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A Sulfated Peptide Segment at the Amino Terminus of PSGL-1 Is Critical for P-Selectin Binding

Dianne Sako,* Kenneth M. Comess,* Karen M. Barone, Raymond T. Camphausen, Dale A. Cumming, and Gray D. Shaw
Genetics Institute
Small Molecule Drug Discovery Group
85 Bolton Street
Cambridge, Massachusetts 02140

Summary

P-selectin glycoprotein ligand 1 (PSGL-1) is a mucin-like glycoprotein expressed on the surface of myeloid cells and serves as the high affinity counterreceptor for P-selectin. The PSGL-1–P-selectin interaction is calcium dependent and requires presentation of sialyl-Lewis^x (sLe^x)-type structures on the O-linked glycans of PSGL-1. We report here the identification of a non-carbohydrate component of the binding determinant that is critical for high affinity binding to P-selectin. Located within the first 19 amino acids, this anionic polypeptide segment contains at least one sulfated tyrosine residue. We propose that this sulfotyrosine-containing segment of PSGL-1, in conjunction with sLe^x presented on O-linked glycans, constitutes the high affinity P-selectin-binding site.

Introduction

The selectins are a family of calcium-dependent adhesion receptors that can mediate the initial "rolling" interaction between leukocytes and vascular endothelium prior to leukocyte extravasation (Lasky, 1992; Varki, 1994). P-selectin, E-selectin, and L-selectin are structurally similar, and each can bind to related fucosylated or sialylated tetrasaccharide structures (or both), such as sialyl-Lewis^x (sLe^x) or sialyl-Lewis^a (Foxall et al., 1992). Additionally, functional binding to P- and L-selectin is maintained after the substitution of sulfate for sialic acid in these oligosaccharides (Yuen et al., 1992; Brandley et al., 1993). Despite this common binding of sLe^x and related structures, it has become clear that the selectins have distinct adhesion patterns and different affinities for sLe^x-bearing glycoprotein ligands (Varki, 1994).

The human myeloid cell line HL-60 has been shown to display sLe^x-conjugated surface molecules capable of binding both P- and E-selectin (Zhou et al., 1991). Protease treatment of HL-60 cells abolished the binding of P-selectin but not E-selectin, suggesting that sLe^x alone is insufficient for P-selectin binding and some additional component is required (Larsen et al., 1992). A unique glycoprotein ligand consisting of a homodimer with two disulfide-linked subunits of apparent molecular mass ~120 kDa was identified from HL-60 cell membrane extracts following affinity purification using immobilized P-selectin (Moore et al., 1992). This ligand appears to share identity with P-selectin glycoprotein ligand 1 (PSGL-1), a glycoprotein cloned and characterized from an HL-60 cell cDNA library by using an expression cloning strategy employing P-selectin as a panning reagent (Sako et al., 1993). Coexpression of the PSGL-1 cDNA with a cDNA encoding α(1,3/1,4)fucosyltransferase (FT3) (Lowe et al., 1990) in COS cells produced functional PSGL-1 that binds to P-selectin in a calcium-dependent manner. Antibodies raised against this recombinant PSGL-1 recognize the major P-selectin-binding protein purified from HL-60 cell membranes (Sako et al., 1993). PSGL-1 also appears to be the critical calcium-dependent ligand for E-selectin in HL-60 cells (Asa et al., 1995).

Glycosidase digestion of myeloid and soluble recombinant forms of PSGL-1 revealed the presence of O-linked and N-linked oligosaccharides (Sako et al., 1993; Moore et al., 1994). These experiments suggested a key role for O-linked, but not N-linked, oligosaccharides in the binding to P-selectin.

At the outset of this study, we sought to identify the unique structural features of PSGL-1 that enable specific recognition by P-selectin. We began by mapping the location of sLe^x-containing O-linked oligosaccharides present on PSGL-1 that are essential for its binding to P-selectin. Inspection of the amino acids encoded by the cDNA indicated that the central portion of the extracellular domain of PSGL-1 contains 15 decameric repeats that are especially rich in prolines and threonines; additional serines and threonines in proximity of prolines extend from the repeats to the amino terminus. Because O-linked oligosaccharide attachment sites are often found at serine and threonine residues proximal to proline residues in glycoproteins (Wilson et al., 1991), we elected to follow a deletion strategy and remove increasing amounts of the putative O-linked oligosaccharide attachment sites. This was done by generating a series of carboxy-terminal truncated mutants of PSGL-1, each of which is fused to a human immunoglobulin G (IgG) Fc domain. The resulting series of soluble dimeric chimeras was used to delineate the minimal domains required for P- and E-selectin binding. We also noted the unusual presence of three potential sites for tyrosine sulfation found near the amino terminus of mature PSGL-1 (Sako et al., 1993). Using site-directed mutagenesis, we have assessed their functional importance in P- and E-selectin binding.

Our results demonstrate that the first 19 amino acids of PSGL-1, containing only a single O-linked oligosaccharide, are capable of binding to P-selectin. We show that this region contains at least one sulfated tyrosine residue that is necessary for high affinity binding to P-selectin. In addition, differential in vitro binding of E- and P-selectin is observed with specific mutated forms of PSGL-1.

*These authors contributed equally to this work.

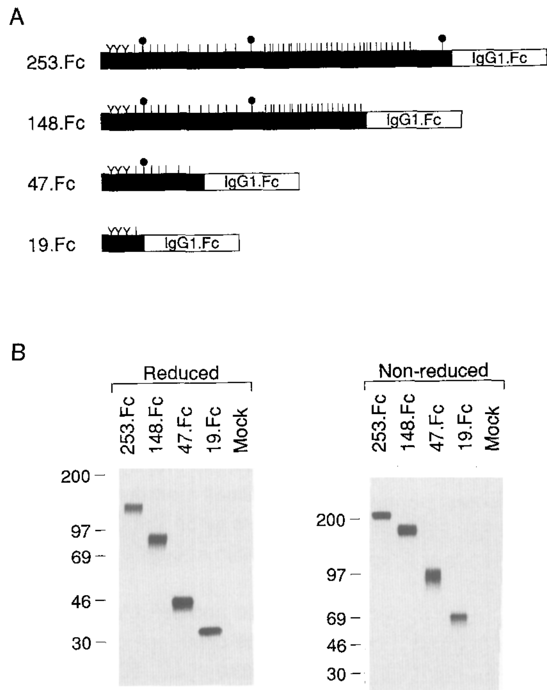

Figure 1. Structure of PSGL-1 Chimeras

(A) Schematic representation of the chimeric PSGL-1–IgG deletion series. The closed bars indicate PSGL-1 segments, and the open bars represent IgG segments. Y, amino-terminal tyrosine. Vertical lines denote the approximate number and location of O-linked oligosaccharides. Vertical lines with closed circles represent locations of potential N-linked glycosylation sites.

(B) Autoradiograph of the series of soluble PSGL-1–IgG chimeras. COS cells were transiently transfected with plasmids encoding sPACE, 3/4FT, and the chimera. Conditioned medium containing the [^{35}S]methionine-labeled chimeras was precipitated using protein A–Sepharose and resolved on SDS–polyacrylamide gels under either reducing or nonreducing conditions as indicated. Mock lanes refer to cells transfected only with sPACE and 3/4FT plasmids. Numbers on the left column correspond to molecular mass markers.

Results

Recombinant PSGL-1-Fc Chimeras Are Processed and Secreted as Soluble Dimers

Previously, we have shown that a soluble form of PSGL-1, sPSGL-1.T7, encoding the extracellular amino-terminal 254 amino acids fused to an epitope tag sequence, is capable of binding both P- and E-selectin in a calcium-dependent fashion (Sako et al., 1993). To define the binding epitope for P-selectin, we fused various lengths of the extracellular domain of PSGL-1 to the heavy chain C_H2–C_H3 region of IgG1 (Capon et al., 1989). This chimeric format facilitated formation of soluble dimers and enabled rapid purification by protein A–Sepharose.

As shown schematically in Figure 1A, 253.Fc is the longest of these chimeric fusions and, like sPSGL-1.T7, contains all potential O- and N-linked glycosylation sites. The shortest chimera, 19.Fc, contains only the first 19 amino acids and no N-linked glycosylation sites. Expression plasmids encoding these chimeras were cotransfected into COS cells with a plasmid encoding an FT3 enzyme (Lowe et al., 1990) and a plasmid encoding a soluble form of the paired basic amino acid converting enzyme (PACE) (Rehemtula and Kaufman, 1992; Wasley et al., 1993). The rationale for including the PACE was predicated on amino-terminal amino acid sequencing results. Purified recombinant PSGL-1.T7 produced from COS cells yields a peptide sequence QATEYE, confirming the utilization of the PACE consensus sequence (R. Ettling, H. White, data not shown). Therefore, the PACE plasmid was included in the cotransfection experiments to ensure complete processing of PSGL-1. Figure 1B demonstrates that all of the truncated forms of PSGL-1 resulting from cotransfections in COS cells were secreted as disulfide-linked soluble homodimers.

Binding of Truncated PSGL-1-Fc Chimeras to P- and E-Selectin

Two different assays were used to monitor the binding of PSGL-1 constructs to P- and E-selectin. The first of these employs soluble chimeric forms of P-selectin or E-selectin covalently coupled to agarose beads and measures the binding of [^{35}S]methionine-labeled PSGL-1 chimeras. Only interactions with sufficient affinity to withstand multiple washing steps yield demonstrable binding. The behavior in this assay of the longest PSGL-1 construct, 253.Fc, is consistent with previous studies of PSGL-1 isolated from human neutrophils (Moore et al., 1992, 1994; Norgard et al., 1993). The protein can be reprecipitated by a polyclonal PSGL-1 antibody after purification on protein A–Sepharose and treatment with sialidase, yet no longer can be affinity captured by P-selectin (Figure 2A). Experiments in which a fucosyltransferase enzyme is omitted from COS cell cotransfections demonstrate the dependence of the protein on fucosylation for activity as well (Figure 2A). In all experiments, recombinant PSGL-1 mutants bound to the P-selectin resin are eluted with EDTA and EGTA, demonstrating cation (presumably calcium) dependence of the interaction. The interaction therefore appears to have a dependence on sLe^x or a related structure. A titration of the amount of 253.Fc-conditioned medium added into this assay indicates a linear dose response over as much as a 40-fold range, as monitored by autoradiography (Figure 2A) and phosphorimager quantitation.

Figure 2B shows that each of the truncated forms of PSGL-1 binds to P-selectin with sufficient affinity to be detected in this assay. Surprisingly, even the 19.Fc chimera, comprising only the first 19 amino acids of PSGL-1, exhibits binding to P-selectin. Phosphorimager analysis indicates that binding of the 19.Fc form to P-selectin occurs to approximately 5% the extent of binding of the 253.Fc form. For the E-selectin chimera, only the 253.Fc and 148.Fc chimeras are observed to bind with comparable affinity. Trace amounts of the 47.Fc chimera are detected to bind E-selectin, and the 19.Fc construct demonstrates no detectable binding. Essentially identical results also are obtained from constructs cotransfected with the recently cloned myeloid α(1,3)fucosyltransferase, FT7 (Natsuka et al., 1994; Sasaki et al., 1994) (data not shown).

In the second assay, CHO cells expressing full-length

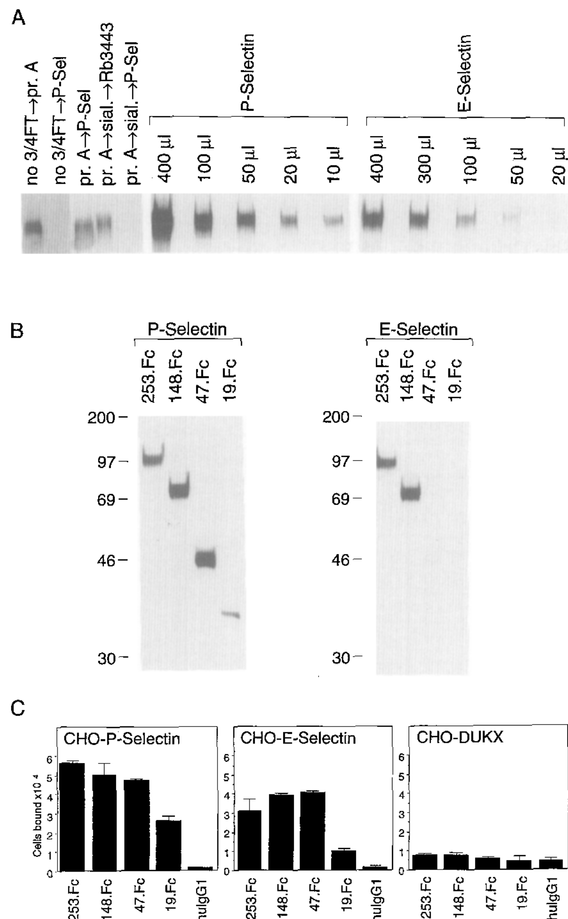

Figure 2. Selectin Binding Studies of PSGL-1 Chimeras

(A) Autoradiograph of 253.Fc protein affinity capture experiments. The left five lanes show the effect on P-selectin affinity capture of cotransfection with and without the vector encoding a fucosyltransferase enzyme (3/4FT), as well as the effect of treatment with sialidase. The [^{35}S]methionine-labeled PSGL-1–IgG chimera was purified from conditioned medium with protein A–Sepharose (pr. A) prior to treatment with sialidase (sial.). The remaining lanes show the effects on P- and E-selectin affinity capture of incubating various amounts of conditioned medium with the capture resin. COS cell–conditioned media containing the protein were precipitated using selectin–IgG chimeras coupled to agarose beads, eluted with EDTA and EGTA, and run on SDS–polyacrylamide gel under reducing conditions.

(B) Autoradiograph of PSGL-1–IgG chimera series affinity captured by P- and E-selectin and eluted with EDTA and EGTA as above. Numbers on the left column correspond to molecular mass markers.

(C) Cell binding assay. Microtiter plate wells were coated with purified PSGL-1–IgG chimeras or human IgG antibody at 2 µg/ml and incubated with ^{3}H-thymidine-labeled CHO cells expressing membrane-bound P-selectin, E-selectin, or negative control (DUKX) cells.

membrane-bound P- and E-selectin (Larsen et al., 1991) were assayed for their binding to microtiter plate wells coated with the PSGL-1 chimeras. This assay allows multivalent interactions; binding events with intrinsic affinities as low as the millimolar range therefore can yield demonstrable binding. Figure 2C shows the results of this assay. As expected, PSGL-1 chimeras that exhibit activity in the higher affinity capture assay also support binding in this assay. In addition, the two chimeras that show little or no higher affinity binding to E-selectin do support binding in this multivalent system. Indeed, in this system the 47.Fc construct is able to support binding at a level indistinguishable from 253.Fc or 148.Fc. Only background levels of binding to any of the PSGL-1 chimeras are observed for control CHO–DUKX cells (Figure 2C). Again, similar results are observed when cotransfections are carried out with FT7 instead of FT3. Taken together, the results from these two binding assays demonstrate that even the first 19 amino-terminal residues of PSGL-1 contain a determinant important for high affinity P-selectin binding and that differential binding of E- and P-selectin could be observed among the various PSGL-1 constructs.

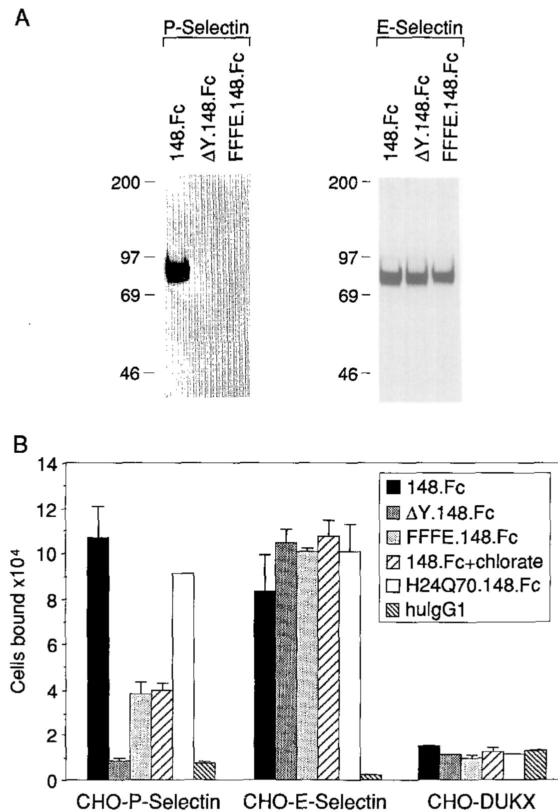

Figure 3. Selectin Binding Studies of PSGL-1 148.Fc Chimera Mutants

(A) Autoradiograph of PSGL-1–IgG chimera mutants affinity captured by P-selectin or E-selectin by method described in Figure 2A.

(B) Cell binding assay of PSGL-1–IgG chimera mutants by method described in Figure 2B.

Sulfation, but Not N-Glycosylation, Is Required for PSGL-1 Binding to P-Selectin

The 148.Fc form of PSGL-1 was chosen for the next series of studies because it is the shortest chimera that supports the higher affinity binding of both P- and E-selectin. To confirm directly that N-linked oligosaccharides on PSGL-1 are not required for selectin binding, we mutated the two remaining putative N-linked glycosylation sites within 148.Fc, producing the mutant H24Q70.148.Fc. The cell-based binding assay was used to show that this mutant remains fully capable of binding to both P- and E-selectin (Figure 3B). This result confirms previous studies em-

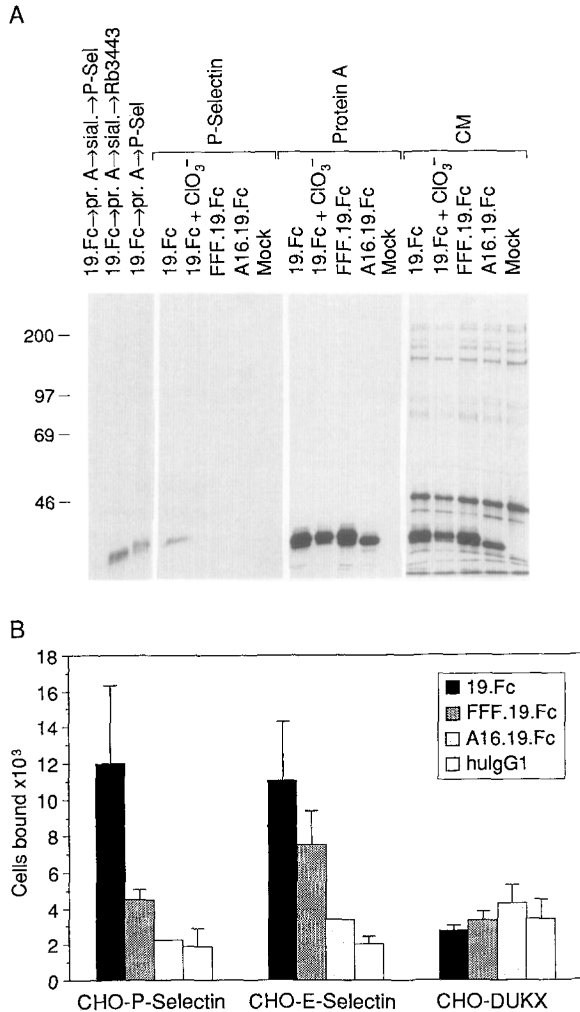

Figure 4. Selectin Binding Studies of PSGL-1 19.Fc Chimera Mutants

(A) Autoradiograph of 19.Fc chimera mutants affinity captured by P-selectin, protein A–Sepharose, or conditioned medium (CM), as indicated above the lanes. Chlorate-treated cells are indicated (ClO_3^-).

(B) Cell binding assay of 19.Fc chimeras, by method described in Figure 2B.

ploying peptide N-glycosidase on both recombinant and natural PSGL-1 (Sako et al., 1993; Moore et al., 1994). In addition, the present results suggest that the N-linked oligosaccharides on PSGL-1 also are not required for E-selectin binding.

Next, we examined whether sulfation of PSGL-1 is important for its binding to P-selectin. A metabolic inhibitor of ATP sulfurylase activity, sodium chlorate (Lipmann, 1958), was used to prevent sulfation of both natural PSGL-1 on myeloid cells and recombinant PSGL-1 in COS cells. Papain-treated HL-60 cells were incubated in the presence or absence of 50 mM chlorate for 48 hr. Analysis by fluorescence-activated cell sorting, employing fluorescently labeled selectin chimeras, demonstrated a significantly decreased level of P-selectin reactivity with PSGL-1 on the surface of chlorate-treated cells. Surface expression of PSGL-1 on these cells was confirmed with a fluorescently labeled monoclonal antibody to PSGL-1 (data not shown).

Sodium chlorate treatment of COS cells producing the 148.Fc protein also yielded PSGL-1 with markedly reduced P-selectin binding (Figure 3B), indicating the importance of sulfation for PSGL-1 function. In contrast, E-selectin binding was completely unaffected by sodium chlorate treatment of myeloid cells or COS cells expressing 148.Fc (Figure 3B).

The Critical Sites of PSGL-1 Sulfation Reside on the Amino-Terminal Tyrosines

Because sodium chlorate inhibits both carbohydrate and tyrosine sulfation, the sites of critical sulfation could be on O-linked oligosaccharides, one or more of these tyrosine residues, or both. Since the first 14 residues of PACE-processed PSGL-1 contain a region with several potential sites of tyrosine sulfation (Huttner and Baeuerle, 1988), the

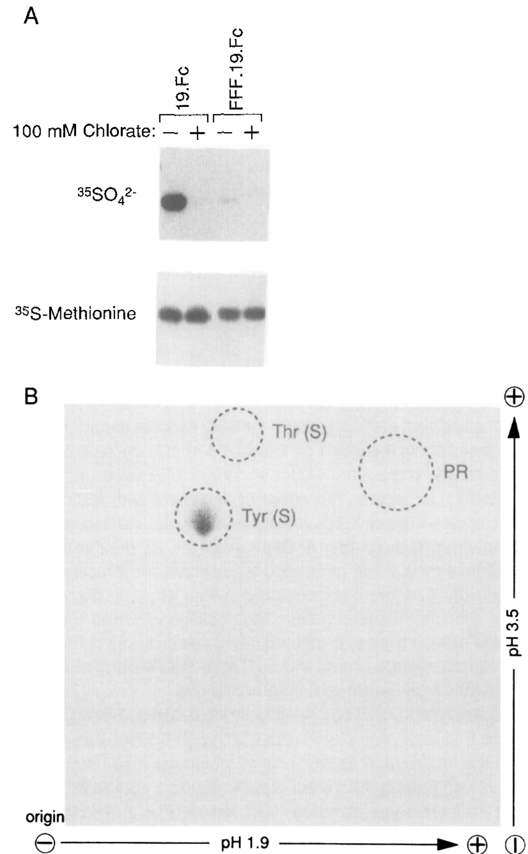

Figure 5. Analysis of Sulfate in PSGL-1 19.Fc Chimeras

(A) Autoradiograph showing sulfate incorporation into 19.Fc chimera proteins. Conditioned medium from cells fed with either [^{35}S]sulfate or [^{35}S]methionine, in the presence and absence of sodium chlorate, was affinity precipitated using protein A–Sepharose, and the proteins were resolved by reducing SDS–polyacrylamide gel electrophoresis.

(B) Thin-layer electrophoresis to detect tyrosine sulfate. A [^{35}S]sulfate-labeled and protein A–purified 19.Fc chimera sample was subjected to alkaline hydrolysis with barium hydroxide and analyzed by two-dimensional gel electrophoresis at pH 1.9 and 3.5. The autoradiograph is shown, and the locations of unlabeled tyrosine sulfate (Tyr(S)), threonine sulfate (Thr(S)), and phenol red (PR) markers are denoted by dashed circles.

148.Fc chimera was used to create two types of specific mutations in the region of putative tyrosine sulfation. An internal deletion mutant, ΔY148.Fc, was created in which the seven amino acids from positions 5–11 of the mature extracellular domain were removed. The deleted region contains three putative tyrosine sulfation sites. This mutant fails to bind P-selectin in either binding assay, yet E-selectin binding is fully maintained (Figures 3A and 3B). The second mutant, FFFE.148.Fc, replaces all three tyrosines, at positions 5, 7, and 10, with phenylalanine and the aspartic acid at position 11 with a glutamic acid. This mutant does show some binding to P-selectin, but only in the multivalent assay. The level of binding is similar to that found after chlorate treatment of the 148.Fc form (Figure 3B). Binding to E-selectin in either assay was completely unaffected by the quadruple mutation. It should be noted that in the lower affinity assay an additional contribution of the acidic residues flanking the tyrosines is indicated (Figure 3B). The data therefore suggest that both the amino-terminal tyrosines and the flanking acidic residues are important specifically for PSGL-1 binding to P-selectin but not E-selectin. To determine whether the amino-terminal tyrosines of PSGL-1 are sulfated, we utilized the 19.Fc chimera in further experiments. Expression of this protein in the presence of chlorate completely abolished binding to P-selectin in the higher affinity assay (Figure 4A). Control precipitations with protein A–Sepharose show that the protein still was synthesized. A triple mutant, termed FFF.19.Fc, also was created to convert each of the three tyrosines at positions 5, 7, and 10 into phenylalanines. Figure 4 shows that, as was seen for FFFE.148.Fc mutant, this mutant fails to bind P-selectin in the higher affinity binding assay. Again, however, a low level of binding is observed in the multivalent cell binding assay. Interestingly, a significant level of E-selectin binding with FFF.19.Fc is still detected in the cell binding assay (Figure 4B).

The residual binding of the FFF.19.Fc mutant to the selectins is likely to be due to an sLe^x-modified O-linked oligosaccharide at Thr-3 or Thr-16. Omitting the plasmid encoding a fucosyltransferase enzyme during COS cell transfection yields a 19.Fc molecule unable to support P- or E-selectin binding (data not shown). Treatment with sialidase also abrogates P-selectin-binding activity (Figure 4A). We created a mutant (A16.19.Fc) in which an alanine residue replaces the threonine at position 16. This mutant exhibits a small shift in gel mobility as compared with 19.Fc, consistent with a loss of oligosaccharide. Moreover, this mutant is unable to support binding to either P- or E-selectin (Figure 4). These data strongly suggest that an O-linked oligosaccharide bearing sLe^x and located at Thr-16 also is essential for 19.Fc binding to P- and E-selectin.

Figure 5 provides evidence that sulfation occurs exclusively on the tyrosines. Examination of [^{35}S]sulfate incorporation by autoradiography shows that far more labeled sulfate is incorporated into 19.Fc than into FFF.19.Fc (Figure 5A). The small amount of label incorporated in the FFF.19.Fc is comparable with levels observed for other unrelated polypeptides fused into the IgG constant region (data not shown) and therefore is likely to represent incorporation into the IgG region rather than into the oligosaccharide at Thr-16. Treatment with sodium chlorate at concentrations that inhibit binding to P-selectin also inhibited the incorporation of sulfate into the 19.Fc chimera. The chlorate effect is specific, as parallel labelings using [^{35}S]methionine indicate that synthesis and secretion of the chimeras in treated or untreated cells are nearly equivalent (Figure 5A).

To confirm directly the presence of sulfated tyrosine, [^{35}S]sulfate-labeled 19.Fc protein was excised from an SDS gel, subjected to alkaline hydrolysis, and then analyzed by two-dimensional thin-layer electrophoresis. Figure 5B shows a single spot from the labeled, hydrolyzed material that comigrates with a sulfated tyrosine standard. At least one sulfated tyrosine therefore is present in PSGL-1.

Discussion

While several glycoproteins have been reported to bind in a specific fashion to L-selectin (Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993), PSGL-1 is the only high affinity P-selectin ligand characterized to date and appears to account for all of the high affinity P-selectin-binding sites on human neutrophils (Moore et al., 1995). Monomeric P-selectin has been reported to bind to ~25,000 sites per neutrophil with an apparent K_d of ~70 nM (Ushiyama et al., 1993). The focus of our work has been to determine what structural features of PSGL-1 differentiate its activity from other sialomucins or glycoproteins.

The results of the mutagenesis studies presented here indicate that an anionic amino-terminal peptide segment of PSGL-1 provides an essential component for the specific high affinity interaction of PSGL-1 with P-selectin. This region is comprised of several acidic amino acid residues and three tyrosines. Furthermore, sulfation of tyrosine residues within this anionic peptide segment is critical for the high affinity binding of P-selectin. This modified region alone is insufficient for high affinity binding, but in concert with an O-linked oligosaccharide modified by sLe^x or a related structure it provides the high affinity recognition determinant for P-selectin. It is noteworthy that sulfation of O-linked glycans rather than tyrosine residues has been shown to be critical for the binding of glycosylation-dependent cell adhesion molecule 1 to L-selectin (Imai et al., 1993; Hemmerich et al., 1994, 1995; Hemmerich and Rosen, 1994).

The profound effect of tyrosine to phenylalanine substitutions on activity may be due solely to removal of the substrate for sulfation or a combination of substrate removal and functional significance of the tyrosine side chain itself. While it is also possible that these mutations are acting indirectly, by altering the sLe^x modification of carbohydrate on the ligand, this is unlikely, since the carbohydrate components of the ΔY148.Fc, FFFE.148.Fc, and FFF.19.Fc mutants are unchanged in their ability to

bind to E-selectin. E-selectin binding indicates the presence of functional sLe^x (or related structures) on the O-linked oligosaccharides of these chimeric proteins, as only glycoproteins modified with sLe^x (or related structures) exhibit binding to E-selectin in vitro.

Our studies are based on PSGL-1 produced in COS cells in the presence of FT3. The use of the myeloid fucosyltransferase, FT7, yields similar results. A potential concern still arises that this COS cell–produced PSGL-1 differs substantially from the native PSGL-1 found on the surface of human neutrophils. While we cannot rule out some structural differences in the oligosaccharides, the validity of the COS cell–produced material for the type of analysis presented here is strongly reinforced by the unique recognition of the recombinant PSGL-1 by P-selectin. Other neutrophil sialoglycoproteins produced in the analogous manner fail to show P-selectin binding (Sako et al., 1993). Polyclonal antisera raised against COS-produced PSGL-1 can block the adherence of HL-60 cells to P-selectin in vitro (Vachino et al., 1995). Chlorate treatment of HL-60 cells reduces the level of binding to P-selectin, but not to E-selectin. Finally, labeled sulfate is incorporated in PSGL-1 isolated from human myeloid U937 cells (R. T. C., J. Flannery, and D. S., unpublished data). Given these similar characteristics between native and recombinant PSGL-1, we believe that the recombinant PSGL-1 is a valid test system and reflects the functional importance of sulfated tyrosines in native PSGL-1.

Previous attempts to assess the molecular determinants of P-selectin ligand recognition have yielded data consistent with unique presentation of oligosaccharides being the sole critical component. One hypothesis, proposed by McEver and colleagues (Norgard et al., 1993) and expanded upon in a recent review (Varki, 1994) is that high affinity selectin–ligand interactions result from a multivalent presentation of sLe^x-modified oligosaccharides. In the specific case of PSGL-1, a "clustered saccharide patch" presented by unique high density O-linked oligosaccharides may be responsible for the observed high affinity and specific binding to P-selectin. While the apparent increase in binding observed between 19.Fc and 148.Fc is consistent with the notion that more than one O-linked oligosaccharide is required for the full binding activity of PSGL-1, this observation is also consistent with the idea that the optimal O-linked oligosaccharide is not localized at Thr-16. Since the ΔY148.Fc and FFFE.148.Fc constructs contain multiple O-links yet fail to bind to P-selectin, clustered oligosaccharides alone cannot constitute the high affinity recognition epitope.

The results presented here argue convincingly for a multicomponent recognition motif. Figure 6 shows a schematic model for this interaction in which both a carbohydrate moiety and an anionic sulfated polypeptide near the amino terminus are necessary for high affinity binding to P-selectin. In contrast, E-selectin binding does not require the anionic peptide portion of the PSGL-1-binding determinant.

The sulfated anionic region resembles that of other sulfotyrosine-containing proteins, such as glycoprotein Ibα

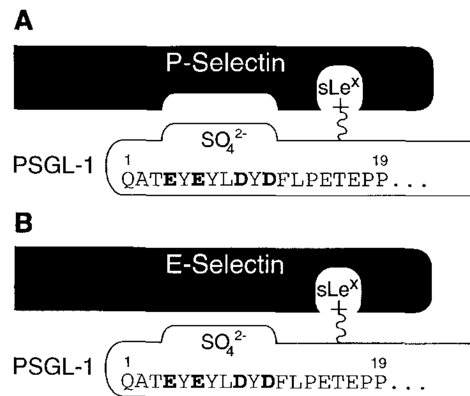

Figure 6. The Sulfated Peptide Segment in PSGL-1 Is Critical for P-Selectin Binding, but Not E-Selectin Binding

The amino-terminal 19 amino acids of mature (PACE-cleaved) PSGL-1 are shown. A bulge over residues 4–11 denotes one recognition component, comprising one or more sulfated tyrosines and four acidic residues. The structure drawn above position 16 indicates the second component, an O-linked oligosaccharide modified with sLe^x or possibly another sialylated fucosylated structure. Both components are necessary for high affinity binding to P-selectin, but only the latter component is critical for E-selectin binding.

(Dong et al., 1994), hirudin (Rydel et al., 1990), and factor VIII (Leyte et al., 1991), yet no direct homology of residues 1–19 of PSGL-1 with any other protein has been found in the GenBank or Swissprot protein database. Each of the above proteins is involved in a ligand- or receptor-binding interaction and requires tyrosine sulfation for full activity. It is interesting to note that the mouse homolog of PSGL-1 also contains a consensus site for tyrosine sulfation at the analogous amino-terminal position, as well as several acidic residues (Yang et al., submitted). Electrostatic binding interactions mediated by the sulfated region of PSGL-1 are consistent with the recently measured high tensile strength of the unimolecular interaction between purified P-selectin and PSGL-1 on neutrophils (Alon et al., 1995). The exact position and number of sulfation sites, as well as the precise structure of the fucosylated oligosaccharide at Thr-16, are the subject of ongoing studies.

The location of the site on P-selectin that interacts with the anionic, sulfated amino-terminal region of PSGL-1 remains to be determined. Several mutagenesis studies suggest that a common site exists on P- and E-selectin for calcium-dependent carbohydrate binding (Erbe et al., 1992, 1993; Hollenbaugh et al., 1993; Bajorath et al., 1994). Crystallographic studies on E-selectin (Graves et al., 1994) indicate that the binding site for sLe^x within the lectin domain is very small. Given this data, it seems unlikely that the binding site on P-selectin for the sulfated peptide closely overlaps the sLe^x binding site. Two reasonable alternatives are, first, a region outside of the sLe^x-binding site but still within the P-selectin lectin domain and, second, the P-selectin epidermal growth factor-like domain. The latter possibility is supported by studies indicating the P-selectin epidermal growth factor domain to be

critical for high affinity binding (Kansas et al., 1994; Gibson et al., 1995) and by the fact that this domain is highly conserved among species. Additional studies are required to define fully the binding sites on P-selectin and resolve this issue.

Lastly, it is interesting to consider the macromolecular structure of PSGL-1 in terms of its physiological function. Experiments using immunogold electron microscopy have indicated that the surface distribution of PSGL-1 on neutrophils is localized at the tips of microvilli (Moore et al., 1995). Our data further localize the critical binding sites to the amino-terminal segment of PSGL-1. The inclusion of the segment from amino acid residues 48–253 did not appreciably enhance the P-selectin-binding properties of our soluble PSGL-1 chimeras. Yet this segment of PSGL-1, which includes the 15 decameric repeats, contains the highest density of O-linked oligosaccharides. Mucin domains having closely packed O-linked carbohydrates are postulated to form rigid rod-like structures extending outward from the cell surface (Jentoff, 1990). It is therefore likely that the high density O-linked segment in PSGL-1 serves to extend the amino-terminal binding determinants outward from the cell surface, where they are readily accessible for selectin binding. In this regard, a rapid association rate constant for P-selectin has been noted (Lawrence and Springer, 1991; Ushiyama et al., 1993). Thus, it is logical that the binding determinants are localized at the amino terminus. Moreover, at physiological shear stress, this configuration is likely to facilitate the efficient tethering and rolling of neutrophils along the blood vessel wall.

Experimental Procedures

Plasmid Constructions

The plasmid pED.253.Fc, expressing a P-selectin ligand protein–IgG chimera, was generated by restricting plasmid pPL85 (Sako et al., 1993) with XbaI and HincII and ligating the purified 950 bp fragment to vector pED.Fc. pED.Fc is expression vector pED (Kaufman et al., 1991) having a cDNA insert encoding the Fc segment of human IgG1 with an adapter sequence immediately 5′ to the hinge region. The fusion of the extracellular portion of PSGL-1 after the valine codon 295 encodes amino acids LRPQSR DKTHTCPPC at the junction. Plasmid constructs pED.148.Fc, pED.47.Fc, and pED.19.Fc were created by standard PCR technique, using pED.253.Fc as template and the following pairs of oligonucleotide primers. Universal upstream primer: 5′-CCAGGTCCAACTGCAGGTCGACTCTAGAGGGCACTTCTT-CTGGGCCCACG. Downstream primer for 148.Fc: 5′-TATTATCT-GTGCGGCCGCCCTCCAGAACCCATGGCTGCTGGTTGCAGTGG. Downstream primer for 47.Fc: 5′-TATTATCTGTGCGGCCGCGCAG-CAGGCTCCACAGTGGTAG. Downstream primer for 19.Fc: 5′-TAT-TATCTGTGCGGCCGCGGAGGCTCCGTTTCTGGCAG. PCR product DNA was digested with PstI and NotI, gel purified, and ligated with pED.Fc vector fragment. Correct constructs were identified by restriction analysis and confirmed by DNA sequencing. Plasmids pED-.ΔY148.Fc, H24Q70.148.Fc, and A16.19.Fc were created by site-directed mutagenesis (Sambrook et al., 1989) using pED.148.Fc or pED.19.Fc as templates. The mutagenesis oligonucleotide for ΔY148 was 5′-CGGAGACAGGCCACCGAATTCCTGCCAGAAACG. Positive colonies were identified by colony hybridization (Sambrook et al., 1989). The pED.FFFE.148.FC vector was constructed by restricting pED.ΔY148.Fc with EcoRI and ligating the following annealed oligonucleotides: 5′-AATTCGAGTTCCTAGATTTTG and 5′-AATTCAAAATC-TAGGAACTCG. The pED.FFF.19.Fc vector was made by restricting pED.ΔY148.Fc with EcoRI and NotI and ligating the following annealed oligonucleotides: 5′-AATTCGAGTTCCTAGATTTCGATTTCCTGCCA-GAAACTGAGCCTCCGC and 5′-GGCCGCGGAGGCTCAGTTTCTG-GCAGGAAATCGAAATCTAGGAACTCG.

Production and Purification of Selectin Chimeras

P-selectin cDNA was fused after the encoded fourth consensus repeat domain to IgG1 Fc to create a soluble P-selectin–IgG chimera in vector pED. The resulting expression plasmid was used to establish a stable CHO cell line. Secreted chimeras were purified from serum-free medium by conventional chromatography. E-selectin–IgG chimera was constructed by fusing coding sequences of complete extracellular domain of E-selectin with human IgG1 Fc of pED.Fc. An AscI fragment containing the fusion cDNA was ligated at polylinker of baculovirus vector pVL1393 (Webb and Summers, 1990), creating plasmid pVLsE.Fc. Recombinant baculovirus was generated by cotransfecting Sf9 cells with 3 μg of pVLsE.Fc plasmid DNA, 1 μg of BaculoGold AcMNPV viral DNA (Pharmingen), followed by plaque purification. High titer virus (108 pfu/ml) was used to infect Trichoplusia ni High Five cells (Invitrogen). The supernatant 72 hr postinfection was collected, and E-selectin–IgG chimera was affinity purified using protein A–Sepharose (Pierce).

Affinity Capture Assays

The E-selectin chimera, P-selectin chimera, and human IgG1 (Sigma) were covalently coupled to a gel resin via imide ester–amide exchange chemistry (Affi-Gel 15; Bio-Rad) using the protocols of the manufacturer. Plasmid DNAs encoding the various forms of sPSGL-1–Fc chimeras were cotransfected with vectors pEA.3/4FT and pEA.sPACE into COS cells using the DEAE–dextran technique (Keown et al., 1990) and labeled metabolically with [^{35}S]methionine. For sulfate incorporation experiments, cells were starved for 1 hr in sulfate-free EMEM medium (Specialty Media), supplemented with 10% of normal concentrations of cysteine and methionine (Gibco) as well as 100 mM sodium chlorate (Fluka). Labeling was carried out for 6–7 hr by adding the same medium, supplemented with 5% dialyzed fetal calf serum, 10 mM HEPES (pH 7.2), and either ^{35}S-labeled sulfate or methionine (New England Nuclear). Control labelings were done in chlorate-free medium. Conditioned media were collected after labeling, aprotinin and sodium azide were added to 0.1% and 0.05%, respectively, and the conditioned media then were centrifuged to remove cell debris and detached cells. Labeled conditioned media, mixed 1:2 with affinity capture buffer (TBS, 5 mg/ml BSA, 2 mM $CaCl_2$, 0.04% Triton X-100, and 0.02% sodium azide), were added to affinity resins, and the mixtures were gently inverted overnight at 4°C. Resins then were washed extensively with BSA-free affinity capture buffer and batch-eluted with TBS containing 5 mM EDTA and 5 mM EGTA.

For control affinity precipitations, protein A–Sepharose 4 fast flow (Pharmacia) or a polyclonal rabbit anti-PSGL-1 antibody, Rb3443, coupled to Affi-Gel 10 was used. The antibody was raised against a peptide comprising the first 15 amino acids of the mature PSGL-1 amino terminus. Quantitative analysis was carried out by using ImageQuaNT software and a phosphorimager (Molecular Dynamics). Data were normalized for differential expression and labeling by using Rb3443 immunoprecipitation data.

Cell Binding Assay

COS cells were transfected with the various PSGL-1–IgG chimera plasmids as for the affinity capture assay. Serum-free medium (50 ml), collected 40–64 hr posttransfection from 1×10^7 COS cells, was purified on a column of 0.25 ml of protein A–Sepharose equilibrated with TBS plus 2 mM $CaCl_2$. After washing with 20 ml of TBS plus 2 mM $CaCl_2$, the bound material was eluted with 0.5 ml of 0.1 M acetic acid, 0.15 M NaCl, 2 mM $CaCl_2$. The eluted material was neutralized with 5% volume of 3 M Tris (pH 9.0) and dialyzed against TBS plus 2 mM $CaCl_2$, 0.1 mM PMSF. The material was quantitated by measuring absorbance at 280 nm and by Coomassie blue staining of SDS-polyacrylamide–Laemmli gels. The protein concentration was adjusted to 2 μg/ml prior to assay.

Microtiter plates (48 well, untreated; Corning) were coated for 16 hr at 4°C with 0.2 ml of purified sPSGL-1–Fc chimeras. The unbound material was removed, and the coated wells were treated with Hanks' buffered salt solution with 10 mg/ml BSA and 2 mM $CaCl_2$ for 1 hr at 4°C. CHO–P-selectin, CHO–E-selectin, and CHO–DUKX cells (Larsen

et al., 1992) were labeled by overnight incubation with ^{3}H-thymidine (New England Nuclear), and counts per minute per number of cells was recorded. Cells (2 × 10^5) were added to the prepared microtiter plates and incubated for 8 min at room temperature. The plates were washed four times with Hanks' buffered salt solution, 2 mM $CaCl_2$ and trypsinized, and the bound cells were quantitated by scintillation counting.

Sialidase Treatment of PSGL-1 Chimeras

[^{35}S]methionine-labeled 253.Fc or 19.Fc mutant constructs, purified from conditioned medium by protein A–Sepharose chromatography, were incubated with sialidase from Arthrobacter ureafaciens (Calbiochem) in a buffer containing 150 mM NaCl, 60 mM sodium acetate, 8 mM calcium chloride, and 0.02% Triton X-100 at pH 5.5. After a 16 hr digestion with 200 mU/ml enzyme at 37°C, the samples were diluted into affinity capture buffer, incubated with either P-selectin coupled to Affi-Gel 15 or rabbit polyclonal antibody Rb3443 coupled to Affi-Gel 10, and processed as described above.

Alkaline Hydrolysis Treatment of 19.Fc

Transfected COS cells were labeled with [^{35}S]sulfate as above, and the conditioned medium was subjected to barium hydroxide hydrolysis as described by Huttner (1984). The following modifications were performed as described previously (Pittman et al., 1992). The ^{35}S-labeled conditioned medium was incubated with protein A–Sepharose, and the bound protein was run on a 12% SDS–polyacrylamide gel and transferred to Immobilon-P (Millipore). The filter was exposed to film, and the labeled band of interest was excised from the membrane and transferred to a Tuftainer (Pierce). Degassed 0.2 M barium hydroxide was added, and the tube was sealed under N_2 and baked for 18 hr at 110°C. Following neutralization and lyophilization, the pellet was resuspended in 7.8% acetic acid, and 2.2% formic acid. We added 5 μg each of unlabeled tyrosine sulfate and threonine sulfate to the sample to run as standards. The sample was spotted onto 20 × 20 cm 100 μm cellulose thin-layer sheets (Kodak) and two-dimensional electrophoresis was performed. The standards were visualized with 1% ninhydrin in acetone, and tyrosine [^{35}S]sulfate was detected by phosphorimager analysis (Fuji) and autoradiography.

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References

Alon, R., Hammer, D.A., and Springer, T.A. (1995). Lifetime of the P-selectin-carbohydrate bond and its response to tensile force in hydrodynamic flow. Nature *374*, 539–542.

Asa, D., Raycroft, L., Ma, L., Aeed, P.A., Kaytes, P.S., Elhammer, A.P., and Geng, J.G. (1995). The P-selectin glycoprotein ligand functions as a common human leukocyte ligand for P- and E-selectins. J. Biol. Chem. *270*, 11662–11670.

Bajorath, J., Hollenbaugh, D., King, G., Harte, W., Eustice, D.C., Darveau, R.P., and Aruffo, A. (1994). Cd62/P-selectin binding sites for myeloid cells and sulfatides are overlapping. Biochemistry *33*, 1332–1339.

Baumhueter, S., Singer, M.S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S.D., and Lasky, L.A. (1993). Binding of L-selectin to the vascular sialomucin CD34. Science *262*, 436–438.

Berg, E.L., Mcevoy, L.M., Berlin, C., Bargatze, R.F., and Butcher, E.C. (1993). L-selectin-mediated lymphocyte rolling on MAdCAM-1. Nature *366*, 695–698.

Brandley, B.K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., and Hasegawa, A. (1993). Structure-function studies on selectin carbohydrate ligands: modifications to fucose, sialic acid and sulphate as a sialic acid replacement. Glycobiology *3*, 633–641.

Capon, D., Charmow, S., Mordenti, J., Marsters, S., Gregory, T., Mitsuya, H., Byrn, R., Lucas, C., Wurm, F., Groopman, G., Broder, S., and Smith, D. (1989). Designing CD4 immunoadhesins for AIDS therapy. Nature *337*, 525–531.

Dong, J., Li, C.Q., and Lopez, J.A. (1994). Tyrosine sulfation of the glycoprotein Ib-IX complex: identification of sulfated residues and effect on ligand binding. Biochemistry *33*, 13946–13953.

Erbe, D.V., Wolitzky, B.A., Presta, L.G., Norton, C.R., Ramos, R.J., Burns, D.K., Rumberger, J.M., Rao, B.N.N., Foxall, C., Brandley, B.K., and Lasky, L.A. (1992). Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. J. Cell Biol. *119*, 215–227.

Erbe, D.V., Watson, S.R., Presta, L.G., Wolitzky, B.A., Foxall, C., Brandley, B.K., and Lasky, L.A. (1993). P- and E-selectin use common sites for carbohydrate ligand recognition and cell adhesion. J. Cell Biol. *120*, 1227–1235.

Foxall, C., Watson, S.R., Dowbenko, D., Fennie, C., Lasky, L.A., Kiso, M., Hasegawa, A., Asa, D., and Brandley, B.K. (1992). The three members of the selectin receptor family recognize a common carbohydrate epitope, the sialyl Lewis X oligosaccharide. J. Cell Biol. *117*, 895–902.

Gibson, R.M., Kansas, G.S., Tedder, T.F., Furie, B., and Furie, B.C. (1995). Lectin and epidermal growth factor domains of P-selectin at physiologic density are the recognition unit for leukocyte binding. Blood *85*, 151–158.

Graves, B.J., Crowther, R.L., Chandran, C., Rumberger, J.M., Li, S., Huang, K.S., Presky, D.H., Familletti, P.C., Wolitzky, B.A., and Burns, D.K. (1994). Insight into E-selectin ligand interaction from the crystal structure and mutagenesis of the lec/EGF domains. Nature *367*, 532–538.

Hemmerich, S., and Rosen, S.D. (1994). 6′-Sulfated sialyl Lewis X is a major capping group of Glycam-1. Biochemistry *33*, 4830–4835.

Hemmerich, S., Bertozzi, C.R., Leffler, H., and Rosen, S.D. (1994). Identification of the sulfated monosaccharides of Glycam-1, an endothelial-derived ligand for L-selectin. Biochemistry *33*, 4820–4829.

Hemmerich, S., Leffler, H., and Rosen, S.D. (1995). Structure of the O-glycans in GlyCAM-1, an endothelial-derived ligand for L-selectin. J. Biol. Chem. *270*, 12035–12047.

Hollenbaugh, D., Bajorath, J., Stenkamp, R., and Aruffo, A. (1993). Interaction of P-selectin (CD62) and its cellular ligand: analysis of critical residues. Biochemistry *32*, 2960–2966.

Huttner, W.B. (1984). Determination and occurrence of tyrosine O-sulfate in proteins. Meth. Enzymol. *107*, 200–223.

Huttner, W.B., and Baeuerle, P.A. (1988). Protein sulfation on tyrosine. Mod. Cell Biol. *6*, 97–140.

Imai, Y., Lasky, L.A., and Rosen, S.D. (1993). Sulphation requirement for GlyCAM-1, an endothelial ligand for L-selectin. Nature *362*, 555–557.

Jentoff, N. (1990). Why are proteins O-glycosylated? Trends Biochem. Sci. *15*, 291–294.

Kansas, G.S., Saunders, K.B., Ley, K., Zakrzewicz, A., Gibson, R.M., Furie, B.C., Furie, B., and Tedder, T.F. (1994). A role for the epidermal growth factor-like domain of P-selectin in ligand recognition and cell adhesion. J. Cell Biol. *124*, 609–618.

Kaufman, R.J., Davies, M.V., Wasley, L.C., and Michnick, D. (1991). Improved vectors for stable expression of foreign genes in mammalian cells by use of the untranslated leader sequence from EMC virus. Nucl. Acids Res. *19*, 4485–4490.

Keown, W.K., Campbell, C.R., and Kucherlapati, R.S. (1990). Methods for introducing DNA into mammalian cells. In Methods in Enzymology: Gene Expression Technology, D.V. Goeddel, ed. (Orlando, Florida: Academic Press Incorporated), pp. 527–537.

Larsen, G.R., Sako, D., Ahern, T.J., Shaffer, M., Erban, J.E., Sajer, S.A., Gibson, R.M., Wagner, D.D., Furie, B.C., and Furie, B. (1992). P-selectin and E-selectin: distinct but overlapping leukocyte ligand specificities. J. Biol. Chem. *267*, 11104–11110.

Lasky, L.A. (1992). Selectins: interpreters of cell-specific carbohydrate information during inflammation. Science *258*, 964–969.

Laskey, L.A., Singer, M.S., Dowbenko, D., Imai, Y., Henzel, W.J., Grimley, C., Fennie, C., Gillett, N., Watson, S.R., and Rosen, S.D.

(1992). An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell *69*, 927–938.

Lawrence, M.B., and Springer, T.A. (1991). Leukocytes roll on a selectin at physiologic flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859–873.

Leyte, A., van Schijndel, H.B., Niehrs, C., Huttner, W.B., Verbeet, M.P., Mertens, K., and van Mourik, J.A. (1991). Sulfation of Tyr^{1680} of human blood coagulation factor VIII is essential for the interaction of factor VIII with von Willebrand factor. J. Biol. Chem. *266*, 740–746.

Lipmann, F. (1958). Biological sulfate activation and transfer. Science *128*, 575–580.

Lowe, J.B., Stoolman, L.M., Nair, R.P., Larsen, R.D., Berhend, T.L., and Marks, R.M. (1990). ELAM-1-dependent cell adhesion to vascular endothelium determined by a transfected human fucosyl-transferase cDNA. Cell *63*, 475–484.

Moore, K.L., Stults, N.L., Diaz, S., Smith, D.F., Cummings, R.D., Varki, A., and McEver, R.P. (1992). Identification of a specific glycoprotein ligand for P-selectin (CD62) on myeloid cells. J. Cell Biol. *118*, 445–456.

Moore, K.L., Eaton, S.F., Lyons, D.E., Lichenstein, H.S., Cummings, R.D., and Mcever, R.P. (1994). The P-selectin glycoprotein ligand from human neutrophils displays sialylated, fucosylated, O-linked poly-N-acetyllactosamine. J. Biol. Chem. *269*, 23318–23327.

Moore, K.L., Patel, K.D., Bruehl, R.E., Fugang, L., Johnson, D.A., Lichenstein, H.S., Cummings, R.D., Bainton, D.F., and McEver, R.P. (1995). P-selectin glycoprotein ligand-1 mediates rolling of human neutrophils on P-selectin. J. Cell Biol. *128*, 661–671.

Natsuka, S., Gersten, K.M., Zenita, K., Kannagi, R., and Lowe, J.B. (1994). Molecular cloning of a cDNA encoding a novel human leukocyte α-1,3-fucosyltransferase capable of synthesizing the sialyl Lewis x determinant. J. Biol. Chem. *269*, 16789–16794.

Norgard, K.E., Moore, K.L., Diaz, S., Stults, N.L., Ushiyama, S., Mcever, R.P., Cummings, R.D., and Varki, A. (1993). Characterization of a specific ligand for P-selectin on myeloid cells: a minor glycoprotein with sialylated O-linked oligosaccharides. J. Biol. Chem. *268*, 22953.

Pittman, D.D., Wang, J.H., and Kaufman, R.J. (1992). Identification and functional importance of tyrosine sulfate residues within recombinant factor VIII. Biochemistry *31*, 3315–3325.

Rehemtulla, A., and Kaufman, R.J. (1992). Protein processing within the secretory pathway. Curr. Opin. Biotech. *3*, 560–565.

Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton, J.W., II (1990). The structure of a complex of recombinant hirudin and human α-thrombin. Science *249*, 277–280.

Sako, D., Chang, X.-J., Barone, K.M., Vachino, G., White, H.M., Shaw, G., Veldman, G.M., Bean, K.M., Ahern, T.J., Furie, B., Cumming, D.A., and Larsen, G.R. (1993). Expression cloning of a functional glycoprotein ligand for P-selectin. Cell *75*, 1179–1186.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, Second Edition (Cold Spring Harbor, New York: Cold Spring Harbor Press).

Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., Hanai, N., and Nishi, T. (1994). Expression cloning of a novel α1,3-fucosyltransferase that is involved in biosynthesis of the sialyl Lewis x carbohydrate determinants in leukocytes. J. Biol. Chem. *269*, 14730–14737.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. Cell *76*, 301–314.

Ushiyama, S., Laue, T.M., Moore, K.L., Erickson, H.P., and McEver, R.P. (1993). Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. J. Biol. Chem. *268*, 15229–15237.

Vachino, G., Chang, X.-J., Veldman, G.M., Kumar, R., Sako, D., Fouser, L.A., Berndt, M.C., and Cumming, D.A. (1995). P-selectin glycoprotein ligand-1 is the major counter-receptor for P-selectin on stimulated T cells and is widely distributed in non-functional form on many lymphocytic cells. J. Biol. Chem. *270*, 21966–21974.

Varki, A. (1994). Selectin ligands. Proc. Natl. Acad. Sci. USA *91*, 7390–7397.

Webb, N.R., and Summers, M.D. (1990). Expression of proteins using recombinant baculoviruses. Technique *2*, 173–188.

Wilson, I.B.H., Gavel, Y., and von Heijne, G. (1991). Amino acid distributions around O-linked glycosylation sites. Biochem. J. *275*, 529–534.

Yuen, C.-T., Lawson, A.M., Chai, W., Larkin, M., Stoll, M.S., Stuart, A.C., Sullivan, F.X., Ahern, T.J., and Feizi, T. (1992). Novel sulfated ligands for the cell adhesion molecule E-selectin revealed by the neoglycolipid technology among O-linked oligosaccharides on an ovarian cystadenoma glycoprotein. Biochemistry *31*, 9126–9131.

Zhou, Q., Moore, K.L., Smith, D.F., Varki, A., McEver, R.P., and Cummings, R.D. (1991). The selectin GMP-140 binds to sialylated, fucosylated lactosaminoglycans on both myeloid and nonmyeloid cells. J. Cell Biol. *115*, 557–564.