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Characterization of the O-linked oligosaccharide structures on P-selectin glycoprotein ligand-1 (PSGL-1)

Paul A. Aeed, Jian-Guo Geng, Darwin Asa, Loretta Raycroft, Li Ma and Åke P. Elhammer*

Pharmacia & Upjohn, 301 Henrietta St., Kalamazoo, MI 49007–4940

P-selectin glypoprotein ligand-1, PSGL-1, a specific ligand for P-, E-, and L-selectin, was isolated from *in vivo* [^{3}H]-glucosamine labeled HL-60 cells by a combination of wheat germ agglutinin and platelet P-selectin- or E-selectin receptor globulin-agarose chromatography. The O-linked oligosaccharides on the ligand were released by mild alkaline sodium borohydride treatment and analyzed by a combination of ion-exchange, size exclusion, lectin, and paper chromatography, together with specific exoglycosidase treatments and chemical modifications. Approximately 91% of the radioactivity released from PSGL-1 was recovered in five O-linked glycans: GalNAc (approximately 4% of the total structures), Galβ, 3GalNAc (36%), and Galβ, 3GalNAc substituted with one (45%), two (6 %), or three (3%) *N*-acetyllactosamine repeat units. None of these structures contained fucose, and the majority were substituted with at least one sialic acid. The *N*-acetyllactosmine-containing structures appeared to be core 2. The remaining 9% of the radioactivity recovered in O-linked oligosaccharides from PSGL-1, eluted in two peaks at 11.8 and 10.2 glucose units, on size-exclusion chromatography. Results from lectin chromatography and chemical and enzymatic degradation experiments suggest that the major portion of the radioactivity in these peaks is associated with sialylated *N*-acetyllactosamine-type oligosaccharides, substituted with fucose at the penultimate residue in the nonreducing end. Since both sialic acid and fucose reportedly are crucial requirements for selectin binding, these results suggest that only a minor portion, approximately 4.5%, of the O-linked oligosaccharides on PSGL-1 are involved in the interaction with the selectins.

Keywords: **Selectin ligands, PSGL-1, O-glycans**

Abbreviations: **PSGL-1, P-selectin glycoprotein ligand-1; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; LacNAc, *N*-acetyllactosamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.**

Introduction

Selectins are a family of C-type lectins involved in the attachment of circulating leukocytes to various target cells [1]. All three selectins described to date, E-selectin, P-selectin, and L-selectin, have demonstrated roles in inflammation type pathologies and are thus of vital interest for the complete understanding of these mechanisms [2]. Although the selectin molecules themselves have been both cloned and extensively characterized, less is known about their ligands. Ligand molecules have been described from several sources for all three selectins, but only a handful have been cloned and characterized to some extent [3–5]. In particular, only incomplete information is available about the domain(s) on the ligand molecules mediating the recognition by the selectin, *i.e.,* the oligosaccharide structures. A characterization of the oligosaccharide structures on the L-selectin ligand, GlyCAM-1, has demonstrated that the majority of O-linked oligosaccharides on this molecule are core 2 based structures containing sulfated derivatives of the blood group type structure sialyl Lewis^x [6–8]. In addition, results from a recent study on the selectin ligand PSGL-1, isolated from the human promyeloid cell line HL-60, suggest that this molecule contains a mixture of mostly core 2 structures, but perhaps more importantly, that approximately 14% of the glycans on the molecule are fucosylated and that the majority of the fucosylated glycans are large multifucosylated structures [9]. The presence of fucosylated structures on PSGL-1 is consistent with a number of reports demonstrating that P-, E-, and L-selectin all are capable of binding to sialylated, fucosylated lactosaminoglycans, including sialyl Lewis^x and related structures [3–5]. Still, contrasting with these observations is the quite low affinity of sialyl Lewis^x-type oligosaccharides for all three selectins, *in vitro* and *in vivo* [10–13]. Data have also appeared suggesting that, although *N*-linked oligosaccharides capable of interacting

*To whom correspondence should be addressed. Tel: (616) 833-9399; Fax: (616) 833-2500; E-mail: ake.elhammer@am.pnu.com

with E- and P-selectin do exist [14–16], in most cases (mucin type) O-linked structures on the ligand molecules are the mediators of selectin-ligand interaction [3–5]. In summary, a considerable body of experimental evidence suggests that O-linked structures substituted with fucose and sialic acid(s) are involved in the binding of all three selectins to their ligands. The data also suggest that both the fucose and the sialic acid(s) are absolute prerequisites for efficient interaction with the selectins.

PSGL-1, a ligand for P-, E-, and L-selectin, is a cell surface mucin expressed in functional form on leukocytes and the human promyeloid cell line HL-60 [3–5, 17–21]. The number of functional ligand molecules on the surface of these two cell types, however, is quite modest [22]. Hence, an analysis of the oligosaccharide structures on PSGL-1 by conventional state-of-the-art methodology is hardly practical. This report presents a characterization of the O-linked oligosaccharides on HL-60 PSGL-1, based on the use of metabolically labeled working material [23].

Experimental Procedures

Materials

D-[6-^{3}H]Glucosamine hydrochloride (32 Ci/mmol) was purchased from Amersham Corp. (U.K.) [^{35}S]Sulfuric acid (1325 Ci/mmol) was from NEN (USA). QAE-Sephadex and CNBr-activated Sepharose 4B were from Pharmacia Biotech (USA). AffiGel 10 and Biogel P-6 were from Bio-Rad (USA). *Xanthomonas manihotis* β-galactosidase was from New England Biolabs (USA). Bovine testis β-galactosidase, jack bean β-galactosidase, jack bean β-*N*-acetylglucosaminidase, and tomato lectin were from Sigma (USA). *Bacteroides fragilis* endo-β-galactosidase was from Boehringer Mannheim (Germany) or Oxford Glycosciences. *Arthrobacter ureafaciens* neuraminidase, *Streptococcus pneumoniae* β-galactosidase, *Streptococcus pneumoniae* β-*N*-acetylglucosaminidase, Newcastle disease virus neuraminidase, almond meal α-fucosidase, chicken liver α-fucosidase, and the linear di-LacNAc oligosaccharide standard were from Oxford Glycosciences (U.K.). *Pasteurella haemolytica* O-sialoglycoprotein endopeptidase was from Cedarlane Laboratories (Canada). *Aleuria aurantia* lectin was from Seikagaku (Japan). The reduced core 2 tetrasaccharide (6.3 GU) standard was a kind gift from Dr. Minoru Fukuda at the LaJolla Cancer Research Foundation. RPMI Medium 1640, heat-inactivated fetal bovine serum, trypsin, penicillin and streptomycin were from GibcoBRL (USA). All other reagents were from standard sources.

Preparation of immobilized lectins

Ten milligrams of tomato lectin were linked to 2 ml AffiGel 10 essentially as outlined by the manufacturer of the matrix. Coupling efficiency, as determined by OD_{280}, was 85%. Six milligrams of *Aleuria aurantia* lectin was conjugated to 2 ml CNBr-activated Sepharose 4B in 0.2 M sodium bicarbonate, pH 8.3 at 4°C for 16 h. The conjugated gel was stored in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl. Coupling efficiency, as determined by Lowry protein assay, was~100%.

Metabolic labeling, isolation of the ligand molecule, and preparation of radiolabeled oligosaccharides and glycopeptides

Labeling of HL-60 cells with [^{3}H]-glucosamine (50 μCi/ml) was carried out in normal RPMI medium for 48 h; labeling with [^{35}S]-sulfur (50 μCi/ml) was done in sulfate-free RPMI medium for 18 h. The radiolabeled ligand molecule was isolated as outlined previously [21, 24].

The purity of the ligand molecule was routinely determined on SDS-PAGE; the purified preparations contained only one radioactive protein band [21]. The purified radiolabeled molecule was precipitated, using the procedure described by Wessel and Flügge [25], to remove salts and detergent, and the O-linked oligosaccharides were released by mild alkaline sodium borohydride treatment, essentially as described by Carlson [26]. The recovery of the ligand molecule in the precipitation step, as determined by SDS-PAGE (sodium dodecylsulfate-polyacrylamide gel electrophoresis), was invariably >90%. The released oligosaccharide structures were purified on SepPak C_{18} cartridges prior to analysis.

Enzyme treatments

Treatment with *A. ureafaciens* neuraminidase (2 U/ml) was done in 100 mM sodium acetate, pH 5.0 for 18 h; Newcastle disease virus neuraminidase (0.2 U/ml) was in 50 mM sodium acetate, pH 5.5 for 1 h (time-limited digestion) or 18 h; bovine testis β-galatosidase (1 U/ml) was in 100 mM citrate-phosphate, pH 4.0 for 18 h; jack bean β-galactosidase (400 mU/ml) was in 50 mM citrate-phosphate, pH 4.6 for 48 h; *S. pneumoniae* β-galactosidase (80 mU/ml) was in 100 mM sodium acetate, pH 6.0 for 18 h; jack bean β-*N*-acetylglucosaminidase (5 U/ml) was in 50 mM sodium citrate, pH 5.6 for 18 h; *S. pneumoniae* β-*N*-acetylglucosaminidase (100 U/ml) was in 100 mM sodium citrate-phosphate, pH 6.0 for 18 h; *X. manihotis* β-galactosidase (167 U/ml) was in 50 mM sodium citrate, pH 4.5 for 18 h; almond meal α-fucosidase (0.2 mU/ml) was in 100 mM sodium citrate, pH 6.0 for 18 h; and chicken liver α-fucosidase (2 U/ml) was in 100 mM sodium citrate-phosphate, pH 6.0 containing 250 μg/ml BSA and 100 mM NaCl for 18 h. *B. fragilis* endo-β-galactosidase (250 mU/ml) was in 50 mM sodium acetate, pH 5.6 for 18 h. All enzyme treatments were carried out at 37°C under a toluene atmosphere.

Chemical degradation procedures

Strong acid hydrolysis was done in 6 M HCl, at 100°C for 4 hours. Chemical cleavage of fucose was done in 0.1 M trifluoroacetic acid, at 100°C for 1 h [27].

Lectin chromatography

Fractionation on tomato lectin-agarose was done essentially as described [28]. Bound material was eluted with 8 mg/ml chitobiose/triose; fraction volume was 2 ml; flow rate was 15 ml/h. Fractionation on *Aleuria aurantia* lectin-Sepharose (0.5 × 10 cm) was done essentially as described [29]. Column buffer was 10 mM sodium phosphate, pH 7.4, 150 mM NaCl. Samples were allowed to interact with the column for 30 min prior to elution in the column buffer. Bound material was eluted with 1 mM fucose; fraction volume was 1 ml; flow rate was 15 ml/h.

Column chromatography

Ion-exchange chromatography on QAE-Sephadex was carried out essentially as described by Varki and Kornfeld [30]. Charged oligosaccharides were eluted step-wise with increasing concentrations of NaCl in 2 mM Tris base; 2 ml fractions were collected; the column bed volume was 1 ml. Sialic acid was removed by treatment with *A. ureafaciens* or Newcastle disease virus neuraminidase.

Size exclusion chromatography and sequential exoglycosidase treatment of desialylated oligosaccharides was carried out on a GlycoMap Chromatograph using a BioGel P-4 type column (Glycan Sizing Column, Oxford GlycoSystems, U.K.) and the High-Resolution Program (30 µl/min for 366 min followed by a linear increase to 200 µl/min over 234 min); 2 drop fractions (approximately 90 µl) were collected.

Paper chromatography

To determine the total radioactive amino sugar composition, samples were subjected to strong acid hydrolysis, reacetylated and separated on borate impregnated papers, as described by Cummings et al. [31]. Small radioactive oligosaccharides were separated on descending paper chromatography in pyridine-ethyl acetate-glacial acetic acid-water (5:5:1:3) for 19–20 h. Sialic acid was separated on descending paper chromatography in isoamyl acetate-glacial acetic acid-water (3:3:1) for 20 h.

Results

Compositional analysis of the oligosaccharide structures on PSGL-1

Separation of radioactive monosaccharides (amino sugars) obtained from strong acid hydrolysis of the total oligosaccharide structures on the HL-60 ligand molecule, by borate paper chromatography, resulted in approximately 64% of the radioactivity co-migrating with the GalNAc (N-acetylgalactosamine) standard; the remaining 36% co-migrated with the GlcNAc (N-acetylglucosamine) standard, producing a GalNAc to GlcNAc ratio of approximately 1.7:1. No other radioactive species were observed (data not shown).

Charge separation of the O-linked oligosaccharides on PSGL-1; determination and distribution of sialic acid

The total O-linked oligosaccharides were cleaved from the purified [^{3}H]-glucosamine labeled ligand by mild alkaline sodium borohydride treatment and fractionated on QAE-Sephadex (Figure 1, panel A). Typically, approximately 13% of the radioactivity loaded on the column did not interact with the ion-exchange matrix and was recovered in the break-through fractions. The remaining radioactivity eluted in fractions containing structures with one and two

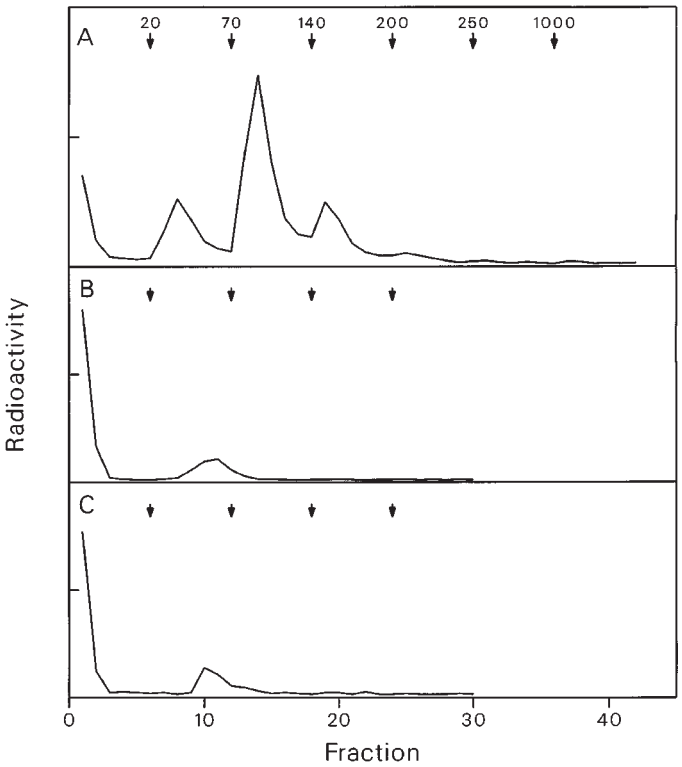

Figure 1. Charge separation of the O-linked oligosaccharides on PSGL-1. Oligosaccharides released from purified, *in vivo* [^{3}H]-glucosamine labeled PSGL-1 were loaded on a QAE-Sephadex column. Following elution of uncharged and charged structures with equilibration buffer and buffer containing increasing concentrations of NaCl, respectively, aliquots from all fractions were counted for radioactivity. Panel A shows separation of the total O-linked oligosaccharides on PSGL-1. Panels B and C show separations of radioactivity recovered from fractions eluted with 20 and 70 mM NaCl (in the experiment shown in Panel A), respectively, following digestion with *Arthrobacter ureafaciens* neuraminidase.

charges (20% and 52%, respectively). Some variation in the distribution of radioactivity in the individual fractions (presumably due to variations in the degree of sialylation of the oligosaccharides in these fractions) was observed in different preparations. By contrast, the distribution of radioactivity in the five major peaks generated by separation of the total desialylated oligosaccharides on the GlycoMap (eluting at 2.5, 3.5, 6.3, 9.8, and 11.8 GU), remained essentially constant (Figure 2, panel A). Analysis (by GlycoMap and paper chromatography) of the radioactivity in the small peak eluting with 140 mM NaCl revealed that 50% to 70% of this material is identical with the later eluting fractions in the 70 mM NaCl eluate and that the remaining 30% to 50% represents (glyco)peptide fragments (data not shown). Rechromatography of the charged fractions after treatment with *A. ureafaciens* or Newcastle disease virus neuraminidase resulted in the majority of the radioactivity in both charged fractions eluting in the column breakthrough and the remaining portion (25% and 31%, respectively) in the fractions containing material with one charge (QAE 20) (Figure 1, panels B and C). More than 95% of the radioactive material in these neuraminidase resistant, charged fractions co-chromatographed with the sialic acid standard on paper chromatography, suggesting (1) that the neuraminidase treatment had completely removed the sialic acids from the oligosaccharides in the samples, (2) that the radioactivity retaining a charge after neuraminidase treatment represented released sialic acid only, and (3) that all sialic acids likely are conjugated in α2,3 linkage. Although NDV sialidase is capable of cleaving sialic acid linked α2,6 to GalNAc-ol, provided galactose is conjugated to the 3-position of the sugar alcohol, this reaction proceeds at a rate considerably slower than the cleavage of sialic acids in α2,3 linkage to galactose [32]. The neuraminidase-treated oligosaccharides were subsequently separated by size-exclusion chromatography. Figure 2, panel A shows a separation of the complete mix of O-linked oligosaccharides released from PSGL-1. This resulted in seven peaks, eluting in the positions of oligomers composed of 12.8, 11.8, 10.2, 9.8, 6.3, 3.5, and 2.5 glucose units (GU), respectively. The distribution of radioactivity in these peaks is shown in Table 1. The radioactivity eluting as a broad elevation of the baseline, ranging from V_0 to approximately 15 GU, likely represents peptide fragments carrying *N*-linked oligosaccharides. A significant portion of the sugar-linked radioactivity incorporated into PSGL-1 is associated with *N*-linked oligosaccharides and analysis of the amino sugar content in the pooled fractions from V_0 to 15 GU, demonstrated that these fractions contained essentially no *N*-acetylgalactosamine, suggesting the absence of mucin type O-linked oligosaccharides. The distribution of sialic acids on the oligosaccharide structures eluting in the peaks obtained by size-exclusion chromatography is shown in Figure 2, panels B, C, and D. This experiment shows separations of the portions of the (total) structures containing none, one, and two charges, respectively; the distribution of radioactivity recovered from the individual peaks in these separations is summarized in Table 1.

Two α2,3-linked (NDV sialidase sensitive) sialic acids requires the presence of branched, i.e., likely core 2 structures on PSGL-1. (The structures containing two sialic acids are all tetrasaccharides and larger. See Figure 2.) Although individual treatment of the three major larger

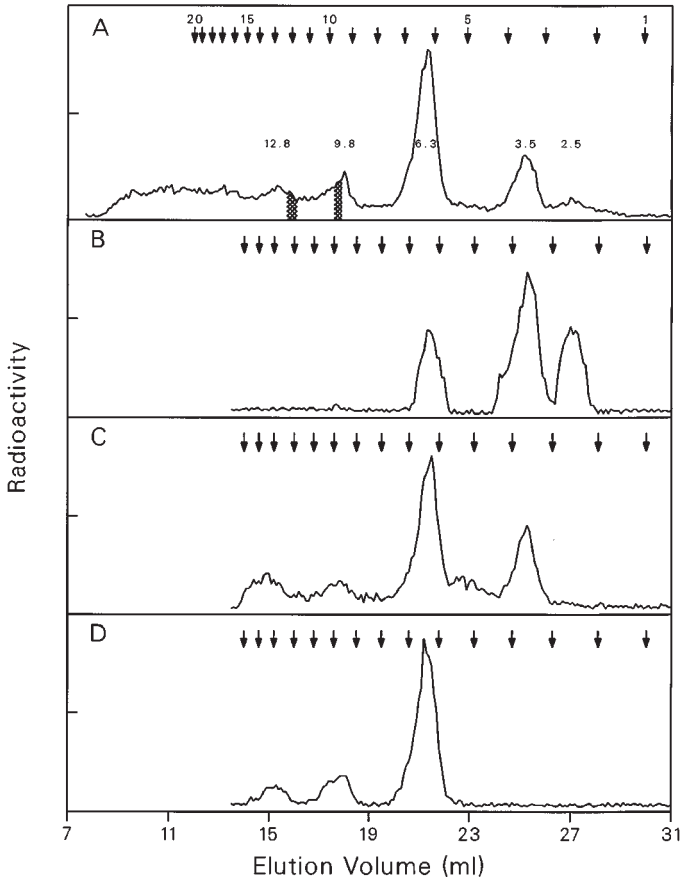

Figure 2. Size-exclusion separation of O-linked oligosaccharides isolated from PSGL-1. Panel A. Separation of the total O-glycans on PSGL-1. The total *in vivo* [^{3}H]-glucosamine labeled O-linked oligosaccharides isolated form PSGL-1 were treated with *Arthrobacter ureafaciens* neuramindase to release sialic acids. Following removal of sialic acids by ion-exchange chromatography, the resulting neutral structures were fractionated on a GlycoMap column, as outlined in Experimental Procedures; aliquots from each eluted fraction were counted for radioactivity. The shaded areas indicate the elution positions (fractions pooled) of the 11.8 and 10.2 GU structures. Panels B-D. Separations of oligosaccharides recovered in subfractions obtained by charge-separation of the total O-linked oligosaccharides on PSGL-1. Total *in vivo* [^{3}H]-glucosamine labeled O-linked oligosaccharides isolated from HL-60 PSGL-1 were fractionated on QAE-Sephadex as outlined in the legend to Figure 1. Following removal of sialic acid by neuraminidase treatment, the resulting neutral oligosaccharides were fractionated on the GlycoMap column. Panels B through D show separations of structures recovered from QAE-Sephadex chromatography fractions eluting with 0, 20 and 70 mM NaCl, respectively. The numbered arrows indicate the elution volumes of dextran oligomers composed of 1–15 glucose units.

Table 1. Distribution of identified O-linked oligosaccharide structures on PSGL-1.

Elution volume (GU)[a]	*# of amino sugar*	*% of total radioactivity*	*% of identified structures in*			
			Total	*QAE 0*[b]	*QAE 20*[b]	*QAE 70*[b]
12.8	4	7.4	3.3	0.0	3.1	2.1
11.8	(4)[c]	4.3	1.9			
10.2	3	4.6	2.8	0.0	3.8	5.4
9.8	3	10.6	6.4			
6.3	2	50.5	45.3	7.1	15.0	23.2
3.5	1	20.2	36.3	23.8	12.5	0.0
2.5	1	2.2	3.9	3.9	0.0	0.0

[a]Designation refers to the elution volume (in GU) for the O-linked oligosaccharide structures identified on PSGL-1.
[b]Designations refer to NaCl concentration (mM) needed to elute the structures when separated on QAE-Sephadex.
[c]Tentative number based on the size of the structures.

structures (12.8, 9.8, and 6.3 GU) with bovine testis β-galactosidase (which cleaves β1,3, β1,4, and β1,6-linked galactose), as well as with jack bean or *S. pneumoniae* β-galactosidase, in all cases, failed to produce more than a one GU shift in elution volume (see below), suggesting the removal of only one terminal galactose. Several investigators have reported that the β1,3-linked galactose in intact, reduced core 2 structures is not efficiently cleaved by these enzymes [e.g., 32–34]. Still, to confirm the presence of core 2 structures on PSGL-1, radioactivity recovered from the 6.3 GU peak was subjected to Smith degradation [35]. Figure 3, panel B shows that degradation of the 6.3 GU oligosaccharide did not produce the 5.2 GU fragment predicted for a linear core 1 oligosaccharide, which was generated by degradation of a linear di-LacNAc standard oligosaccharide (Figure 3, panel A). Instead a smaller fragment, eluting at approximately 3.2 GU was produced (Figure 3, compare panels A and B). Smith degradation of the linear oligosaccharide is predicted to result in the destruction of the terminal galactose and in the cleavage of the reduced *N*-acetyglucosamine to produce the structure GlcNAcβ1,3Galβ1,4xylosaminitol; degradation of a linear 6.3 GU oligosaccharide should have produced the only slightly smaller fragment GlcNAcβ1,3Galβ1,4threosaminitol. By contrast, degradation of a 6.3 GU core 2 structure is predicted to result in the destruction of two terminal galactoses and cleavage of the reduced *N*-acetylgalactosamine to produce the much smaller radioactive fragment GlcNAcβ1,2glycol. (Since the ^{3}H-label on GalNAc-ol is at the 6-position, the threosaminitol also generated by the reaction will not be labeled, and hence not detected.) Degradation of an authentic core 2 tetrasaccharide standard resulted in a fragment pattern identical to that produced by the 6.3 GU oligosaccharide (Figure 3, compare panels B and C). Taken together, these results suggest that the 6.3 GU oligosaccharide is a core 2 structure. Although the small amounts of available radioactivity did not allow us to directly identify the core(s) in the larger 9.8 and 12.8 GU oligosaccharides, it appears likely that they also are core 2 structures, given the fact that no indication was found for

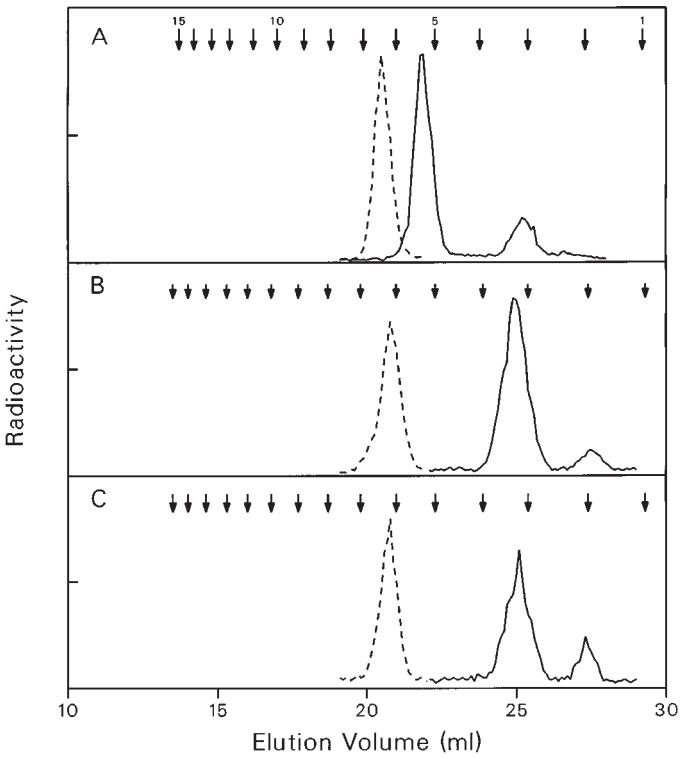

Figure 3. Smith degradation of the 6.3 GU oligosaccharide isolated from PSGL-1. A reduced, radioactively labeled di-LacNAc standard oligosaccharide (Panel A), radioactivity recovered from the 6.3 GU peak generated by separation of the total O-linked oligosaccharides on PSGL-1 (Panel B), and a reduced, radioactively labeled standard 6.3 GU core 2 oligosaccharide were subjected to Smith degradation; the resulting degradation products were separated on the GlycoMap column. Dashed lines indicate the elution positions of the untreated oligosaccharides. The numbered arrows indicate the elution volumes of dextran oligomers composed of 1–15 glucose units.

structures containing NDV sialidase-resistant (i.e., α2,6-linked) sialic acids (see above), and the fact the PSGL-1 molecule analyzed was synthesized by HL-60 cells, which are known to synthesize predominantly core 2 oligosaccharides [36]. Results from MS analysis of degradation products from standard oligosaccharides suggest that the smaller additional peaks in the chromatograms shown in Figure 3 represents products generated by a partial cleavage of the linkage between the nonreducing amino sugar and the remaining oligosaccharide (data not shown).

Sequential exoglycosidase degradation of the O-linked oligosaccharides on PSGL-1.

To determine the structure(s) of the oligosaccharides isolated from PSGL-1, radioactivity recovered from three of the earlier eluting GlycoMap peaks (6.3, 9.8, and 12.8 GU) were subjected to sequential exoglycosidase treatment and rechromatography on the GlycoMap. Figure 4, panel A shows that treatment of the 6.3 GU oligosaccharide with *S. pneumoniae* β-galactosidase resulted in a 1 GU shift in elution volume, suggesting the removal of one terminal β1,4-linked galactose. Treatment with *Xanthomonas manihotis* β-galactosidase did not result in a shift, whereas treatment with jack bean β-galactosidase again resulted in a 1 GU shift in elution volume, confirming the reported specificity of the *S. pneumoniae* enzyme [37]; treatment with bovine testis β-galactosidase again yielded a 1 GU shift in elution volume (data not shown). Subsequent treatment (of the *S. pneumoniae* β-galactosidase degradation product) with *S. pneumoniae* β-*N*-acetylglucosaminidase produced an additional 2 GU shift, suggesting the removal of one *N*-acetylglucosamine; treatment with jack bean β-*N*-acetylglucosaminidase produced identical results, suggesting that the cleaved *N*-acetylglucosamine was linked β1,3 or β1,6 to reducing monosaccharide (see below). The radioactivity released by the β-*N*-acetylglucosaminidase treatment eluted at 2 GU (Figure 4, panel A) and was identified as *N*-acetylglucosamine on paper chromatography (data not shown). The remaining 3.5 GU fragment (of the 6.3 GU oligosaccharide) was identified as Ga1β1-3Ga1NAc-ol by treatments with jack bean and bovine testis β-galactosidases followed by separation of the reaction products on paper chromatography, as outlined below for the 3.5 GU structure (data not shown).

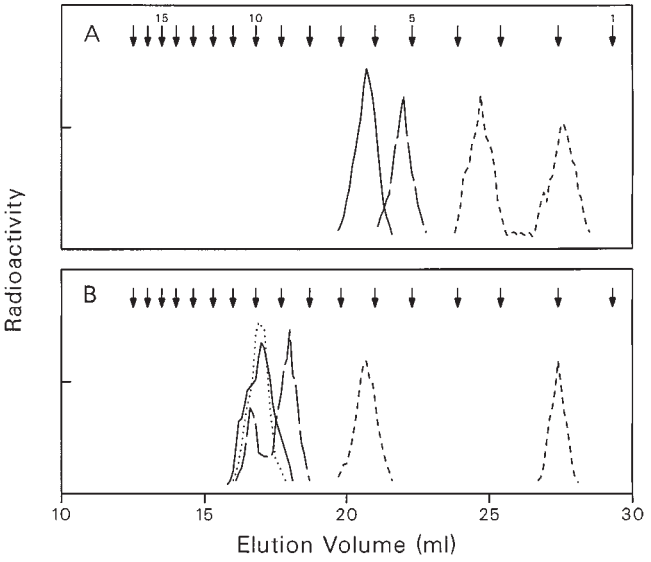

Figure 4. Sequential exoglycosidase treatment of PSGL-1 oligosaccharides. Radioactivity recovered from the peaks eluting at 6.3 (Panel A) and 9.8 GU (Panel B) in the initial separation of the O-linked oligosaccharides on PSGL-1 (see Figure 3, panel A) was subjected to sequential exoglycosidase treatment and rechromatography on the GlycoMap column, as outlined in Experimental Procedures. Lines: (—) the untreated oligosaccharide; (. . . .) product from treatment with almond meal α-fucosidase; (— —) product from treatment with jack bean or *S. pneumoniae* β-galactosidase; (- - -) product from treatment with jack bean or *S. pneumoniae* β-galactosidase followed by treatment with jack bean or *S. pneumoniae* β-*N*-acetylglucosaminidase. The numbered arrows indicate the elution volumes of dextran standard oligomers composed of 1–17 glucose units.

Treatment of the radioactivity recovered from the peak eluting at approximately 9.8 GU with *S. pneumoniae* β-galactosidase or jack bean β-galactosidase resulted, for both enzymes, in a shift to 8.8 GU for approximately 70% of the radioactivity (Figure 6, panel B). The remaining 30% appeared as a peak at approximately 10.2 GU. Although poorly resolved, the 10.2 GU peak can be seen in the chromatography profile of the untreated radioactivity, at the leading edge of the 9.8 GU peak (Figure 4, panel B). The 10.2 GU peak was resistant to treatment with *S. pneumoniae* β-galactosidase or jack bean β-galactosidase, as well as to treatment with *S. pneumoniae* β-*N*-acetylglucosaminidase (data not shown). It also appeared resistant to digestion with almond meal α-fucosidase (Figure 4, panel B). The low levels of radioactivity recovered from the 10.2 GU peak, and its resistance to exoglycosidase digestions did not allow a complete characterization. Still, the following experiments were carried out: A portion of the radioactivity recovered from the peak was treated with 0.1M TFA (trifluoro acelic acid) at 100°C for 1 h [27], and subsequently rechromatographed on the GlycoMap column; this resulted in a shift of approximately 0.6 GU, in elution volume (Figure 5, panel A). Further, digestion of portions of the radioactivity recovered from the 10.2 GU peak, before and after treatment with 0.1M TFA, with a mixture of jack bean β-galactosidase and jack bean β-hexosaminindase, followed by analysis on paper chromatography, revealed that although the untreated radioactivity was resistant to degradation by these glycosidases, radioactivity treated with 0.1M TFA was efficiently degraded (Table 2). Finally, in contrast to radioactivity eluting at 9.8 GU (and 12.8 GU), the radioactivity in the untreated 10.2 GU peak was retarded on an *Aleuria aurantia* lectin-Sepharose column (Table 2). Taken together, these results suggest that the

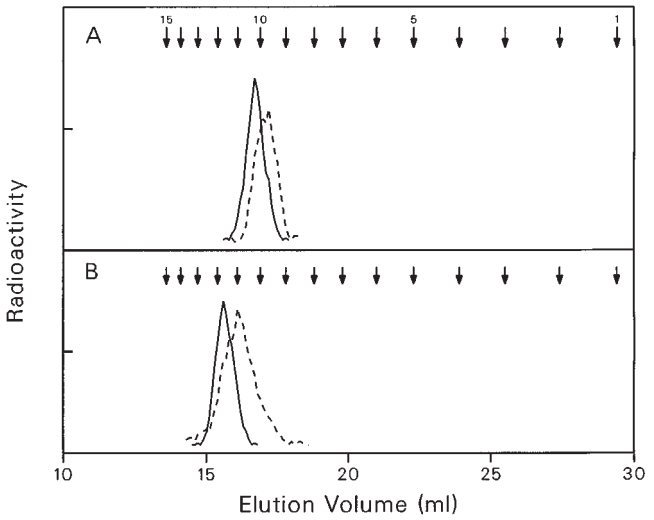

Figure 5. 0.1M TFA treatment of the fucosylated structures on PSGL-1. Radioactivity eluting at 10.2 (Panel A) and 11.8 GU (Panel B) during the separation of the total O-linked oligosaccharides on PSGL-1 was treated with 0.1M TFA at 100°C for 1 hour [27], and rechromatographed on the GlycoMap column. Lines: (——), the untreated oligosaccharide(s); (- - -) product from 0.1M TFA treatment. The numbered arrows indicate the elution volumes of dextran standard oligomers composed of 1–15 glucose units.

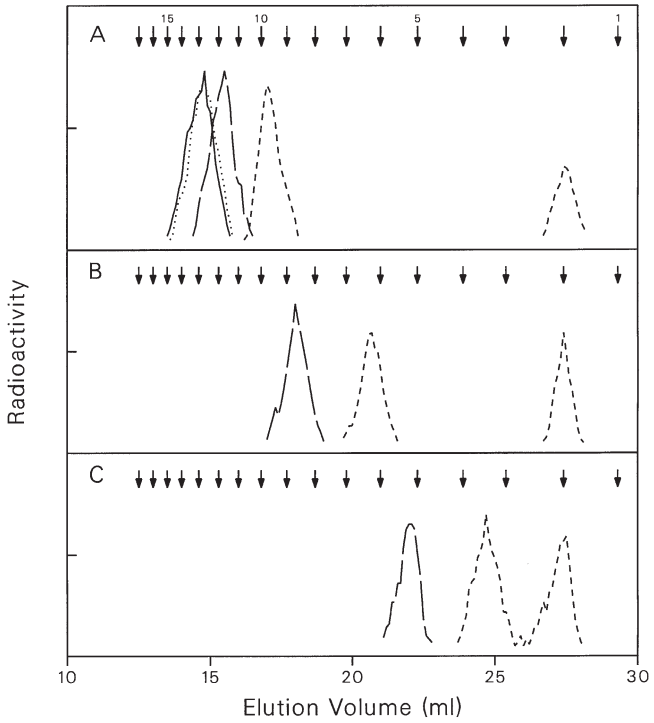

Figure 6. Sequential exoglycosidase treatment of PSGL-1 oligosaccharides. Radioactivity recovered from the peak eluting at 12.8 GU in the initial separation of the O-linked oligosaccharides on PSGL-1 (see Figure 3, panel A) was subjected to sequential exoglycosidase treatment and rechromatography on the GlycoMap column, as outlined in Experimental Procedures. Panels A, B, and C show the sequential removal of three *N*-acetyllactosamine units. Lines: (—) the untreated oligosaccharide; (. . . .) product from treatment with almond meal α-fucosidase; (— —) product from treatment with jack bean or *S. pneumoniae* β-galactosidase; (- - -) product from treatment with jack bean or *S. pneumoniae* β-galactosidase followed by treatment with jack bean *S. pneumoniae* β-*N*-acetylglucosaminidase. The numbered arrows indicate the elution volumes of dextran standard oligomers composed of 1–17 glucose units.

radioactivity in the 10.2 GU peak likely constitutes a LacNAc-type oligosaccharide containing a fucose linked to the penultimate monosaccharide (Table 3).

Further sequential degradation of the β-galactosidase sensitive radioactivity in the 9.8 GU peak with *S. pneumoniae* β-*N*-acetylglucosaminidase, followed by *S. pneumoniae* β-galactosidase, resulted in the 2 and 1 GU shifts predicted for a poly-*N*-acetyllactosamine type oligosaccharide; treatments with jack bean β-*N*-acetylglucosaminidase, followed by jack bean β-galactosidase (instead of the *S. pneumoniae* enzymes) produced identical results (data not shown). The β-*N*-acetylglucosaminidase treatments produced the predicted additional 2 GU degradation product (Figure 4, panel B), which was identified as *N*-acetylglucosamine on paper chromatography. For the continued degradation of the structure, due to the low remaining levels of radioactivity, the 6.3 GU fragment generated by sequential exoglycosidase treatment of the 9.8 GU oligosaccharide was pooled with the corresponding exoglycosidase degradation fragment (6.3 GU) from the 12.8 GU oligosaccharide (see below), and the combined radioactivity was sequentially treated with jack bean β-galactosidase and jack bean β-*N*-acetylglucosaminidase. This yielded the 1 and 2 GU shifts in elution volume predicted for degradation of a structure containing a terminal *N*-acetyllactosamine unit (Figure 6, panel C). The identity of the final 3.5 GU degradation product with Galβ1,3GalNAc-ol was confirmed by jack bean and bovine testis β-galactosidase treatments in combination with paper chromatography as outlined below. To acquire additional information about the linkages in the 6.3 GU fragment, the 5.3 GU glycosidase degradation product (from the 9.8 and 12.8 GU oligosaccharide; see above) was also degraded with *S. pneumoniae* β-*N*-acetylglucosaminidase, and the resulting products were separated on paper chromatography; this produced two fragments which co-chromatographed with the Galβ1,3GalNAc-ol and GlcNAc standards, respectively (data not shown). Taken together, the results from these experiments suggest that the radioactivity eluting at 9.8 GU represents a structure containing two *N*-acetyllactosamine repeat units, a conclusion supported by results from treatments with *B. fragilis* endo-β-galactosidase. Digestion of radioactivity from the intact 9.8 GU peak (including the 10.2 GU peak) with this enzyme resulted in approximately 50% of the radioactivity being degraded by the enzyme and recovered in the two fragments, 5.3 and 4

Table 2. Characterization of 10.2 and 11.8 GU oligosaccharides on PSGL-1.

Elution volume (GU)	*Susceptible to βGal:ase/βHex:ase digestion*	*Susceptible to βGal:ase/βHex:ase digestion following treatment with 0.1 M TFA*	*Retarded on AAL*[a]
12.8	+	+	−
11.8	−	+	+
10.2	−	+	+
9.8	+	+	−

[a]AAL, *Aleuria aurantia* lection-Sepharose.

GU, predicted for an O-linked poly-*N*-acetyllactosamine structure containing two repeat units. The radioactivity that was not degraded by the endo-β-galactosidase treatment presumably represents the 10.2 GU fucosylated structure included in the 9.8 GU peak (see above) and was not investigated further (data not shown).

The radioactive peak eluting at 11.8 GU was resistant to (repeated) treatments with *S. pneumoniae* or jack bean β-*N*-acetylglucosaminidase, as well as to treatment with *S. pneumoniae* β-galactosidase and/or jack bean β-galactosidase (Table 2 and data not shown). Moreover, the radioactivity in the combined intact 11.8 and 12.8 GU peaks appeared resistant to treatments with almond meal α-fucosidase (Figure 6, panel A). Again, due to the low levels of radioactivity recovered, it was not possible to sequentially degrade the structure(s) in the 11.8 GU peak. Instead, a set of experiments similar to those described above for the 10.2 GU oligosaccharide(s) were carried out, with a similar outcome: Treatment of radioactivity form the 11.8 GU peak with 0.1 M TFA at 100°C for 1 h resulted in a 0.6 GU shift in elution volume upon rechromatography on the GlycoMap (Figure 5, panel B). And the product formed was degraded with by mixture of jack bean β-*N*-acetylglucosaminidase and jack bean β-galactosidase (Table 2). Chromatography of untreated radioactivity from the 11.8 GU peak on *A. aurantia* lectin-Sepharose again resulted in radioactivity being retarded on the column (Table 2); by contrast, the TFA-treated radioactivity was not. Taken together, these results suggest that, in analogy with the 10.2 GU oligosaccharide(s), the radioactivity in the 11.8 GU peak likely constitutes a structure(s) containing a fucose linked to the penultimate monosaccharide.

Treatment of the radioactivity eluting at 12.8 GU with *S. pneumoniae* or jack bean β-galactosidase produced, for

Table 3. Summary of the O-linked oligosaccharide structures identified on PSGL-1.

	Elution Volume (GU)	*Percent of Structures*
±Siaα2,3(Galβ1,4GlcNAcβ1,3)$_2$Galβ1,4GlcNAcβ1,6 ±Siaα2,3Galβ1,3GalNAc-ol	12.8	3.5
Fucα1,3 ±Siaα2,3Galβ1,4GlcNAc - - - - - - - - - - - - - - - - - GalNAc-ol	11.8	1.9
Fucα1,3 ±Siaα2,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,6 ±Siaα2,3Galβ1,3GalNAc-ol	10.2	2.8
±Siaα2,3Galβ1,4GlcNAcβ1,3Galβ1,4GlcNAcβ1,6 ±Siaα2,3Galβ1,3GalNac-ol	9.8	6.4
±Siaα2,3Galβ1,4GlcNAcβ1,6 ±Siaα2,3Galβ1,3GalNAc-ol	6.3	45.3
±Siaα2,3Galβ1,3GalNAc-ol	3.5	36.3
GalNAc-ol	2.5	3.9

±Indicates that this portion is conjugated with sialic acid on a portion of the structures.
[a]Sia, sialic acid.

both enzymes, a 1 GU shift in elution volume. Subsequent treatment of the 11.8 GU β-galactosidase degradation product with *S. pneumoniae* or jack bean β-*N*-acetylglucosaminidase resulted, again for both enzymes, in a 2 GU shift in elution volume; the treatment produced the predicted additional 2 GU degradation product (Figure 6, panel A), which was identified as *N*-acetylglucosamine on paper chromatography. Further sequential treatments of the 9.8 GU β-*N*-acetylglucosaminidase degradation product (from the 12.8 GU peak) with *S. pneumoniae* β-galactosidase or jack bean β-galactosidase and *S. pneumoniae* β-*N*-acetylglucosaminidase or jack bean β-*N*-acetylglucosaminidase produced additional shifts in elution volume of 1 and 2 GU, respectively, yielding a final 6.3 GU product (Figure 6, panel B). At this stage, the low levels of remaining radioactivity in the sample made continued analysis by size exclusion chromatography difficult. The following experiments were carried out to identify the remaining portion of the structure. A portion of the radioactivity recovered from 6.3 GU fragment was mixed with the radioactivity from the corresponding fragment from the 9.8 GU structure, and the combined material was subjected to further degradation and analysis on the GlycoMap column as outlined above (Figure 6, panel C). In addition, radioactivity from the 6.3 GU fragment was treated with a mixture of *S. pneumoniae* β-galactosidase and *S. pneumoniae* β-*N*-acetylglucosaminidase, and the products were separated on paper chromatography. This yielded two fragments which co-migrated with the Galβ1,3GalNAc-ol and GlcNAc standards, respectively (data not shown). Additional experiments to confirm the identity of the putative Galβ1,3GalNAc-ol fragment generated in this experiment were not done, due to the very low remaining levels of radioactivity. However, the identity of the corresponding fragment produced by sequential degradation of the combined radioactivity from the 9.8 and 12.8 GU structures was confirmed (see above). Thus, our results suggest that the radioactivity in the 12.8 GU peak represents a poly-*N*-acetyllactosamine structure containing three repeat units. This conclusion was confirmed by an endo-β-galactosidase treatment experiment, as outlined above for the 9.8 GU oligosaccharide (see above). When radioactivity from the intact 12.8 GU peak was treated with *B. fragilis* endo-β-galactosidase, the radioactivity was recovered in three peaks eluting at approximately 5.8, 4 and 3 GU, respectively, consistent with the degradation of an O-linked poly-*N*-acetyllactosamine structure containing three repeat units (data not shown).

The presence of poly-*N*-acetyllactosamine containing structures in the O-linked oligosaccharide preparation isolated from PSGL-1 was further supported by results from a lectin chromatography experiment. Fractionation of the intact (sialic acid containing) total O-glycans from PSGL-1, on tomato lectin-agarose, resulted in approximately 18% of the radioactivity binding to the column (data not shown). This is consistent with the amounts of radioactivity recovered in the poly-*N*-acetyllactosamine containing oligosaccharides identified in the 9.8 and 12.8 GU peaks from size exclusion chromatography (compare above).

Separation of radioactivity from the 3.5 GU peak by paper chromatogaphy resulted in only one radioactive peak which co-migrated with the Galβ1,3GalNAc-ol standard. The identity of this structure was subsequently confirmed by treatment with jack bean and bovine testis β-galactosidase in combination with separation on paper chromatography, essentially as described [31]. All the radioactivity in the 2.5 GU peak co-migrated with the GalNAc-ol standard on paper chromatography (data not shown).

Sulfation of the O-linked oligosaccharides on PSGL-1

Metabolic labeling with [^{35}S]-sulfur prior to isolation of PSGL-1 resulted in no detectable radioactivity in the O-linked oligosaccharides on the molecule (data not shown). In addition, no charged, neuraminindase-resistant structures were detected (see above). Taken together, these results suggest that the O-linked oligosaccharides on PSGL-1, including the partly characterized structures eluting at 11.8 and 10.2 GU, are not sulfated.

Discussion

The data presented in this report suggest that PSGL-1 predominantly contains the O-linked structures summarized in Table 3. A major portion of the oligosaccharides on the ligand, i.e., the structures eluting at 12.8, 9.3 and 6.3 GU, are *N*-acetyllactosamine containing core 2 structures varying only in the number of *N*-acetyllactosamine repeat units and in the sialylation pattern. By contrast, a small but significant portion (approximately 9% of the total radioactivity and eluting at 10.2 and 11.8 GU) appear to be branched oligosaccharides that contain a fucose likely linked to the penultimate monosaccharide at the nonreducing end. The 0.6 GU shift in the elution volume, observed on treatment of these structures with 0.1M TFA, is consistent with removal of one fucose. The approximate size of fucose on the GlycoMap column is 0.4-0.6 GU. Other neutral monosaccharide typically yield larger shifts in elution volume: glucose and galactose approximately 1 GU; mannose 0.8-0.9 GU.

In addition, the mild hydrolysis conditions used for this experiment cleave other neutral saccharide linkages quite inefficiently, and the fact that the structures are retarded on *A. aurantia* lectin-Sepharose provides further support of the presence of a fucose branch (Table 2). It is not clear why no change in elution volume was observed on digestion (of these structures) with almond meal α-fucosidase. However, working with model oligosaccharides, we have noticed that in our hands, cleavage (by this enzyme) of

even a modest amount of fucose from Le^x-containing structures requires prolonged incubation times in the presence of considerable amounts of enzyme (P.A. Aeed & Å.P.Elhammer, unpublished observations). The large fucosylated structure identified on PSGL-1 by Wilkins et al., also appeared to a large extent resistant to α-fucosidase digestion [9].

Fucosylated oligosaccharides on PSGL-1 are consistent with a large body of data suggesting the presence of sialylated $Lewis^x$ or $Lewis^a$ containing structures on the endogenous ligands for E-, P-, and L-selectin (e.g., 1, 3, 8, 9). It is also consistent with the apparent functional requirement for oligosaccharides with this type of substitution on PSGL-1 expressed in heterologous cells [20, 38]. In addition, the size and LacNAc content of the structures is consistent with observations made by Stroud and coworkers [39, 40], that structures containing several LacNAc repeat units bind more efficiently (to E-selectin) than shorter structures.

The fucosylated O-linked structures described in this report differ in several aspects from those reported in a previous characterization of oligosaccharides recovered from HL-60 PSGL-1 [9]. First, in the investigation reported here no indications were found for a disiallylated, fucosylated tetrasaccharide. Second, the large fucosylated structures identified in the current investigation contain only one fucose, instead of three, as reported by Wilkins et al. [9]. And finally, and perhaps most interestingly, the number of fucosylated structures found in the current investigation is quite low, only approximately 4.5% (percentage calculated assuming that the fucosylated structures recovered from the 11.8 and 10.2 GU peaks contain 4 and 3 amino sugars, respectively). Wilkins et al. reported 14% fucosylated structures [9].

The fucosylated tetrasaccharide (described by Wilkins et al. [9]), if present in the current investigation, should have eluted at approximately 6.9 GU on the GlycoMap column, well resolved from the unfucosylated tetrasaccharide eluting at 6.3 GU. If for some reason it had remained unresolved from the 6.3 GU peak, it would have appeared immediately on exoglycosidase degradation of this peak. On the other hand, the amount of the fucosylated tetrasaccharide (approximately 2% of total) reported by Wilkins et al., is quite modest. Hence, it is possible that subtle variations in (the glycosylation machinery of) the cell lines and/or culture conditions may be the reason why the structure was absent (or below detection level) in the investigation reported here. Further, an important consideration when comparing the results from the current investigation with those of Wilkins et al. is the different methodologies used. In the current investigation, each structure was individually purified and disassembled, one monosaccharide at a time, on a calibrated size exclusion (GlycoMap) column. This column has a resolution of 0.2–0.5 GU (depending on the size of the oligosaccharide analyzed). Wilkins et al., subjected radioactivity (obtained from group separations of labeled oligosaccharides on BioGel P-4 and P-10 columns) to mixed glycosidase digestions followed by analysis of the resulting fragment patterns by paper chromatography; the fragments were identified by their migration positions (in the paper chromatography system) in relation to known standards. Although the pyridene-ethyl acetate-acetic acid-water (5:5:1:3) chromatography system used is usually capable of efficiently resolving smaller (1-6 GU) structures, it is (in our hands) much less efficient for separation of larger oligosaccharides. In fact, using this system, separations of the 6.3, 9.8, 10.2, 11.8, and 12.8 GU structures, described in this report, demonstrated that although the 6.3 GU structure is well resolved (migrating at approximately 20 cm, as reported by Wilkins et al.,) all the larger oligosaccharides (9.8–12.8 GU) migrate in the same (heterogenous) radioactivity peak, at approximately 6 cm (Å Elhammer, unpublished observations). The trifucosylated structure described by Wilkins et al., is larger than any of the structures described in this report.

In the current investigation, the number of fucoses on the fucosylated oligosaccharides was determined by comparing the size of the structures before and after chemical cleavage (see above). Wilkins et al., [9] measured the percentage of radioactivity released by α-fucosidase digestion of *in vivo* ^{3}H-fucose labeled material recovered from BioGel P-10 chromatography; the one-third radioactivity released suggested that the structure contained three fucoses.

The low number (4.5%) of fucosylated structures recovered from PSGL-1 carries interesting implications. Given that PSGL-1 contains 53 predicted sites for O-glycosylation [41] and assuming that most of these sites are occupied, an assumption that is supported by published data suggesting large amounts of O-linked oligosaccharides on PSGL-1 [8, 20, 21], a simple calculation reveals that each PSGL-1 molecule could contain as few as two fucosylated oligosaccharides. The fact that the migration position of PSGL-1 on SDS-PAGE, remains unchanged on α-fucosidase digestion is consistent with a low number of fucosylated structures [42]. A small number of functional ligand oligosaccharides on a heavily glycosylated molecule such as PSGL-1 suggest that the attachment and/or assembly of the ligand structures is restricted to specific sites, a notion that is supported by recent data suggesting that one specific glycosylation site, Thr16 (in the mature, processed molecule), close to the amino-terminal end of the PSGL-1 molecule, carries the oligosaccharide structure(s) responsible for P-selectin recognition [43–45]. A site-specific arrangement of the ligand oligosaccharides is of interest, not only from the point of view of understanding the molecular architecture of selectin-ligand interaction, but also because it suggests that the glycosyltransferase(s) involved in the synthesis of the ligand structure(s) on PSGL-1 (and perhaps other selectin ligands) have acceptor specificities that include not only the oligosaccharide primer, but perhaps also an underlying

polypeptide motif. Several examples of "outer chain" glycosyltransferases interacting with the polypeptide portion of a glycoprotein acceptor have been reported previously [e.g., 46–51]. In conclusion, available data suggest that only a limited subset of the O-glycans on PSGL-1 are fucosylated and hence likely involved in the interactions with the selectins. Moreover, the assembly of the fucosylated structures appears to be confined to certain sites on the ligand; sites that may be specifically recognized by the transferases involved in ligand glycan synthesis.

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