Peptide Inhibition of Catalytic and Noncatalytic Activities of Matrix Metalloproteinase-9 Blocks Tumor Cell Migration and Invasion*

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Migration of invasive cells appears to be dependent on matrix metalloproteinases (MMPs) anchored on the cell surface through integrins. We have previously demonstrated an interaction between the integrin α-subunit I domain and the catalytic domain of MMP-9. We now show that there is also an interaction between the integrin β subunit and MMP-9. Using phage display, we have developed MMP-9 inhibitors that bind either to the MMP-9 catalytic domain, the collagen binding domain, or the C-terminal hemopexin-like domain. The C-terminal domain-binding peptide mimics an activation epitope in the stalk of the integrin β chain and inhibits the association of MMP-9 C-terminal domain with αvβ3 integrin. Unlike other MMP-9 binding peptides, it does not directly inhibit catalytic activity of MMP-9, but still prevents proenzyme activation and cell migration in vitro and tumor xenograft growth in vivo. We also find an association between MMP-9 and urokinase-plasminogen activator receptor and find that urokinase-plasminogen activator receptor is cleaved by MMP-9. Collectively, we have defined molecular details for several interactions mediated by the different MMP-9 domains.

Matrix metalloproteinases 2 and 9 (MMP-2 and -9), also known as gelatinases, play an important role in cell migration and tissue remodeling during development but also in pathological conditions such as inflammation and cancer (1). We have identified a highly selective peptide inhibitor of gelatinases, CTTHWQFTLCL (CTT) by phage display (2), whereas others have developed gelatinase-selective small molecule inhibitors (3) to specifically target these enzymes.

The unique structural feature of the gelatinases is the collagen-binding domain (CBD) within the catalytic domain (4). The CBD is composed of three fibronectin type II repeats and is an intriguing target to develop gelatinase-specific compounds.

Like most MMPs, the gelatinases also contain a C-terminal hemopexin/vitronectin-like domain (C domain or PEX), which contains the binding site for tissue inhibitors for matrix metalloproteinases (TIMPs) and is responsible for the dimerization of MMP-9 (5).

Although MMP-2 and MMP-9 are closely related enzymes, they do have differences in the regulation of expression, activation, and glycosylation and in substrate selectivity (1, 4). Of these two enzymes, MMP-2 has been investigated in a more detailed. For example, the activation of pro-MMP-2 has been thoroughly characterized and involves interactions of TIMP-2, MT1-MMP, and αvβ3 integrin on the cell surface (6, 7). MMP-9 has not been found to be activated via the same mechanism, and several proteinases including the plasmin/MMP-3 cascade (8) and trypsin-2 (9) can activate MMP-9 in vitro.

Relatively little is known about the molecular details of the MMP-9 interactions on the cell surface and how these regulate cell migration. MMP-9 has been found to interact with the αvβ3 integrin, the α chain of type IV collagen, and the hyaluronan receptor CD44 (10, 11). We have recently identified the leukocyte specific β3-integrins as a binding partner for pro-MMP-9. The phage display peptide ADGACILWMDGDGGCAAG (DDGW) competed with pro-MMP-9 binding to the ligand-binding domain of αvβ3 integrin subunit and inhibited migration of leukocytes (12). Here we have isolated MMP-9 binding peptides, which inhibit either substrate binding or proenzyme activation, leading to an inhibition of cell migration and invasion. Using these peptides, we identify MMP-9 interaction sites in fibronectin, vitronectin, and αvβ3 integrin.

EXPERIMENTAL PROCEDURES

Phage Display—Phage display selections were made using random peptide libraries CX7-12C and X9-10 (13). Purified human pro-MMP-9 (9) or recombinant MMP-9 C domain (2 μg/ml) was immobilized on microtiter wells, and the wells were blocked with BSA. The phage were added in 50 mM Hepes (pH 7.5), 5 mM CaCl2, 1 mM ZnCl2, 150 mM NaCl, 2% BSA. After three rounds of selection, the phage sequences were determined (14). The phage binding specificity was tested with pro-MMPs or the recombinant domains (20 ng/well). The phage (109 transducing units/well) were allowed to bind in the absence or presence of competitor peptides (20 μM), gelatin (2.5 μg/ml), or TIMP-1 (2.5 μg/ml; Calbiochem) followed by washings with PBS plus 0.05% Tween 20 (PBST). The phage were detected with a peroxidase-conjugated anti-phage antibody (Amersham Biosciences).

Peptide Synthesis—The peptide phages were initially prepared in a recombinant form using intein fusions (12, 15). Chemical peptide synthesis was then done using Fmoc (9-fluorenylmethoxy carbonyl) chemistry, and the purity and integrity of the peptides were verified by mass spectroscopy (15). The peptides were dissolved in water, except the CRVYGYLLC (CRV) and DDGW peptides, which were dissolved in 50 mM NaOH at a 10 mM concentration and then diluted into PBS to neutralize the pH. The TTRNNLLVSWQPPRARIT and ADIMINF-GREWHDGDGYPF peptides were synthesized on a cellulose membrane. The membrane was blocked with 3% BSA in TBS plus 0.05% Tween 20 and incubated with 0.2 μg/ml biotinylated CBD. Bound CBD was de-
Expression of the MMP and Integrin Domains—CDD (amino acids Gly361–Gly773) was amplified from MMP-9 cDNA with the oligonucleotides 5′-GGCCGGCATAAGGGGACGACATGCGGCGG-3′ and 5′-GGTCGAGATTTGCTGGCGGCAAGA-3′ incorporating NdeI and PstI restriction sites. The PCR product was ligated into pTWIN vector (New England Biolabs). CDD was expressed in Escherichia coli and purified using gelatin-Sepharose (Amersham Biosciences). For some constructs, CDD was cloned from β integrin cDNA using oligonucleotides 5′-GGTCGAGGCTTCACTGGATTGGGGAGTCCAGGAGGAGTGCCAGGATGGGG-3′ and 5′-GGTGGTGGCCGCCGTGCATTCACTGGATTGGGGAGTCCAGGAGGAGTGCCAGGATGGGG-3′. The protein with an N-terminal His6 tag was digested with XhoI and NotI, and ligated into the pTWIN vector. The protein was purified from inclusion bodies by solubilization with urea, refolded in the presence of gelatin, and purified with gelatin-Sepharose. The β integrin-epidermal growth factor-like domain 2 + 3 (1-EGF2-3) fragment (Glu96–Asn170) was cloned from β integrin cDNA using oligonucleotides 5′-GGTGGTGGCCGCCGTGCATTCACTGGATTGGGGAGTCCAGGAGGAGTGCCAGGATGGGG-3′ and 5′-GGTGGTGGCCGCCGTGCATTCACTGGATTGGGGAGTCCAGGAGGAGTGCCAGGATGGGG-3′. The β integrin-epidermal growth factor-like domain 2 + 3 fragment (Glu96–Asn170) was cloned and expressed in E. coli and purified in a soluble form using Ni⁺⁺ affinity chromatography. The K542A and Y544A mutant βi 1-EGF2-3 constructs were prepared by site-directed mutagenesis. The integrity of all constructs was verified by DNA sequencing.

Gelatinase Inhibition Assay—Gelatin-Sepharose (Amersham Biosciences) was covalently coupled to a 1-mL column (50–500 μg/ml) with 2% glutaraldehyde in PBS. The column was washed three times with PBS, incubated with 50 mM glycine HCl (pH 2.1) to neutralize the column, and then washed with PBS. Gelatin-Sepharose (1 mg/mg) was allowed to bind for 30 min at room temperature. Bound gelatin was detected with streptavidin-peroxidase.

Domain-specific Peptide Inhibitors of MMP-9—Activation of MMP-9—THP-1 cells (40,000 cells/100 μl) or confluent HT1080 cells were incubated for 16 h in the presence or absence of 2.5 μg/ml plasminogen, 0.5 μg/ml pro-MMP-3 (Oncogene Research Products), or fibronectin (2 μg/ml), or a monoclonal anti-uPAR (H-129; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a control IgG. Integrins were immunoprecipitated with 2 μl of anti-integrin cytoplasmic domain antisera (20). The immunoprecipitates were resolved in 8% SDS-polyacrylamide gel, blotted, and detected with anti-MMP-9 antibodies. The MMP-9 C domain was preincubated with the peptides for 30 min in 1% BSA-PBST and then added to the wells. After a 2-h incubation, the wells were washed.

Cell Adhesion—HT1080 cells were allowed to adhere on vitronectin (10 μg/ml) in serum-free Dulbecco’s modified Eagle’s medium. The cells were stimulated by mitogens to enhance the cells with 0.5% agarose in Dulbecco’s modified Eagle’s medium and adding 5 μl of fetal bovine serum with PDGF (20 ng/ml final concentration) to the one end of the wells. Overnight cultured cells were washed with PBS, fixed with paraformaldehyde, permeabilized, and stained with the monoclonal anti-uPAR antibody (Ab3937, 2 μg/ml; American Diagnostica) or anti-β5 integrin IAP (2 μg/ml (21)) and polyclonal MMP-9 antibody (H-129; 10 μg/ml). The primary antibodies were detected with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 antibodies. uPAR Cleavage on the surface of HT1080 cells was studied using biotinylated CBD (5 μg/ml) and allowed to bind for 30 min at room temperature. Bound gelatin was studied as described (12). Gelatinase binding to immobilized pro-MMP-9 was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay (Roche Applied Science).

Cell Migration and Invasion—The cell migration assay was conducted using transwell migration chambers (8-μm pore size; Costar) in 10% serum-containing medium (2, 14). Briefly, the membranes were coated on both sides with 40 μg/ml GST or with the βi integrin ligand peptide CPFLILLAAGC-GST fusion (GST-LL-CA) and blocked with 10% FBS. HT1080 cells (50,000 cells) or THP-1 cells (50,000 cells) were mixed with the peptides for 1 h in serum-containing medium. The cells were allowed to migrate for 16 h and were then stained with crystal violet and counted (14). The HT1080 (20,000 cells/100 μl) invasion assay was performed as the THP-1 migration, except that matrigel-coated transwells (BD Biosciences) were used.

Phage Display—Microtiter wells were coated with a mixture of fibronectin (10 μg/ml) and fluorescein isothiocyanate-labeled gelatin (100 μg/ml) followed by saturation with 1% BSA in PBS. HT1080 cells (50,000 in 100 μl of 0.1% BSA/Dulbecco’s modified Eagle’s medium) were incubated in the presence of 20 μg 4-phorbol-12,13-dibutyrate (PDBu) (Sigma) and the peptides or the MMP-9/MMP-8-selective inhibitor InhI (Calbiochem). As a control, nonactivated cells and medium without the cells were used. Gelatinolysis after 48 h was measured as the increase of fluorescence from a 50-μl aliquot of the conditioned medium using a Wallac Victor ii reader.

Pro-MMP-9 and Gelatin Binding to Leukocyte α9β5 Integrin—Pro-MMP-9 binding to the α9 domain in the presence of peptides was studied as described (12). Gelatin binding to the pro-MMP-9 was measured by gelatin complex was studied by immobilizing the integrin α9β5; (12) or α5β1, as a control (Enzyme Research Laboratories, South Bend, IN) (1 μg/well) in TBS plus 1 μM CaCl₂ and 1 mM MgCl₂ followed by saturation of the wells with 1% BSA in PBS. Pro-MMP-9 (100 μg/well) was incubated for 2 h, and the unbound pro-MMP-9 was washed away. Gelatin was allowed to bind to α9β5 for 2 h, washed in PBS and separated in a nonreducing 12% SDS-PAGE followed by Western blotting with anti-uPAR antibodies (99R; American Diagnostica, Greenwich, CT) or anti-MMP-9 (H-129; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or a control IgG. Integrins were immunoprecipitated with 2 μl of anti-integrin cytoplasmic domain antisera (20). The immunoprecipitates were resolved on an 8% SDS-polyacrylamide gel, blotted, and detected with anti-MMP-9 antibodies.

Immunofluorescence—HT1080 cells were allowed to adhere on vitronectin (10 μg/ml) in serum-free Dulbecco’s modified Eagle’s medium. The cells were stimulated by mitogens to enhance the cells with 0.5% agarose in Dulbecco’s modified Eagle’s medium and adding 5 μl of fetal bovine serum with PDGF (20 ng/ml final concentration) to the one end of the wells. Overnight cultured cells were washed with PBS, fixed with paraformaldehyde, permeabilized, and stained with the monoclonal anti-uPAR antibody (Ab3937, 2 μg/ml; American Diagnostica) or anti-β5 integrin IAP (2 μg/ml (21)) and polyclonal MMP-9 antibody (H-129; 10 μg/ml). The primary antibodies were detected with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 555 antibodies. uPAR Cleavage on the surface of HT1080 cells was studied using biotinylated CBD (5 μg/ml) of recombinant soluble human uPAR (R&D Systems) was digested with 50 ng of trypsin-activated MMP-9 in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂, 1 μM ZnCl₂, 0.02% NaN₃, 0.01% Tween 20 for 1 h at +37 °C in the presence or absence of 200 μM CRV or the scrambled peptide. The samples were incubated for 16 h in the presence or absence of 20 μM InhI, 200 μM CTI or W-A CTI control peptide, 25 μg/ml aprotinin, or 20 μM benzamidine. The cells were washed three times with PBS, incubated with 50 mM glycine HCl (pH 3.0) plus 100 mM NaCl to extract cell surface-bound urokinase-plasminogen activator (uPA) and MMPs, and neutralized with 500 mM Hepes (pH 7.5) plus 100 mM NaCl. Membrane proteins were enriched with 100 mM KCl and 0.1% CHAPS. Membrane (M) (120 μg of protein) or cytoplasmic (C) (150 μg of protein) (ThP-1) cells of protein was separated on 12% SDS-PAGE and analyzed by Western blotting with anti-uPA antibodies (99R; 1:1000 dilution). uPA Cleavage on the surface of HT1080 cells was studied using biotinylated CBD (5 μg/ml) and allowed to bind for 30 min at room temperature. Bound gelatin was studied as described (12). Gelatinase binding to immobilized pro-MMP-9 was measured using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay (Roche Applied Science).
in a 200-μl volume on ice for 30 min with the competitors, 125I-labeled C domain (1 × 10⁶ cpm) was added and incubated for 3 h on ice. The cells were transferred to tubes containing 200 μl of dibutyl phthalate/cyclohexane mixture (23:2, v/v), centrifuged 7500 × g for 10 min, and snap-frozen (24). The bottom of the tube containing the cells was cut and analyzed with a γ-counter.

Tumor Growth in Vivo—The animal studies were approved by the ethical committee of Helsinki University. HSC-3 tumors were established by administering 5 × 10⁶ tumor cells in a 100-μl volume in PBS in both flanks of the Hsd:Athymic Nude-nu mice. After 3 days, the mice received five daily injection of 0.8 mg/ml CRV or the scrambled peptide or the vehicle (PBS) via the tail vein in a 200-μl volume (20). The tumor volumes were calculated. Mice were sacrificed when the tumor volume reached 1000 mm³. For the staining of the tumor vasculature, 7-μm frozen tissue sections were stained with anti-CD31 antibody (MEC 13.3; BD Biosciences) and anti-rat Alexa Fluor 488 antibodies. Statistical significance was calculated with the t test or with log rank test in Kaplan-Meier survival analysis.

RESULTS

Identification of Peptide Probes to Different Domains of MMP-9—In order to understand gelatinase-mediated cell migration in depth, we searched for putative MMP-9-binding proteins by phage display of random peptide libraries. The pro-MMP-9 and its recombinant domains were used in biopanning, since the active MMP-9 primarily bound peptides with a WGF motif (2). Two groups of pro-MMP-9 binding peptides were found (Table I). Group I had a motif, CGArGArS/S/Q PPC, where Ar represents an aromatic amino acid. These peptides show similarity to sequences found in the gelatinase substrates fibronectin and vitronectin (4). Group II had a CRX-YGPXXXC motif. In this group, the CRVYGYPYLLC peptide was obtained by biopanning with pro-MMP-9, whereas the other sequences were obtained with a recombinant C-terminal domain. The CGYGRFSPPC (PPC) and CRV peptides were chosen for further studies as representatives of the two groups.

To identify the binding sites of these peptide motifs, we carried out phage binding experiments. Binding of PPC peptide-bearing phage to pro-MMP-9 was inhibited by a soluble recombinant 18-mer ADGACGYGRFSPPCGAAG (PPC) peptide and gelatin, but not with CTT or a recombinant ADGACRYYGYPYLLCGAAG (CRV) peptide (Fig. 1A). Conversely, binding of the CRV-phage was inhibited by CRV and not by PPC, CTT, or gelatin, indicating nonoverlapping binding sites for these peptides. Inhibition of PPC phage binding by gelatin implied that that PPC binds to the CBD of MMP-9. Furthermore, phage selection with the MMP-2 CBD has also yielded a PPC-like peptide AGTYTYPHYPPCARLT (25). The PPC peptide, but not CTT or CRV, inhibited gelatin binding to immobilized CBD in a dose-dependent manner (Fig. 1B) but had no effect on gelatin binding to fibronectin (data not shown), suggesting that PPC is specific for the fibronectin type II repeats of gelatinases. In a gelatin degradation assay, PPC inhibited both MMP-9 and MMP-2 activity (Fig. 1C), the scrambled control peptide ADGACPSYGRFCGAAG (scr. PPC) having no effect. The CRV peptide did not inhibit gelatinase activity, consistent with the inability of gelatin to compete with CRV. The PPC peptide was a weaker gelatinase inhibitor than CTT, which completely inhibits gelatin degradation at a 100 μM concentration in this assay (15).

To study whether the PPC-like sequences of fibronectin and vitronectin bind MMP-9, we examined the binding of CBD to these proteins in a solid phase binding assay. CBD bound to both fibronectin and vitronectin, but not to the 110-kDa fragment of fibronectin lacking the C-terminal heparin-binding domain and thus the suspected gelatinase-binding site (Fig. 1D). PPC, but not the scrambled peptide, inhibited the CBD binding. Similar results were obtained in a pepspot membrane assay, where biotinylated CBD bound to the PPC-like fibronectin peptide TTPNSLLVSWQP PRARIT but not to an 18-mer control peptide (Fig. 1D, inset). When different MMPs were compared, the CRV phage showed a CRV peptide-inhibitable binding only to pro-MMP-9, and not to pro-MMP-2 or pro-MMP-3 (Fig. 2A). The scrambled CRV peptide CGYLPLRYVC had no effect. MMP-9 selectivity was also observed with the recombinant MMP-9 and MMP-2 C domains. The CRV phage recognized the MMP-9 C domain strongly in comparison with the MMP-2 C domain (Fig. 2B).

| Table I | Pro-MMP-9 binding peptide sequences |
|---------|------------------------------------|
| **Group I: CBD-binding sequences** | **Group II: C domain-binding sequences** |
| CGYGRFSPPC (6) | CRVYGYPYLLC |
| CGWGRYSPPC | CRVYGYPILWC (2) |
| CGFGRWQPPC | CRVYGPMWALC |
| FN TPNSSLLVSWQPPRARIT | CRVYGPSWVWC |
| VN PETLHPGRGFCNPC | CRVYGAWLLC |
| CRWYGPWVWC | CRVYGCPSSIC |
| CRWYGPWVWC | CRVYGPMVFC |
| CRWYGWVVC | CRVYGWLTVC |
| CRWYGLPFLC | CRWSYGFPVVC |
| CRWYGNPRVC | CRWYGFPRVVC |

FIG. 1. Characterization of peptide ligands to MMP-9. A, phage carrying PPC, CRV, or a control peptide (GPEELWLLC) were allowed to bind to pro-MMP-9 with the indicated peptides (20 μM) or gelatin (2.5 μg/ml) as a competitor. Bound phage was quantified with an anti-phage antibody. The results are means ± S.D. of triplicate samples in this and other figures unless otherwise stated. **, statistically significant difference (p < 0.001) in Student’s t test in this and other figures. B, PPC, but not CTT or CRV, inhibits binding of gelatin to immobilized CBD. Biotinylated gelatin was detected using a streptavidin-peroxidase conjugate. C, the enzymatic activity of MMP-2 and MMP-9 in a gelatin degradation assay is inhibited by the PPC peptide but not the CRV peptide or the scrambled PPC peptide (scr. PPC). D, binding of the biotinylated CBD to intact fibronectin (FN), the 110-kDa cell-binding fragment of fibronectin, vitronectin (VN) (1 μg/well), or BSA in the presence or absence of 20 μM peptides was measured. D, inset, binding of the biotinylated CBD to fibronectin-derived peptide TTPNSLLVSWQP PRARIT or a control peptide on a pepspot filter. CBD binding was detected with enhanced chemiluminescence.
Domain-specific Peptide Inhibitors of MMP-9

Fig. 2. CRV peptide selectively binds to the C-terminal domain of MMP-9 and inhibits homodimerization. Binding of the CRV phage to pro-MMPs (A) and the recombinant C-terminal domains of MMP-2 and -9 (B) was studied in the presence or absence of 20 μM soluble peptides. C, phage binding to the recombinant C domain or MMP-9 or the CBD was assayed in the presence or absence of peptides (20 μM) or TIMP-1 (2.5 μg/ml). D, binding of the 125I-labeled C domain to unlabeled C domain, CBD, or BSA was studied in the presence or absence of CRV or scrambled (scr.) CRV peptide. Bound radioactivity was measured with a γ-counter. *p < 0.01, statistically significant difference in Student’s t test. n.s., not significant. cntrl, control.

TIMP-1 could not compete with the CRV phage binding to the MMP-9 C domain (Fig. 2C) or pro-MMP-9 (not shown). The CRV phage did not bind to the CBD (Fig. 2C) or a pro-MMP-9 lacking the hinge region and the C-terminal domain (pro-MMP-9ΔHC; not shown). We next examined the effect of CRV on the dimerization of MMP-9 C domain. 125I-labeled C domain was preincubated with CRV or scrambled peptide and then added to wells coated with unlabeled C domain. Dimerization of the C domain was inhibited by CRV but not by the scrambled peptide (Fig. 2D).

Cell Migration and Invasion Are Inhibited by Blocking the Domain-specific Interactions of the Gelatinases—We studied the role of the gelatinase domains in cell migration and invasion using the CTT, PPC, and CRV peptides. The binding site of CTX maps to the catalytic domain, but not to CBD (Fig. 1B (12)). As indicated above, PPC and CRV are probes for the CBD and the C domain, respectively. All three peptides inhibited HT1080 fibrosarcoma invasion into matrigel. At a 200 μM concentration of CRV or CTX, 50% inhibition was observed.

The PPC peptide required a 500 μM concentration to achieve the same efficacy (Fig. 3A). The scrambled control peptides were inactive. Similar results were obtained with THP-1 monocytic cells, which migrate on a synthetic GST-LLG-C4 substrate (14) in a β3 integrin- and gelatinase-dependent manner. PPC, CRV, and CTX, but not the scrambled peptides, had an inhibitory effect (Fig. 3B). The inhibition of cell migration was not due to toxicity as there was no effect on cell viability when the cells were cultured for 48 h with the peptides at a 500 μM concentration (not shown). Surprisingly, CRV inhibited peri-
cellular gelatinolysis similarly as did CTX and PPC, as measured by a release of fluorescent gelatin fragments into the conditioned medium (Fig. 3C). In this assay, HT1080 cells were cultured for 48 h in the presence of PDBu on a fibronectin/fluorescein isothiocyanate-labeled gelatin coating. The gelatinase-selective small molecule inhibitor (Inh1) also inhibited gelatinolysis, but the scrambled peptides did not. These results indicated that not only the direct MMP enzyme inhibitors but also CRV affects cell migration and pericellular proteolysis. We also tested that the CRV and PPC peptides do not affect the interaction of MMP-9 with the leucocyte α5 integrin I domain, which is blocked by DDGW (Fig. 3D). In fact, PPC stabilized pro-MMP-9 binding to the I domain as shown by typically 20–50% higher binding in the presence of PPC. Antibody binding to pro-MMP-9 in the absence of the I domain was not affected by PPC (not shown). The data suggested that the α5β3 integrin-bound MMP-9 could bind its substrates using CBD to generate a triple molecular complex between an integrin, MMP-9, and a ligand/substrate. To directly test this, pro-MMP-9 was allowed to bind to immobilized α5β3 integrin, and binding of biotinylated gelatin, an MMP-9 substrate, was examined. Gelatin bound to the pro-MMP-9-α5β3 integrin complex but not the α5β3 integrin alone. The platelet integrin αIIbβ3 did not support pro-MMP-9/gelatin binding (Fig. 3E).

MMP-9 Associates with the Urokinase-Plasminogen Activator Receptor—We next investigated the effects of the peptides on plasmin/MMP-3-mediated pro-MMP-9 activation in PDBu-activated HT1080 and THP-1 cells. The conditioned medium from the cells incubated in the presence of the peptides was analyzed by gelatin zymography. Of the three peptides, only CRV was capable of inhibiting pro-MMP-9 activation. In HT1080 cells, CRV peptide inhibited pro-MMP-9 activation strongly and the activation of pro-MMP-2 partially (Fig. 3F). The addition of plasminogen was sufficient in activating pro-MMP-9 in HT1080 cells, and pro-MMP-3 did not promote activation any further. In THP-1 cells, pro-MMP-9 activation required pro-MMP-3 and plasminogen added together, and the activation was blocked by CRV but not by the other peptides (Fig. 3G). In fact, pro-MMP-9 activation was augmented in the presence of PPC or DDGW, and there were higher levels of released MMP-9 as previously observed with DDGW (12). CRV did not inhibit the activation of purified pro-MMP-9 by MMP-3 in vitro (Fig. 3H).

Since the plasminogen activation cascade is involved in pro-
MMP-9 activation, we considered the possibility that the uroki-

nase receptor associates with MMP-9. Immunoprecipitations from PDBu-activated HT1080 cells showed that pro-MMP-9 co-precipitated with anti-uPAR antibodies but not with the control antibodies (Fig. 4A). The association of uPAR and pro-
MMP-9 was similarly found in THP-1 cells and was not affected by prior PDBu activation (Fig. 4A). Several proteases are able to cleave uPAR (26, 27); we thus asked whether MMP-9 also does so. Using purified proteins, we observed that MMP-9 cleaved the domain 1 (D1) from uPAR similarly as does chymotrypsin (Fig. 4B). The uPAR cleavage by MMP-9 occurred in the presence of aprotinin and was inhibited by the metallopro-
teinease inhibitor EDTA. uPAR cleavage occurs on the surface of phorbol-ester-activated cells (26). To study the contribution of gelatinases in this process, we incubated HT1080 cells with proteinase inhibitors and analyzed the membrane protein-en-
riched lysates by Western blotting with antibodies to uPAR. The gelatinase-selective inhibitor Inh1, but not the serine pro-
teinease inhibitors aprotinin or benzamidine, inhibited uPAR cleavage (Fig. 4C). The inhibition of uPAR cleavage was accom-
panied with reduced gelatinase levels in the conditioned me-
dium and on the cell surface. In the conditioned medium,
MMP-9 occurred in higher levels than MMP-2, whereas the opposite was true for the cell surface. The cell surface-bound MMP-9 was in the latent form, as previously observed (28). In addition, the level of cell surface-bound uPA was reduced in the presence of InhA. uPAR cleavage on the THP-1 cells was similarly inhibited by InhI and CTT but not by the inactive W→A CTT mutant peptide (15) or aprotinin (Fig. 4D). In the absence of PDBu, the THP-1 cells cultured in a serum-free medium expressed hardly detectable levels of uPAR.

The CRV Peptide Is a Mimic of an Integrin β Chain Epitope—In nonleukocytic cells, uPAR is able to associate with β1, β3, and β5 integrins (29–32). We thus investigated which integrin(s) could interact with MMP-9 in HT1080 cells. Immunoprecipitations were performed with antibodies against α5, α3, αβ1, β3, and β5 integrins. Pro-MMP-9 associated with the α5 and β3 integrins, indicating that α5β3 and αβ5 are the major integrins involved in pro-MMP-9 binding in HT1080 cells grown on a tissue culture-treated plastic (Fig. 5A). MMP-1 and -2 can interact with integrins through their C-terminal domains (6, 33). Interestingly, a database search revealed that the CRV peptide bears a similarity to sequences found in the stalk of the β5 integrin chain. Seven of the CRV amino acid residues had a matching or a similar residue in the β5 sequence (Fig. 5B). These sequences are located in the cysteine-rich I-EGF-like domain 2 and become exposed in the activated integrins, as shown by the reactivity of activation state-specific antibodies (34, 35). Indeed, the antibody KIM127 epitope maps to the CRV-like sequence in the β5 integrin chain (34). To study whether MMP-9 binds to this integrin activation epitope, we first assessed the effect of the MMP-9 C domain on cell adhesion to vitronectin and fibronectin. Neither the C domain nor the pro-MMP-9–JH3 (40 μg/ml) or the CRV peptide (200 μM) inhibited HT1080 cell adhesion to vitronectin or fibronectin (Fig. 5C). Adhesion to vitronectin occurred in a αvβ5-dependent manner as demonstrated by inhibition with the αvβ5 integrin-blocking antibody P1F6 (25 μg/ml). We did not observe specific adhesion of HT1080 cells to the immobilized C domain (not shown). These results indicated that the putative interaction site of the MMP-9 C domain in αvβ5 and αvβ3 is not the major RGD ligand-binding site or a cell adhesion determinant. This prompted us to express the I-EGF domains 2 and 3 (6) from the β5 integrin. Interestingly, biotinylated β5 I-EGF2+3 protein specifically bound to the MMP-9 C domain in a CRV-peptide-inhibitable manner (Fig. 5D). The β5 I-EGF2+3 fragment did not bind to MMP-9 CBD, vitronectin, or itself (Fig. 5D) or the C domain of MMP-2 (not shown). The binding was cation-independent (not shown) and could be inhibited with unlabeled β5 I-EGF2+3. We next mutated the Lys562 and Tyr544 residues of the β5 I-EGF2+3 to alanines to study the importance of the CRV-like sequence. This resulted in a decrease of activity, the K542A and Y544A proteins competing less efficiently for the binding of biotinylated β5 I-EGF2+3 to the MMP-9 C domain (Fig. 5E). The Y544A mutation also decreased the ability of β5 I-EGF2+3 to inhibit HT1080 invasion through matrigel (Fig. 5F). β3 I-EGF2+3, MMP-9 C domain, and MMP-2 C domain each inhibited HT1080 invasion with a similar potency, whereas GST had no effect.
cells. 125I-Labeled MMP-9 C domain showed a specific binding we studied the binding of MMP-9 C domain to HT1080 by a chemical gelatinase inhibitor InhI (20 μM). D2D3 fragment, is shown as a control. inhibited with 10 mM EDTA. Chymotrypsin cleavage, which yields a WB blotting (WB). From BDBu-activated HT1080 cells and nonactivated were performed from BDBu-activated HT1080 cells and nonactivated cells. Pro-MMP-9 was detected with Western blotting (WB). 2, MMP-9 cleaves soluble uPAR in vitro. The cleavage is inhibited with 10 mM EDTA. Chymotrypsin cleavage, which yields a D2D3 fragment, is shown as a control. C, inhibition of uPAR cleavage on THP-1 cells is similarly inhibited by the gelatinase inhibitors InhI (20 μM). MeSO (DMSO) was used as vehicle for the InhI. Aprotinin (aprot.) (25 μg/ml) and benzamidine (benz.) (20 μM) were used as controls. Equal amounts of membrane fractions were separated on SDS-PAGE and analyzed with antibodies to uPAR. The conditioned medium was analyzed by gelatin zymography. The cell surface gelatinases and uPA were analyzed from acid eluates.

To find further evidence for the MMP-β3 integrin interaction, we studied the binding of MMP-9 C domain to α5β3 expressing cells. 125I-Labeled MMP-9 C domain showed a specific binding to β3 integrin-transfected, but not to the untransfected CS-1 melanoma cells (Fig. 6A). The binding was competed with unlabeled MMP-9 C domain, the β3-EGF2+3 fragment, and to a lesser extent the β3-EGF2+3 fragment decreased its activity. The CRV peptide, which is identical to the CRV-like site of the integrin CRV peptide, did not affect the MMP-9 C-domain interaction, which occurs in the presence of calcium and presumably maintains pro-MMP-9 inactive, the C domain/β3 subunit interaction requires activated integrins and appears to play a dynamic role in mediating MMP-9 activation and pericellular gelatinolysis.

The C-terminal domain-binding CRV peptide did not affect the enzymatic activity of MMP-9 but inhibited dimerization of the MMP-9 C domain, activation of the pro-MMP-9 via plasminogen/MPM-3-dependent pathway, and pericellular gelatinolysis. Several findings indicate that CRV is a mimic of the activation epitope in the integrin β3 subunit, preferentially the β3 subunit. The C domain of MMP-9 inhibited leukocyte adhesion to the KIM127 antibody, which recognizes the CRV homologous site in the β3 integrin. The recombinant β3 integrin-EGF2+3 fragment specifically bound to the C domain in a CRV-dependent manner, and the single alanine mutations of the Lys542 and Tyr544 residues in the β3 integrin-EGF2+3 decreased its activity. The β3 integrin-

![Image](https://example.com/image.png)

**Fig. 4. MMP-9 interacts with and cleaves uPAR.** A, immunoprecipitations (IP) with antibodies to uPAR (399R) and MMP-9 (H-129) were performed from BDBu-activated HT1080 cells and nonactivated and activated THP-1 cells. Pro-MMP-9 was detected with Western blotting (WB). B, MMP-9 cleaves soluble uPAR in vitro. The cleavage is inhibited with 10 mM EDTA. Chymotrypsin cleavage, which yields a D2D3 fragment, is shown as a control. C, inhibition of uPAR cleavage on HT1080 by a chemical gelatinase inhibitor InhI (20 μM). MeSO (DMSO) was used as vehicle for the InhI. Aprotinin (aprot.) (25 μg/ml) and benzamidine (benz.) (20 μM) were used as controls. Equal amounts of membrane fractions were separated on SDS-PAGE and analyzed with antibodies to uPAR. The conditioned medium was analyzed by gelatin zymography. The cell surface gelatinases and uPA were analyzed from acid eluates. D, uPAR cleavage on THP-1 cells is similarly inhibited by the gelatinase inhibitors InhI (20 μM) and CTT (200 μM).

DISCUSSION

We have developed domain-specific peptide probes to the gelatinases and examined molecular interactions important for these enzymes. Each of the domain-specific peptides inhibited cell migration, indicating that the three major domains of MMP-9 (the catalytic domain, CBD, and the C domain) each play a distinct role. We have previously shown that in leukocytes, pro-MMP-9 interacts with the α5M and α5 integrin I domains through the catalytic domain (12). Here we have found another integrin interaction for MMP-9, where the C domain of MMP-9 binds to the integrin β3 subunit. In contrast to the I domain interaction, which occurs in the presence of calcium and presumably maintains pro-MMP-9 inactive, the C domain/β3 subunit interaction requires activated integrins and appears to play a dynamic role in mediating MMP-9 activation and pericellular gelatinolysis.

Of the MMP-9 binding peptides identified in this study, the CBD-binding peptide PPC functioned as an exosite inhibitor of MMP-2 and -9 inhibiting gelatin binding and degradation but had no inhibitory effect on the MMP-9 interactions with integrins. We identified a PPC-like sequence in the heparin-binding domain of fibronectin as a CBD recognition site. Vitronectin had a similar but apparently lower affinity binding site for MMP-9, where the C domain of MMP-9 inhibits matrix metalloproteinases. The CRV peptide, which is identical to the CRV-like site of the integrin CRV peptide, did not affect the MMP-9 C-domain interaction, which occurs in the presence of calcium and presumably maintains pro-MMP-9 inactive, the C domain/β3 subunit interaction requires activated integrins and appears to play a dynamic role in mediating MMP-9 activation and pericellular gelatinolysis.

The C-terminal domain-binding CRV peptide did not affect the enzymatic activity of MMP-9 but inhibited dimerization of the MMP-9 C domain, activation of the pro-MMP-9 via plasminogen/MPM-3-dependent pathway, and pericellular gelatinolysis. Several findings indicate that CRV is a mimic of the activation epitope in the integrin β3 subunit, preferentially the β3 subunit. The C domain of MMP-9 inhibited leukocyte adhesion to the KIM127 antibody, which recognizes the CRV homologous site in the β3 integrin. The recombinant β3 integrin-EGF2+3 fragment specifically bound to the C domain in a CRV-dependent manner, and the single alanine mutations of the Lys542 and Tyr544 residues in the β3 integrin-EGF2+3 decreased its activity. The β3 integrin-
transfected cells, but not the untransfected cells, bound the C domain of MMP-9. In HT1080 cells, pro-MMP-9 was co-precipitated with antibodies to \( \beta_5 \) integrins, and the \( \beta_5 \)-I-EGF2+3 fragment and the C domain both inhibited invasiveness of this cell line. MMP-9 and \( \beta_5 \) integrins similarly localized to the leading edge of the HT1080 cells. However, we cannot exclude the possibility that the CRV peptide inhibits also other C domain-mediated interactions.

We did not observe association of MMP-9 with \( \alpha_5\beta_3 \) in HT1080 cells, although a functional linkage between MMP-9 and the active \( \alpha_5\beta_3 \) integrin has been found (37). This may reflect the fact that HT1080 cells utilize the \( \alpha_5\beta_3 \) integrin for vitronectin adhesion. MMP-9 binding to \( \alpha_5\beta_3 \) may be physiologically more relevant, since \( \alpha_5\beta_3 \) and MMP-9 expression are under similar transcriptional regulation (8, 38). In turn, \( \alpha_5\beta_3 \) and MMP-2 appear to be co-regulated (39). In our studies, the CRV peptide only weakly inhibited pro-MMP-2 activation, and the C domains of MMP-2 and MMP-9 did not compete with each other in binding assays.

The finding that CRV mimics an integrin activation epitope provides an explanation for the requirement of ligand-engaged integrins in pro-MMP-9 activation (37, 40). We also demonstrate that uPAR, which is required for MMP-9 activation, associates with pro-MMP-9 in HT1080 and THP-1 cells. uPAR was a substrate for MMP-9 in vitro, and the cellular cleavage of uPAR was gelatinase-dependent. Cleavage by MMP-9 resulted in the release of the D1 domain of uPAR, which has also been observed with other MMPs such as MMP-12 (27). Functionally, uPAR cleavage causes loss of uPA binding and the dissociation of uPAR and integrins (41). Thus, MMP-9 not only regulates its own activation but also uPAR function. Interestingly, cooperation of MMP-9 and uPAR has been shown to be essential for the extravasation of tumor cells (42). Also, uPA/uPAR and gelatinases co-exist in transport vesicles in migrating cells (43, 44).

Inhibition of tumor growth by CRV suggests an important function for the MMP-9/\( \alpha_5\beta_3 \) pair in primary tumor growth and/or angiogenesis. However, increased tumor growth rather than inhibition is observed in both the \( \beta_3 \) and \( \beta_5 \) integrin knockout mice (45) and also in mice with low plasma levels of MMP-9 (46). The ability of MMP-9 to generate angiotatin or tumstatin (47) may explain these contradictory findings, and perhaps \( \alpha_5\beta_3 \)-bound MMP-9 is also used for angiotatin generation. Furthermore, the cleavage of uPAR by MMP-9 could also inhibit tumor spreading. Since tumor therapies aimed at direct inhibition of MMP activity have not been very successful, the noncatalytic means to inhibit MMPs may be more attractive (6). It is encouraging that our phage display-developed peptides specifically interfere with different integrin-mediated interactions blocking either the MMP catalytic or the C-terminal domain binding, suggesting that specific drugs can be developed that locally prevent gelatinase function but not the enzymatic activity. Supporting this conception, also the \( \beta_2 \)-integrin ligand DDGW peptide, which blocks the \( \alpha_5\beta_2 \) integrinpro-MMP-9 complex, is active in vivo, inhibiting neutrophil recruitment in an acute inflammation model in mice (48).

Our model of the MMP-9 interactions with integrins is based on a “peptidoscopic” view obtained with phage display peptides and suggests that pro-MMP-9 can interact with
integrins in two ways. In leukocytes, the interaction between the integrin I domain and the MMP-9 catalytic domain is dominant and apparently keeps pro-MMP-9 in an inactive form. However, our data do not exclude the possibility that both α and β subunit-mediated interactions occur at the same time. Ligand binding activates the integrin and exposes the activation epitope in the β chain, which can act as a docking site for the C domain of MMP-9. MMP-9 may then be activated by proteases or becomes catalytically competent by direct binding to a substrate (49). In integrins that lack an I domain in the α subunit, the MMP-9 C domain-directed interaction may be the dominant interacting site.

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