Post-translational Modifications of Recombinant P-selectin Glycoprotein Ligand-1 Required for Binding to P- and E-selectin*

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P-selectin is a lectin-like ligand for P- and E-selectin on human leukocytes. P-selectin requires sialylated, fucosylated O-linked glycans and tyrosine sulfate to bind P-selectin. Less is known about the determinants that P-selectin requires to bind E-selectin. To further define the modifications required for P-selectin to bind P- and E-selectin, we transfected Chinese hamster ovary (CHO) cells with cDNAs for P-selectin and specific glycosyltransferases. CHO cells synthesize only core 1 O-linked glycans (Galβ1-3GalNAcα1-Ser/Thr); they lack core 2 O-linked glycans (Galβ1-3(Galβ1-4GlcNAcβ1-6)GalNAcα1-Ser/Thr) because they do not express the core 2 β1-6-N-acetylgalactosaminyltransferase (C2GnT). CHO cells also lack α1-3 fucosyltransferase activity. P-selectin was expressed on transfected CHO cells bound P- and E-selectin only when it was co-expressed with both C2GnT and an α1-3 fucosyltransferase (Fuc-TIII, Fuc-TIV, or Fuc-TVII). Chromatography of β-eliminated O-linked glycans from P-selectin co-expressed with C2GnT confirmed synthesis of core 2 structures. Tyrosine residues on P-selectin-1 expressed in CHO cells were shown to be sulfated. Phenylalanine replacement of three tyrosines within a consensus sequence for tyrosine sulfation abolished binding to P-selectin but not E-selectin. These results demonstrate that P-selectin requires core 2 O-linked glycans that are sialylated and fucosylated to bind P- and E-selectin. P-selectin also requires tyrosine sulfate to bind P-selectin but not E-selectin.

The selectins are a group of three Ca2+-dependent lectins that mediate rolling adhesion of leukocytes on the vessel wall during inflammation (reviewed in McEver et al. (1)). L-selectin, expressed on leukocytes, binds to constitutively or inducibly expressed carbohydrate ligands on endothelial cells. E-selectin, expressed on cytokine-activated endothelium, and P-selectin, expressed on thrombin-activated endothelial cells and platelets, bind to carbohydrate ligands on myeloid cells and subsets of lymphocytes. Leukocyte rolling requires that selectins rapidly associate and then dissociate from their ligands in a manner largely independent of shear stress (2). The selectins interact weakly with sialylated and fucosylated oligosaccharides such as sialyl Lewis x (sLex),1 but bind with higher affinity/avidity to only a few glycoproteins (1). Studies with mAbs support a physiologic role for one of these glycoproteins, P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 accounts for all the high affinity binding sites for P-selectin on human leukocytes (3). PSGL-1 must interact with P-selectin in order for neutrophils to roll on P-selectin under physiologic shear forces (3, 4), and it also contributes to the rolling of neutrophils on E-selectin (5).

PSGL-1 is a membrane glycoprotein with two identical disulfide-linked subunits (6). Each subunit has at most three N-linked glycans, but has many clustered, sialylated O-linked glycans (6, 7), some with polylactosamine terminating in sLex (8). PSGL-1 is a type I membrane protein with an extracellular domain that is rich in serines, threonines, and prolines (9). PSGL-1 binds both E-selectin and P-selectin (8–11). The structural requirements for binding are not well characterized, although there is evidence that PSGL-1 does not interact identically with P- and E-selectin (5, 8). PSGL-1 must be sialylated and fucosylated to bind both molecules (6, 9, 10). Enzymatic removal of N-linked glycans from human neutrophil PSGL-1 does not affect binding to P-selectin, suggesting that O-linked glycans are required for binding (6). It is not known if O-linked glycans are required for binding to E-selectin. Fab fragments of PL1, an IgG mAb to PSGL-1, block binding of PSGL-1 to P-selectin but only partially inhibit binding to E-selectin (3, 5). The PL1 epitope is located near the N terminus (3), near three clustered tyrosines within a consensus motif for tyrosine sulfation (9, 12). PSGL-1 is sulfated on tyrosine rather than on carbohydrate, and enzymatic removal of sulfate from tyrosine eliminates binding of PSGL-1 to P-selectin (13). These data demonstrate that tyrosine sulfation functions in conjunction with sialylated and fucosylated glycans to mediate high affinity binding of PSGL-1 to P-selectin. It is not known if tyrosine sulfation is required for PSGL-1 to bind E-selectin.

To further characterize the post-translational modifications that confer binding of PSGL-1 to P-selectin and E-selectin, we transfected CHO cells, which have well-characterized oligosaccharide structures, with cDNAs for PSGL-1 and various glycosyltransferases. PSGL-1 was co-expressed with a branching enzyme for O-linked glycans (core 2 β1-6-N-acetylgalactosaminyltransferase [C2GnT]), and/or an α1-3 fucosyltransferase (Fuc-TIII, Fuc-TIV, or Fuc-TVII). Recombinant PSGL-1 co-ex-

1 The abbreviations used are: sLex, sialyl Lewis x; CHO, Chinese hamster ovary; C2GnT, core 2 β1-6-N-acetylgalactosaminyltransferase; DHFR, dihydrofolate reductase; Fuc-T, fucosyltransferase; HBSS, Hank's balanced salt solution; PSGL-1, P-selectin glycoprotein ligand-1; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis.

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pressed in CHO cells with the appropriate glycosyltransferases bound avidly to both P- and E-selectin.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free [35S]sulfate (1–1.6 Ci/mmol) and [3H]glucosamine (20–45 Ci/mmol) were purchased from DuPont NEN. [3H]Thymidine (5 Ci/mmol) was obtained from Amersham Corp. Sialidase from Arthrobacter ureafaciens and Galβ1-3GalNAc were obtained from Boehringer Mannheim. α-1-3/4-fucosidase was obtained from Takara Biochemicals. Phycocyanin-streptavidin was purchased from Molecular Probes, Inc. Lipofectamine® and all cell culture reagents were purchased from Life Technologies, Inc. Other chemicals were ACS grade or better; unless stated otherwise, they were obtained from Fisher.

Antibodies—The anti-human P-selectin mAbs S12 and G1 were prepared as described previously (24, 15). G1, but not S12, blocks binding of P-selectin to leukocytes. The anti-human E-selectin mAbs ES1 and ES2 were prepared as described previously (5). ES1, but not ES2, blocks binding of E-selectin to leukocytes. The anti-human PSGL-1 mAbs PL1 and PL2 were prepared as described previously (3). PL1, but not PL2, blocks binding of PSGL-1 to leukocytes. These mAbs are all of the IgG1 subclass. The hybridoma secreting the IgM anti-sLex mAb CSLEX-1 was obtained from Bioclon Laboratories. [3H]Thymidine (5 Ci/mmol) was obtained from Amersham Corp. Sialidase from Arthrobacter ureafaciens and Galβ1-3GalNAc were obtained from Boehringer Mannheim. α-1-3/4-fucosidase was obtained from Takara Biochemicals.

Mutagenesis—The PSGL-1 cDNA was amplified by the polymerase chain reaction from genomic DNA. The amplified products were sequenced to confirm that they matched the published sequences (22, 23) and were then ligated into the expression vector pRC/RSV (Invitrogen). The cDNA encoding human Fuc-T VII in the plasmid pCDM8 (24) was a generous gift from Dr. J. John Lowe.

Transfections—CHO DHFR(-) cells and COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum at 37 °C in an atmosphere containing 5% CO2. CHO DHFR(-) cells express sLex® when transfected with cDNAs for Fuc-TIII or Fuc-TIV (25). CHO cells were transfected with Fuc-TIII or Fuc-TIV cDNA in pRC/RSV using the Stratagene Transfection MBS kit according to the suggested protocol. CHO cells were transfected with C2GnT cDNA in pRC/RSV or C2GnT cDNA plus Fuc-TIII cDNA in pRC/RSV by the calcium phosphate precipitation. The transfected cells were selected in medium containing 400 μg/ml of G418 (Life Technologies, Inc.). Individual colonies were isolated and ranked by limiting dilution. In this manner, permanently transfected CHO cells were isolated that expressed Fuc-TIII, Fuc-TIV, C2GnT, or Fuc-TIII plus C2GnT. Portions of these cells were then transfected with PSGL-1 cDNA in pZeoSV using Lipofectamine®. Some of the Fuc-TIV-expressing cells were transfected with both PSGL-1 cDNA and C2GnT cDNA. All cells transfected a second time were selected in medium containing both G418 (400 μg/ml) and Zeocin® (250 μg/ml). Individual colonies were expanded and, in some cases, ranked by limiting dilution. In this manner, permanently transfected CHO cells were isolated that expressed various combinations of PSGL-1, C2GnT, and/or Fuc-TIV.

Fuc-TIV cDNA was transiently transfected into CHO cells permanently transfected with PSGL-1 cDNA, or with PSGL-1 cDNA plus C2GnT cDNA. In other experiments, wild-type or mutated PSGL-1 cDNA was transiently transfected into CHO cells permanently transfected with Fuc-TIII cDNA or C2GnT cDNA plus Fuc-TIII cDNA. Transiently transfected CHO cells were analyzed 2 days after transfection.

COS-7 cells were transfected with Fuc-TIII or Fuc-TIV cDNA using Lipofectamine® and selected in medium containing 400 μg/ml G418 to obtain cells permanently expressing Fuc-TIII or Fuc-TIV. COS cells expressing Fuc-TIII were transiently transfected with PSGL-1 cDNA, or PSGL-1 plus C2GnT cDNA using Lipofectamine®. The cells were analyzed 2 days after transfection.

C2GnT Assay—Cells were detached with 0.02% EDTA in phosphate-buffered saline, washed twice with phosphate-buffered saline, centrifuged, and stored as cell pellets at −20 °C until they were analyzed. Frozen cell pellets were typically resuspended in 100 μl of 10 mM sodium cacodylate, pH 7.2, containing 0.1 mg/ml leupeptin (Sigma), 0.2 mM sodium pyruvate, 10% fetal calf serum at 37°C in an atmosphere containing 5% CO2. CHO DHFR(-) cells were transfected with the expression plasmid pCDM8 (24) and then selected in medium containing 25 μg/ml sodium cacodylate, pH 6.5, 5 μg/ml EDTA, 333 μg/ml G418, 10% glycerol, 0.8 mM UDP-GlcNAc (100,000 cpm/mmol) (unlabeled UDP-GlcNAc from Sigma, and UDP-[3H]GlcNAc from American Radiolabeled Chemicals, Inc.), and 8 mM benzyl-2-acetamido-2-deoxy-3-O-α-D-galactopyranosyl-α-D-galactopyranoside from Sigma. Mixtures were incubated at 37 °C for 4 h. Each reaction mixture was diluted with 0.3 ml of water and loaded onto a C18 SepPak cartridge (Millipore) on a vacuum manifold. The cartridge was washed with 25 ml of water to remove unreacted radiolabeled sugar-nucleotide. The hydrophobic glycosylated product was then eluted with 3 ml of methanol and counted in a scintillation counter. Blank values were obtained by carrying out assays without acceptor and were subtracted from the values obtained with acceptor.

Flow Cytometry—HL-60 cells were maintained in RPMI 1640 containing 20% fetal calf serum, 4 mM l-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Binding of mAbs to intact HL-60, CHO, or COS cells was measured as described previously (3). Binding of fluid-phase P-selectin to intact cells was assessed as described previously (27).

Cell Adhesion Assay—Confluent or near-confluent monolayers of CHO cells were labeled for 16 h in medium containing 10 μg/ml [3H]thymidine. The cells were washed with Hanks’ balanced salt solu-

3 G. Zhou, T. Fujimoto, R. P. McEver, and R. D. Cummins, manuscript in preparation.
tion (HBSS, Life Technologies, Inc.) and then detached from the substratum by incubation in 0.02% EDTA for 10 min. The cells were washed twice in serum-free Dulbecco’s modified Eagle’s medium and then suspended at 2 × 10⁶ cells/ml in Dulbecco’s modified Eagle’s medium containing 1% bovine serum albumin and 1% fetal bovine serum. The cells were labeled to a specific activity of 2–7 × 10⁻⁹ μCi/ml; all the cells were centrifuged, greater than 95% of the radioactivity remained associated with the cell pellet.

Microtiter wells (Immulon I Removable™ strips, Dynatech Laboratories) were coated with soluble P-selectin or soluble E-selectin (each at 2.5 μg/ml) in 100 μl of HBSS overnight at 4°C. The wells were then blocked with 1% human serum albumin in HBSS for 2 h at room temperature. Labeled CHO cells (100 μl, 2 × 10⁵ cells) were added to each well and incubated on an orbital shaker for 20 min at room temperature. In some experiments, the cells were preincubated with 10 μg/ml of the mAbs PL1, PL2, G1, or S12. After aspirating the unbound cells, the wells were gently washed three times with HBSS. Each well was then counted, and the bound radioactivity was determined by liquid scintillation counting. The number of bound cells was derived from a standard curve generated with known concentrations of cells.

Metabolic Labeling and Immunoprecipitation of PSGL-1—CHO cells were metabolically labeled with [3H]glucosamine or [35S]sulfate as described previously for HL-60 cells (6, 13). Cell extracts were immunoprecipitated as described previously (8, 13), using the rabbit antiserum to a dinitrophenylated residue of PSGL-1, which does not cross-react with normal rabbit serum. The immunoprecipitates were analyzed by SDS-PAGE under reducing or nonreducing conditions, followed by fluorography. In some gels, 3H-labeled PSGL-1 from human neutrophils was analyzed in parallel.

Glycosidase Treatment of PSGL-1—Immunoprecipitates of [3H]glucosamine-labeled PSGL-1 were either mock-treated or treated with 100 units/ml sialidase, alone, or with 0.1 unit/ml endo-β-galactosaminidase, in 20 μl of 50 mM sodium acetate, pH 5.5, for 18 h at 37°C. The samples were then analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography.

Structural Analysis of PSGL-1 Oligosaccharides—Following identification of [3H]glucosamine-labeled PSGL-1, by fluorography, the dried gel was aligned with the exposed x-ray film, and the bands from the nonreducing lane were subjected to β-elimination (28). The released oligosaccharides were treated with sialidase (2.5 units/ml) in 20 μl of 50 mM sodium acetate, pH 5.5, for 18 h at 37°C (28). In some experiments, neutral β-eliminated oligosaccharides from PSGL-1 co-expressed with C2GnT and Fus-TIV were treated with 0.25 mg/ml of a 1–3 fucosidase in 20 μl of 50 mM KH₂PO₄, pH 6.0, 0.1% NaN₃ at 37°C for 18 h. A portion of the oligosaccharides was analyzed by anion-exchange high performance liquid chromatography on a Varian AX-5 column (4 mm × 30 cm). The column was equilibrated in 65:35 (v/v) acetonitrile:deionized water and was eluted with a linear gradient of increasing water (0.33%/min) (29). Fractions (0.5 ml) were collected, and radioactivity in each fraction was determined by scintillation counting.

RESULTS

PSGL-1 Expressed on CHO Cells Binds Fluid-phase P-selectin—When it is Co-expressed with C2GnT and an α⁻³ Fucosyltransferase—To study the modifications required for recombinant PSGL-1 to bind P- and E-selectin, we used CHO cells because they have well characterized oligosaccharide structures. CHO cells synthesize complex N-linked glycans with type 2 polylactosamine sequences (30–32). The α⁻³ linked glycans synthesized by CHO cells are sialylated or nonsialylated core 1 structures (Galβ₁⁻³GalNAcβ₁⁻⁴SerThr) (33–35). A core 2 structure (Galβ₁⁻³Galβ₁⁻⁴GlcNAcβ₁⁻⁶GalNAcβ₁⁻³SerThr) (36), which is expressed by human myeloid cells (36), will allow fusocysylation or chain extension with polylactosamine (35, 36). Formation of the core 2 structure is catalyzed by C2GnT (21). CHO cells have no endogenous α⁻¹ fucosyltransferase or C4GnT activity (35, 37, 38).

To determine whether both C2GnT and an α⁻¹ fucosyltransferase must modify PSGL-1 for it to bind P- or E-selectin, we first isolated clones of permanently transfected CHO cells that expressed various combinations of PSGL-1, C2GnT, and/or an α⁻¹ fucosyltransferase (Fuc-TIII or Fuc-TV). C2GnT activity was detected only in lysates of CHO cells that were transfected with the C2GnT cDNA (Table I). Flow cytometric analysis with the anti-sLex mAb CSLEX-1 indicated that CHO cells expressing PSGL-1 accounts for all the high binding sites of PSGL-1. Stably transfected CHO cells expressing PSGL-1 plus C2GnT exhibited greater than 95% of the binding of the anti-sLex mAb CSLEX-1, but not by the non-blocking mAb PL2 (3) (Fig. 1F). P-selectin did not bind CHO cells lacking PSGL-1, even when they expressed both C2GnT and an α⁻¹ fucosyltransferase (Fig. 2A and E). P-selectin also did not bind CHO cells expressing PSGL-1 plus C2GnT (Fig. 2B), or CHO cells expressing PSGL-1 plus either Fuc-TIII or Fuc-TV (Fig. 2, C and G). In contrast, P-selectin bound avidly to CHO cells expressing PSGL-1 plus C2GnT and either Fuc-TIII or Fuc-TV (Fig. 2, D and H). PL1, but not PL2, completely inhibited the binding, suggesting that P-selectin interacted with a similar region on PSGL-1 expressed by CHO cells or leukocytes. These results establish that CHO cells require both C2GnT and an α⁻¹ fucosyltransferase in order for PSGL-1 to bind P-selectin.

We also transiently expressed Fuc-TVIII in CHO cell lines that stably expressed PSGL-1, or PSGL-1 and C2GnT. Like Fuc-TIII and Fuc-TV, Fuc-TVIII caused expression of sLex on the cell surface (Fig. 3, A and B). Fuc-TVIII also conferred binding of PSGL-1 to P-selectin, but only when the cells also expressed C2GnT (Fig. 3, C and D). Thus, CHO cells express a functional form of PSGL-1 when it is co-expressed with C2GnT and either of the fucosyltransferases normally expressed in leukocytes, Fuc-TIV or Fuc-TVIII.

| Cell line | Transfected cDNAs | C2GnT activity (pmol/min/mg) |
|-----------|-------------------|-----------------------------|
| CHO       | C2GnT, Fus-TIII    | 250 ± 30                     |
| CHO       | Fus-TIII, PSGL-1   | ND*                         |
| CHO       | Fus-TIV, PSGL-1    | ND                          |
| CHO       | C2GnT, Fus-TIII, PSGL-1 | 50 ± 7               |
| CHO       | C2GnT              | 730 ± 120                   |
| CHO       | C2GnT, Fus-TIV, PSGL-1 | 1300 ± 270              |
| COS       | Fus-TIII, PSGL-1   | 68 ± 7                      |
| COS       | C2GnT, Fus-TIII, PSGL-1 | 930 ± 150               |
| HL-60     |                   | 120 ± 30                     |
Fluid-phase P-selectin Does Not Bind PSGL-1 Co-expressed with C2GnT and Fuc-TIII on COS Cells—The oligosaccharide structures are less well characterized in COS cells than in CHO cells. However, as measured by a cell adhesion assay, P-selectin binds PSGL-1 that is co-expressed with Fuc-TIII in transfected COS cells (9). We used flow cytometry to determine whether co-expression of PSGL-1 with Fuc-TIII in transfected COS cells resulted in appearance of high affinity binding sites for fluid-phase P-selectin on the cell surface. PSGL-1 cDNA was transiently transfected into COS cells that had been permanently transfected with Fuc-TIII cDNA. The transfected COS cells expressed both PSGL-1 and sLe\(^\alpha\), as measured by binding of the mAbs PL1, PL2, and CSLEX-1 (Fig. 4A). However, fluid-phase P-selectin did not bind to the COS cells (Fig. 4C). PSGL-1 co-expressed with Fuc-TIII in COS cells has many core 1 O-linked glycans that are removed by sialidase and endo-\(\alpha\)-galactosaminidase (8, 9). This might be due to the fact that COS cells have slightly lower levels of endogeneous C2GnT activity than HL-60 cells (Table I). To determine whether a deficiency of C2GnT accounted for the relatively poor binding of P-selectin to PSGL-1 on COS/Fuc-TIII cells, we transiently transfected COS/Fuc-TIII cells with cDNAs for both C2GnT and PSGL-1. These cells also expressed PSGL-1 and sLe\(^\alpha\) (Fig. 4B), and lysates of the cells had significantly increased C2GnT activity (Table I). However, fluid-phase P-selectin still failed to bind to these cells (Fig. 4D). In parallel experiments, P-selectin bound avidly to PSGL-1 on HL-60 cells and on CHO cells expressing PSGL-1, C2GnT, and Fuc-TIII (data not shown). These results demonstrate that P-selectin binds with relatively lower affinity to PSGL-1 co-expressed with Fuc-TIII and C2GnT on COS cells than to PSGL-1 co-expressed with the same glycosyltransferases on CHO cells. The mechanisms accounting for this difference are not known. However, the data reinforced the decision to use CHO cells for these experiments, because the oligosaccharide structures are much better characterized in CHO cells than in COS cells.

PSGL-1 Expressed on CHO Cells Mediates Adhesion to P- and E-selectin Only When It Is Co-expressed with C2GnT and an \(\alpha\)-3 Fucosyltransferase—We next used a cell adhesion assay to measure the modifications required for PSGL-1 expression on CHO cells to bind P- and E-selectin. CHO cells transfected with an \(\alpha\)-3 fucosyltransferase express sLe\(^\alpha\) on the cell surface, and adhere to immobilized P- or E-selectin under certain experimental conditions (23, 25, 38). To measure PSGL-1-dependent adhesion, we developed conditions in which CHO cells expressing an \(\alpha\)-3 fucosyltransferase did not adhere to P- or E-selectin. Using these experimental conditions, CHO cells adhered to immobilized P-selectin or E-selectin in a PSGL-1-dependent manner, but only when the cells also expressed C2GnT and Fuc-TIII (Fig. 5, A and C). Adhesion of the cells was Ca\(^{2+}\)-dependent and was inhibited by a blocking, but not a nonblocking, mAb to the appropriate selectin (Fig. 5, B and D). Cell adhesion to P-selectin was blocked by PL1, but not by PL2 (Fig. 5B). In contrast, cell adhesion to E-selectin was not inhibited by PL1 or PL2 (Fig. 5D). CHO cells adhered in a similar PSGL-1-dependent manner to immobilized P- and E-selectin when they expressed C2GnT and Fuc-TIV (data not shown). These data demonstrate that PSGL-1 expressed on CHO cells binds both P- and E-selectin, but only when it is co-expressed with both C2GnT and an \(\alpha\)-3 fucosyltransferase.

PSGL-1 Expressed in CHO Cells Is Modified with Core 2 O-linked Glycans When It Is Co-expressed with C2GnT—To determine if co-expression of C2GnT caused the addition of core 2 O-linked glycans to PSGL-1, we metabolically labeled CHO cell lines with \(^{3}H\)glucosamine, which is incorporated as amino sugars in oligosaccharides. Radiolabeled PSGL-1 was immunoprecipitated from cell lysates, resolved by SDS-PAGE under reducing and nonreducing conditions, and detected by fluorography (Fig. 6A). We analyzed CHO cells expressing PSGL-1 with either Fuc-TIII or Fuc-TIV, in the presence or absence of C2GnT. PSGL-1 expressed by all the CHO cells migrated as a disulfide-linked homodimer, as did \(^{125}\)I-PSGL-1 from human neutrophils. The mobilities of PSGL-1 from all...
cells were similar. Sialidase treatment of PSGL-1 from CHO cells, like sialidase treatment of PSGL-1 from leukocytes (6), prevented binding to a P-selectin affinity column (data not shown). Sialidase treatment decreased the electrophoretic mobility of PSGL-1 from CHO cells (Fig. 6B), an effect similar to that observed with PSGL-1 from leukocytes (6). Addition of endo-\(\alpha\)-N-acetylgalactosaminidase had no further effect on the mobility of PSGL-1 co-expressed with C2GnT. This lack of effect is similar to that observed with PSGL-1 from leukocytes (8), and is consistent with the resistance of core 2 O-linked glycans to this enzyme. In contrast, addition of endo-\(\alpha\)-N-acetylgalactosaminidase to PSGL-1 expressed without C2GnT resulted in loss of a detectable radiolabeled band on the gel. This indicates that all of the labeled O-linked glycans were core 1 structures that were removed by the enzyme.

To confirm that co-expression of C2GnT caused addition of core 2 O-linked glycans to PSGL-1, immunoprecipitated \(^{[3}H\)glucosamine-labeled PSGL-1 was resolved by SDS-PAGE under nonreducing conditions. The gel slice containing PSGL-1 was excised and subjected to \(\beta\)-elimination. The released glycans to this enzyme.
Fig. 4. Fluid-phase P-selectin does not bind PSGL-1 co-expressed with C2GnT and Fuc-TIII on COS cells. COS cells permanently transfected with cDNA for Fuc-TIII (A and C) or C2GnT and Fuc-TIII (B and D) were transiently transfected with cDNA for PSGL-1. Two days after transfection, the cells were analyzed for binding of fluid-phase P-selectin as in Fig. 2. In parallel assays, fluid-phase P-selectin bound avidly to HL-60 cells and to CHO cells expressing PSGL-1, Fuc-TIII, and C2GnT as in Fig. 1. The data are representative of at least three independent experiments.

Fig. 5. PSGL-1 co-expressed with C2GnT and Fuc-TIII in CHO cells mediates cell adhesion to immobilized P- and E-selectin. Adhesion of CHO cells permanently transfected with the indicated cDNAs was measured in wells containing immobilized P-selectin (A) or E-selectin (C). Adhesion of CHO cells expressing C2GnT, Fuc-TIII, and PSGL-1 to immobilized P-selectin (B) or E-selectin (D) was measured in the presence of the indicated mAb or in buffer containing EDTA. The data represent the mean ± S.D. of triplicate wells and are representative of three independent experiments.
[H]glucosamine-labeled O-linked glycans were desialylated and analyzed by amine-adsorption high performance liquid chromatography. The radiolabeled oligosaccharides from PSGL-1, when co-expressed only with Fuc-TIII or Fuc-TIV, eluted as the GalNAcitol and Galβ1–3GalNAcitol standards, consistent with their predicted core 1 structures (Fig. 7). In contrast, most of the radiolabeled oligosaccharides from PSGL-1, when co-expressed with C2GnT plus either Fuc-TIII or Fuc-TIV, co-eluted with the tetrasaccharide core 2 standard, Galβ1–3(Galβ1–4GlcNAcβ1–6)GalNAcitol (Fig. 7). Some of the radioactivity in cells co-expressing C2GnT and Fuc-TIV eluted as a pentasaccharide, indicating the presence of a larger core 2 structure (29). This peak was no longer observed after treatment of the labeled glycans from these cells with an α1–3-specific fucosidase (Fig. 7). This suggests that the larger structure is a core 2 tetrasaccharide modified with a fucose in an α1–3 linkage to the β1–6 linked N-acetylglucosamine. These data confirm that co-expression of C2GnT results in addition of core 2 O-linked glycans to PSGL-1 in CHO cells.

PSGL-1 co-expressed on CHO Cells with C2GnT and a Fuscosyltransferase Must Also Be Sulfated on Tyrosine to Bind P-selectin but Not E-selectin—PSGL-1 on leukocytes must be sulfated to bind P-selectin (13). To determine whether PSGL-1 expressed by CHO cells was sulfated, we metabolically labeled CHO cells co-expressing PSGL-1, C2GnT, and either Fuc-TIII or Fuc-TIV with [35S]sulfate. PSGL-1 was immunoprecipitated from cell lysates, resolved by SDS-PAGE under non-reducing conditions, and analyzed by fluorography. A radiolabeled protein with the expected mobility for PSGL-1 was immunoprecipitated from cells co-expressing C2GnT and either Fuc-TIII or Fuc-TIV, indicating that PSGL-1 was sulfated in CHO cells (Fig. 8A). To confirm that the sulfate was on tyrosine, the gel slice containing the [35S]sulfate-labeled PSGL-1 from CHO cells was subjected to strong base hydrolysis and analyzed by anion exchange chromatography. The radioactivity was recovered in a single peak that co-migrated with the authentic tyrosine sulfate standard (Fig. 8B). These data demonstrate that PSGL-1 expressed on CHO cells, like PSGL-1 on leukocytes, is sulfated on tyrosine rather than on carbohydrate.
PSGL-1 has three tyrosines at residues 46, 48, and 51 that are located within a consensus sequence for tyrosine sulfation (9, 12). To test the importance of these residues for binding P-selectin and E-selectin, we prepared a construct of PSGL-1 in which all three tyrosines were replaced with phenylalanines. Wild-type or mutant PSGL-1 cDNA was transiently transfected into CHO cells that permanently expressed C2GnT and Fuc-TIII. The use of the same permanently transfected cells for transient expression of wild-type or mutant PSGL-1 ensured equivalent levels of C2GnT activity (Table I) and surface expression of sLeα (Fig. 9). Furthermore, surface levels of wild-type and mutant PSGL-1 were comparable (Fig. 9). However, fluid-phase P-selectin bound to CHO cells expressing wild-type PSGL-1, but not to cells expressing mutant PSGL-1 (Fig. 9). Furthermore, CHO cells expressing mutant PSGL-1 did not adhere to immobilized P-selectin, although they did adhere to immobilized E-selectin (Fig. 10). These data demonstrate that PSGL-1 expressed in CHO cells requires at least one of the three N-terminal tyrosines to bind P-selectin, presumably because this residue(s) must be sulfated. In contrast, appropriately glycosylated PSGL-1 does not require the tyrosines, and therefore tyrosine sulfate, to bind E-selectin.

**DISCUSSION**

Because their oligosaccharide structures are well characterized, CHO cells are an excellent model for analyzing the effects of glycosylation on protein structure and function. CHO cells synthesize complex-type bi-, tri-, and tetra-antennary N-glycans with type 2 lactosamine (Galβ1–4GlcNAc) and polylactosamine; the only other modification is terminal sialylation as NeuAcα2–3Galβ4GlcNAcR. CHO cells synthesize O-glycans with simple core 1 structures that can be mono- or disialylated. Our results establish that PSGL-1 expressed in CHO cells binds P- and E-selectin only when it is co-expressed with C2GnT and an α1–3 fucosyltransferase. These enzymes mediate branching and fucosylation of the O-linked glycans, modifications that are critical for PSGL-1 to bind P-selectin. PSGL-1 expressed in CHO cells is also sulfated on tyrosine, a modification required for binding P-selectin but not E-selectin.

**Fig. 8. PSGL-1 expressed in CHO cells is sulfated on tyrosine.** CHO cells expressing PSGL-1, C2GnT, and either Fuc-TIII or Fuc-TIV were metabolically labeled with [35S]sulfate. A, [35S]-PSGL-1 was immunoprecipitated with a specific rabbit antiserum to PSGL-1 (anti-42–56) or with normal rabbit serum (NRS). The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography. B, [35S]-PSGL-1 from CHO cells co-expressing C2GnT and Fuc-TIV was hydrolyzed with strong base. The hydrolysate was analyzed by ion exchange chromatography using a Na2PO4, pH 3.0, gradient (dashed line). The retention times of tyrosine, tyrosine sulfate, Gal-6-sulfate, and free sulfate are indicated. Other sulfated monosaccharides (GlcNAc-6-sulfate, GalNAc-6-sulfate, and GalNAc-4-sulfate) eluted with similar retention times between 14 and 15 min.

**Fig. 9. Fluid-phase P-selectin does not bind CHO cells expressing PSGL-1 in which phenylalanines replace three tyrosines in a consensus sequence for tyrosine sulfation.** CHO cells permanently transfected with cDNAs for C2GnT and Fuc-TIII were transiently transfected with cDNA for wild-type or mutant PSGL-1. Two days after transfection, the cells were analyzed for binding of mAbs as in Fig. 1 or for binding of fluid-phase P-selectin as in Fig. 2. The data are representative of at least four independent experiments.
Post-translational Modifications of Recombinant PSGL-1

Fig. 10. CHO cells expressing PSGL-1 in which phenylalanines replace three tyrosines do not adhere to immobilized E-selectin. Aliquots of the same transfected cells used in the flow cytometry experiment of Fig. 9 were used in the cell adhesion assay. As an additional control, some cells permanently transfected with cDNAs for C2GnT and Fuc-TII were not transiently transfected with cDNA for PSGL-1. One day after transfection, the cells were labeled with [3H]thymidine. Two days after transfection, adhesion of the CHO cells was measured in wells containing immobilized P-selectin (A) or E-selectin (B). The data represent the mean ± S.D. of triplicate wells and are representative of two independent experiments.

These studies extend previous findings on PSGL-1 from human leukocytes that 1) sialic acids on PSGL-1 are required for binding P-selectin (6); 2) enzymatic removal of N-linked glycans from PSGL-1 does not affect binding to P-selectin (6); 3) PSGL-1 has many clustered, sialylated O-linked glycans (7), some with polygalactosamine terminating in sLex (8); and 4) enzymatic removal of sulfate from tyrosine on PSGL-1 abrogates binding to P-selectin (13).

We demonstrated previously that Fab fragments of PL1, a mAb to a membrane-distal epitope on PSGL-1, block binding of P-selectin to PSGL-1 on human leukocytes (3). Here we show that PL1 also blocks binding of P-selectin, but does not affect adhesion to E-selectin. Furthermore, substitution of the N-terminal tyrosines, which eliminates adhesion to P-selectin, does not affect adhesion to E-selectin. These data demonstrate that, like leukocyte PSGL-1, PSGL-1 expressed in CHO cells binds differently to E-selectin than to P-selectin, although binding to both selectins requires sialylated, fucosylated, core 2 O-linked glycans.

The O-glycosylation sites and specific structures of the core 2 O-linked glycans required for native or recombinant PSGL-1 to optimally bind P-selectin or E-selectin need further study. For example, native PSGL-1 has polygalactosamine (8), which can be extended from core 2 structures (35), but the actual structures of the O-linked glycans on native PSGL-1 have not been determined. It is possible that polygalactosamine extension is required for a terminal sLeX structure to interact optimally with P-selectin or E-selectin. Fucosylation of internal N-acetylgalactosamine residues might also be required for optimal binding (39). Internal fucosylation renders polygalactosamine resistant to cleavage by endo-β-galactosidase and may explain why treatment of native PSGL-1 with this enzyme does not eliminate binding to P-selectin (8).

Although fucosyltransferases have been intensively studied with regard to their actions on small oligosaccharide acceptors and N-linked glycans (22–25, 40–44), little is known about how fucosyltransferases modify O-linked glycans. We found that PSGL-1 binds P- and E-selectin when it is co-expressed in CHO cells with C2GnT and either Fuc-TII, Fuc-TIV, or Fuc-TVII. However, the data do not demonstrate that these fucosyltransferases modify specific O-linked glycans in the same manner. Furthermore, it is not known whether Fuc-TIV and Fuc-TVII modify PSGL-1 similarly in leukocytes, where the two enzymes are normally expressed (23, 24, 42). Chromatography of the β-eliminated glycans of PSGL-1 co-expressed with C2GnT and Fuc-TVII in CHO cells revealed an oligosaccharide eluting at a retention time for a tetrasaccharide that was not observed in β-eliminated O-linked glycans from CHO cells co-expressing C2GnT and Fuc-TII. Based on the change in elution position after digestion with an α1–3-specific fucosidase, the tetrasaccharide is probably a core 2 tetrasaccharide with a fucose linked α1–3 to the N-acetylgalactosamine. It should be noted that larger O-linked structures with polygalactosamine could be present but not be detected if they do not elute from the amine-adsorption column under the conditions used. The fucosylated polygalactosamine-containing N-linked glycans synthesized by CHO cells expressing Fus-TIV are smaller than those synthesized by CHO cells expressing Fus-TII, suggesting that fucosylation by Fus-TIV competes with polygalactosamine chain extension (37).
Perhaps Fuc-TIV and Fuc-TIII also differentially modify the core 2 O-linked glycans in CHO cells. If so, Fuc-TIV might produce a core 2 glycan with a single terminal sLe\(^\alpha\); this structure would be detected as a core 2 pentasaccharide after sialidase treatment, as observed in Fig. 7. Fuc-TIII might preferentially fucosylate core 2 O-linked glycans with longer polylactosamine that are not readily identified by amine-adsorption high performance liquid chromatography under the conditions used.

The importance of appropriate core 2 O-linked glycans is underscored by the observation that P-selectin bound better to PSGL-1 co-expressed with C2GnT and Fuc-TIII on CHO cells than to PSGL-1 co-expressed with C2GnT and Fuc-TIV on COS cells. It is possible that PSGL-1 requires modification of only a few specific O-linked glycans to bind optimally to P-selectin. Modifications of other O-linked glycans may ensure optimal binding to E-selectin. PSGL-1 expressed on CHO cells shares several structural and functional features with PSGL-1 on leukocytes. However, detailed characterization of the O-linked structures at specific sites on native and recombinant PSGL-1 is needed to define the requirements for optimal binding to P- and E-selectin.

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Note Added in Proof—Several structural and functional features with PSGL-1 on leukocytes. However, detailed characterization of the O-linked structures at specific sites on native and recombinant PSGL-1 is needed to define the requirements for optimal binding to P- and E-selectin.