A Novel Glycosulfopeptide Binds to P-selectin and Inhibits Leukocyte Adhesion to P-selectin*

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P-selectin glycoprotein ligand-1 (PSGL-1) is a dimeric membrane mucin on leukocytes that binds selectins. The molecular features of PSGL-1 that determine this high affinity binding are unclear. Here we demonstrate the in vitro synthesis of a novel glycosulfopeptide (GSP-6) modeled after the extreme N terminus of PSGL-1, which has been predicted to be important for P-selectin binding. GSP-6 contains three tyrosine sulfate (TyrSO₃) residues and a monosialylated, core 2-based O-glycan with a sialyl Lewis x (C2-O-sLex) motif at a specific Thr residue. GSP-6 binds tightly to immobilized P-selectin, whereas glycopeptides lacking either TyrSO₃ or C2-O-sLex do not detectably bind. Remarkably, an isomeric glycosulfopeptide to GSP-6, termed GSP-6′, which contains sLeᵪ on an extended core 1-based O-glycan, does not bind immobilized P-selectin. Equilibrium gel filtration analysis revealed that GSP-6 binds to soluble P-selectin with a Kᵦ of ~350 nM. GSP-6 (<5 μM) substantially inhibits neutrophil adhesion to P-selectin in vitro, whereas free sLeᵪ (5 mM) only slightly inhibits adhesion. In contrast to the inherent heterogeneity of post-translational modifications of recombinant proteins, glycosulfopeptides permit the placement of sulfate groups and glycans of precise structure at defined positions on a polypeptide. This approach should expedite the probing of structure-function relationships in sulfated and glycossylated proteins, and may facilitate development of novel drugs to treat inflammatory diseases involving P-selectin-mediated leukocyte adhesion.

The interactions between selectins and their carbohydrate-based ligands initiate adhesion of leukocytes to the vascular wall during inflammation. Although L-, E-, and P-selectin can bind a simple glycan containing sialyl Lewis x (sLeᵪ)¹

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The abbreviations used are: sLeᵪ, sialyl Lewis x; PSGL-1, P-selectin glycoprotein ligand-1; sPS, soluble P-selectin; GP, glycosulfopeptide; Gly, GlcNAcT, core 1 β1,3-galactosyltransferase; β1,6-GlcNAcT, core 2 β1,6-N-acetylglucosaminyltransferase; β1,3-GlcNAcT, β1,3-N-acetylglucosaminyltransferase; TPST-1, tyrosyl-protein sulfotransferase-1; β1,4-GaIT, β1,4-galactosyltransferase; FuCT, α1,3-fucosyltransferase; α2,3-(N)-sialylT, α2,3-(N)-sialyltransferase; PAGE, polyacrylamide gel electrophoresis; C2-O-sLeᵪ, core 2-based O-glycan with sLeᵪ; C1-O-sLeᵪ, extended core 1-based O-glycan with sLeᵪ; TyrSO₃, tyrosine sulfate; HPLC, high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 4-[N-morpholino]propanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; mAb, monoclonal antibody; APP, amyloid precursor protein; Fnoc, N-(9-fluorenylmethoxycarbonyl); CHO, Chinese hamster ovary; HIV-1, human immunodeficiency virus-1.
Specific Glycosulfopeptide Binding to P-selectin

The interaction of P-selectin with simple sLe\textsubscript{x}-containing glycans is relatively weak (26), and concentrations of sLe\textsubscript{x} in the millimolar range are required to inhibit adhesion of cells to P-selectin (27). In contrast, surface plasmon resonance measurements indicate that monomeric P-selectin binds to neutrophil-derived PSGL-1 with relatively high affinity ($K_d \sim 300$ nM) and rapid on/off kinetics (28). This affinity is particularly high relative to typical carbohydrate-binding proteins, where the $K_d$ for monovalent carbohydrate ligands is usually in the range of 0.1–1 mM (29). Indeed, s-selectin binds to GlyCAM-1, a heavily sialylated and sulfated mucin, with a $K_d$ of $\sim 100$ $\mu$M (30).

We sought to directly address the functional significance of the extreme N-terminal domain of PSGL-1 and its putative post-translational modifications in binding to P-selectin. To this end, we used purified and recombinant enzymes to modify synthetic peptides based on the N-terminal sequence of human PSGL-1. The resultant glycosulfopeptides contain tyrosine sulfate residues and O-glycans with defined carbohydrate residues that allow a direct exploration of their importance in binding to P-selectin. This new strategy of glycosulfopeptide synthesis was chosen because it has several advantages over the expression of recombinant glycoproteins. Recombinant glycoproteins expressing the post-translational modifications described here are difficult, if not impossible, to produce, because of the uncertain requirements of glycosyltransferases for site-specific initiation and modification of O-glycan structure. Here we use synthetic glycosulfopeptides to show that both tyrosine sulfate residues and a specific core 2-based O-glycan with sLe\textsubscript{x} (C2-O-sLe\textsubscript{x}) on a nearby Thr residue are required for high affinity binding to P-selectin. The binding affinity of one of these glycosulfopeptides is similar to that of native neutrophil-derived PSGL-1. Moreover, micromolar concentrations of this glycosulfopeptide are sufficient to block neutrophil attachment to immobilized P-selectin. These results help define the molecular nature of the interaction between P-selectin and PSGL-1 and pave the way for novel glycosulfopeptides that may be useful in treating inflammatory diseases.

**EXPERIMENTAL PROCEDURES**

**Decacylation of GP-1**—Crude glycopeptide 1 (GP-1) was synthesized at the Protein Resource Facility of Oklahoma State University. Tri-O-acetylated GalNAc was incorporated into the peptide during the solid phase synthesis using tri-O-acetyl-GalNAc-Fmoc Thr derivative (Oxford GlycoSciences, Oxford, United Kingdom). The crude GP-1 (2 mg) was de-O-acetylated with 6 mM methanolic sodium methoxide as described (31). The deacetylated peptide was purified by reversed phase HPLC. The retention time of deacylated GP-1 (34.6 min) was clearly different from the tri-O-acetylated GP-1 (45.3 min) (Fig. 3). The yield of the pure GP-1 was 1.1–1.5 mg. In matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra, the observed $m/z$ for the [M + H$^+$] molecular ion was 2952.9 (calculated $m/z$ 2953.2) (Fig. 4). In addition the [M + H$^+$] molecular ion of oxidized GP-1 ($m/z$ 2969.5) was present where the methionine residue had been oxidized.

**Enzymatic Synthesis of GSP-6, GSP-5, and GSP-2**—Core 1 $\beta$1,3-GalT has not been purified, and its $\alpha$1,3-fucosyltransferase FucT-VII bind weakly to P-selectin (17, 18). Finally, P-selectin binds to chimeric glycoproteins containing only a small N-terminal domain of PSGL-1 that lacks N-glycans (10, 11, 19).

The yield of the core 1 $\beta$1,3-GalT was 32.9 min (Fig. 3). The degree of galactosylation was 100–200-nmol aliquots by using 3–4 times molar excess of UDP-Gal (Sigma) and 13 nmol/h of purified core 1 $\beta$1,3-GalT—Until recently the core 1 $\beta$1,3-GalT has not been purified, and its cDNA had not been cloned. In the course of these studies, we developed a strategy to purify the core 1 $\beta$1,3-GalT from rat liver. The enzyme, purified over 70,000-fold to near homogeneity, was highly efficient in catalyzing the formation of GP-2 (Fig. 2). The purification of this novel enzyme and cloning of its cDNA has been accomplished and will be described elsewhere. GP-1 was galactosylated overnight at 37°C in 100–200-nmol aliquots by using 3–4 times molar excess of UDP-Gal (Sigma) and 13 nmol/h of purified core 1 $\beta$1,3-GalT in a total volume of 100 $\mu$l of 50 mM MES, pH 6.5, 2 mM ATP, 15 mM MnCl\textsubscript{2}, 0.2% Triton X-100. After removing proteins and Triton X-100 by chloroform-methanol (2:1) extraction (deproteinization), the reaction mixture was analyzed by HPLC. The retention time of the galactosylated product, GP-2, was 32.9 min (Fig. 3), and the degree of galactosylation was $\sim$95%.

![Schematic illustration of the dimeric structure of human PSGL-1](image-url)
MALDI-TOF analysis of GP-2 revealed \( m/z \) 3115.4 for the \([M - H]^-\) molecular ion (calculated \( m/z \) 3115.3) (Fig. 4).

Core 2 \( \beta1,6\)-GlcNAcT—GP-2 (0.4–0.6 mM) was incubated at 37 °C with 1–2 mM UDP-GlcNAc (Sigma) and affinity purified recombinant core 2 \( \beta1,6\)-GlcNAcT (100 nmol/h) in a total volume of 50 μl of 50 mM sodium cacodylate, pH 7.0. After 24 h of incubation, a small aliquot from the reaction mixture was analyzed by HPLC. GP-2 was converted quantitatively into a faster moving product, GP-3 (retention time 31.3 min) (Fig. 3). MALDI-TOF mass spectrum of GP-3 showed \( m/z \) 3318.2 for the \([M - H]^-\) molecular ion (calculated \( m/z \) 3318.5) (Fig. 4). The reaction mixture was taken directly to a \( \beta1,4\)-GalT reaction. Alternatively, UDP-[\(^3\)H]GlcNAc (American Radiolabeled Chemicals Inc., St. Louis, MO) (12,000 cpm/nmol) was used as a donor in the core 2 \( \beta1,6\)-GlcNAcT reaction to get \([\(^3\)H]GP-3.

\( \beta1,4\)-GalT—Unlabeled GP-3 (0.4 mM) (core 2 \( \beta1,6\)-GlcNAcT reaction mixture) was galactosylated using 125 milliunits of bovine milk \( \beta1,4\)-GalT (Sigma) and UDP-Gal (1.5 mM) in a total volume of 40 mM sodium cacodylate, pH 7.0, 20 mM MnCl₂, and 0.02% NaN₃. After 20 h of incubation at 37 °C, a sample from the reaction mixture was analyzed by HPLC, which showed that all GP-3 had been converted into a faster moving product, GP-4 (retention time 30.4 min) (Fig. 3). In MALDI-TOF analysis, the observed \( m/z \) for the \([M - H]^-\) molecular ion of GP-4 was 3480.4 (calculated \( m/z \) 3480.7) (Fig. 4). Glycopeptide samples were deproteinated and desalted in a Sephadex G-50 column (10 ml, 0.7 × 25 cm) using distilled water or 25 mM NH₄HCO₃ as an eluant. 0.5-ml fractions were collected, and the glycopeptides were detected by measuring either UV absorbance at 215 nm or radioactivity of the fractions. After desalting and deproteinization, the sample was taken directly to an \( \alpha2,3\)-(N)-sialylT (Calbiochem, La Jolla, CA) and 3 mM CMP-NeuAc (Sigma) in a total volume of 50 μl of 50 mM MOPS, pH 7.4, 0.1% bovine serum albumin, and 0.02% NaN₃. After 14 h of incubation at 37 °C, a 1-μg sample was analyzed by HPLC, which showed that GP-4 had been converted completely into a faster moving product, GP-5 (retention time 29.1 min) (Fig. 3). In MALDI-TOF analysis, the observed \( m/z \) for the \([M - H]^-\) molecular ion of GP-5 was 3770.6 (calculated \( m/z \) 3771.9) (Fig. 4). The reaction mixture was used directly for the \( \alpha1,3\)-FucT reaction. Radiolabeled \([\(^3\)H]GP-4 (0.1 mM) was also sialylated using the donor CMP-[\(^3\)H]NeuAc (0.2 mM, 31,500 cpm/nmol) (NEN Life Science Products).

\( \alpha1,3\)-FucT—GP-4 (0.4 mM) was fucosylated for 16 h at 37 °C with 2 milliunits of \( \alpha1,3\)-FucT-VI (Calbiochem, La Jolla, CA) and GDP-Fuc (0.8 mM) (Calbiochem) in a total volume of 120 μl of 50 mM MOPS, pH 7.4, 20 mM MnCl₂, and 0.02% NaN₃. Deproteinated and desalted sample was analyzed by HPLC, which showed that GP-5 was converted completely into the product GP-6 (retention time 29.1 min) (Fig. 3). In MALDI-TOF analysis, the observed \( m/z \) for the \([M - H]^-\) molecular ion of GP-6 was 3917.5 (calculated \( m/z \) 3918.1) (Fig. 4). Starting with 185 μg of GP-2, the overall recovery of GP-6 was 86 μg, as determined by UV absorbance at 275 nm during the HPLC runs. Radiolabeled \([\(^14\)C]GP-4 was fucosylated using GDP-[\(^14\)C]Fuc (83,000 cpm/nmol) (Amersham Pharmacia Biotech) as the donor.

**Fig. 2.** Synthesis of glycosulfopptide-6 (GSP-6). Each step in the synthesis is illustrated, starting with a glycopeptide (GP-1) containing GalNAc₁₋₋Thr at position 57.
Electrospray mass spectrum analysis showed the molecular mass of GSP-6 as 4158.0 (calculated 4158.2), confirming that three sulfate groups were present (Fig. 4). Alternatively, a radiolabeled form of GSP-6 was generated by incubating GP-6 (0.01 mM) for 14–17 h with 0.6 mM [35S]PAPS (Sigma) and 4.8 nmol/h of affinity-purified recombinant TPST-1 in a total reaction volume of 400 μl. The conversion of GP-6 to 35SO3-GSP-6 had a retention time of 18.7 min in HPLC. Partially sulfated slower moving products were not present.

**Reversed Phase High Performance Liquid Chromatography—**Glycopeptide samples were filtered on a Spin-X membrane (Corning Costar, Cambridge, MA) and were subsequently analyzed in a reversed phase C-18 HPLC column (Vydac, Hesperia, CA) on a Beckman System Gold instrument. The following solvent system was used at a flow rate of 1 ml/min: 10 cm, 2 ml) equilibrated with 4 ml of 20 mM MOPS, pH 7.5, containing 5 mM ATP, 15 mM MnCl2, and 0.5% bovine serum albumin. After deproteination and desalting, the reaction mixture was analyzed by HPLC. The retention time of the product was 21.4 min (Fig. 3), and the conversion of GP-2 to GSP-2 was 98%. Electrospray mass spectra of GSP-2 showed the molecular mass as 3356.0 (calculated 3356.5), which confirmed that three sulfate groups were present (Fig. 4). Alternatively, GP-2 (0.04 mM) was sulfated for 18 h at 37 °C using [35S]PAPS (0.2 mM, 30300 cpm/nmol) (Sigma) and TPST-1 (0.7 nmol/h) in a total volume of 56 μl. The conversion of GP-2 to 35SO3-GSP-2 was >98%. GP-5 was sulfated in a similar fashion as GP-6 using [35S]PAPS (30300 cpm/nmol) as a donor. The conversion of GP-5 to 35SO3-GSP-5 was >99%. The retention time of 35SO3-GSP-5 was 17.5 min in HPLC (data not shown).

**Enzymatic Synthesis of GSP-6—β1,3-GlCNAcT—**Extension of GP-2 was carried out using purified recombinant β1,3-GlCNAcT from Neisseria meningitidis IgA (34). Acceptor GP-2 (0.1 mM) was incubated for 20 h at 37 °C in the presence of 1 mM UDP-GlcNAc and 8 nmol/h of β1,3-GlCNAcT (activity assayed using lactose as an acceptor) in a total volume of 100 μl in 100 mM sodium cacodylate, pH 7.5, containing 5 mM ATP, 15 mM MnCl2, and 0.5% bovine serum albumin. After deproteination and desalting, the reaction mixture was analyzed by HPLC. The retention time of the product GP-3’ was 31.9 min (data not shown). The conversion of GP-2 to GP-3’ was 62%. In MALDI-TOF analysis, the observed m/z for the [M – H]+ molecular ion of GP-3’ was 3317.8 (calculated m/z 3318.5) (data not shown). β1,4-GaIT, α2,3-sialylT, and α1,3-FucT-VI reactions were carried out essentially as described above. Each glycosyltransferase reaction was followed by HPLC. GP-6’ had a retention time of 30.3 min in HPLC (data not shown). In MALDI-TOF analysis, the observed m/z for the [M – H]+ molecular ion of GP-6’ was 3917.6 (calculated m/z 3918.1) (data not shown). The TPST-1 reaction using GP-6’ as an acceptor and [35S]PAPS (400,000 cpm/nmol) as a donor was performed as described for GP-6. The retention time of 35SO3-GSP-6’ was 18.7 min in HPLC. Partially sulfated slower moving products were not present.
a flow rate of 70 μl/min. Radioactivity in the fractions was determined by liquid scintillation counting. Control experiments to test inhibitors of binding between sPS and GSP-6 were performed using 400 pmol of sPS and 5,000 cpm/ml of 35SO3-GSP-6. The EDTA concentration was 1 mM in 20 mM MOPS, pH 7.5, 150 mM NaCl, 0.02% NaN3. Anti-P-selectin monoclonal antibodies (G1 and S12) (800 pmol each) were preincubated for 30 min with sPS in buffer A before 35SO3-GSP-6 was added. Elution was performed by using buffer A with 35SO3-GSP-6.

P-selectin Affinity Chromatography—Soluble P-selectin was coupled to Ultrapak2TM biosupport medium (Pierce) according to the manufacturer’s instructions. P-selectin columns (0.8 ml, 0.6 × 2.7 cm) of different densities (0, 1.0, 1.3, 1.6, and 2.0 mg/ml) were equilibrated with 25 mM of buffer A. Radiolabeled glyco(sulfo)peptides (800–1,000 cpm, 1–10 pmol) were dissolved in 200 μl of buffer A and applied to the sPS-columns. Bound material was eluted with buffer B (20 mM MOPS, pH 7.5, containing 10 mM EDTA, 150 mM NaCl, 0.02% NaN3). Fraction size was 0.5 ml, and the flow rate was 200–250 μl/min. All fractions were counted for radioactivity.

Mass Spectrometric Analysis—MALDI-TOF mass spectrometry was performed in the linear negative ion delayed extraction mode with a BiflexTM time-of-flight instrument (Bruker-Franzen Analytik, Germany) equipped with a nitrogen laser operating at 337 nm. HPLC-purified glycopeptide samples, except GSP-2 and GSP-6, were dissolved in 30% aqueous acetonitrile, and a 0.5-mM solution (about 2.5 pmol) was mixed with 0.5 μl of 2,4,6-trihydroxyacetophenone matrix (3 mg/ml in acetonitrile, 20 mM ammonium citrate, pH 5.5) to prepare the sample for MALDI-TOF analysis. The reaction mixture was desalted and deproteinized before analysis by HPLC, followed by liquid scintillation counting. Core 2 b1,6-GlcNAcT with the 12-amino acid HPC4 epitope (33) and used to transform E. coli strain DH5α. The construct was released from pCR-TOPO 2.1 vector by digestion with BamHI and EcoRV and purified by agarose gel electrophoresis. The construct (1.2 kilobase pairs) was cloned into pCR-TOPO 2.1 vector (Invitrogen, Carlsbad, CA) and used to transform E. coli strain JM109 for plasmid preparation. The construct was released from pCR-TOPO 2.1 vector by digestion with BamHI and EcoRV and purified by agarose gel electrophoresis. The construct (1.2 kilobase pairs) was ligated into a BamHI/EcoRV site of modified pcDNA 3.1(+) vector (pcDNA 3.1(+)IT-H), which contains an Nterminal transfer signal sequence and HPC4 epitope (CCS-L) and used to transform E. coli. Stable clones of cells expressing core 2 b1,6-GlcNAcT activity in the media (50 nmol/h/ml) were selected and grown to 100% confluence. The medium was changed to Dulbecco’s modified Eagle’s medium (Cellgro, Herndon, Virginia) containing 10% fetal calf serum and G418 (600 mg/ml). Stable clones of cells expressing core 2 b1,6-GlcNAcT activity in the media (50 nmol/h/ml) were selected and grown to 100% confluence. The medium was changed to Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum and incubated for 2–3 days. The medium was collected and adjusted to 1 mM CaCl2 and 5 mM benzamidine. Soluble core 2 b1,6-GlcNAcT containing an HPC4 epitope tag was purified from the conditioned medium (60 ml) using a HPC4-mAb affinity column (5 ml) column of 5 mg/ml affinity matrix.
HPC-mAb coupled to Ultralink™ biosupport medium) at 4 °C as described (41). The purified enzyme was stabilized by adding 0.1% bovine serum albumin, and the enzyme was concentrated using Centricon-30 ultrafiltration tubes (Amicon, Beverly, MA). The purified enzyme was used directly or aliquoted and stored at −20 °C. The activity (8.2 μmol/h/ml) was stable at −20 °C for at least 2 months. Core 2 β1,6-GlcNAc and Galβ1–3GlcNAc–p-nitrophenyl (Toronto Research Chemicals Inc., Canada) and 1 mM UDP-[3H]GlcNAc (specific activity 1000 cpm/μmol). The assays were carried out at 37 °C with 2.5–10 μl of the purified enzyme for 30 min or 25 μl of cell culture medium for 2–3 h in a total volume of 50 μl of 50 mM sodium cacodylate, pH 7.0. The radiolabeled reaction product was separated from the radiolabeled donor using Sep Pak cartridges (Waters, Milford, MA).

**Neutrophil Isolation and Labeling—** Human neutrophils were isolated from healthy volunteers as described (42) and labeled with Calcein-AM (Molecular Probes, Inc., Eugene, OR) according to the manufacturer’s instructions.

**Neutrophil Adhesion Assay—** The adhesion assay was performed essentially as described (37), with the following modifications. Calcein-labeled neutrophils were used. sPS was coated directly on wells of Immulon 1 microtiter plates by incubating the wells with 2 μg/ml sPS in 0.1 x sodium carbonate buffer at 4 °C overnight (100 μl/well). For GSP-6 inhibition, the wells were preincubated with 50 μl of different dilutions of GSP-6 in Hank’s balanced salt solution containing 0.1% human serum albumin at room temperature for 15 min. In control experiments, wells were preincubated with mAbs against P-selectin. In other controls, mAbs against PSGL-1 or fluid-phase sPS were preincubated with 25 μl of the cell suspension at room temperature for 15 min. The neutrophils (25 μl) were then added to the sPS-coated wells. The number of adherent cells was quantified using an Fmax fluorescence plate reader (Molecular Devices).

**RESULTS**

**Synthesis of Glyco(sulfo)peptides—** The possibility that the N-terminal region of PSGL-1 is independently capable of high affinity interactions with P-selectin was explored by synthesizing the target glycosulfopeptide designated GSP-6. The synthetic route to GSP-6 is shown schematically in Fig. 2. This glycosulfopeptide was targeted to test the hypothesis that a single O-glycan in conjunction with TyrSO3 is required for high affinity binding to P-selectin. The synthesis of this complex glycosulfopeptide has not been described previously, partly because current chemical methods for synthesis are exceedingly complicated. In addition, the sulfotransferase and glycosyltransferases capable of modifying a peptide to generate GSP-6 have only recently become available.

A key enzyme catalyzing the first step in the biosynthesis of O-glycans is a polypeptide:N-acetylgalactosaminyltransferase (α-GalNAcT) that adds GalNAc from UDP-GalNAc to Ser and Thr residues. A large family of these enzymes has recently been discovered (43–45). Some demonstrate recognition of Ser/Thr residues within specific polypeptide domains, and only one (α-GalNAcT-4) appears to recognize the Thr residue within the N-terminal peptide domain of PSGL-1 (44). Rather than using a polypeptide:N-acetylgalactosaminyltransferase, we used an isolated enzyme (α-GalNAcT) that adds GalNAc to a specific Ser or Thr on a peptide, we incorporated an acetylated Fmoc derivative of GalNAc-Thr at a specific site during peptide synthesis. We recently observed the conversion of Thr to Ala, but not of Thr to Ala, blocks binding of full-length recombinant PSGL-1 to P-selectin (13). Thus, we initiated O-glycan synthesis on the Thr residue corresponding to Thr57 rather than Thr44 (Fig. 2). This deacetylated glycopeptide, designated GP-1, served as the starting material for the synthesis.

A key precursor enzyme for formation of core 2 O-glycans in animal cells is the core 1 β1,3-galactosyltransferase (core 1 β1,3-GalT) that creates the core 1 structure Galβ1–3GalNAc-R. We used purified core 1 β1,3-GalT from rat liver to generate GP-2. Core 1 structures, as on GP-2, serve as acceptors for the core 2 β1–6 N-acetylgalactosaminyltransferase (core 2 β1,6-GlcNAc) to allow synthesis of the branched core 2 structure Galβ1–3GlcNAcβ1–6GalNAc-R (39). We used an epitope-tagged, soluble, recombinant form of core 2 β1,6-GlcNAcT to generate GP-3. The other glycosyltransferases important for the sequential synthesis of GP-6 from GP-3 were obtained commercially.

The stable synthesis of peptides containing multiple tyrosine sulfate residues (TyrSO3) is chemically complex. Preliminary studies demonstrated inefficient synthesis of such peptides when Fmoc derivatives of TyrSO3 were utilized in the solid-phase synthesis of peptides. Therefore, a soluble recombinant form of tyrosyl-protein sulfotransferase-1 (TPST-1) (32, 33) was used to add sulfate to Tyr residues to form the glycosulfopeptide GSP-6.

At each step of the synthesis, the glycopeptide products were analyzed by reversed phase HPLC (Fig. 3). The addition of each monosaccharide caused a significant reduction in retention time for the glycopeptides, allowing the completeness of each reaction step to be easily monitored. At each step of the reactions shown in Fig. 2, the glycopeptide products were purified by reversed phase HPLC, and mass spectral analyses were performed to verify the sizes of the synthesized products. As shown in Fig. 4, the predicted and observed masses for each glycopeptide were identical within experimental error. Thus, a purified form of each of the glycopeptides (GP-1 through GP-6) and the glycosulfopeptide (GSP-6) was obtained. In control studies some of the glycopeptide intermediates were enzymatically sulfated by TPST-1. For example, GP-2 was enzymatically sulfated to generate GSP-2. Only microgram quantities of each glyco(sulfo)peptide were required for the current studies. Because of the yield and efficiency of synthesis of these compounds, it is possible to synthesize larger quantities.

**GSP-6 Binds to Immobilized P-selectin—** To test whether these synthetic glyco(sulfo)peptides interact with P-selectin, a series of affinity columns containing recombinant sPS at different coupling densities were prepared. The different column densities allow estimation of the relative affinities of glyco(sulfo)peptides for P-selectin.

Radiolabeled glyco(sulfo)peptides were applied to immobilized sPS in Ca2+ containing buffer (Fig. 5). In columns containing sPS at densities ranging from 1.0 to 2.0 mg/ml, the only glyco(sulfo)peptide retarded or bound was GSP-6. The elution of GSP-6 was retarded on columns containing 1.3 and 1.6 mg/ml sPS. GSP-6 bound to the column containing 2.0 mg/ml sPS and could be eluted with EDTA. GSP-6 lacking the sulfates on tyrosines and GSP-2 lacking the αLeα determinant had no detectable affinity for sPS. The results demonstrate the dual importance of sulfated tyrosines and αLeα for binding. Interestingly, neither GSP-5, which lacks the fucosyl residue, nor the desialylated form of GSP-6 bound detectably to immobilized sPS. These results demonstrate that both sialic acid and fucose in αLeα are necessary for high affinity binding of GSP-6 to immobilized sPS.

An Isomer of GSP-6 Does Not Bind to Immobilized P-selectin—To test whether a core 2-based O-glycan is essential for binding of GSP-6 to immobilized sPS, we synthesized a novel glycosulfopeptide that is isomeric in structure to GSP-6. This glycosulfopeptide, designated GSP-6′, has αLeα on an extended core 1-based O-glycan (C1-O-αLeα) rather than on a core 2-based O-glycan (Fig. 6A). It was synthesized by a series of steps as outlined under “Experimental Procedures.” A key step in the synthesis of GSP-6′ is the addition of GlcNAc in β1–3 linkage to the Gal residue in the core 1 O-glycan by a recombinant β1,3-GlcNAcT from N. meningitidis IgTα (34). This glycopeptide, designated GP-3′, was subsequently modified by the action of β1,4-GalT, α2,3-sialylT, and α1,3-FucT to generate a glycopeptide designated GP-6′, which has αLeα on the extended
core 1 O-glycan. GP-6' was converted to GSP-6' by action of TPST-1. Mass spectral analysis confirmed the predicted size of the final product. Unexpectedly, GSP-6 did not bind to immobilized sPS (Fig. 6B). To confirm the presence of sLe\(^a\) on the extended core 1 O-glycan, enzyme-linked immunosorbent assays were performed using 2H5, a monoclonal antibody that recognizes the sLe\(^a\) determinant (46). 2H5 bound to immobilized GP-6 and GP-6', but not to the control glycopeptide GP-2, which lacks sLe\(^a\) (data not shown). This verifies the expression of sLe\(^a\) determinants on both GP-6 and GP-6'. Taken together, these results demonstrate that sLe\(^a\) must be expressed on a core 2-based O-glycan for GSP-6 to bind immobilized sPS.

GSP-6 Binds with Relatively High Affinity to Soluble P-selectin—The dissociation constant (K\(_d\)) for binding of GSP-6 to soluble sPS was determined using an equilibrium gel filtration technique (35, 36). Different amounts of fluid-phase sPS (25–1000 pmol) were loaded into a small gel filtration column equilibrated with \(^{35}\)SO\(_3\)-GSP-6 in Ca\(^{2+}\)-containing buffer (Fig. 7A). The binding data were plotted to derive the equilibrium binding constant, yielding an estimated K\(_d\) of \(-350\) nM (Fig. 7B). Binding of GSP-6 to sPS was inhibited with EDTA and with the inhibitory anti-P-selectin mAb G1, which binds to the lectin domain of P-selectin (Fig. 7B, inset). Binding was not inhibited with anti-P-selectin mAb S12, which binds to one of the consensus repeats of P-selectin (47, 48). These results demonstrate that GSP-6 binds with relatively high affinity to sPS in a Ca\(^{2+}\)-dependent manner.

GSP-6 Is a Potent Inhibitor of Neutrophil Adhesion to Immobilized Soluble P-selectin—The ability of GSP-6 to inhibit neutrophil adhesion to P-selectin was tested in microtiter wells coated with sPS (Fig. 8). We first validated the specificity of adhesion. Adhesion was inhibited by EDTA and the anti-P-selectin mAb G1, but not by the anti-P-selectin monoclonal antibody S12. Adhesion was also inhibited by PL1, a mAb directed to an N-terminal epitope of PSGL-1 that blocks binding of PSGL-1 to P-selectin. In contrast, PL2, which recognizes an epitope within the mucin decapetide repeats of PSGL-1, did not inhibit adhesion. These results demonstrate that adhesion in this assay requires binding of PSGL-1 to sPS. Low concentrations of fluid-phase sPS (5.67 \(\mu\)M) inhibited neutrophil adhesion. A similar concentration of GSP-6 (4.7 \(\mu\)M) also significantly inhibited neutrophil adhesion to immobilized sPS. In marked contrast, a pure sLe\(^a\)-containing tetrasaccharide (NeuAc\(^\alpha\)2\(\beta\)3Gal\(^\beta\)1\(\beta\)3\[Fuc\(^\alpha\)1\(\beta\)3\]GlcNAc) only minimally inhibited neutrophil adhesion even at very high concentrations (5.3 mM). Taken together, these results demonstrate that GSP-6 binds specifically to P-selectin and strongly inhibits PSGL-1-dependent neutrophil adhesion to P-selectin.

**DISCUSSION**

This study demonstrates that the small glycosulfopeptide GSP-6 binds with high affinity to P-selectin. GSP-6 represents a portion of the primary sequence of the extreme N terminus of PSGL-1 and was modified to contain a specific C2-O-sLex on Thr\(^{57}\) and Tyr\(^{59}\) residues at Tyr\(^{46}\), Tyr\(^{48}\), and Tyr\(^{51}\). Comparison of binding of various glyco(sulfo)peptides demonstrates that both C2-O-sLex and Tyr\(^{59}\) are required for high affinity binding of GSP-6 to P-selectin. GSP-6 binds to soluble P-selectin with a K\(_d\) of \(-350\) nM and inhibits PSGL-1-dependent adhesion of neutrophils to immobilized P-selectin.

**Requirement of Tyrosine Sulfation for Glycosulfopeptide Binding to P-selectin**—The inability of glycopeptides lacking Tyr\(^{59}\) to bind immobilized P-selectin demonstrates that Tyr\(^{59}\) plays a critical role in glyco(sulfo)peptide recognition by P-selectin. Previously, indirect evidence suggested an im-
important role of TyrSO$_3$ residues in promoting high affinity binding of PSGL-1 to P-selectin. Treatment of native PSGL-1 with bacterial arylsulfatase blocks its binding to immobilized sPS (14). Blockade of overall sulfation of PSGL-1 by treating cells with chlorate to prevent formation of the phosphoadenosine phosphosulfate, the substrate for sulfation reactions, reduces adhesion of cells to P-selectin (10, 11). Replacement of the three tyrosine residues at positions 46, 48, and 51, which fall within the tyrosine sulfation motif, with phenylalanines inhibits binding of recombinant PSGL-1 to P-selectin (10–13). However, replacement of two of the three Tyr residues with Phe within the extreme N-terminal domain of PSGL-1 permits binding of recombinant PSGL-1 to P-selectin (13). The present study demonstrates directly that sulfation of tyrosine residues 46, 48, and 51 in GSP-6 promotes high affinity binding of GSP-6 to P-selectin. The GSP technology will allow testing of the importance of the number and relative locations of TyrSO$_3$ to binding of glycosulfopeptides to P-selectin.

Requirement of C2-O-sLe$^\alpha$ for Glycosulfopeptide Binding to P-selectin—Our results directly demonstrate that an sLe$^\alpha$-bearing O-glycan on Thr$^{57}$ in GSP-6 is required for high affinity binding to P-selectin. Previous indirect evidence suggested a requirement for an O-glycan in this region. Chimeric glycoproteins containing only the extreme N-terminal region of PSGL-1 and lacking N-glycosylation sites bind to P-selectin (10, 11). Substitution of Thr$^{57}$, but not Thr$^{44}$, blocks binding of full-length recombinant PSGL-1 to P-selectin (12, 13). Other observations suggested that a1,3-fucosylation of PSGL-1 is also required for binding to P-selectin. Expression of recombinant human PSGL-1 in COS cells capable of binding P-selectin required co-expression of an a1,3-fucosyltransferase (7). Leukocytes from mice lacking FucT-VII also fail to bind P-selectin (18).

Unexpectedly, we found that sLe$^\alpha$ on a core 2-based O-glycan, but not an extended core 1-based O-glycan, promotes high affinity binding of GSP-6 to P-selectin. Previous indirect evidence suggested that expression of recombinant human PSGL-1 in CHO cells capable of binding P-selectin required co-expression of the core 2 $\beta$1,6-GlcNAcT (12, 16). HL-60 cell-derived PSGL-1 contains many O-glycans with the core 2 motif, but only a minor subset of these O-glycans contain the sLe$^\alpha$ antigenic determinant (15). Core 2-based O-glycans may also be important for neutrophil adhesion to P-selectin in vivo, since leukocytes from mice lacking expression of the
core 2 β1,6-GlcNAcT fail to bind to fluid-phase P- or E-selectin (17). Our results directly demonstrate that the expression of a C2-O-sLe± requires high affinity binding of GSP-6 to P-selectin.

It is remarkable that GSP-6, which has C1-O-sLe±, does not bind P-selectin. This result suggests that the β1,6 GlcNAc residue of the core 2 branch may enhance accessibility of the sLe± moeity to P-selectin, perhaps through a more flexible linkage (49). It is also possible that the β1,6 GlcNAc residue subtly alters the secondary structure of the glycosulfopptide, thereby enhancing the exposure of the TyrSO3 along with the sLe± moiety. Alternatively, it is possible that the underlying core 2 O-glycan may bind directly to P-selectin. The availability of GSPs with various core O-glycan modifications will now allow a direct testing of these possibilities.

Taken together, these results suggest a model in which α1,3-fucosyl and α2,3-sialyl residues within C2-O-sLe± plus one or more TyrSO3 residues on GSP-6 interact with the lectin domain of P-selectin (Fig. 9). Such interactions may not occur efficiently if sLe± is present in an extended core 1 O-glycan, as for GSP-6, where the α1,3-fucosyl and α2,3-sialyl residues may be displaced in their orientations relative to the TyrSO3 residues (Fig. 9). This model suggests that the lectin domain of P-selectin has independent binding sites for these determinants on GSP-6 and that a specific stereochemical interaction occurs. This possibility is indirectly supported by the observation that the affinity of GSP-6 for P-selectin far exceeds that of the simple tetrasaccharide sLe±. Mapping of the specific binding sites in the lectin domain of P-selectin for GSP-6 will require structural characterization by crystallography and other approaches.

Previous structural analyses demonstrated that some core 2-based O-glycans from PSGL-1 from HL-60 cells contain a polyfucosylated, poly lactosamine sequence (α3Galβ1→4(Fucα1→3)GlcNAcβ1→) , capped with sLe± (15). The present results show that a glycosulfopptide lacking this polyfucosylated, polylactosamine sequence binds P-selectin with high affinity. Core 2 O-glycans with polyfucosylated, polylactosamine sequences in PSGL-1 may bind to P-selectin with altered kinetics or affinity compared with core 2-based O-glycans lacking this feature. Alternatively, the polyfucosylated polylactosamine may promote binding of PSGL-1 to other selectins. The synthesis of GSPs with core 2-based O-glycans containing polyfucosylated, polylactosamine sequences may help to address these possibilities. In vitro, FucT-VII can generate a terminal sLe± moiety on a polylactosamine sequence, but cannot add internal fucosyl residues to generate the polyfucosylated, polylactosamine sequence found in the O-glycans of PSGL-1 (50, 51). Human and murine leukocytes contain a second α1,3-fucosyltransferase, termed FucT-IV, that adds fucosyl residues to internal sequences of polylactosamine (50). The combined actions of both FucT-VII and FucT-IV may be necessary to efficiently synthesize such polyfucosylated polylactosamines.

**Comparison of Glycosulfopptide and PSGL-1 Binding to P-selectin**—Our results indicate that a synthetic glycosulfopptide binds to P-selectin with an affinity equivalent to that of neutrophil-derived PSGL-1, which occurs as a disulfide-bonded dimer in the membrane. These results contrast with recent proposals that covalent dimerization of PSGL-1 is required for binding to fluid-phase P-selectin (24, 25). Surface plasmon resonance measurements demonstrate that monomeric, soluble recombinant P-selectin binds immobilized neutrophil-derived PSGL-1 with a Kₘ of ~300 nM (28). This compares favorably with that of GSP-6, which binds to P-selectin with a Kₘ of ~350 nM, as determined by Hummel-Dreyer equilibrium gel filtration. The discrepancies between our results and those suggesting a requirement for covalent dimerization of PSGL-1 for binding to P-selectin are unclear. The use of recombinant molecules synthesized in a cellular environment presents special difficulties, since the precise post-translational modifications required for binding of recombinant PSGL-1 to P-selectin are
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