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Selective Hydrolysis of Triple-helical Substrates by Matrix Metalloproteinase-2 and -9*

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The role of proteases in the tumor cell invasion process is multifaceted. Members of the matrix metalloproteinase (MMP) family have been implicated in primary and metastatic tumor growth, angiogenesis, and degradation of extracellular matrix (ECM) components. Differentiating between the up-regulation of MMP production and the presence of activated MMPs can be difficult but may well dictate which MMPs are critical to invasion. Because the hydrolysis of collagens is one of the committed steps in ECM turnover, we have investigated selective MMP action on collagenous substrates as a means to evaluate active MMPs. Two triple-helical peptide (THP) models of the MMP-9 cleavage site in type V collagen, α1(V)436–450 THP and α1(V)436–447 fTHP, were hydrolyzed by MMP-2 and MMP-9 at the Gly-Val bond, analogous to the bond cleaved by MMP-9 in the corresponding native collagen. Kinetic analyses showed k_{cat}/K_m values of 14,002 and 5,449 $s^{-1}M^{-1}$ for MMP-2 and -9 hydrolysis of α1(V)436–447 fTHP, respectively. These values, along with individual k_{cat} and K_m values, are comparable with collagen hydrolysis by MMP-2 and -9. Neither THP was hydrolyzed by MMP-1, -3, -13, or -14. α1(V)436–447 fTHP and a general fluorogenic THP were used to screen for triple-helical peptidase activity in $\alpha_2\beta_1$ integrin-stimulated melanoma cells. Binding of the $\alpha_2\beta_1$ integrin resulted in the production of substantial triple-helical peptidase activity, the majority (>95%) of which was non-MMP-2/-9. THPs were found to provide highly selective substrates for members of the MMP family and can be used to evaluate active MMP production in cellular systems.

The role of proteases, particularly metalloproteases, in the tumor cell invasion process is multifaceted. Members of the matrix metalloproteinase (MMP)[1] family have been implicated in primary and metastatic tumor growth, angiogenesis, and degradation of ECM components during tumor cell invasion (1–4). MMP expression is widespread in common human tumors, with MMP-1, -2, -3, -7, -9, -11, and/or -14 found most often (3). Of these MMPs, a great deal of attention has been focused on the two gelatinases, MMP-2 and MMP-9. MMP-2 expression is found in the greatest variety of stromal tissue surrounding neoplasms (5), and cleavage of certain ECM components by MMP-2 may reveal "cryptic sites" that aid cell migration and growth and angiogenesis (6, 7). Cell surface activation and association of MMP-2 correlates to enhanced ECM remodeling by tumors (8, 9). MMP-9 has also been shown to induce angiogenesis during carcinogenesis (10). Activation of MMP-9 enhances tumor cell invasion (11) and increases shedding of intercellular adhesion molecule 1 (ICAM-1) from tumor cell surfaces (12), which in turn decreases tumor cell susceptibility to natural killer cell-mediated cytotoxicity. MMP-9 expression has been shown to increase in advanced-stage melanoma cells (13).

Although the vast majority of studies on MMP regulation examine factors that up-regulate MMP production, the ability to differentially examine actual MMP activity is necessary to discern which MMPs are critical to the invasion process. The hydrolysis of collagen is one of the committed steps in ECM turnover, and it has long been demonstrated that tumor extracts can possess collagenolytic activity (14). The triple-helical structure of collagen renders it resistant to most mammalian proteases, with the exception of cathepsin K and MMPs. Thus, triple-helical collagen-like substrates may provide an effective screening tool for activated tumor cell proteases. Such substrates could be used to determine whether different triple-helical sequences can be cleaved selectively by MMP family members and, if so, how the kinetics of hydrolysis compare.

Several approaches have been described for construction of THP substrates (recently reviewed in Refs. 15 and 16). Noncovalent self-assembly of lipophilic molecules, N-terminal linked to a peptide, can be used to form stable triple-helical "peptide-amphiphiles" for mechanistic evaluation of MMP activity (17–19). Fluorescence resonance energy transfer triple-helical peptide-amphiphile substrates have been constructed

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[1] The abbreviations used are: MMP, matrix metalloproteinase; α1(IV)402–413, C_{16}-(Gly-Pro-Hyp)$_4$-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Glu-Lys-(Gly-Pro-Hyp)$_4$-NH$_2$; Dnp, 2,4-dinitrophenyl; ECM, extracellular matrix; fTHP-3, (C_6-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH$_2$)$_3$; fTHP-4, ((Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH$_2$)$_3$; Hyp, 4-hydroxy-L-proline; MALDI-MS, matrix-assisted laser desorption ionization-mass spectrometry; Mca, (7-methoxycoumarin-4-yl)acetyl; NFF-1, Mca-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Lys(Dnp)-Gly; NFF-3, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH$_2$; Nva, norvaline; PTH, phenylthiohydantoin; RP-HPLC, reversed-phase high performance liquid chromatography; SSP, single-stranded peptide; THP, triple-helical peptide; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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by combining a sequence based on the type II collagen 769–783 region, a fluorophore (Lys(Mca)) in the P_5 subsite, and a quencher (Lys(Dnp)) in the P_5' subsite (19). Individual kinetic parameters for MMP hydrolysis were then determined using a continuous fluorometric assay (19).

Fluorogenic substrates can be used to examine other triple-helical sequences for selective MMP hydrolysis. As a starting point, we can consider the five triple-helical collagen sequences hydrolyzed by MMPs that have been identified.[2] Types I-III collagens are hydrolyzed by MMP-1, -8, -13, -14, -18, and -22 at the 775–776 bond (20–27). MMP-2 hydrolyzes type I collagen at the same single locus (20). The Gly^{439}-Val^{440} bonds in type V collagen are cleaved by MMP-9, along with a similar cleavage site sequence in type XI collagen (28).

For the present study, we have constructed THP models of the MMP-9 cleavage sites in types V and XI collagen. The THPs use the α1(V)436–450 sequence Gly-Pro-Pro-Gly↓Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro as a template. We have compared the susceptibility of the THPs to several MMP family members (MMP-1, -2, -3, -9, -13, and -14) and determined individual kinetic parameters by continuous monitoring of a fluorogenic THP. Mechanistic aspects of MMP hydrolysis have been examined by comparing activities toward THPs and the analogous SSPs. Ultimately, fluorogenic THPs were used to assess MMP-2/-9 and general MMP activity in $\alpha_2\beta_1$ integrin-stimulated metastatic melanoma cells.

EXPERIMENTAL PROCEDURES

Materials—All standard peptide synthesis chemicals were analytical reagent grade or better and purchased from Novabiochem (San Diego, CA) or Fisher Scientific. 9-Fluorenylmethoxycarbonyl-amino acid derivatives were obtained from Novabiochem. Amino acids are of the L-configuration (except for Gly). Hexanoic acid (CH_3-$(CH_2)_4$-CO_2H, designated C_6) was purchased from Aldrich. THPs were synthesized and purified by methods previously described in our laboratory (19, 29, 30).

Peptide Analyses—Analytical RP-HPLC was performed on a Hewlett Packard 1100 liquid chromatograph equipped with a Hypersil small-pore, narrow-bore C_{18} reversed-phase column (5-μm particle size, 120-Å pore size, 100 × 2.1 mm). Eluants were 0.1% trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B). The elution gradient was 0–100% B in 20 min with a flow of 0.3 ml/min. Detection was at λ = 229, 324, and 363 nm. Edman degradation sequence analysis was performed on an Applied Biosystems 477A protein sequencer/120A analyzer as described (31) for "embedded" (non-covalent) sequencing. MALDI-MS was performed on a Hewlett-Packard G2025A mass spectrometer using either a sinapinic acid or 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxy-benzoic acid (9:1, v/v) matrix (32). Single-chain mass values were as follows: α1(V)436–450 THP, $[M+H]^+$ 3636.2 Da (theoretical 3639.9 Da); α1(V)436–450 SSP, $[M+H]^+$ 1402.9 Da (theoretical 1403.5 Da); α1(V)436–447 fTHP, $[M+H]^+$ 4487.4 Da (theoretical 4490.7 Da); and α1(IV)402–413, $[M+H]^+$ 3609.3 Da (theoretical 3609.0 Da).

Circular Dichroism Spectroscopy—Circular dichroism spectra were recorded over the range λ = 190–250 nm on a JASCO J-600 using a 10-mm path length quartz cell. Thermal transition curves were obtained by recording the molar ellipticity ([Θ]) at λ = 225 nm while the temperature was continuously increased in the range of 5–80 °C at a rate of 0.2 °C/min. Temperature was controlled using a JASCO PTC-348WI temperature control unit. For samples exhibiting sigmoidal melting curves, the reflection point in the transition region (first derivative) is defined as the melting temperature (T_m).

Cells—M14 human melanoma cells were propagated as described previously (33–35). Briefly, melanoma cells were cultured in Eagle's minimum essential medium or RPMI 1640 supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate, 0.1 mg/ml gentamycin (Roche Applied Science), 50 units/ml penicillin, and 0.05 mg/ml streptomycin. Cells were passaged 8 times and then replaced from frozen stocks of early passage cells to minimize phenotypic drift. All cells were maintained at 37 °C in a humidified incubator containing 5% CO_2. All media reagents were purchased from Fisher Scientific.

Matrix Metalloproteinases—ProMMP-1 and proMMP-3 were expressed in *Escherichia coli* and folded from the inclusion bodies as described previously (36). ProMMP-2 was purified from the culture medium of human uterine cervical fibroblasts (37). ProMMP-2 was activated by reacting with 1 mM 4-aminophenylmercuric acetate at 25 °C for 2 h. ProMMP-1 was activated by reacting with 1 mM 4-aminophenylmercuric acetate and a 0.1 molar amount of MMP-3 at 37 °C for 6 h. After activation, MMP-3 was completely removed from MMP-1 by affinity chromatography using an anti-MMP-3 IgG Affi-Gel 10 column. ProMMP-3 was activated to the 45-kDa MMP-3 by reacting with 5 μg/ml chymotrypsin at 37 °C for 2 h. Chymotrypsin was inactivated with 2 mM diisopropyl fluorophosphate. ProMMP-13 was a generous gift from Dr. Maureen Horrocks, AstraZeneca Pharmaceuticals. ProMMP-9 and MMP-14 were purchased from Chemicon (Temecula, CA). ProMMP-9 and -13 were activated with 1 mM 4-aminophenylmercuric acetate. The amounts of active MMP-1, -2, and -3 were determined by titration with recombinant N-terminal domain of tissue inhibitor of metalloproteinases-1 (38) over a concentration range of 0.1–3 μg/ml.

Assays—Two different assay methods were utilized, the first for discontinuous fluorometric analyses and the second for continuous fluorometric analyses. For the discontinuous assay method, α1(V)436–450 THP was prepared as 270 μM stock solution in "fluorometric assay" buffer (50 mM Tricine, pH 7.5, 50 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35). A 0.5-M stock solution of *o*-phenanthroline was prepared in Me_2SO, followed by dilution with assay buffer to a concentration of 20 mM. MMP assays were carried out in assay buffer by incubating a range of substrate concentrations with 40 nM enzyme at 30 °C. Enzymatic activity was terminated by the addition of 20 μl of the enzyme/substrate solution to 30 μl of *o*-phenanthroline (20 mM) at appropriate times. Rates of hydrolysis were monitored by the addition of 200 μl of fluorescamine solution. Fluorescamine solution was prepared by first dissolving fluorescamine in acetone at a concentration of 40 mM, then diluting to 5 mM with assay buffer minus Brij-35. Fluorescamine reacts with free amino groups, resulting in a fluorophore with $\lambda_{excitation}$ = 387 nm and $\lambda_{emission}$ = 480 nm. Fluorescence was measured on a Molecular Devices SPECTRAmax Gemini dual-scanning microplate spectrofluorometer.

For continuous fluorometric analyses, substrate α1(V)436–447 fTHP was prepared as 270 μM stock solution in fluorometric assay buffer. MMP assays were carried out in assay buffer by incubating a range of substrate concentrations (1–50 μM) with 40 nM enzyme at 30 °C. Fluorescence was measured using $\lambda_{excitation}$ = 325 nm and $\lambda_{emission}$ = 393 nm. Initial velocities were obtained from plots of fluorescence *versus* time, using only data points corresponding to less than 40% full hydrolysis. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain initial velocity in units of μM/s. The fluorogenic substrates NFF-1, NFF-3, and fTHP-4 were used for control assays of MMP activity as described (19, 39, 40).

Cellular MMP Assay—Melanoma cell adhesion to substrate-coated non-tissue culture-treated plates (BD Biosciences) was performed as described previously (35). The α1(IV)402–413 ligand was dissolved in phosphate-buffered saline, diluted in 70% ethanol, added to the 96-well plate, and allowed to adsorb overnight at room temperature with mixing. Plates were rinsed three times with sterile phosphate-buffered saline to remove all traces of ethanol. Cells were released with 5 mM EDTA in phosphate-buffered saline and washed two times with adhesion medium (20 mM HEPES, RPMI 1640). Cells were then resuspended in adhesion medium and added to the plate. The plate was incubated for 60 min at 37 °C. Non-adherent cells were removed by washing three times with adhesion medium, and fresh medium was added. Conditioned media were collected at various time points, centrifuged to remove cell debris, and stored at −20 °C. One volume of the appropriate fluorogenic substrate was added to each well in a 96-well plate. Where applicable, EDTA was added to each well. The plate was incubated at 30 °C in a humidified atmosphere for 30 min, and one volume of conditioned media, adhesion medium (as a negative control), or MMP (as a positive control) was added to each well. The plate was incubated at 30 °C in a humidified atmosphere for at least 18 h. Fluorescence readings ($\lambda_{excitation}$ = 325 nm and $\lambda_{emission}$ = 393 nm) were taken and a standard curve created by plotting the increase in fluorescence *versus* concentration of MMP standard. The standard curve was used to calculate the active enzyme concentration in the conditioned media.

[2] Although MMP-3 and -9 cleavage sites within the triple-helical region of type IV collagen have been identified (57), it was not determined whether these sites were cleaved while the triple helix was intact or after the triple-helical region had denatured because of other MMP-3 or MMP-9 cleavages.

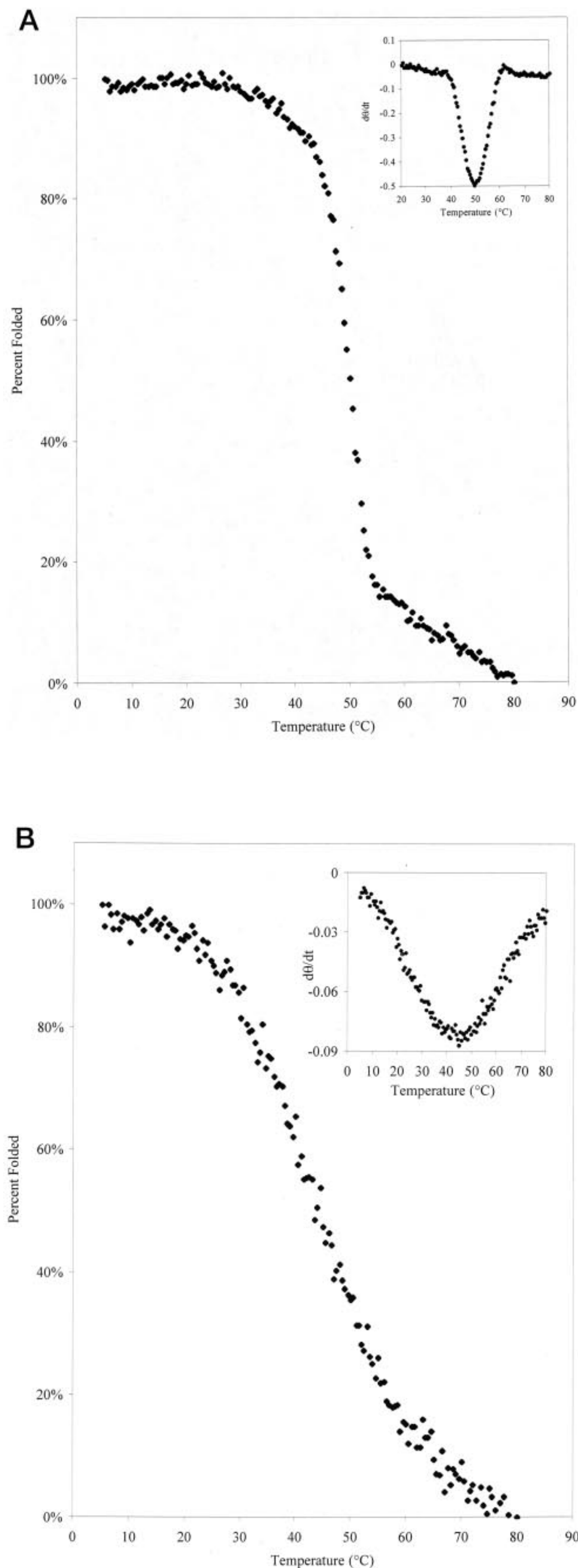

FIG. 1. **Thermal transition curves for purified (*A*) α1(V)436–450 THP and (*B*) α1(V)436–447 fTHP in 1.0% (v/v) fluorometric assay buffer at substrate concentrations of 10 μM.** Molar ellipticities ([θ]) were recorded at λ = 225 nm while the temperature was increased from 10 to 80 °C. *Inserts* are the first derivative of the transition curves, from which T_m values are determined.

RESULTS AND DISCUSSION

The first THP constructed as a possible MMP-2 and -9 substrate was based upon the MMP-9 cleavage site in types V and XI collagen. It incorporated the α1(V)436–450 sequence between N- and C-terminal $(Gly\text{-}Pro\text{-}Hyp)_4$ repeating tripeptides and an N-terminal C_6 alkyl chain (C_6-$(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Pro-Pro-Gly ↓ Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro(Gly-Pro-Hyp)$_4$-NH_2). The α1(V)436–450 THP had a T_m value of 49.5 °C in 1.0% (v/v) fluorometric assay buffer (Fig. 1), which is a desirable thermal stability for an MMP substrate. The composition and homogeneity were confirmed by Edman degradation sequence analysis (prior to addition of the C_6 alkyl chain), RP-HPLC, and MALDI-MS.

MMP-1 and MMP-9 hydrolysis of α1(V)436–450 THP was studied at 30 °C using the discontinuous fluorometric assay with 40 μM substrate. MMP-9 rapidly hydrolyzed the substrate within 1 h, whereas MMP-1 did not cleave the substrate even after 24 h. MMP-9 hydrolysis of the α1(V)436–450 THP was analyzed by MALDI-MS. Each intact chain of the α1(V)436–450 THP has a mass of 3638.9 Da. If the Gly-Val bond is cleaved, the two products generated in assay buffer are the single-stranded peptides C_6-$(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Pro-Pro-Gly ($[M+Na]^+$ = 1515.6 Da) and Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro-$(Gly\text{-}Pro\text{-}Hyp)_4$-$NH_2$ ($[M+Na]^+$ = 2187.3 Da). Mass spectrometric analysis of MMP-9 hydrolysis showed two products, one of $[M+Na]^+$ = 1516.2 Da and one of $[M+Na]^+$ = 2189.8 Da. No hydrolysis of the substrate by MMP-1 was detected using MALDI-MS analysis. The α1(V)436–450 THP is the first synthetic substrate that shows complete selectivity between MMP-1 and MMP-9. Subsequent treatment of α1(V)436–450 THP with MMP-2 and MALDI-MS analysis indicated cleavage at the Gly-Val bond. Edman degradation sequence analysis of the α1(V)436–450 THP cleavage products also showed that MMP-2 hydrolysis occurred exclusively at the Gly-Val bond, because the only amino acids seen in the first two cycles are PTH-Val and PTH-Val (emanating from the C-terminal fragment of the cleaved THP). Thus, both Edman degradation sequence analysis and MALDI-MS analyses indicated that MMP-2 and MMP-9 cleaved the α1(V)436–450 THP exclusively at the Gly-Val bond. This is the analogous bond cleaved by MMP-9 in types V and XI collagen (28). Although the cleavage sites for MMP-2 hydrolysis of type V collagen have not been determined, the digestion pattern is very similar to that of MMP-9 (41), suggesting that the Gly^{439}-Val^{440} bond could be cleaved by MMP-2 in intact type V collagen.

To determine whether primary structure alone accounted for the observed MMP-2/-9 selectivity, an SSP analog of the α1(V)436–450 sequence (Gly-Pro-Pro-Gly ↓ Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro-NH_2) was constructed and incubated with several different MMPs. RP-HPLC and MALDI-MS indicated that the α1(V)436–450 SSP was very slowly cleaved by MMP-3 and -9. Interestingly, MMP-9 hydrolyzed the α1(V)436–450 SSP at a considerably slower rate than the α1(V)436–450 THP. Thus, primary structure was not the sole determinant for MMP-2/-9 selectivity of the α1(V)436–450 THP, and substrate triple-helical conformation greatly enhanced hydrolysis rates. These results suggest that MMP-9 acts as a true "collagenase," preferentially cleaving a specific sequence in triple-helical conformation, whereas MMP-3 has limited triple-helical peptidase activity.

Based on the results with α1(V)436–450 THP, a fluorogenic substrate was designed and designated α1(V)436–447 fTHP ($(Gly\text{-}Pro\text{-}Hyp)_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly ↓ Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-$(Gly\text{-}Pro\text{-}Hyp)_5$-$NH_2$). The Gln in the P_5' subsite from α1(V)436–450 THP was replaced with Lys(Dnp), whereas Hyp in the P_5 subsite was replaced with

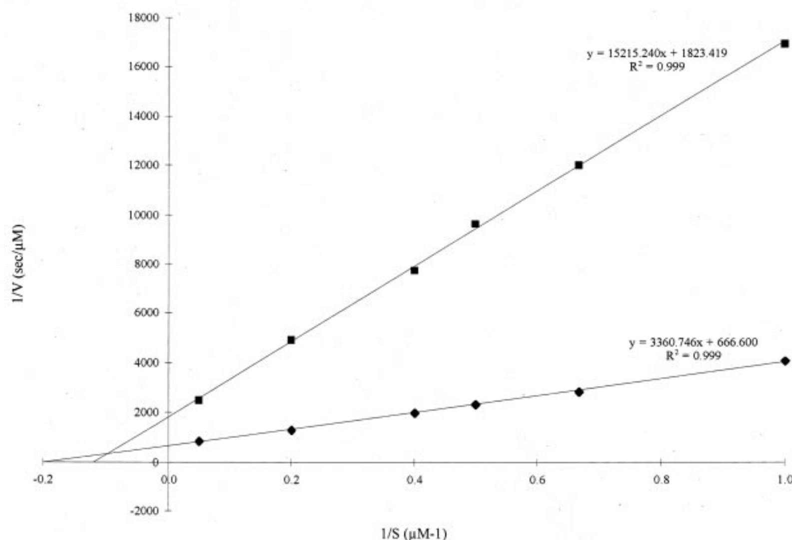

FIG. 2. **Lineweaver-Burk analysis for MMP-2 (*closed triangles*) and MMP-9 (*closed squares*) hydrolysis of α1(V)436–447 fTHP in fluorometric assay buffer at 30 °C.** The substrate concentration range is 1–50 μM.

Lys(Mca). Prior studies indicated that MMP-2 and -9 well tolerate substitution of a P_5 subsite Hyp by a bulky aromatic residue (42). To improve substrate solubility while not sacrificing thermal stability, (*a*) the C_6 alkyl chain was replaced by two Gly-Pro-Hyp repeats on the N terminus and (*b*) an additional Gly-Pro-Hyp repeat was added to the C terminus. The α1(V)436–447 fTHP has a T_m value of 45.0 °C in 1.0% (v/v) fluorometric assay buffer (Fig. 1), which is a desirable thermal stability for an MMP substrate. The composition and homogeneity were confirmed by Edman degradation sequence analysis, RP-HPLC, and MALDI-MS.

Edman degradation sequence analysis of the α1(V)436–447 fTHP cleavage products showed that MMP-2 hydrolysis occurred exclusively at the Gly-Val bond, because the only amino acids seen in the first three cycles are PTH-Val, PTH-Val, and PTH-Gly (emanating from the C-terminal fragment of the cleaved THP). MMP-2 and -9 hydrolysis of the α1(V)436–447 fTHP was also examined by MALDI-MS. Each intact chain of the α1(V)436–447 fTHP has a mass of 4489.7 Da. If the Gly-Val bond is cleaved, the two products generated are the single-stranded peptides (Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly ($[M+H]^+$ = 2162.3 Da) and Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2 ($[M+H]^+$ = 2347.5 Da). Mass spectrometric analysis of MMP-2 or MMP-9 hydrolysis showed two products of $[M+H]^+$ = 2159.6 and 2346.4 Da. Thus, both Edman degradation sequence analysis and MALDI mass spectrometric analyses indicated that MMP-2 and -9 cleaved the α1(V)436–447 fTHP exclusively at the Gly-Val bond. No hydrolysis of the substrate by MMP-1, -3, -13, or -14 was detected using MALDI-MS analysis.

Individual kinetic parameters for MMP hydrolysis of α1(V)436–447 fTHP were evaluated by Lineweaver-Burk (Fig. 2), Hanes-Woolf, and Eadie-Hoftsee analyses. The relative order of apparent k_{cat}/K_m values is MMP-2 > MMP-9 ≫ MMP-14 ~MMP-13 > MMP-1 ~MMP-3 (Table I). MMP-2 has the highest k_{cat}/K_m value for hydrolysis of α1(V)436–447 fTHP, because of both a lower K_m value (4.4 μM for MMP-2 *versus* 8.1 for MMP-9) and a higher k_{cat} value. For MMP-2, α1(V)436–447 fTHP is a 13-fold better substrate than the previously described triple-helical substrate fTHP-3 (Table II), which is modeled after the type II collagen MMP cleavage site. MMP-2 prefers α1(V)436–447 fTHP to type I collagen (Table II). MMP-2 cleaves type V collagen very efficiently, although at a 4-fold lower rate than MMP-9 (41). Thus, some differences exist between triple-helical peptidase and collagenolysis specificity, as previously observed (18, 19).

TABLE I
Kinetic parameters for α1(V)436–447 fTHP hydrolysis by MMPs at 30 °C

Enzyme	k_{cat}/K_m	k_{cat}	K_m
	s^{-1} M^{-1}	s^{-1}	μM
MMP-1	<48[a]	ND[b]	ND[b]
MMP-2	14,002	0.0616	4.4
MMP-3	<48[a]	ND[b]	ND[b]
MMP-9	5,449	0.0441	8.1
MMP-13	434[c]	ND[b]	ND[b]
MMP-14	579[c]	ND[b]	ND[b]

[a] Indicates the minimal measurable amount of fluorescence detected, and thus no kinetic parameters could be calculated.
[b] ND, not determined.
[c] Although an increase over background fluorescence was detected, no substrate cleavage was seen by MALDI-MS analysis. See “Results and Discussion” for details.

Incubation of α1(V)436–447 fTHP with MMP-14 or -13 resulted in a slight increase in fluorescence (Table I). However, MALDI-MS analysis (see above) indicated that neither of these MMPs cleaves α1(V)436–447 fTHP. The slight increase in fluorescence appears to result from perturbation of the substrate structure by MMP-13 and -14, but not from hydrolysis. MMP-1 and -3 do not cleave α1(V)436–447 fTHP under the conditions studied here. Simultaneous studies using the fluorogenic substrates NFF-1, NFF-3, and fTHP-4 indicated that MMP-1, -3, -13, and -14 were active. For example, treatment of NFF-3 with MMP-3 resulted in a rapid increase in substrate fluorescence and MALDI-MS detection of two peptide products corresponding to Mca-Arg-Pro-Lys-Pro-Val-Glu and Nva-Trp-Arg-Lys (Dnp)-NH_2.

The present work has demonstrated that triple-helical sequences are selectively hydrolyzed by members of the MMP family. Thus, depending upon the invasion pathway and the type of collagen encountered, different MMPs may be required to facilitate metastasis. Similar conclusions could be drawn from prior studies on collagenolysis. For example, a subset of the MMP family are capable of cleaving type I collagen (see earlier discussion). In similar fashion, type IV collagen is hydrolyzed by MMP-2, -3, -7, -9, -10, and -12, but not by MMP-1, -8, or -13 (43). However, the prior collagenolysis studies consider several MMP actions, including (*a*) the binding and manipulation of collagen, (*b*) unwinding of the triple-helix, and (*c*) hydrolysis of individual collagen strands. The utilization of THPs eliminates effects due to the binding and manipulation of collagen, allowing for direct comparisons of triple-helical se-

TABLE II
Kinetic parameters for triple-helical substrate hydrolysis by MMP-2 and MMP-9 at 30 °C

Substrate	Enzyme	k_{cat}/K_m	K_m	k_{cat}
		s^{-1} M^{-1}	μM	s^{-1}
α1(V)436–447 fTHP	MMP-2	14,002	4.4	0.0616
α1(V)436–447 fTHP	MMP-9	5,449	8.1	0.0441
fTHP-3[a]	MMP-2	1,082	17.2	0.017
Type I collagen (rat)[b]	MMP-2	529	8.5	0.0045
Type V collagen (human)[c]	MMP-2	430[d]	ND	0.0022
Type V collagen (human)[c]	MMP-9	2,200[d]	ND	0.011

[a] From Ref. 19.
[b] From Ref. 20. Assay performed at 25 ° C.
[c] From Ref. 41. Assay performed at 32 ° C.
[d] Calculated assuming mass = 335 kDa for $[\alpha\ 1(V)]_2\alpha2(V)$ type V collagen and $K_m \approx 5\ \mu M$.

TABLE III
Fluorogenic THP analysis of melanoma cell supernatants following treatment with the $\alpha_2\beta_1$ integrin ligand α1(IV)402–413 THP

Sample	Substrate	Relative fluorescence units
Media	fTHP-3[a]	312.5
Conditioned media	fTHP-3	1453.9
Conditioned media + EDTA[b]	fTHP-3	65.7
Media + MMP-1[c]	fTHP-3	1408.2
Media + MMP-1[c] + EDTA[b]	fTHP-3	−8.2
Media + MMP-2[d]	fTHP-3	1266.3
Media + MMP-2[d] + EDTA[b]	fTHP-3	19.9
Media	α1(V)436–447 fTHP	164.9
Conditioned media	α1(V)436–447 fTHP	301.3
Conditioned media + EDTA[b]	α1(V)436–447 fTHP	−24.3
Media + MMP-2[e]	α1(V)436–447 fTHP	463.1
Media + MMP-2[e] + EDTA[b]	α1(V)436–447 fTHP	39.9

[a] fTHP-3 is modeled after the human type II collagen 769-783 sequence.
[b] 10 mM EDTA.
[c] 5 nM MMP-1.
[d] 5 nM MMP-2.
[e] 1 nM MMP-2.

quences and MMP susceptibility. Thus, studies with the α1(V)436–450 THP and α1(V)436–447 fTHP are the first to show discrimination of substrate hydrolysis by collagenolytic MMPs (*i.e.* MMP-1 and -2) based on triple-helical sequence specificity. These results complement prior studies showing that selectivity between collagenolytic MMPs (*i.e.* MMP-1, -2, -8, etc.) and non-collagenolytic MMPs (*i.e.* MMP-3) can be based on triple-helical sequence specificity (18, 44). Interestingly, the selectivities of α1(V)436–450 THP and α1(V)436–447 fTHP are not solely because of the primary structure of the substrates. A single-stranded analog of the α1(V)436–450 sequence is cleaved by several MMPs. Also, MMP-3 cleaves the single-stranded substrate Gly-Pro-Gln-Gly↓Val-Ala-Gly-Gln 2-fold faster than MMP-2 or -9 (45). Thus, triple-helical structure appears to effect the susceptibility of Gly-Val bonds to cleavage by MMP-3.

α1(V)436–447 fTHP is the first truly selective fluorogenic substrate described for gelatinases (MMP-2 and -9). Of the previously studied gelatinase substrates, Dnp-Pro-cyclohexylalanyl-Gly↓Cys(CH_3)-His-Ala-Lys(*N*-methylanthranilic acid)-NH_2 is cleaved by MMP-1 and -9, Dnp-Pro-Leu-Gly↓Leu-Trp-Ala-D-Arg-NH_2 is cleaved by MMP-1, -2, -3, and -9, and Mca-Arg-Pro-Lys-Pro-Tyr-Ala↓Nva-Trp-Met-Lys(Dnp)-NH_2 is cleaved by MMP-2, -3, and -9 (45). The (cyanine fluorochrome)-Gly-Pro-Leu-Gly↓Val-Arg-Gly-Lys(fluorescein isothiocyanate)-Cys-NH_2 substrate, used for near-infrared fluorescent imaging of MMP-2 positive tumors, is additionally hydrolyzed by MMP-1, -7, -8, and -9 (46).

The results described herein suggest that triple-helical substrates could be utilized to discriminate among MMP family members for a given cell or tissue type. We have previously described a general MMP fluorogenic THP substrate (19) and now complement this with a selective gelatinase substrate. To test whether these substrates can be used to discriminate between MMP family members, we have examined MMP production by highly metastatic M14 melanoma cells. Melanoma cells are known to express a variety of MMPs, including MMP-1, -2, and -9, when in a highly metastatic state (47, 48). In addition, the $\alpha_2\beta_1$ integrin, which is abundant on M14 melanoma cell surfaces (49), is a positive regulator for MMP-1 gene expression (50) and has been implicated in MMP-9 regulation (51). It has not been determined previously whether $\alpha_2\beta_1$ integrin-mediated signaling results in the production of active MMPs nor whether specific MMP-2/-9 activity is present. To induce $\alpha_2\beta_1$ integrin signaling, we constructed a triple-helical model of α1(IV)402–413. This region of type IV collagen contains the Gly-Phe-Hyp-Gly-Glu-Arg motif which, in triple-helical conformation, binds to the $\alpha_2\beta_1$ integrin (52–54). The α1(IV)402–413 THP ligand was used to engage the $\alpha_2\beta_1$ integrin of M14 melanoma cells, and MMP production was quantitated. Significant triple-helical peptidase activity was detected in melanoma cell-conditioned media, because an increase of ~1140 fluorescence units was observed (Table III). This activity was completely inhibited by EDTA, indicating metalloproteinase activity. At an enzyme concentration of 5 nM, MMP-1 hydrolysis of fTHP-3 results in an increase of ~1100 fluorescence units, whereas MMP-2 shows an increase of ~950 fluorescence units (Table III). Thus, although fTHP-3 indicates significant activity, the nature of MMP(s) present is unknown. Based on comparison with standard curves (data not shown) and correction for dilution, the increase in fluorescence generated by the melanoma-conditioned media correlates to 24.3 nM MMP-2 or 11.7 nM MMP-1 if either of these enzymes was the sole MMP present. Subsequent analysis of melanoma-conditioned media with the α1(V)436–447 fTHP showed an increase of ~135 fluorescence units (Table III). This activity was also completely inhibited by EDTA. MMP-2 hydrolysis of α1(V)436–447 fTHP results in an increase of ~300 fluorescence units at an enzyme

concentration of 1 nM (Table III). Based on comparison with a standard curve (data not shown) and correction for dilution, the increase in fluorescence generated by the melanoma-conditioned media correlates to 1.02 nM MMP-2 if this enzyme was the sole MMP present. To a very rough approximation, this indicates that only ~4% of the triple-helical peptidase activity in melanoma cell-conditioned media was because of MMP-2. These results suggest that melanoma binding via the $\alpha_2\beta_1$ integrin produces active metalloproteinases and that only a small percentage of the activity is because of gelatinases. The relative distribution of specific MMPs produced in response to $\alpha_2\beta_1$ integrin binding, at both the gene and protein level, will be described elsewhere.[3]

By varying the sequence within THPs, we can hope to design additional selective fluorogenic substrates that incorporate triple-helical structures. It may also be possible to manipulate the P_2 subsite to obtain selectivity between MMP-2 and -9 (45, 55). Selective THP substrates would allow for a better understanding of the specific functions of MMP domains and subsequently could be used to design specific inhibitors of these enzymes. Finally, the fluorogenic triple-helical substrate described herein allows for continuous monitoring of MMP-2 and MMP-9 activity and thus has distinct advantages over enzyme-linked immunosorbent assay and zymographic methods for analysis for gelatinases (56).

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