Structures of the O-Glycans on P-selectin Glycoprotein Ligand-1 from HL-60 Cells*

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P-selectin glycoprotein ligand (PSGL-1) is a disulfide-bonded homodimeric mucin-like glycoprotein on leukocytes that interacts with both P- and E-selectin. In this report we describe the structures of the Ser/Thr-linked O-glycans of PSGL-1 synthesized by HL-60 cells metabolically radiolabeled with 3H-sugar precursors. In control studies, the O-glycans on CD43 (leukosialin), a mucin-like glycoprotein also expressed by HL-60 cells, were analyzed and compared to those of PSGL-1. O-Glycans were released from Ser/Thr residues by mild base/borohydride treatment of purified glycoproteins, and glycan structures were determined by a combination of techniques. In contrast to expectations, PSGL-1 is not heavily fucosylated; a majority of the O-glycans are disialylated or neutral forms of the core-2 tetrasaccharide Galβ1–4GlcNAcβ1→3Galβ1–4(Fucα1→3)GlcNAcβ1→6(Galβ1–3)GalNAcOH. A minority of the O-glycans are α-1,3-fucosylated that occur as two major species containing the sialyl Lewis x antigen; one species is a disialylated, monofucosylated glycan,

\[
\begin{align*}
Fucα1\rightarrow3 \\
NeuAcα2→3Galβ1→4GlcNAcβ1 \\
\downarrow \\
NeuAcα2→3Galβ1→3GalNAcOH
\end{align*}
\]

and the other is a monosialylated, trifucosylated glycan having a polylactosamine backbone.

\[
\begin{align*}
Fucα1\rightarrow3 \\
NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1 \\
\downarrow \\
Galβ1→3GalNAcOH
\end{align*}
\]

CD43 lacks the fucosylated glycans found on PSGL-1 and is enriched for the nonfucosylated, disialylated core-2 hexasaccharide. These results demonstrate that PSGL-1 contains unique fucosylated O-glycans that are predicted to be critical for high affinity interactions between PSGL-1 and selectins.

P-selectin is a Ca\(^{2+}\)-dependent cell adhesion molecule expressed by activated platelets and endothelium and is a member of the selectin family of receptors that also includes L- and E-selectin. During early steps in the inflammatory response, P-selectin, which is rapidly redistributed to the surface of activated endothelial cells, initiates attachment and rolling events between these cells and circulating leukocytes (1, 2). E-selectin, which is inducibly synthesized and expressed on activated endothelial cells in a delayed fashion, also binds leukocytes and mediates rolling (1, 2). P-selectin is similar to other selectins in its ability to interact weakly with a variety of sialylated, fucosylated, and sulfated glycans, including those containing the sialyl Lewis x antigen (SLEx)\(^{1}\). NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R (3). However, P-selectin binds with high affinity to a specific cell surface glycoprotein on human leukocytes, termed P-selectin glycoprotein ligand (PSGL-1) (4). Studies with mAbs indicate that PSGL-1 is the primary determinant mediating rolling of leukocytes on P-selectin under physiological shear forces (5–7). PSGL-1 is also recognized by E-selectin, although the binding sites for E-selectin are not identical to those for P-selectin (7–11).

PSGL-1 is a mucin-like, homodimeric, disulfide-bonded glycoprotein with ~120-kDa subunits (4). The cDNA for PSGL-1 isolated from a cDNA library of HL-60 cells predicts a trans-

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1 The abbreviations used are: SLEx, sialyl Lewis x; PSGL-1, P-selectin glycoprotein ligand-1; NDV, Newcastle disease virus; CHO, Chinese hamster ovary; GlcN, glucosamine; GalN, galactosamine; GalNOH, galactosaminidase; GalNAcOH, N-acetylgalactosaminidase; FT, fucosyltransferase; C2GnT, core 2-β-1,6-N-acetylgalactosaminyltransferase; mAb, monoclonal antibody; HPAEC, high pH anion exchange chromatography; PAGE, polyacrylamide gel electrophoresis.
membrane glycoprotein of 402 amino acids (9). The mature polypeptide, after signal sequence removal and amino-terminal processing, has a predicted extracellular domain of 267 amino acids with three consensus sites for N-glycosylation (9). In this extracellular domain are also 56 Thr and 14 Ser residues, many of which may be O-glycosylated (4, 12). A number of experimental results have indicated that the O-glycans on PSGL-1 are responsible for high affinity interactions between PSGL-1 and P-selectin. Enzymatic removal of N-glycans from PSGL-1 does not affect binding to P-selectin (4). Recombinant forms of PSGL-1 lacking N-glycans still bind P-selectin, whereas mutations in Thr residues in the amino-terminal domain of PSGL-1 decrease binding to P-selectin (13, 14).

Interestingly, PSGL-1 shares features with CD43 (leukosialin), a monocarboxylic acid sialomucin expressed on leukocytes that contains numerous Ser/Thr-linked O-glycans in the amino-terminal domain of PSGL-1 and P-selectin. Enzymatic removal of N-glycans from PSGL-1 and P-selectin. Enzymatic removal of N-glycans from PSGL-1 does not affect binding to P-selectin (4). Recombinant forms of PSGL-1 lacking N-glycans still bind P-selectin, whereas mutations in Thr residues in the amino-terminal domain of PSGL-1 decrease binding to P-selectin (13, 14).

The structures of the glycans on PSGL-1 responsible for binding to P- and E-selectins are not known. Both sialylation and fucosylation of PSGL-1 are important for interactions with P- and E-selectins (4, 9, 10, 18). Recent studies on the post-translational glycosylation of recombinant PSGL-1 indicate that O-glycans containing the core-2 motif GlcNAcβ1→6-(Galβ1→3)GalNAcα1→Ser/Thr are necessary for high affinity binding of PSGL-1 to P- and E-selectin (19). However, proper glycosylation of PSGL-1 is necessary but not sufficient for high affinity binding to P-selectin. PSGL-1 also contains tyrosine sulfate residues near the amino terminus that are essential for high affinity interactions with P-selectin, but not with E-selectin (13, 14, 19, 20).

Although carbohydrates on PSGL-1 are critical for binding to selectins, no detailed chemical structures of the glycans are available. Much of the information about the glycosylation of the molecule has been obtained by enzymatic treatments of the native ligand and by studies on recombinant forms of PSGL-1 expressed in various cell types. While these indirect methods can provide valuable information about critical determinants on the ligand, detailed structural information on O-glycans from native PSGL-1 is essential to identify glycans that are important for ligand function and to provide a clearer understanding of why PSGL-1 is a ligand for P- and E-selectin, whereas other mucins such as CD43 are not. Here we describe the structures of the O-glycans on PSGL-1 synthesized by HL-60 cells that were metabolically radiolabeled with 3H-sugar precursors. We have compared the glycosylation of PSGL-1 with that of CD43 to determine whether two sialomucins expressed by the same cells are O-glycosylated differently. The HL-60 cell line was used in these studies because the post-translational modifications known to be important for binding to P- and E-selectin on both HL-60 and neutrophil PSGL-1 are comparable (4, 5, 9, 20).

Our studies demonstrate that the majority of O-glycans of PSGL-1 are disialylated or neutral forms of the core-2 tetrasaccharide. Less than 15% of the O-glycans are fucosylated and contain these structural determinant for the slE antigen. These results demonstrate that PSGL-1 is glycosylated differently from CD43 and that PSGL-1 contains unique O-glycans that are likely to be critical for high affinity interactions with P- and E-selectin.

EXPERIMENTAL PROCEDURES

Materials—The following chemicals and reagents were purchased: Protein A-Sepharose, QAE-Sephadex, Arthrobacter ureafaciens neuraminidase, jack bean β-galactosidase, jack bean β-N-acetylhexosaminidase, and Galβ1→3GalNAc (Sigma); Pronase and l-1-tosyl-amido-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington Biochemicals); Escherichia freundii (V-labs); Streptomyces sp. 142 α-1,3,4-fucosidase (Takara); Newcastle disease virus neuraminidase (Oxford Glycosystems); [6-3H]glucosamine hydrochloride (20–45 Ci/mmol), [3H]mannose (20–30 Ci/mmol), and [6-3H]fucose (70–90 Ci/mmol) (DuPont NEN); NaFb1H1 (40–60 Ci/mmol) (ARC); rabbit antiserum IgG1 (Zymed); Emphazon affinity support resin (Pierce); Bio-Gel P-4 and P-10 resins and molecular weight markers (Bio-Rad); all cell culture reagents (Life Technologies, Inc.). Other chemicals were ACS grade or better (Fisher Scientific).

Isolation of Metabolically Radiolabeled PSGL-1—[1H]GlcN, [3H]Man, and [3H]Fuc metabolic radiolabeling of HL-60 cells was performed essentially as described previously (4, 20). [3H]PSGL-1 was purified from cell extracts using affinity chromatography on a column of immobilized, recombinant soluble P-selectin (21) coupled to Emphazon at a density of 5 mg/ml (1.0 ml bed volume) (8). The enriched EDTA-eluted samples were rechromatographed on the P-selectin column after dialysis into Ca2+-containing buffer. The twice purified PSGL-1 represented 0.07% of the [3H]GlcN radioactivity in the cell extract. Purified [3H]PSGL-1 was analyzed by SDS-PAGE under reducing or nonreducing conditions (22) in a 7.5% polyacrylamide gel, followed by fluorography.

Immunoprecipitation of CD43—CD43 was immunoprecipitated from 3H-sugar-labeled HL-60 cells using a CD43-specific mAb, H5H5 (IgG1). The H5 hybridoma cell line was produced by Dr. T. August and obtained from The Developmental Hybridoma Bank (The Johns Hopkins University School of Medicine). CD43 was immunoprecipitated using described procedures (8). The immunoprecipitates were analyzed by SDS-PAGE under nonreducing conditions, followed by fluorography. β-Elimination of Radiolabeled O-Glycans from [3H]PSGL-1 and [3H]CD43 and Chromatography of Glycans on Bio-Gel and QAE-Sephadex—The gel slices containing radiolabeled PSGL-1 and CD43 were digested with mild base/borohydride as described elsewhere (23, 24). Chromatography was performed on Bio-Gel P-4 (medium mesh) in a 1 x 90 cm column and on Bio-Gel P-10 (medium mesh) in a 1 x 48 cm column in 0.1 M pyridylacetate buffer, pH 5.5, and 1-M fractions were collected. The anionic character and sialylation of glycans was assessed by paper chromatography on a column of QAE-Sephadex, with step elution in 2 ml Tris-base containing 20, 70, 140, 200, and 250 mM NaCl (24, 25).

Preparation of Radiolabeled O-Glycan Standards—The simple core-1 O-glycan Galβ1→3GalNAcβ1→6-(Galβ1→3)GalNAcβ1→Ser/Thr is a ligand for P- and E-selectin, whereas other mucins such as CD43 are not. Here we describe the structures of the O-glycans on PSGL-1 synthesized by HL-60 cells that were metabolically radiolabeled with 3H-sugar precursors. We have compared the glycosylation of PSGL-1 with that of CD43 to determine whether two sialomucins expressed by the same cells are O-glycosylated differently. The HL-60 cell line was used in these studies because the post-translational modifications known to be important for binding to P- and E-selectin on both HL-60 and neutrophil PSGL-1 are comparable (4, 5, 9, 20).

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period, the relative molar ratios for these residues should be close to unity. Longer labeling times do not affect the distribution of radioactivity between GlcN, GalN, and NeuAc, confirming that equilibrium has been attained. To determine the relative molar ratio for GlcNac and GalNac residues, this $^{3}H$GlcN-hexasaccharide was desialylated to generate a $^{3}H$GlcN-tetrasaccharide; this tetrasaccharide was hydrolyzed in strong acid and the released radioactivity was identified by high pH anion exchange chromatography (HPAEC) as described below. All radioactivity was recovered in $^{3}H$GlcN and $^{3}H$GalN in the ratio of $^{3}H$GlcN:$^{3}H$GalN of 1:0.8. This ratio was used as a correction factor for calculating relative molar ratios of isolated glycans, i.e. the radioactivity recovered in $^{3}H$GalN/OH in a sample after hydrolysis was divided by 0.8. The relative molar ratio we observed is consistent with other studies on HL-60 cells metabolically radioiodinated with the $^{3}H$GlcN precursor (17). To determine the relative molar ratio for NeuAc and GlcNac residues, the $^{3}H$GlcN-hexasaccharide was desialylated with neuraminidase, and the released radioactivity in NeuAc was determined. The radioactivity recovered in $^{3}H$GlcN and NeuAc was in the ratio of 1:0.14, respectively. Since NeuAc radioactivity was derived from two residues of the disialylated hexasaccharide, this gave a final value for the relative molar ratio for GlcN:NeuAc of 1:0.07.

Determination of Monosaccharide Composition—The ratios of GlcN: GalN and Man:Fuc in PSGL-1 and CD43 were determined following strong acid hydrolysis of excised gel slices containing purified PSGL-1 and CD43. The gel slices were treated with 250 µl of 2 n trifluoroacetic acid at 122 °C for 2 h. The released, radiolabeled monosaccharides in the hydrolysate were identified by HPAEC on a Carbopac PA-1 column (4 × 250 mm) in a Dionex system and elution with 16 m sodium hydroxide for 30 min. The relative molar ratio for GlcN:GalN in glycans was calculated by dividing the radioactivity in the GlcN peak by the radioactivity in the GalN peak and correcting for differences in specific activity of $^{3}H$GlcN versus $^{3}H$GalN. The Man:Fuc ratio in $^{3}H$Man-glycans was also determined following acid hydrolysis as described above, using a Man:Fuc ratio of 1:0.10, which is typically observed after equilibrium radiolabeling of cells with $^{3}H$Man (24, 32, 33).

Miscellaneous Procedures—Enzymatic treatments of glycans with β-N-acetylhexosaminidase, β-galactosidase, Arthrobacter neuraminidase, Streptomyces α-1,3-4-fucosidase, and E. freundii endo-β-galactosidase were performed as described previously (34, 35). Digestion with Newcastle disease virus (NDV) neuraminidase was performed in 20 µl of 10 µM phosphate buffer, pH 7.0, with 200 units of enzyme for 24 h at 37 °C, followed by addition of another 200 units of enzyme and further incubation for 24 h. O-Glycans were analyzed and purified by descending paper chromatography on Whatman filter paper for the (1 → 6)GalNAc(1 → 3)GalNAcOH (15, 17). These results demonstrate that the glycans of PSGL-1 contain higher amounts of GlcNac relative to GalNac than the glycans of CD43.

The presence of the sLex determinant on PSGL-1 has led to expectations that PSGL-1 might be heavily fucosylated (8). We assessed the amount of Fuc present on PSGL-1 and CD43 isolated from HL-60 cells metabolically radioiodinated with $^{2}H$Man. This labeled precursor is metabolized by cells to $^{2}H$Fuc, and the relative specific activity of Man and Fuc after equilibrium labeling is equivalent (32). $^{2}H$Man-glycoproteins and -CD43 were isolated by SDS-PAGE and fluorography. The corresponding bands were subjected to strong acid hydrolysis, and the released monosaccharides were separated by HPAEC on a Dionex system. The Man:Fuc ratio was determined to be 3:5 for PSGL-1 and 32 for CD43. As a control, the Man:Fuc ratio was also determined for the total unpurified glycoproteins from HL-60 cells and found to be 3:1. Thus, PSGL-1 contains more Fuc residues than CD43 and more Fuc residues than average glycoproteins in HL-60 cells.

Using this information it is possible to estimate the number of Fuc residues on PSGL-1. The cDNA sequence of PSGL-1 predicts that PSGL-1 has three potential N-glycosylation sites (9). PSGL-1 contains only complex-type N-glycans, each of which should have 3 Man residues (4, 8, 12). Thus, 3 complex-type N-glycans on PSGL-1 represent 9 Man residues per mol and, correspondingly, there are ~7 Fuc residues per mol of PSGL-1. In contrast, CD43 contains only a single N-linked glycan (16) and has much less Fuc in comparison to PSGL-1. Taken together, the compositional analyses from $^{3}H$GlcN- and $^{2}H$Man-glycopeptides demonstrate that PSGL-1 is glycosylated differently than CD43.

β-Elimination of O-Glycans from PSGL-1 and CD43 and Chromatography on Bio-Gel P-4 and P-10—The O-glycans of PSGL-1 and CD43 were directly released by treating gel slices containing purified glycoproteins with mild base/borohydride to effect β-elimination. The released $^{3}H$GlcN-glycans were sized by column chromatography on Bio-Gel P-4 (Fig. 2A). The

**RESULTS**

Purification of PSGL-1 and CD43—PSGL-1 and CD43 were purified from HL-60 cell extracts that remained after the first chromatography (1X) was repurified by a second chromatography (2X) on the P-selectin column. Virtually all (90-99%) of the radioactivity in the 2X-purified material was bound to the P-selectin column. This two-step procedure was necessary to remove a contaminating glycoprotein of ~120 kDa under nonreducing conditions that remained after the first step. The purified PSGL-1 behaved as a dimer of ~250 kDa in nonreducing conditions and ~120 kDa in reducing conditions. CD43 was immunoprecipitated using a CD43 mAB, and electrophoretically separated under nonreducing conditions. A single band of 120 kDa for CD43 was observed in both nonreducing (Fig. 1B) and in reducing conditions (data not shown).

Composition of Radiolabeled Sugars in PSGL-1 and CD43—In the initial assessment of the glycosylation of PSGL-1 and CD43, we determined the ratio of GlcN:GalN in the purified glycoproteins. $^{3}H$GlcN is metabolized by animal cells into radiolabeled GlcNac, GalNac, and sialic acids (32). Gel slices containing the $^{3}H$GlcN-glycoproteins were treated with strong acid (which results in destruction of sialic acids), and the radiolabeled GlcN and GalN were identified by Dionex HPAEC. The GlcN:GalN ratio was determined to be ~2:1 for PSGL-1 and ~1:1 for CD43. The GlcN:GalN ratio of ~1:1 for CD43 is consistent with published evidence that a majority of the glycans in CD43 have the simple core-2 motif Gal(1 → 4)GlcNAc(1 → 6)Galβ1 → 4GlcNAc(1 → 3)GalNacOH (15, 17). Thus, PSGL-1 contains more GlcN: GalN ratios than CD43 and more GlcN: GalN ratios than average glycoproteins in HL-60 cells.

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β-Elimination of O-Glycans from PSGL-1 and CD43 and Chromatography on Bio-Gel P-4 and P-10—The O-glycans of PSGL-1 and CD43 were directly released by treating gel slices containing purified glycoproteins with mild base/borohydride to effect β-elimination. The released $^{3}H$GlcN-glycans were sized by column chromatography on Bio-Gel P-4 (Fig. 2A). The
Bio-Gel P-10 in a position slightly smaller (designated P-10-3) than the disialylated core-2 hexasaccharide standard (Fig. 2B). In contrast, most of the material in both the P-4-I and P-4-II fractions of CD43 eluted identically on Bio-Gel P-10 as the disialylated core-2 hexasaccharide standard (Fig. 2B).

The glycans recovered in P-4-I from CD43 were analyzed using exoglycosidase treatments and anion exchange chromatography, as described previously (15). The glycans were shown to be the expected disialylated core-2 hexasaccharide (data not shown). A small fraction of the P-4-I sample from CD43 was recovered in larger sized glycans on Bio-Gel P-10 (Fig. 2B), consistent with previous studies showing that a small fraction of O-glycans from CD43 have an extended poly lactosamine structure on the core-2 motif (17). Since the structures of glycans in CD43 have been described (15, 17), they were not further analyzed.

Compositional Analyses of P-10-1, P-10-2, and P-10-3 Glycans from PSGL-1—O-Glycans released from Sera/Thr residues by β-elimination should contain [3H]GalNAcOH at the reducing terminus, which is recoverable as [3H]GalNOH following strong acid hydrolysis. To identify which glycans in the mixture from PSGL-1 represent the O-glycans, the GalNOH, GalN, and GlcN content of each glycan from Bio-Gel P-10 was determined by HPAEC on a Dionex system following strong acid hydrolysis. More than 95% of the total GalNOH in PSGL-1 P-10-2 and P-10-3 was recovered as GalNOH, demonstrating that β-elimination was efficient and that the O-glycans of PSGL-1 are represented in these fractions. The ratio of GlcN to GalNOH in P-10-2 and P-10-3 was 1:2.1 and 0.8:1, respectively. The glycans in P-10-1 lack GalNOH and do not represent O-glycans released by β-elimination. Instead, the P-10-1 fraction contains N-glycans still attached to peptide. This was confirmed by the presence of [3H]Man recovered in these glycans from [3H]Man-PSGL-1 (data not shown). P-10/phenyl contained a small amount of unreduced GalN, which might arise from GalNAc residues present in N-glycans (37), or from a small amount of residual O-glycans still linked to peptide and not released during β-elimination procedures.

Sialylation of P-10-1, P-10-2, and P-10-3 Glycans from PSGL-1—The sialylation patterns of the O-glycans in P-10-2 and P-10-3 and of the N-glycans in P-10-1 were determined by anion-exchange column chromatography on QAE-Sephadex, before and after neuraminidase treatment. In this system, glycans with 1 negative charge (1 sialic acid) elute with 20 mM NaCl, those with 2 negative charges (2 sialic acids) elute with 70 mM NaCl, and those with 3 negative charges (3 sialic acids) elute with 140 mM NaCl (24, 25). The glycans in the P-10-1 fraction were heterogeneously charged, consistent with the occurrence of N-glycans in glycopeptides in this fraction (Fig. 3A). The P-10-2 glycans were mono- and disialylated species (Fig. 3C) and the P-10-3 glycans were a mixture of neutral and monosialylated species (Fig. 3E).

To determine whether the anionic character of the glycans was due to sialic acid and to define the linkage of sialic acid, portions of the [3H]GlcN-labeled O-glycans were treated with neuraminidase from NDV. This enzyme displays high specificity for α2,3-linked sialic acid residues and will not efficiently cleave sialic acid in other linkages (38). After NDV neuraminidase treatment, the glycans in P-10-1 were less charged, consistent with a loss of sialic acid (Fig. 3B). However, the presence of residually charged glycans is indicative of the profile expected for N-glycans in glycopeptides. In contrast, the glycans in P-10-2 and P-10-3 became neutral following NDV neuraminidase treatment, demonstrating that all sialic acids in these glycans are α2,3-linked (Fig. 3, D and F). The peak of material eluting with 20 mMEDTA following NDV...
neuraminidase treatment was quantitatively recovered as free [3H]NeuAc, as shown by its co-elution with standard NeuAc on descending paper chromatography (Fig. 3, D and F). In a previous report we established that the sialic acid on PSGL-1 from [3H]GlcN-labeled HL-60 cells is Neu5Ac (12). These results demonstrate that the O-glycans in P-10-2 are mono- and disialylated species and that the O-glycans in P-10-3 are a combination of neutral and sialylated species, with sialic acid in α2,3-linkage to glycans. Furthermore, these results demonstrate that the O-glycans of PSGL-1 are not sulfated, since neutral species result following treatment with NDV neuraminidase.

Descending Paper Chromatography of P-10-2 Glycans from PSGL-1—The glycan in P-10-2 was further purified using preparative descending paper chromatography and two major species were recovered (Fig. 4A). One peak contained larger-sized glycans that migrated slowly from the origin (designated P-10-2a). The other peak contained smaller sized glycans that migrated further (designated P-10-2b). A small peak of faster migrating material (~24 cm) was also observed (Fig. 4A), which represented some residual glycans derived from the P-4-II peak (Fig. 2A). Upon anion-exchange chromatography on QAE-Sephadex, the P-10-2a material eluted as monosialylated species, whereas the P-10-2b material eluted as disialylated species (Fig. 4B).

Exoglycosidase Treatments of P-10-2b Glycans—The smaller sized, disialylated glycans in P-10-2b were desialylated by treatment with NDV neuraminidase, and released sialic acid was removed by chromatography on QAE-Sephadex. The desialylated glycans were analyzed by descending paper chromatography, before and after sequential exoglycosidase treatments (Fig. 5). Following treatment with neuraminidase, the desialylated P-10-2b glycans fractionated as two species (Fig. 5A). A minor peak (~10%) co-migrated with the fucosylated pentasaccharide standard Galβ1→4(Fucα1→3)GlcNAcβ1→6-(Galβ1→3)GalNAcOH and was designated P-10-2b1. A major peak (~90%) co-migrated with the standard core-2 tetrasaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH and was designated P-10-2b2.

The Galβ1→3GalNAcOH structure is resistant to jack bean β-galactosidase, since the enzyme does not efficiently cleave terminal galactosyl residues in β1,3-linkage (39). We confirmed this in control studies, in which no cleavage of the glycans, whereas the P-10-2b material eluted as disialylated species (Fig. 4B).

Exoglycosidase Treatments of P-10-2b Glycans—The smaller sized, disialylated glycans in P-10-2b were desialylated by treatment with NDV neuraminidase, and released sialic acid was removed by chromatography on QAE-Sephadex. The desialylated glycans were analyzed by descending paper chromatography, before and after sequential exoglycosidase treatments (Fig. 5). Following treatment with neuraminidase, the desialylated P-10-2b glycans fractionated as two species (Fig. 5A). A minor peak (~10%) co-migrated with the fucosylated pentasaccharide standard Galβ1→4(Fucα1→3)GlcNAcβ1→6-(Galβ1→3)GalNAcOH and was designated P-10-2b1. A major peak (~90%) co-migrated with the standard core-2 tetrasaccharide Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH and was designated P-10-2b2.

The Galβ1→3GalNAcOH structure is resistant to jack bean β-galactosidase, since the enzyme does not efficiently cleave terminal galactosyl residues in β1,3-linkage (39). We confirmed this in control studies, in which no cleavage of the glycans, whereas the P-10-2b material eluted as disialylated species (Fig. 4B).
P-10-2b2 glycans contain the SL£ antigen NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→R and have the heptasaccharide structure NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→6-(NeuAcα2→3Galβ1→3)GalNAcOH (Table I).

To further confirm the presence of fucose in these glycans and to facilitate the identification of Fuc in other glycans, HL-60 cells were metabolically radiolabeled with [3H]Fuc. The [3H]Fuc-labeled O-glycans were recovered by β-elimination as described for the [3H]GlcN-glycans, following the same procedures shown in Figs. 1 and 2. The [3H]Fuc recovered in the desialylated P-10-2b fraction co-migrated with the desialylated [3H]GlcN-P-10-2b, glycans (Fig. 5E). Taken together, these results demonstrate that the P-10-2b2 glycans from PSGL-1 are fucosylated and contain the SL£ structure.

Endo- and Exoglycosidase Treatments of P-10-2a Glycans—Because of their relatively large size, we considered the possibility that the P-10-2a glycans contain poly lactosamine (3Galβ1→4GlcNAcβ1)4N. To assess this possibility, the P-10-2a glycans were desialylated and the sialic acid was removed by QAE-Sephadex chromatography. The resulting neutral glycans were treated with endo-β-galactosidase, an enzyme that cleaves internal β1→4 galactosyl residues within a type 2 poly lactosamine (40). The glycans were resistant to this treatment (Fig. 6A). The desialylated P-10-2a glycans were also resistant to combined treatment with β-galactosidase and β-N-acetylhexasaminidase (Fig. 6A). We then considered the possibility that the P-10-2a glycans might contain a poly lactosamine backbone in which all internal GlcNAc residues are fucosylated. Such poly fucosylated, poly lactosamine structures are resistant to endo-β-galactosidase (41). We characterized polyfucosylated poly lactosaminos in the parasitic helminth Schistosoma mansoni and found that complete defucosylation was necessary before endo-β-galactosidase could digest the chains (35).

The desialylated P-10-2a glycans were chemically defucosylated and then treated with endo-β-galactosidase. After defucosylation, the enzyme quantitatively digested the P-10-2a glycans to release three major compounds in an approximate equimolar ratio identified as the trisaccharide Galβ1→4GlcNAcβ1→3Gal, the residual core-2 trisaccharide GlcNAcβ1→6Galβ1→3GalNAcOH, and the disaccharide GlcNAcβ1→3Gal (Fig. 6B). The generation of such products from the specific action of endo-β-galactosidase is predicted for a glycan with the backbone structure (Structure 1),

\[
\text{Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→6(Galβ1→3)GalNAcOH}
\]

**Structure 1**

where the arrows indicate specific cleavage sites for endo-β-galactosidase of a defucosylated poly lactosamine-containing glycan (40). The length of the poly lactosamine chain can be deduced from the radioactivity recovered in the disaccharide GlcNAcβ1→3Gal relative to the other fragments. The recovery of the trisaccharide GlcNAcβ1→6Galβ1→3GalNAcOH following endo-β-galactosidase treatment demonstrates that the poly lactosamine is extended from the GlcNAc in β1→6 linkage to the GalNAcOH residue. The recovery of the trisaccharide Galβ1→4GlcNAcβ1→3Gal demonstrates that Gal residues are present in the non reducing terminus of the poly lactosamine. As expected, treatment of the desialylated and defucosylated P-10-2a glycans with a combination of β-galactosidase and β-N-acetylhexasaminidase resulted in complete digestion to free [3H]GlcNac and the 3H-labeled disaccharide Galβ1→3GalNAcOH (Fig. 6B).

The resistance of the desialylated P-10-2a glycans to endo-β-
galactosidase prior to defucosylation is consistent with the possibility that each GlcNAc residue within the glycan contains an α-1,3-linked fucosyl residue in the structure Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4(Fucα1→3)GlcNAcβ1→6(Galβ1→3)GalNAcOH and the remainder co-migrated with the core-1 disaccharide Galβ1→3GalNAcOH (Fig. 9). Treatment of the desialylated P-10-3 glycans with β-galactosidase caused a shift in the migration of the major peak to that of the expected trisaccharide standard (Fig. 9). Treatment with a combination of β-galactosidase and β-N-acetylhexosaminidase generated free [3H]GlcNAc and [3H]Galβ1→3GalNAcOH (Fig. 9). Approximately 20% of the radiolabel in the P-10-3 glycans was found in predominately monosialylated species that were converted to neutral species by neuraminidase treatment (Fig. 3F). These results demonstrate that the P-10-3 glycans are primarily neutral core-2 tetrasaccharides and some monosialylated core-2 pentasaccharides (Table I). In addition, some of the neutral glycans have the core-1 disaccharide structure (Table I).

Relative Percentages of O-Glycans in PSGL-1 from HL-60 Cells—The relative percentages of O-glycans in PSGL-1 can be calculated from the amount of radioactivity recovered in each O-glycan and from the relative molar ratio for [3H]GlcN: [3H]GalNAcOH:[3H]NeuAc of 1:0.8:0.7 (Table I). This type of analysis was successfully used in previous studies on CD43 (15, 17). The majority of O-glycans in PSGL-1 contain the core-2 structure. A minority of the glycans, recovered in P-10-2b and P-10-2a, contain the sLeX determinant.

**DISCUSSION**

This study demonstrates that PSGL-1 from human HL-60 cells contains O-glycans with a core-2 motif. A majority of the glycans are not fucosylated and are mixtures of neutral and sialylated species. A minority of the O-glycans (∼14%) are fucosylated and contain the terminal sLeX structure. Two types of fucosylated O-glycans are present; one type is a disialylated heptasaccharide lacking polyactosamine and the other is a unique monosialylated, trifucosylated glycan that contains polyactosamine. The presence of core-2 O-glycans on PSGL-1 from HL-60 cells is consistent with results of studies on the glycosylation of PSGL-1 from human neutrophils. Desialylated PSGL-1 from both human neutrophils and HL-60 cells is resistant to treatment with endo-α-N-acetylgalactosaminidase (O-glycanase), which cleaves only desialylated core-1 glycans (4, 8). The direct demonstration of sLeX determinants and polyactosamine on O-glycans of HL-60-derived PSGL-1 reinforces indirect evidence that these structures are present on O-glycans from neutrophil-derived PSGL-1 (8, 12).

Significant differences were observed in the O-glycans on PSGL-1 and CD43 from HL-60 cells (this study, Ref. 17). Although both proteins have primarily core-2 O-glycans, PSGL-1...
has many neutral core-2 tetrasaccharides, whereas CD43 has mostly disialylated, core-2 hexasaccharides. The core-2 structure is a precursor for polylactosamine synthesis in O-glycans (43), but only PSGL-1 has significant amounts of polylactosamine. This indicates that the core-2 structure is necessary but not sufficient for polylactosamine addition. Furthermore, CD43 lacks the two species of fucosylated O-glycans found in PSGL-1. Although a monofucosylated O-glycan was identified in CD43, this species represents only 0.5% of the O-glycans in CD43 (17). The trifucosylated monosialylated O-glycan we have identified on PSGL-1 is not found on CD43.

The basis for the differential glycosylation of PSGL-1 and CD43 is not known. Differential fucosylation and polylactosamine extension of CD43 and PSGL-1 could result from differential recognition by α-2,3-sialyltransferases, α-1,3-fucosyltransferases, and the polylactosamine extension enzyme β-1,3-N-acetylgalactosaminyltransferase. Although the trifucosylated, monosialylated core-2 O-glycan found in PSGL-1 is novel, the simple disialylated core-2, sLeα-containing heptasaccharide, like that in P-10-2b1, has been observed in other glycoproteins (44–46). Some of the core-2 fucosylated O-glycans on PSGL-1 must be essential for interactions with P- and E-selectin. PSGL-1 requires sialylation and fucosylation to bind P- and E-selectin (4, 9, 10), but it does not require N-glycans (4, 13). When expressed in Chinese hamster ovary (CHO) cells, PSGL-1 binds P-selectin only when it is co-expressed with an α-1,3-fucosyltransferase and with the core-2 β-1,6-N-acetylgalactosaminyltransferase (C2GnT), which is necessary for core-2 O-glycan synthesis (19). The current data do not reveal, however, whether P- or E-selectin recognize one or both types of the fucosylated O-glycans in PSGL-1 or whether P- or E-selectin bind a small cluster of these glycans. E-selectin may have affinity for glycoprotein ligands containing N-glycans (47). Interestingly, N-glycans containing difucosylated polylactosamine bind to immobilized E-selectin (48).

The pathways for biosynthesis of the two types of fucosylated O-glycans on PSGL-1 are not known. Myeloid cells express both

| O-Glycan Structures | Fraction | % |
|---------------------|----------|---|
| Gal[β1,3]GalNAcOH   | P-10-3   | 14 |
| Gal[β1,4]GlcNAc     | P-10-3   | 52 |
| Gal[β1,6]GalNAcOH   | P-10-3   | 6  |
| NeuAc[α2,3]Gal[β1,4]GlcNAc | P-10-2b1 | 14 |
| NeuAc[α2,3]Gal[β1,3]GalNAcOH | P-10-2b1 | 2 |
| Fuc[α1,3]Gal[β1,4]GlcNAc | P-10-2b1 | 2 |
| Fuc[α1,3]Gal[β1,3]GalNAcOH | P-10-2a  | 12 |
FTIV and FTVII (28–30, 49, 50), and both enzymes can synthesize the sLex determinant when expressed in appropriate cells (49–51). In studies on recombinant PSGL-1 expressed in CHO cells, co-expression of C2GnT with either FTIII, FTIV, or FTVII generates a functional ligand that promotes static adhesion of transfected cells to immobilized P-selectin and binding to fluid-phase P-selectin (19). However, the specificities of these fucosyltransferases for O- versus N-glycans and for terminallactosaminyl units versus polylactosamine have not been well studied. FTIV does not efficiently fucosylate internal GlcNAc residues in long polylactosamines on N-glycans when expressed in CHO cells, whereas FTIII, the Lewis enzyme, does efficiently fucosylate such long polylactosamines (52). We analyzed the glycans on [3H]GlcN-PSGL-1 synthesized by transfected CHO cells co-expressing C2GnT and FTIV; among the neutral glycans generated by neuraminidase treatment was a fucosylated core-2 pentasaccharide lacking polylactosamine (similar to the P-10-2b1 glycan) (19). Perhaps the short sLe\textsuperscript{a} containing O-glycan (P-10-2b\textsubscript{1}) of PSGL-1 is generated by FTIV, and the longer sLe\textsuperscript{a}-containing glycan (P-10-2a) is generated by FTVII. Studies on the structures of the O-glycans on recombinant PSGL-1 expressed in CHO cells with either FTIV or FTVII will determine whether these two enzymes differ in the fucosylation of polylactosamine sequences in O-glycans. P-selectin can bind weakly to a variety of sulfated glycans,
and these glycans inhibit binding of P-selectin to human myeloid cells (53–56). However, the O-glycans of PSGL-1 are not sulfated. Instead, PSGL-1 contains tyrosine sulfate that is required for interactions with P-selectin but not with E-selectin (13, 14, 19, 20). Three consensus sites for tyrosine sulfation occur at the amino terminus of PSGL-1 at residues 46, 48, and 51. P1, a mAb to PSGL-1, blocks binding to P-selectin and recognizes an epitope spanning residues 49–62 that overlaps the tyrosine sulfation sites (57). Near the tyrosine sulfation sites are two Thr residues that represent potential O-glycosylation sites at residues 44 and 57. Mutations in these residues reduce binding of PSGL-1 to P-selectin when PSGL-1 is co-expressed in COS cells with either FTII or FTVII (13, 14). These results suggest that only one or two O-glycans in conjunction with tyrosine sulfate residues may be sufficient to promote high affinity binding of PSGL-1 to P-selectin. However, O-glycans in other regions of the molecule may also contribute to interactions with P- and E-selectin.

A fraction of the O-glycans of PSGL-1 is similar to those found on GlyCAM-1, the sulfated, mucin-like glycoprotein ligand for L-selectin (58–61). The O-glycans of GlyCAM-1 contain a disialylated tetrasaccharide like P-10,2b; in PSGL-1, however, these glycans from GlyCAM-1 also contain Gal-6-sulfate and GlcNAc-6-sulfate residues. In addition, GlyCAM-1 is not reported to contain poly-Lactosamine sequences. Interestingly, when co-expressed in COS cells with FTVII, the mucin-like glycoproteins GlyCAM-1, CD34, CD43, and PSGL-1 are all sulfated, but only PSGL-1 is able to bind P-selectin (14).

It was originally suggested that mucin-like glycoproteins act as convenient scaffolds upon which many O-glycans can be clustered for recognition by selectins (1). However, our data, in conjunction with other studies, indicate that mucin-like glycoproteins are differentially glycosylated. Further studies are required to identify the factors regulating differential glycosylation of sialomucins and to address the possibility that there are site-specific differences in the structures of O-glycans in these mucins. With regard to PSGL-1, further studies are needed to identify whether the fucosylated O-glycans occur at specific sites and to determine which structural features of the fucosylated O-glycans are required for recognition by P- and E-selectin.
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