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 method to evaluate active MMPs. Two triple-helical peptide (THP) models of the MMP-9 cleavage site in type V collagen, α(V)436–450 THP and α(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 α(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. α(V)436–447 fTHP and a general fluorogenic THP were used to screen for triple-helical peptidase activity in αvβ3 integrin-stimulated melanoma cells. Binding of the αvβ3 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.

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† The abbreviations used are: MMP, matrix metalloproteinase; α(V)402–413, Cε-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Phe-Hyp; Gly-Glu-Arg-Gly-Glu-Lys-(Gly-Pro-Hyp) 5-NH2; Dnp, 2,4-dinitrophenyl; αvβ3 integrin; THP, triple-helical peptide; Tricine, N,N,N′-tris(hydroxymethyl)methylglycine.

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Gln-Gly-Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Gly-Arg-(Gly-Pro-Hyp) 402–413, Cε-Gly-Pro-Hyp-Gly-Ala-Hyp-Gly-Phe-Hyp-Gly-Glu-Arg-Gly-Glu-Lys(Dnp)-Gly-Gly-Arg-(Gly-Pro-Hyp)-NH2; Hyp, 4-hydroxyproline; MALDE-MS, matrix-assisted laser desorption ionization-mass spectrometry; Mca, (7-methoxycoumarin-4-yl)acetyl; NFF-1, Mca-Pro-Lys-Pro-Gln-Gly-Phe-Gly-Leu-Lys(Dnp)-Gly; NFF-3, Mca-Pro-Lys-Pro-Gln-Gly-Phe-Gly-Leu-Lys(Dnp)-Gly; NFF-9, Mca-Pro-Lys-Pro-Gln-Gly-Phe-Gly-Leu-Lys(Dnp)-Gly; NFF-15, Nva, norvaline; PTH, phenylthiohydantoin; RP-HPLC, reversed-phase high performance liquid chromatography; SSP, single-stranded peptide; THP, triple-helical peptide; Tricine, N,N,N′-tris(hydroxymethyl)methylglycine.
by combining a sequence based on the type II collagen 769–783 region, a fluorophore (Lys(Mca)) in the P3 subsite, and a quencher (Lys(Dnp)) in the P3' 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 determined the five triple-helical collagen sites at various positions. MALDI-MS was performed on a Hewlett-Packard 2800 mass spectrometer. The samples of peptide were analyzed using nitrocellulose membranes. Fluorescence emission spectra were recorded with a SPECTRAmax Gemini dual-scanning microplate spectrofluorometer.

**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 (CH3-(CH2)4-CO2H, desalted by reverse-phase HPLC and lyophilized) was purchased from Aldrich. THPs were synthesized and purified by methods previously described in our laboratory (19, 29, 30). Hexanoic acid (CH3-(CH2)4-CO2H, desalted by reverse-phase HPLC and lyophilized) was purchased from Aldrich.

**Cellular MMP Assay—**Melanoma cell adhesion to substrate-coated non-tissue culture-treated plates (BD Biosciences) was performed as described previously (35). The α(IV)402–413 fragment of type IV collagen 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 was collected at various time points. Cells were also collected at 30 °C in a humidified atmosphere for at least 18 h. Fluorescence readings (λex = 325 nm and λem = 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.

**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-3 was activated by reacting with 1 mM 4-aminophenylmercuric acetate and a 0.1 mM 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 Horrock, 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 μM.

**Assays—**Two different assay methods were utilized, the first for discontinuous fluorometric analyses and the second for continuous fluorometric analyses. For the continuous assay, 20 mM sodium acetate (pH 5.0) with 40 μM enzyme at 30 °C, and Edman degradation sequence analysis was performed on an Applied Biosystems 477A protein sequence/120A analyzer as described (31) for “encoded” (non-covalent) quenching..addr.
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-Pro-Hyp)4 repeating tripeptides and an N-terminal C6 alkyl chain (C6-(Gly-Pro-Hyp)4-Gly-Pro-Pro-Gly [Val-Val-Gly-Glu-Gln-Gly-Pro-Pro-(Gly-Pro-Hyp)4-NH2]). The α1(V)436–450 THP had a Tm 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 C6 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 C6-(Gly-Pro-Hyp)4-Gly-Pro-Pro-Gly (M+Na) (= 2187.3 Da). Mass spectrometric analysis of MMP-9 hydrolysis showed two products, one of [M+Na]+ = 1515.6 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 Gly439-Val440 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-Pro-Pro-NH2]) 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 THP ((Gly-Pro-Hyp)4-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly [Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-Gly-Pro-Hyp)4-NH2]). The Gln in the P1 position of the α1(V)436–450 THP was replaced with Lys(Dnp), whereas Hyp in the P9 position was replaced with

Fig. 1. Thermal transition curves for purified (A) α1(V)436–450 THP and (B) α1(V)436–447 THP 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 Tm values are determined.
Lys(Mca). Prior studies indicated that MMP-2 and -9 well tolerate substitution of a P5 subsite Hyp by a bulky aromatic residue (42). To improve substrate solubility while not sacrificing thermal stability, (a) the C6 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(IV)36–447 fTHP has a Tm 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(IV)36–447 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(IV)36–447 fTHP was also examined by MALDI-MS. Each intact chain of the α1(IV)36–447 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)2-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly ([M+H]+ = 2162.3 Da) and Val-Pro-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)2-NH2 ([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(IV)36–447 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(IV)36–447 fTHP were evaluated by Lineweaver-Burk (Fig. 2), Hanes-Woolf, and Eadie-Hofstee analyses. The relative order of apparent kcat/Km values is MMP-2 > MMP-9 ≫ MMP-14 – MMP-13 > MMP-1 – MMP-3 (Table I). MMP-2 has the highest kcat/Km value for hydrolysis of α1(IV)36–447 fTHP, because of both a lower Km value (4.4 μM for MMP-2 versus 8.1 for MMP-9) and a higher kcat value. For MMP-2, α1(IV)36–447 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(IV)36–447 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).

Incubation of α1(IV)36–447 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(IV)36–447 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(IV)36–447 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-Glu and Nva-Trp-Arg-Lys(Dnp)-NH2.

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-
quences and MMP susceptibility. Thus, studies with the α1(V)436–450 THP and α1(V)436–447 THP 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 THP 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 \_{\text{Val-Ala-Gly-Gln}} \text{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.}

\[ \alpha1(V)436–447 \text{ THP is the first truly selective fluorogenic substrate described for gelatinases (MMP-2 and -9). Of the previously studied gelatinase substrates, Dnp-Pro-cyclohexylalanyl-Gly} \_\text{Cys(\text{CH}_3)_{\text{His-Ala-Lys(\text{N-methylantranilic acid})-NH}_2 \text{is cleaved by MMP-1 and -9, Dnp-Pro-Leu-Gly} \_\text{Leu-Trp-Ala-\text{d-Arg-NH}_2 \text{is cleaved by MMP-1, -2, -3, and -9, and Mca-Arg-Pro-Lys-Pro-Tyr-Ala} \_\text{Nva-Trp-Met-Lys(Dnp)-NH}_2 \text{is cleaved by MMP-2, -3, and -9 (45). The (cyanine fluorochrome)-Gly-Pro-Leu-Gly} \_\text{Val-Arg-Gly-Lys} \text{is (fluorescein isothiocyanate)-Cys-NH}_2 \text{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 α2β1 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 α2β1 integrin-mediated signaling results in the production of active MMPs nor whether specific MMP-2/9 activity is present. To induce α2β1 integrin signaling, we constructed a triple-helical model of α1(V)402–413. This region of type IV collagen contains the Gly-Phe-Hyp-Gly-Arg motif which, in triple-helical conformation, binds to the α2β1 integrin (52–54). The α1(V)402–413 THP ligand was used to engage the α2β1 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 20 nM, whereas fTHP-3 results in an increase of ~1140 fluorescence units at an enzyme concentration of 5 nM.}

### TABLE II

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

| Substrate | Enzyme | \( k_{\text{cat}}/K_m \) s\(^{-1}\) μM | \( K_m \) μM | \( k_{\text{cat}} \) s\(^{-1}\) μM |
|-----------|--------|--------------------------|-----------|--------------------------|
| 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 | MMP-2 | 1,052 | 17.2 | 0.0107 |
| 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.  
\(^c\) From Ref. 41.  
\(^d\) Calculated assuming mass ~335 kDa for [α1(V)\_α2(V)] type V collagen and \( K_m \sim 5 \text{ μM.} \)

### TABLE III

**Fluorogenic THP analysis of melanoma cell supernatants following treatment with the α2β1 integrin ligand α1(V)402–413 THP**

| Sample | Substrate | Relative fluorescence units |
|--------|-----------|----------------------------|
| Media  | fTHP-3   | 321.5                      |
| Conditioned media | fTHP-3 | 1453.9 |
| Conditioned media + EDTA\(^a\) | fTHP-3 | 65.7 |
| Media + MMP-1\(^b\) | fTHP-3 | 1408.2 |
| Media + MMP-1\(^b\) + EDTA\(^b\) | fTHP-3 | 8.2 |
| Media + MMP-2\(^b\) | fTHP-3 | 1296.3 |
| Media + MMP-2\(^b\) + 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\(^a\) | α1(V)436–447 fTHP | 24.3 |
| Media + MMP-2\(^b\) | α1(V)436–447 fTHP | 465.1 |
| Media + MMP-2\(^b\) + EDTA\(^b\) | α1(V)436–447 fTHP | 39.9 |

\(^a\) fTHP-3 is modeled after the human type II collagen 769-783 sequence.  
\(^b\) 5 nM EDTA.  
\(^c\) 5 nM MMP-1.  
\(^d\) 5 nM MMP-2.  
\(^e\) 1 nM MMP-2.
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 peptide activity in melanoma-cell-conditioned media was because of MMP-2. These results suggest that melanoma binding via the α₅β₃ 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 α₅β₃ 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 P₃ 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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