Binding of Glycosulfopeptides to P-selectin Requires Stereospecific Contributions of Individual Tyrosine Sulfate and Sugar Residues*

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P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin on leukocytes that binds to selectins. P-selectin binds to an N-terminal region of PSGL-1 that requires sulfation of at least one of three clustered tyrosines (TyrSO3) and an adjacent core-2-based O-glycan expressing sialyl Lewis x (C2-O-sLex). We synthesized glycosulfopeptides (GSPs) modeled after this region of PSGL-1 to explore the roles of individual TyrSO3 residues, the placement of C2-O-sLex relative to TyrSO3, the relative contributions of fucose and sialic acid on C2-O-sLea, and the function of the peptide sequence for binding to P-selectin. Binding of GSPs to P-selectin was measured by affinity chromatography and equilibrium gel filtration. 2-GSP-6, which has C2-O-sLea at Thr-57 and TyrSO3 at residues 46, 48, and 51, bound to P-selectin with high affinity (Kd ~ 650 nM), whereas an isomeric trisulfated GSP containing C2-O-sLea at Thr-44 bound much less well. Non-sulfated glycopeptide (2-GP-6) containing C2-O-sLea at Thr-57 bound to P-selectin with ~40-fold lower affinity (Kd ~ 25 μM). Proteolysis of 2-2GP abolised detectable binding of the residual C2-O-sLea-Thr to P-selectin, demonstrating that the peptide backbone contributes to binding. Monosulfated and disulfated GSPs bound significantly better than non-sulfated 2-2GP-6, but sulfation of Tyr-48 enhanced affinity (Kd ~ 6 μM) more than sulfation of Tyr-46 or Tyr-51. 2-GSP-6 lacking sialic acid bound to P-selectin at ~10% that of the level of the parent 2-GSP-6, whereas 2-GSP-6 lacking fucose did not detectably bind; thus, fucose contributes more than sialic acid to binding. Reducing NaCl from 150 to 50 mM markedly enhanced binding of 2-GSP-6 to P-selectin (Kd ~ 75 nM), demonstrating the charge dependence of the interaction. These results reveal a stereospecific interaction of P-selectin with PSGL-1 that includes distinct contributions of each of the three TyrSO3 residues, adjacent peptide determinants, and fucose/sialic acid on an optimally positioned core-2 O-glycan.

Selectins and their glycoconjugate ligands promote the tethering and rolling of circulating leukocytes on blood vessel endothelial cells, platelets, and other leukocytes. L-, E-, and P-selectin weakly bind to the sialyl Lewis x (sLe x) tetrasaccharide determinant NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc-R, but each selectin binds with higher affinity to specific sialylated and fucosylated macromolecular ligands (1–3). The best characterized ligand for selectins is P-selectin glycoprotein ligand-1 (PSGL-1), a dimeric mucin present on the surface of all leukocytes (4–7). PSGL-1 binds with relatively high affinity (Kd ~ 300 nM) to P-selectin expressed on the surface of activated endothelial cells and platelets (8). Antibody blockade studies (9) and targeted disruption of the gene for PSGL-1 in mice (10) confirm that PSGL-1 is the primary ligand for P-selectin on leukocytes.

The N terminus of mature human PSGL-1 begins at residue 42, after removal of the signal peptide from residues 1–18 and a propeptide from residues 19–41 (11). Sulfation of tyrosine residues and O-glycosylation in the mature N-terminal region of PSGL-1 appears necessary for high affinity binding of PSGL-1 to P-selectin (4), but the importance of modifications at specific amino acid residues is not well understood. Studies employing site-directed mutagenesis of recombinant PSGL-1, which was co-expressed in Chinese hamster ovary cells or COS cells with specific glycosyltransferases, suggested that a core-2 based O-glycan with the sialyl Lewis x (C2-O-sLea) antigen at Thr-57 and at least one of the Tyr residues at Tyr-46, -48 or -51 are required for measurable binding of PSGL-1 to P-selectin (12–14). Whether the individual Tyr residues make distinct contributions to P-selectin recognition has not been examined. Mutation of Thr-44 to alanine did not inhibit binding of recombinant PSGL-1, suggesting that an O-glycan at this position does not contribute to binding. However, this residue might be O-glycosylated in hematopoietic cells but not in transfected Chinese hamster ovary cells.

To obtain more precise information about the interaction of P-selectin with O-glycans and TyrSO3 residues on PSGL-1, we recently used purified and recombinant glycoconjugates and a tyrosyl-protein sulfotransferase to generate synthetic glycosulfopeptides (GSPs) modeled after the mature N-termi-
nal region of PSGL-1. A glycosulfopeptide-6 (GSP-6) was synthesized to contain three TyrSO₃ residues at Tyr-46, -48, or -51 and a nearby C2-O-SLe⁰ at Thr-57 (16). GSP-6 binds to P-selectin with an affinity (Kᵅ = 350 nM) close to that of native, neutrophil-derived PSGL-1 (Kᵅ ~ 300 nM) (8, 16). Consistent with this finding, monomeric forms of full-length PSGL-1 and a tryptsin-digested glycosulfopeptide from the N terminus of HL-60 cell-derived PSGL-1 also bind with high affinity to P-selectin (17).

Very little is known about the stereospecific contributions of tyrosine sulfation and O-glycosylation to binding of PSGL-1 to P-selectin. Substitution of any two of the three tyrosines with phenylalanines in recombinant PSGL-1 impairs the mechanical properties of its bonds with P-selectin in shear flow, but these substitutions could affect peptide structure as well as prevent sulfation (18). Synthetic conjugates that express sLe⁰ on different types of O-glycans bind differentially to P-selectin (16, 19). Notably, a GSP modeled after the N terminus of PSGL-1 that contains sLe⁰ expressed on a core-2-based O-glycan binds much better to P-selectin than an isomeric GSP that contains sLe⁰ expressed on an extended core-1 based O-glycan (16). It is not clear whether the position of the O-glycan relative to TyrSO₃ residues, i.e. at Thr-44 versus Thr-57, contributes to binding.

The interpretation of the effects of amino acid substitutions on post-translational modifications of PSGL-1 has significant limitations because it is difficult to structurally define specific post-translational modifications on recombinant glycoproteins. There is usually microheterogeneity of glycosylation that complicates the interpretation of binding affinities. The use of synthetic, homogeneously glycosylated glycosulfopeptides offers marked advantages, but the tyrosyl-protein sulfotransferase adds sulfate from PAPS donor to all available Tyr residues within the consensus sequence. Thus, enzymatic sulfation does not readily allow sulfation of individual Tyr residues within a peptide that contains multiple Tyr residues (16).

To gain more insight into the contributions of individual TyrSO₃ residues, the peptide backbone, specific sugar residues, and their site-specific contributions, we generated synthetic GSPs modeled after the mature N terminus of human PSGL-1 that contain TyrSO₃ residues and O-glycans at specific sites. Our results demonstrate that P-selectin differentially recognizes GSPs containing one, two, or three TyrSO₃ residues and that the relative contributions of each TyrSO₃ residue differ. P-selectin binds weakly to non-sulfated anionic peptides containing C2-O-SLe⁰, but the O-glycan must be placed at Thr-57 rather than at the alternative site at Thr-44. These studies further extend our knowledge about the roles of peptide, sulfate, and carbohydrate determinants in binding of PSGL-1 to P-selectin.

**EXPERIMENTAL PROCEDURES**

**Chemical Synthesis of the Glycosulfopeptides, Cleavage from the Resin, and Deacetylation**—Glycosulfopeptides were synthesized on an Applied Biosystems 433A peptide synthesizer using Fmoc chemistry. The preloaded Wang resin (p-hydroxybenzylphenoxymethyl resin) and liquid reagents and solvents were purchased from Applied Biosystems, Warrington, Great Britain. Tri-O-acetyl-GalNAc and tyrosine sulfates were incorporated into the peptide during the solid phase peptide synthesis using tri-O-acetyl-GalNAc-Fmoc-Thr (Glycotech, Rockville, MD) and Fmoc-Tyr(SO₃H)-H sodium salt (Bachem California Inc., Torrance, CA). Other Fmoc amino acid derivatives were from Calbiochem-Novabiochem. Sulfated peptides 2-GSP(46)-1, 2-GSP(48)-1, 2-GSP(51)-1, 2-GSP(46,48)-1, 2-GSP(46,51)-1, 2-GSP(48,51)-1, and 2-GSP-1 were cleaved from the resin as described (20). Briefly, 70–100 mg of resin containing peptide was cleaved in 700 μl of 90% aqueous trifluoroacetic acid containing 2.5% (v/v) m-cresol for 5 h at 4 °C under continuous stirring. Nonsulfated 2-GP-1 was cleaved from the resin in 700 μl of 90% aqueous trifluoroacetic acid containing 5% (v/v) thionai-solute and 2.5% (v/v) ethanethiol for 15 min on ice, and incubation was continued for 1 h 45 min at room temperature under stirring. The cleaved peptides were then precipitated and washed with tert-butyl methyl ether. The dried peptides were dissolved in 30% aqueous aceto-nitrile to a concentration of 2 mg/ml. An equal volume of methanol was added, and the free sulfhydryl of the C-terminal cysteine was converted into S-S-CH₃ by incubating the peptides in 4 mM methyl methanesulfonate (Pierce) for 1 h at room temperature. The complete derivatization was confirmed by reversed phase HPLC. Tri-O-acetyl-GalNAc was deacetylated using 18 mM sodium methylate as described (16). The deacetylated peptides were purified by reversed phase HPLC and analyzed by electrospray mass spectrometry (see Table I). The sialyltransferease products served as acceptors for the synthesis of radiolabeled peptides.

Radiolabeled glycosulfopolypeptides (2-GP-6, 2-GSP(46)-6, 2-GSP(48)-6, 2-GSP(51)-6, 2-GSP(46,48)-6, 2-GSP(46,51)-6, 2-GSP(48,51)-6, and 2-GSP-6) were synthesized using glycosulfopeptide products (2-GSP-6 series) as acceptors and either GDP-[3H]Fuc (1000 cpm/pmol) (American Radiolabeled Chemicals Inc., St. Louis, MO) or GDP-[14C]Fuc (96,000 cpm/nmol) (Amersham Pharmacia Biotech) as a donor in the a1,3-fucosyltransferase VI (Calbiochem) reaction. [3H]2-GP-2 was synthesized using 2-GP-1 as an acceptor and UDP-[3H]Gal (1000 cpm/pmol) (Amersham Pharmacia Biotech) as a donor in the core-1 b1,3-galactosyltransferase reaction. [3H]2-GP-5 was synthesized using 2-GP-4 as an acceptor and CMP-[3H]NueAc (1000 cpm/pmol) (NEN Life Science Products, Inc.) as a donor in the α2,3-2,3-sialyltransferase (Calbiochem) reaction. Radiolabeled products were purified by HPLC.

**Enzymatic Synthesis of Isomeric Glycosulfopeptides 3-GSP-6 and 3-GSP-6**—Isomeric glycosulfopeptides 3-GP-6 and 3-GP-6 were synthesized as described (16). Matrix-assisted laser desorption ionization/time of flight mass spectrometric analysis confirmed the masses of the HPLC-purified products (observed m/z 3637.3 for 3-GP-6 and observed m/z 3637.7 for 3-GP-6, calculated m/z 3637.7 for 3-GP-6 and 3-GP-6). The glycosulfopeptides 3-GP-6 and 3-GP-6 were then electrosprayed and analyzed by HPLC. The masses of the final products (3-GP-6 series) and of peptide products after sialyltransferase reaction (3-GP-5 series) were verified by electrospray mass spectrometry (see Table I). The sialyltransferease products served as acceptors for the synthesis of radiolabeled peptides.
150 mM or 50 mM NaCl in 20 mM MOPS, pH 7.5, containing 2 mM CaCl$_2$, 2 mM MgCl$_2$, and 0.02% NaN$_3$. Inhibition experiments with EDTA were conducted using 1 mM EDTA with 150 mM or 50 mM NaCl in 20 mM MOPS, pH 7.5, containing 0.02% NaN$_3$. The amount of soluble P-selectin (sPS) (21) used was 500 pmol in high salt buffer or 50 pmol in low salt buffer. Inhibition experiments with anti-P-selectin monoclonal antibody G1 (22) were performed using the same molar amount of G1 and sPS (500 pmol or 50 pmol) in buffers containing Ca$^{2+}$.

**P-selectin Affinity Chromatography**—sPS was coupled to Ultralink Biosupport Medium (Pierce) at a density of 6.5 mg/ml. An sPS column (0.9 ml, 0.5 × 4.5 cm) was equilibrated with 20 ml of 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 0.02% NaN$_3$. Radiolabeled peptides dissolved in 200 ml of equilibration buffer (1000–2000 cpm, 1–10 pmol) were chromatographed in the P-selectin column by collecting 0.5-ml fractions at a flow rate of 250 ml/min. Bound peptides were eluted using 10 mM EDTA instead of divalent cations in buffer. In some experiments, 50 mM NaCl was used instead of 150 mM NaCl in buffers containing Ca$^{2+}$ or EDTA (1 mM). To derive the $D$ value for each bound peptide, the elution volume of unbound peptide (2 ml) was subtracted from the elution volume of bound peptide.

**Desialylation of 2-GSP-6**—[3H]2-GSP-6 (506,000 cpm, 0.5 nmol) was desialylated by adding 13 milliunits of *Arthrobacter ureafaciens* neuraminidase (Sigma) in 400 ml of 0.2 M sodium acetate, pH 5.5, for 14 h at 37 °C. The reaction mixture was deproteinized by chloroform-methanol (2:1) extraction and purified by HPLC.

**Pronase Digestion of 2-GP-6**—[3H]2-GP-6 (10,000 cpm, 10 pmol) was digested with *Streptomyces griseus* Pronase E (2 mg/ml) (Sigma) in 100 ml of 0.1% Tris, pH 8.0, for 1 h at 60 °C. Pronase was inactivated by boiling for 30 min and was removed by chloroform-methanol (2:1) extraction. The aqueous phase was dried under vacuum and dissolved by 100 ml of 20 mM MOPS, pH 7.5, containing 50 mM NaCl and 0.02% NaN$_3$. An aliquot of Pronase-digested [3H]2-GP-6 (1, 300 cpm) was chromatographed on a P-selectin column using 20 mM MOPS, pH 7.5, containing 50 mM NaCl, 2 mM CaCl$_2$, 2 mM MgCl$_2$, 0.02% NaN$_3$.

**Mass Spectrometric Analysis of Glyco(sulfo)peptides**—Electrospray mass spectra were collected in the negative ion mode using an API365 triple quadrupole mass spectrometer (PerkinElmer Sciex Instruments, Thornhill, Ontario, Canada). Samples were dissolved in 1% triethylamine in 50% aqueous methanol to a concentration of 5 pmol/µl and injected into the mass spectrometer with a nanoelectrospray ion source (MDS Protana A/S, Odense M, Denmark).

**RESULTS**

**Synthesis of Glyco(sulfo)peptides**—We explored the possibility that only one or two TyrSO$_3$ residues together with a nearby $O$-glycan can support high affinity binding to P-selectin. To this end we synthesized a series of glyco(sulfo)peptides corresponding to the extreme N terminus of human PSGL-1 (residues 45–61) with one, two, or three TyrSO$_3$ residues at defined positions or without TyrSO$_3$ residues (Fig. 1). Each glyco(sulfo)peptide contained a C-terminal Cys residue for future coupling of the peptide to artificial supports. Each glyco(sulfo)peptide was subsequently modified by purified or recombinant glycosyltransferases to generate a core-2-based $O$-glycan expressing the sLe$^a$ antigen at Thr-57 (Fig. 2). To compare the roles of sialic acid and fucose for binding to P-selectin, peptides with three TyrSO$_3$ residues but having incomplete glycosylation were also synthesized (Fig. 1).

Peptides with one, two, or three TyrSO$_3$ residues (Tyr-46, -48, and/or -51) and an α-linked GalNAc residue at Thr-57 were
snytransferase; a sylhydryl into S-S-CH₃ to prevent oxidation and dimerization of Cys of each peptide was protected by converting the free sulf-

TABLE I

| Glyco(sulfo)peptide | Calculated mass | Observed mass |
|---------------------|----------------|--------------|
| 2-GP-1              | 2501.7         | 2501.0       |
| 2-GSP(46)-1         | 2558.0         | 2558.0       |
| 2-GSP(48)-1         | 2558.0         | 2558.0       |
| 2-GSP(51)-1         | 2558.0         | 2558.0       |
| 2-GSP(46,48)-1      | 2661.7         | 2661.7       |
| 2-GSP(46,51)-1      | 2661.7         | 2661.7       |
| 2-GSP(46,51)-1      | 2661.7         | 2661.7       |
| 2-GSP-1             | 2741.7         | 2741.7       |
| 2-GP-5              | 3320.4         | 3319.8       |
| 2-GSP(46)-5         | 3400.4         | 3400.1       |
| 2-GSP(48)-5         | 3400.4         | 3400.5       |
| 2-GSP(51)-5         | 3400.4         | 3400.0       |
| 2-GSP(46,48)-5      | 3480.4         | 3480.0       |
| 2-GSP(46,51)-5      | 3480.4         | 3480.0       |
| 2-GSP(46,51)-5      | 3480.4         | 3480.0       |
| 2-GSP-5             | 3560.5         | 3559.9       |
| 2-GP-6              | 3466.5         | 3466.0       |
| 2-GSP(46)-6         | 3546.4         | 3546.3       |
| 2-GSP(48)-6         | 3546.4         | 3546.2       |
| 2-GSP(51)-6         | 3546.4         | 3546.2       |
| 2-GSP(46,48)-6      | 3626.5         | 3626.4       |
| 2-GSP(46,51)-6      | 3626.5         | 3626.7       |
| 2-GSP(46,51)-6      | 3626.5         | 3626.3       |
| 2-GSP-6             | 3706.6         | 3706.3       |
| 3-GSP-6             | 3978.8         | 3978.3       |
| 3-GSP-6′            | 3978.8         | 3978.4       |

FIG. 2. Enzymatic synthesis of glycosulfopeptide 2-GSP-6. Glyco(sulfo)peptides containing tyrosine sulfate at specified position(s) and GalNAc at Thr-57 were acceptors for glycosyltransferase reactions. The synthesis of each peptide in Fig. 1 was performed as indicated for 2-GSP-6, β1,3-GalT, core-1 β1,3-galactosyltransferase, β1,6-GlcNAcT, core-2 β1,6-N-acetylglucosaminyltransferase, β1,4-GalT, β1,6-galacto-
syltransferase, α2,3-(N)-sialylT, α2,3-(N)-sialyltransferase; a1,3-FucT, a1,3-fucosyltransferase.

A Glycosulfopeptide with C2-O-sLex at Thr-57 but Not at Thr-44 Binds to P-selectin—Two isomeric 35SO3-labeled glyco-
sulfopeptides containing three TyrSO3 residues and a C2-O-
sulfopeptide (GSP-6) or Thr-44 (3-GSP-6) (Fig. 1). O-Glycans were synthe-
sized enzymatically as shown in Fig. 2, starting from the non-
sulfated peptides. As a final step, all three Tyr residues of 3-GP-6 and 3-GP-6′ were enzymatically sulfated using recombinant human tyrosyl-protein sulfttransferase-1. Electrospray mass spectrometer verified the masses of the fully sulfated glycosulfopeptides, 3-GSP-6 and 3-GSP-6′ (Table I).

Electrospray mass spectrometric analysis of HPLC-purified glyco(sulfo)peptide samples

Shown are calculated and observed masses for the indicated glyco-
sulfopeptides (for structures, see Fig. 1). In some cases minor signals (peptide mass minus 80 mass units) that represent partially desulfated peptides were present (not shown).

are 2-GSP-2 (core-1 O-glycan), desialylated 2-GSP-6, and 2-GSP-5, which lacks a fucose residue (Fig. 1).

We previously showed that a core-2-based O-glycan containing sLeα at Thr-57 together with three TyrSO3 residues sup-
ported high affinity binding of a glycosulfopeptide (GSP-6) to P-selectin (16). However, the extreme N terminus of PSGL-1 contains another potential glycosylation site at Thr-44. To ex-

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suled by mass spectrometry, and used as acceptors for enzymatic glyco-
ysyltransferase. sLeα on a core-2-based O-glycan was synthesized on each peptide at Thr-57 using recombinant or highly purified glycosyltransferases (Fig. 2). The completeness of each gly-
cosyltransferase reaction was easily monitored by HPLC, be-
cause the addition of each monosaccharide reduced the reten-
tion time for the peptides by ~0.5–2 min (data not shown). Electrospray mass spectrometry was used to analyze the masses of the final products. The mass of each glyco(sulfo)peptide matched the calculated mass (Table I). Radiolabeled peptides were synthesized using either GDP-[3H]Fuc or GDP-
[14C]Fuc in the reactions with α1,3-fucosyltransferase, which is the final step in the synthesis. In addition, radiolabeled glyco-
sulfopeptides with three TyrSO3 residues but with incomplete O-glycan structure were synthesized. These glycosulfopeptides

synthesized chemically using Fmoc derivatives of TyrSO3 and tri-O-acetyl-GalNAc-Thr during the solid phase peptide syn-
thesis. The sulfated peptides were cleaved from the solid sup-
port under mild conditions because of the acid lability of TyrSO3 residues (20). After peptide cleavage, the C-terminal Cys of each peptide was protected by converting the free sulf-
hydryl into S-S-CH3 to prevent oxidation and dimerization of the peptide. After deacetylation of tri-O-acetyl-GalNAc, the peptides were purified by reversed phase HPLC, characterized by mass spectrometry, and used as acceptors for enzymatic glyco-
ysyltransferase. α2,3-sialyltransferase.

Each Additional TyrSO3 Residue Enhances the Binding Af-
finity of GSPs for Immobilized P-selectin—As an initial test to determine whether incompletely sulfated GSPs containing an intact sLe\(^\alpha\) O-glycan interact with P-selectin, \[^{3}H\]Fuc-labeled GSPs were chromatographed on a sPS affinity column (Fig. 4). At physiological salt concentration (150 mM NaCl) nonsulfated peptide 2-GP-6 had only a slight affinity for immobilized sPS (Fig. 4C, \(\Delta = 0.2\)); the \(\Delta\) reflects the difference in elution volume between the test sample and that of a control peptide, with no affinity for P-selectin or the test peptide analyzed in the presence of 1 mM EDTA). The nonsulfated peptide 2-GSP(48)-6 had higher affinity for immobilized P-selectin (\(\Delta = 1.2\)) than the other isomeric peptides 2-GSP(46)-6 and 2-GSP(51)-6 (\(\Delta = 0.7\)) (Fig. 4A). Disulfated peptides were more retarded in their elution from the sPS column than monosulfated peptides (Fig. 4B). Two of the disulfated peptides (2-GSP(46,48)-6, \(\Delta = 4.3\); 2-GSP(48,51)-6, \(\Delta = 3.7\)) had higher affinity for P-selectin than 2-GSP(46,51)-6 (\(\Delta = 2.2\)). This indicates that Tyr(48)-SO\(_3\) is more important for binding of disulfated as well as monosulfated peptides to P-selectin. Trisulfated 2-GSP-6 had the highest affinity for immobilized P-selectin and was eluted with EDTA (Fig. 4C). Because of the reversible nature of the binding of the ligands to immobilized P-selectin, bound ligands eventually eluted from the column without the necessity of chelating Ca\(^{2+}\). When chromatography was continued without the addition of EDTA, 2-GSP-6 eluted as a very symmetrical broad peak beginning at fraction 20 (\(\Delta = 14\), the inset in Fig. 4C). In contrast, replacement of divalent cations in the buffer with 1 mM EDTA completely inhibited the binding of 2-GSP-6 to P-selectin (\(\Delta = 0\), dashed line in Fig. 4), demonstrating that binding of 2-GSP-6 to P-selectin is dependent on Ca\(^{2+}\). These results demonstrate that each additional TyrSO\(_3\) residue enhances the binding affinity of glycosulfopeptides to P-selectin and that Tyr-48 is the most optimal tyrosine sulfate position for binding.

\(\alpha 1,3\)-Fucose Contributes More than \(\alpha 2,3\)-Sialic Acid to Binding of 2-GSP-6 to P-selectin—The binding of tri sulfated but incompletely glycosylated GSPs to immobilized P-selectin was also studied (Fig. 4, D–F). These peptides were desialylated 2-GSP-6, 2-GSP-5 (no fucose), and 2-GSP-2 (core-1 O-glycan only). Desialylated 2-GSP-6 showed a weak affinity for sPS (\(\Delta = 0.3\), Fig. 4D), whereas the other two GSPs did not detectably bind. These results indicate that \(\alpha 1,3\)-fucose in the absence of sialic acid contributes to binding, whereas \(\alpha 2,3\)-sialic acid in the absence of fucosylation does not detectably contribute to binding.

Three TyrSO\(_3\) Residues Increase the Binding Affinity of 2-GSP-6 for P-selectin by 40-Fold—Hummel-Dreyer equilibrium gel filtration (23, 24) was used to directly measure the dissociation constants (\(K_d\)) for binding of GSPs to soluble P-selectin. We examined those mono-, di-, and trisulfated glycosulfopeptides that clearly interacted with immobilized P-selectin (\(\Delta > 1\)). Experiments were conducted using a constant amount of [\(^3H\)]Fuc-labeled sample in Ca\(^{2+}\)-containing buffer and different concentrations of sPS. The \(K_d\) was derived from the binding data as shown for 2-GSP-6 (Fig. 5A) and 2-GSP(46,48)-6 (Fig. 5C). In typical equilibrium gel filtration experiments (23, 24), a plateau is usually observed between the peak elution position of a large-sized complex of protein-bound ligand and the trough elution of the small-sized free ligand. However, we did not observe a significant plateau between the peak and trough in equilibrium binding studies with sPS and the GSPs (Fig. 5, A and C). This was due to the close elution positions of the sPS versus the GSPs (Fig. 5A, inset), thus resulting in an absence of a significant plateau. This absence of a plateau does not materially affect the results, since the elution of the complex of sPS with GSP is clearly distinguishable from the elution of the free GSPs. 2-GSP-6 bound to sPS with a \(K_d\) of \(-650\) nM (Fig. 5B). The binding was Ca\(^{2+}\)-dependent, since it was blocked by inclusion of EDTA, and specific to the lectin domain of P-selectin (not shown). Scatchard analysis of the binding data with the assumption that each molecule of sPS binds a single monomeric protein (21). \(K_d\) values of 1.5 and 1.9 \(\mu\)M were determined for the disulfated peptides 2-GSP(46,48)-6 and 2-GSP(48,51)-6, respectively, which showed the highest affinity for immobilized sPS relative to 2-GSP-6 (Fig. 5, C–E). The disulfated 2-GSP(46,51)-6 bound to P-selectin with a \(K_d\) of \(-5.9\) \(\mu\)M (Fig. 5F). The binding affinity for 2-GSP(48)-6, which exhibited the highest affinity for immobilized sPS among the monosulfated peptides, was approximately the same as for 2-GSP(46,51)-6 (\(K_d = 6.1\) \(\mu\)M, Fig. 5G). The dissociation constant for the nonsulfated peptide 2-GP-6 was estimated by Hummel-Dreyer equilibrium gel filtration using a single concentration of sPS (3.2 nmol). The concentration of bound 2-GP-6 (2 nmol) and free sPS (7.6 \(\mu\)mol) were compared with the equilibrium gel filtration data for 2-GSP-6, 2-GSP(46,48)-6, and 2-GSP(48,51)-6 (Fig. 5, A–E), yielding an estimated \(K_d\) of \(-20–30\) \(\mu\)M for 2-GP-6. These results show that the three TyrSO\(_3\) residues increase the binding affinity of 2-GSP-6 for P-selectin by 40-fold over the non-sulfated glycopeptide.

A direct linear relationship was found between elution positions (\(\Delta\)) of GSPs on immobilized P-selectin and the association constants (\(K_d\)) derived from equilibrium gel filtration data (Fig. 5H). This direct relationship demonstrates that the elution position on affinity chromatography is a direct reflection of the equilibrium binding in solution and shows that the
immobilized P-selectin column can be used to estimate dissociation constants by calibrating the column with GSPs having known dissociation constants.

Sub-physiological Concentration of NaCl Enhances the Binding Affinity of 2-GSP-6 for P-selectin—The binding of selectins to sLeα is inhibited by increasing the concentration of NaCl (25). To study the effect of sub-physiological salt concentration on the binding affinity of 2-GSP-6 to sPS, we determined the $K_d$ for binding of 2-GSP-6 to sPS at 50 mM NaCl concentration (Fig. 6A). The $K_d$ was significantly lowered to 7.76 nM, which is nearly a 9-fold higher affinity than that observed at 150 mM NaCl (Fig. 5B). The binding of 2-GSP-6 to sPS at 50 mM NaCl was completely inhibited by EDTA and by monoclonal antibody G1, demonstrating that binding was Ca2+-dependent and involved the lectin domain of P-selectin (Fig. 6B).

a1,3-Fucose Contributes More than a2,3-Sialic Acid in Binding of 2-GSP-6 to sPS in Equilibrium Gel Filtration—We exploited the finding that low salt concentrations enhanced the binding affinity of GSPs to P-selectin. The binding affinities of glyco(sulfo)peptides that showed very weak or no interaction with immobilized P-selectin ($\Delta < 0.3$) at physiological salt concentration (150 mM NaCl) were estimated relative to 2-GSP-6 using 50 pmol of sPS and low salt (50 mM NaCl) in equilibrium gel filtration (Fig. 6B). For example, desialylated 2-GSP-6 ($\Delta = 0.3$) and 2-GP-6, which lacks TyrSO3 residues ($\Delta = 0.2$), showed a weak affinity for immobilized sPS under physiological salt concentration (Fig. 4, C and D). In equilibrium gel filtration at low salt concentration, 2-GP-6 had $-4%$ relative binding to P-selectin (Fig. 6B), compared with the control 2-GSP-6, which contained all three TyrSO3 residues. Trisulfated 2-GSP-2 and 2-GSP-5 did not show any detectable affinity for soluble P-selectin (not shown), but desialylated 2-GSP-6 had $-10%$ relative binding to P-selectin compared with the control 2-GSP-6 (Fig. 6B). EDTA and monoclonal antibody G1 specifically inhibited the binding of 2-GSP-6, desialylated 2-GSP-6, and 2-GP-6 to P-selectin (Fig. 6B). These results, along with those from affinity chromatography, demonstrate that the a1,3-fucosyl residue of 2-GSP-6 contributes more than a2,3-sialic acid to binding to P-selectin.

Monosulfated GSPs as Well as Nonsulfated 2-GP-6 Bind to Immobilized P-selectin in a Ca2+-dependent Manner at Sub-physiological Salt Concentration—We further explored the binding of GSPs to immobilized P-selectin using a sub-physiological salt concentration to aid in comparing the affinities of relatively weak ligands. When affinity chromatography on immobilized P-selectin was performed in Ca2+-containing buffer with 50 mM NaCl, all isomeric monosulfated glycosulfoproteptides were more retarded in their elution on the column compared with conditions employing 150 mM NaCl (Fig. 7A). 2-GSP(48)-6 clearly bound with higher affinity to immobilized P-selectin than the two other isomeric GSPs. These results are consistent with the results obtained using 150 mM NaCl, as described above in Fig. 4A. Binding of monosulfated peptides to P-selectin was Ca2+-dependent, since replacement of divalent cations with 1 mM EDTA in the elution buffer completely inhibited the binding (Fig. 7B). Monosulfated GSPs had higher affinity for immobilized P-selectin in comparison to non-sulfated 2-GP-6 under low salt conditions. However, 2-GP-6 was detectably retarded in its elution under low salt conditions, and the binding was inhibited with 1 mM EDTA (Fig. 7C). These data confirm the results obtained at 150 mM NaCl, which indicated that monosulfated GSPs bind to immobilized P-selectin with...
**DISCUSSION**

A set of glycosulfopeptides based on the primary sequence of the N-terminal region of human PSGL-1 were synthesized to study the effect of site-specific tyrosine sulfation as well as the structures of O-glycans and their peptide locations for binding to P-selectin (Fig. 1). The results indicate that each TyrSO3 residue contributes to the binding affinity of glycosulfopeptides for P-selectin, although their contributions are not equivalent (Fig. 8). For example, the disulfated 2-GSP(46,51)-6 binds only slightly better than the monosulfated 2-GSP(48)-6. The three TyrSO3 residues together increase the binding affinity of 2-GSP-6 for P-selectin 40-fold over the non-sulfated glycopeptide. The ability of the non-sulfated 2-GP-6, but not its proteolytic fragments, to bind P-selectin, indicates that the highly anionic peptide backbone of the peptide contributes to the interaction. Quantitative measurements of binding affinities indicate that the α1,3-fucosyl residue of 2-GSP-6 has a contribution to P-selectin binding that is equivalent to the contributions of the combined three TyrSO3 residues. By contrast, the α2,3-sialic acid residue has an important, but subordinate role, in comparison to either α1,3-fucose or tyrosine sulfation. Finally, an isomeric GSP containing a C2-O-sLe^x^ at Thr-57, but not at Thr-44, binds to P-selectin. Thus, the stereospecific placement of both the O-glycan and the TyrSO3 residues on the peptide is critical for optimal binding to P-selectin.

Although previous studies indicated that TyrSO3 residues and a specific C2-O-sLe^x^ are required for PSGL-1 to bind to P-selectin, the contributions of individual TyrSO3 residues and the peptide backbone had not been quantitatively evaluated. Replacement of the three Tyr residues at positions 46, 48, and 51 with Phe in recombinant PSGL-1 abrogated its binding to P-selectin (12–15). Site-directed replacement of any two of the three Tyr residues with Phe did not eliminate binding, indicating that a single TyrSO3 residue may be sufficient for binding (14). But recombinant PSGL-1 expressing single TyrSO3 residues is not functionally equivalent to recombinant wild-type PSGL-1, because cells expressing P-selectin roll more irregularly and with less mechanical strength on single-Tyr forms of PSGL-1 (18).

Our results quantify the contributions of sulfation of each of the three Tyr residues at positions 46, 48, and 51. The tri-sulfated 2-GSP-6 binds with high affinity to P-selectin ($K_d \sim 650 \text{ nM}$), whereas mono- or disulfated GSPs bind with significantly lower affinity ($K_d \sim 1.5–10 \text{ μM}$) (Fig. 8). The non-sulfated 2-GP-6 also binds to P-selectin, but with even lower affinity ($K_d \sim 25 \text{ μM}$); thus, sulfation of all three Tyr residues on 2-GSP-6 increases binding affinity by 40-fold. Only 25% of $^{35}$S-labeled tryptic fragments of PSGL-1 from HL-60 cells bind to a P-selectin affinity column (17). The bound fraction may represent...
the fully sulfated and glycosylated peptides, and the nonbound material may represent inefficiently sulfated and/or glycosylated peptides. Partial characterization of P-selectin-bound 35S-labeled tryptic fragments of recombinant PSGL-1 co-expressed with specific glycosyltransferases in Chinese hamster ovary cells indicated that the fragments have only minor structural differences from that of GSP-6 (17). The differences are mainly due to charge heterogeneity among the tryptic fragments. Taken together, these studies suggest that native or recombinant PSGL-1 might be partly sulfated or represent a population of mono-, di-, and trisulfated species. Differential sulfation of PSGL-1 might affect the kinetics of leukocyte rolling during inflammation (18).

The method used here to generate GSPs is particularly advantageous to explore contributions of individual TyrSO3 residues and O-glycan positioning in interactions with P-selectin. Previously we used recombinant human tyrosyl-protein sulfotransferase-1 to enzymatically sulfate the three Tyr residues in a glycopeptide. However, tyrosyl-protein sulfotransferases do not readily sulfate specific Tyr residues, and the separation and characterization of partially sulfated isomeric peptides is extremely difficult. Here we incorporated TyrSO3 residues into the glycopeptide chain using Fmoc-Tyr(SO3Na)-OH and tri-O-acetyl-GalNAc-Fmoc-Thr as building blocks during the solid phase peptide synthesis. Fmoc-Tyr(SO3Na)-OH was utilized earlier to generate mono- and disulfated peptides, but these peptides lacked O-glycosylated residues (20). The disadvantage of this method is that the acidic deprotection conditions must be mild because of the acid lability of the TyrSO3 residues, which can lead to inefficient peptide cleavage from the solid support. The recoveries of the sulfated peptides can be improved using the acid-sensitive 2-chlorotrityl resin for the synthesis of TyrSO3-containing peptides (26).

FIG. 6. Equilibrium binding affinities of glyco(sulfo)peptides for sPS at subphysiological salt concentration. A, equilibrium binding affinity of 2-GSP-6 for sPS at subphysiological salt concentration. Equilibrium gel filtration experiments were carried out by loading 12.5, 25, 50, and 100 pmol of sPS into a gel filtration column equilibrated with [3H]2-GSP-6 in buffer (8000 cpm/ml in 20 mM MOPS, pH 7.5, containing 50 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 0.02% NaN3). The bound GSP and free sPS concentrations were calculated from the equilibrium gel filtration data, and the dissociation constant (76 nM) was derived as in Fig. 5. B, relative equilibrium binding affinities of glyco(sulfo)peptides for sPS. Experiments were carried out by loading 50 pmol of sPS into a gel filtration column equilibrated with the indicated [3H]glycosulfopeptides in buffer (see panel A). The amounts of bound peptides were calculated relative to 2-GSP-6. The specificity of binding under these conditions was validated using 1 mM EDTA instead of divalent cations in buffer or anti-P-selectin monoclonal antibody G1 (50 pmol) in Ca2+-containing buffer. All experiments except 2-GP-6 + EDTA/G1 were carried out in duplicates. The results shown are representative of duplicate measurements.

FIG. 7. Affinity chromatography of glyco(sulfo)peptides on immobilized sPS at subphysiological salt concentration. Approximately 1,300 cpm (~13 pmol) of the indicated radiolabeled monosulfated glycosulfopeptides were loaded into the P-selectin column. A, chromatography in buffer containing Ca2+ (20 mM MOPS, pH 7.5, 50 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 0.02% NaN3). B, chromatography in buffer containing EDTA (20 mM MOPS, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.02% NaN3). C, radiolabeled 2-GP-6 was loaded into the P-selectin column in buffers containing either Ca2+ or EDTA (see panels A and B). D, radiolabeled 2-GP-6 digested with Pronase was loaded into the sPS column in buffer containing Ca2+ (see panel A). When duplicate analyses were performed, elution profiles were identical.
P-selectin Binding to Sulfated Tyrosine and to Sugars in GSPs

P-selectin is ~350 nm, as determined by equilibrium gel filtration (16). Our present results show that the $K_d$ for binding of 2-GSP-6 to P-selectin is ~650 nm. The differences in the amino acid sequences between GSP-6 (23-mer) and 2-GSP-6 (18-mer) are three extra amino acids (QAT) at the N terminus and two extra amino acids (ML) at the C terminus of GSP-6 (Met is replaced with Cys in 2-GSP-6). The difference in $K_d$ between GSP-6 and 2-GSP-6 may result from small conformational and/or charge differences that make the binding of GSP-6 to P-selectin more favorable than that of 2-GSP-6.

Salt concentrations above the physiological level inhibit binding of selectins to isolated sLex tetrasaccharides, whereas low salt concentrations enhance binding (25, 27). Our present data show that lowering the NaCl concentration by 3-fold (from 150 mM to 50 mM NaCl) enhances the binding affinity of 2-GSP-6 for P-selectin by 9-fold. EDTA blocks the binding of 2-GSP-6 to P-selectin at low salt as well as at physiological salt concentrations, demonstrating the importance of Ca$^{2+}$ for binding under both conditions. EDTA also blocks the binding of nonsulfated 2-GP-6 to P-selectin, which indicates the involvement of Ca$^{2+}$ for binding of C2-O-sLe$^x$ to P-selectin. Because we were unable to detect binding of the trisulfated peptide with only a core-1 O-glycan (2-GSP-2) to P-selectin ($K_d > 30 \mu M$), we currently do not know whether the binding of TyrSO$_3$ residues to P-selectin is dependent on Ca$^{2+}$. These interactions may not require Ca$^{2+}$, since glycosaminoglycan ligands have been shown to bind P-selectin in a cation-independent manner (28).

Indirect experiments have suggested that sialic acid and fucose residues are critical monosaccharides for PSGL-1 binding to P-selectin, but the distinct contributions of sialic acid and fucose residues have not been quantitatively evaluated. Neuraminidase treatment of human leukocytes dramatically reduced, but did not eliminate, their binding to P-selectin, but it was not determined whether the residual binding was due to weak recognition of the resulting Le$^a$ determinant (8, 29, 30). Neutrophil binding to P-selectin on stimulated platelets was partially inhibited by a Le$^a$-containing oligosaccharide, but inhibition was not compared with sLe$^x$-containing oligosaccharides (31). Interestingly, treatment of HL-60 cells with high concentrations of a-fucosidase was more potent in blocking adhesion to P-selectin than treatment of the cells with neuraminidase (32). These earlier results suggested that P-selectin might bind weakly to Le$^a$-containing ligands. Our current study quantitatively validates this suggestion. The desialylated 2-GSP-6, which expresses the Le$^a$ determinant, binds to P-selectin with measurable affinity ($K_d < 10–20 \mu M$) (Fig. 8). In contrast, a sialic acid-containing glycosulfopptide without fucose (2-GSP-5) does not detectably bind to P-selectin ($K_d > 30 \mu M$). Thus, an a1,3-fucose contributes more than an a2,3-sialic acid to glycosulfopptide binding to P-selectin. Collectively, the binding studies with GSPs suggests that the lectin domain of P-selectin has a binding site for a1,3-fucose, a secondary site for a2,3-sialic acid, and a binding site for peptide determinants and TyrSO$_3$ residues.

A model depicting these stereospecific interactions is illustrated in Fig. 9. The model suggests that the position of a C2-O-sLe$^x$ relative to tyrosine sulfation and other peptide determinants has a major impact on binding of PSGL-1 to P-selectin. The stereospecific recognition of these sugar residues is consistent with our previous observation that P-selectin binds to a glycosulfopptide containing the sLe$^x$ epitope on a core-2 O-glycan, but not on an extended core-1 O-glycan, at Thr-57 (16). The location, orientation, and distance of the TyrSO$_3$ groups relative to the C2-O-sLe$^x$ in PSGL-1 are critical for optimal binding affinity. In this context it should be noted that the mature N terminus of murine PSGL-1 has only two
Tyr residues that are potential sites for sulfation (33). Furthermore, the positions of these Tyr residues relative to Thr residues that are potential sites of O-glycosylation are also very different from those of human PSGL-1. Thus, the model in Fig. 9 suggests that murine PSGL-1 may bind to human P-selectin with lower affinity than does human PSGL-1, because the stereochemistry of the interaction would be different. In our assays, binding of P-selectin to the peptide and TyrSO₄ determinants is not detectable in the absence of a C2-O-sLex. However, very low affinity binding of P-selectin to these determinants may be analogous to low affinity binding of P-selectin to the platelet glycoprotein Ib-IX-V complex, which requires multiple TyrSO₄ residues, but not glycosylation, on GPIbα (34). In shear flow, the very high density of GPIbα on platelets may allow it to support platelet translocation on P-selectin despite its low binding affinity.

The specific residues within the P-selectin lectin domain that recognize the TyrSO₄ residues, peptide determinants, and sugar residues on PSGL-1 are not known. Mutagenesis and chemical modification experiments have suggested that P-selectin requires conserved lysine residues at positions 111 and 113 to bind to sLeα-containing ligands (27, 35–38). The crystal structure of the lectin and epidermal growth factor domains of E-selectin has been determined (39), but no structure of E-selectin bound to a selectin ligand has been reported. However, the structure of an sLeα oligosaccharide bound to a chimeric form of mannose-binding protein has been determined (40).

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