure of the pro-MMP-2 (5). In the proenzyme, the prodomain interacts intramolecularly with the putative gelatin-binding site of Col-3 via contacts that involve propeptide amino acid residues Ile-35, Phe-37, and Asp-40. As these studies indicate, the ligand specificity of the Col domains is not restricted to collagen-like peptides. It would be useful to gain more information as to the range of structural diversity acceptable for peptides to interact with the Col-binding sites.

In the context of MMP-2 involvement in tumor invasion, metastasis and other physiopathological processes (reviewed in Ref. 9), it is highly desirable to identify agents that could block its activity. The suitability of MMP-2 as an anticancer target is supported by the finding that MMP-2-deficient mice display reduced angiogenesis and tumor progression (10). However, very few inhibitors specific for MMP-2 have been described to date (11). The most potent inhibitors also inhibit several other MMP family members (12–14). Although generic MMP inhibitors prevent tumor dissemination and formation of metastases in animal models (14–19), they tend to elicit too broad a spectrum of response and often exhibit side effects. It can be speculated that active site inhibitors that also bind to the unique FN2 domains of MMP-2 may be more MMP-2-specific. As a platform for such studies, we have screened random 6-mer and

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**Materials and Methods**

Selection of Peptides from Phage Display Libraries—Microtiter plates (Greiner Labortechnik) were coated with the first (βgalCol-1), second (βgalCol-2), or third (βgalCol-3) FN2 modules from human MMP-2 (20) or with the three domains in tandem (βgalCol-123) (21). The recombinant proteins, consisting of the appropriate FN2 module(s) and an amino-terminal peptide derived from the β-galactosidase moiety of the expression vector, were prepared as described previously (20, 21). The plates were incubated with the proteins (20 µg/ml) in 100 mM NaHCO₃ buffer for 2 h at 37 °C, after which they were blocked with 30 mg/ml serum albumin in 100 mM NaHCO₃ buffer for 2 h at 37 °C and washed six times with TBS buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl) and 0.5% Tween 20. βgalCol-123-Sepharose was prepared using cyanogen bromide-activated Sepharose 4B (Amersham Biosciences) and βgalCol-123 according to instructions from the manufacturer.

Two phage fUSE5 libraries, which express a foreign 15- or 6-mer random peptide library at the amino-terminal end of all five copies of its pIII coat protein (22) and the *Escherichia coli* strain K91Kan (thi/HfrC), carrying a “mini-kan hopper” element inserted in the *lacZ* gene, were obtained from Prof. G. Smith (University of Missouri-Columbia). The number of primary clones in the 15-mer library is 2.5 × 10⁸ (23), and in the case of the 6-mer library it is 2 × 10⁹ (22). Approximately 10¹⁵ phage/well in 100 µl of TBS buffer were incubated for 60 min at room temperature. Nonspecifically adsorbed phage were washed away with 12 times with 200 µl of TBS buffer containing 5 mg/ml serum albumin and 0.5% Tween 20. Unless otherwise indicated, bound phage were eluted with 2 times with 200 µl of TBS buffer containing 1 mg/ml gelatin type A from porcine skin type I collagen (Sigma). In some experiments with immobilized βgalCol-123, elution was performed with buffer containing 2 mg/ml βgalCol-123. Alternatively, −10¹⁵ phage in 200 µl of TBS buffer were incubated with 50 µl of βgalCol-123-Sepharose for 60 min. The resin was then washed with 10 ml of TBS buffer containing 5 mg/ml serum albumin and 0.5% Tween 20, and the bound phage were eluted with 3 × 200 µl of TBS buffer containing 1 mg/ml gelatin. The eluted phages were amplified, and a portion was used in the next biopanning cycle (24). After three rounds, individual phage were isolated, and the DNA sequence of the 5' end of the gene III was determined using a primer complementary to the positions 1663–1680 of the wild type gene.

The peptides ACGYTYHPPCARLTV (ACG), WFPGPITFIPRPWSS (WF), WHHRHRIPLLAAGR (WHW), THSHQWRHHQFPAPT (THS), and HASHFRFRHSHVYGV (HAS) were synthesized on an FMoc (N-(9-fluorenylmethoxy carbonyl) chemistry.

**NMR-Monitored Peptide Binding**—15N-labeled Col-12 and Col-23 modules from human MMP-2 (residues 223–337 and 278–394 respectively) (Fig. 1) were expressed in *E. coli* and purified as described previously (3, 4).

**Fig. 1.** Primary structures of Col-12 (A) and Col-23 (B). Numbering of residues is as previously published (2–4). Residues of module 2 in Col-12 and Col-23 (Col-12/2, Col-23/2) are primed (’) and of module 3 in Col-23 (Col-23/3) are double-primed (”) to distinguish them from those in module 1 (Col-12/1) (unprimed). Extraneous residues stemming from the expression vectors are shown as lowercase letters.

**Fig. 2.** 1H/15N HSQC spectra of Col-12 (A) and Col-23 (B) ligand-free (black) and in the presence of excess WHW (red). The assignment of 1H/15N amide resonances has been reported (2–4). The cross-peaks are labeled according to the residue numbering convention described in Fig. 1. Signals from extraneous residues at the amino terminus of Col-12 (N) have not been specifically assigned.

**Table I**

| Peptide | β-GalCol-U plates/gelatin | β-GalCol-2/ plates/gelatin | β-GalCol-3/ plates/gelatin | β-GalCol-12/ plates/gelatin | β-GalCol-123/ plates/gelatin |
|---------|---------------------------|---------------------------|---------------------------|-----------------------------|-----------------------------|
| ACG     | 3                         | 71                        | 25                        | 88                          | 16                          |
| WFP     | 16                        | 13                        | 6                         | 37                          | 6                           |
| WHW     | 6                         | 55                        | 6                         | 6                           | 6                           |
| WHV     | 9                         | 6                         | 17                        | 6                           | 6                           |
| HAS     | 6                         | 6                         | 6                         | 6                           | 6                           |

The frequency (%) of phage display peptides selected under various conditions.
To monitor ligand-induced resonance shifts, small aliquots of WHW or THS stock solutions in 90% H\textsubscript{2}O, 10% D\textsubscript{2}O, pH 7.0, were added to samples of 0.35 mM \textsuperscript{15}N-labeled Col-12 and Col-23 in 90% H\textsubscript{2}O, 10% D\textsubscript{2}O, pH 7.0, and \textsuperscript{1}H-\textsuperscript{15}N HSQC experiments (25–27) were recorded at each step. All of the data were acquired at 25 °C on Bruker Avance DMX-500 spectrometer equipped with a 5-mm triple resonance three-axis gradient probe. The spectra were processed and analyzed with the programs Felix 95 and Felix 98 (Molecular Simulations, Inc., San Diego, CA) on a Silicon Graphics Indy R-5000 work station. Protein and peptide ligand concentrations were determined spectrophotometrically (28). Values of the equilibrium association constant ($K_a$) were determined by a combination of linear and non-linear least squares fitting of the chemical shift changes, as described previously (29, 30).

### RESULTS AND DISCUSSION

#### Selection of Peptides That Interact with FN2 Domains from MMP-2

To identify peptides that bind to the FN2 modules of MMP-2, phage display 6-mer or 15-mer random peptide libraries were screened with $\beta$galCol-1, $\beta$galCol-2, $\beta$galCol-3 and $\beta$galCol-123. Nonspecifically adsorbed phage were washed away with buffer containing 5 mg/ml serum albumin, then-in most experiments-specifically bound phage was eluted with buffer containing gelatin.

In the case of the 6-mer library- unlike in the case of the 15-mer library—there was no enrichment of bound phage after three rounds of biopanning. This observation suggested that a six-residue-long peptide may be too short for unique recognition of FN2 domains, therefore we concentrated on the 15-mer.
library. From the latter library, after three rounds of biopanning, individual clones were sequenced and the following peptides were identified: ACGTYTYPPCARLTV (ACG), WFPGPITFIPRPWSS (WFP), WHWRHRIPLQLAAGR (WHW), THSHQWRHHQFPAPT (THS), WHVSPRHQRLFHGLF (WHV) and HASHFRFRHSHYGV (HAS). The frequencies of the peptides selected under various conditions are summarized in Table I. Interestingly, the peptides are not collagen-like and do not share a common, obvious sequence motif. However, their sequences exhibit biased amino acid composition. For example, although His accounts for only 2% of residues in protein databases, in the selected peptides, their proportion is 16%. This trend is even more pronounced for peptides selected on single FN2 domains, which contain 22% His. There also is bias in the aromatic amino acid content: whereas Tyr, Trp and Phe account for 8% of residues in protein databases, in the selected peptides, their proportion is 16%. Interestingly, peptide inhibitors of the whole gelatinases that have been selected previously from phage display library (11) are similarly enriched in aromatic residues whereas those from combinatorial (31) library contain multiple histidines. Our results suggest that not only interaction with the active site but also binding to FN2 modules may contribute to activity of these inhibitors. It is also noteworthy that peptide WFP contains sequence FPG which is found in the pro-domain (residues 37–39); F37 from this motif inserts into the hydrophobic gelatin-binding pocket of Col-3 in the x-ray structure of pro-MMP-2 (5).

The clones selected on βgalCol-1, βgalCol-2 and βgalCol-3 exhibit identical sequences with only moderate differences in

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**Fig. 5.** Contact surface of Col-12/1, residues 5–59 (A), Col-12/2 (B), Col-23/2 (C), and Col-23/3, residues 3–58 (D) colored according to Col-12 or Col-23 backbone amide chemical shift changes induced by WHW binding. Front (left) and back (right) views are shown. The gelatin-binding site comprising hydrophobic cleft and a protruding loop (residues 33–38) on its right-hand rim is revealed in the front view. The description of the figure is the same as for Fig. 4.

**Fig. 6.** Contact surface of Col-12/1, residues 5–59 (A), Col-12/2 (B), Col-23/2 (C), and Col-23/3, residues 3–58 (D), colored according to Col-12 or Col-23 backbone amide chemical shift changes induced by THS binding. Front (left) and back (right) views are shown. The description of the figure is the same as for Figs. 4 and 5.
their relative distributions. In turn, this indicates that despite the sequence differences between the modules, the ligand-binding sites of the three domains are likely to possess common features.

The ACG peptide, which was recovered with the greatest frequency on immobilized βgalCol-123, has two cysteines that may form a cystine bridge, thus constraining the structure. It is a common observation with phage display peptides that those with the highest affinity tend to be cyclic (11, 32–34). Interestingly, ACG was rarely selected on single Col domains, which suggests that the complementary binding surface on βgalCol-123 comprises multiple modules.

There is a striking difference between clones eluted from βgalCol-123 with gelatin and those eluted with βgalCol-123. Although gelatin will primarily elute phage that interacts with the gelatin-binding site of Col-123, βgalCol-123 is likely to release phage bound to any part of the protein. Therefore, peptides that become enriched upon elution with βgalCol-123 relative to elution with gelatin (WHW and, to a lesser extent, WHW) are likely to interact with a region situated at least partially outside of the gelatin-binding surface.

NMR Characterization of the Interaction between FN2 Modules and Selected Peptides—The interaction of the peptides with Col-12 and Col-23 was investigated using NMR (Fig. 1). Because of solubility problems, only WHW and THS were found suitable for this study. Col-12 and Col-23 chemical shift changes induced by WHW and THS were monitored in 1H-15N HSQC spectra (Fig. 2), and affinity constants ($K_a$) were calculated from the ligand titration curves (Fig. 3, Table II). The first and second modules of the Col-12 construct bind to WHW with $K_a = 1.9 \pm 0.2$ mM (Col-12/1) and $K_a = 2.6 \pm 0.3$ mM (Col-12/2). Within experimental errors, the latter agrees with the value derived for Col-23/2, $K_a = 2.8 \pm 0.4$ mM. For Col-23/3, $K_a = 3.0 \pm 0.5$ mM was determined. The affinity of THS for the Col modules is somewhat weaker, with $K_a = 0.9 \pm 0.2$ mM for Col-12/1, $K_a = 0.9 \pm 0.3$ mM for Col-12/2 and Col-23/2, and $K_a = 0.3 \pm 0.1$ mM for Col-23/3.

Previously (2–4), we mapped the gelatin binding surface of the Col modules by localizing spectral perturbations induced by the synthetic gelatin-like peptides (PPG)$_6$ and (PPG)$_{12}$ on the three-dimensional structures of the modules (Fig. 4). This approach proved to be less straightforward in the current study. Backbone amide resonances stemming from the termini and the linking segment of the two-domain constructs are affected by WHW (Figs. 2 and 5) and THS (Fig. 6); similar effects were observed during titration of Col-23 with the synthetic gelatin mimics (compare against Fig. 4, C and D). However, in the latter study, the shifts arising from the linking peptide were negligible relative to those marking the gelatin-binding pocket. In the current experiments, on the other hand, most spectral perturbations were of similar magnitude or smaller than those localized in the termini and linker. Hence, it is difficult to distinguish the effects caused by direct contact with the ligand from shifts related to altered conformation or dynamics due to peptide binding elsewhere. Chemical shift perturbations at locations distant from ligand contact sites have been observed previously in other systems (35).

The gelatin-binding surfaces of Col modules comprise an aromatic cluster and its surrounding region, in particular the loop comprising residues 33–38, at the front face of the domains. The distribution of WHW- and THS-induced shifts on the three-dimensional structures of Col modules can be summarized as follows (Figs. 5 and 6). In the first FN2 module within the Col-12 construct (Col-12/1), WHW predominantly affects residues neighboring the gelatin-binding pocket, most notably residues Gly-33, Arg-34 and Trp-40. In the second FN2 module within both the Col-12 and Col-23 constructs (Col-12/2 and Col-23/2), WHW perturbs mainly the termini, whereas the loop on the front face, which is involved in gelatin binding, is affected to a lesser extent. In the third FN2 module in Col-23 (Col-23/3), WHW-induced resonance shifts are limited to the back of the domain and include the termini and exposed hydrophobic patch, which encompasses, among others, residues Cys-15 to Phe-17; the front side is affected only minimally. Thus, it may be concluded that WHW interacts with the gelatin-binding pocket of Col-1 (4) and possibly Col-2. In agreement with such an interpretation, WHW has been selected most frequently by gelatin elution from plates coated with βgalCol-1 and less so from those coated with βgalCol-2 (Table I). THS perturbs amide resonances stemming from residues on the right-hand rim of the gelatin-binding pocket in all Col modules (Fig. 6), suggesting that THS contacts Col modules via this site. Signals from the termini are also affected.

CONCLUSIONS

Design of inhibitors that act on MMP-2 but not on other metalloproteases is a challenging task. Because MMP-2 (together with MMP-9) is unique in having FN2 domains positioned next to its catalytic cleft, active site inhibitors that also interact with FN2 domains should be much more specific. Hence, we have screened random 6- and 15-mer phage display libraries and identified several peptides from the latter library that interact with the FN2 modules of MMP-2.

The selected peptides were found to contain a high proportion of aromatic residues and no acidic side chains. Enrichment in aromatic amino acids may reflect the fact that such peptides are more likely to have a fixed conformation and thus may have higher affinity than more flexible peptides. The same reasoning may explain why no significant enrichment was observed in the case of shorter peptides.

The interaction of two peptides, WHW and THS, with FN2 modules was characterized by NMR. Both peptides bound to the Col domains with $K_a$ in the mM$^{-1}$ range. Perturbation of NMR signals from the termini and the linker upon peptide binding may reflect direct contact with the ligands or indirect effects on conformation and dynamics of these flexible regions. The NMR data also suggest that the contacts in most cases involve the gelatin-binding site, particularly the protruding loop on its right-hand rim that contains residues Gly-33 and Arg-34. The latter may account for the competition between these peptides and gelatin for binding to FN2 domains. Interestingly, residues Gly-33 and Arg-34 are also involved in intramolecular interactions between the Col-3 module and the propeptide domain (3, 5).

It is a well-established strategy in drug design to chemically link two low-affinity ligands to generate a high-affinity, high-specificity compound (36). As an application, peptides binding to FN2 modules can be linked to active site inhibitors with the aim of significantly increasing the affinity and specificity of the interaction. It is our hope that the peptide ligands we have identified will provide useful leads for the development of more potent gelatinase inhibitors.

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