Novel Sulfated Ligands for the Cell Adhesion Molecule E-Selectin Revealed by the Neoglycolipid Technology among O-Linked Oligosaccharides on an Ovarian Cystadenoma Glycoprotein†

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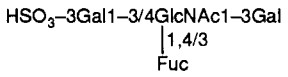
ABSTRACT: E-selectin is the inducible adhesion protein on the surface of endothelial cells which has a crucial role in the initial stages of recruitment of leucocytes to sites of inflammation. In addition, it is almost certainly involved in tumor cell adhesion and metastasis. This report is concerned with identification of a new class of oligosaccharide ligand—sulfate-containing—for the human E-selectin molecule from among oligosaccharides on an ovarian cystadenoma glycoprotein. This has been achieved by application of the neoglycolipid technology to oligosaccharides released from the glycoprotein by mild alkaline β-elimination. Oligosaccharides were conjugated to lipid, resolved by thin-layer chromatography, and tested for binding by Chinese hamster ovary cells which had been transfected to express the full-length E-selectin molecule. Several components with strong E-selectin binding activity were revealed among acidic oligosaccharides. The smallest among these was identified by liquid secondary ion mass spectrometric analysis of the neoglycolipid, in conjunction with methylation analysis of the purified oligosaccharide preparation as an equimolar mixture of the Le^a- and Le^x/SSEA-1-type fucotetrasaccharides sulfated at position 3 of outer galactose:



To our knowledge this is the first report of a sulfofucooligosaccharide ligand for E-selectin. The binding activity is substantially greater than those of lipid-linked Le^a and Le^x/SSEA-1 sequences and is at least equal to that of the 3′-sialyl-Le^x/SSEA-1 glycolipid analogue.

The inducible endothelial adhesion molecule E-selectin (formerly termed ELAM-1)[1] has an established role in the regulation of leucocyte recruitment to inflammatory sites (Bevilacqua et al., 1989). E-selectin binding to human tumor cell lines has also been documented (Rice et al., 1989), raising the possibility of the involvement of this adhesion molecule in tumor cell adhesion and metastasis in vivo. E-selectin is a modular transmembrane protein with an N-terminal domain (Bevilacqua et al., 1989) similar in amino acid sequence to those of known Ca^{2+}-dependent carbohydrate-binding domains (Drickamer, 1988). E-selectin clearly functions as a carbohydrate-binding protein, and it recognizes oligosaccharides terminating in the 3′-fucosyl-*N*-acetyllactosamine sequence (Le^x/SSEA-1 antigen) or the 4′-fucosyl isomer (Le^a antigen) that bear sialic acid 3-linked to galactose. These conclusions have been reached as a result of multiple experimental approaches: (i) monitoring carbohydrate expression on cells that adhere to E-selectin using monoclonal antibodies to specific carbohydrate sequences or inhibiting adherence with the antibodies (Lowe et al., 1990; Phillips et al., 1990; Walz et al., 1990), (ii) comparing E-selectin binding to Chinese hamster ovary (CHO) cells and to glycosylation mutants which differ in their expression of α1–3 fucosyltransferase products (Phillips et al., 1990), (iii) transfecting specific fucosyltransferases into cell lines that lack these normally and testing the transfected cells for E-selectin binding (Lowe et al., 1990), (iv) evaluating several structurally defined glycolipids and oligosaccharides as inhibitors of E-selectin binding to myeloid cells (Phillips et al., 1990; Takada et al., 1991; Tyrrell et al., 1991), and (v) performing E-selectin binding experiments with certain glycolipids extracted from myelogenous leukemia cells (Tiemeyer et al., 1991) and with a series of structurally defined fucooligosaccharides conjugated to protein (Berg et al., 1991) or to lipid (neoglycolipids) (Larkin et al., 1992). The binding studies with neoglycolipids have shown that E-selectin binds also to oligosaccharides terminating with nonsialylated Le^a and Le^x sequences and that 10 times or greater amounts of the Le^a sequence and even greater amounts of Le^x sequence are required to give binding intensities equivalent to those observed with the sialylated analogues (Larkin et al., 1992). A special feature of the neoglycolipid technology is that it is

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[1] Abbreviations: BSA, bovine serum albumin; BSA-α, α medium containing 5% w/v bovine serum albumin; CHO, Chinese hamster ovary; DHPE, L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; DPPE, L-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; ELAM-1, endothelial leucocyte adhesion molecule 1; dHex, deoxyhexose; GC-MS, gas chromatography–mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; LNT, lacto-*N*-tetraose; LNFP-II, lacto-*N*-fucopentaose II; LNFP-III, lacto-*N*-fucopentaose III; LNFT-Cer, lacto-*N*-fucotetraose ceramide; Le^a, blood group Lewis^a antigen; Le^x, Lewis^x antigen; LSIMS, liquid secondary ion mass spectrometry; PBS, 10 mM phosphate-buffered saline, pH 7.4; SSEA-1, stage-specific embryonic antigen; TLC, thin-layer chromatography.

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applicable to hitherto uncharacterized oligosaccharide mixtures released from glycoproteins (Tang et al., 1985; Tang & Feizi, 1986; Stoll et al., 1988; Feizi, 1989a). The resulting neoglycolipids may be resolved on silica gel chromatograms, for example, and overlaid with whole cells or soluble proteins in order to identify individual components bound. The excellent ionization property of neoglycolipids in mass spectrometry allows concomitant determination of the molecular weights and monosaccharide sequences of the oligosaccharide moieties directly from the chromatogram surface (Lawson et al., 1990). In the present study this approach has been made to characterize the minimum E-selectin-binding oligosaccharide fraction among acidic oligosaccharides released by β-elimination from a glycoprotein derived from a human ovarian cystadenoma. A novel class of oligosaccharide ligand is thus revealed that is sulfated rather than sialylated.

MATERIALS AND METHODS

Glycoproteins and Oligosaccharides. A mucin-type glycoprotein fraction, isolated (Pusztai & Morgan, 1961) by phenol extraction [phenol-insoluble fraction (Morgan, 1967)] from the cyst fluid of a pseudomucinous ovarian cystadenoma of a blood group A_1 Le^a person, was a gift from Dr. Winifred M. Watkins (Royal Postgraduate Medical School, London). This glycoprotein preparation, designated cyst 350 glycoprotein, had been noted (Pusztai & Morgan, 1961) to have blood group Le^a activity and to contain 18% sialic acid and receptor activity for influenza hemagglutinins types A and B. Hog gastric mucin was from Sigma Chemical Co., Poole, Dorset, U.K.

Lacto-*N*-tetraose (LNT), Galβ1–3GlcNAcβ1–3Galβ1–4Glc, and lacto-*N*-fucopentose II (LNFP-II), Galβ1–3(Fucα1–4)GlcNAcβ1–3Galβ1–4Glc, were gifts from the late Dr. A. S. R. Donald (Research Sugars, Aylesbury, Bucks); the sulfated oligosaccharide HSO_3-6GlcNAcβ1–3Gal was isolated from keratan sulfate (Scudder et al., 1986), and the phosphated oligosaccharide H_2PO_3–6Manα1–3Manα1–3Manα1–3Manα1–2Man was isolated (Bretthauer et al., 1973) by Paula Green (Glycoconjugates Section, Clinical Research Centre, Harrow, U.K.) from *Hansenula holstii* phosphomannan (a gift from Dr. M. Slodki, Agricultural Research Service, Peoria, IL). Maltopentaose was from Sigma.

O-Linked oligosaccharides were released from cyst 350 glycoprotein by a mild alkaline β-elimination procedure (W. Chai, T. Feizi, and A. M. Lawson, unpublished) which will be described in detail elsewhere. In brief, the glycoprotein (1.5 g) was treated at 65 °C with 150 mL of 70% w/v ethylamine for 6 h, desalted with the cation-exchange resin AG50W X12 (hydrogen form), deproteinated by passing over C18 SepPak cartridges (Waters, Milford, MA), and chromatographed on a column of Bio-Gel P6 (1.5 × 100 cm) in 10 mM ammonium acetate buffer, pH 5.0. The included fractions from the Bio-Gel P6 column, 84.9 mg of total hexose (assayed by orcinol staining of aliquots applied onto a silica gel plate using galactose as standard), and 86.9 mg of sialic acid (assayed by resorcinol staining on a silica gel plate using *N*-acetylneuraminic acid as standard) were fractionated by DEAE A25 Sephadex chromatography into a neutral and an acidic fraction, 22 and 54.2 mg of hexose, respectively. The neutral fraction was not investigated further. The acidic oligosaccharide fraction was rechromatographed on a DEAE A25 Sephadex column. A major oligosaccharide peak (fraction A), 2.1 mg of hexose, eluting with 0.1 M ammonium acetate buffer, pH 5.0, was desalted with AG50W X12 and chromatographed on a Bio-Gel P4 column (1.5 × 100 cm) in 10 mM ammonium acetate, pH 5.0, and separated into four subfractions, A1–A4, with elution positions corresponding to 11–15, 8–11, 5–8, and 2–5 hexose units, respectively. Aliquots of the fractions were converted to neoglycolipids (see below) and resolved on aluminum-backed, silica gel 60 high-performance TLC plates (Merck, from BDH, Poole, Dorset, U.K.), using the solvent system chloroform/methanol/water, 60/35/8 by volume. Chemical detection of neoglycolipids was with primulin or orcinol stains. The oligosaccharide fraction A4 giving rise to the fastest migrating neoglycolipid with E-selectin binding (see Results) was further fractionated by HPLC on a TSK gel amide-80 column, 4.6 mm × 25 cm (Anachem, Luton, U.K.), in 20 mM sodium acetate, pH 5.3. The E-selectin-binding component (designated A4E) amounted to 70 μg measured as hexose. An aliquot of the A4E oligosaccharide preparation (approximately 3 μg of hexose) was digested with 10 milliunits of *Arthrobacter ureafaciens* sialidase (Nakarai Chemicals, Kyoto, Japan) in 10 mM ammonium acetate buffer, pH 5, and desalted with ion-exchange resins AG50W X12 and AG3-4A. A further aliquot (15 μg of hexose) was subjected to acid treatment in 0.1 M trichloroacetic acid for 2 h at 100 °C and taken to dryness under a stream of nitrogen; following addition of methanol, the material was reevaporated three times further.

Lipid-Linked Oligosaccharides. Neoglycolipids were prepared (Stoll et al., 1988) by conjugation of oligosaccharides to L-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) from Sigma, unless otherwise stated. Oligosaccharide A4E was also conjugated to L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) from Fluka Chemical Ltd., Glossop, Derbyshire, U.K. (Pohlentz et al., 1992). E-selectin-binding activities of the derivatives using DPPE and DHPE are identical (unpublished observations); mobility on TLC is slightly faster with the DHPE derivative. For mild acid treatment of the neoglycolipids derived from fraction A4E, an aliquot of the oligosaccharide preparation (15 μg of hexose) was conjugated to DHPE. Unlike DPPE, which tends to lose the acyl chains upon acid treatment, DHPE is unaffected (unpublished observations); the neoglycolipid was isolated from the conjugation mixture by preparative thin-layer chromatography, heated in 0.02 M sulfuric acid for 30 min at 100 °C, neutralized with 0.2 M ammonium hydroxide, and dried under nitrogen.

The glycosphingolipids 3′-sialyllacto-*N*-fucotetraose ceramide, NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAcβ1–3Gal–Cer (3′-sialyl-LNFT-Cer), and 3′-sialyllacto-*N*-fucopentaose III ceramide, NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAcβ1–3Galβ1–4Glc–Cer (3′-sialyl-LNFP-III-Cer), were synthesized chemically (Hasegawa et al., 1992; Kameyama et al., 1991) and were gifts of Drs. A. Hasegawa and M. Kiso.

Quantitations for matching the A4E neoglycolipid and 3′-sialyl-LNFT-Cer and 3′-sialyl-LNFP-III-Cer bands on thin-layer chromatograms, with respect to picomoles used in E-selectin binding assays, were made by orcinol staining, using maltopentaose neoglycolipid as a standard. Orcinol does not stain sialic acid or *N*-acetylhexosamines; orcinol stains fucose with approximately 0.8 the intensity of galactose and glucose in this assay. In separate experiments (unpublished) it has been established that intensities of orcinol staining of the hexoses in this neoglycolipid and the glycolipids are equivalent. Also, galactose and galactose-3-sulfate attached to ceramide stain equivalently with orcinol, as does galactose on a lactose and a 3′-sialyllactose neoglycolipid.

Cells. CHO cells (designated SC2) expressing full-length recombinant E-selectin in high density (Larsen et al., 1992;

Larkin et al., 1992) were cultured at 37 °C in an atmosphere of 50% CO_2 in air in MEM α medium (Gibco Ltd., Uxbridge, U.K.) containing 10% dialyzed fetal calf serum, 10 mM L-glutamine, 60 μg/mL benzylpenicillin, 100 μg/mL streptomycin, 10 mM sodium bicarbonate, and 25 nM methotrexate. The culture medium for nontransfected CHO cells, designated DUKX, contained in addition adenosine, deoxyadenosine, and thymidine, 10 μg/mL of each, but not methotrexate.

E-Selectin Binding Assays. E-selectin binding was detected by nitrocellulose overlay or chromatogram overlay assays (Larkin et al., 1992) using [^{3}H]thymidine-labeled SC2 cells and, as a negative control, the nontransfected DUKX cells. Specific radioactivities were approximately 10 μCi/cell. In brief, serial dilutions of glycoproteins were applied onto a nitrocellulose sheet as 1-μL spots, 1–100 ng applied per spot; lipid-linked oligosaccharides (amounts are indicated in individual experiments) were resolved on silica gel chromatograms, and the air-dried chromatograms were then treated with 0.001% Plexigum P28 (Cornelius Chemical Co., Romford, U.K.) in *n*-hexane. Thereafter, the nitrocellulose sheet or chromatograms were equilibrated in α medium containing 5% w/v bovine serum albumin (BSA-α) and overlaid with ^{3}H-labeled cell suspensions, 5×10^5 cells in BSA-α, approximately 100 μL/cm^2, and incubated at 20 °C for 15 min in a covered chamber. Unbound cells were removed by washing with 10 mM phosphate-buffered saline, pH 7.4 (PBS), containing 0.5 mM Ca^{2+} and 0.4 mM Mg^{2+}, avoiding sheer forces (Larkin et al., 1992) at the nitrocellulose or chromatogram surface. Bound cells were fixed in the presence of glutaraldehyde, 1.5% v/v in PBS, and binding was detected by fluorography. In this assay system, using 100 and 10 pmol of lipid-linked oligosaccharides, equivalent binding intensities are given by the 3′-sialyl-LNFP-II neoglycolipid, the 3′-sialyl-LNFP-III-Cer, and the 3′-sialyl-LNFP-III neoglycolipid, while binding to 3′-sialyl-LNFT-Cer is weaker (Larkin et al., 1992; C-T. Yuen, A. Hasegawa, M. Kiso, T. J. Ahern, and T. Feizi, unpublished observations[2]).

For antibody inhibition experiments, the binding assays were performed with SC2 cells suspended in BSA-α medium containing an E-selectin blocking antibody, HEL 3/2 (IgG class), at 20 μg/mL or an irrelevant IgG antibody as described (Larkin et al., 1992).

Liquid Secondary Ion Mass Spectrometry. Neoglycolipid bands were analyzed directly from the chromatogram surface by liquid secondary ion mass spectrometry (LSIMS) as described previously (Lawson et al., 1990). A phosphated and a sulfated oligosaccharide were selected as model compounds in order to identify distinctive features in the fragmentation of their neoglycolipid derivatives, knowing that diagnostic features have been observed for sulfated and phosphated peptides (Gibson & Cohen, 1990), reflecting the relative stability of the phosphate linkage under conditions of LSIMS. For the neoglycolipid of the phosphated oligosaccharide H_2PO_3–6Manα1–3Manα1–3Manα1–3Manα1–2Man, the quasimolecular ions $[M - H]^-$ and $[MH]^+$ were the main peaks, and the respective dephosphated fragment ions $[M - H_2PO_3]^-$ and $[MH - HPO_3]^+$ were both of low abundance, less than 5%. For the neoglycolipid of the sulfated oligosaccharide HSO_3–6GlcNAcβ1–3Gal, when examined in the negative ion mode, the quasimolecular ion $[M - H]^-$ was the main peak in the spectrum, and the relative intensity of the desulfated fragment ion $[MNa - HSO_3]^-$ was 20%. In the positive ion mode the main peak in the spectrum was the desulfated fragment ion $[M - NaSO_3 + H]^+$, the quasimolecular ion $[MH]^+$ was absent, and the relative intensity of the sodium adduct ion $[MNa]^+$ was only 35%.

Methylation Analysis. For methylation analysis of oligosaccharides, permethylation (Ciucanu & Kerek, 1984), hydrolysis, reduction, acetylation, and GC–MS analysis were performed as described previously (Scudder et al., 1987).

Molecular Modeling. The minimum energy conformation for each glycosidic linkage in the Le^x trisaccharide [Galβ1–4(Fucα1–3)GlcNAc] and the 3′-sialyl-Le^x tetrasaccharide [NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc] was calculated using the HSEA potential in the program CCM (Thørgensen et al., 1982). For modeling of the 3-sulfated Le^x trisaccharide [HSO_3–3Galβ1–4(Fucα1–3)GlcNAc] the requisite bond lengths, valence angles, and torsion angles of the sulfate group were from crystallographic data (Kanters et al., 1991), with subsequent adjustment of the torsion angles (O–S–O–C3, S–O–C3–C4). Carbohydrate coordinates were imported for display into the modeling package Quanta using a CCM to Quanta conversion routine (A. C. Stuart, unpublished).

RESULTS

In initial experiments the E-selectin-expressing SC2 cells were found to bind strongly to cyst 350 glycoprotein immobilized on nitrocellulose; binding was detected to as little as 3 ng of this glycoprotein, but there was no binding to the immobilized hog gastric mucin at the highest level tested, 100 ng (not shown).

Isolation of Oligosaccharide Component A4E with E-Selectin Binding Activity. Neoglycolipids derived from the acidic oligosaccharide fractions A1–A4 were resolved by TLC and tested for E-selectin binding. SC2 cell binding was observed to one or more components in each of these neoglycolipid fractions (Figure 1A, panel b, lanes 1–4). No binding was detected when DUKX cells were used (not shown). Oligosaccharide fraction A4 gave the fastest migrating neoglycolipid band with SC2 cell binding activity, and judging from the intensities of binding in relation to lipid staining with primulin (lanes 4 and 5 in Figure 1A, panels a and b), this neoglycolipid component was substantially more active per mole of neoglycolipid than the Le^a-active LNFP-II neoglycolipid which was used as a standard. From fraction A4, the component with SC2 cell binding activity was isolated by HPLC and designated A4E. As predicted for E-selectin binding, SC2 cell binding to A4E neoglycolipid was cation-dependent; it was abolished when 10 mM EGTA was included in the SC2 cell suspension; in addition, the binding was inhibited specifically in the presence of anti-E-selectin antibody HEL 3/2 but not an irrelevant IgG antibody (not shown).

Identification of A4E as a Sulfated Fucotetrasaccharide by LSIMS Analysis of the Neoglycolipid. The acidic oligosaccharide component A4E with E-selectin binding activity was identified as a tetrasaccharide by negative ion LSIMS analysis of the neoglycolipid (Figure 2A). The ions at m/z 1445 and 1467 could be assigned as the quasimolecular ion $[M - H]^-$ and the sodium adduct ion $[M - 2H + Na]^-$, respectively, of a lipid-linked oligosaccharide with the composition deoxyhexose–hexose$_2$–*N*-acetylhexosamine (dHex–Hex$_2$–HexNAc) and an additional 80-Da equivalent to either a sulfate or phosphate group. The negative ion mass spectrum of the free oligosaccharide, $[M - H]^-$ at m/z 770 (data not shown), was consistent with this composition. The ion at m/z 1365, in the neoglycolipid spectrum in the negative mode (Figure 2A), could have arisen from (a) a phosphated

[2] Presented at Keystone Meeting Glycobiology II, March 1992.

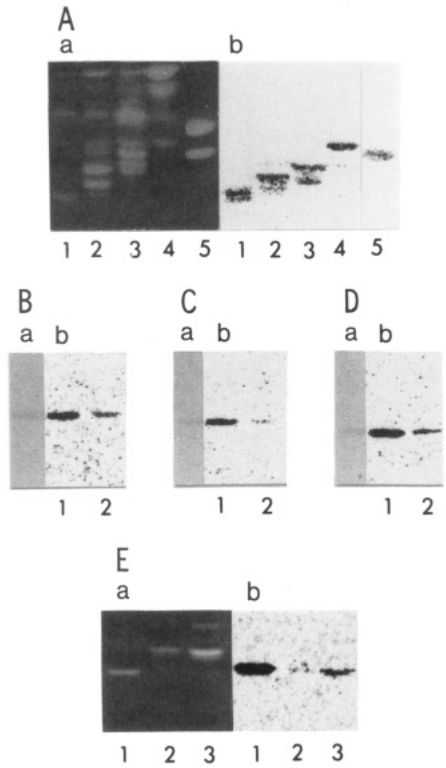

FIGURE 1: Chromatogram overlay assays showing E-selectin (SC2 cell) binding to lipid-linked oligosaccharides. The lipid-linked oligosaccharides were chromatographed on silica gel plates and revealed chemically (parts a) with primulin (panels A and E) or orcinol (panels B–D) or they were overlaid with ^{3}H-labeled SC2 cells as described under Materials and Methods, and binding was detected by fluorography (parts b, panels A–E). In panel A, lanes 1–4 contained mixtures of neoglycolipids made from oligosaccharide fractions A1–A4, respectively; lane 5 contained neoglycolipid standards derived from LNT (upper band) and LNFP-II (lower band), 1 nmol of each. In panels B–D parts a contained A4E neoglycolipid, 3′-sialyl-LNFT-Cer, and 3′-sialyl-LNFP-III-Cer at 110, 110, and 75 pmol, respectively; in parts b, lanes 1 contained 10 pmol and lanes 2 contained 5 pmol of the three lipid-linked oligosaccharides, respectively. In panel E, lanes 1 contained the A4E neoglycolipid, 100 pmol; lanes 2 and 3 contained mild-acid-treated (defucosylated) A4E neoglycolipid, 100 and 300 pmol, respectively; the presence of a trace amount of the original fucosylated neoglycolipid remaining after acid treatment is indicated by the SC2 cell binding in part b, lanes 2 and 3. The neoglycolipids in panels B and E were made with DHPE. Chromatography was upward. Panel A, panels B–D, and panel E show results of three separate experiments.

oligosaccharide by elimination of hydrogen phosphite from the $[M-H]^-$ ion species or from fragmentation of the sodiated molecular ions or (b) a sulfated oligosaccharide by elimination of sulfite from the $[M - H]^-$ species or by loss of sodium sulfite from the sodiated molecular ion with hydrogen rearrangement. The fragmentations of the molecule in both the positive and negative ion modes were consistent with those of a sulfated oligosaccharide (see Materials and Methods): first, in the positive mode (Figure 2B), an ion corresponding to the quasimolecular ion $[MH]^+$ was lacking, and the sodium adduct ion $[MNa]^+$ at m/z 1469 had an intensity only 25% of that of the ion at m/z 1367 which could be assigned as the desulfated sodium adduct ion $[MNa - NaSO_3 + H]^+$; second, in the negative mode (Figure 2A), the fragment ion at m/z 1365, with an intensity 30% of that of the quasimolecular ion $[M - H]^-$, indicated loss of sulfite.

Information on the monosaccharide sequence in tetrasaccharide A4E was obtained from the additional fragment ions in the negative ion spectrum of the neoglycolipid derivative (Figure 2A). Fragment ions from glycosidic cleavage, equivalent to loss from m/z 1365 of hexose (m/z 1203), deoxyhexose (m/z 1219), and hexose–deoxyhexose–*N*-acetylhexosamine (m/z 854), together with their satellite ions from associated cleavages (Lawson et al., 1990) indicated a branched oligosaccharide sequence Hex–(dHex)HexNAc–Hex. The single ion at m/z 1023 (180 Da less than m/z 1203) is characteristic of a dHex branch [unpublished observations; see also Lawson et al. (1990)]. Results with mild-acid-treated neoglycolipid (DHPE derivative), where the lipid contributes 661 Da, indicated that only the deoxyhexose residue had been removed: $[M - H]^-$ and $[M - HSO_3]^-$ for the untreated neoglycolipid were m/z 1417 and 1337, respectively, and for the acid-treated neoglycolipid, m/z 1271 and 1191, respectively (spectra not shown).

Monosaccharide and Linkage Assignments for A4E by Methylation Analysis. Monosaccharide composition and linkage information on tetrasaccharide A4E were obtained by methylation analysis of the free oligosaccharide preparation and of the acid-treated preparation. Results with the untreated oligosaccharide showed the presence of Fuc1–, –3Gal1–, and –3,4GlcNAc1– [partially methylated alditol acetates 2,4,6-tri-*O*-methylfucitol, 2,4,6-tri-*O*-methylgalactitol, and 6-mono-*O*-methyl-2-(*N*-methylacetamido)glucitol, respectively] in the approximate ratio of 1:2:1. Together with the sequence information obtained above, these results indicated the sequence HSO_3–3Gal1–3(or 4)(Fuc1–3(or 4))GlcNAc1–3Gal.

Results with the acid-treated oligosaccharide preparation showed a major product lacking fucose, the monosaccharide linkages detected being –3Gal1–, –3GlcNAc1–, and –4GlcNAc1– [partially methylated alditol acetates 2,4,6-tri-*O*-methylgalactitol, 3,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol, and 4,6-di-*O*-methyl-2-(*N*-methylacetamido)glucitol, respectively] in the approximate ratio of 4:1:1. A minor product was also isolated lacking fucose and containing a terminal galactose rather than a 3-substituted galactose, indicating the additional loss of the 3-linked sulfate. From these results, and the absence of an *N*-acetylgalactosamine residue at the reducing end, the oligosaccharide preparation A4E is identified as an equimolar mixture of the 3-sulfated Le^a- and Le^x/SSEA-1-type, β-eliminated and peeled products:

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HSO3-3Gal1-3GlcNAc1-3Gal        HSO3-3Gal1-4GlcNAc1-3Gal
           |1,4                            |1,3
           Fuc                             Fuc
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An E-Selectin Binding Activity in A4E Neoglycolipid at Least Equal to That of the 3′-Sialyl-Le^x/SSEA-1 Glycolipid Analogue. A comparison was made of the intensities of E-selectin binding to A4E neoglycolipid and to the 3′-sialyl-Le^x/SSEA-1 glycolipid analogue with a trisaccharide backbone, 3′-sialyl-LNFT-Cer, over a range of glycolipid levels (30–2 pmol). The intensities of binding of these two lipid-linked oligosaccharides were comparable overall; but near the limits of detectable binding, for example, at 10 and 5 pmol, the intensities of binding to A4E neoglycolipid were greater than to 3′-sialyl-LNFT-Cer (cf. Figure 1B,C) and were close to those observed with 3′-sialyl-LNFP-III-Cer (Figure 1D) which has a tetrasaccharide backbone and elicits stronger E-selectin binding[2] than 3′-sialyl-LNFT-Cer. Neither the chromatographic mobility nor the E-selectin binding activity of A4E neoglycolipid was affected by sialidase treatment of A4E oligosaccharide (not shown).

Requirement for the Fucose in A4E Neoglycolipid for E-Selectin Binding. The E-selectin binding was abolished, however (Figure 1E), after mild acid treatment of A4E neoglycolipid under conditions which removed fucose residues

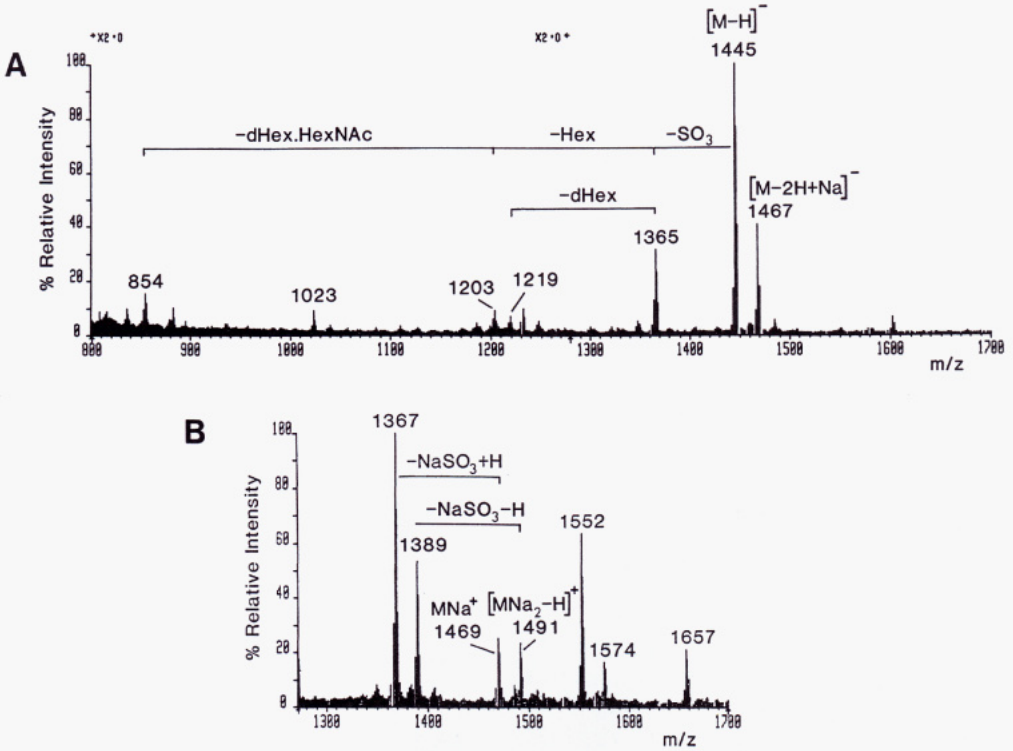

FIGURE 2: Liquid secondary ion mass spectra for the neoglycolipid derivative of the E-selectin binding oligosaccharide preparation, A4E. The negative ion mass spectrum is shown in panel A. The quasimolecular ion $[M - H]^-$ and the sodium adduct species $[M - 2H + Na]^-$ are indicated as well as fragment ions. All ions contain the DPPE moiety which contributes 689 Da. The presence of a sulfate rather than a phosphate substituent is deduced by comparing the relative intensities of (a) ions in the molecular ion region in the negative ion mode shown here, (b) ions in the positive ion mode shown in panel B, and (c) ions from known sulfate-containing or phosphate-containing oligosaccharides (see Materials and Methods). The molecular ion region of the positive ion mass spectrum is shown in panel B. Sodium-cationized quasimolecular ions ($[MNa]^+$ and $[MNa_2 - H]^+$) and fragment ions are indicated. The ions at m/z 1552, 1574, and 1657 are matrix (diethanolamine, DEA) adduct ions ($[MH + DEA]^+$, $[MNa + DEA]^+$, and $[MH + 2DEA]^+$).

as shown by LSIMS analysis of the neoglycolipid (as described above). Thus, the 3-sulfated oligosaccharide backbone cannot support E-selectin binding in the absence of fucosylation at *N*-acetylglucosamine.

Molecular Modeling of 3-Sulfated Le^x and 3′-Sialyl-Le^x. Molecular modeling studies of the 3-sulfated Le^x sequence, HSO_3–Galβ1–4(Fucα1–3)GlcNAc, and the 3′-sialyl-Le^x sequence, NeuAcα2–3Galβ1–4(Fucα1–3)GlcNAc, have been performed. From these it is predicted that the relative spatial dispositions of the charged moieties in the two molecules, sulfate and carboxyl, respectively, are similar (Figure 3).

DISCUSSION

These results show the power of the neoglycolipid technology (Tang et al., 1985; Stoll et al., 1988) to detect specific oligosaccharide ligands among the highly heterogeneous oligosaccharides derived from a mucin-type glycoprotein. Thus, 3-sulfated fucooligosaccharides of Le^a and Le^x/SSEA-1 type are established here as ligands for this adhesion molecule, and the intensity of binding is equal if not superior to that observed with the corresponding 3′-sialyl-Le^x/SSEA-1 glycolipid analogue. Further studies are underway to characterize the spectrum of sulfated and sialylated oligosaccharides on cyst 350 glycoprotein.

It is now clear that 3-sulfated Le^x/Le^a and 3′-sialyl-Le^x and -Le^a sequences have substantially higher E-selectin binding activities relative to the nonacidic Le^x and Le^a analogues. This knowledge, together with the present molecular modeling studies (Figure 3) and other modeling studies (Berg et al., 1991; Tyrrell et al., 1991; A. C. Stuart, F. X. Sullivan, and Dale Cumming, unpublished observations) which indicate that the carboxyl groups of 3′-sialyl-Le^x and 3′-sialyl-Le^a have similar topological dispositions, suggests that the spatial positioning of the negatively charged groups is crucial for generation of the high-affinity fucooligosaccharide ligands. This further suggests that the primary contribution of sialic

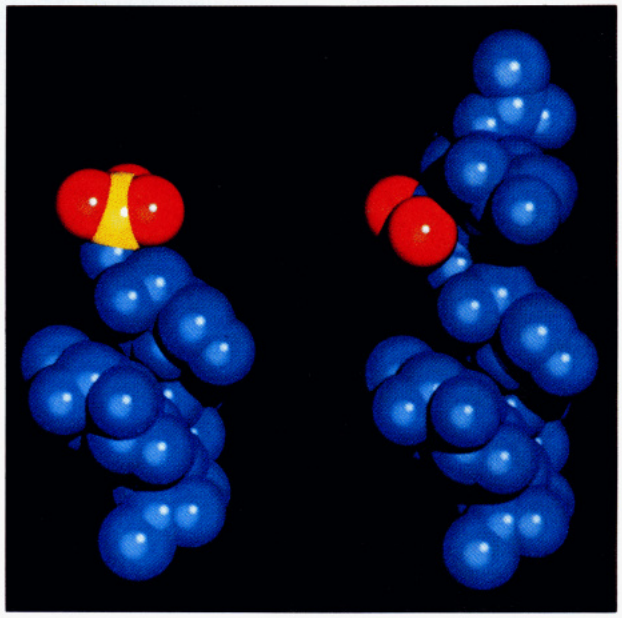

FIGURE 3: Molecular models of the 3-sulfated trisaccharide HSO_3–3Galβ1–4(Fucα1–3)GlcNAc (left) and 3′-sialyl-Le^x tetrasaccharide. This figure depicts the charge groups SO_3^- and CO_2^- with the oxygens shown in red and sulfur in yellow; all other atoms are shaded blue. The relative orientations of the carbohydrate moieties shared between the two molecules are identical. The topological disposition of the charged groups in the two molecules is similar.

acid in 3′-sialyl-Le^x and 3′-sialyl-Le^a is the negatively charged carboxyl group. This suggestion is supported by the observation (Tyrrell et al., 1991) that other molecular features in the sialic acid moiety of 3′-sialyl-Le^x have no effect on E-selectin recognition: for example, the presence of *N*-glycolyl rather than *N*-acetyl groups or the trimming of the sialic acid polyol tail by periodate treatment. Thus high-affinity E-selectin ligands are formed by oligosaccharides which present a negative charge in a similar spatial orientation, although differing in molecular detail. This implies that biosynthetically distinct E-selectin ligands may exist in various tissues and cells.

Sulfate-containing sequences of the *N*-acetyllactosamine series, with sulfate residues 6-linked to galactose or to *N*-acetylglucosamine, are the major repeating structural components and antigenic elements of the glycosaminoglycan keratan sulfate and behave as differentiation antigens of chondrocytes (Feizi, 1989b). Fucooligosaccharides of Le^a and Le^x/SSEA-1 type and their 3′-sialyl analogues are members of a family of blood group-related oncodevelopmental antigens that have been defined with the aid of monoclonal antibodies (Feizi, 1985). It will be interesting to investigate whether the expression of 3-sulfated analogues similarly changes during fetal development and oncogenesis in the light of the present study and a previous report (Capon et al., 1989) describing the sequence HSO_3–3Galβ1–4(Fucα1–3)GlcNAc linked to core *N*-acetylgalactosamine among O-linked oligosaccharides from human fetal meconium glycoproteins.

Major resources are being directed at the design of compounds that are related to saccharide ligands of the selectins, serve as inhibitors of selectin binding, and may be useful as therapeutic substances in the management of undesirable or life-threatening instances of the adhesive events that they mediate. Thus far, the emphasis has been on the synthesis of 3′-sialyl-Le^x/SSEA-1 (Hodgson, 1991; Edgington, 1992). The present study renders the 3-sulfated analogues strong contenders as inhibitory compounds for E-selectin-mediated adhesion. It will be important therefore to consider chemical, biochemical, and biosynthetic strategies for their large-scale production.

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