Isolation and Characterization of Natural Protein-Associated Carbohydrate Ligands for E-Selectin

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ABSTRACT: A comparative analysis of carbohydrate 'libraries' derived from cell lines binding E-selectin with differing avidity identified endogenous protein-associated carbohydrate ligand candidates for E-selectin. Three unusual structures, which constitute less than 3% of cell surface protein-associated carbohydrate, were unique to the E-selectin-binding cells, including neutrophils and the monocytic cell line U937. All are tetraantennary N-linked structures with a NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAcβ1→3Galβ1→4-(Fucα1→3)GlcNAc lactosaminoglycan extension (diSLex) on the arm linked through the C4 residue on the mannose. While all contained the expected SLex [NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc] moiety, these structures have an additional fucosylated lactosamine unit. Direct evidence that these diSLex-containing structures are, indeed, high-affinity ligands for E-selectin came from the use of recombinant soluble E-selectin−agarose affinity chromatography. We found that these three carbohydrate structures bound specifically to the E-selectin column. SLex itself does not bind under identical conditions. In summary, these related structures: (1) all possess an unusual 3-sialyl di-Lewis x extension on one arm of an N-linked tetraantennary glycan; (2) of the cells tested, are present only on E-selectin-binding leukocytes and leukocytic cell lines; (3) bind to E-selectin with a relatively high affinity (K_d < μM) and one greater than that of 3-sialyl Lewis x or 3-sialyl Lewis a; and (4) represent a very small percentage of the protein-associated carbohydrate. These carbohydrate structures appear to be present on only a very small number of cell surface proteins and may alone be responsible for the specificity of E-selectin-dependent adhesion.

The specificity of cell−cell recognition and adhesion derives in large part from selective and controlled expression of cell surface glycoconjugates and their cognate receptors. A number of these receptors have been identified and classified into distinct families on the basis of sequence homology (Osborn, 1990; Springer, 1990, 1994; Bevilacqua & Nelson, 1993). One family of cell adhesion molecules is the selectins, of which there are three members reported to date, designated L-, P-, and E-selectin (Bevilacqua & Nelson, 1993; Springer, 1994). All three are glycoproteins with a similar and distinctive molecular architecture, including a calcium-dependent carbohydrate recognition domain at their N-terminus. E-selectin is an inducible protein expressed on the surface of endothelial cells *in vitro* in response to cytokines such as IL-1 and TNF and *in vivo* at inflammatory sites (Bevilacqua et al., 1987; Pober & Cotran, 1991). Using *in vitro* assays, cells have been identified which can bind to E-selectin including peripheral blood cells such as neutrophils, monocytes, eosinophils, NK cells, and CTA^+ T-cells and myeloid-derived cell lines such as U937, HL60, and THP1 (Bevilacqua et al., 1989; Bochner et al., 1991; Carlos et al., 1991; Lobb et al., 1991; Picker et al., 1991a; Weller et al., 1991).

Recent studies have identified several putative cell surface ligands for selectins. On the basis of *in vitro* assays, three ligands for L-selectin have been characterized and cloned, the mucins GlyCAM-1 and CD34 (Lasky, 1992; Baumhueter et al., 1993) and the mucin-containing molecule MadCAM (Berg et al., 1993; Briskin et al., 1993). Blotting of HL60 cell extracts with labeled P-selectin and affinity chromatography studies with immobilized P-selectin identified specific 120 and 160 kDa glycoprotein ligands (Moore et al., 1992; Norgard et al., 1993). Recent expression cloning studies have shown the 120 kDa protein to be a novel homodimeric mucin designated PSGL-1 (Sako et al., 1993). Finally, specific glycoprotein ligands for murine E-selectin have been identified using E-selectin affinity chromatography (Levinovitz et al., 1993; Lenter et al., 1994), although to date no E-selectin ligands have been cloned. These results argue for a very restricted set of high-affinity ligands for selectins.

There is substantial evidence that specific carbohydrate structures can function as ligands for selectins, interacting, at least in part, with their lectin domains (Lasky, 1992; Bevilacqua & Nelson, 1993; Kansas et al., 1994). In general, these ligands are acidic derivatives of the Lewis x and Lewis a trisaccharide structures, in which sialic acid (i.e., SLex[1] and SLea) or inorganic sulfate is attached to the C3 position of the galactose residue. It has been shown that the surface expression of these structures is regulated by key fucosyltransferases (Goelz et al., 1990, 1994; Lowe et al.,

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[1] Abbreviations: ELFT, ELAM-ligand fucosyltransferase; rsE-selectin, recombinant soluble E-selectin; PMSF, phenylmethane sulfonyl fluoride; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; MALD-MS, matrix-assisted laser desorption mass spectrometry; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; SLex, NeuAcα2→3Galβ1→4(Fucα1→3)-GlcNAc; SLea, NeuAcα2→3Galβ1→3(Fucα1→4)GlcNAc; SA, sialic acid; gu, glucose units.

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1990). Their expression in cells that do not normally bind E-selectin can allow their subsequent adhesion to E-selectin-expressing cells via new sialylated, fucose-containing structures on the cell surface. However, the acidic carbohydrates (such as SLex) defined to date block adhesion *in vitro* with low affinity, with apparent K_d's usually on the order of 0.1−2 mM, depending on assay format (Phillips et al., 1990; Nelson et al., 1993).

Given the low affinities of the acidic carbohydrates defined to date, and an apparently restricted set of high-affinity selectin glycoprotein ligands (Lenter et al., 1994), there remains uncertainty as to whether these acidic carbohydrates alone constitute the set of natural ligands for selectins. While multivalent expression of low-affinity carbohydrates on heavily O-glycosylated mucin-like structures provides reasonable ligand candidates for L- and perhaps P-selectin (Lasky, 1992; Norgard et al., 1993), no direct experimental approaches have been taken to address this issue. In this report we have directly identified the responsible carbohydrate structures on cells that bind E-selectin with high avidity (human neutrophils and U937 cells). We have characterized three related structures which (1) all possess an unusual 3-sialyl di-Lewis x extension on one arm of an N-linked tetraantennary glycan; (2) in the cells tested, are present only on E-selectin-binding leukocytes and leukocytic cell lines; (3) bind to E-selectin with a relatively high affinity (K_d < μM) and one greater than that of 3-sialyl Lewis x or 3-sialyl Lewis a; and (4) represent a very small percentage of the protein-associated carbohydrate. These carbohydrate structures are likely present on only a very small number of cell surface proteins and may alone be responsible for the specificity of E-selectin-dependent adhesion.

METHODS

Cells. U937, RAMOS, HL60, and COS7 cells were obtained from the American Type Culture Collection. U937, RAMOS, and HL60 cells were grown in RPMI, 10% fetal bovine serum (FBS), 2 mM glutamine. COS7 cells were grown in HEPES-buffered DMEM, 10% FBS, 2 mM glutamine. Using a modification of the procedure described by Boyum (1968), polymorphonuclear cells (PMNs) were isolated from fresh human blood by 3% dextran sedimentation followed by gradient separation with Ficoll-Paque (Pharmacia) and then hypotonic lysis. The cell line that transiently expresses ELFT, (COS7.2) was made by electroporation of ELFT/CDM8 into COS7 cells essentially as described by Goelz et al., (1990).

Plasma Membrane Preparation. The adherent cell lines (COS7 and COS7.2) were detached from the culture flasks by incubation with PBS, 5 mM EDTA for 5 min and washed twice with PBS, and the pellet was lysed using a N_2 cavitation as follows. Cells were resuspended in cold 10 mM HEPES buffer, pH 7.0, containing 5 mM $MgCL_2$, 5 mM $CaCl_2$, PMSF (35 μg/mL), aprotinin (10 μg/mL), and leupeptin (1 mM) at a concentration of about 10^8 cells/mL. This mixture was placed in a chilled N_2 cavitation bomb, and the cells were lysed using 700 psi for 25 min. The nuclei were removed by contrifugation at 10000*g* at 4 °C for 5 min, and the supernatant was fractionated by further centrifugation at 100000*g* at 4 °C for 1 h. The pellet containing crude plasma membranes was resuspended in PBS (containing aprotinin and leupeptin) and the protein concentration determined using the BCA assay (Pierce). The membranes were frozen in liquid N_2 and stored at −8 °C until use.

rsE-Selectin− and BSA−Agarose. rsE-selectin was made as described (Lobb et al., 1991). rsE-selectin and bovine serum albumin (BSA) were coupled to Affigel as follows: 7.2 mg of rSE-selectin (1.2 mg/mL) or 6 mg of BSA (2 mg/mL) was dialyzed against three changes of 0.1 M MOPS buffer, pH 7.5, overnight and concentrated to 1 mL by centrifugation at 2000*g* for 45 min with an Amicon Centriprep-30 instrument. The rsE-selectin or BSA was coupled to Affigel 15 (BioRad) according to the manufacturer's instructions (approximately 1 mL of packed gel was used and coupled overnight at 4 °C). The resin was washed thoroughly with PBS and was ready for use.

Carbohydrate Analysis. Release, isolation, and labeling of carbohydrates were performed as previously described (Patel et al., 1993). Fractionation of radilabeled oligosaccharide alditols by QAE anion-exchange chromatography, gel filtration, and HPAEC-PAD (Dionex BioLC instrument) as well as analysis of oligosaccharides by glycosidase (Oxford GlycoSystems) digestion, MALD-MS (Finnegan MAT instrument), and controlled acetolysis were all performed as described previously (Parekh et al., 1989a).

Affinity Chromatography Using a rsE-Selection−Agarose Column. Radiolabeled oligosaccharide alditols (approximately 2 nmol) were applied at 4 °C in 10 μL of loading buffer (TBS, pH 7.4, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$) to a column of rsE-selectin−Affigel 15 (0.15 mL bed volume, ≈3 mg of rsE-selectin/mL bed volume) equilibrated in loading buffer and flow suspended for 15 min. Unbound alditols were then eluted using loading buffer, and 0.1 mL fractions were collected. Elution of unbound alditols continued until no radioactivity was detected in four consecutive fractions by liquid scintillation counting. Bound alditols were then eluted using 5 × 0.15 mL of TBS, pH 7.4, containing 10 mM EDTA, and 0.1 mL fractions were again collected. Fractions containing bound alditols were pooled, the total radioactivity in the eluted pool was determined, and the pool was then desalted prior to further analysis. As a control, radiolabeled alditols of 3-sialyl Lewis x (SLex) and 3-sialyl Lewis a (SLea) were fractionated as above, and neither oligosaccharide was retained by the rsE-selectin−agarose column. As a further control, each radiolabeled oligosaccharide alditol pool was passaged in the same way through a column of BSA−Affigel 15 (≈3 mg of BSA/mL bed volume). In no case were any alditols retained by the BSA column (to an estimated limit of detection of 0.01%).

Iodination. PMN, HL60, or U937 cells (1×10^7) were washed three times with PBS, resuspended in 0.5 mL of PBS, and added to a tube coated with 50 μg of 1,2,3,4,6-tetrachloro-3α,6α-diphenylglycouril (Iodogen) (Sigma Chemical Co.). To this was added 1 mCi of ^{125}I, and the mixture was incubated for 30 min at 0 °C with occasional mixing. The labeled cells were transferred to a tube containing 10 mL of RPMI, 10% FBS, centrifuged at 1000*g* for 5 min, and then washed with another 10 mL of RPMI, 10% FBS and finally with 2 mL of PBS. The cells were pelleted in an Eppendorf tube by centrifugation at 1000*g* for 2 min and were then lysed by addition of 1.0 mL of PBS containing 1% NP40, 2 mM PMSF, 1 mM EDTA, soybean trypsin inhibitor (50 mg/mL), and Leupeptin (1 mM) (Sigma Chemical Co.). After vortexing, the mixture was incubated

for 30 min at 0 °C and then centrifuged for 1 min at 10000*g* to remove particulate matter. The supernantant containing labeled solubilized membrane proteins was precleared with 10 μL of Protein A Sepharose 4B (Zymed) for 2 h at 0 °C, and the supernantant was stored at 4 °C. ARX beads were used to immunoprecipiate the labeled C_2E_5 antigens from the cell lysates.

Preparation of ARX Resin. ARX resin used for the immunoprecipitations was made as follows: 100 μL (50% slurry) of Protein A Sepharose 4B (Zymed), 100 μL (200 μg) of rabbit antimouse IgM, μ-chain specific (Jackson ImmunoResearch), and 10 μL of 1 M Tris-HCl, pH 8.0, were mixed together in an Eppendorf tube and rocked at room temperature for 30 min. The supernatant was removed, and the resin was washed two times with 500 μL of 0.1 M Tris-HCl, pH 8.0, and then two times with 500 μL of 0.01 M Tris-HCl, pH 8.0 (between washes the resin was pelleted by centrifuging 10000*g* for 1 min). To the washed resin was added 0.5 mL of C_2E_5 ascites (~2.5 mg of IgM), and the mixture was rocked at room temperature for 1 h. The supernatant was removed, and the resin was washed two times with Tris-HCl, pH 8.0, and then resuspended in 1.0 mL of 0.2 M sodium borate, pH 9.0. The antibodies were cross-linked to the Protein A resin and to each other by addition of 5.18 mg of dimethylpimelimidate to give a final concentration of 20 mM and rocked at room temperature for 30 min. The mixture was centrifuged at 3000*g* for 3 min, the supernatant removed, and the resin washed three time with 1 mL of 0.2 M ethanolamine, pH 8.0 (the final wash was incubated with the resin for 30−60 min at room temperature before removing the supernatant). The ARX resin was resuspended in 0.5 mL of PBS, 0.01% merthiolate and stored at 4 °C.

Preparation of C_2E_5 Ascites. This preparation was obtained as described previously (Goelz et al., 1990).

Immunoprecipitation. Immunoprecipitations were performed by adding 10 μL of ARX beads to 50−100 μL of precleared labeled cell lysate and incubating for 2 h at 4 °C. The Sepharose was then washed four times with 2 mL of PBS containing 0.75% NP40, 0.2% DOC, and 1 mM EDTA. The ARX beads were resuspended in 25 μL of nonreducing SDS sample buffer, and the sample was heated for 10 min at 85 °C. The mixture was briefly centrifuged (30 s, 10000*g*) and the supernatant removed. To this supernatant was added β-mercaptoethanol to 3%, and the sample was heated at 85 °C for another 5 min and then separated on a 10% SDS−polyacrylamide gel.

RESULTS

Comparative Analysis of Carbohydrate Libraries

Analytical Strategy. Carbohydrate 'libraries' containing both N- and O-linked glycans were prepared from the pool of total plasma membrane glycoproteins isolated from cell lines that normally bind E-selectin avidly (PMN and U937 cells), that bind to E-selectin due to the expression of an exogenous added fucosyltransferase (COS cells transiently expressing ELFT), and that do not bind E-selectin at all (RAMOS and untransfected COS7 cells). Each such carbohydrate library was fractionated chromatographically, and the primary structure was determined for those carbohydrates that are conserved, as judged by chromatographic identity, in the cell lines that bind E-selectin. These primary structures were then compared to those of carbohydrates affinity-purified from the individual carbohydrate libraries using an rsE-selectin−agarose column.

Fractionation of the Desialylated Oligosaccharide Alditols Recovered from the Plasma Membrane Preparations of U937, COS7, COS7.2, PMN, and Ramos Cells. An initial comparison of the carbohydrate structures on E-selectin-binding cells with those found on nonbinding cells was performed as follows. An aliquot of the total pool of oligosaccharide alditols recovered from plasma membrane preparations of U937, COS7, COS7.2, PMN, or Ramos cells was desialylated by exhaustive incubation with a broad specificity neuraminidase (*Arthrobacter ureafaciens*), and each pool was fractionated by gel filtration chromatography. The resulting chromatograms are shown in Figure 1A−E (inclusive). In each chromatogram, the single major oligosaccharide alditol (marked 0−2) corresponds to galactose β1→3 *N*-acetylgalactosaminitol. The regions of each chromatogram eluting between V_0 and 7 gu are shown enlarged in Figure 1A′−E′ (inclusive). A comparison of these chromatograms reveals that, as judged by hydrodynamic volume, many oligosaccharide alditols are common to all the cell lines (though each occurs at a particular molar incidence in each pool), while some are not. The relative molar content of asialo oligosaccharide alditol fractions in the pool derived from each cell line is summarized in Table 1A. In Table 1B are summarized the data for those fractions not common to all cell lines. From Table 1B, it is clear that asialo oligosaccharide alditols of hydrodynamic volume 24.4 gu (U4, P3, 7.2-3), 27.3 gu (U6, P4, 7.2-4), and 29.5 gu (U8, P5, 7.2-5) are found only in the oligosaccharide pools from cells that bind E-selectin (U937, PMNs, COS7.2). These asialo oligosaccharide alditols together with those eluting at 23.7 gu (U3), 26.4 gu (U5), 28.7 gu (U7), and 31.6 gu (U9) were therefore recovered for further structural analysis from the asialo oligosaccharide pool derived from U937 cells.

Structural Analysis of the Asialo Oligosaccharide Alditols U3−U9, Inclusive. Primary structural analysis of asialo oligosaccharide alditols U3−U9 was performed using a combination of sequential exoglycosidase digestion, controlled acetolysis (previously optimized to maximize the selective cleavage of the mannose α1→6 bond), and matrix-assisted laser desorption mass spectrometry (MALD-MS) to determine the molecular mass of the underivatized form of each alditol. During sequential exoglycosidase digestion and controlled acetolysis, P4 gel filtration chromatography was used to determine the change in hydrodynamic volume induced by each exoglycosidase or by the acetyolysis procedure. From the induced change in hydrodynamic volume, the number of monosaccharide residues cleaved is readily calculated. The results of the controlled acetolysis and MALD-MS are presented in Table 2 and of the sequential exoglycosidase digestion in Figure 2.

Together, these data support the structural assignments of asialo oligosaccharide alditols U1−U9 shown in Figure 2. Interestingly, no simple SLex structures were found in the membrane glycoproteins of these E-selectin-binding cells, and all outer arm fucose α1→3 residues are found on the GlcNAcβ1→4Manα1→3 branch and only in di-Lewis x structures. The assignment of the lactosaminoglycan extension to the GlcNAcβ1→4Manα1→3 is based primarily on data from sequential exoglycosidase digestion and controlled

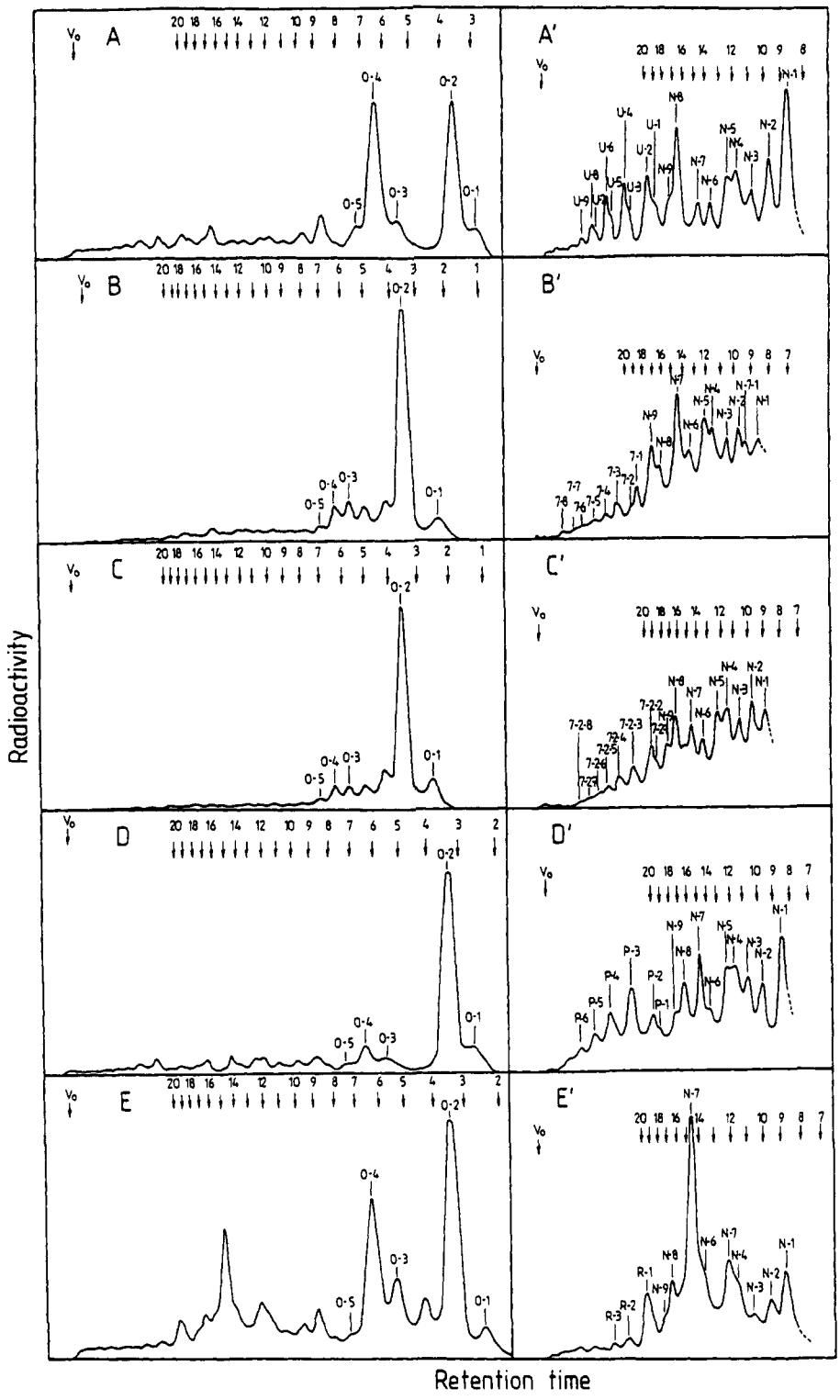

FIGURE 1: Bio-Gel P4 (~400 mesh) gel filtration chromatograms of the oligosaccharides recovered from the plasma membranes of U937 (A), COS7 (B), COS7.2 (C), PMN (D), and RAMOS (E) cells. The corresponding regions of the chromatograms between V_o and the glucose octamer (eluting at gu = 8) are shown expanded in parts A′–E′, respectively.

acetolysis. Insufficient material was available to confirm the assignment by ^{1}H-NMR. The sialylation of the lactosamine-bearing arm(s) of each of the asialo ogligosaccharide alditols U3–U9 (inclusive) in the authentic oligosaccharide pool recovered from U937 was next determined.

Sialylation Patterns of the Lactosamine-Bearing Arm of Each of U3–U9 in the Authentic Oligosaccharide Alditol Pool Recovered from U937 Cell Plasma Membranes. An aliquot of the total pool of oligosaccharide alditols derived from the plasma membranes of U937 cells was fractionated by QAE anion-exchange chromatography. The chromatogram is shown in Figure 3A. In order to locate the sialylated forms of U3–U9, individual fractions from the chromatogram shown in Figure 3A were pooled as indicated and desalted, a small aliquot was desialylated by incubation with neuraminidase (*A. ureafaciens*), and the desialylated oligosaccharides were fractionated by P4 gel filtration chromatography (not shown). This analysis showed that

Table 1

A: Relative Incidence of Individual Oligosaccharides Recovered from the Plasma Membranes of U937, COS7.2, COS7, PMN, and RAMOS Cells

fraction	hydrodynamic volume (gu)	U937	COS7	COS7.2	PMN	RAMOS
0-1 + 0-2	2.5, 3.5	31.9	40.8	42.2	51.3	36.0
0-3 + 0-4 + 0-5	5.5, 6.5, 7.3	36.2	22.8	24.1	16.6	27.6
N-1	8.8	5.6	2.7	2.9	5.8	4.1
N-2	9.8	3.1	4.3	4.1	2.1	2.4
N-3	10.9	1.9	2.1	2.4	2.2	1.8
N-4 + N-5	11.8, 12.5	4.1	6.0	6.5	5.0	6.4
N-6	13.6	1.1	2.3	1.9	2.7	10.5
N-7	14.6	1.3	5.2	2.4		
N-8 + N-9	16.3, 17.3	4.4	5.5	4.6	3.9	4.6
U1 + U2	18.5, 19.5	3.1	–	–	–	–
U3 + U4	23.7, 24.4	2.3	–	–	–	–
U5 + U6	26.4, 27.3	1.8	–	–	–	–
U7 + U8	28.7, 29.7	1.1	–	–	–	–
U9	(31.6)	0.6	–	–	–	–
V_0 → U9	>32	1.5	–	–	–	–
7-1 + 7-2	18.5, 19.5	–	2.8	–	–	–
7-3	23.6	–	1.7	–	–	–
7-4	26.3	–	1.2	–	–	–
7-5	28.5	–	0.9	–	–	–
V_0-7-5	>30	–	1.7	–	–	–
7.2-1 + 7.2-2	18.5, 19.5	–	–	2.7	–	–
7.2-3	24.6	–	–	1.7	–	–
7.2-4	27.5	–	–	1.4	–	
7.2-5	29.7	–	–	0.9	–	–
V_0 → 7.2-5	>32	–	–	2.2	–	–
P-1 + P-2	18.5, 19.5	–	–	–	2.0	–
P-3	24.5	–	–	–	3.7	–
P-4	27.5	–	–	–	2.3	–
P-5	29.8	–	–	–	1.3	–
V_0 → P-5	>32	–	–	–	1.1	–
R1	19.5	–	–	–	–	3.2
R2	23.4	–	–	–	–	1.3
R3	2-5.4	–	–	–	–	0.6
V_0 → R3	>30	–	–	–	–	1.5

B: Summary from the Data in Part A of Oligosaccharide Fractions That Are Not Common to the Cell Lines Investigated

hydrodynamic volume of fraction (gu)	cell line				
	U937	PMN	COS7.2	COS7	RAMOS
18.5	+[a]	+	+	+	–
19.5	+	+	+	+	+
23.7	+	–	–	+	+
24.4 (U4, 7.2-3, P-3)	+	+	+	–	–
26.4 (U5, 7-4)	+	–	–	+	–
27.3 (U6, 7.2-4, P-4)	+	+	+	–	–
28.7 (U7, 7-5)	+	–	–	+	–
29.5 (U8, 7.2-5, P-5)	+	+	+	–	–

[a] + indicates the presence and – the absence of an oligosaccharide fraction centered at the hydrodynamic volume indicated.

oligosaccharide alditols U3–U9 were contained primarily within pool X (~85% of the total U3–U9 content in the unfractionated pool was contained in pool X) as judged by elution volume. The oligosaccharide alditols within pool X were fractionated by HPAEC-PAD (Figure 3B). A portion of the 11 resulting sialylated oligosaccharide alditols was desialylated, and each of U3–U9 were identified by P4 gel filtration chromatography (not shown). Fractions were pooled as indicated in Figure 3B, desalted, and further fractionated by HPAEC-PAD to homogeneity (not shown). These sialylated forms are now referred to as AU3–AU9, respectively. The position and nature of the sialic acid linkages on the lactosamine-bearing arm of each of AU3–AU9 were determined, as is now described in detail for AU4.

The oligosaccharide alditol AU4 was incubated with endo-β-galactosidase (e.g., *Escherichia freundii*) under exhaustive conditions, determined using standard oligosaccharide alditols. The reaction mixture was desalted and rotary-evaporated to dryness and the new nonreducing terminus radiolabeled by reduction by alkaline sodium borotritide. The radiolabeled fragments were then separated by P4 gel filtration chromatography using water as eluant, under which conditions acidic oligosaccharide alditols elute in the void (Figure 3C). All radioactive oligosaccharide alditols were recovered in the void indicating that these were all still sialylated. The void pool, A-U4-V_0 was fractionated by Mono Q anion-exchange chromatography. The chromatogram is shown in Figure 3D. Pools A-U4-1 and A-U4-2 were desalted and incubated first with the α2→3 specific neuraminidase (from Newcastle disease virus) and the products fractionated by P4 gel filtration chromatography. In the case of pool A-U4-2, all products eluted in the void indicating that at least some of the SA was a 2,6 linkage (not shown). In the case of pool A-U4-1, the gel filtration chromatogram (Figure 3E) shows that some (82%) of the total radioactive pool was included (A-U4-1-2) and the remainder (18%) continued to elute in the void (A-U4-1-1). When pool A-U4-1-1 was incubated with the broader specificity neuraminidase (e.g., *A. ureafaciens*), all radiolabeled oligosaccharides coeluted with A-U4-1-2, at 5.3 gu. Desialylated pools A-U4-1-2 and the desialylated A-U4-1-1 were identified as galactose β1→4(fucose α1→3) *N*-acetylglucosamine β1→3 galactitol by sequential exoglycosidase analysis (not shown). It is therefore concluded that 82% of the lactosamine-bearing arms in A-U4 are capped by NeuNAcα2→3 and the remaining 18% by NeuNAcα2→6. The results of similar analysis for A-U6 and A-U8 are summarized in Table 3. To understand whether the presence of outer-arm α1→3-linked fucose correlated with one type of sialic acid linkage, A-U3 and A-U4 (structures where other lactosamine-bearing arms which might confuse the analysis are not present) were compared for sialic acid linkage (Figure 4). The fucosylated structure (A-U4) contains relatively more (82 ÷ 18 = 4.6) α2→3-linked sialic acid than their nonfucosylated equivalents [A-U3 (33 ÷ 67 = 0.49)], by a factor of almost 10 times. This finding may reflect either a difference in how the two sialyl transferases recognize fucosylated structures or a preference of the fucosyl transferase for sialic acid α2→3-containing substrates over α2→6 substrates.

Thus, the analysis of the protein-linked carbohydrates from cells that bind to E-selectin to those from cells that do not bind identifies three structures that are unique to the E-selectin-binding cells. These structures (A-U4, A-U6, A-U8),

Table 2: Analysis by Partial Acetolysis and MALD-MS of Oligosaccharide Alditols U3–U9

alditol[a] (gu)	observed change in hydrodynamic volume after acetolysis (gu)[b]	observed molecular mass of native alditol[b]
U3 (23.7)	7.1	2885.9
U4 (24.4)	6.8	3176.2
U5 (26.4)	10.2	3249.3
U6 (27.3)	10.1	3541.6
U7 (28.7)	10.3	3450.1
U8 (29.7)	9.8	3746.4
U9 (31.6)	12.7	3813.7

[a] Fractions for analysis were obtained by collecting *individual* fractions (not pools) across the chromatogram shown in Figure 1B. This maximized the purity of each fraction and was particularly important in collecting U3, U5, and U7. No fraction was completely pure, but each contained, as the major alditol, a structure of the hydrodynamic volume indicated. [b] Refers to the major alditol in each fraction.

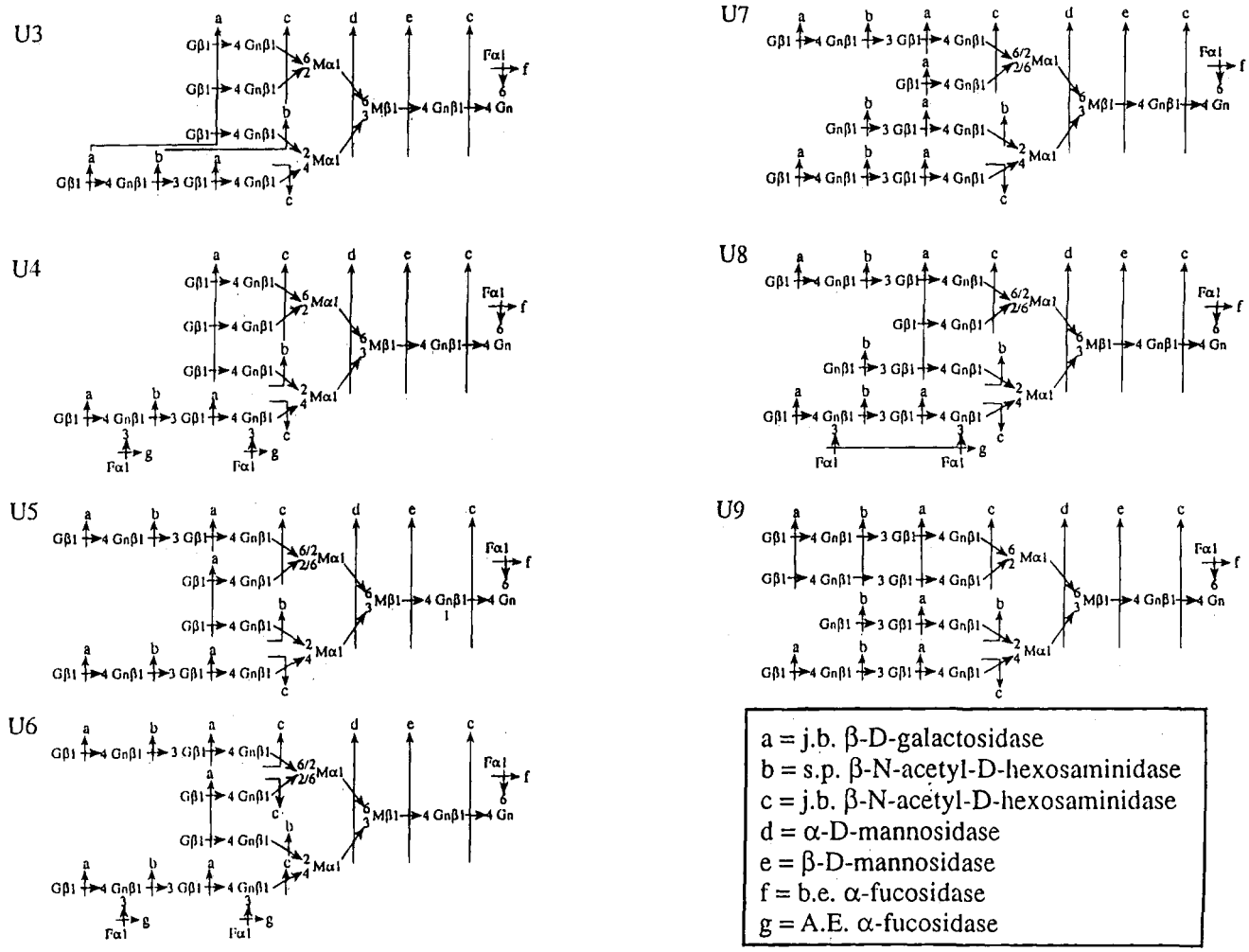

FIGURE 2: Structures of the desialylated oligosaccharides U3−U9. Structural analysis was performed on individual fractions by using a combination of sequential exoglycosidase analysis, MALD-MS (see Table 2), and partial acetolysis (see Table 2) and assuming conservation of the biosynthetic pathway for N-linked oligosaccharide in each cell line. Changes in the hydrodynamic volume of oligosaccharide structures were effected by exoglycosidases when used in the following order: U3, a−b−a−c−d−e−c−f; U4, a−b−c−g−a−b−a−c−d−e−c−f; U5, a−b−a−c−d−e−c−f; U6, a−b−c−a−c−g−a−b−a−c−d−e−c−f; U7, b−a−b−a−c−d−e−c−f; U8, b−a−b−c−a−c−g−a−b−a−c−d−e−c−f; U9, b−a−b−a−c−d−e−c−f. The deduced points of hydrolysis of each structure by individual exoglycosidases are indicated.

identified on U937 cells but also present on neutrophils and COS7.2 cells, are candidates for natural, high-affinity E-selectin ligands. They share a number of features including an SLex-related structure. Somewhat surprising, however, this is not simply SLex but a difucosylated version of SLex, diSLex. In addition the diSLex structure is always found on the C4 mannosyl arm of a tetraantennary structure.

Identification of Specific E-Selectin Binding Structures

Fractionation of the Total Oligosaccharide Alditol Pools Recovered from the U937 Cell Plasma Membranes Using rsE-Selectin Agarose. To provide direct evidence that these structures can bind to E-selectin and thus may represent naturally occurring E-selectin ligands, an affinity column was made with recombinant soluble E-selectin. An aliquot (estimated at ~2 nmol) of the total oligosaccharide alditol pool from the U937 cell plasma membrane was passaged through a column of rsE-selectin−agarose. Unbound and weakly retarded material was completely washed through in loading buffer, and bound alditols were then eluted using buffered EDTA. The elution chromatogram is shown in Figure 5A′. The retained oligosaccharide alditol fraction (~0.7% of the total) was desalted, and this fraction labeled "bound" was analyzed as described below. As a control, the U937 oligosaccharide pool was also passaged through a column to which BSA had been coupled. No detectable material was bound (not shown). An aliquot of the E-selectin-binding material from the U937 cells was desialylated by incubation with neuraminidase (e.g., *A. ureafaciens*) and the desialylated pool fractionated by P4 gel filtration chromatography (Figure 5B). Three alditol peaks are detected of elution volume 24.5, 27.2, and 29.6 gu. The identity of these peaks with U4, U6, and U8, respectively, was confirmed by sequential exoglycosidase digestion (not shown). The remainder of pool "bound" was fractionated into its (three) main components by HPAEC-PAD, and the sialic acid linkage to the arm carrying di-Lewis x was determined for each fraction (as described above for A-U4) by analyzing Lewis x-bearing monosialylated fragments released using endo-β-galactosidase. It was found that the di-Lewis x extension was capped only with NeuAcα2→3 in the three main components of pool "bound". From these results, it is concluded that the major oligosaccharide structures released from the U937 cell plasma membranes that are bound by rsE-selectin−agarose correspond to AU4, AU6, and AU8 (Figure 2) in which the lactosamine-bearing

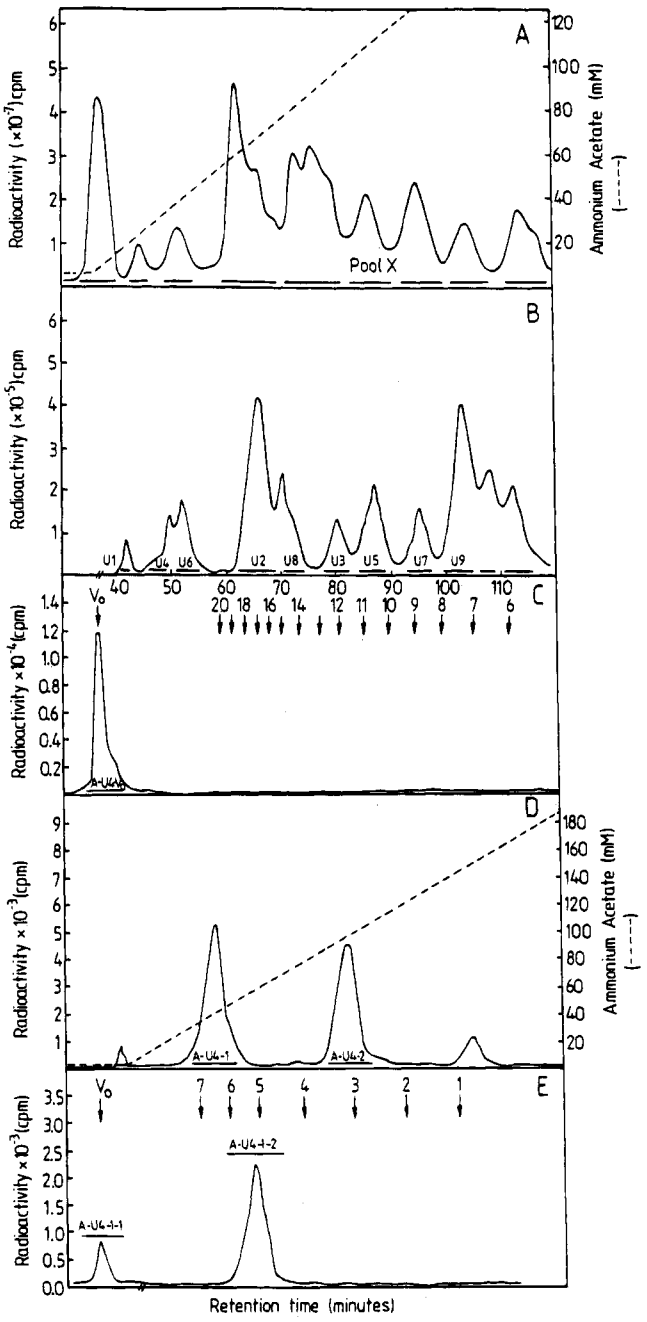

FIGURE 3: Aliquot of the total pool of oligosaccharide alditols from U937 separated by QAE-A25 anion-exchange chromatography (A), using a linear gradient of ammonium acetate. Pool X, shown separately to be particularly enriched in structures AU3−AU9 (inclusive), was further fractionated by HPAEC-PAD (B). Each fraction (B) was separately collected, desalted, and refractionated by HPAEC-PAD to homogeneity (not shown) and an aliquot desialylated and analyzed by gel filtration (not shown), so as to identify fractions AU3−AU9, inclusive. Analysis of each of AU3−AU9, inclusive, was performed in an analogous way, and data are therefore shown for AU4, by way of example. An aliquot of AU4 was incubated with endo-β-galactosidase, the products were radiolabeled and separated by gel filtration (C). The void fraction, A-U4-V_o (C), was fractionated by Mono Q anion-exchange chromatography (D). The two fractions, A-U4-1 and A-U4-2 (D), were individually incubated with α2→3 specific neuraminidase and again separated by gel filtration (data shown for A-U4-1) (E). For A-U4-1, the relative radioactivity included in the gel represents the fraction of lactosaminoglycan chains carrying α2→3NeuNAc and the relative radioactivity in the void represents the fraction of lacotsaminoglycan chains carrying α2→6NeuNAc.

arm is capped with NeuNAcα2→3. Authentic 3-SLex and 3-SLea were also passaged through the E-selectin affinity column, and under the chromatographic conditions used for the U937 membranes, neither showed any binding (Figure 5A″) indicating that A-U4, A-U6, and A-U8 bind E-selectin with higher affinity.

Table 3: Analysis of the Sialylation of Fucosylated Lactosaminoglycan Chains in Certain Sialylated Oligosaccharide Alditols Recovered from U937 Cells

	percent of di-Lewis x arms capped with	
fraction[a]	NeuNAcα2→3	NeuNAcα2→6
A-U4[b]	82	18
U-U6[c]	78	22
A-U8[c]	84	16

[a] Fractions were purified by repetitive use of a combination of QAE-A25 anion-exchange chromatography and HPAEC-PAD. [b] Contains lactosamine extensions with only outer-arm fucose. [c] Contains two types of lactosaminoglycan extensions, one with and one without outer-arm fucose.

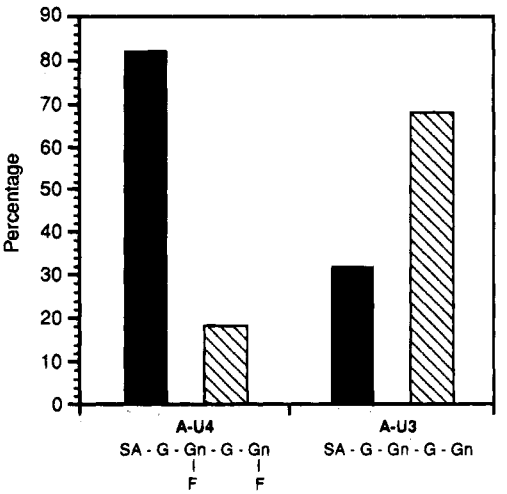

FIGURE 4: Representation of the relative percentage of lactosaminoglycan extensions capped with α2→3-linked sialic acid (solid bars) and α2→6-linked sialic acid (hatched bars) in the structures A-U3 and A-U4. Structure A-U3 carries a lactosaminoglycan chain with no outer-arm fucose. A-U4 carries a lactosaminoglycan extension with outer-arm fucose.

Fractionation of the total Oligosaccharide Alditol Pools Recovered from the Plasma Membranes of U937, COS7, COS7.2, and PMN Cells Using rsE-Selectin−Agarose. An aliquot of the total oligosaccharide alditol pool from the plasma membrane glycoproteins of PMN, COS7.2, and COS7 cells was fractionated using rsE-selectin−agarose exactly as described above for the oligosaccharide alditol pool from U937 cells. The relative percentages of bound oligosaccharide alditols is 0.6, 0.2, and nondetectable (<0.1%), respectively. Bound oligosaccharide alditol fractions in the pools from COS7.2 and PMN cells were analyzed exactly as described above for the fraction "U-bound" derived from U937 cells, and the results (not shown) indicate that the oligosaccharide alditols bound to rsE-selectin in both the COS7.2 and PMN-derived oligosaccharide pools correspond to AU4, AU6, and AU8 (Figure 2) in which the lactosamine-bearing arm is capped with NeuNAcα2→3. Additional minor components (corresponding to ~15% of the total rsE-selectin-bound fraction) in the U937-, COS7.2-, and PMN-derived oligosaccharide pools were not analyzed. Thus the major oligosaccharides on the plasma membrane of U937, COS7.2, and PMN cells that are bound most strongly to rsE-selectin are the three structures indicated in

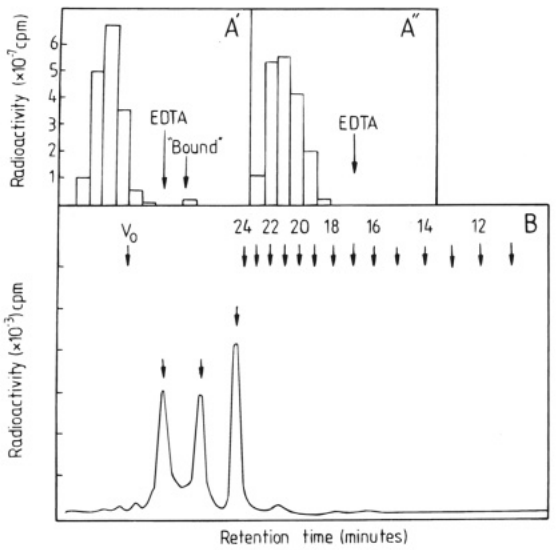

FIGURE 5: Fractionation (A′) and analysis (B) of the oligosaccharide alditols from the plasma membrane glycoproteins of U937 cells after affinity chromatography using rsE-selectin−agarose. An aliquot of the oligosaccharide alditol pool was passaged through an affinity column of rsE-selectin (A′). Bound alditols were eluted using EDTA. Authentic 3-SLex and 3-SLea were not retained by this affinity column (A″). An aliquot of the bound alditols from A′ was desialylated and fractionated by gel filtration (B).

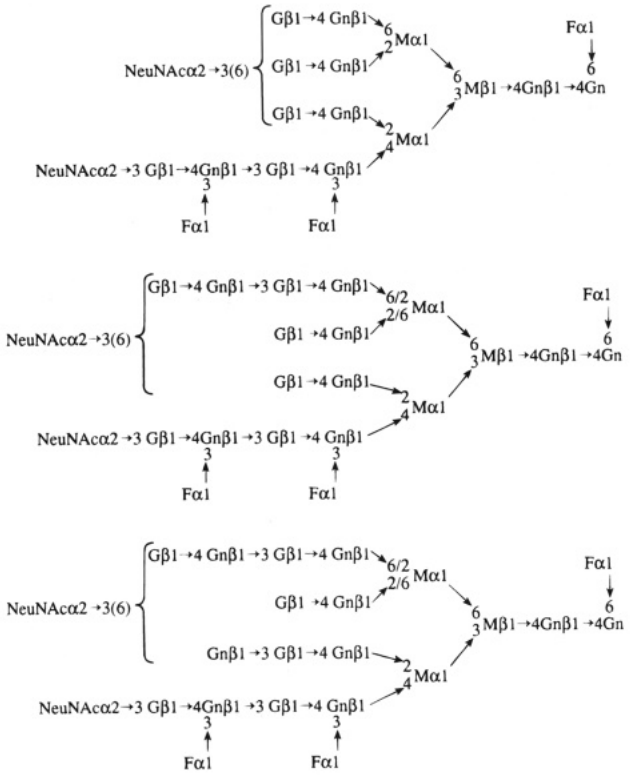

FIGURE 6: Summary of the oligosaccharide structures proposed to be ligands for E-selectin. Assignment of these structures as ligands for E-selectin is based on the association of these structures with cell lines that are able to bind E-selectin and on the binding of these structures to a column to rsE-selectin−agarose.

Figure 6. The oligosaccharides identified as binding to E-selectin all contain the trimannosyl chitobiosyl core that is characteristic of protein-associated *N*-glycans. They are, therefore, presumed to be naturally associated only with proteins. These results suggest that PMN, U937, and COS7 cells can synthesize the same basic complex carbohydrate

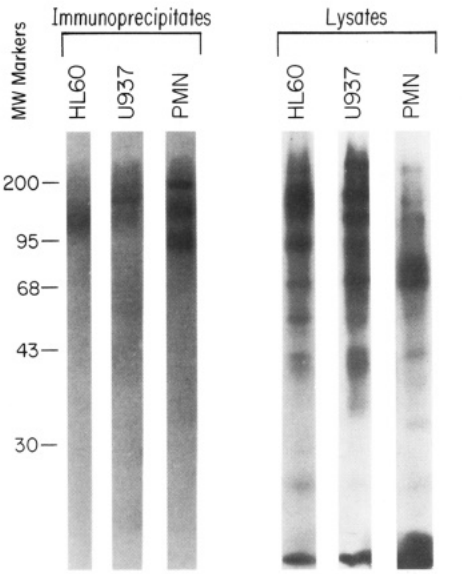

FIGURE 7: Immunoprecipitation of ^{125}I-labeled proteins from E-selectin-binding cells. C_2E_5, a monoclonal antibody that recognizes Lex-, Slex-, and diSlex-containing structures, was used to isolate glycoproteins from PMN, HL60, and U937 cells. The visualized proteins are cell surface proteins that have been labeled wtih ^{125}I. The autoradiogram shown is a 16 h exposure at −80 °C.

structures and that PMN and U937 cells possess a naturally expressed ELFT-like activity.

While it could be argued that the binding specificity of immobilized rsE-selectin differs from that of native E-selectin and that therefore the oligosaccharides isolated by affinity chromatography are not bound with high affinity by native E-selectin, we consider this unlikely for the following reasons: First, U937 cells bind similarly well to both cytokine-activated human endothelial cells and immobilized recombinant E-selectin (not shown), indicating a similarity in binding properties between the two forms of E-selectin. Second, the comparative analysis of carbohydrate libraries points to the identified oligosaccharides as being the only structures unambiguously common to and confined to E-selectin binding cells. Third, on both U937 cells and PMNs, Lewis x structures appear principally as part of diSlex. Fourth, the wider literature relating to immobilized and/or recombinant carbohydrate-binding proteins indicates that the essential binding specificities are generally retained.

Glycoproteins Containing DiSlex Oligosaccharides. Since the diSLex structures constitute less than 2% of the protein associated glycans from U937 cells (see Figure 1 and Table 1), it seemed likely that they might be expressed on only a small number of cell surface glycoproteins. To examine this possibility, we labeled the cell surface proteins from U937 cells, HL60 cells, and PNMs with ^{125}I and isolated diSlex-containing glycoproteins by immunoprecipitation with the monoclonal C_2E_5. A required part of the epitope for C_2E_5 is an outer-arm α1→3 fucose residue (Goelz et al., 1990) which, in U937 cells and PMNs, occurs principally in the form of diSLex. Furthermore, this antibody can inhibit the binding of HL60 cells, PMNs (Goelz et al., 1990), and U937 cells (unpublished observations) to both rsE-selectin and activated human endothelial cells. Figure 7 shows that relative to the unfractionated, labeled lysate few proteins are specifically precipitated from the E-selectin-binding cells HL60, U937, and PMNs by C_2E_5.

DISCUSSION

The two complementary strategies used here to identify endogenous high-affinity protein-associated carbohydrate ligands for E-selectin lead us to the following conclusions: (1) three related carbohydrate structures (Figure 6) constitute the set of endogenous high-affinity protein-associated carbohydrate ligands for E-selectin; (2) all possess an unusual 3-sialyl di-Lewis x extension on one arm of an N-linked tetraantennary glycan; (3) on the cells tested, all are present only on E-selectin-binding leukocytes and leukocytic cell lines; (4) all bind to E-selectin with a relatively high affinity (estimated $K_d < 1$ μM), greater than that of 3-sialyl Lewis x or 3-sialyl Lewis a; and (5) these structures represent a very small percentage of the protein-associated carbohydrate. Our data provide a critical piece of information on selectin/carbohydrate interactions that has to date been lacking, namely the identity of high-affinity endogenous carbohydrate ligands for E-selectin. We suggest that these carbohydrate structures are likely present on only a very small number of cell surface proteins and may alone be responsible for the specificity of E-selectin-dependent adhesion.

Our belief that the three identified carbohydrates have a high affinity for E-selectin is based on the observation that the rsE-selectin−agarose column retained these structures, but not SLex nor SLea, under the chromatographic conditions employed. These three carbohydrates were loaded onto the affinity column at a concentration of approximately 1.0 μM (1−2% of 4 μM) and, based on column size and flow rate, a crude estimated for the on-column K_d between these carbohydrates and E-selectin is <1 μM. If the significant difference in affinity between the carbohydrate structures reported here and 3-SLex and 3-SLea holds *in vivo*, it is very likely that the carbohydrates identified here would be physiologically relevant ligands for E-selectin.

The acidic carbohydrates defined to date as interacting with selectins are highly expressed on the surface of myeloid cells (Fukuda et al., 1984; Ohmori et al., 1989; Macher & Beckstead, 1990; Berg et al., 1991) and block adhesion *in vitro* with millimolar affinities (Phillips et al., 1990; Nelson et al., 1993). At the same time, recent studies have uncovered an apparently highly restricted set of high-affinity selectin glycoprotein ligands (Lasky, 1992; Moore et al., 1992; Baumhueter et al., 1993; Berg et al., 1993; Bevilacqua & Nelson, 1993; Levinovitz et al., 1993; Sako et al., 1993; Lenter et al., 1994). Several explanations for this apparent paradox have been suggested. Many of the well-characterized selectin ligands appear to be heavily O-glycosylated mucins, suggesting that adhesion occurs via multivalent expression of low-affinity carbohydrates on extended O-linked core structures (Lasky, 1992; Baumhueter et al., 1993; Berg et al., 1993; Norgard et al., 1993; Sako et al., 1993). However, studies on the P-selectin ligand PSGL-1 show that cotransfection of COS cells with a fucosyltransferase and alternative scaffolds, such as leukosialin/CD43, failed to generate P-selectin ligands under conditions where PSGL-1 was functional, despite expression of both CD43 and SLex (Sako et al., 1993). These results argue that the protein backbone, either indirectly by regulating the expression of specific carbohydrate structures or directly, plays a key role in defining PSGL-1 as a P-selectin ligand (Sako et al., 1993). These data also argue that multivalent expression of low-affinity carbohydrates is not sufficient to generate functional high-affinity selectin ligands. This result is consistent with a variety of fucosyltransferase transfection experiments, including those where cell lines with quite variable selectin adhesion were generated (Goelz et al., 1990, 1994; Lowe et al., 1990) and where poorly secreted selectin inhibitors built upon novel scaffolds were produced (Meier et al., 1993). One alternative explanation is that protein−protein as well as protein−carbohydrate interactions are critical to high-affinity binding (Kansas et al., 1994), providing a natural explanation for restricted ligand specificities. A third explanation relies on 3-dimensional arguments. Neutrophil but not lymphocyte L-selectin expresses SLex and has been shown to bind E-selectin *in vitro* (Picker et al., 1991b). Neutrophil L-selectin is found in a highly restricted distribution on cellular microvilli, the sites of initial cell contact, and it has been argued that the ability of SLex on L-selectin, rather than other glycoprotein scaffolds, to interact with E- and P-selectin derives from this positional information (Picker et al., 1991b).

These explanations implicitly assume weak carbohydrate/selectin interactions, and we can now offer an alternative hypothesis. We show that in fact unique carbohydrate structures exist on myeloid cells which bind with high affinity to E-selectin even when monovalent, under conditions where SLex itself does not bind. Therefore a single such structure expressed on a cell surface glycoprotein could provide sufficient affinity to bind E-selectin, without requirement for protein interactions. The expression of specific carbohydrate structures or glycoforms on cell surface glycoproteins is known to be both cell type specific and dependent upon the protein scaffold, which subtly alters glycosylation patterns in the endoplasmic reticulum (Parekh et al., 1989b; Cumming, 1991). Therefore we propose that a highly restricted set of glycoprotein ligands for E-selectin derives from specific structural features important for directing the construction of high-affinity glycoforms during intracellular carbohydrate processing. In addition, protein−protein interactions may play an important role in the binding of selectins to their respective ligands (Kansas et al., 1994). This hypothesis is not mutually exclusive with those described above and needs to be further explored.

Sulfofucosyl oligosaccharide ligands for E-selectin have been isolated from an ovarian cystadenoma protein (Yuen et al., 1992). Our data suggest that sulfated carbohydrates are not endogenous protein-associated high-affinity ligands for E-selectin, at least on myeloid cells, as sulfated monosaccharides would have been recovered intact during the analytical process adopted here. This is consistent with most data on selectin/carbohydrate interactions, which suggest sulfated carbohydrates are important only in L-selectin interactions with high endothelial venules in peripheral nodes (Lasky, 1992). However, the experimental approach adopted here was designed specifically to detect N-linked or O-linked protein-associated carbohydrate ligands and would not have led to the discovery of glycolipid- or proteoglycan-associated ligands, which may be sulfated (Aruffo et al., 1991; Needham & Schnaar, 1993).

Although SLex itself is a low-affinity ligand for selectins, SLex-based carbohydrate structures of higher affinity have been synthesized, and extension of the SLex structure at its reducing terminus with an aliphatic C8 carbon chain provides significantly increased affinity (Nelson et al., 1993). Such structures may mimic the extended diSLex structure we describe here and suggest that selectins may have an extended

groove or binding pocket capable of multiple interaction points. Analysis of the crystal structure of E-selectin reveals a potential binding domain (somewhat more like a plateau than a pocket) for SLex that could also accommodate diSLex (Graves et al., 1994) (B. Graves, personal communication). The use of the diSLex structure may have significant advantages over SLex itself in examining E-selectin-binding interactions, including use in crystallographic approaches.

In summary, we have demonstrated the feasibility both of comparative carbohydrate analysis and of selectin affinity chromatography in the characterization of specific glycoforms important in mammalian lectin/carbohydrate interactions. Our results support the view that physiological interactions between native carbohydrates and carbohydrate-binding proteins can be selective and of high affinity. Importantly, selectin-based affinity fractionation of total cell carbohydrates should be a method of broad applicability. For example, this technique should prove valuable in comparing high-affinity carbohydrates on cells which bind different selectins (e.g., neutrophil or HL60 binding to E- and P-selectin) and on different cells which bind the same selectin (e.g., neutrophil and CLA^+ T lymphocyte binding to E-selectin). The results of such experiments, combined with an analysis of glycoforms present on already characterized mucin-like selectin ligands, should provide considerable insight into the complexities of selectin/ligand interactions.

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