Glycosulfopeptides with O-Glycans Containing Sialylated and Polyfucosylated Polylactosamine Bind with Low Affinity To P-Selectin

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SUMMARY

P-selectin glycoprotein ligand-1 (PSGL-1), a dimeric mucin on leukocytes, is the best characterized ligand for selectins. P-selectin binds stereospecifically to the extreme N-terminus of PSGL-1, which contains three clustered tyrosine sulfates (TyrSO3⁻) adjacent to a Thr residue with a core-2 based O-glycan expressing sialyl Lewis x (C2-O-sLe⁴). GSP-6, a synthetic glycosulfopeptide modeled after the N-terminus of PSGL-1, containing three TyrSO₃⁻ residues and a short, monofucosylated C2-O-sLe⁴ bound to P-selectin with high affinity (Kₐ ~ 650 nM). However, PSGL-1 from human HL-60 cells contains higher levels of O-glycans that are sialylated andpolyfucosylated polylactosamines (PFPL). Furthermore, studies with fucosyltransferase-deficient mice suggest that sialylated PFPL structures contribute to binding to P-selectin. To resolve whether sialylated PFPL O-glycans participate in binding of PSGL-1 to human P-selectin, we synthesized glycosulfopeptides, designated GSP-6' and GSP-6", with three TyrSO₃⁻ residues and either difucosylated polylactosamine (C2-O-Le⁴-sLe⁴) or trifucosylated polylactosamine (C2-O-Le⁴-Le⁴-sLe⁴). Binding of the GSPs to P-selectin was measured by affinity chromatography, fluorescence solid-phase assays, and equilibrium gel filtration. Unexpectedly, both GSP-6' and GSP-6" bound to P-selectin with low affinity (Kₐ ~ 37 µM for GSP-6' and Kₐ ~ 50 µM for GSP-6"). Binding of GSP-6' and GSP-6" to P-selectin required fucosylation and, to a lesser extent, sialylation, as well as the sulfated peptide backbone of GSP-6' and GSP-6". These results demonstrate that contrary to expectations, a core-2 O-glycan containing sialylated PFPL does not promote high affinity binding of PSGL-1 to P-selectin.
INTRODUCTION

The selectin family of cell adhesion molecules comprising P-, L- and E-selectin and their glycoconjugate ligands play an important role in the initial events of the inflammatory cascade, by initiating the tethering and rolling of circulating leukocytes on endothelial cells, platelets, and other leukocytes (1). Selectins bind with high affinity to specific macromolecular ligands in the vascular system containing the sialyl Lewis x epitope (sLe\(^x\), NeuAc\(\alpha\)2-3Gal\(\beta\)1-4(Fuc\(\alpha\)1-3)GlcNAc-R), but binding to the sLe\(^x\)-containing glycan alone is weak (2,3). The best characterized ligand for selectins is P-selectin glycoprotein ligand-1 (PSGL-1), a disulfide-linked homodimeric mucin on leukocytes. PSGL-1 binds with relatively high affinity (K\(_d\)~300 nM) to P-selectin (4). By contrast, L-selectin binds to the sulfated mucin GlyCAM-1 with a K\(_d\) of ~108 \(\mu\)M (5) and E-selectin binds to ESL-1 with a K\(_d\) of 62 \(\mu\)M (6).

The extreme N-terminus of mature human PSGL-1 begins at amino acid residue 42 and contains three potential tyrosine sulfation sites at Tyr46, -48 and -51. Sulfation of these tyrosine residues, along with a core-2 O-glycan capped with sLex (C2-O-sLex\(^x\)) at Thr57, are necessary for high affinity binding of PSGL-1 to P-selectin (7-10). Direct interaction of P-selectin with this domain was demonstrated by utilizing synthetic glycosulfopeptides. Monomeric glycosulfopeptide-6 (1-GSP-6), corresponding to an N-terminal segment of PSGL-1 containing three TyrSO\(_3\) residues at Tyr46, -48 and -51 and a C2-O-sLex\(^x\) at Thr57, binds to P-selectin with high affinity (K\(_d\)~350 nM) (11), comparable to that of the native ligand from human neutrophils. In addition, trypsin treatment of PSGL-1 from HL-60 cells generates a structurally related glycosulfopeptide that binds to P-selectin with high affinity (12).

However, there is evidence that more complex O-glycans in PSGL-1 might mediate high affinity binding to P-selectin. PSGL-1 contains ~70 potential O-glycosylation sites at Ser and
Thr residues (7). Endo-β-galactosidase treatment of PSGL-1 from human neutrophils suggested that PSGL-1 carries sialylated and fucosylated polylactosamines \([-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-\]_n in O-glycans that promote high affinity binding to P-selectin (13). Subsequent detailed structural analysis of O-glycans on PSGL-1 from the human promyelocytic cell line HL-60 showed that a majority of the fucose-containing O-glycans on PSGL-1 from HL-60 cells were sialylated and polyfucosylated polylactosamines (PFPL) (10). Surprisingly, however, only 14% of the total O-glycans in PSGL-1 from HL-60 cells were α1,3-fucosylated and contained sLe^x (10). Two fucosylated O-glycans were identified: a core-2 based monosialylated, trifucosylated structure with a polylactosamine backbone (C2-O-Le^x-Le^x-sLe^x, 12%), and a less abundant core-2 based disialylated, monofucosylated structure lacking polylactosamine (C2-O-sLe^x, 2%). While synthetic glycosulfopeptides containing the simple monosialylated monofucosylated C2-O-sLe^x bind to P-selectin, the role of sialylated PFPL O-glycans in binding to P-selectin has not been studied. Efficient generation of sialylated PFPL in vitro requires the participation of two human α1,3-fucosyltransferases expressed in leukocytes – FucT-IV and FucT-VII (14,15). FucT-IV appears to preferentially fucosylate internal GlcNAc residues within the polylactosamine backbone, whereas FucT-VII fucosylates a GlcNAc residue of the non-reducing sialylated N-acetyllactosamine unit to generate the sLe^x terminus (14). Studies in mice containing null mutations in FucT-VII or in both FucT-IV and FucT-VII have led support to the possibility that both enzymes contribute to selectin ligands, possibly by cooperating to generate the sialylated PFPL (16-18).

To gain direct insight into the contribution of O-glycans with extended polylactosamine in PSGL-1 for binding to P-selectin, we synthesized two novel GSPs corresponding to the N-terminal amino acid sequence of human PSGL-1. These contained the sLe^x epitope on an
extended core-2 based O-glycan at Thr57 with either two (C2-O-Le\textsuperscript{x}-sLe\textsuperscript{x}) or three (C2-O-Le\textsuperscript{x}-Le\textsuperscript{x}-sLe\textsuperscript{x}) fucosylated lactosamine repeats, plus three TyrSO\textsubscript{3}\textsuperscript{-} residues at Tyr46, -48 and -51 (Fig. 1). Using these synthetic GSPs we directly examined the contribution of the extended O-glycan for binding to P-selectin.

EXPERIMENTAL PROCEDURES

Enzymatic synthesis of GSP-6' and GSP-6": Approach I (see Fig. 2A) — Glycopeptide-1 (GP-1) corresponding to amino acid residues 45-61 of human PSGL-1 with a GalNAc\textsubscript{α} residue at T57 was synthesized on an automated peptide synthesizer as described (19). In Approach I reversed phase HPLC-purified GP-1 served as an acceptor for enzymatic synthesis of GSP-6' and GSP-6". GP-4 was synthesized and purified as described (11,19). GP-4 was extended using partially purified β1,3-GlcNAcT from human serum (precipitated from human serum by ammonium sulfate at 25-50 % saturation) (20). In a typical reaction, GP-4 (20 nmol), UDP-GlcNAc (560 nmol), MnCl\textsubscript{2} (160 nmol), 0.04 % (w/v) NaN\textsubscript{3} and a concentrate of β1,3-GlcNAcT (700 µg protein) were incubated for nine days at 37\textdegree C in 20 µl of 50 mM Tris-HCl, pH 7.5. Then 100 µl of 50 mM NH\textsubscript{4}HCO\textsubscript{3} was added, the solution was clarified by centrifugation, and the clear supernatant was subjected to gel filtration HPLC on a column of Superdex 75 HR (Pharmacia, Uppsala, Sweden). The glycopeptide fraction was analyzed by MALDI mass spectrometry (MS) as described (11). MALDI MS revealed a major [M-H\textsuperscript{+}]\textsuperscript{-} molecular ion peak at \textit{m/z} 3231.7 that was assigned to glycopeptide GP-4.1 (calculated \textit{m/z}, 3232.3). Three small signals were also present. One of these, representing ~5 % of the major peak, had the same \textit{m/z} as the acceptor GP-4. Another small signal was assigned as anhydro-GP-4.1 (~10-15 %) and the third satellite signal could not be assigned. Glycopeptide GP-4.1 was then galactosylated by
incubation with bovine milk β1,4-GalT (11). MALDI analysis of the HPLC-purified glycopeptide fraction showed a major signal at \( m/z \) 3393.7 that was assigned to the \([\text{M-H}^+]\) molecular ion of the galactosylated product GP-4.2 (calc. \( m/z \) 3394.4). A minor signal of a putative anhydro-GP-4.2 was also present. Glycopeptide GP-4.2 (100 nmol) was extended using serum β1,3-GlcNAcT, as described for the extension of GP-4. MALDI analysis of the HPLC-purified glycopeptide fraction revealed a major signal at \( m/z \) 3597.2 that was assigned to the \([\text{M-H}^+]\) molecular ion of GP-4.3 (calc. \( m/z \) 3597.6). Small signals (4 % and ~20 %) represented the \([\text{M-H}^+]\) molecular ions of the acceptor GP-4.2 and the anhydro form of GP-4.3, respectively. GP-4.3 was galactosylated with bovine milk β1,4-GalT. MALDI MS of the HPLC-purified glycopeptide fraction showed a major signal at \( m/z \) 3759.4, which was assigned to the \([\text{M-H}^+]\) molecular ion of GP-4.4 (calc. \( m/z \) 3759.8). The MALDI spectrum also revealed several minor signals; the largest one (~30 %) was assigned to anhydro-GP-4.4. Glycopeptides GP-4.2 (8.2 nmol) and GP-4.4 (10 nmol) were sialylated using α2,3-(N)-sialylT (Calbiochem, La Jolla, CA) (11). The reaction mixtures were purified by HPLC, and glycopeptide fractions were analyzed by MALDI MS. A major \([\text{M-H}^+]\) molecular ion for the sialylT product of GP-4.2 was at \( m/z \) 3686.3 that represented GP-5' (calc. \( m/z \) 3685.3). A minor signal at \( m/z \) 3669.2 was assigned to anhydro-GP-5' (~20 %). MALDI analysis of the sialylT product of GP-4.4 gave a major signal at \( m/z \) 4049.9 that was assigned to the \([\text{M-H}^+]\) molecular ion of GP-5'' (calc. \( m/z \) 4050.9). The minor signal of anhydro-GP-5'' was also present. Glycopeptides GP-5' (5 nmol) and GP-5'' (6 nmol) were fucosylated using α1,3-FucTVI (Calbiochem, La Jolla, CA) (11). The reaction mixtures were purified by HPLC, and the glycopeptide fractions were analyzed by MALDI MS. MALDI analysis of the FucT product of GP-5' showed a major signal at \( m/z \) 3977.9 which was assigned to the \([\text{M-H}^+]\) molecular ion of GP-6' (calc. \( m/z \) 3977.5). The minor signals of anhydro-GP-6'
and monofucosylated GP-6' (~5 %) were also present. MALDI MS of the FucT product of GP-5" revealed the major signal at m/z 4488.9 which was assigned to the [M-H⁺]⁻ molecular ion of GP-6" (calc. m/z 4489.1). MALDI analysis showed that partially fucosylated structures were very minor but the signal of anhydro-GP-6" was ~50 %. Glycopeptide products GP-6' and GP-6" were further purified in reversed phase HPLC where GP-6' and GP-6" were separated from anhydro-GP-6' and -GP-6", respectively. The masses of GP-6' and GP-6" were verified by electrospray MS which showed the molecular masses of 3977.8 for GP-6' and 4489.1 for GP-6". Sodium adducts of the peptides were also present in the mass spectra. Glycopeptides GP-6' and GP-6" were sulfated enzymatically using human tyrosyl-protein sulfotransferase-1 and [³⁵S]PAPS (1470 cpm/pmol) (NEN Life Science Products, Inc. Boston, MA) as a sulfate donor as described (11). The fully sulfated glycosulfopeptides, GSP-6' and GSP-6" were separated from partially sulfated glycosulfopeptides using HPLC.

Enzymatic synthesis of GSP-6' and GSP-6": Approach II (see Fig. 2B) — Glycosulfopeptide-1 (GSP-1), corresponding to amino acid residues 45-61 of human PSGL-1 with three TyrSO₃ residues (Y46, 48 and 51) and a GalNAcα residue at T57, was synthesized on an automated peptide synthesizer as described (19). HPLC-purified GSP-1 served as an acceptor for enzymatic synthesis of GSP-6' and GSP-6". The glycosyltransferase reactions were carried out by adding one glycosyltransferase and one donor at a time, and the completeness of each reaction was followed by HPLC. After the reaction was 95-100 % complete, a new glycosyltransferase and a new donor were added. The first steps in the synthesis have been described in detail (11,19). Briefly, the GalNAc residue in GSP-1 served as an acceptor for core-1 β¹,³-GalT that was purified from rat liver (21). The resulting GSP-2 was used as an acceptor for recombinant core-2 β¹,⁶-GlcNAcT (11). The product GSP-3 (322 nmol) was then sialylated.
at 37°C for 18 h using CMP-NeuAc (650 nmol) and 30 mU of α2,3-(O)-sialylT (Calbiochem) in reaction mixture (700 µl) containing 50 mM Na-cacodylate, pH 7.0, 10 mM MnCl₂, 1 U of alkaline phosphatase, 0.6 % BSA, 0.02 % NaN₃. The α2,3-(O)-sialylT reaction mixture containing the product (GSP-3S, 294 nmol) was galactosylated for 15 h at 37°C using 450 nmol of UDP-Gal and 50 mU of bovine milk β1,4-GalT (Sigma). The reaction mixture containing the galactosylated product (GSP-4S, 281 nmol) was diluted to a final volume of 2 ml of 50 mM Na-cacodylate, pH 7.4, 15 mM MnCl₂ and 0.02 % NaN₃. The galactosylated core-2 branch of GSP-4S was extended by incubating the reaction mixture with 1.2 µmol of UDP-GlcNAc and 2.7 mU of recombinant (Nm)β1,3-GlcNAcT from Neisseria meningitidis lgtA (22) for 46 h at 25-30°C. The reaction mixture containing the extended glycosulfopeptide (GSP-4S.1, ~266 nmol) was galactosylated by incubation for 49 h at 37°C using 650 nmol of UDP-Gal and 150 mU of β1,4-GalT in a total reaction volume of 3 ml of 50 mM Na-cacodylate, pH 7.4, 15 mM MnCl₂ and 0.02 % NaN₃. The reaction mixture containing the galactosylated glycosulfopeptide (GSP-4S.2) was divided into two. One half was treated with neuraminidase as described below, and the other half was extended by one N-acetyllactosamine unit using (Nm)β1,3-GlcNAcT and β1,4-GalT. The resulting GSP-4S.4 was treated with neuraminidase. The neuraminidase-treated samples (GSP-4.2 and GSP-4.4, 40-50 nmol each) were purified by HPLC and sialylated for ~40 h at 37°C using 500 nmol of CMP-NeuAc and 80 mU of α2,3-(N)-sialylT (Calbiochem) in a total reaction volume of 500 µl of 50 mM Na-cacodylate, pH 7.4 containing 15 mM MnCl₂, 0.5 mg/ml BSA and 1U alkaline phosphatase. The newly sialylated samples were divided into two. One half of the reaction mixture containing GSP-5' or GSP-5” was purified by HPLC and analyzed by MS. The other half was fucosylated for ~21 h at 37°C using 3 times molar excess of GDPFuc and 10 mU of α1,3-FucTVI (Calbiochem). The reaction products (GSP-6' and GSP-6")
were purified by HPLC and analyzed by MS. Electrospray MS analysis showed the following molecular masses for the purified glycosulfopeptides: GSP-5', 3925.7 (calc. 3925.8); GSP-5", 4290.9 (calc. 4290.9); GSP-6', 4217.8 (calc. 4218.1); GSP-6", 4729.8 (calc. 4729.3). During the enzymatic synthesis of GSPs the molecular masses of other intermediate peptide products shown in Fig. 2 were also verified by MS (data not shown). The enzymatic synthesis of GSP-6 and GP-6 has been described earlier (19).

Radiolabeled glyco(sulfo)peptides were synthesized using GP-5, GSP-5, GSP-5' and GSP-5" as acceptors and GDP-[3H]Fuc (American Radiolabeled Chemicals Inc., St. Louis, MO) as a donor in the $\alpha$1,3-FucTVI reaction. $[^3H]$GSP-5' and $[^3H]$GSP-5" were synthesized using GSP-4.2 and GSP-4.4 as acceptors and CMP-$[^3H]$NeuAc (7130 cpm/pmol) (American Radiolabeled Chemicals Inc.) as a donor in the $\alpha$2,3-(N)-sialyltransferase reaction. The following specific activities of GDP-$[^3H]$Fuc were used in the $\alpha$1,3-FucTVI reactions: GP-5, 6300 cpm/pmol; GSP-5, 1000 cpm/pmol; GSP-5' and GSP-5", 2800 cpm/pmol. The radiolabeled products were purified by HPLC.

Reversed phase high performance liquid chromatography — Glyco(sulfo)peptide samples were filtered in a Spin-X membrane (Costar Corning Incorporated, Corning, NY) and purified in a semipreparative reversed phase C-18 HPLC column (10 $\mu$m, 10 mm x 250 mm) (Vydac, Hesperia, CA). Small aliquots from the glycosyltransferase reaction mixtures were analyzed in an analytical reversed phase C-18 HPLC column (5 $\mu$m, 4.6 mm x 250 mm) (Vydac) on a Beckman System Gold instrument. The following solvent systems were used in analytic separations at a flow rate of 1 ml/min: (I) 1-10 min, isocratic 15 % aqueous acetonitrile containing 0.1 % TFA; 10-70 min, linear gradient 15-40 % aqueous acetonitrile containing 0.1 % TFA; (II) 1-5 min, isocratic 15 % aqueous acetonitrile containing 0.1 % TFA; 5-70 min, linear
gradient 15-30 % aqueous acetonitrile containing 0.1 % TFA. Semipreparative HPLC separations were carried out at a flow rate of 3 ml/min using a linear 10-30 % aqueous acetonitrile gradient containing 0.1 % TFA (1-80 min). The UV absorbance at 215 nm was followed, and/or the radioactivity of the collected fractions (1 min) was measured. To prevent desulfation, GSP products eluted from HPLC were neutralized by adding NH₄HCO₃ before drying in vacuum.

Neuraminidase digestion — The glycosyltransferase reaction mixtures containing the extended glycosulfopeptides GSP-4S.2 and GSP-4S.4 (approximately 100 nmol each) were treated overnight at 37°C with 10 mU of neuraminidase from *Vibrio cholerae* (Roche Diagnostics Corp., Indianapolis, IN) in 50 mM Na-cacodylate, pH 5.0 and 2 mM CaCl₂. Reactions were terminated by boiling for 5 min. The samples were filtered through a Spin-X membrane, purified by HPLC, and analyzed by electrospray MS. [³H]GSP-6' and [³H]GSP-6" were desialylated using 2 mU of neuraminidase and purified by HPLC.

P-selectin affinity chromatography — Soluble P-selectin (sPS) was coupled to Ultralink Biosupport Medium (Pierce, Rockford, IL) at a density of 2 mg/ml. Radiolabeled glyco(sulfo)peptides (~2000 cpm, 0.3-1 pmol) were chromatographed in the sPS column (0.9 ml, 0.5 x 4.5 cm) using physiological salt concentration (20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.02 % NaN₃) as described (19).

Equilibrium gel filtration chromatography — Hummel-Dreyer equilibrium gel filtration experiments (23,24) were conducted in a 2 ml Sephadex G-100 column (0.5 x 10 cm) at a subphysiological salt concentration (20 mM MOPS, pH 7.5, containing 50 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 0.02 % NaN₃) as described (11). ³H- and ³⁵S-labeled peptides were used in the buffer at a concentration of 1.2-1.3 pmol/ml, except in the experiment of Fig. 6A where the
concentration of $[^{3}H]GP-6$ in the buffer was 1.1 pmol/ml. The specific radioactivities of peptides were as follows: $[^{3}H]GP-6$ and pronase digested $[^{3}H]GP-6$, 6330 cpm/pmol; $[^{3}H]GSP-6'$ and desialylated $[^{3}H]GSP-6'$, 5640 cpm/pmol; $[^{3}H]GSP-6''$ and desialylated $[^{3}H]GSP-6''$, 8460 cpm/pmol; $[^{3}H]GSP-5'$ and $[^{3}H]GSP-5''$, 7130 cpm/pmol; $[^{35}S]GSP-6'$ and $[^{35}S]GSP-6''$, 3420 or 3730 cpm/pmol.

Pronase digestion of GP-6 — $[^{3}H]GP-6$ (6330 cpm/pmol) was digested with *Streptomyces griseus* pronase E (2 mg/ml) (Sigma, St. Louis, MO) as described (19).

Biotinylation of glyco(sulfo)peptides — The protected C-terminal Cys of glyco(sulfo)peptides (GSP-6, GSP-6', GSP-6'', desialylated GSP-6', desialylated GSP-6'', GSP-5', GSP-5'', GSP-1, GP-6 and GP-4) was deprotected by treating each peptide with 2.5 mM or 5 mM DTT in 100 µl of 0.1 M NH$_4$HCO$_3$ for 1-2 h at room temperature. Peptides were purified in HPLC and dried down in vacuum. Reduced peptides were biotinylated by incubating the peptides with 10-20 molar excess of Biotin-HPDP (Pierce) in 100 µl of PBS containing 1 mM EDTA for 1-2 h at room temperature or overnight at 4°C. Biotinylated peptides were purified by HPLC. Alternatively, HPLC-purified, reduced peptides were biotinylated by incubating them with ~2.5 molar excess of Biotin-HPDP in 100 µl of 20 mM MOPS, pH 7.5 containing 150 mM NaCl and 1 mM EDTA. The completeness of the reaction was confirmed by HPLC, and the biotinylated peptides were directly used in a fluorescence solid phase assay without purification.

Fluorescence solid phase assay — Biotinylated glyco(sulfo)peptides were dissolved in 20 mM MOPS, pH 7.5 containing 150 mM NaCl, and the concentration of each peptide solution was determined by UV absorbance at 215 nm of a sample subjected to HPLC. Streptavidin-coated black 96-well microtiter plates (Pierce) were washed 3 times with 200 µl of 20 mM MOPS, pH 7.5 containing 50 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 0.02 % NaN$_3$ (buffer A) or
20 mM MOPS, pH 7.5 containing 50 mM NaCl, 5 mM EDTA, 0.02 % NaN₃ (buffer B) and coated for 1 h with 1 pmol of individual glyco(sulfo)peptide in 100 µl of buffer A or B. The wells were incubated for 1 h with 100 µl of P-selectin IgG chimera (3 µg/ml) or anti-PSGL-1 mAb PL1 (25) (2 or 5 µg/ml) in buffer A or B containing 0.05% Tween-20 and 1% BSA. The wells were subsequently incubated for 1 h with 100 µl of 5 µg/ml dilution of Alexa Fluor™ 488 goat anti-human or -mouse IgG (H+L) (Molecular Probes, Inc., Eugene, OR) in buffer A or B containing 0.05% Tween-20 and 1% BSA. After final washing 100 µl of buffer A or B was added to each well and the fluorescence was measured using Tecan Ultra384 microtiter plate reader (Tecan U.S., Durham, NC) with excitation wavelength 485 nm and emission wavelength 535 nm. Experiments with different dilutions of P-selectin IgG chimera were carried out using P-selectin IgG chimera concentrations of 0, 0.1, 0.2, 0.3, 0.5, 1, 2 and 3 µg/ml in buffer A or B containing 0.05% Tween-20 and 1% BSA. Peptide coating and all incubations were performed at room temperature, and the wells were washed 3 times using buffer A or B containing 0.05% Tween-20. The assays were performed in triplicate, and the results represent averages of three determinations where background fluorescence reading without peptide coating was subtracted from each sample (typically 100-600 RFU depending on the P-selectin IgG chimera concentration used in the assay).

Mass spectrometric analysis of glyco(sulfo)peptides — Electrospray mass spectra were collected in the negative ion mode using a Q-STAR triple-quadrupole MS (PerkinElmer Sciex Instruments, Thornhill, Ontario, Canada). Samples were dissolved in 10 mM ammonium acetate in 50 % aqueous acetonitrile to a concentration of ~2 pmol/µl and injected into the MS with a nanoelectrospray ion source.
RESULTS

Synthesis of glycosulfopeptides with sialylated and PFPL O-glycan — To define the possible contributions of sialylated PFPL O-glycan for binding of PSGL-1 to P-selectin, we synthesized glyco(sulfo)peptides (GSPs) corresponding to the N-terminal amino acid residues 45-61 of human PSGL-1 (Fig. 1). All sulfated GSPs contained three TyrSO₃⁻ residues at Tyr46, 48 and 51, whereas some control peptides were not sulfated (GP-6 and GP-4). GSP-6 and GP-6 contained simple C2-O-sLe⁺ at Thr57, whereas GSP-6' and GSP-6" contained two (C2-O-Le⁺-sLe⁺) or three (C2-O-Le⁺-Le⁺-sLe⁺) fucosylated lactosamine repeats at Thr57, respectively. Desialylated GSP-6' and GSP-6" were prepared by treating GSP-6' and GSP-6" by neuraminidase. GSP-5' and GSP-5" were nonfucosylated derivatives of GSP-6' and GSP-6", respectively. Incompletely glycosylated GSP-1 and GP-4 contained a GalNAc residue only or galactosylated core-2 tetrasaccharide at Thr57, respectively (Fig. 1).

Glyco(sulfo)peptides GSP-6 and GP-6 lacking polylactosamine sequences were synthesized as described (19). Sialylated PFPL O-glycans of GSP-6' and GSP-6" were synthesized using two different approaches (Fig. 2). In Approach I, nonsulfated GP-1 with an α-linked GalNAc at Thr57 was synthesized on an automated peptide synthesizer as described (19). GP-1 served as an initial acceptor for further glycosylation with purified or recombinant glycosyltransferases (Fig. 2A). The galactosylated core-2 branch of GP-4 was an acceptor site for the polylactosamine extension enzyme from human serum, (hs)β1,3-GlcNAcT. The alternating action of (hs)β1,3-GlcNAcT and β1,4GalT yielded nonsulfated glycopeptides with two (GP-4.2) or three (GP-4.4) polylactosamine repeats. GP-4.2 and GP-4.4 were further α2,3-sialylated and α1,3-fucosylated, and the resulting nonsulfated glycopeptides GP-6' and GP-6" were purified by reversed phase HPLC and characterized by electrospray MS. Tyrosine residues
of GP-6' and GP-6" were enzymatically sulfated using recombinant human TPST-1 and
[35S]PAPS. Trisulfated radiolabeled glycosulfopeptides GSP-6' and GSP-6" were isolated and
purified by HPLC (not shown).

In **Approach II** TyrSO₃⁻ residues were incorporated into the peptide during the solid-
phase peptide synthesis (19). GSP-1 served as an initial acceptor for glycosyltransferases (Fig.
2B). The core-1 Gal residue of GSP-3 was α2,3-sialylated to prevent modification of the Gal
residue by recombinant (Nm)β1,3-GlcNAcT from *Neisseria meningitidis* *lgtA*. N-
acetyllactosamine extensions were synthesized by the alternating action of recombinant
(Nm)β1,3-GlcNAcT and β1,4-GalT. The extended glycosulfopeptides (GSP-4S.2 and GSP-4S.4)
were then treated with neuraminidase to remove the protecting sialic acid from the core-1
branch. After HPLC purification, desialylated GSP-4.2 and GSP-4.4 were α2,3-sialylated and
α1,3-fucosylated on the core-2 branch. Electrospray MS was used to verify the masses of the
final products, GSP-6' and GSP-6". The completeness of each glycosyltransferase reaction in
both approaches was monitored by HPLC. To verify that the linkages of the newly synthesized
N-acetyllactosamine units were as expected (Galβ1,4GlcNAcβ1,3), the nonfucosylated
glycosulfopeptides GSP-5' and GSP-5" were digested with endo-β-galactosidase. In both cases
the HPLC retention times of the products were identical and as expected for the cleavage
product, GSP-3 (not shown). Radiolabeled [³H]GSP-6' and [³H]GSP-6" were synthesized using
nonlabeled GSP-5' and GSP-5" as acceptors, respectively and GDP-[³H]Fuc as a donor in the
α1,3-fucosyltransferase reaction.

**GSPs with sialylated PFPL O-glycan have impaired affinity for immobilized P-selectin**
— We initially tested binding of GSP-6' and GSP-6" to P-selectin by affinity chromatography of
radiolabeled GSPs on immobilized soluble P-selectin (sPS) at physiological salt concentration
(150 mM NaCl). GSP-6’ and GSP-6” did not detectably interact with immobilized sPS, whereas the control compound GSP-6 bound to immobilized sPS (Fig. 3). These results indicate that sLe\(^x\) presented on an extended fucosylated polylactosamine O-glycan, as in GSP-6’ and GSP-6”, decreases binding of glycosulfoproteptides to P-selectin.

The affinity of P-selectin to GSP-6’ and GSP-6” is similar to the affinity of P-selectin to GP-6 — Selectins bind to their ligands with higher affinity at subphysiological salt concentration than at physiological salt concentration (26). Therefore, the binding affinities of GSPs that show weak or no interaction to P-selectin at physiological salt concentration can be estimated relative to a "standard" glycopeptide at subphysiological salt concentration (19). To define whether there are weak interactions of GSP-6’ and GSP-6” with P-selectin, we developed a sensitive fluorescence-based solid phase assay. Equimolar amounts of biotinylated glyco(sulfo)peptides were first captured quantitatively on Streptavidin-coated 96-well plates. P-selectin-Ig chimera (P-sel-Ig) was incubated with the wells, and bound P-sel-Ig was detected with fluorescently labeled anti-human IgG. To confirm equal capture of each biotinylated GSP, we measured the binding of monoclonal antibody PL1, which recognizes a peptide epitope in PSGL-1 that is also present in the GSPs (25). The fluorescence assay was performed under reduced salt concentration (50 mM NaCl) to enhance the binding of relatively weak ligands. P-sel-Ig showed high affinity binding to GSP-6; binding increased with a linear correlation to increased concentrations of P-sel-Ig chimera (Fig. 4A). P-sel-Ig showed linear, but much weaker, binding to GSP-6’ and GSP-6”. The binding of P-sel-Ig to GSP-6’ and GSP-6” was similar to the binding of P-sel-Ig to the nonsulfated GP-6 (Fig. 4A). P-sel-Ig did not detectably bind to the non-sulfated GP-4, which also lacks fucose and sialic acid. The coating with each GSP was in a similar range,
as determined by binding of PL1 (Fig. 4B). The results indicate that P-selectin binds with significantly lower affinity to GSP-6' and GSP-6" than to GSP-6.

The binding of P-selectin to GSP-6' and GSP-6" is Ca$^{2+}$-dependent and partially dependent on $\alpha_{2,3}$-sialic acid, $\alpha_{1,3}$-fucose and the sulfated peptide backbone — The relative contributions of Ca$^{2+}$, sialic acid, and fucose for binding of GSP-6' and GSP-6" to P-selectin was next examined. A fixed concentration of P-sel-Ig (3 $\mu$g/ml) was incubated with a series of immobilized glyco(sulfo)peptides (Fig. 5A) Similar amounts of mAb PL1 bound to each peptide, demonstrating that the peptides were immobilized at equivalent densities (Fig. 5B). GP-6 was used as a reference peptide, because the dissociation constant for its binding to P-selectin ($K_d \sim 3.1$ $\mu$M, see below) was determined using equilibrium gel filtration under subphysiological salt concentration. P-sel-Ig bound equally well to GSP-6" and the non-sulfated GP-6, whereas P-sel-Ig bound $\sim$2 times better to GSP-6' (Fig. 5A). Desialylation of GSP-6' by neuraminidase treatment reduced its binding to P-sel-Ig by $\sim$50%, whereas desialylation of GSP-6" did not affect its binding (Fig. 5A). As a result P-sel-Ig bound equally to desialylated GSP-6' and GSP-6"; thus, the sialic acid of GSP-6', but not of GSP-6", contributes to its weak binding.

P-sel-Ig bound equally to the sialylated but non-fucosylated GSP-5' and GSP-5"; this binding was weaker than to desialylated GSP-6' and GSP-6". These results indicate that fucose contributes more than sialic acid to binding of GSP-6' and GSP-6" to P-selectin. The binding of P-sel-Ig to GSPs was only partially inhibited by EDTA (Fig. 5A). This observation suggests that Ca$^{2+}$-independent residual binding of P-sel-Ig to GSP-6' and GSP-6" and to their desialylated and defucosylated derivatives is through recognition of the sulfated peptide backbone rather than the polylactosamine O-glycan. The binding of P-sel-Ig to the nonsulfated GP-6 was completely
inhibited by EDTA, demonstrating that the binding of P-sel-Ig to the sLe\textsuperscript{x}-containing O-glycan is strictly Ca\textsuperscript{2+} dependent.

\textit{GSP-6' and GSP-6" have 50-80-fold impaired affinity for P-selectin in comparison to GSP-6.} Lowering the NaCl concentration from 150 mM to 50 mM increases the binding affinity of GSP-6 to sPS by 9-fold (19). Therefore, the binding affinities of GSPs that show weak or no interaction to immobilized P-selectin at physiological salt can be estimated relative to known "standard" glyco(sulfo)peptides at subphysiological salt in equilibrium gel filtration. We used nonsulfated GP-6 as a reference glycopeptide, because the fluorescence-based solid phase assay indicated that P-sel-Ig interacts with GSP-6', GSP-6" and GP-6 with weak, but similar affinity under reduced salt concentration (Figs. 4,5). We first determined the \( K_d \) for binding of GP-6 to sPS at 50 mM NaCl concentration in equilibrium gel filtration (Fig. 6A). The \( K_d \) was 3.1 \( \mu \)M, which is ~40-fold lower than that of GSP-6 for sPS (\( K_d \sim 76 \text{ nM} \)) at subphysiological salt concentration (19). The binding affinities of GSP-6' and GSP-6" for sPS were estimated relative to GP-6 using 500 pmol of sPS and subphysiological salt concentration in equilibrium gel filtration (Fig. 6B). GSP-6' and GSP-6" bound to P-selectin with ~71% and ~53%, respectively, of the levels of GP-6 (Fig. 6C, inset). The binding affinities of GSP-6', GSP-6" and GP-6 were ~1.7%, ~1.3% and ~2.5% relative to GSP-6, respectively (Fig. 6C). In this assay, the binding of GSP-6' and GSP-6" to P-selectin was independent of sialic acid, because sialidase treatment of GSP-6' and GSP-6" did not affect binding (Fig. 6C, inset). However, the binding of GSP-6' and GSP-6" to P-selectin was partially dependent on \( \alpha 1,3\)-fucosylation because nonfucosylated GSP-5' and GSP-5" showed reduced binding (Fig. 6C, inset). Pronase-digested GP-6 did not detectably bind to sPS even at subphysiological salt concentration, showing that the C2-O-sLe\textsuperscript{x} O-glycan without the peptide backbone has extremely low affinity for P-selectin.
DISCUSSION

Expression of sLe\(^x\) in a core-2-based O-glycan along with adjacent multiple TyrSO\(_3^-\) residues at the extreme N-terminus of PSGL-1 or within synthetic glycosulfopeptides are required for high affinity binding to P-selectin. The results of this study demonstrate that glycosulfopeptides containing three TyrSO\(_3^-\) residues and O-glycans with sLe\(^x\) in sialylated PFPL (GSP-6' and GSP-6") have 50-80-fold reduced affinity to P-selectin in comparison to GSP-6, which has a simple C2-O-sLe\(^x\) O-glycan. The relatively poor binding of the sialylated PFPL compounds was consistently observed in equilibrium binding assays, solid-phase fluorescence-based assays, and affinity chromatography on immobilized P-selectin. These results shed new light on the contributions of sLe\(^x\) and PFPL-containing O-glycans to high affinity interactions of PSGL-1 with P-selectin.

Earlier studies had indicated that expression of sLe\(^x\) by cells was required for their binding to P-selectin (27,28), but expression of sLe\(^x\) was not sufficient for high affinity interactions with P-selectin (28). Subsequent identification of a high-affinity mucin ligand for P-selectin on leukocytes, now termed PSGL-1 (7,29), raised the possibility that multiple clusters of sLe\(^x\) on O-glycans might enhance binding to P-selectin. Surprisingly, however, analysis of PSGL-1 from human HL-60 cells revealed that relatively few of the O-glycans contained fucose (~14%). A majority of fucose residues in PSGL-1 occur in novel sialylated PFPL (C2-O-Le\(^x\)-Le\(^x\)-sLe\(^x\)), whereas only ~2% of the glycans are short, sialylated C2-O-sLe\(^x\) structures (10). The extracellular domain of human PSGL-1 contains 70 Ser and Thr residues that are potential sites for O-glycosylation, but analysis of the sequence for the likelihood of O-glycosylation, using the NetOGlyc 2.0 Prediction Server (http://www.cbs.dtu.dk/services/NetOGlyc/), predicts that only 49 of these sites may be used. If this were true, each PSGL-1 polypeptide might contain
approximately 6 sialylated, PFPL O-glycans but only 1 sialylated, monofucosylated O-glycan. Because sialylated PFPL was more abundant, it was postulated that the sialylated PFPL sequences in PSGL-1 may be particularly important for binding to P-selectin (10).

Additional indirect evidence in support of the importance of polylactosamine sequences for binding of PSGL-1 to P-selectin came from studies of the human α1,3-fucosyltransferases expressed in leukocytes, FucT-VII and FucT-IV, which differed in their ability to fucosylate polylactosamine sequences on synthetic acceptors in vitro (14,15,30). FucT-IV acts preferentially on internal GlcNAc residues distal from the non-reducing terminus, whereas FucT-VII acts preferentially on the GlcNAc residue at the extreme non-reducing terminal sialylated N-acetylactosamine unit. These results suggest that biosynthesis of the sialylated PFPL sequence might occur through the cooperative action of these two enzymes. The possibility that both enzymes are required to produce an optimal ligand for P-selectin was strengthened in studies in mice containing null mutations in either FucT-VII (17) or both FucT-VII and FucT-IV (16,31). Deletion of FucT-VII alone dramatically reduced, but did not eliminate, binding of leukocytes to selectins, whereas deletion of both FucT-VII and FucT-IV totally eliminated binding. Such results were consistent with the proposal (10,14,16,17) that a polylactosamine sequence modified by both FucT-VII and FucT-IV might be required for high affinity binding of PSGL-1 to P-selectin. However, the studies reported here indicate that such sialylated PFPL are not high affinity ligands for P-selectin.

Several studies have shown that singly fucosylated core 2-based O-glycans lacking PFPL on a tyrosine sulfated peptide modeled after PSGL-1 bind with high affinity to P-selectin (11,19,32). The crystal structure of a recombinantly-derived glycosulfopeptide containing a disialylated, monofucosylated O-glycan and multiple TyrSO3⁻ residues bound to the C-type lectin
domain of human P-selectin revealed interactions with the single fucose and the sialic acid residue within the sLe\textsuperscript{x} moiety plus interactions with the adjacent TyrSO\textsubscript{3\textsuperscript{-}} residues and other flanking amino acids (32).

The GSP-6\textsuperscript{”} containing sialylated PFPL is probably too large to allow accommodation of both the fucose and sialic acid determinants and sulfated peptide determinants within the binding site of P-selectin, as depicted in Fig. 8. It is likely that the terminal sialic acid and fucose residues in the sLe\textsuperscript{x} at the non-reducing end of the sialylated PFPL O-glycans are extended far from the peptide and thus not in close proximity to the required TyrSO\textsubscript{3\textsuperscript{-}} residues and flanking amino acids. Our results indicate that the weak binding of GSP-6\textsuperscript{”} to P-selectin is not dependent on sialic acid, but is dependent on fucose and sulfated tyrosine residues. Thus, it is likely that the weak binding observed results from interactions shown in Fig. 8B, rather than Fig. 8C. The predicted model in Fig. 8B is also consistent with the slightly better binding observed for GSP-6\textsuperscript{’} compared to GSP-6\textsuperscript{”} to P-selectin (Fig. 7). This may occur because of the sialic acid moiety in GSP-6\textsuperscript{’} is closer to the peptide and fucose determinants than seen for the sialic acid in GSP-6\textsuperscript{”}.

As discussed above, it is possible that PSGL-1 from HL-60 cells contains on average a single C2-O-sLe\textsuperscript{x} O-glycan and ~6 PFPL O-glycans per polypeptide. The K\textsubscript{d} of native PSGL-1 from human neutrophils for human P-selectin is 320 nM, as determined by surface plasmon resonance (4), which is comparable to the K\textsubscript{d} of 350 nM observed for binding of a slightly longer synthetic glycosulfopeptide containing the simple C2-O-sLe\textsuperscript{x} O-glycan, as determined by equilibrium gel filtration (11). The similarity in binding affinities of the native ligand and the synthetic glycosulfopeptide suggest that PSGL-1 is preferentially modified with a short C2-O-sLe\textsuperscript{x} O-glycan at Thr57 in the extreme N-terminus of the molecule. The observation that such a short C2-O-sLe\textsuperscript{x} O-glycan is required for high affinity binding of GSPs to P-selectin and the fact
that native PSGL-1 may contain only a single residue of this glycan, further suggest that the Thr57 is a preferential site on PSGL-1 for expression of this short C2-O-sLe\(^\alpha\) O-glycan. Such a specific modification at Thr57 is consistent with the identification of a tryptic glycosulfopeptide from PSGL-1 on HL-60 cells (12). The nature and cause of this specific modification of Thr57 is unknown. It is possible that the \(\alpha\)1,3-fucosyltransferases (FucT-VII and FucT-IV) preferentially recognize core 2-based O-glycans at this site, the \(\alpha\)2-3 sialyltransferase preferentially sialylates core 2-based O-glycans at this site, and/or the \(\beta\)1,3-N-acetylglcosaminyltransferase responsible for polylactosamine extension is unable to efficiently elongate the core 2-based O-glycans at this site. Studies on the specificity of these enzymes with native PSGL-1 will be required to address such possibilities. The potential preferential and/or competitive action of glycosyltransferases on O-glycans at Thr57 could be important in regulating the formation of properly glycosylated forms of PSGL-1 in hematopoietic cells thereby regulating their interactions with P-selectin.

Since the O-glycans containing sialylated PFPL do not contribute to high affinity binding of PSGL-1 to P-selectin, what could be their functions? L-selectin, which binds to the same extreme N-terminal region of the molecule recognized by P-selectin (33-36), also binds to certain types of sialylated PFPL in the absence of their sulfation (37). While purely speculative, L-selectin might recognize sialylated PFPL with a higher affinity than P-selectin, thus contributing to the specificity of binding of L-selectin to PSGL-1. Of course, if PSGL-1 preferentially contains a short C2-O-sLe\(^\alpha\) O-glycan at Thr57 in the N-terminus, then this might not be the case. However, it is also possible that PSGL-1 may occur in various glycoforms, with some glycoforms of PSGL-1 containing sialylated PFPL at Thr57, thus promoting binding to L-selectin. To address such possibilities in the future, it will be necessary to evaluate the binding of L-selectin to all the modifications of the GSPs described to date.
PSGL-1 also binds to E-selectin (13,38,39) and contributes significantly to the initial tethering of flowing leukocytes to E-selectin (40), but the nature of the glycan ligands of PSGL-1 recognized by E-selectin are poorly understood. E-selectin appears to bind better to glycans containing PFPL than to short glycans (41-43). Thus, E-selectin might bind preferentially to the sialylated PFPL O-glycans of PSGL-1. The polylactosamine sequences on PSGL-1 may also serve as a precursor to other modifications. Sulfated polylactosamine chains on PSGL-1, recognized as the PEN5 epitope, have been reported on PSGL-1 on some natural killer cells and in the human and rat central nervous systems (44,45). It is also possible that the sialylated PFPL glycans on PSGL-1 could interact with as yet unidentified lectins. For example, the human granulocytic ehrlichiosis bacterium binds in a sialic acid- and fucose-dependent manner to PSGL-1 on human neutrophils (46), but the preferred glycans on PSGL-1 have not been defined. The availability of the GSPs containing sialylated PFPL should allow these possibilities to be addressed.

The molecular interaction of glycosulfopeptides with P-selectin has many novel aspects. The tripartite nature of the recognition involving carbohydrate, peptide determinants, and TyrSO₃⁻ promotes a high degree of specificity and high affinity. Sialic acid has an important role for binding of GSP-6 to P-selectin, since desialylation of GSP-6 reduces its binding to P-selectin by ~90% (19). However, the binding of GSP-6' and GSP-6" to P-selectin appears to be largely sialic acid independent. Desialylated GSP-6' binds to P-selectin with only ~ 20% lower affinity than GSP-6', and the binding of GSP-6" to P-selectin is sialic acid independent. This suggests that sLeˣ on an extended fucosylated polylactosamine backbone of GSP-6' and GSP-6" is displaced relative to the three TyrSO₃⁻ residues, preventing simultaneous binding of TyrSO₃⁻ residues and sLeˣ to the lectin domain of P-selectin. The internal Leˣ of GSP-6' and GSP-6" may
instead contribute to binding (Fig. 8). The observation that desialylated GSP-6 interacts with P-selectin with higher affinity than desialylated GSP-6' and GSP-6" is intriguing. It is possible that conformational differences between terminal Le^x in desialylated GSP-6 and internal Le^x in GSP-6' and GSP-6" account for this observation. Our earlier results have shown that α1,3-fucose contributes more than α2,3-sialic acid to binding of GSP-6 to P-selectin (19). Similarly, the present study indicates that fucose contributes more than sialic acid to binding of GSP-6' and GSP-6" to P-selectin (Fig. 6,7). Nonfucosylated GSP-5' and GSP-5" show weak residual binding to P-selectin both in the fluorescence-based solid phase assay (Fig. 5) and in equilibrium gel filtration (Fig. 6). The sulfated peptide backbone likely contributes partly to binding of GSP-5' and GSP-5" to P-selectin, because GSP-1 shows weak binding to P-selectin (Fig. 5). The present results indicate that binding of GSP-1 to P-selectin is largely Ca^{2+} independent (Fig. 5). The result is similar to other studies indicating that P-selectin binds to sulfatide, sulfoglucuronyl glycolipids and heparan sulfate in a Ca^{2+} independent manner (26,47,48). This is in agreement with the crystal structure of P-selectin complexed with a PSGL-1-derived glycosulfopeptide, which indicates that TyrSO_3^- residues bind in a Ca^{2+}-independent manner to a site different from the Ca^{2+}-containing carbohydrate-binding site in the lectin domain of P-selectin (32). Our results also indicate that binding of nonsulfated GP-6 to P-selectin is Ca^{2+} dependent (Fig. 5).

This study, which used some of the largest glycopeptides synthesized in vitro to date, explored two novel approaches for synthesis. In **Approach I** (Fig. 2A), the extension of the polylactosamine chain was performed using partially purified β1,3-GlcNAcT from human serum (49). Because serum β1,3-GlcNAcT does not extend the core-1 galactose (49), core-1 galactose was not protected before extension of the core-2 branch. However, **Approach II** (Fig. 2B) uses recombinant (Nm)β1,3-GlcNAcT from *Neisseria meningitidis lgtA* (22), which can extend
galactose from both core-1 and core-2 glycans (22). Therefore the core-1 galactose was
sialylated to prevent its extension before extension of the core-2 branch. The core-1 sialic acid
was removed after the synthesis of the polylactosamine chain. An earlier study suggested that
(Nm)β1,3-GlcNAcT from N. meningitidis lgtA cannot efficiently extend polylactosamine
structures on simple oligosaccharide acceptors (22). However, we found that (Nm)β1,3-
GlcNAcT acts efficiently on the core-2 branch of our glycopeptide acceptors. This suggests that
the peptide part of the acceptor may induce the optimal conformation of the carbohydrate
acceptor for the enzyme.

We developed a fluorescence-based solid phase assay to measure P-selectin binding to
biotinylated glyco(sulfo)peptides immobilized on Streptavidin-coated microtiter plates. The
sensitivity of the assay was improved by reducing the salt concentration to enhance binding. The
relative binding affinities determined by the solid-phase assay and the more quantitative
equilibrium gel filtration under low salt conditions are very similar (Fig. 6C). The availability of
reliable solid-phase fluorescence-based assays should provide new approaches for measuring
lectin-glycoconjugate interactions, including the binding of human and murine selectins to
various glycoconjugates.
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ABBREVIATIONS

1The abbreviations used are: PSGL-1, P-selectin glycoprotein ligand-1; sPS, soluble P-selectin; P-sel-Ig, P-selectin IgG chimera; sLe^x, sialyl Lewis x; Le^x, Lewis x; GP, glycopeptide; GSP, glycosulfopeptide; β1,3-GalT, core-1 β1,3-galactosyltransferase; β1,6-GlcNAcT, core-2 β1,6 N-acetylglcosaminyltransferase; β1,3-GlcNAcT, β1,3-N-acetylglcosaminyl-transferase; (Nm), Neisseria meningitidis lgtA; (hs), human serum; β1,4-GalT; β1,4-galactosyltransferase; α1,3-FucT, α1,3-fucosyltransferase; α2,3-(N)-sialylT, α2,3-(N)-sialyltransferase; α2,3-(O)-sialylT, α2,3-(O)-sialyltransferase; PFPL, polyfucosylated polylactosamine; C2-O-sLe^x, core-2 based O-glycan with sLe^x; C2-O-Le^x-sLe^x, core-2 based O-glycan with Le^x-sLe^x; C2-O-Le^x-Le^x-sLe^x, core-2 based O-glycan with Le^x-Le^x-sLe^x; TyrSO_3^-, tyrosine sulfate; HPLC, high-performance liquid chromatography; K_d, dissociation constant; MOPS, 4-[N-morpholine]propanesulfonic acid; PAPS, adenosine 3’-phosphate 5’-phosphosulfate; TPST-1, tyrosyl-protein sulfotransferase-1; RFU, relative fluorescence units; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry.
REFERENCES:

FIGURE LEGENDS

Fig. 1. The structures of glyco(sulfo)peptides synthesized in the present study.

Fig. 2. Enzymatic synthesis of glycosulfoproteptides containing sialylated PFPL O-glycans using two different approaches. A, (Approach I) the enzymatic synthesis starts from nonsulfated GP-1. B, (Approach II) sulfated GSP-1 serves as an initial acceptor for glycosyltransferases. The end products GSP-6' and GSP-6" are the same in both approaches. The circled numbers refer to the specific enzymes used in each step, 1, core-1 β1,3-galactosyltransferase; 2, core-2 β1,6-N-acetylglucosaminyltransferase; 3, β1,4-galactosyltransferase; 4, β1,3-N-acetylglucosaminyltransferase (from human serum in A and from Neisseria meningitidis lgtA in B); 5, α2,3-(N)-sialyltransferase; 6, α1,3-fucosyltransferase; 7, tyrosylprotein sulfotransferase-1; 8, α2,3-(O)-sialyltransferase; 9, neuraminidase.

Fig. 3. Affinity chromatography of glyco(sulfo)peptides on immobilized sPS at physiological salt concentration. The indicated 3H- or 35S-labeled glyco(sulfo)peptides (~2000 cpm ~ 0.3-1 pmol) were chromatographed on a sPS column (2 mg/ml) in 20 mM MOPS, pH 7.5 containing 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2 and 0.02% NaN3.

Fig. 4. Binding of P-selectin IgG chimera to immobilized glyco(sulfo)peptides in a fluorescence-based solid phase assay at subphysiological salt concentration. Biotinylated glyco(sulfo)peptides (1 pmol) were immobilized on Streptavidin-coated microtiter wells. A. Various concentrations of P-selectin IgG chimera (P-sel-Ig) were incubated with the
immobilized GSPs in 20 mM MOPS, pH 7.5 containing 50 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% BSA, 0.05% Tween-20 and 0.02% NaN₃. Fluorescently labeled anti-human IgG was used to detect the bound P-sel-Ig. B. Monoclonal antibody PL1 was used to confirm that each glyco(sulfo)peptide was immobilized on microtiter wells. A saturating concentration of PL1 was incubated with the immobilized GSPs, and fluorescently labeled anti-mouse IgG was used to detect the bound PL1. All assays were performed in triplicate, and the results represent the mean ± SD of three determinations.

Fig. 5. Comparison of binding of P-selectin IgG chimera to immobilized glyco(sulfo)peptides in a fluorescence-based solid phase assay at subphysiological salt concentration. Biotinylated glyco(sulfo)peptides (1 pmol) were immobilized on Streptavidin coated microtiter wells. A. P-selectin IgG chimera (3 µg/ml) was incubated with the immobilized GSPs in 20 mM MOPS, pH 7.5 containing 50 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% BSA, 0.05% Tween-20 and 0.02% NaN₃ (dark gray bars) or in 20 mM MOPS, pH 7.5 containing 50 mM NaCl, 5 mM EDTA, 1% BSA, 0.05% Tween-20 and 0.02% NaN₃ (light gray bars). Fluorescently labeled anti-human IgG was used for detection. B, Monoclonal antibody PL1 was used to confirm that each GSP was immobilized on microtiter wells. PL1 was incubated with the immobilized GSPs in buffer containing Ca²⁺ (dark gray bars) or EDTA (light gray bars) (see panel A), and fluorescently labeled anti-mouse IgG was used to detect the bound PL1. All assays were performed in triplicate and the results represent the mean ± SD of three determinations.

Fig. 6. Equilibrium binding affinities of glyco(sulfo)peptides for sPS at subphysiological salt concentration. A, equilibrium binding of GP-6 for sPS. Equilibrium gel filtration experiments
were carried out by loading 100, 200, 400, 800, 1600 and 2200 pmol of sPS on a gel filtration column equilibrated with $[^3]H$GP-6 in buffer (6900 cpm/ml in 20 mM MOPS, pH 7.5, containing 50 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$ and 0.02% NaN$_3$). The bound GP-6 and free sPS concentrations were calculated from the equilibrium gel filtration data (not shown) by dividing the molar amounts of GP-6 and sPS by the peak volume of sPS-GP-6 complex. The dissociation constant (3.1 μM) was calculated using a rectangular hyperbola equation to derive the nonlinear curve fitting. B, equilibrium binding affinities of glyco(sulfo)peptides for sPS at subphysiological salt concentration. Experiments were carried out by loading 500 pmol of sPS on a gel filtration column equilibrated with 1.3 pmol/ml of the indicated $^3$H- or $^{35}$S-labeled glyco(sulfo)peptides in low salt buffer (see panel A). Representative results are shown for each peptide. C, relative equilibrium binding affinities of glyco(sulfo)peptides for sPS at subphysiological salt concentration. Experiments were carried out as described in panel B except for GSP-6, where the dissociation constant was determined earlier under both subphysiological (76 nM) and physiological (650 nM) salt concentrations (19). The amounts of bound peptides were calculated relative to GSP-6 or GP-6 (inset). The results shown represent the mean ± SD of four determinations for $^3$H- or $^{35}$S-labeled GSP-6' and GSP-6", three determinations for $[^3]H$GP-6, and two determinations for desialylated $[^3]H$GSP-6', desialylated $[^3]H$GSP-6", $[^3]H$GSP-5', $[^3]H$GSP-5", and pronase-digested $[^3]H$GP-6.

Fig. 7. **Relative binding affinities and estimated dissociation constants of glycosulfopeptides for P-selectin.** Relative binding affinities and dissociation constants for each glyco(sulfo)peptides were derived from equilibrium gel filtration data under subphysiological salt concentrations. Physiological $K_d$s were estimated from the results obtained at
subphysiological salt concentration by multiplying subphysiological $K_d$ by 8.5, which is the difference between the subphysiological and physiological $K_d$ for GSP-6. The hyphens indicate that binding was not detectable (physiological $K_d > 400 \mu M$; subphysiological $K_d > 50 \mu M$).

Fig. 8. A model depicting the possible interaction between the C-type lectin domain of P-selectin with glycosulfopeptides in this study. The predicted and defined interactions between GSP-6 and P-selectin are indicated in (A), based on previous studies (11,19,32). The predicted reduced interaction with the glycosulfopeptide GSP-6” containing sLe$^\alpha$ in sialylated PFPL are shown in B and C. In one hypothetical model of binding, the peptide moiety and fucose, but not sialic acid, residues are involved in the interaction of GSP-6” with P-selectin (B), whereas in the other model, fucose and sialic acid residues, but not the peptide moiety, are involved in the interaction (C).
Leppanen et al., Fig. 4

A

![Graph A](image)

B

![Bar Chart B](image)
A

GP-6 bound [nM]

GP-6

$K_d = 3.1 \pm 0.9 \, \mu M$

sPS free [µM]

B

Peptide (pmol)

GSP-6'  
GSP-6"  
GP-6  
Pronase dig. GP-6

C

Binding relative to GSP-6 (%)
<table>
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<tr>
<th>Glyco(sulfo)peptide</th>
<th>Estimated $K_d$ (µM)</th>
<th>Relative binding (%)</th>
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**KEY**
- **Peptide**
- **S** Sialic acid
- **Core-2 O-glycan**
- **LeX** Lewis x trisaccharide
- **Tyr-SO_3**
- **LN** N-acetyllactosamine disaccharide
Glycosulfopeptides with O-glycans containing sialylated and polyfucosylated polylactosamine bind with low affinity to P-selectin
Anne Leppanen, Leena Penttila, Ossi Renkonen, Rodger P. McEver and Richard D. Cummings

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