Melanoma Cell CD44 Interaction with the α1(IV)1263-1277 Region from Basement Membrane Collagen is Modulated by Ligand Glycosylation*

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Running Title: Glycosylation Modulation of Melanoma Cells.

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ABSTRACT: Invasion of the basement membrane is believed to be a critical step in the metastatic process. Melanoma cells have been shown previously to bind distinct triple-helical regions within basement membrane (type IV) collagen. Additionally, tumor cell binding sites within type IV collagen contain glycosylated hydroxylysine residues. In the present study, we have utilized triple-helical models of the type IV collagen α1(IV)1263-1277 sequence to (a) determine the melanoma cell receptor for this ligand and (b) analyze the results of single-site glycosylation on melanoma cell recognition. Receptor identification was achieved by a combination of methods, including (a) cell adhesion and spreading assays using triple-helical α1(IV)1263-1277 and an Asp^{1266}Abu variant, (b) inhibition of cell adhesion and spreading assays, and (c) triple-helical α1(IV)1263-1277 affinity chromatography with whole cell lysates and glycosaminoglycans. Triple-helical α1(IV)1263-1277 was bound by melanoma cell CD44/chondroitin sulfate proteoglycan (CSPG) receptors, and not by the collagen-binding integrins or MPG. Melanoma cell adhesion to and spreading on the triple-helical α1(IV)1263-1277 sequence was then compared for glycosylated [replacement of Lys^{1265} with Hyl(βD-galactopyranosyl)] versus non-glycosylated ligand. Glycosylation was found to strongly modulate both activities, as adhesion and spreading were dramatically decreased due to the presence of galactose. CD44/CSPG did not bind to glycosylated α1(IV)1263-1277. Overall, this study (a) is the first demonstration of the prophylactic effects of glycosylation on tumor cell interaction with the basement membrane, (b) provides a rare example of an apparent unfavorable interaction between carbohydrates, and (c) suggests that sugars may mask “cryptic sites” accessible to tumor cells with cell-surface glycosidase activities.
Tumor cell invasion, a key step in the metastatic process, involves a complex series of correlated macromolecular interactions. These include interaction with, and movement through, collagen, most often type I and/or basement membrane (type IV) collagen. In general, invasion of the basement membrane is believed to be a critical step in the metastatic process. Human melanoma cells have been shown to bind distinct triple-helical regions within type IV collagen (1-5). Melanoma receptors for triple-helical collagen fall into one of two categories: members of the integrin heterodimeric protein family (α1β1, α2β1, and α3β1 integrins) or cell surface proteoglycans [such as CD44 and melanoma-associated proteoglycan/melanoma chondroitin sulfate proteoglycan (MPG/MCSP/NG2)]. Specific ligands from type IV collagen have been described for the α1β1, α2β1, and α3β1 integrins. The α1β1 integrin simultaneously binds Asp441 from two α1(IV) chains and Arg458 from the α2(IV) chain (6,7). The Gly-Phe-Hyp-Gly-Glu-Arg motif, in triple-helical conformation, has been shown to bind to the α2β1 integrin (8-10). This motif is found within type IV collagen at α1(IV)405-410; a triple-helical model of α1(IV)402-413 is bound by melanoma cells.² The melanoma cell α3β1 integrin binds to α1(IV)531-543 (2,4,11).

In addition to the integrin binding sites, the α1(IV)1263-1277 region from type IV collagen (gene-derived sequence Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro, designated [IV-H1]), promotes melanoma cell adhesion, spreading, and signaling (1,3,12-14). Affinity chromatography studies with a single-stranded [IV-H1] peptide resulted in the isolation of melanoma cell CD44 receptors, in the chondroitin sulfate proteoglycan (CSPG) form (15,16). Subsequently, several triple-helical constructs incorporating the [IV-H1] sequence have been described (1,14,17). One of these, a “peptide-amphiphile” of general structure Cₙ-(Gly-Pro-Hyp)₄-[IV-H1]-(Gly-Pro-Hyp)₄-NH₂, has undergone extensive biophysical characterization by CD and one- and two-dimensional NMR spectroscopies (18-20). The [IV-H1] region within the peptide-amphiphile forms a continuous triple-helix (19,20). Loss of triple-helical structure dramatically reduces melanoma cell adhesion, spreading, and signaling modulated by this ligand (1,14,17). However, whether the triple-helical [IV-H1] ligand is bound by CD44, in analogous fashion to the linear version, has not been addressed. Cells may engage different receptors depending upon the conformational state of collagen (21-27).

The [IV-H1] sequence contains a glycosylated hydroxylysine (Hyl) residue in position 1265 (28). Hyl is the major glycosylation site within mammalian collagens. The 5-hydroxyl group may be posttranslationally modified by the monosaccharide galactose (β-D-galactopyranosyl) or the disaccharide glucose-galactose [α-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl] (29,30). Interest in glycosylation of collagen stems from
the recent reports of activation of specific receptor tyrosine kinases by glycosylated type I collagen (31), T-cell recognition of a glycosylated sequence within type II collagen (32), and the identification of melanoma and breast carcinoma binding sites within type IV collagen that contain glycosylated Hyl residues (1,2,4,11,12,14,33). Most secreted and cell surface eukaryotic proteins are found glycosylated in vivo. Glycosylation is believed to have three important biological roles (34-38). First, glycosylation can serve as a recognition marker for a cell, both in the context of cell-cell and cell-extracellular matrix interactions. A second role concerns the alteration glycosylation has on the physical properties of the protein. Frequently, glycosylation will render a protein resistant to hydrolysis, significantly increase solubility of a protein, or even drastically affect the overall folding and/or physical bulk of a protein. In the case of collagen-like triple-helices, the addition of β-D-galactose to Thr in the Yyy position of either (Gly-Pro-Yyy)_{10} or (Gly-Hyp-Yyy)_{10} greatly enhances triple-helical stability compared to Thr alone (39,40). A third role for glycosylation is in signal transduction, and may be analogous to or compete with protein phosphorylation (37). For example, addition of O-linked β-N-acetylglucosamine to insulin receptor substrate-1 and -2 apparently decreases phosphorylation and affects insulin-mediated homeostasis.

Prior studies have focused on the carbohydrate structures expressed on the tumor cell surface (34,41,42). Cell surface oligosaccharides may play a critical role in tumor cell angiogenesis, growth, and metastasis (43-45). It is presently unknown, but of great interest, as to what effect ligand glycosylation has on melanoma cell binding and signaling. The effects of glycosylated triple-helical structure on cellular systems have not been addressed.

In the present study, we sought to (a) determine the melanoma cell receptor for triple-helical [IV-H1] and (b) analyze the results of [IV-H1] single-site glycosylation on triple-helical stability and melanoma cell recognition of this ligand. We have used collagen-model triple-helical peptides of the general sequence (Gly-Pro-Hyp)_n-[IV-H1]- (Gly-Pro-Hyp)_m (where n = 4 or 6 and m = 4 or 0) for both goals. Affinity chromatography and melanoma cell adhesion/inhibition assays were utilized to determine the cellular receptor for triple-helical [IV-H1]. To evaluate the effects of glycosylation, the desired peptides and peptide-amphiphiles were assembled, and biophysical comparisons were first performed using CD spectroscopy to determine the conformational effects of single site glycosylation. Melanoma cell adhesion and spreading on the respective peptide-amphiphile ligands were then quantitated to evaluate the biological consequences of glycosylation.
MATERIALS AND METHODS

General. All standard peptide synthesis chemicals were peptide synthesis grade or better and purchased from FisherBiotech (La Jolla, CA). HOAt and HATU were purchased from Perkin Elmer (Foster City, CA) and DIEA from Fisher Scientific (Atlanta, GA). Fmoc-4-((2',4'-dimethoxyphenyl)aminomethyl)phenoxy resin (substitution level = 0.55 mmol/g) and Fmoc-amino acid derivatives were purchased from Novabiochem/Calbiochem (La Jolla, CA). Amino acids are of the L-configuration (except for Gly). Palmitic acid [CH$_3$-(CH$_2$)$_{14}$-CO$_2$H, designated C$_{16}$] was purchased from Fisher.

Purification of 5-Hydroxy-L-lysine. Hyl was isolated from porcine gelatin by hydrolysis followed by multiple passages over an ion-exchange column (46). The aqueous Hyl solution is acidified with HCl and evaporated to obtain crystals of Hyl. The Hyl is recrystallized when necessary. Analysis was performed using a Crownpak CR$^+$ column (150 x 4 mm; Daicel Chemical Industries, Ltd.) at 0 °C at a flow rate of 0.4 mL/min. The mobile phase was 0.13 M aqueous perchloric acid. Detection was at $\lambda = 200$ nm.

Peptide Synthesis, Purification, and Characterization. Fmoc-Hyl[ε-Boc,O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)], branched α1(IV)1263-1277 THP, and branched [Hyl(Gal)$_{1265}$]-α1(IV)1263-1277 THP were prepared as previously described (47,48). Branched THP compositions were confirmed by MALDI-MS analysis of the branch and Edman degradation sequence analysis of the intact THP (47,49). All other peptides were synthesized as C-terminal amides to prevent diketopiperazine formation (50). Peptide-resin assembly was performed by Fmoc solid-phase methodology on a Perkin Elmer/ABD 433A Peptide Synthesizer by methods previously described in our laboratory (18,19). For incorporation of the glycosylated amino acid, the H$_2$N-peptidyl-resin was removed from the instrument. Fmoc-Hyl[ε-Boc,O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)] was coupled manually in an orbital shaker using 3-fold molar excesses of Fmoc-amino acid and HOAt, a 2.7-fold molar excess of HATU, and a 6-fold molar excess of DIEA in 10 ml DMF for 18 h. Subsequent amino acids were coupled on the instrument. Peptide-resins were characterized by Edman degradation sequence analysis as described previously for “embedded” (non-covalent) sequencing (51) on an Applied Biosystems 477A Protein Sequencer/120A Analyzer. Peptide-resins were then either (a) cleaved or (b) acylated with the C$_{16}$ alkyl tail (19) and then cleaved. Cleavage and side-chain deprotection of the peptide-resin proceeded for 2 h using ethanedithiol–thioanisole–phenol–water–TFA (2.5:5:5:5:82.5) as described (52). The cleavage solution was extracted with methyl tert-butyl ether prior to purification. The
glycosylated peptide was deacetylated with methanolic sodium methoxide (2 M) for 1 h at 20 °C (53).

RP-HPLC purification was performed on a Rainin AutoPrep System. Peptides were purified with a Vydac 218TP152022 C\textsubscript{18} column (15-20 µm particle size, 300 Å pore size, 250 x 25 mm) at a flow rate of 5.0 mL/min. The elution gradient was 0-85% B in 85 min where A was 0.1% TFA in water and B was 0.1% TFA in acetonitrile. Detection was at 229 nm. Analytical RP-HPLC was performed on a Hewlett Packard 1100 Liquid Chromatograph equipped with an ODS Hypersil C\textsubscript{18} RP column (5 µm particle size, 120 Å pore size, 100 x 2.1 mm). Eluants were the same as for peptide purification. The elution gradient was 0-100% B in 30 min with a flow of 0.3 ml/min. Diode array detection was at 220, 254, and 280 nm.

MALDI-MS was performed on a Hewlett-Packard G2025A LD-TOF mass spectrometer using either a sinapinic acid or 2,5-dihydroxybenzoic acid/2-hydroxy-5-methoxy-benzoic acid (9:1, v/v) matrix (54). Peptide mass values were as follows: (Gly-Pro-Hyp)\textsubscript{4}[IV-H1](Gly-Pro-Hyp)\textsubscript{4}-NH\textsubscript{2}, [M+H]\textsuperscript{+} 3573.3 Da (theoretical 3574.9 Da); [Hyl(Gal)\textsuperscript{1265}](Gly-Pro-Hyp)\textsubscript{4}[IV-H1](Gly-Pro-Hyp)\textsubscript{4}-NH\textsubscript{2}, [M+H]\textsuperscript{+} 3752.4 Da (theoretical 3751.1 Da); C\textsubscript{16}(Gly-Pro-Hyp)\textsubscript{4}[IV-H1](Gly-Pro-Hyp)\textsubscript{4}-NH\textsubscript{2}, [M+H]\textsuperscript{+} 3811.5 Da (theoretical 3813.3 Da); C\textsubscript{16}[Hyl(Gal)\textsuperscript{1265}](Gly-Pro-Hyp)\textsubscript{4}[IV-H1](Gly-Pro-Hyp)\textsubscript{4}-NH\textsubscript{2}, [M+H]\textsuperscript{+} 3992.0 Da (theoretical 3989.1 Da); and C\textsubscript{16}[Abu\textsuperscript{1267}](Gly-Pro-Hyp)\textsubscript{4}[IV-H1](Gly-Pro-Hyp)\textsubscript{4}-NH\textsubscript{2}, [M+H]\textsuperscript{+} 3781.9 Da (theoretical 3783.4 Da).

Carbazole Test. A sample of peptide was dissolved in 85% H\textsubscript{2}SO\textsubscript{4} and heated at 80 °C for 35 min. After cooling, carbazole (1 mg/mL in ethanol) was added and incubated at room temperature for 2 h. The presence of carbohydrate was monitored by an increase in absorbance at \( \lambda = 490 \) nm (55).

CD Spectroscopy. CD spectra were recorded over the range \( \lambda = 190-250 \) nm on a JASCO J-600 using a 10 mm path-length quartz cell. The peptide concentration (14 µM in water) was kept constant for all the experiments. Thermal transition curves were obtained by recording the molar ellipticity ([\( \Theta \)]) at \( \lambda = 225 \) nm while the temperature was continuously increased in the range of 5 - 80 °C at a rate of 12 °C/h. 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 (\( T_m \)). Alternatively, \( T_m \) was evaluated from the midpoint of the transition.

Cells. SK-Mel2, M14P, M14#5, and M14#11 human melanoma cells were propagated as described previously (2,4,11). Briefly, melanoma cells were cultured in EMEM or RPMI-1640 supplemented with 10% fetal bovine sera, 1 mM sodium pyruvate,
0.1 mg/mL gentamycin (Boehringer Mannheim, Indianapolis, IN), 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% CO₂. All media reagents were purchased from Fisher Scientific.

**ELISA Analysis of CD44 or β1 Integrin Subunit Concentration.** The cell surface CD44 or β1 integrin subunit concentration was evaluated for M14P, M14#5, and M14#11 human melanoma cells by ELISA. Briefly, cells were diluted in PBS and plated at various concentrations on a 96-well plate. The plate was incubated at 4 °C for 2 h and the PBS removed. The cells were fixed with methanol and the plate blocked with BSA at 4 °C. Either anti-CD44 mAb, anti β1-integrin subunit mAb, or an equivalent concentration of IgG was diluted in PBST (PBS with 0.05% tween-20) containing 2 mg/mL BSA and incubated at 4 °C. The plate was washed with PBST and subsequently incubated with goat anti-mouse IgG conjugated to HRP in PBST and 2 mg/mL BSA. The plate was washed and HRP detected using TMB (Pierce, Rockford, IL).

**Cell Adhesion Assays.** Melanoma cell adhesion to substrate-coated Pro-Bind™ 96-well plates (Becton Dickenson, Franklin Lakes, NJ) was performed as described previously (4). Peptide-amphiphiles dissolved in PBS were diluted in 70% ethanol and added to the 96-well plate and allowed to adsorb overnight at room temperature with mixing. Nonspecific binding sites were blocked with 2 mg/mL BSA in PBS for 2 h at 37 °C. Cells were released with 5 mM EDTA in PBS, washed 2x with adhesion medium (20 mM HEPES, 2 mg/mL albumin in EMEM or RPMI-1640), and labeled with 5- or 6-carboxyfluorescein diacetate. Unincorporated fluorophore was removed by repeated washings with adhesion medium. Cells were then resuspended in adhesion medium and added to the plate. The plate was incubated 60 min at 37 °C. Non-adherent cells were removed by washing three times with adhesion medium. Adherent cells were lysed with 0.2% SDS and quantitated with a SpectraMAX Gemini, 96-well plate spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Inhibition of Cell Adhesion Assays.** Cells were labeled with 5- or 6-carboxyfluorescein diacetate and Immulon 96-well plates were coated with peptides or proteins as in the adhesion assay. The cells were preincubated with various concentrations of mAbs (Chemicon, Temecula, CA), GAGs, or chondroitinase ACII in the presence of 10 µg/mL aprotinin, leupeptin, pepstatin A, and phenyl methyl sulfonyl fluoride for 60 min at 37 °C after they had been harvested. The cells were added to the wells to evaluate the adhesion to coated peptides or proteins in the continued presence of
mAbs or GAGs. Cells were allowed to adhere for 30 min at 37 °C, and cell adhesion was quantified as in the adhesion assay.

**Cell Spreading Assays.** These assays are performed exactly as the adhesion assays with the exception of the last step, cell lysis. After washing unbound cells, the remaining cells are fixed with 2.5% glutaraldehyde dissolved in formalin, and stained with R-250 Coomassie Blue. Digital photos of each well are taken and the area of the cells quantitated with the assistance of Quantity One Software (BioRad, Hercules, CA).

**Affinity Chromatography and Immunoprecipitation and Blotting Analysis.** Branched α1(IV)1263-1277 THP or branched [Hyl(Gal)1265]-α1(IV)1263-1277 THP was coupled to activated CH-Sepharose according to the instructions of the supplier (Amersham/Pharmacia Biotech, Piscataway, NJ). In addition, a mock-coupled column was made without the peptide. M14 and SK-Mel2 melanoma cells were extracted in OGS lysis buffer (50 mM Tris•HCl, 15 mM NaCl, pH 7.2, 0.5 mM CaCl2, 0.5 mM MnCl2, 1 μM PMSF, 10 μg/mL aprotinin, 10 μg/mL, 10 μg/mL leupeptin, and 50 mM octyl-β-glucoside) by shaking 30 min at 4 °C. The lysates were cleared by centrifugation at 36,500 x g for 60 min at 4 °C. Cell lysates were shaken with the mock beads for 4 h at 4 °C. The unbound materials were collected and incubated with the peptide-Sepharose beads by rocking overnight at 4 °C. The beads were washed with 3 volumes of OGS lysis buffer, and the α1(IV)1263-1277 THP bound proteins were eluted with IP lysis buffer (0.25% triton x-100, 75 mM NaCl, 25 mM Tris•HCl, 0.5 mM vanadate, 2.5 mM EDTA) supplemented with 50 mM EDTA and 1 M NaCl. The eluate was concentrated and the buffer was exchanged with IP lysis buffer using Microsep Centrifugal Concentrators. The samples were then immunoprecipitated with 5 μg/mL anti-CD44 (Zymed, South San Francisco, CA) or anti-β1 integrin subunit (Chemicon) mAbs or mouse IgG (Chemicon). The CD44 samples were digested with 2.5 U/mL chondroitinase ACII for 3 h at 37 °C. All samples were electrophoresed on a 4-20% gradient polyacrylamide gel (BioRad, Hercules, CA) and transferred to nitrocellulose (Micron Separations, Inc, Westboro, MA). The nitrocellulose was incubated in TBST (10 mM Tris•HCl, pH 7.6, 200 mM NaCl, 0.5% tween-20) with 2% BSA for at least 4 h and incubated in anti-CD44 or anti-β1 mAb or mouse IgG diluted in TBST with 2% BSA overnight at 4 °C. The membrane was washed with TBST and incubated with fluorescein conjugated secondary antibody diluted in TBST with 2% BSA for 1 h and washed with TBST. Fluorescence was monitored using a FluorS Multi-imager (BioRad).

**Affinity Chromatography and GAG Analysis.** Branched α1(IV)1263-1277 THP was coupled to activated CH-Sepharose according to the instructions of the supplier (Pharmacia Biotech). In addition, a mock-coupled column was made without the peptide.
Chondroitin-4-sulfate, chondroitin-6-sulfate, or dermatan sulfate (all from Calbiochem) were dissolved in carbonate buffer, pH 9.0. A 4-fold molar excess of FITC dissolved in the same buffer was added to each GAG and incubated overnight at 4 °C with mixing. The goal was to achieve a 10-15% labeling of amino groups. Unreacted FITC was removed by quenching with a 10-fold excess of Gly followed by performing repeated buffer exchanges with Microsep Centrifugal Concentrators. The final product was dissolved in an OGS lysis buffer in which the OGS was omitted (-OGS lysis buffer). The columns were also equilibrated in this buffer. The desired GAG (2.5 µmoles) was incubated with the mock-coupled column for 2 h at 4 °C. The unbound portion was then added to the peptide column and the column was mixed overnight at 4 °C. The column was washed with 4 volumes of -OGS lysis buffer and bound GAGs were removed by washing sequentially with 3 volumes of 0.25, 0.5, 0.75, 1.0, and 2.0 M NaCl, while saving 2-3 mL fractions. The fluorescence of 200 µL from each fraction was measured at λ_{excitation} = 485 nm and λ_{emission} = 538 nm.

RESULTS

Construction and Characterization of Ligands

Design of Potential Ligands. To determine the receptor binding to the α1(IV)1263-1277 region and evaluate the role of glycosylation on melanoma activities, triple-helical models incorporating collagen sequences of interest needed to be constructed. In addition, in order to properly evaluate biological effects, the triple-helices of these “mini-collagens” needed to be stable to assay conditions. We have previously described two methods for assembling THPs of desirable thermal stabilities. One method uses a C-terminal covalent branch (1,47), while the other uses self-assembly driven by pseudo-lipids (18-20). Both approaches were used in the present study to create either the “branched” α1(IV)1263-1277 THP (Figure 1, top) or the α1(IV)1263-1277 “peptide-amphiphile” (Figure 1, bottom). In addition, one variant of the branched α1(IV)1263-1277 THP was created in which Lys^{1265} was replaced with Hyl(Gal), and two variants of the α1(IV)1263-1277 peptide-amphiphile were created in which Asp^{1267} was replaced with Abu or Lys^{1265} was replaced with Hyl(Gal).

Construction and Characterization of Glycosylated Peptides. Pure L-Hyl was prepared from porcine gelatin by hydrolysis and ion-exchange chromatography. The Fmoc-Hyl[ε-Boc,O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)] derivative was used for the synthesis of the collagen-model sequence (Gly-Pro-Hyp)$_4$-Gly-Val-Hyl(Gal)-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro-(Gly-Pro-Hyp)$_4$-NH$_2$, which is
designated [Hyl(Gal)]_{1265}-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$. The analogous sequence containing Lys$_{1265}$ instead of Hyl(Gal)$_{1265}$ has been synthesized previously and structurally characterized by CD and NMR spectroscopies (18-20). Synthesis of the peptide proceeded without difficulty, and Edman degradation sequence analysis of the peptide-resin indicated a highly efficient assembly. The peptide were subsequently purified by RP-HPLC, and characterized by MALDI-MS. The Fmoc-Hyl[ε-Boc,O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)] derivative was also used for the synthesis of the branched α1(IV)1263-1277 THP, where Lys$_{1265}$ was replaced with Hyl(Gal)$_{1265}$. Due to difficulties in obtaining mass spectra for large branched peptides, branched [Hyl(Gal)$_{1265}$]-α1(IV)1263-1277 THP was characterized by MALDI-MS analysis of the branch, sequence analysis of the intact THP following RP-HPLC purification, and reaction with carbazole-sulfuric acid to confirm the presence of carbohydrate.

**Biophysical Characterization of Potential Ligands.** CD spectra characteristic of triple-helices exhibit a positive molar ellipticity at $\lambda = 222-227$ nm and a negative molar ellipticity at $\lambda = 195-200$ nm (56). Also, a triple-helical assembly can be distinguished from a simple, non-intercoiled polyPro II structure by its thermal denaturation behavior. A triple-helix is relatively sensitive to temperature, and thus triple-helical melts are highly cooperative (56). The CD spectra for (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ and [Hyl(Gal)]$_{1265}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ at 5 °C were compared (Figure 2). The CD spectra for (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ and [Hyl(Gal)]$_{1265}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ are indicative of triple-helical structure. To examine the thermal stability of the two peptides, the molar ellipticity at $\lambda = 225$ nm was monitored as a function of increasing temperature. Both (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ and [Hyl(Gal)]$_{1265}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ exhibited sigmoidal transitions, consistent with the melting of a triple-helical to single-stranded structure (Figure 3). The $T_m$ values for (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ and [Hyl(Gal)]$_{1265}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ were 39 and 42 °C, respectively (Table 1). Thus, glycosylation appeared to slightly increase triple-helical stability.

To assess the biological effects of glycosylation, we prepared the peptide-amphiphile models of (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$, [Abu$_{1267}$]-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$, and [Hyl(Gal)]$_{1265}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$. Prior work has shown that construction of peptide-amphiphiles, whereby an alkyl chain is incorporated onto the N-terminus of a peptide, results in enhanced thermal stability of peptide conformation and improved binding to hydrophobic surfaces (3,14,18,19,57). The melting temperatures of the peptide-amphiphiles were 45.0 and 48.5
°C for \( C_{16}^{\alpha}(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) and \( C_{16}^{\beta}[\text{Hyl(Gal)}]^{1265}_{12}-(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \), respectively (Table 1). These \( T_m \) values are sufficient for analysis of cellular activities. The \( T_m \) of 45.0 °C for \( C_{16}^{\alpha}(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) is considerably lower than the \( T_m \) value reported previously for this peptide-amphiphile (19). However, the peptide-amphiphile concentration for the earlier study was 0.5 mM (19), which causes more extensive aggregation and a correspondingly higher \( T_m \) value (57). The peptide-amphiphile concentration used for the CD analysis described herein (14 µM) approximates the concentration range required for biological studies (see below).

**Evaluation of the Melanoma Receptor for Triple-Helical \( \alpha1(IV)1263-1277 \)**

Melanoma receptors for triple-helical collagen include members of the integrin heterodimeric protein family (\( \alpha1\beta1, \alpha2\beta1, \) and \( \alpha3\beta1 \) integrins) and cell surface proteoglycans. To examine the involvement of integrins for mediating melanoma cell adhesion to \( \alpha1(IV)1263-1277 \), a peptide-amphiphile analog was prepared in which Asp\(^{1267} \) was replaced with Abu. The collagen-binding integrins require either a Glu or Asp residue for ligand binding (2,9-11,58,59). Replacement of the only Asp/Glu residue within \( \alpha1(IV)1263-1277 \) by a sterically similar, but uncharged, residue (Abu), allowed us to specifically examine the possible role of integrin interaction with this sequence. The \( T_m \) value for \( C_{16}^{\beta}[\text{Abu}^{1267}]^{1265}_{12}-(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) was 27 °C (Table 1), and thus cell adhesion assays were performed at 20 °C to ensure that this ligand was primarily in triple-helical conformation. Melanoma cell adhesion to \( C_{16}^{\alpha}(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) and \( C_{16}^{\beta}[\text{Abu}^{1267}]^{1265}_{12}-(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) was virtually identical over the concentration range of 0.1-10 µM (Figure 4). Both peptide-amphiphiles exhibited EC\(_{50}\) values of ~0.5 µM. Thus, the lack of a negatively charged residue had no effect on melanoma cell binding to \( \alpha1(IV)1263-1277 \), suggesting a lack of integrin involvement.

Inhibition of melanoma cell adhesion assays were designed to discriminate between integrin and proteoglycan involvement. Melanoma cells were treated with 10 µg/mL of anti-\( \alpha2 \) or 20 µg/mL anti-\( \beta1 \) integrin subunit mAbs prior to adhesion to \( C_{16}^{\alpha}(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \). Neither mAb inhibited melanoma adhesion (data not shown). Treatment of melanoma cells with GAGs prior to adhesion to \( C_{16}^{\alpha}(\text{Gly-Pro-Hyp})_4^{-}[\text{IV-H1}]-(\text{Gly-Pro-Hyp})_4^{-}\text{NH}_2 \) resulted in substantial inhibition by chondroitin-4-sulfate or chondroitin-6-sulfate (Figure 5). Consistent with these results, treatment of melanoma cells with chondroitinase ACII inhibited cell adhesion to \( C_{16}^{\alpha-} \)
(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ in similar fashion as chondroitin-4-sulfate or chondroitin-6-sulfate (Figure 5).

Since the adhesion and inhibition assays indicated that a CSPG was potentially involved in melanoma cell interaction with $\alpha_1$(IV)1263-1277, we needed to determine if CD44/CSPG or MPG/MCSP/NG2 was involved. Two clones from the M14 parental cell line have been created (M14#5 and M14#11) based on repeated cell sorting using the 9.2.27 MPG/MCSP/NG2 mAb. $^3$ M14#5 expresses CD44/CSPG but does not express MPG/MCSP (60). ELISA studies were performed to determine the relative cell surface concentrations of CD44 and $\beta_1$ integrins. M14#5 cells had higher levels of CD44 than M14P, while M14#11 cells had lower levels of CD44 than M14P (Figure 6). In contrast, the levels of the $\beta_1$ integrin subunit were similar for M14P, M14#5, and M14#11 (Figure 6). We then examined the relative levels of melanoma adhesion to and spreading on C$_{16}$ (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ by M14, M14#5 and M14#11 (Figure 7). The highest levels of adhesion and spreading were achieved by M14#5, followed by M14P, and lastly M14#11. Thus, melanoma adhesion and spreading activities were not due MPG/MCSP/NG2 and most likely mediated by CD44/CSPG.

Results from the cell adhesion/inhibition assays suggested that CD44/CSPG was responsible for melanoma cell adhesion to triple-helical $\alpha_1$(IV)1263-1277. Affinity chromatography was performed to further characterize the receptor for triple-helical $\alpha_1$(IV)1263-1277. Branched $\alpha_1$(IV)1263-1277 THP was immobilized to CH-Sepharose, and precleared human melanoma cell lysates were added to the beads. Following application of the cell lysates, the column was washed with 3 volumes of OGS lysis buffer, and then bound materials were eluted with IP lysis buffer. Eluants were incubated with mAbs against either CD44 or the $\beta_1$ integrin subunit, followed by immunoprecipitation and immunoblotting with the respective mAb. A protein of ~85-90 kDa was immunoprecipitated by the anti-CD44 mAb (Figure 8). This apparent molecular weight corresponded to melanoma CD44s core protein following chondroitinase treatment (60,61). No corresponding proteins were observed using an anti-$\beta_1$ integrin subunit mAb immunoprecipitation (data not shown; see later discussion). Immunoprecipitation analysis of whole cell lysates showed the presence of both CD44 and the $\beta_1$ integrin subunit (Figures 8 and 13), consistent with prior studies (60). Incubation of the column-bound materials or whole cell lysates with IgG resulted in the detection of only IgG proteins (data not shown).

To further examine the role of CS in the binding of melanoma cells to $\alpha_1$(IV)1263-1277, affinity chromatography was performed using branched $\alpha_1$(IV)1263-1277 THP and chondroitin-4-sulfate, chondroitin-6-sulfate, and dermatan sulfate. Both
chondroitin-4-sulfate and chondroitin-6-sulfate were found to specifically bind to α1(IV)1263-1277 THP, while dermatan sulfate did not (Figure 9). The relative elution profiles of chondroitin-4-sulfate and chondroitin-6-sulfate make it appear that chondroitin-4-sulfate has a greater ability to bind branched α1(IV)1263-1277 THP, but a significant amount (>4000 RFU) of chondroitin-6-sulfate remains bound to the THP and elutes only with successive washes with acetate buffer, pH 4.0, and Tris•HCl buffer, pH 8.0 (data not shown).

Effects of Glycosylation on Melanoma Activities

Human melanoma cell adhesion was examined for C16-(Gly-Pro-Hyp)4-[IV-H1]- (Gly-Pro-Hyp)4-NH2 and C16-[Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 at 37 °C (Figure 10). The C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 peptide-amphiphile promoted significant adhesion of melanoma cells, with an EC50 value of ∼2.5 µM. The glycosylated peptide-amphiphile promoted very low levels of adhesion of melanoma cells at all concentrations tested. Neither the [IV-H1] peptide nor the C16 tail alone produced significant adhesion over the concentration range studied (14). Prior studies had shown that the single-stranded [IV-H1] peptide promotes adhesion at concentrations greater than 50 µM (EC50 ~170 µM) (1,14).

The ability of C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 and C16-[Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 to promote cell spreading was next studied. Spreading was quantitated over a ligand concentration range of 0.01 – 50 µM (Figure 11). Melanoma cell spreading was more extensive on C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 compared with C16-[Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2. Representative microscopic images of melanoma cell spreading on 10 µM C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 and 10 µM C16-[Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 (Figure 12) illustrate the modulation of cell activity based on glycosylation.

The dramatic decrease in cellular activities upon ligand glycosylation could be due to decreased binding by CD44/CSPG. To address this possibility, affinity chromatography experiments were repeated, this time using the branched, glycosylated triple-helical ligand. Branched [Hyl(Gal)1265]-α1(IV)1263-1277 THP was immobilized to CH-Sepharose, and precleared human melanoma cell lysates were added to the beads. Following application of the cell lysates, the column was washed with 3 volumes of OGS lysis buffer, and then bound materials were eluted with IP lysis buffer. Eluants were incubated with mAbs against either CD44 or the β1 integrin subunit, followed by immunoprecipitation and immunoblotting with the respective mAb. No proteins were
immunoprecipitated by either the anti-CD44 or the anti-β1 integrin subunit mAb (Figure 13). The result for the β1 integrin subunit is identical to that observed when using the non-glycosylated ligand (see earlier discussion). Immunoprecipitation analysis of whole cell lysates showed the presence of both CD44 and the β1 integrin subunit (Figure 13).

DISCUSSION

The development of model triple-helical peptide ligands has led to a better understanding of the role of the triple-helix as a modulator of biological function. In the present study, triple-helical models of α1(IV)1263-1277 have been used to define the roles of both triple-helicity and glycosylation on tumor cell interactions with basement membrane (type IV) collagen. Prior studies had shown that CD44/CSPG from melanoma cells binds directly to single-stranded α1(IV)1263-1277 (15,16), and that CD44/CSPG binds to type IV collagen (60). However, cells may engage different receptors depending upon the conformational state of collagen (21-27), and thus we needed to determine the receptor for triple-helical α1(IV)1263-1277. A variant of α1(IV)1263-1277 was constructed in which the single Asp residue was replaced with Abu. The three collagen-binding integrins, α1β1, α2β1, and α3β1, require a negatively charged residue (Asp or Glu) for binding (2,9-11,58,59). Replacement of the one negatively charged residue in α1(IV)1263-1277 had no effect on melanoma cell binding, indicating that melanoma cell interaction with this triple-helical ligand is not integrin mediated. Inhibition of adhesion assays showed that (a) anti-integrin mAbs had no effect on melanoma cell adhesion, (b) chondroitin-4-sulfate and chondroitin-6-sulfate interfered with melanoma cell adhesion, and (c) removal of chondroitin and chondroitin sulfate GAG chains by chondroitinase ACII inhibited melanoma cell adhesion. MGP/MCSP was ruled out as a potential receptor, since M14#5 melanoma cells, which do not express MGP/MCSP, efficiently adhered to and spread on triple-helical α1(IV)1263-1277.

Affinity chromatography with the branched α1(IV)1263-1277 THP indicated that melanoma cell CD44 bound directly to this ligand, while the β1 integrin subunit did not. Melanoma cell binding to type IV collagen uses integrins of only the β1 family (62,63), so interaction to the α1(IV)1263-1277 region of this collagen does not appear to be integrin mediated. In addition, CD44 is believed to be in the chondroitin sulfate form, based on (a) removal of the chondroitin sulfate glycosaminoglycan chains from the receptor by chondroitinase ACII, (b) binding of chondroitin-4-sulfate and chondroitin-6-sulfate to triple-helical α1(IV)1263-1277, (c) binding of melanoma CSPGs to type IV
collagen (60) and the linear form of α1(IV)1263-1277 (5,15,16), and (d) virtually all of the melanocyte CD44 proteoglycans being CSPGs (64).

Triple-helical α1(IV)1263-1277 represents the second distinct extracellular matrix ligand described for CD44. CD44 has long been recognized for the ability to bind hyaluronic acid (HA). HA binds to the CD44 amino-terminal globular domain (65). The HA binding motif consists of two basic amino acids separated by seven non-acidic amino acids (B[X7]B) (65). In CD44, HA binding motifs are found within residues 21-45, with Arg41 of particular importance (65). Several distal residues also contribute to HA binding, such as Lys158 and Arg162 (65). Since CS is required for CD44 binding to α1(IV)1263-1277, but interferes with CD44 binding to HA (65), it appears that α1(IV)1263-1277 and HA bind to different regions of CD44.

Position 1265 of the α1(IV) collagen chain can be glycosylated (28). The effects of this glycosylation on either triple-helical structure or CD44 binding are unknown. CD spectroscopic studies have shown that glycosylation at residue 1265 of either the triple-helical peptide or peptide-amphiphile increased the melting temperature by 3.0-3.5 °C compared to the non-glycosylated ligands. Prior work had demonstrated that β-D-galactose glycosylation of Thr in the Yyy position of (Gly-Hyp-Yyy)10 enhanced triple-helical stability by 32 °C compared to Thr (40). This corresponds to 3.2 °C per glycosylated residue. Thus, both studies have come to similar conclusions as the role of glycosylation in stabilizing the triple-helix. However, in the case of Hyl glycosylation, this stabilization effect is most likely localized to a specific sequence, and is not a general mechanism by which collagen thermal stability is enhanced. Type II collagen, whether in fully glycosylated (10 residues/1016 total) or lowly glycosylated (2 residues/1016 total) form, has the same $T_m$ value (66).

The role of Hyl glycosylation in the CD44 recognition processes was first studied by comparing melanoma cell adhesion to the glycosylated and non-glycosylated ligands. A dramatic reduction in cell adhesion was observed due to the presence of the single galactose residue, suggesting significant biological consequences of even subtle changes in collagen carbohydrate content. Promotion of melanoma cell spreading had similar, although not identical, trends as seen for cell adhesion. Subsequent affinity chromatography experiments indicated that CD44 no longer bound to the α1(IV)1263-1277 sequence once carbohydrate was present. The exquisite sensitivity of cell interaction with glycosylated ligand has only rarely been observed. T cell hybridoma response to type II collagen fragments has been shown to depend upon contacts from a single glycosylated Hyl with the CD3 loops of the T cell receptor (32).
We have found that glycosylation inhibits CD44 interaction with the α1(IV)1263-1277 region derived from basement membrane collagen. This result is unexpected, as prior studies had shown that melanoma cell binding to α1(IV)1263-1277 is primarily via electrostatic interactions with Lys_{1265} and Lys_{1268} (67). While it is possible that the glycosylation may mask the side-chain charge of residue 1265, such behavior seems unlikely given the small size of the carbohydrate. It is more likely that we have observed a specific, unfavorable carbohydrate-carbohydrate interaction between the CD44 CS and the α1(IV)1263-1277 galactose residue. Overall, little is known about how carbohydrates interact with cell surface receptors, particularly in the case of unfavorable associations (68,69). More often, such interactions are favorable, as when carcinoma cell surface mucins associate with platelet P-selectin, creating a platelet “cloak” surrounding the tumor cells that aid in the metastatic process (45). While CD44 does bind certain carbohydrates (HA), this interaction requires a minimum of six sugar residues (three repeating disaccharide units), with affinity increasing for longer HA molecules (K_d ~ 0.3 nM) (65). The present study suggests that glycosylation can be used for modulating tumor cell behaviors, based on carbohydrate structure and chain length.

The reduced binding of CD44/CSPG due to ligand glycosylation presents a possible “cryptic sites” mechanism by which tumor cells may invade the basement membrane. In the native, glycosylated state, regions within type IV collagen may have minimal interaction with receptors such as CD44/CSPG. After tumor cells bind to type IV collagen (presumably via integrins such as α2β1), cell surface glycosidases could liberate the collagen-bound carbohydrates. This process would expose “cryptic sites” for interaction with CD44/CSPG and/or other cell surface receptors (such as the α3β1 integrin, which also binds to a glycosylated region within type IV collagen (2,11,28)). Galactosylation has been shown previously to mask Lewis X antigens (70). Specific enzymes have been characterized for (a) removal of glucose from disaccharide-modified Hyl [2-O-α-D-glucopyranosyl-O-β-D-galactopyranosyl-Hyl glucohydrolase [Hamazaki, 1980 #2024;Ishii, 1987 #2025]] and (b) transfer of galactose to Hyl [UDP-galactose:hydroxylsine-collagen (basement membrane) galactosyltransferase [Spiro, 1971 #2031]]. In addition, a cell surface galactosyltransferase that binds to type IV collagen has been described (71). While a deglycosylation/cryptic sites mechanism provides interesting speculation, it should also be noted that not all Lys residues in type IV collagen are fully hydroxylated and glycosylated (28,72), and thus receptor interaction may just occur with the sub-population of type IV collagen that does not contain carbohydrate.
CD44/CSPG interaction with α1(IV)1263-1277, and subsequent promotion of signaling and spreading activities, is dependent upon triple-helical conformation and level of glycosylation. However, the role of CD44 in tumor cell invasion is just beginning to be unraveled (65,73). CD44 and several isoforms have been characterized on a variety of tumor cell surfaces (74-77), and have been suggested to be prognostic indicators of malignant melanoma (78,79). Although CD44 binds to types I, IV, VI, and XIV collagen, it is not a primary receptor for cell adhesion to collagen (60,80-82). The CD44 cytoplasmic domain binds to ankyrin and members of the ezrin-radixin-moesin (ERM) family of cytoskeletal proteins (83). CD44 is also directly linked to two Tyr kinases, p185HER2 and c-Src kinase (83). We have found that signaling via the CD44/α1(IV)1263-1277 interaction results in autophosphorylation of p125FAK (14), while others have shown that CD44 mediates phosphorylation of ZAP-70 and the activation of PLCγ, Ras, PKCζ, and NF-κB binding activity (84). One result of CD44 “outside-in” signaling is up regulation and activation of integrins (85) and the expression of matrix metalloproteinases (61). It is possible that CD44 works in concert with another receptor, such as the α2β1 integrin, to efficiently bind to type IV collagen and subsequently upregulate cell signaling pathways. Amongst the products of these pathways are proteases and growth factors that aid in compromising the basement membrane. Such a mechanism is consistent with our “collagen structural modulation” model previously proposed for tumor cell invasion (4), and will be explored in future studies.

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FOOTNOTES
1Abbreviations used are: Abu, 2-aminobutyric acid; Boc, tertiary-butylxycarbonyl; BSA, bovine serum albumin; CD, circular dichroism; CSPG, chondroitin sulfate proteoglycan; DIEA, N,N-diisopropyl-ethylamine; DMF, N,N-dimethylformamide; Fmoc, 9-fluorenylmethoxy-carbonyl; EMEM, Eagle's Minimum Essential Medium; FITC, fluorescein isothiocyanate; GAG, glycosaminoglycans; HA, hyaluronic acid; HATU, O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; Hyl, 5-hydroxy-L-lysine; Hyp, 4-hydroxy-L-proline; [IV-H1], α1(IV)1263-1277 collagen sequence Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MPG/MCSP/NG2, melanoma-associated proteoglycan/melanoma...
chondroitin sulfate proteoglycan; NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; THP, triple-helical peptide; TFA, trifluoroacetic acid.

2J.A. Borgia, J.L. Lauer-Fields, and G.B. Fields, manuscript in preparation.

3B. Mueller, personal communication.
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Table 1: $T_m$ Values for Triple Helix $\leftrightarrow$ Coil Transitions.

| Peptide or Peptide-Amphiphile$^a$                                      | $T_m$ (°C) |
|-----------------------------------------------------------------------|------------|
| (Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$                    | 39.0       |
| [Hyl(Gal)$_{1265}$)-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ | 42.0       |
| $C_{16}$-(Gly-Pro-Hyp)$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$            | 45.0       |
| $C_{16}$-[Hyl(Gal)$_{1265}$]-[Gly-Pro-Hyp]$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ | 48.5       |
| $C_{16}$-[Abu$_{1267}$]-[Gly-Pro-Hyp]$_4$-[IV-H1]-(Gly-Pro-Hyp)$_4$-NH$_2$ | 27.0       |

$^a$[IV-H1] = Gly-Val-Lys-Gly-Asp-Lys-Gly-Asn-Pro-Gly-Trp-Pro-Gly-Ala-Pro.
FIGURE LEGENDS

Figure 1: Structures of the branched α1(IV)1263-1277 THP (top) and C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 peptide-amphiphile (bottom). Ahx is 6-aminohexanoic acid and Hyp is 4-hydroxy-L-proline.

Figure 2: Circular dichroism spectra of (A) (Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 and (B) [Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 at 5 °C. Peptide concentrations were 14 μM in H2O.

Figure 3: Temperature dependence of molar ellipticity at λ = 225 nm for (A) (Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 and (B) [Hyl(Gal)1265]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2. Peptide concentrations were 14 μM in H2O.

Figure 4: Human melanoma cell adhesion to C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 (closed triangles, solid line), C16-[Abu1267]-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 (closed triangles, solid line), or BSA (dashed line), at 20 °C. Peptide-amphiphile concentrations were 0.01 - 10 μM.

Figure 5: Inhibition of human melanoma cell spreading on C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 by chondroitin-4-sulfate (closed circles, solid line), chondroitin-6-sulfate (closed circles, dashed line), or chondroitinase ACII (closed triangles, dashed line), at 37 °C. The solid line is spreading on bovine serum albumin. The peptide-amphiphile concentration was 10 μM.

Figure 6: ELISA of human melanoma M14, M14#5, and M14#11 CD44 (top) and β1 integrin subunit (bottom) cell surface protein levels.

Figure 7: Human melanoma M14 (closed squares, solid line), M14#5 (closed squares, dashed line), and M14#11 (open squares, dashed line) cell spreading on C16-(Gly-Pro-Hyp)4-[IV-H1]-(Gly-Pro-Hyp)4-NH2 or BSA (solid line) at 37 °C. The peptide-amphiphile concentration was 0.01 - 50 μM.

Figure 8: Immunoprecipitation of immunoblot analysis of melanoma cell surface proteins eluted from the α1(IV)1263-1277 THP affinity column. Lane 1 contains the MW markers
Lane 2 contains proteins eluted by EDTA + NaCl from the THP column, immunoprecipitated with an anti-CD44 mAb, and then treated with chondroitinase ACII. Lanes 3 and 4 contain lysis buffer immunoprecipitated with an anti-CD44 mAb, which serves as a negative control. Lane 5 contains melanoma whole cell lysate proteins immunoprecipitated with an anti-CD44 mAb followed by treatment with chondroitinase ACII. In lanes 2 and 5, one protein of ~85-90 kDa, corresponding to CD44, was immunoprecipitated. In lanes 2-5, the CD44 mAb appears at ~50 and ~25 kDa.

Figure 9: Affinity chromatographic analysis of chondroitin-4-sulfate (closed diamonds, solid line), dermatan sulfate (closed squares, solid line), or chondroitin-6-sulfate (closed circles, dashed line) binding to the α1(IV)1263-1277 THP affinity column. The GAGs were eluted by increasing NaCl concentrations, and detected by fluorometric analysis (where RFU = relative fluorescence units; see Materials and Methods). Chondroitin-4-sulfate and chondroitin-6-sulfate bound specifically to the α1(IV)1263-1277 THP, while dermatan sulfate did not.

Figure 10: Human melanoma cell adhesion to C₁₆-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ (open triangles, solid line), C₁₆-[Hyl(Gal)¹²⁶⁵]-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ (open triangles, dashed line), or BSA (dashed line) at 37 °C. Peptide-amphiphile concentrations were 0.01 – 10 µM.

Figure 11: Human melanoma cell spreading on C₁₆-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ (open triangles, solid line) C₁₆-[Hyl(Gal)¹²⁶⁵]-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ (open triangles, dashed line), or BSA (dashed line) at 37 °C. Peptide-amphiphile concentrations were 0.01 – 50 µM.

Figure 12: Human melanoma cell spreading on (a) 10 µM C₁₆-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ or (b) 10 µM C₁₆-[Hyl(Gal)¹²⁶⁵]-(Gly-Pro-Hyp)₄-[IV-H₁]-(Gly-Pro-Hyp)₄-NH₂ at 37 °C.

Figure 13: Immunoprecipitation of immunoblot analysis of melanoma cell surface proteins eluted from the [Hyl(Gal)¹²⁶⁵]-α1(IV)1263-1277 THP affinity column. For gel (A), lane 1 contains the MW markers (208 kDa doublet, 126, 97, and 48 kDa). Lane 2 contains melanoma whole cell lysate proteins immunoprecipitated with an anti-CD44 mAb, followed by treatment with chondroitinase ACII. Lane 3 contains proteins eluted
by EDTA + NaCl from the glycosylated THP column, immunoprecipitated with an anti-CD44 mAb, and then treated with chondroitinase AC II. Lane 4 contains lysis buffer immunoprecipitated with an anti-CD44 mAb, which serves as a negative control. In lane 2, one protein of ~85-90 kDa, corresponding to CD44, was immunoprecipitated. In lane 3, no proteins were immunoprecipitated from the column. In lanes 2-4, the CD44 mAb appears at ~50 kDa.

For gel (B), lane 1 contains the MW markers (108, 97, 48, 35, 28, and 20 kDa). Lane 2 contains melanoma whole cell lysate proteins immunoprecipitated with an anti-β1 integrin subunit mAb. Lane 3 contains proteins eluted by EDTA + NaCl from the glycosylated THP column and immunoprecipitated with an anti-β1 integrin subunit mAb. Lane 4 contains lysis buffer immunoprecipitated with an anti-β1 integrin subunit mAb, which serves as a negative control. In lane 2, one protein of ~145 kDa, corresponding to the β1 integrin subunit, was immunoprecipitated. In lane 3, no proteins were immunoprecipitated from the column. In lanes 2-4, the anti-β1 integrin subunit mAb appears at ~50 and ~25 kDa.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9
Figure 10
Figure 11
Figure 12
Figure 13
Melanoma cell CD44 interaction with the α1(IV)1263-1277 region from basement membrane collagen is modulated by ligand glycosylation
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