Molecular Basis of Leukocyte Rolling on PSGL-1

PREDOMINANT ROLE OF CORE-2 O-GLYCANS AND OF TYROSINE SULFATE RESIDUE 51*

Michael Pierre Bernimoulin†, Xian-Lu Zeng‡, Claire Abbàl*, Sylvain Giraud*, Manuel Martinez†, Olivier Michielin‡‡, Marc Schapira‡, and Olivier Spertini‡‡

From the †Division and Central Laboratory of Hematology, Centre Hospitalier Universitaire Vaudois, Bugnon 46, 1011 Lausanne, Switzerland, the ‡Ludwig Institute for Cancer Research, Lausanne Branch, chemin des Boveresses 155, Epalinges, and the *Swiss Institute for Bioinformatics, chemin des Boveresses 155, Epalinges, Switzerland

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Interactions between the leukocyte adhesion receptor L-selectin and P-selectin glycoprotein ligand-1 play an important role in regulating the inflammatory response by mediating leukocyte tethering and rolling on adherent leukocytes. In this study, we have examined the effect of post-translational modifications of PSGL-1 including Tyr sulfation and presentation of sialylated and fucosylated O-glycans for L-selectin binding. The functional importance of these modifications was determined by analyzing soluble L-selectin binding and leukocyte rolling on CHO cells expressing various glycoforms of PSGL-1 or mutant PSGL-1 targeted at N-terminal Thr or Tyr residues. Simultaneous expression of core-2 β1,6-N-acetylglucosaminyltransferase and fuscosyltransferase VII was required for optimal L-selectin binding to PSGL-1. Substitution of Thr-57 by Ala but not of Thr-44, strongly decreased L-selectin binding and leukocyte rolling on PSGL-1. Substitution of Tyr by Phe revealed that PSGL-1 Tyr-51 plays a predominant role in mediating L-selectin binding and leukocyte rolling whereas Tyr-48 has a minor role, an observation that contrasts with the pattern seen for the interactions between PSGL-1 and P-selectin where Tyr-48 plays a key role. Molecular modeling analysis of L-selectin and P-selectin interactions with PSGL-1 further supported these observations. Additional experiments showed that core-2 O-glycans attached to Thr-57 were also of critical importance in regulating the velocity and stability of leukocyte rolling. These observations pinpoint the structural characteristics of PSGL-1 that are required for optimal interactions with L-selectin and may be responsible for the specific kinetic and mechanical bond properties of the L-selectin-PSGL-1 adhesion receptor-counterreceptor pair.

Selectins play a major role in regulating leukocyte migration in inflammatory lesions by mediating leukocyte rolling along vascular wall at site of inflammation (1–8). L-selectin is expressed by most leukocytes whereas P-selectin and E-selectin expression is induced on activated platelets and/or endothelial cells (1, 2, 4, 5, 7, 9). E-, P-, and L-selectin function at different sites in the vascular wall at site of inflammation (1–8). L-selectin is expressed at the earliest phase of inflammation (11–13). Several studies have indicated that L-selectin mediates both primary leukocyte-endothelial interactions (14, 15) and secondary interactions between circulating and adherent leukocytes, which both participate in leukocyte recruitment in inflammatory lesions. Secondary interactions are mainly supported by the interaction of PSGL-1 with L-selectin (16–18).

PSGL-1 is a mucin-like glycoprotein expressed as a homodimer on leukocyte microvilli (4, 19–21). P-selectin binds with relatively high affinity (Kd ~300 nM) (22) to PSGL-1 by reacting with N-terminal tyrosine sulfate residues and with the sLex tetrasaccharide determinants presented by core-2 O-glycans attached to Thr-57 (23–33). Binding studies performed with glycosulfopeptides indicated a contribution of each tyrosine sulfate residue in supporting P-selectin binding with a predominant role of Tyr-48 (34). The molecular contacts between P-selectin and PSGL-1 were identified by the analysis of the crystal structure of P-selectin co-complexed with the N-terminal peptide of PSGL-1 (23). These studies revealed the involvement of Tyr-48 and -51, but no interaction was observed between P-selectin and Tyr-46 (23).

Previous observations indicated the involvement of O-glycans attached to Thr-57 and tyrosine sulfate residues in supporting L-selectin- and P-selectin-mediated rolling (35). In the present study, we characterized the PSGL-1 determinants that interact with L-selectin. Adhesion studies indicated that O-glycosylation of Thr-57 and sulfation of Tyr-46 and -51 play a critical role in supporting recombinant L-selectin binding to PSGL-1 and leukocyte rolling on PSGL-1. In addition, these determinants were shown to play a major role in stabilizing rolling velocity, a key feature for the regulation of leukocyte exposure to chemotactic stimuli that lead to cell arrest and firm adhesion. By contrast, Tyr-48 played only a minor role in supporting L-selectin-mediated adhesion whereas it was shown to be critical for P-selectin-mediated interactions with PSGL-1 (23, 34).

EXPERIMENTAL PROCEDURES

Antibodies and Chimeric Selectins—The anti-L-selectin monoclonal antibodies (mAbs) LAM1–3, LAM-14 (36), HECA–452 (ATCC HB 11485), and CSLEX-1 (ATCC number: HB-10135) were purified from hybridoma culture medium. mAbs PL1 and PL2 were purchased from Coulter Immunotech (Marseille, France) and KPL1 from BD Bioscience PharMingen (Heidelberg, Germany). L-selectin/FcM heavy chain (L-FcM) was purified from CHO cells transfected with L-FcM DNA by protein A affinity chromatography (22). Selectins were purified from histidine tagged proteins and concentrated using Xcess SP Midi (Amersham Biosciences). P-selectin was purified from plasma of P-selectin transgenic mice by protein A affinity chromatography and concentrated using Xcess SP Midi (Amersham Biosciences). L-selectin/IgM heavy chain (L-FcM) was purified from CHO cells transfected with L-FcM DNA by protein A affinity chromatography (22). Selectins were purified from histidine tagged proteins and concentrated using Xcess SP Midi (Amersham Biosciences).
<p>Molecular Basis of PSGL-1 Interactions with L-selectin</p>

**Table 1. N-terminal amino acid sequences of wild-type and mutant PSGL-1.** The first 21 amino acids of mature PSGL-1 are indicated. Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.</p>

| Wild-type PSGL-1 | PSGL-1 Y46F | PSGL-1 Y46F | PSGL-1 Y46F | PSGL-1 Y46F | PSGL-1 Y46F | PSGL-1 Y46F | PSGL-1 Y46F |
|------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM | GATEY EYLDV DFLP ETEPPEM |

**Fig. 1.** Terminal amino acid sequences of wild-type and mutant PSGL-1. The first 21 amino acids of mature PSGL-1 are indicated. Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 2.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 3.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 4.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 5.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 6.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 7.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 8.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 9.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 10.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 11.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 12.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 13.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 14.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 15.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 16.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 17.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.

**Fig. 18.** Amino acid substitutions at Tyr-46, -48, and -51 by Phe and at Thr-57 and -44 by Ala are shown in bold.
cells every 0.002 a are illustrated in Figs. 7a and 8a and were used to assess the mean velocity ± S.D. of each tracked cell over 2–5 s observation periods. The mean velocity of frame-by-frame tracked cells was included between percentile 25–75 of the velocity of each cell population illustrated in Fig. 6. The S.D. value of the mean velocity of each tracked cell was then used to calculate the mean ± S.D. of cell-rolling velocities of each cell population. The mean ± S.D. was used as an indicator of the variation of cell-rolling velocity. 373–1477 independent determinations of frame-by-frame velocity were measured for each tested conditions.

The requirement in Sialylated, Fucosylated, Core-2 O-Glycans Are Essential to Support Leukocyte Rolling on PSGL-1—The requirement in sLe\(^\text{a}\)/CLA and core-2 O-glycans to support L-selectin-mediated interactions with PSGL-1 was examined by analyzing L-selectin/-μ chimera binding and leukocyte rolling on CHO cells co-transfected with PSGL-1 cDNA in pcDNA3.1 vector and/or FucT-VII cDNA in pZeo SV and/or C2GnT and FucT-VII cDNAs in pIREs Zeo SV vector. Five different transfecants containing cDNA sequences of (1) FucT-VII alone, (2) C2GnT and FucT-VII, (3) PSGL-1 alone, (4) PSGL-1 and FucT-VII, or (5) PSGL-1, C2GnT and FucT-VII were obtained. CHO cells stably expressed similar levels of PSGL-1 and/or sLe\(^\text{a}\)/CLA as ascertained by determination of antibody binding site density (mean ± S.D.: 247 ± 19 PL2 binding sites/μm\(^2\)) and immunostaining of CHO cell monolayers with mAbs PL2 (anti-PSGL-1), CSLEX-1 (anti-sLe\(^\text{a}\)), or HECA-452 (anti-CLA) (Fig. 2a). L-selectin/-μ chimera weakly interacted with CHO cells expressing FucT-VII cDNA alone or co-expressing C2GnT and FucT-VII cDNAs (Fig. 2b, upper panels). L-selectin/-μ bound to a much higher percentage of CHO cells when PSGL-1 was co-expressed with both FucT-VII and C2GnT (Fig. 2b, lower right panel).

Neutrophil rolling was studied under flow conditions at a constant shear stress of 1.25 dyn/cm\(^2\). Mock-transfected CHO cells or CHO cells transfected with PSGL-1 cDNA alone did not support neutrophil rolling (mean number of rolling cells/min/μm\(^2\) ± S.E.: 1 ± 0.3, n = 4 (not illustrated) versus 1 ± 0, n = 4). Although CHO cells transfected with the pIREs vector containing C2GnT and FucT-VII cDNA sequences expressed sLe\(^\text{a}\)/CLA (Fig. 2a) and bound L-selectin/-μ (Fig. 2b), the expression of C2GnT and FucT-VII was not sufficient to confer to CHO cells the ability to support neutrophil rolling (3 ± 2 rolling cells/min/μm\(^2\), n = 4, Fig. 3a).

Interestingly, neutrophil rolling was observed on CHO cells co-transfected with PSGL-1 and FucT-VII cDNAs even in the absence of C2GnT expression (64 ± 18 rolling cells/min/μm\(^2\), n = 5, p = 0.02, Fig. 3a, shaded box). The presentation of sLe\(^\text{a}\)/CLA residues at the termini of core-2 O-glycans attached to PSGL-1 strongly increased neutrophil recruitment at the surface of CHO cells. Neutrophil rolling on CHO cells co-expressing PSGL-1, C2GnT, and FucT-VII cDNA sequences was increased by 6-fold over the data obtained without C2GnT expression (416 ± 45 rolling neutrophils/min/μm\(^2\) versus 64 ± 48 rolling cells/min/μm\(^2\), n = 5, p = 0.0008, Fig. 3a, black box).
This observation emphasizes the key role played by core-2 O-glycans in supporting PSGL-1 binding to L-selectin. The L-selectin specificity of this interaction was indicated by the complete inhibition of neutrophil rolling in presence of LAM1, another potential site of Tyr residues by Phe (Fig. 5 lower panels). This observation strongly suggests that Thr-44 plays a major role in supporting neutrophil tethering and rolling on CHO cells expressing PSGL-1 Y46F/Y48F; Fig. 4a, left and center panels) whereas it only weakly bound to PSGL-1 T57A (Fig. 4a, right panel). L-selectin/µ binding was not completely inhibited by the replacement of Thr-57 by Ala indicating that additional structures support L-selectin/µ binding to PSGL-1. The N-terminal tyrosine sulfation consensus is the most likely alternate potential binding site. Sialyl Leα/CLA determinants expressed at the surface of CHO cells may also play a role (Fig. 2b, upper panel).

Replacement of Thr-44 by Ala did not impair neutrophil rolling on CHO-PSGL-1 T44A cells (335 < 28, n = 4 versus 322 ± 58 rolling neutrophils/mm²/min, n = 9) whereas Thr-57 replacement by Ala decreased neutrophil rolling by 74% (335 ± 28 versus 7 ± 2 rolling neutrophils/mm²/min, n = 8; p < 0.001). These observations indicate that O-glycans attached to Thr-57 play a major role in supporting neutrophil tethering and rolling on PSGL-1 (Fig. 4b). Similar results were obtained with peripheral blood lymphocytes. The replacement of Thr-57 by Ala decreased lymphocyte recruitment on CHO-PSGL-1 cells by 74 ± 3% (1596 ± 177 versus 412 ± 43 rolling lymphocytes/mm²/min, n = 4, p < 0.001, not shown).

Regulation of L-selectin Interaction with PSGL-1 by N-terminal Tyrosine Sulfate Residues—Point mutations were introduced in PSGL-1 cDNA to exchange Tyr-46, -48 and -51 by Phe and Thr-57 by Ala. Five constructs (PSGL-1 Y46F/Y48F; PSGL-1 Y46F/Y51F, PSGL-1 Y48F/Y51F, PSGL-1 Y46F/Y48F/Y51F, and PSGL-1 Y46F/Y48F/Y51F/T57A; Fig. 1) were stably co-expressed in CHO cells with piresZeo SV vector containing C2GnT/FucT-VII cDNA sequences. CHO cells exhibited similar levels of PSGL-1 (mean ± S.D.: 115 ± 37 PL2 binding sites/μm²), sLeα, and CLA (not shown). L-selectin/µ strongly bound to CHO cells co-expressing C2GnT/FucT-VII and wild-type PSGL-1 cDNAs (Fig. 5a, upper left panel). The interaction of L-selectin/µ chimera was reduced by the replacement of two Tyr residues by Phe (Fig. 5a, lower panel). Interestingly, L-selectin/µ bound more efficiently to mutant PSGL-1

O-Glycans Attached to Thr-57 Are Essential to Support L-selectin Binding and Neutrophil Rolling on PSGL-1—The role of core-2 O-glycans attached to Thr-57 was evaluated by replacing Thr-57 by Ala (PSGL-1 T57A, Fig. 1). Similarly, Thr-44, another potential site of O-glycosylation, was replaced by Ala (PSGL-1 T44A). CHO cells stably expressed similar levels of PSGL-1 (mean ± S.D.: 115 ± 37 PL2 binding sites/μm²), sLeα, and CLA. L-selectin/µ strongly reacted with wild-type PSGL-1 or PSGL-1 T44A (Fig. 4a, right panel). L-selectin/µ binding was not completely inhibited by the replacement of Thr-57 by Ala indicating that additional structures support L-selectin/µ binding to PSGL-1. The N-terminal tyrosine sulfation consensus is the most likely alternate potential binding site. Sialyl Leα/CLA determinants expressed at the surface of CHO cells may also play a role (Fig. 2b, upper panel).

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**Fig. 3.** Role of core-2 O-glycans and sLeα/CLA in regulating neutrophil recruitment and velocity on PSGL-1 expressing cells: a, neutrophils were perfused under a constant shear stress of 1.25 dyn/cm² on CHO cells stably co-transfected with C2GnT/FucT-VII/PSGL-1 cDNAs, as indicated. Neutrophil rolling was analyzed by video-microscopy at 2–4 min of perfusion. Results represent the mean ± S.E. of four experiments. b, velocity of neutrophil rolling on CHO cells stably co-transfected with C2GnT/FucT-VII cDNAs with or without PSGL-1 cDNA, after 2–4 min of perfusion. Curves were constructed using 720 independent determinations of rolling velocity and are representative of four experiments.
expressing a single tyrosine residue at position 46 or 51 (Fig. 5a, lower left and right panels) than at position 48 (Fig. 5a, lower central panel).

The N-terminal Tyrosine Sulfation Consensus of PSGL-1 Regulates Neutrophil and Lymphocyte Recruitment at CHO Cell Surface—The role of Tyr-46, -48, -51 in supporting neutrophil and T-lymphocyte rolling was assessed under laminar flow conditions using a constant shear stress of 1.25 dyn/cm². Replacement of Tyr-46, -48, and -51 by Phe decreased by 82% neutrophil rolling on CHO-PSGL-1/C2GnT/FucT-VII cells. Replacement of Tyr-46 and -51 by Phe decreased by 56% 13% in the absence of Tyr-48 and -51 (180 ± 50 neutrophils/min/mm², n = 5, p < 0.0001); 2) by 72 ± 8% after replacement of Tyr-46 and -51 by Phe (115 ± 29 neutrophils/min/mm², n = 5, p < 0.0001); and 3) only by 35 ± 8% after exchange of Tyr-46 and -48 by Phe (266 ± 29 neutrophils/min/mm², n = 5, p < 0.01). These results suggest, like observations made with L-selectin/µ chimera, that Tyr-51 plays a predominant role in regulating L-selectin interactions with PSGL-1.

Similar results were obtained with peripheral blood lymphocytes, which rolled efficiently on wild-type PSGL-1 (1594 ± 74 cells / min/mm²) and were replaced by Phe leaving a single Tyr residue also strongly affected neutrophil recruitment. Neutrophil rolling decreased: 1) by 56 ± 13% in the absence of Tyr-48 and -51 (180 ± 50 neutrophils/min/mm², n = 5, p < 0.0001); 2) by 72 ± 8% after replacement of Tyr-46 and -51 by Phe (115 ± 29 neutrophils/min/mm², n = 5, p < 0.0001); and 3) only by 35 ± 8% after exchange of Tyr-46 and -48 by Phe (266 ± 29 neutrophils/min/mm², n = 5, p < 0.01). These results suggest, like observations made with L-selectin/µ chimera, that Tyr-51 plays a predominant role in regulating L-selectin interactions with PSGL-1.

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lymphocytes/min/mm², n = 3, Fig. 5c). Lymphocyte rolling was reduced by 32 ± 5\% on mutants expressing Tyr-51 as single sulfated Tyr residue (PSGL-1 Y46F/Y48F: 1085 ± 67 lymphocytes/min/mm², n = 3, p < 0.001), by 69 ± 3\% on mutants expressing Tyr-48 (PSGL-1 Y46F/Y51F: 481 ± 45 lymphocytes/min/mm², n = 3, p < 0.001) and by 52 ± 4\% on mutants expressing Tyr-46 (PSGL-1 Y48F/Y51F: 754 ± 52 lymphocytes/min/mm², n = 3, p < 0.001, Fig. 5c). These observations confirm: 1) that the presence of a single Tyr residue is less efficient than three Tyr residues to support leukocyte rolling on PSGL-1 and 2) that Tyr-51 plays a predominant role in supporting lymphocyte interactions with PSGL-1.

Additional experiments were performed to examine whether the predominant role of Tyr-51 in supporting L-selectin interaction with PSGL-1 was dependent on wall shear stress. At all tested shear stress (1.0, 2.0, and 3.0 dyn/cm²), the recruitment of 300.19 cells on PSGL-1 Y46F/Y51F, which express Tyr-48, was lower than on mutants expressing Tyr-51 (number of cells/min/mm²) that rolled on PSGL-1 Y46F/Y51F at 1.0 dyn/cm² (mean ± S.E.): 138 ± 12; at 2.0 dyn/cm²: 171 ± 19; at 3.0 dyn/cm²: 201 ± 29). The number of rolling cells was significantly higher on PSGL-1 Y46F/Y48F (number of rolling cells at 1.0 dyn/cm²: 290 ± 23; at 2.0 dyn/cm²: 374 ± 20; at 3.0 dyn/cm²: 627 ± 35, p < 0.001). These observations confirmed that Tyr-51 plays a predominant role in mediating L-selectin-dependent rolling on PSGL-1 and indicate that this property is not dependent on shear stress. In contrast, a predominant role for Tyr-46 was observed only at 1.0 dyn/cm² (number of rolling cells/min/mm² on PSGL-1 Y48F/Y51F at 1 dyn/cm²: 205 ± 18, p < 0.05 versus 138 ± 12 on PSGL-1 Y46F/Y51F). These results indicated that tyrosine residues distinctly contribute to support L-selectin-dependent rolling. Since experiments performed with PSGL-1 glycosylpeptides previously showed that tyrosine residues distinctly contribute to support P-selectin binding (34), we compared side-by-side the role of tyrosine sulfate residues in supporting L-selectin and P-selectin-dependent rolling. Adhesion studies were performed with 300.19-L-selectin cells and K-562 P-selectin cells under a constant shear stress of 2.0 and 3.0 dyn/cm². Results were expressed as percentage of rolling cells. Under a constant shear stress of 2.0 dyn/cm², 476 ± 35 (mean ± S.E.) 300.19-L-selectin cells (% of rolling cells: 100 ± 6) and 433 ± 7 K-562-P-selectin cells (100 ± 10\%), rolled on wild-type PSGL-1 (Fig. 5, d and c). Tyrosine replacement by Phe leaving Tyr-48 as single tyrosine residue strongly decreased L-selectin-dependent rolling (% of rolling cells: 33 ± 3, p < 0.001, n = 3, Fig. 5d) whereas P-selectin-mediated rolling was not significantly reduced (96 ± 8\%, n = 3, Fig. 5e).

Similar results were obtained under a shear stress of 3.0 dyn/cm² (% of 300.19-L-selectin-rolling cells on PSGL-1 Y46F/Y51F: 30 ± 3, p < 0.001, n = 3 versus 82 ± 6 K-562-P-selectin-rolling cells, n = 3) confirming that tyrosine sulfate residues distinctly contribute to support L-selectin and P-selectin-dependent rolling, Tyr-51 playing a major role in supporting L-selectin-dependent rolling whereas Tyr-48 has an essential role in mediating P-selectin-dependent rolling (Fig. 5, d and e).

Leukocyte Rolling Velocity on PSGL-1 Is Regulated by N-terminal Tyr Sulfate Residues and O-Glycans Attached to Thr-57—Neutrophils rolled significantly faster on PSGL-1 Y46F/Y48F/Y51F (median-rolling velocity: 80 µm/s, P25: 29 µm/s; P75 = 155 µm/s; n = 3, Fig. 6a) than on wild-type PSGL-1 (median: 44 µm/s, P25 = 23 µm/s; P75 = 69 µm/s; n = 3, p < 0.001, Fig. 6a) emphasizing the key role played by tyrosine residues in supporting neutrophil rolling. Higher rolling velocities were also observed on mutants expressing a single N-terminal tyrosine residue (Fig. 6a). Among these mutants, lower rolling velocities were observed on PSGL-1 Y48F/
Y51F and PSGL-1 Y46F/Y48F presenting Tyr-46 or -51 as single sulfated tyrosine residue (median-rolling velocity on Tyr-46: 43 μm/s; P25 = 18 μm/s; P75 = 92 μm/s; p < 0.001, n = 3; median-rolling velocity on Tyr-51: 47 μm/s; P25 = 22 μm/s, P75 = 92 μm/s; p < 0.01, n = 3, Fig. 6c) than on PSGL-1 Y46F/Y51F expressing only Tyr-48 (median-rolling velocity: 61 μm/s; P25 = 25 μm/s, P75 = 132 μm/s, p < 0.001, n = 3).

Lymphocytes rolled faster than neutrophils on wild-type PSGL-1 (median rolling velocity of lymphocytes: 58 μm/s, P25 = 48 μm/s, P75 = 71 μm/s, n = 4 versus 44 μm/s, P25 = 23 μm/s, P75 = 69 μm/s for neutrophils, n = 5, p < 0.0001). The replacement of all three Tyr by Phe strongly increased lymphocyte-rolling velocity (median-rolling velocity on PSGL-1 Y46F/Y48F/Y51F: 163 μm/s, P25 = 135 μm/s, P75 = 198 μm/s, n = 4, p < 0.0001, Fig. 6b). The replacement of 2 N-terminal Tyr residues by Phe also significantly increased lymphocyte-rolling velocities on PSGL-1 (p < 0.0001, n = 4, Fig. 6b). Lower rolling velocities were observed on PSGL-1 mutants expressing Tyr-51 or -46 than on mutants expressing only Tyr-48 (p < 0.001, n = 4, Fig. 6b). Lymphocyte-rolling velocities on mutants expressing Tyr-51 (PSGL-1 Y46F/Y48F, median-rolling velocity: 123 μm/s, P25 = 99 μm/s, P75 = 150 μm/s, n = 4) or Tyr-46 (PSGL-1 Y48F/Y51F, median: 116 μm/s, P25 = 92 μm/s, P75 = 145 μm/s) were not statistically different. On the other hand, higher rolling velocities were observed on PSGL-1 Y46F/Y51F, which expressed Tyr-48 (median-rolling velocity 150 μm/s, P25 = 119 μm/s, P75 = 190 μm/s, n = 4, p < 0.001). Additional experiments were performed with 300.19 cells expressing L-selectin to show that the predominant role of Tyr-51 in regulating L-selectin-dependent rolling velocity was not cell type-specific. The rolling velocity of 300.19-L-selectin cells was significantly lower on PSGL-1 Y46F/Y48F (median-rolling velocity: 89 μm/s; P25: 75 μm/s; P75 = 129 μm/s) than on PSGL-1 Y46F/Y51F (median-rolling velocity: 173 μm/s; P25: 135 μm/s; P75 = 195 μm/s, p < 0.001) or PSGL-1 Y48F/Y51F (median-rolling velocity: 121 μm/s; P25: 98 μm/s; P75 = 159 μm/s, p < 0.05) confirming that Tyr-51 plays a key role in regulating L-selectin-dependent rolling on PSGL-1 whereas Tyr-48 has a less important role. The distinct contribution of Tyr sulfate residues in regulating cell rolling velocity was not dependent on shear stress. Thus, 300.19 cells rolled faster on PSGL-1 Y46F/Y51F than on PSGL-1 Y46F/Y48F at 1.0, 2.0 and 3.0 dyn/cm² (median cell rolling velocity on PSGL-1 Y46F/Y51F versus PSGL-1 Y46F/Y48F at 1.0 dyn/cm²: 140 μm/s versus 92 μm/s; at 2.0 dyn/cm²: 173 μm/s versus 88 μm/s; at 3.0 dyn/cm²: 174 μm/s versus 92 μm/s, p < 0.001).

The contribution of tyrosine sulfate residues in regulating L-selectin- and P-selectin-dependent rolling velocity was examined in experiments comparing side-by-side 300.19-L-selectin and K-562-P-selectin rolling under a constant shear stress of 2.0 dyn/cm². Rolling velocities of 300.19-L-selectin cells on mutant PSGL-1 was strongly increased by the replacement of Tyr-46 and -51 by Phe, leaving Tyr-48 as single tyrosine residue (median-rolling velocity on wild-type versus PSGL-1 Y46F/Y51F: 41 μm/s (P25: 30 μm/s; P75 = 80 μm/s) versus 174 μm/s (P25: 135 μm/s; P75 = 195 μm/s), p < 0.001, n = 3, Fig. 6c). In contrast, rolling velocity of K-562-P-selectin cells was not significantly increased on PSGL-1 Y46F/Y51F (median-rolling velocity on wild-type versus PSGL-1 Y46F/Y51F: 8 μm/s (P25: 4 μm/s; P75 = 18 μm/s) versus 11 μm/s (P25: 5 μm/s; P75 = 20 μm/s); n = 3, Fig. 6d) whereas significantly higher rolling velocity were observed on PSGL-1 Y48F/Y51F (median-rolling velocity on wild-type versus PSGL-1 Y48F/Y51F: 43 μm/s (P25: 23 μm/s; P75 = 71 μm/s) versus 132 μm/s (P25: 25 μm/s; P75 = 132 μm/s), p < 0.001, n = 3, Fig. 6e) confirming that Tyr-51 plays a key role in regulating P-selectin-dependent rolling on PSGL-1. The contribution of Tyr-48 and -51 to rolling velocity was examined in experiments comparing side-by-side 300.19-L-selectin and K-562-P-selectin rolling under a constant shear stress of 2.0 dyn/cm². Rolling velocities of 300.19-L-selectin cells on mutant PSGL-1 was strongly increased by the replacement of Tyr-46 and -51 by Phe, leaving Tyr-48 as single tyrosine residue (median-rolling velocity on wild-type versus PSGL-1 Y46F/Y51F: 41 μm/s (P25: 30 μm/s; P75 = 80 μm/s) versus 174 μm/s (P25: 135 μm/s; P75 = 195 μm/s), p < 0.001, n = 3, Fig. 6c).
velocity: 19 μm/s (P25: 12.5 μm/s; P75 = 26 μm/s); n = 3, p < 0.001, Fig. 6d). The key role played by Tyr-48 in regulating P-selectin-dependent cell-rolling velocity contrasts with its minor role in supporting L-selectin-dependent rolling on PSGL-1. These differences indicate that N-terminal tyrosine sulfate residues are distinctly involved when they support L-selectin or P-selectin interactions with their common ligand.

O-glycans presented by Thr-57 played an essential role in regulating lymphocyte-rolling velocity. The replacement of Thr-57 by Ala strikingly increased rolling velocities (median-rolling velocity on PSGL-1 T57A: 176 μm/s, P25 = 137 μm/s, P75 = 238 μm/s, n = 4, p < 0.0001, Fig. 6b). Surprisingly, lymphocyte rolling at very high velocities was still observed on PSGL-1 Y46F/Y48F/Y51F/T57A mutants, which lack core-2 O-glycans attached to Thr-57 and N-terminal tyrosine residues (PSGL-1 Y46F/Y48F/Y51F/T57A: 202 μm/s, P25 = 153 μm/s, P75 = 280 μm/s, n = 4, p < 0.001, Fig. 6b).

Additional analysis was performed to examine the frame-by-frame velocity of 300.19-L-selectin cells rolling on PSGL-1 mutants under a constant shear stress of 1.25 dyn/cm². The velocity of tracked cells was determined by measuring cell displacements within successive video frames (0.032 s) in the flow direction. Each increase in velocity is represented by a peak and each decrease by a valley (Fig. 7a). The replacement of tyrosine sulfate residues by Phe strongly increased the variations of cell-rolling velocity indicated by higher irregularity in “peaks” and “valleys”, as illustrated in Fig. 7a. The observed instability of cell rolling was quantified by calculating the S.D. of the mean velocity of each tracked cell. The pooled data obtained from the whole cell population were used to determine the mean S.D. of rolling velocities on wild-type and each PSGL-1 mutant. More irregular rolling velocities were observed on PSGL-1 mutants devoid of tyrosine sulfate residues or expressing Tyr-48 as single tyrosine sulfate residue (mean ± S.D. of 300.19-L-selectin cell-rolling velocities on PSGL-1 Y46F/Y48F/Y51F: 170 μm/s versus 163 μm/s on PSGL-1 Y46F/Y51F, n = 5, p < 0.001) than on wild-type PSGL-1 (mean ± S.D.: 60 μm/s, n = 5, p < 0.001, Fig. 7a). The stability of rolling velocity was less affected on mutant PSGL-1 expressing Tyr-46 (mean ± S.D. on PSGL-1 Y48F/Y51F: 111 μm/s, n = 5) or Tyr-51 (mean ± S.D. on PSGL-1 Y46F/Y48F: 117 μm/s, n = 6) than Tyr-48 (Fig. 7a).

The distribution of travel distances illustrated in Fig. 7b was assessed by measuring cell displacements within successive video frames (0.032 s; 891–1477 determinations for each cell category). Data obtained for each cell category were pooled and illustrated in Fig. 7b. The replacement of tyrosine sulfate residues strongly affected cell displacements. A significantly higher percentage of 300.19-L-selectin cells rolled > 4.1 μm on PSGL-1 Y46F/Y48F/Y51F, within a video frame, than on wild-type PSGL-1 (53.2 versus 15.7%, p < 0.004, Fig. 7b). A broader range of travel distances was observed on mutants expressing Tyr-48 as single sulfated tyrosine. Thus, the 300.19-L-selectin cells more frequently traveled > 4.1 μm on PSGL-1 Y46F/Y51F than on PSGL-1 Y48F/Y51F (37.3 versus 11.0%, number of observed events: ≥891, p < 0.001) or PSGL-1 Y46F/Y48F (18.6%, p < 0.003).

The analysis of the variation of velocity and travel distances of 300.19-L-selectin cells on PSGL-1 T57A lead to similar observations. In the absence of O-glycans attached to Thr-57 (PSGL-1 T57A), 300.19-L-selectin cells exhibited a very unstable rolling velocity (mean ± S.D. of rolling velocities on PSGL-1 T57A: 198 μm/s (n = 7) versus 60 μm/s on wild-type PSGL-1 (n = 5), p < 0.001, Fig. 8a). In contrast, the replacement of Thr-44 by Ala had no effect (mean ± S.D. on PSGL-1 T44A: 77 μm/s, n = 7). Similarly to observations made on PSGL-1 T57A, the rolling velocity of 300.19-L-selectin cells was very unstable in the absence of sLeα/CLA presentation by core-2 O-glycans (mean ± S.D. on CHO cells co-transfected with PSGL-1 and FucT-VII cDNAs without C2GnT cDNA: 162 μm/s, n = 11, p < 0.001, not illustrated). A higher percentage of cells rolled on longer distances on PSGL-1 T57A than on wild-type PSGL-1 (no. of cells that rolled > 4.1 μm: 64.9 versus 20.2%, no. of determinations: n ≥ 440, p < 0.001) or on PSGL-1 T44A (64.9 versus 16.5%, n ≥ 373, p < 0.001, Fig. 8b).

Lack of Inhibition of L-selectin-dependent Rolling on PSGL-1 by CSLEX-1 and HECA-452 mAbs.—The anti-CLA mAb HECA-452 was reported to inhibit by >90% L-selectin-dependent lymphocyte rolling on the human vascular endothelial cell line EA hy926 transfected with FucT-VII cDNA suggesting that CLA, a sLeα determinant, is a major determinant of endothelial L-selectin ligand(s) (55). In contrast to these observations, neutrophil rolling was not significantly reduced by
HECA-452 mAb (no. of neutrophils rolling on PSGL-1 in presence of HECA-452 mAb versus control mAb, mean ± S.E.: 239 ± 21 versus 213 ± 49 neutrophils/min/mm², n = 6).

**Molecular Modeling of L-selectin Interactions with PSGL-1**

The RMSD calculated between all P-selectin structures (23) for each amino acids allowed for the identification of the regions of high and low mobility (52). Loops 42–48 and 108–114 of L-selectin and P-selectin, which interact with PSGL-1-sulfated Tyr-48, have low mobility and are surrounded by low mobility regions. In contrast, loop 64–89 of L-selectin and P-selectin, which interacts with PSGL-1-sulfated Tyr-51, exhibits high mobility. Thus, we analyzed only the interactions of loops 42–48 and 108–114 of L-selectin with sulfated Tyr-48 of PSGL-1. Hydrogen-bonding pattern between L-selectin or P-selectin and Tyr-48 was calculated using the HBPLUS program, based on a homology model obtained with the MOD-ELLER program (45, 50). Hydrogen bonds involved in the interactions of sulfated Tyr-48 with L-selectin and P-selectin are indicated in Table I and Fig. 9. Two potential hydrogen bonds were disclosed between Ser-47 of L-selectin and the sulfate group of Tyr-48, whereas P-selectin binding to PSGL-1-sulfated Tyr-48 is supported by 4 hydrogen bonds located between Ser-47, Ser-48, His-114, and the sulfate group of Tyr-48 and by an additional hydrogen bond located between Ser-46, Ser-47, His-114, and the sulfate group of PSGL-1. Hydrogen-bonding pattern between L-selectin or P-selectin and Tyr-48 was calculated using the HBPLUS program (69). The donor (D) and the acceptor (A) atoms are defined by the three letter code amino acid, residue numbers and atom types (in the X-ray structure (23), the Tyr-SO₃⁻-48 corresponds to Tyr-SO₃⁻-51 to Tyr-610). D-A distance: distance between the donor and the acceptor atoms. DHA: angle centered on the hydrogen (H) and linking the donor and acceptor atoms.

| Protein | Donor atom | Acceptor atom | D-A distances [Å] | DHA angle |
|---------|------------|---------------|------------------|----------|
| L-selectin | Ser-47 N | Tyr-SO₃⁻-48 O3 | 2.66 | 126.0 |
|  | Ser-47 OG | Tyr-SO₃⁻-48 O3 | 3.17 | 121.7 |
| P-selectin | Ser-46 OG | Tyr-SO₃⁻-48 O3 | 3.34 | 172.4 |
|  | Ser-47 N | Tyr-SO₃⁻-48 O3 | 3.08 | 152.5 |
|  | Ser-47 OG | Tyr-SO₃⁻-48 O3 | 3.02 | 164.2 |
|  | Lys-114 N | Tyr-SO₃⁻-48 O0 | 3.13 | 160.0 |
|  | His-114 NE2 | Tyr-SO₃⁻-48 O2 | 2.72 | 155.2 |

**DISCUSSION**

The determinants of PSGL-1 that mediate L-selectin and P-selectin binding include tyrosine sulfate residues and O-glycans attached to Thr-57 (24, 33, 34, 56–62). The crystal structure analysis of P-selectin co-complexed with the N-terminal peptide of PSGL-1 showed that O-glycans terminated by sLeα/CLA as well as Tyr-48 and -51 play an essential role in supporting P-selectin binding. In addition, although this interaction had not been highlighted in the crystallographic study mentioned above, rolling adhesion assays and glycosylpeptide binding studies have suggested a role for PSGL-1-Tyr-46 in adhesion to P-selectin (23, 24, 34, 35). The results presented here show that P-selectin and L-selectin use similar mechanisms to bind to PSGL-1. However, the three tyrosine sulfate residues of PSGL-1 do not contribute in an equal fashion to L- and P-selectin binding. Specifically, our results indicate that Tyr-48 is of key importance in supporting P-selectin-mediated rolling on PSGL-1 whereas it only plays a minor role in mediating L-selectin binding. In addition, the present study shows that: 1) sialylated and fucosylated core-2 O-glycans attached to Thr-57 are essential to allow optimal L-selectin binding to PSGL-1 and 2) to support and stabilize leukocyte rolling on CHO-PSGL-1 cells; 3) at least 2 or 3 N-terminal Tyr sulfate residues are required to optimally support leukocyte recruitment and rolling; and 4) Tyr-51 plays a predominant role in recruiting and stabilizing leukocyte rolling on PSGL-1.

We have defined here the minimal molecular requirements supporting L-selectin interactions with PSGL-1. L-selectin/µ weakly bound to sLeα/CLA-expressing CHO cells in the absence of PSGL-1 (Fig. 2b, upper panel). However, this interaction was not sufficient to support neutrophil rolling (Fig. 3a). This observation is consistent with the notion that low affinity interactions between L-selectin and sLeα cannot efficiently support L-selectin-mediated rolling (13). Interestingly, a low number of neutrophils rolled on CHO-PSGL-1/FucT-VII cells even in the absence of C2GnT suggesting that the presentation of sLeα/CLA by core-2 O-linked glycans is not essential to support L-selectin-dependent rolling (13). The strong reduction in neutrophil rolling and L-selectin/µ binding observed after the replacement of Thr-57 (but not of Thr-44) by Ala confirmed the essential role played by Thr-57 in presenting O-glycans involved in L-selectin binding (Fig. 4). Interestingly, leukocyte rolling was less affected by the replacement of the whole tyrosine sulfation consensus by Phe residues, suggesting a predominant role for core-2 O-glycans (Fig. 5, b and c). A critical role for sialic acid residues was indicated by the abrogation of neutrophil rolling on PSGL-1 after sialidase treatment of CHO cells. Similar observations were previously reported for P-selectin (22, 34, 37, 63). Sialic acid residues presented by sLeα/CLA determinants most likely play a key role in this interaction.

C2GnT co-expression with FucT-VII and PSGL-1 strongly increased leukocyte recruitment and decreased rolling velocity (Fig. 3). Similar observations were made by others who studied PSGL-1 interactions with P-selectin in vitro using leukocyte isolated from C2GnT-deficient mice or in vivo in C2GnT-deficient mice (25, 28). Higher rolling velocities on P-selectin were observed in the absence of C2GnT suggesting that core-2 O-linked glycans have a major role in regulating leukocyte-rolling.
velocity. The present study extends these observations to L-selectin. By stabilizing and reducing leukocyte-rolling velocity on L-selectin or P-selectin, core-2 O-glycans may improve the exposure of rolling cells to cytokines or chemokines at site of inflammation and promote leukocyte arrest and firm adhesion following integrin activation.

Whereas the results of this study confirm that L-selectin interaction with PSGL-1 is dependent on the expression of O-glycans attached to Thr-57 and of N-terminal tyrosine sulfated residues (Figs. 4 and 5) (35), they also revealed that PSGL-1 mutants exhibiting a single tyrosine sulfated residue were not as efficient as wild-type PSGL-1 in supporting leukocyte rolling. Moreover, these results showed that tyrosine sulfated residues did not contribute equally to L-selectin interactions with PSGL-1. A predominant role for Tyr-51 was indicated by: 1) the lower rolling velocities (Fig. 6), 2) the more efficient recruitment of leukocytes on PSGL-1 Y46F/Y48F expressing Tyr-51 as single tyrosine sulfated residue (Fig. 5), 3) the more efficient binding of L-selectin/μ to PSGL-1 Y46F/Y48F (Fig. 5a), and 4) the increased stability of leukocyte-rolling velocity on this mutant PSGL-1 (Fig. 7).

Experiments performed with glycosulfopeptides previously indicated that Tyr-48 plays a major role in mediating the interactions of the N-terminal peptide of PSGL-1 with P-selectin (34). Adhesion studies performed with K-562-P-selectin cells demonstrated with whole cells that Tyr-48 plays a predominant role in supporting P-selectin-mediated rolling interactions with PSGL-1 whereas it had only a minor role in mediating L-selectin-dependent rolling (Fig. 6). These observations are in keeping with crystal structure analysis that revealed a major role for His-114 in mediating P-selectin binding to PSGL-1 Tyr-48 (23). The analysis of hydrogen-bonding pattern disclosed additional potential bonds, which may strengthen P-selectin interactions with Tyr-48 (Fig. 9 and Table I). The absence of a basic amino acid residue at position 114 of L-selectin and the lower number on hydrogen bonds may explain why Tyr-48 of PSGL-1 plays only a minor role in supporting L-selectin binding to PSGL-1 (Fig. 9 and Table I). Of note, molecular modeling analysis of L-selectin interactions with Tyr-48 is consistent with results of adhesion studies performed under flow at various shear stress and validate this model.

L-selectin and P-selectin are likely to bind to PSGL-1 in a similar fashion because of the conservation of residues within the sLeX binding site and the presence of a basic residue (Lys) at position 85 (23). Electrostatic interactions most likely play a major role in supporting the negatively charged Tyr-51 binding to L-selectin Lys-85 and to P-selectin Arg-85. An important role for Lys-85 in supporting L-selectin binding to PSGL-1 is suggested by the elevated partial charge of the ammonium group of Lys-85 (+0.69) (64). In comparison, a lower charge is associated to the iminium group of Arg-85 (+0.12) of P-selectin. The absence of a basic amino acid residue at position 114 of L-selectin and the presence of Lys-85 may explain why sulfated Tyr-51 plays a predominant role in supporting L-selectin interactions with PSGL-1 whereas sulfated Tyr-48 is less important.

L-selectin/μ binding studies and adhesion assays indicated that Tyr-46 plays an important role in supporting L-selectin interactions with PSGL-1. Leukocyte rolling was more stable and slower on PSGL-1 Y48F/Y51F than on PSGL-1 Y46F/Y51F (Figs. 6 and 7). Interestingly, the involvement of Tyr-46 in P-selectin binding was not revealed by crystal structure analysis (23) whereas binding studies of PSGL-1 glycosulfopeptides to P-selectin (34) and adhesion studies performed with K-562-P-selectin cells (Figs. 5c and 6d) indicate that Tyr-46 supports this reaction. The role of Tyr-46 in mediating L-selectin and P-selectin binding was recently further supported by the ability of the anti-PSGL-1 mAb PS-4, which reacts with Tyr-46 but not with Tyr-48 or -51, to inhibit L-selectin-dependent rolling on PSGL-1.5

Rolling is an important step during which leukocytes are exposed to chemotactracts at sites of inflammation, a reaction that leads to integrin activation and leukocyte firm adhesion. Frame by frame analysis of cell displacements indicates that cell rolling occurs through a series of steps or jerks that appear to represent receptor-ligand dissociation events (65–67). Rolling through selectins is unaffected by alterations in selectin density and hydrodynamic forces acting on the cell (65). This surprising stability of rolling has been explained by the ability of leukocyte to reach a dynamic balance between formation and breakage of bonds between selectins and their ligands over a wide range of wall shear stress and ligand densities (66, 67). The stereospecific interactions of tyrosine sulfate and O-glycans with P-selectin and L-selectin create a high affinity binding site (23, 27, 34), which contributes to rolling stabilization. The highly irregular cell rolling observed on PSGL-1 mutants devoid of tyrosine sulfates residues or expressing Tyr-48 as single tyrosine sulfated residue (Fig. 7a) indicates that Tyr-46 and -51 play a major role in the ability of PSGL-1 to stabilize L-selectin-mediated rolling. Similar observations were made for core-2 O-glycans attached to Thr-57 which present sLeX/CLA to L-selectin (Fig. 8c). Post-translational modifications of PSGL-1 may facilitate endothelium surveillance for signs of inflammation and thereby represent important additional levels of regulation of leukocyte traffic.

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Molecular Basis of Leukocyte Rolling on PSGL-1: PREDOMINANT ROLE OF CORE-2 O-GLYCANS AND OF TYROSINE SULFATE RESIDUE 51
Michael Pierre Bernimoulin, Xian-Lu Zeng, Claire Abbal, Sylvain Giraud, Manuel Martinez, Olivier Michielin, Marc Schapira and Olivier Spertini

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