Model Glycosulfoproteptides from P-selectin Glycoprotein Ligand-1 Require Tyrosine Sulfation and a Core 2-branched O-Glycan to Bind to L-selectin*

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Anne Leppänen¶, Tadayuki Yagosh, Vivianne I. Ottos, Rodger P. McEver¶§, and Richard D. Cummings¶¶

From the ¶Department of Biochemistry and Molecular Biology, Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center and the ¶¶Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104

L-selectin expressed on leukocytes is involved in lymphocyte homing to secondary lymphoid organs and leukocyte recruitment into inflamed tissue. L-selectin binds to the sulfated sialyl Lewis x (6-sulfo-sLex) epitope present on O-glycans of various glycoproteins in high endothelial venules. In addition, L-selectin interacts with the dimeric mucin P-selectin glycoprotein ligand-1 (PSGL-1) expressed on leukocytes. PSGL-1 lacks 6-sulfosialyl Lewis x but contains sulfated tyrosine residues (Tyr-SO3) at positions 46, 48, and 51 and sLex in a core 2-based glycan. This paper is available online at http://www.jbc.org

Selectins are a family of cell adhesion molecules that act in concert with their glycoconjugate ligands to regulate lymphocyte recirculation and leukocyte recruitment into inflammatory sites (1). P-selectin is expressed on activated endothelial cells and activated platelets. E-selectin is expressed on activated endothelial cells, and L-selectin is constitutively expressed on various leukocyte subtypes. L-selectin mediates lymphocyte homing into secondary lymphoid organs and neutrophil recruitment into inflamed tissue (2). High endothelial venules (HEV) of secondary lymphoid organs express various glycoproteins that are bound by L-selectin and may be involved in lymphocyte homing (3). These include GlyCAM-1 (4, 5), CD34 (6), MadCAM-1 (7), Sgp200 (8), and podocalyxin (9). L-selectin also binds to PSGL-1 present on the surfaces of other leukocytes, thus mediating leukocyte attachment to already adherent cells, and increasing overall leukocyte recruitment to inflammatory sites (10–13).

PSGL-1 is a dimeric, mucin-type glycoprotein ligand originally identified as a ligand for P-selectin (14), but PSGL-1 also interacts with L- and E-selectin (15, 16). To date, however, detailed biochemical binding studies have only been carried out for P-selectin and PSGL-1. These studies have shown that P-selectin binds to the extreme N terminus of PSGL-1 by interacting stereospecifically with clustered tyrosine sulfates (Tyr-SO3) and a nearby core 2 O-glycan with a sialyl Lewis x epitope (C2-O-sLe\(^\alpha\)) (17–19). The use of synthetic glycosulfoproteptides modeled after the N-terminal region of PSGL-1 was a key factor in elucidating the molecular requirements for P-selectin binding (17, 18, 20). By contrast, the interaction between L-selectin and PSGL-1 has been studied less directly with blocking monoclonal antibodies and site-directed mutagenesis of recombinant PSGL-1.

Early studies with blocking monoclonal antibodies indicated that L-selectin, like P-selectin, bound to the extreme N terminus of PSGL-1 (10–12, 21). This region contains sulfate on tyrosine residues but no sulfate on glycans. However, L-selectin does bind to sulfated carbohydrate ligands on various HEV glycan.
Glycosulfopeptide Binding to L-selectin

Fluorescence-based solid phase assay was performed essentially as described (20). Briefly, streptavidin-coated black 96-well microtiter plates (Pierce) were washed 3 times with 200 μl of 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2, 0.02% NaN3 (buffer A) or 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 0.02% NaN3 (buffer B) and coated for 1 h with 1–30 pmol of GSls in 50 μl of buffer A or B. The wells were then incubated for 1 h with 50 μl of P-sel-Ig chimera (30–200 μg/ml) L-selectin chimera (5–30 μg/ml), or anti-P-selectin mAb PL1 (2 or 5 μg/ml) (30) in buffer A or B containing 0.05% Tween 20 and 1% BSA. The wells were subsequently incubated for 1 h with 50 μl of 10–50 μg/ml Alexa Fluor™ 488 goat anti-human IgG (H+L) or with 50 μl of 5 or 10 μg/ml Alexa Fluor™ 488 goat anti-mouse IgG (H+L) (Molecular Probes, Inc., Eugene, OR) in buffer A or B containing 0.05% Tween 20 after gentle washing, 100 μl of buffer A or B was added to each well and the fluorescence was measured using a Victor® (Wallac, Turku, Finland) or Tecan Ultra384 (Tecan U.S., Durham, NC) microtiter plate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm. 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 of Figs. 2 and 3 were performed in duplicate, of Figs. 6 and 7 in triplicate, and the results represent averages of two or three determinations, respectively. Background fluorescence reading without peptide coating was subtracted from each sample in each experiment.

RESULTS

Synthesis of Glycosulfopeptides—To study the role of tyrosine sulfation and O-glycosylation at Thr-57 for binding of L-selectin to PSGL-1, utilizing synthetic glyco-(sulfo)peptides (GSls) modeled after the N terminus of human PSGL-1, and to measure the binding affinity between the GSls and L-selectin. To this end, we synthesized a set of GSls containing one, two, three, or no sulfated tyrosine residues and C2-O-sLeX at Thr-57. This approach not only allowed us to study the role of tyrosine sulfation but also the sterosepecific contribution of individual Tyr-SO3 residues for binding to L-selectin. We also synthesized GSls containing three Tyr-SO3 residues and a modified O-glycan at Thr-57 to study the role of specific monosaccharide residues of C2-O-sLeX, of sialylated polyfucosylated polylactosamine O-glycan and of sialyl Lewis x on extended core 1 O-glycan (C1-O-sLeX) for binding to L-selectin. The interaction of L-selectin with GSls was studied using multiple approaches, including a fluorescence-based solid phase assay, equilibrium gel filtration, and in vitro rolling experiments. Our results demonstrate that L-selectin binds with relatively high affinity to GSls that contain sulfate on all three tyrosines and that present sLeX on a core 2 rather than on an extended core 1 O-glycan.

EXPERIMENTAL PROCEDURES

Enzymatic Synthesis of Glycosulfopeptides—Glyco(sulfo)peptide precursors corresponding to amino acid residues 45–61 of human PSGL-1 with a GalNAc residue at Thr-57 and no, one, two or three Tyr-SO3 residues (Tyr-46, -48, and -51) were synthesized on an automated peptide synthesizer as described (17). The glycan at Thr-57 of each peptide was synthesized enzymatically using highly purified or recombinant glycosyltransferases. GP-4, GP-6, GP-1, GP-5, GP-6, GP(46)-6, GP(48)-6, GP(51)-6, GP(46,48)-6, GP(46,51)-6, GP(48,51)-6, and DS-GP-6 were synthesized and characterized as described (17). GP-6 and GP-6’ were synthesized and characterized as described (20). sLeX on extended core 1 in C1-GSP-6 and C1-GP-6 were synthesized as described (18). Radiolabeled [3H]GP-6 was synthesized using unlabeled GP-5 as an acceptor and GDP-[3H]Fuc (American Radiolabeled Chemicals Inc., St. Louis, MO) (specific activity 950 or 8480 cpm/pmol) as a donor in a 1:3 FucTIV (Calbiochem) reaction. [3H]GP-6 was purified from the reaction mixture by reversed phase HPLC.

Recombinant Selectin-Ig Chimeras—The vectors that were used to express soluble P- and L-selectin-Ig chimeric proteins were a gift from Dr. Ajit Varki (University of California, San Diego) (28). P- and L-selectin-Ig chimeric proteins were expressed in 293 cells and purified from the media using Protein A-Septarose as described (29). The purity and homogeneity of the purified selectin-Ig chimeras were analyzed by reducing and non-reducing SDS-PAGE, followed by Coomassie Blue staining and Western blotting. All preparations of P- and L-selectin-Ig chimeras were found to be >90% pure and homogeneously dimeric, showing a molecular weight of ~190,000.

Biotinylation of Glycosulfopeptides and Fluorescence-based Solid Phase Assay—Biotinylation of the P-terminal Cys of each GSls was performed using biotin-HDFP (N-(6-biotinamidohexyl)-3-(2-carboxy-aldehyde)dithiothreitol) (Pierce) as described (20). Biotinylated GSls 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.

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Fig. 1. The structures of glyco(sulfo)peptides used in the present study.
Glycosulfopptide Binding to L-selectin

Fig. 2. Binding of L-sel-Ig and P-sel-Ig to immobilized GSP-6 in a fluorescence-based solid phase assay. Biotinylated GSP-6 was immobilized on streptavidin-coated microtiter wells at different coating densities (see figure). Various concentrations of L-sel-Ig (A) and P-sel-Ig (B) were incubated with the immobilized GSP-6 in 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% BSA, 0.05% Tween 20, and 0.02% NaN₃. Fluorescently labeled anti-human IgG (50 µg/ml) was used to detect the bound selectin-Ig chimeras. All assays were performed in duplicate, and the results represent the average of two determinations.

Fig. 3. Binding of L-sel-Ig and P-sel-Ig to different densities of immobilized GSP-6 in a fluorescence-based solid phase assay. Biotinylated GSP-6 was immobilized on streptavidin-coated microtiter wells at different coating densities (see figure). A fixed concentration of L-sel-Ig (A, 10 µg/ml) or P-sel-Ig (B, 5 µg/ml) was incubated with the immobilized GSP-6 in 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl₂, 2 mM MgCl₂, 1% BSA, 0.05% Tween 20, and 0.02% NaN₃. Fluorescently labeled anti-human IgG (50 µg/ml) was used to detect the bound selectin-Ig chimeras. All assays were performed in duplicate, and the results represent the average of two determinations.

Cosylated but fully sulfated GSPs were derivatives of GSP-6 (DS-GSP-6, GSP-5, and GSP-1). Fully sulfated C1-GSP-6 and nonsulfated C1-GP-6 were isomers of GSP-6 and GP-6, respectively, containing the sLeα epitope on an extended core 1 branch instead of a core 2 branch.

L-selectin Binds to Immobilized GSP-6 and Binding Affinity Is Dependent on the Ligand Density—We first used a newly developed and sensitive fluorescence-based solid phase assay to compare the relative binding affinities of L-selectin and P-selectin to GSP-6. Biotinylated GSP-6 (0.25–10 pmol/well) was first captured quantitatively on streptavidin-coated 96-well plates. Different amounts of L-sel-Ig or P-sel-Ig were incubated in the wells, and bound L-sel-Ig and P-sel-Ig were detected with fluorescently labeled anti-human IgG. At the highest GSP-6 coating density (10 pmol/well), binding of L-sel-Ig to GSP-6 increased linearly with increased concentration of L-sel-Ig, reaching a plateau at 10–30 µg/ml of L-sel-Ig (Fig. 2A). However, at lower GSP-6 densities, no plateau was observed even at the highest concentrations of L-sel-Ig. This shows that the affinity of L-sel-Ig for immobilized GSP-6 is dependent on the ligand density. By contrast, P-sel-Ig generated a saturated binding curve with all GSP-6 coating densities used (Fig. 2B), indicating that the affinity of P-sel-Ig for GSP-6 is less dependent on the density of the immobilized ligand.

L-selectin Binds to Immobilized GSP-6 with ~10-fold Reduced Affinity Compared with P-selectin at Physiological Salt Concentration—Binding affinities of L-sel-Ig and P-sel-Ig for immobilized GSP-6 were first compared using the fluorescence-based solid phase assay. Biotinylated GSP-6 was immobilized on streptavidin-coated microtiter plates at different densities, and fixed concentrations of L-sel-Ig (10 µg/ml) and P-sel-Ig (5 µg/ml) were incubated with wells containing varying amounts of GSP-6. Binding of L-sel-Ig to increasing densities of GSP-6 formed a semi-sigmoidal binding curve (Fig. 3A), suggesting that L-sel-Ig binding to immobilized GSP-6 may be cooperative. By contrast, P-sel-Ig bound to GSP-6 forming a typical rectangular hyperbola binding curve (Fig. 3B). Comparison of the GSP-6 densities that give half-maximal binding for L-sel-Ig (~10 pmol) and P-sel-Ig (~1 pmol) indicates that L-sel-Ig has...
In contrast, binding to the isomers of disulfated GSPs was not as strong as to the monosulfated GSPs, and the affinity of L-sel-Ig to GSP-6 was 10-25-fold lower than the affinity of P-sel-Ig for GSP-6. This is in good agreement with the results from the solid phase assay which indicates that L-sel-Ig binds to immobilized GSP-6 with 10-fold lower affinity than P-sel-Ig (Fig. 3A). Taken together, the results of the solid phase and equilibrium gel filtration binding experiments demonstrate that L-sel-Ig binds to GSP-6 with relatively high affinity ($K_d$ between 2 and 5 μM) under physiological conditions.

The Binding of L-selectin to GSP-6 Is Highly Dependent on Tyrosine Sulfation—The role of tyrosine sulfation and the positional importance of Tyr-SO$_3^-$ residues of GSP-6 for binding of L-selectin to GSP-6 was studied using the fluorescence-based solid phase assay. Equimolar amounts of different biotinylated GSPs (10 pmol/well in experiments with L-sel-Ig and 1 pmol/well with P-sel-Ig) were immobilized on streptavidin-coated 96-well plates. Fixed concentrations of L-sel-Ig (5 μg/ml) and P-sel-Ig (1 μg/ml) were incubated in the wells in the presence of either Ca$^{2+}$ or EDTA, and bound L-sel-Ig and P-sel-Ig were detected with fluorescently labeled anti-human IgG. L-sel-Ig showed high affinity binding to GSP-6 containing three Tyr-OSO$_3^-$ residues and very weak binding to nonsulfated GP-6 (Fig. 6A). L-sel-Ig did not show clear preferential binding to any isomer of the monosulfated GSPs, and the affinity of L-sel-Ig to monosulfated GSPs was 6-11% relative to GSP-6 (Fig. 6A). In contrast, binding to the isomers of disulfated GSPs was not equal, and L-sel-Ig preferred binding to GSP(48,51)-6 with 50% affinity relative to GSP-6. Binding to the other two isomers of disulfated GSPs was weaker, 15-19% relative to GSP-6. In agreement with earlier data (17, 19), P-sel-Ig pre-
L-sel-Ig binding

A

Ca\(^{2+}\)

5 mM EDTA

RFU

GSP-6

GSP-4

GSP-3

GSP-2

GSP-1

GSP-0

B

Ca\(^{2+}\)

5 mM EDTA

RFU

GSP-6

GSP-4

GSP-3

GSP-2

GSP-1

GSP-0

Fig. 6. Comparison of binding of L-sel-Ig and P-sel-Ig to different immobilized glyco(sulfo)peptides in a fluorescence-based solid phase assay. Biotinylated glyco(sulfo)peptides were immobilized on streptavidin-coated microwells (A, 10 pmol/well; B, 1 pmol/well). L-sel-Ig (A, 10 μg/ml) or P-sel-Ig (B, 1 μg/ml) was incubated with the immobilized GSPs in either 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 2 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1% BSA, 0.05% Tween 20, and 0.02% NaN\(_3\), (light gray bars) or in 20 mM MOPS, pH 7.5, containing 150 mM NaCl, 5 mM EDTA, 1% BSA, 0.05% Tween 20, and 0.02% NaN\(_3\), (dark gray bars). Fluorescently labeled anti-human IgG was used for detection. Monoclonal antibody PL1 was used to confirm that equal amounts of each GSP was immobilized on microwells (not shown). The data of panel A is from one representative experiment of three independent experiments. All assays were performed in triplicate, and the results represent the mean ± S.D. of three determinations.

L-sel-Ig was more efficiently bound to GSPs containing tyrosine sulfate at position 48 (Fig. 6B). Moreover, P-sel-Ig bound more strongly to disulfated GSPs than to monosulfated GSPs, which is in agreement with earlier equilibrium gel filtration data (Fig. 6B and Ref. 17). Binding of L-sel-Ig and P-sel-Ig to all GSPs was strictly Ca\(^{2+}\)-dependent, because 5 mM EDTA completely inhibited binding (Fig. 6). Taken together, these results indicate that GSP-6 requires multiple sulfated tyrosine residues to bind with high affinity to L-selectin. L-selectin binds to mono- and disulfated GSPs with reduced affinity but binding to one disulfated iso-mer, GSP(48,51)-6, is stronger than to other disulfated GSPs or monosulfated GSPs.

The Binding of L-selectin to GSP-6 Is Highly Dependent on Fucose and Sialic Acid Residues—The role of fucose and sialic acid residues of GSP-6 for binding to L-selectin and P-selectin was studied using the fluorescence-based solid phase assay. L-sel-Ig bound to desialylated GSP-6 (DS-GSP-6) and nonsialylated GSP-5 with ~3–6% affinity relative to GSP-6 (Fig. 6A). This low residual binding of the L-sel-Ig is likely toward the sulfated peptide backbone of DS-GSP-6 and GSP-5, because L-sel-Ig showed similar binding to GSP-1 compared with both DS-GSP-6 and GSP-5. By comparison, P-sel-Ig bound to DS-GSP-6 with ~7% affinity relative to GSP-6, whereas binding to GSP-5 was undetectable (Fig. 6B). This confirms our earlier results showing that fucose is more important than sialic acid for P-selectin binding to GSP-6 (17). Our present results show that fucose and sialic acid residues of GSP-6 are equally important for L-selectin binding.

L-selectin Binds to GSPs Containing Sialylated Polysulfated Polyfucosylated O-Glycan with Low Affinity—Most of the fucosylated O-glycans on PSGL-1 from human HLL-60 cells contain a core 2-based sialylated polysulfated polyfucosylated (PFPL). We recently showed that P-selectin binds very poorly to GSPs containing the PFPL structure. Instead, a monosulfated and monosialylated core 2 O-glycan is required for high affinity recognition by P-selectin (20). However, it is possible that L-selectin might prefer such PFPL-containing O-glycans. The potential recognition of sialylated PFPL O-glycans by L-selectin was studied using a fluorescence-based solid phase assay and the constructs GSP-6’ and GSP-6” (Fig. 6B). Both GSP-6’ and GSP-6” contain three Tyr-SO\(_3\) residues, but they differ in the length of the PFPL chains, which contain two (C2-O-Le\(^3\)-SlE\(^3\)) or three (C2-O-Le\(^4\)-SlE\(^3\)) fucosylated lactosamine repeats, respectively, at Thr-57. L-sel-Ig showed weak but detectable affinity for GSP-6” and GSP-6” (8–12% relative to GSP-6) (Fig. 6A). Binding to GSP-6” was slightly better than to GSP-6”. Binding of P-sel-Ig to GSP-6” was very weak and to GSP-6” undetectable under these conditions, confirming our earlier results that P-selectin binds to GSP-6” and GSP-6” with very low affinity (Fig. 6B and Ref. 20).

L-selectin Binds with Low Affinity to a Glycosulfopeptide Presenting sLe\(^\text{x}\) on an Extended Core 1 O-Glycan—The role of the O-glycan core structure of GSPs for binding to L-selectin was evaluated using the fluorescence-based solid phase assay. Fully sulfated C1-GSP-6 and nonsulfated C1-GP-6 containing the sLe\(^\text{x}\) epitope on an extended core 1 O-glycan (C1-O-SLe\(^\text{x}\)) at Thr-57 were immobilized on streptavidin-coated microwells. The interactions of L-sel-Ig and P-sel-Ig with C1-GSP-6 and C1-GP-6 were compared with the isomeric structures GSP-6 and GP-6 containing the sLe\(^\text{x}\) epitope on a core 2 O-glycan (C2-O-SLe\(^\text{x}\)) at Thr-57 (Fig. 7). L-sel-Ig showed weak but detectable affinity for fully sulfated C1-GSP-6 (~6.4% relative to GSP-6) but undetectable binding to nonsulfated C1-GP-6 as well as to GP-6 and GSP-1 (Fig. 7A). By comparison, P-sel-Ig did not bind detectably to C1-GSP-6, C1-GP-6, and GSP-1 and bound only weakly to GP-6 (Fig. 7B). The poor interaction of P-selectin with C1-GSP-6 is consistent with our earlier results employing affinity chromatography (18). These data show that L-selectin binds only weakly to C1-GSP-6. This weak binding is dependent on tyrosine sulfation of the glycopeptide.

Rolling of Neutrophils on Glyco(sulfo)peptides—A recent study reported that L-selectin-expressing cells rolled much better on COS cells expressing recombinant PSGL-1 if the latter cells also expressed a sulfotransferase for carbohydrate 6-sulfation. Under these conditions, mutation of the three N-terminal tyrosines of PSGL-1 did not inhibit rolling. The authors concluded that tyrosine sulfation of PSGL-1 was not required to support rolling interactions with L-selectin (24). To directly address the molecular requirements for PSGL-1 to support L-selectin-dependent leukocyte rolling under flow, we perfused human neutrophils over streptavidin-captured, biotinylated GSP-6 in a parallel flow chamber at different wall shear stresses. Neutrophils required a minimum wall shear stress to roll, with peak accumulation at 1 dyn/cm\(^2\) (Fig. 8A). This shear threshold for L-selectin-dependent rolling closely resembled the shear threshold observed on other L-selectin ligands (27, 36). Anti-L-selectin mAb DREG-56 or anti-PSGL-1 mAb PL1,
but not control mAbs, inhibited rolling, demonstrating the specificity of the interactions (Fig. 8B). Rolling neutrophils did not accumulate on similar densities of GP-6, which lacks sulfate on tyrosines, or of GSP-1, which contains sulfated tyrosines but not C2-O-sLex (Fig. 8B), although neutrophils formed some transient tethers to these structures (data not shown). All assays were performed in triplicate, and the results represent the mean ± S.D. of three determinations.

**DISCUSSION**

Earlier studies suggested that L-selectin binds to leukocytes and that sulfation of leukocyte ligands is important for their interactions with L-selectin. Pretreatment of leukocyte cell lines with chlorate, an inhibitor of PAPS biosynthesis that is required for sulfation of macromolecules, blocked cell binding to L-selectin (12). Also, treatment of cells with either neuraminidase or O-sialoglycoprotein endopeptidase, a protease that cleaves sialylated mucins, abrogated cell binding to L-selectin (12), indicating that in addition to sulfation, sialic acid and a mucin-type glycoprotein ligand on cells are required for L-selectin recognition. Independently, it was shown that PSGL-1, a minor surface mucin on human neutrophils first identified as a potential ligand for P-selectin, can also bind to L-selectin (10) and that leukocyte binding to L-selectin was blocked by the monoclonal antibody PL1 (10, 11), which recognizes the extreme N-terminal domain of mature PSGL-1 and blocks binding of PSGL-1 to P-selectin (30, 35). These earlier results strongly suggested that P- and L-selectin bind to the extreme N-terminal region of PSGL-1.

The extreme N terminus of human PSGL-1 contains Thr residues at positions −44 and −57, both potential sites for O-glycan addition, which are near three potential sites for tyrosine sulfation at positions −46, −48, and −51 (37, 38). Enzymatic removal of sulfate from Tyr residues in intact PSGL-1 (39) or Tyr replacement by site-directed mutagenesis of recombinant PSGL-1 (37, 40) demonstrated that at least one of the Tyr residues at positions −46, −48, or −51, is required for binding of recombinant PSGL-1 to P-selectin. Similarly,
site-directed mutagenesis indicated that the Thr at position −57, but not −44, is essential for PSGL-1 recognition by P-selectin (41). Using synthetic glycosulfopeptides, we previously demonstrated that all three Tyr-SO₃ and a core 2-based O-glycan with a sLeα determinant at Thr-57 are required for high affinity binding to P-selectin (18). Site-directed mutagenesis revealed that PSGL-1 requires at least one Tyr sulfate residue to bind to L-selectin (25, 27). These studies strongly suggested, but did not directly prove, that tyrosine sulfation of PSGL-1 is required for significant binding to L-selectin.

To more precisely explore the specificity and binding affinity of L-selectin for determinants within the extreme N-terminal domain of PSGL-1, we synthesized a large set of glycosulfopeptides modeled after that domain that varied in degree and position of sulfation of tyrosine and in the type and structure of the O-glycan Thr-57. Our studies indicate that three Tyr-SO₃ residues at positions −46, −48, and −51 and a sLeα determinant on a core 2 but not a core 1 O-glycan are required for high affinity binding to L-selectin. The observed Kₘ of binding of the optimal GSP-6 to L-selectin was ~5 μM. This represents a relatively high affinity interaction and compares very favorably to the high affinity binding of this glycopeptide to P-selectin (Kₘ = 650 nM) (17). Very weak binding of L-selectin was observed to glycosulfopeptides lacking Tyr-SO₃ and to glycosulfopeptides containing a single Tyr sulfate residue at any position. We did not observe preferential binding of any of the monosulfated glycosulfopeptides to L-selectin. This agrees with a previous study in which the different single Tyr forms of recombinant PSGL-1 supported similar L-selectin-dependent cell rolling (24). These combined results do not support the suggestion that PSGL-1 preferentially uses Tyr-51 to support L-selectin-dependent rolling (25). Interestingly, an isomeric disulfated GSP containing Tyr-SO₃ at positions −48 and −51 bound more strongly than other disulfated isomers. By contrast, sulfation of Tyr-48 contributes relatively more than sulfation of Tyr-46 or Tyr-51 to binding of glycosulfopeptides to P-selectin (17, 19).

Engineered Chinese hamster ovary cells expressing the recombinant 6-sulfotransferase that generates the GlcNAc-6-O-sulfate within the sLeα determinant (6-sulfo-sLeα) bind to L-selectin independently of PSGL-1 (24). This study of transfected cells confirms the ability of L-selectin to bind to the 6-sulfo-sLeα determinant on mucins expressed by lymph node HEV (42, 43). However, human leukocytes lack the 6-sulfotransferase and do not express appreciable amounts of 6-sulfo-sLeα determinants (44). Moreover, sulfate residues within human PSGL-1 are expressed primarily in tyrosine sulfate rather than on sulfated glycans (39, 45). Thus, tyrosine sulfation is critically important for leukocyte PSGL-1 to interact with L-selectin. Our results with sulfated and nonsulfated glycopeptides modeled after PSGL-1 definitively demonstrate the importance of tyrosine sulfation for L-selectin binding in a system that lacks 6-sulfo-sLeα glycan determinants. Direct binding studies reveal that human L-selectin binds to GSP-6 with a Kₘ in the range of ~5 μM, which is especially interesting in light of previous studies showing that monomeric L-selectin binds to immobilized GlyCAM-1 with a much lower Kₘ of ~108 μM (46). HEV mucin selectin ligands such as GlyCAM-1 and CD34 contain multiple copies of the 6-sulfo-sLeα epitopes that appear to increase avidity, which may compensate for the low affinity of such determinants for L-selectin. We also observed that binding of L-selectin, but not P-selectin, to the immobilized glycosulfopeptides is affected by ligand density (Figs. 2 and 3). While it is premature to speculate on the biological significance of this observation at present, the differential effects of ligand density might relate to differential association and dissociation kinetics of L-selectin binding compared with P-selectin. The kinetics of L-selectin binding to the glycosulfopeptides could be very rapid and it may be advantageous for L-selectin to bind cooperatively to the multiple determinants within glycosulfopeptides to stabilize relatively labile interactions. Whether this binding feature of L-selectin relates to its role in shear-dependent rolling of leukocytes as observed in Fig. 8 is not known. It will be interesting in the future to prepare homogeneous glycopeptides modeled after PSGL-1 and other mucins known to be bound by L-selectin, in which sulfate residues are presented in glycan moieties and/or in tyrosine residues, and directly measure their binding affinity and kinetics to L-selectin. It is possible that some HEV mucins express Tyr-SO₃-containing glycoconjugates that function as L-selectin ligands, as noted for endoglycan, an endothelial CD34 family member that contains two potential N-terminal tyrosine sulfation sites (47).

We observed that expression of the sLeα determinant on a core 2, but not a core 1, O-glycan is required for high affinity binding of glycosulfopeptides to L-selectin, as noted earlier for binding to P-selectin (18). Unlike binding to P-selectin, small residual binding of C1-GSP-6 to L-selectin was observed (Fig. 9). Our findings provide direct support for previous strong but indirect evidence that core 2-based O-glycans are important for recognition by both P- and L-selectin. To bind P- or L-selectin with high affinity, recombinant PSGL-1 requires co-expression with an α1,3-fucosyltransferase and the core 2 β1,6-N-acetylgalactosaminyltransferase (Core2GlcNAcT-I) that forms the core 2 O-glycan from the core 1 O-glycan precursor (25, 45). Mice lacking the gene encoding Core2GlcNAcT-I have significantly reduced neutrophil rolling on E-, L-, and P-selectins and reduced neutrophil recruitment to sites of inflammation (48). A recent study reported that human neutrophils and lymphocytes expressed low levels of transcripts encoding β1,3GlcNAcT-3, an enzyme that extends core 1-based O-glycans (26). Chinese hamster ovary cells expressing recombinant PSGL-1 and FucT-VII, plus either β1,3GlcNAcT-3 or Core2GlcNAcT-I, acquired sLeα determinants on O-glycans. Furthermore, cells expressing PSGL-1 and sLeα on extended core 1 O-glycans supported L-selectin-dependent tethering and rolling of neutrophils and lymphocytes, although much less well than Chinese hamster ovary cells expressing PSGL-1 and sLeα in core 2-branched O-glycans. Although these results suggested that sLeα in extended core 1 O-glycans can support L-selectin-dependent rolling, the specific contribution of PSGL-1 to this rolling was not tested (26). Regardless, it is questionable whether extended core 1 O-glycans on leukocytes function as physiologically relevant selectin ligands. Extended core 1 O-glycans have not been detected in either human leukocytes (49) or in the human promyelocytic leukemic cell line HL-60 (50, 51). Furthermore, leukocytes from Core2GlcNAcT-I-deficient mice have severe defects in rolling on L- and P-selectin (48). Our finding that L-selectin binds much better to a PSGL-1-derived glycosulfopeptide with sLeα presented on a core 2 O-glycan rather than on an extended core 1 O-glycan strongly supports previous structural and functional evidence for the importance of leukocyte core 2 O-glycans for binding to L-selectin.

L-selectin also binds to asubpopulation of PSGL-1 molecules carrying a sulfated polylactosamine, known as the PEN5 epitope, expressed on activated NK cells (52). This binding was considered to be independent of tyrosine sulfation, based on the ability of NK cells to tether and roll on L-selectin after monocarboxylin treatment, a snake venom protease that cleaves the extreme N terminus of PSGL-1 and eliminates binding to P-selectin (12, 53). We found that the unsulfated polylactosamine structures in the glycosulfopeptides GSP-6 and GSP-6α supported only low affinity, albeit detectable, binding to L-selectin.

Glycosulfopeptide Binding to L-selectin
P-selectin also binds to some extent to 6-sulfo-sLe^x (54). The glyco(sulfo)peptides shown in Figs. 6 and 7 where background (no coating, typically 200 as a reference (100% binding). The data are derived from experiments GSPs were derived from fluorescence-based solid phase assay using GSP-6 PSGL-1-derived glycosulfopeptide indicates that Tyr-SO_3

- sulfation of GlcNAc residues
- and sLex expressed on a core 2-based carbohydrate sulfation together with tyrosine sulfation promote even higher affinity binding of GSPs to L-selectin.

In summary, our results directly demonstrate that both P- and L-selectin require dual recognition of Tyr-SO_3 residues and sLe^x expressed on a core 2-based O-glycan at Thr-57 for high affinity binding to PSGL-1. However, P-selectin binds to the same glycosulfopeptide (GSP-6) with ~10-fold higher affinity than L-selectin. It is interesting that L-selectin is able to recognize sulfate residues both within tyrosine-sulfated peptides and in sulfated GlcNAc residues such as 6-sulfo-sLe^x. P-selectin also binds to some extent to 6-sulfo-sLe^x (54). The crystallographic structure of P-selectin complexed with a PSGL-1-derived glycosulfopeptide indicates that Tyr-SO_3 residues 48 and 51 form direct contacts with the lectin domain of P-selectin (19). Crystal structures of L-selectin complexed with the sulfated tyrosine-containing glycosulfopeptides described here and with glycans containing sulfate in 6-sulfo-sLe^x will provide further insights into how L-selectin can bind different forms of sulfate in cooperation with sLe^x.

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![Fig. 9](image-url)
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Model Glycosulfopeptides from P-selectin Glycoprotein Ligand-1 Require Tyrosine Sulfation and a Core 2-branched O-Glycan to Bind to L-selectin
Anne Leppänen, Tadayuki Yago, Vivianne I. Otto, Rodger P. McEver and Richard D. Cummings

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