Visualization of P-selectin Glycoprotein Ligand-1 as a Highly Extended Molecule and Mapping of Protein Epitopes for Monoclonal Antibodies*

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P-selectin glycoprotein ligand-1 (PSGL-1), a sialomucin on human leukocytes, mediates rolling of leukocytes on P-selectin expressed by activated platelets or endothelial cells under shear forces. PSGL-1 requires both tyrosine sulfate and O-linked glycans to bind P-selectin. Electron microscopy of rotary-shadowed PSGL-1 purified from human neutrophils indicated that it is a highly extended molecule with an extracellular domain that is ~50 nm long. Both individual PSGL-1 molecules and rosettes composed of several molecules presumably attached at their transmembrane segments were observed. The extracellular domain of PSGL-1 has 318 residues, including a signal peptide from residues 1-18 and a propeptide from residues 19-41. Using bacterially expressed fusion proteins and synthetic peptides derived from the extracellular domain, we mapped the epitopes for two IgG anti-PSGL-1 monoclonal antibodies, PL1 and PL2. PL2 bound to a region within residues 188-235 that is located in a series of decameric consensus repeats. PL1, which blocks binding of PSGL-1 to P-selectin, recognized an epitope spanning residues 49-62. This sequence overlaps the tyrosine sulfation sites at residues 46, 48, and 51 that have been implicated in binding of PSGL-1 to P-selectin. Our results demonstrate that PSGL-1 is a long, extended molecule and suggest that the P-selectin binding site is located near the N terminus, well above the membrane. This location may facilitate interactions of PSGL-1 with P-selectin under shear stress.

The selectins are a family of Ca\(^{2+}\)-dependent lectins that mediate rolling adhesion of leukocytes on the vessel wall during inflammation (reviewed in Refs. 1 and 2). L-selectin is expressed on leukocytes, whereas E- and P-selectin are expressed on activated endothelial cells or platelets. All three selectins bind sialylated and fucosylated oligosaccharides such as sialyl Lewis x, and L- and P-selectin also recognize many polylactosamine terminating in sialyl Lewis x (5). PSGL-1 is a type 1 membrane protein with an extracellular domain rich in sulfate and many clustered, sialylated O-sialoglycans that render the protein susceptible to digestion with O-sialoglycoprotein endopeptidase (4, 9). Some of the O-linked glycans have polylactosamine terminating in sialyl Lewis x (5). PSGL-1 is a highly extended molecule with an extracellular domain rich in sialine, threonine, and proline, including a series of decameric repeats (15 in HL-60 cells and 16 in human leukocytes) (8, 10, 11). Following an 18-residue signal peptide, there is a putative propeptide extending from residues 19 to 41 (8). If the propeptide is cleaved, the extracellular domain begins at residue 42 and extends to residue 318 of PSGL-1 on human leukocytes. The sequence concludes with a 25-residue transmembrane domain and a 69-residue cytoplasmic tail.

PSGL-1 must be sialylated and fucosylated to interact with both P- and E-selectin (4, 8). Recombinant PSGL-1 expressed on CHO cells binds P- and E-selectin when it is co-expressed with an α1,3-fucosyltransferase and with core 2 O-acetylglucosaminyltransferase, a glycosyltransferase that creates core 2 O-linked glycans (12). This indicates that PSGL-1 requires sialylated and fucosylated core 2 O-linked glycans to bind both P- and E-selectin. However, the interactions of PSGL-1 with P- and E-selectin are not identical. Fab fragments of PL1, an IgG mAb to PSGL-1, abolish binding of purified PSGL-1 to P-selectin, suggesting that PL1 recognizes an epitope that overlaps a specific binding site for P-selectin on PSGL-1 (10). In contrast, PL1 only partially inhibits binding of PSGL-1 to E-selectin, suggesting that PSGL-1 has an additional binding site(s) for E-selectin (13). PL2, another IgG mAb to PSGL-1, has no effect on binding to either P- or E-selectin (10, 13). Treatment of leukocytes with O-sialoglycoprotein endopeptidase prevents binding of PL1 but not PL2, indicating that the PL1 epitope on PSGL-1 is located farther from the membrane than the PL2 epitope (10). PSGL-1 has three potential tyrosine sulfation sites near the N terminus (8). Enzymatic removal of sulfate from tyrosine and mutational analysis indicate that sulfation of at least one of these tyrosines is required for binding of PSGL-1 to P-selectin but not to E-selectin (12, 16).

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14–16). Collectively, these data demonstrate that N-terminal tyrosine sulfate functions with core 2 sialylated and fucosylated O-linked glycans to mediate high affinity binding of PSGL-1 to P-selectin.

PSGL-1 is localized on the tips of microvilli of leukocytes (10), where it is positioned to interact optimally with P-selectin under flow (17). Projection of the P-selectin-binding site well above the cell surface might also facilitate rapid interactions with P-selectin. Mucins usually have extended structures (18, 19), and rotary-shadowed electron micrographs of CD43, another mucin-like protein on leukocytes, confirm that it has a long extracellular domain (20). Here we employ electron microscopy to demonstrate that PSGL-1 from human neutrophils is also highly extended. Using bacterially expressed fusion proteins and synthetic peptides, we have mapped the epitopes for PL1 and PL2 on PSGL-1. The epitope for PL1 is located near the N terminus, further supporting a specific membrane-distal location for the P-selectin-binding site.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Proteins**—The mAbs PL1 and PL2 (both IgG1) to human PSGL-1 were prepared as described previously (10). A rabbit polyclonal antiserum to a synthetic peptide encoding residues 42–56 of PSGL-1 was prepared as described previously (5). A rabbit antiserum to a synthetic peptide encoding residues 19–37 of PSGL-1 was prepared by the same protocol used for generating the anti-42–56 serum (5). PSGL-1 was purified from human neutrophils as described previously (5).

**Electron Microscopy—** Samples of PSGL-1 were purified by zone centrifugation on 15–40% glycerol in 0.2 M ammonium bicarbonate. The 0.2-ml sample was layered on a 5-ml gradient and centrifuged at 38,000 rpm for 15 h at 20°C in a SW-50.1 rotor. Electron microscopy of rotary-shadowed specimens of PSGL-1 was performed as described previously (21).

**PSGL-1 Fusion Proteins—** A cDNA encoding a full-length form of PSGL-1 from human leukocytes was isolated as described previously (10). The cDNA was excised with XbaI and SalI and ligated into Bluescript™ SK(+) (Stratagene). Constructs were prepared that encoded proteins with overlapping segments of the extracellular domain of PSGL-1 fused to dihydrofolate reductase (DHFR). cDNAs encoding overlapping portions of the extracellular domain of PSGL-1 were amplified from the full-length PSGL-1 cDNA by standard polymerase chain reaction protocols (22). The primers, prepared in the Molecular Biology Resource Facility of the University of Oklahoma Health Sciences Center, were designed to introduce an XbaI site and a SalI site at the 5′ end and a HindIII site at the 3′ end. Each product amplified by the polymerase chain reaction was excised from agarose gels with the QIAEX II gel extraction kit (Qiagen), digested with XbaI and HindIII, and ligated into Bluescript. The fragment was then excised from Bluescript with StuI and HindIII and ligated in-frame with the murine DHFR cDNA in the PQE-42 vector (Qiagen) that had been digested with SmaI and HindIII. All constructs were verified by dideoxynucleotide sequencing.

Each construct in PQE-42 was transformed into M15[pREP4] competent cells (Qiagen), and the resultant fusion protein was expressed as described (23). In brief, overnight cultures were diluted 1:5 in fresh medium and incubated for 30 min at 37°C. Isopropyl-1-thio-β-D-galactopyranoside (2 mM) was then added, and the cells were incubated for an additional 4 h at 37°C. The cells were then pelleted and lysed, and the fusion protein was purified on nickel-nitriotriacetic acid resin (Qiagen) according to the instructions of the manufacturer. Aliquots were boiled in SDS sample buffer for analysis by SDS-PAGE.

**Western Blotting—** Aliquots of the fusion proteins, or the expressed DHFR protein with no fused PSGL-1 sequence, were resolved by SDS-PAGE under reducing conditions. A sample of neutrophil membrane proteins was extracted from a 30-μl droplet of a detergent suspension on a glass slide using detergent (9-fluorenyl)methylcarbonylpeptide (SmB/B) or lupus autoantigens (24, 25). Wash steps and incubations were carried out in sealed plastic containers. para-Nitrophenyl phosphate disodium was used as a substrate for alkaline phosphatase, and plates were read at 410 nm with a Microelisa Reader (Dynatech, Alexandria, VA). Results for each plate were then standardized by comparison with positive control pins.

**RESULTS**

**Visualization of PSGL-1 by Electron Microscopy—** PSGL-1 purified from human neutrophils was used to prepare rotary-shadowed specimens for electron microscopy. The initial specimen was made from PSGL-1 in 0.1 M NaCl, 0.02 M MOPS, pH 7.4, containing 0.1% Brij 58. Although the detergent obscured most of the specimen, many areas showed strongly bound flexible molecules that frequently crossed each other or attached in small aggregates. Strands that appeared to be single molecules could also be found; these were typically ~50–60 nm long (data not shown).

Much better specimens were obtained after centrifuging PSGL-1 through a glycerol gradient in ammonium bicarbonate free of detergent. The protein eluted as a fairly sharp peak at about 6 s. Rotary-shadowed specimens prepared from the peak fractions showed single molecules and rosettes (Fig. 1). Most were single molecules, identified by a uniform thickness and length. The molecules were all aligned in some areas of the grid, probably by shear flow as the droplet moved on the mica. These straight molecules were selected for length measurements, giving an average length of 54 ± 0.7 nm (n = 41). The 54-nm length should represent the ectodomain plus some portion of the transmembrane and cytoplasmic domains. These images demonstrate that PSGL-1 is a thin, highly extended molecule.

Rosettes consisted of two to five molecules emanating from a central globular hub. These rosettes are typical of membrane proteins, which are thought to aggregate at their hydrophobic, transmembrane-spanning ends to form the hubs (20, 21, 28,
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The specimen areas showing the best rosettes were away from those where molecules were aligned by shear, and the protruding ectodomains were usually bent, making length measurements more difficult. The longest and straightest molecules had a length of \( \sim 50 \) nm from the edge of the hub to the end of the molecule. Therefore, the ectodomain of PSGL-1 was estimated to be 50 nm long.

Mapping of Epitopes on PSGL-1 Using Fusion Proteins—We previously showed that the anti-PSGL-1 mAbs PL1 and PL2 bind to PSGL-1 after it is treated with sialidase (10). Furthermore, PL1 and PL2 bind to PSGL-1 expressed on CHO cells, which have only simple core 1 O-linked glycans (10). These data suggested that PL1 and PL2 recognize protein-dependent epitopes on PSGL-1 that are not significantly affected by glycosylation. To test this hypothesis and to map the epitopes for PL1 and PL2, we expressed in bacteria a series of fusion proteins containing residues 19–78 of PSGL-1, whereas PL2 bound to the protein containing residues 188–258 (Fig. 3). These data confirm that PL1 and PL2 recognize protein-dependent epitopes on PSGL-1 and suggest that each mAb binds to a single epitope. As expected, PL1 and PL2 also bound native PSGL-1 from human neutrophils (Fig. 3).

Polyclonal antisera generated against residues 19–37 (in the putative propeptide) and 42–56 (at the N terminus immediately following the propeptide) both bound to the 19–78 fusion protein (Fig. 3). The anti-42–56 serum recognized native PSGL-1, as observed previously (5, 10). In contrast, the anti-19–37 serum did not bind native PSGL-1 on the Western blot, suggesting that the propeptide, which includes residues 19–37, had been removed.

To further localize the epitope for PL1, we expressed a series of smaller fusion proteins containing sequences within residues 19–78 (Fig. 2B). Western blotting revealed that PL1 bound to a fusion protein containing residues 51–63, but not to fusion proteins containing residues 42–56 or 56–63 (Fig. 4). The epitope for PL2 was studied less thoroughly. However, PL2 did not bind a fusion protein containing residues 235–266 (Figs. 2C and 4), indicating that the PL2 epitope requires residues located between positions 188 and 235.

Mapping of the PL1 Epitope Using Synthetic Peptides—To independently map the PL1 epitope, we synthesized a series of octamer peptides spanning the sequence between residues 19 and 77. Each octamer overlapped the sequence of its neighbors by one residue. The peptides were synthesized by a solid-phase procedure on the tips of polyethylene pins in a microtiter plate format. Using an enzyme-linked immunosorbent assay, we confirmed that peptides with sequences between residues 42 and 56 were recognized by the polyclonal anti-42–56 serum (data not shown). The PL1 mAb bound specifically to octamer peptides spanning residues 49–62 (Fig. 5). PL1 bound most strongly to peptides within residues 51–61, which share the sequence LPETE at positions 54–58. These data indicate that PL1 binds optimally to a long 14-residue epitope that spans amino acids 49–62. However, the antibody can bind to shorter octamer peptides within this sequence, particularly those that include the LPETE motif.

The Propeptide Is Cleaved from Native PSGL-1 but Not from Recombinant PSGL-1—Expressed by CHO Cells—We demonstrated previously that PSGL-1 expressed in CHO cells binds both P- and E-selectin when it is co-expressed with an α1,3-fucosyltransferase and core 2 β1,6-N-acetylglucosaminyltransferase (12). Western blot analysis indicated that the anti-42–56 serum recognized both native PSGL-1 and recombinant PSGL-1 expressed in CHO cells (Fig. 6). The anti-19–37 serum also specifically recognized recombinant PSGL-1, but not the native protein (Fig. 6, see also Fig. 3). This result demonstrates that CHO cells, unlike myeloid cells, do not remove the propeptide from newly synthesized PSGL-1.

DISCUSSION

PSGL-1 is a sialomucin on human leukocytes that binds both P- and E-selectin. Using electron microscopy, we have established that the extracellular domain of PSGL-1 is highly extended, like other heavily O-glycosylated proteins. We have also mapped protein-dependent epitopes on PSGL-1 for two IgG mAbs, one of which completely blocks binding of PSGL-1 to P-selectin. The rotary-shadowed images of PSGL-1 reported here are remarkably similar to those described previously for CD43, another sialomucin on leukocytes (20). Both human PSGL-1 and rat CD43 are visualized as thin, highly extended molecules. Both proteins also form rosettes; these are assumed to result from interactions of the transmembrane domains to form the central hubs, with the extracellular domains extending outward from the hubs (20, 21, 28, 29). The extended structures of the ectodomains of PSGL-1 and CD43 are thought to result from interactions of the Ser/Thr-linked GalNAc residues with adjacent amino acids in the peptide core (18, 19). We estimate that the ectodomain of PSGL-1 is 50 nm long, which is only slightly longer than the 45-nm length estimated for the ectodomain of CD43 (20). The ectodomain of rat CD43 (assuming cleavage of the signal peptide) has 224 amino acids or 4.98 residues/nm. The ectodomain of human PSGL-1 (assuming cleavage of the signal peptide and propeptide) has 276 amino acids or 5.52 residues/nm. Therefore, the extracellular domain of PSGL-1 appears to be slightly more compact than that of CD43. The ectodomain of PSGL-1 has 72 serines and threonines, whereas the ectodomain of CD43 has 85 serines and threonines. Although it is not known how many of these residues are O-glycosylated, the ectodomain of CD43 may have a

![Electron micrographs of rotary-shadowed PSGL-1 molecules.](image-url)
higher percentage of O-glycosylated amino acids than that of PSGL-1, accounting for its more extended structure relative to the total number of residues.

The individual molecules of PSGL-1 and CD43 appear to be monomers; this seems to contradict the apparent disulfide-linked homodimeric structure of PSGL-1 observed by SDS-PAGE (4). It is possible that disulfide bonding of PSGL-1 occurs only during preparation of samples for SDS-PAGE; however, the dimers are still observed when samples are alkylated with iodoacetamide before they are added to SDS denaturing buffer. Because PSGL-1 is so extended, two closely associated subunits in a dimer might be visualized as an apparent monomer. If so, it is possible that CD43 forms noncovalently associated dimers that are also visualized as monomers.

We found that the IgG mAbs PL1 and PL2 bind to bacterially expressed fusion proteins and/or synthetic peptides containing portions of the extracellular domain of PSGL-1. Thus, these mAbs clearly recognize protein-dependent epitopes, as do many IgG mAbs to CD43 (20). Unlike the binding of some IgG mAbs to CD43 (20), however, the binding of PL1 or PL2 to PSGL-1 is not obviously affected by glycosylation. IgM mAbs to PSGL-1 have also been described (30). Binding of these antibodies is eliminated when PSGL-1 is treated with sialidase. Like other IgM antibodies, these antibodies probably recognize epitopes that include carbohydrate components. If so, each IgM mAb may bind to more than one site on PSGL-1 and may also cross-react with carbohydrate epitopes on proteins other than PSGL-1.

The PL2 epitope includes residues within positions 188–235, located in decameric repeats 7–12. Therefore, the epitope is still positioned a considerable distance from the membrane. When PSGL-1 in solution is treated with O-sialylglycoprotein endopeptidase, it is digested into very small fragments that cannot be detected by SDS-PAGE (9). In contrast, when leukocytes are treated with O-sialylglycoprotein endopeptidase, a relatively large fragment of PSGL-1 that includes the PL2 epitope is retained on the cell surface (10). This suggests that, on the intact cell, O-sialylglycoprotein endopeptidase cannot cleave PSGL-1 at sites between the membrane and the PL2 epitope because of steric hindrance or other mechanisms.

PL1 blocks binding of PSGL-1 to P-selectin, suggesting that the PL1 epitope overlaps the P-selectin-binding site (10). The PL1 epitope is located near the extreme N terminus of PSGL-1 (Fig. 7). The complete epitope spans residues 49–62, but PL1 binds strongly to octamer peptides sharing the LPETE sequence at residues 54–58. The complete epitope includes Tyr-51 and is adjacent to Tyr-46 and Tyr-48. Sulfation of one or more of these residues is required for PSGL-1 to bind P-selectin (12, 14–16), and a cobra venom protease that cleaves PSGL-1 between Tyr-51 and Asp-52 eliminates binding to P-selectin (31). Because PL1 blocks binding of PSGL-1 to P-selectin, it clearly recognizes tyrosine-sulfated forms of PSGL-1. However, the interaction of PL1 with synthetic peptides demonstrates that sulfation is not required for binding. Furthermore, PL1

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2 F. Li, K. L. Moore, and R. P. McEver, unpublished observations.
recognizes a recombinant form of PSGL-1 in which the three tyrosines at residues 46, 48, and 51 are replaced with phenylalanines (12). The LPETE sequence in the PL1 epitope includes Thr-57, a potential site for O-glycosylation. An Ig chimera containing only residues 42–60 of PSGL-1, when co-expressed with an α1,3-fucosyltransferase in COS cells, binds P-selectin, although not as well as longer PSGL-1 constructs (16). Substitution of Thr-57 with alanine in the short construct eliminates binding. However, binding is only partially reduced when Thr-44 and Thr-57 are substituted with alanine in longer PSGL-1 constructs that are co-expressed with an α1,3-fucosyltransferase in COS cells (15). The next closest potential O-glycosylation sites are Thr-69 and Thr-70, which may also be masked by binding of PL1. There is a potential N-linked glycosylation site at Asn-65, but enzymatic removal of N-linked glycans does not obviously affect binding of PSGL-1 to P-selectin (4). More distant O-linked glycans might participate in P-selectin recognition, but may be incapable of supporting binding if PL1 masks the critical N-terminal recognition site.

De Luca et al. (31) reported that rabbit polyclonal antibodies raised to a peptide encoding residues 42–56 (1–15 if the sequence is renumbered after cleavage of the propeptide) blocks binding of P-selectin to neutrophils. In agreement with this result, we observed that our anti-42–56 serum blocks binding of P-selectin to purified PSGL-1 or to human HL-60 cells. However, De Luca et al. (31) also reported that rabbit polyclonal

**Fig. 3. Western blot analysis of PSGL-1-DHFR fusion proteins shown in Fig. 2A.** The left panel shows a Coomassie Blue-stained gel of the PSGL-1-DHFR fusion proteins, following SDS-PAGE under reducing conditions. In the other four panels, identical aliquots of the proteins were electrophoresed under the same conditions, transferred to Immobilon membranes, and probed with the mAbs PL1 or PL2, or with the polyclonal anti-19–37 or anti-42–56 sera. DHFR with no attached PSGL-1 sequence was analyzed as a negative control. A neutrophil membrane protein fraction enriched in PSGL-1 was also analyzed.

**Fig. 4. Western blot analysis of PSGL-1-DHFR fusion proteins shown in Fig. 2, B and C.** Proteins were resolved by SDS-PAGE under reducing conditions, followed by Coomassie Blue staining or Western blotting as in Fig. 3.
antibodies to a peptide spanning residues 50–64 (9–23 in the new numbering system) have no effect on binding of P-selectin to neutrophils. Since the sequence of the peptide used to raise these polyclonal antibodies is almost identical with the sequence of the PL1 epitope, it is difficult to explain why the polyclonal antibodies did not inhibit binding. No data were provided to confirm that the polyclonal anti-50–64 antibodies actually bind to intact PSGL-1 or to neutrophils (31). Perhaps these antibodies are of low titer and/or low affinity.

Residues 19–41 of PSGL-1 (Fig. 7) comprise a putative propeptide that might be cleaved by proteases in the trans-Golgi network of some cells (8). We found that a polyclonal antiserum to residues 19–37 does not bind native PSGL-1 from human neutrophils. This suggests that human myeloid cells do cleave the propeptide, in agreement with conclusions made using a different immunologic assay (32). In contrast, the anti-19–37 serum binds strongly to recombinant PSGL-1 expressed in CHO cells, indicating that the propeptide had not been removed from PSGL-1 in these cells. Lysates of CHO cells that did not express PSGL-1 did not react with either antiserum.

Like PSGL-1, P-selectin is an extended molecule, with an ectodomain that is ~38 nm long (21). The PSGL-1-binding site of P-selectin includes the membrane-distal lectin domain, and the P-selectin-binding site of PSGL-1 includes the N-terminal, membrane-distal region. The interaction sites of both molecules, therefore, are projected well above their respective cell surfaces, which may optimize the opportunities for contact under shear forces. Indeed, shortening P-selectin by deletion of its internal short consensus repeats markedly diminishes its ability to mediate PSGL-1-dependent rolling of neutrophils on transfected CHO cells under shear forces (33). Most of the O-linked glycans of PSGL-1 may function indirectly by extending the N-terminal P-selectin-binding site far above the membrane, rather than by interacting directly with P-selectin.

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