Identification of the Sulfated Monosaccharides of GlyCAM-1, an Endothelial-Derived Ligand for L-Selectin†

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ABSTRACT: L-Selectin, a receptor bearing a C-type lectin domain, mediates the initial attachment of lymphocytes to high endothelial venules of lymph nodes. One of the endothelial-derived ligands for L-selectin is GlyCAM-1 (previously known as Sgp50), a mucin-like glycoprotein with sulfated, sialylated, and fucosylated O-linked oligosaccharide chains. Sialylation, sulfation, and fucosylation appear to be required for the avid interaction of this ligand with L-selectin, but the exact carbohydrate structures involved in recognition remain undefined. In this study, we examine the nature of the sulfate-modified carbohydrates of GlyCAM-1. GlyCAM-1 was metabolically labeled in lymph node organ culture with $^{35}SO_4$ and a panel of tritiated carbohydrate precursors. Mild hydrolysis conditions were established that released sulfated oligosaccharides without cleavage of sulfate esters. Low molecular weight and singly charged fragments, obtained by a combination of gel filtration and anion-exchange chromatography, were analyzed. The structural identification of the fragments relied on the use of a variety of radiolabeled sugar precursors, further chemical and enzymatic hydrolysis, and high-pH anion-exchange chromatography analysis. Sulfated constituents of GlyCAM-1 were identified as Gal-6-SO_4, GlcNAc-6-SO_4, (SO_4-6)Galβ1→4GlcNAc, and Galβ1→4(SO_4-6)GlcNAc. In the accompanying paper [Hemmerich, S., & Rosen, S. D. (1994) *Biochemistry 33,* 4830–4835] evidence is presented that (SO_4-6)Galβ1→4GlcNAc forms the core of a sulfated sialyl Lewis x structure that may comprise a recognition determinant on GlyCAM-1.

L-Selectin is a primary lymphocyte adhesion molecule involved in lymphocyte binding to high endothelial venules (HEV)[1] of lymph nodes during lymphocyte recirculation (Gallatin et al., 1983). The widespread distribution of L-selectin on all classes of leukocytes underlies its more general function in a broad spectrum of leukocyte–endothelial interactions [reviewed in Picker and Butcher (1992) and Rosen (1993a)]. The other two selectins, E- and P-selectin, are expressed on endothelial cells where they mediate attachment to leukocytes [reviewed in Bevilacqua and Nelson (1993) and Lasky (1992)]. The selectins perform their adhesive functions by virtue of C-type lectin domains at their amino termini (Drickamer, 1988). Reflecting a high degree of sequence similarity among these domains (60–70%), the biological ligands for L-selectin on HEV and for E- and P-selectin on leukocytes share a requirement for sialic acid [reviewed in Rosen (1993b)]. Moreover, each selectin is capable of recognizing sialyl Lewis x [sLe^x, i.e., Neu5Acα2→3Galβ1→4 (Fucα1→3)GlcNAc] and related structures [reviewed in Stoolman (1992) and Varki (1992)], although these compounds bind with low affinity. The activity of carbohydrate-specific antibodies has implicated the presence of sLe^x-related structures in the actual biological ligands for the selectins (Phillips et al., 1990; Walz et al., 1990; Norgard et al., 1993; Sawada et al., 1993). Nonetheless, each selectin appears to have a set of preferred biological ligands, although information is lacking on what distinguishes the ligands for the different selectins (Berg et al., 1992; Larsen et al., 1992).

Two HEV-associated ligands for L-selectin, GlyCAM-1 and CD34 (formerly known as Sgp50 and Sgp90, respectively), have been identified in mouse by direct precipitation of lymph node extracts with a soluble L-selectin/immunoglobulin chimera (Imai et al., 1991; Lasky et al., 1992; Baumhueter et al., 1993). GlyCAM-1 and CD34 are a subset of the molecules recognized by the adhesion-blocking antibody known as MECA 79 (Berg et al., 1991). GlyCAM-1 is released into conditioned medium of cultured lymph nodes as an intact molecule (Lasky et al., 1992; Brustein et al., 1992), suggesting that it is a secreted product and/or a loosely associated peripheral membrane component. In contrast, CD34 possesses a classical transmembrane domain (Brown et al., 1991; Suda et al., 1992). Both GlyCAM-1 and CD34 are mucin-like glycoproteins, which are likely to function as ligands through

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[1] Abbreviations: C-type, calcium-type; Gal, galactose; GlcN, glucosamine; GalN, galactosamine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; Fuc, fucose; Gal-6S, galactose 6-sulfate; GlcNAc-6S, *N*-acetylglucosamine 6-sulfate; GlcU-S, glucuronic acid monosulfate; HEV, high endothelial venule; HPAEC, high-pH anion-exchange chromatography; *N*-acetyllactosamine, Galβ1→4GlcNAc; Neu5Ac, *N*-acetylneuraminic acid; sialyl Lewis x or sLe^x, Neu5Acα2→3Galβ1→4(Fucα1→3)GlcNAc; Lewis x or Le^x, Galβ1→4(Fucα1→3)GlcNAc; Lewis a or Le^a, Galβ1→3(Fucα1→4)GlcNAc; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. With the exception of fucose, which is in the L-configuration, all sugars are in the D-configuration.

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the presentation of highly clustered O-linked chains to L-selectin on the leukocyte cell surface (Lasky et al., 1992; Baumhueter et al., 1993).

GlyCAM-1 and CD34/Sgp90 are sulfated, fucosylated, and sialylated glycoproteins (Imai et al., 1991). The O-linked chains of GlyCAM-1 are heterogeneous in both size and charge (Imai & Rosen, 1993). The interaction of both GlyCAM-1 and CD34 with L-selectin depends on their sialylation (Imai et al., 1991), confirming earlier findings that sialidase treatment of lymph node HEV significantly reduces lymphocyte attachment (Rosen et al., 1985, 1989). However, exhaustive desialylation does not completely abrogate the ligand activity of GlyCAM-1, suggesting that a sialic acid-independent mode of recognition also exists (Imai et al., 1992). The critical sialic acids of GlyCAM-1 appear to be in $\alpha 2 \rightarrow 3$ linkages, since the linkage-specific sialidase from Newcastle disease virus partially inactivates ligand activity (Imai et al., 1992). Furthermore, sLe^x-type oligosaccharides manifest ligand activity for L-selectin, whereas the Lewis x-type structures with $\alpha 2 \rightarrow 6$-linked Neu5Ac are relatively inactive (Imai et al., 1992; Foxall et al., 1992). An essential contribution from fucose is suspected, since sLe^x is more active than sialyllactose (i.e., Neu5Ac$\alpha 2 \rightarrow$3Gal$\beta 1 \rightarrow$4Glc) as a competitor of L-selectin binding (Imai et al., 1992). Moreover, fucose has been shown to be a critical determinant for the neutrophil ligands for P- and E-selectin (Larsen et al., 1992).

Prior to the discovery that HEV ligands are, in fact, sulfated, the potential importance of sulfation for their function was suspected on the basis of the potent ligand activity of a number of sulfated carbohydrates (e.g., fucoidin, sea urchin egg jelly fucan, and sulfatide) for L-selectin (Stoolman & Rosen, 1983; Imai et al., 1990; True et al., 1990). Subsequently, it was shown that 3′-sulfated Le^x/Le^a (i.e., sulfated on the 3-OH of galactose), sulfoglucuronyl glycolipids, and other sulfated structures are also capable of binding to L-selectin (Green et al., 1992; Needham & Schnaar, 1993; Norgard-Sumnicht et al., 1993; Suzuki et al., 1993). The essential role of sulfation in the activity of these various carbohydrates has focused attention on the contribution of sulfation to GlyCAM-1 function. The use of chlorate as a metabolic inhibitor of sulfation has confirmed that sulfation of GlyCAM-1, independent of its overall sialylation and fucosylation, is necessary for ligand activity (Imai et al., 1993).

The crucial role of sulfate in the recognition of GlyCAM-1 by L-selectin necessitates the structural characterization of its sulfated carbohydrates. Here, we report the analysis of mild acid hydrolysis products of metabolically radiolabeled GlyCAM-1. Using a combination of analytical techniques including high-pH anion-exchange chromatography, we identify two GlyCAM-1 constituents as galactose 6-sulfate and *N*-acetylglucosamine 6-sulfate in the context of *N*-acetyllactosamine.

EXPERIMENTAL PROCEDURES

Metabolic Labeling of Murine Lymph Nodes. For metabolic labeling (Imai et al., 1991), axillary, brachial, cervical, and mesenteric lymph nodes were dissected from five ICR mice, diced with a razor blade, and incubated in 1 mL of RPMI-1640, supplemented with penicillin (100 units/mL) and streptomycin (0.1 mg/mL). The tissue was cultured for 4 h (37 °C) in the presence of D-[6-^{3}H]galactose, D-[6-^{3}H]-glucosamine, D-[2-^{3}H]mannose (at 0.5 mCi/mL, all from DuPont-New England Nuclear, Boston, MA), L-[5,6-^{3}H]fucose, or $Na_2{}^{35}SO_4$ (at 1 mCi/mL, both from ICN, Costa Mesa, CA). Inhibition of sulfation with chlorate was carried out as previously described (Imai et al., 1993). Conditioned medium was collected from cultures and centrifuged briefly (5 min at 10000*g*).

Immunoprecipitation of GlyCAM-1. All steps were carried out at 4 °C. GlyCAM-1 was immunoprecipitated from the conditioned medium by the addition of 20 μL of protein A–Sepharose 4B (Zymed, So. San Francisco, CA; 2.5 mg of recombinant protein A/mL of gel) derivatized with rabbit polyclonal antibody (CAM02) directed to the peptide CK-EPSIFREELISKD (peptide 2) from the deduced GlyCAM-1 protein core (Lasky et al., 1992). The matrix was washed five times with Tris-buffered saline (TBS: 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl), and bound ligand was eluted from the matrix by the addition of 200 μL of TBS containing free peptide 2 (1 mg/mL). One-tenth of the preparation (20 μL) was subjected to Laemmli SDS–gel electrophoresis on a 10% polyacrylamide gel, followed by fluorography using Enhance (New England Nuclear).

Isolation and Fractionation of O-Linked Chains from GlyCAM-1. Immunoprecipitated ligand was treated with alkaline borohydride to release chains, as described previously (Imai & Rosen, 1993). O-Linked chains were desialylated by treatment with *Arthrobacter ureafaciens* sialidase (Imai & Rosen, 1993) and fractionated by anion-exchange chromatography as described in the caption to Figure 2.

Mild Acid Hydrolysis of GlyCAM-1. Immunoprecipitated GlyCAM-1 was treated with 0.2 M H_2SO_4 (final volume, 250 μL), overlaid with clear mineral oil (Sigma), and incubated at 100 °C for 30 min.

Thin-Layer Chromatography. After neutralization of the hydrolysate with 50 μL of 2 M NH_4OH, two 1-μL aliquots were applied to a 100 × 50 mm silica gel coated glass plate (60 F254; Merck, Darmstadt, Germany). Two 0.5-μL aliquots of 1 mM $Na_2{}^{35}SO_4$ solution (5000 cpm each) were then applied to the same plate, with one aliquot overlaid onto the hydrolyzed material and the second in a separate lane. The plate was developed in *n*-BuOH/*N*,*N*-dimethylformamide (DMF)/1 M sodium borate, pH 9, 50:20:25, followed by fluorography with Enhance spray (New England Nuclear).

Initial Gel Filtration of Mild Acid Hydrolysate. The neutralized hydrolysate was combined with 50 μL of 2.5% hemoglobin (Sigma) in 0.1 M pyridine–acetate, pH 5.4, and loaded onto a column of Bio-Gel P4 (Bio-Rad Laboratories, Richmond, CA; 200–400 mesh, 112 × 1 cm , bed volume = 88 mL) in pyridine–acetate (0.1 M, pH 5.4). The column was eluted at 22 °C with the same pyridine–acetate buffer at a rate of 5 mL/h. The void volume of the column (V_0 = 28.4 mL) was defined by the first appearance of the hemoglobin standard in the eluate. Material eluting between V_0 + 20 mL and V_0 + 80 mL was pooled, lyophilized, and redissolved in 200 μL of H_2O.

Anion-Exchange Chromatography of P4-Included Fraction from Mild Acid Hydrolysate. The P4-included fragments generated by mild acid hydrolysis (in 200 μL of H_2O) were loaded onto three parallel DEAE-Sepharose columns (Sigma; acetate form, 80 × 5 mm, bed volume = 1.6 mL) equilibrated with 2 mM pyridine–acetate (pH 5.0). The columns were eluted with 8.5 mL of 2 mM pyridine–acetate, followed by 85 mL of a 2–1000 mM linear gradient of pyridine–acetate (pH 5.0). Fractions (30 drops, ~0.85 mL) were collected, and a 30-μL sample of each fraction was mixed with 5 mL of Ultima Gold scintillation cocktail (Packard, Downers Grove, IL) and counted in a Beckman LS-8000 liquid scintillation counter. An identical column was calibrated with the indicated standards. Fractions eluting from the DEAE-Sepharose

columns between 50 and 150 mM pyridine–acetate (singly charged material) were pooled and lyophilized.

Gel Filtration of the Singly Charged Fraction. The singly charged fragments obtained above were redissolved in 200 μL of water and fractionated by gel filtration on the P4 column described above. The column was eluted at 22 °C with pyridine–acetate buffer at a rate of 5 mL/h, and 30-drop fractions (0.83–0.87 mL) were collected. A 40-μL sample of each fraction was counted as described above. Elution volume is given relative to the first appearance of hemoglobin (void volume), which occurred at 28.4 mL. Fractions were pooled as follows: peak I (55–60 mL), peak II (49–54 mL), peak III (43–48 mL), and peak IV (36–42 mL). Pooled fractions were lyophilized, and the residues were redissolved in 20 μl of water and stored at −80 °C until further analysis.

Carbohydrate Standards Employed in Anion-Exchange Chromatography and Gel Filtration Analysis. The following standards were utilized for calibration of the DEAE anion-exchange and P4 gel filtration columns. [^{3}H]Sialic acid was obtained by sialidase treatment (*Arthrobacter ureafaciens*, Calbiochem, La Jolla, CA; 0.3 unit/mL, pH 5.5, 30 min, 37 °C) of GlyCAM-1 metabolically labeled with [^{3}H]glucosamine.

Glucuronic acid monosulfate (GlcU-S, mixture of isomers) was prepared as follows. A solution of glucuronic acid (1.0 g, 5.2 mmol) and SO_3·trimethylamine (1.45 g, 10.4 mmol) (Westerduin et al., 1990) in 10 mL of anhydrous DMF was stirred at 25 °C for 4 h under an inert atmosphere. The excess reagent was quenched with saturated $NaHCO_3$, and the solution was concentrated. The resulting residue was purified on a 25 × 2 cm column of DEAE-Sephadex (acetate form) eluting with a linear gradient of 0–0.5 M pyridine–acetate (pH 5.4) at a flow rate of 1.5 mL/min. Fractions (9 mL) were collected and analyzed by TLC (5:3:2 *n*-butanol/acetic acid/water). A total of 25 fractions were collected, with unreacted glucuronic acid eluting in fractions 10–12 and glucuronic acid monosulfates eluting in fractions 21–25. The monosulfate fractions were combined and concentrated, and the pyridinium salts were converted to the sodium salts by passage over Bio-Rad AG 50W-X8 resin (Na^+ form). The stoichiometry of sulfate modification was confirmed by negative ion FAB mass spectrometry (m/z = 259.0, $C_6H_8O_{10}SNa$, $[M-Na]^-$). The monosulfate isomers eluted as a single peak in DEAE chromatography and were therefore not purified further.

Galactose disulfate and galactose trisulfate (mixtures of isomers) were prepared in a similar fashion. A solution of galactose (1.0 g, 5.6 mmol) and SO_3·trimethylamine (3.9 g, 28 mmol) in 50 mL of anhydrous DMF was stirred at 40 °C for 6 h. The solution was cooled to room temperature, and the excess reagent was quenched with saturated $NaHCO_3$. The solution was concentrated, and the crude mixture of galactose sulfates was purified into mono-, di-, and trisulfate fractions by DEAE-Sephadex chromatography eluting with a linear gradient of 0–2 M pyridine–acetate (pH 5.4). A total of 40 fractions (9 mL each) were collected at a flow rate of 1.5 mL/min. Monosulfate isomers eluted in fractions 4 and 5, disulfate isomers in fractions 15–20, and trisulfate isomers in fractions 30–40. The respective fractions were combined and concentrated, and the pyridinium salts were converted to sodium salts as previously described. The yields of mono-, di-, and trisulfates were 0.76 g (52%), 0.77 g (36%) and 0.27 g (10%), respectively. The stoichiometry of sulfate modification for the di- and trisulfates was confirmed by negative ion FAB mass spectrometry: galactose disulfate isomers (m/z = 361.0, $C_6H_{10}O_{12}S_2Na$, $[M-Na]^-$); galactose trisulfate isomers (m/z = 463.1, $C_6H_9O_{15}S_3Na_2$, $[M-Na]^-$; 441.1, $C_6H_{10}O_{15}S_3Na$, $[M-2Na+H]^-$). The galactose disulfate and trisulfate isomers eluted as single peaks in DEAE and P4 chromatography and were therefore not purified further.

N-Acetylglucosamine 3-sulfate (GlcNAc-3S, sodium salt) and galactose 6-sulfate (Gal-6S, sodium salt) were from Sigma. GlcNAc-3S was detected in the fractions eluted from the P4 column with the Elson Morgan reaction (Reissig et al., 1955). The galactose sulfates were detected with the phenol–sulfuric acid assay (Dubois et al., 1956). GlcU-S was detected with the carbazole reaction (Bitter & Muir, 1962).

Total Hydrolysis of Sulfated Carbohydrates. Hydrolysis of the sulfated carbohydrates to yield desulfated and deacetylated monosaccharide units was accomplished by heating samples of peaks I, II, and III in 6 M HCl (4 h, 100 °C). After hydrolysis, samples were evaporated repeatedly from water and redissolved in water for monosaccharide compositional analysis by high-pH anion-exchange chromatography (HPAEC).

Desulfation. Peaks II and III were desulfated (without concomitant deacetylation) by incubation in 50 mM methanolic HCl/5% H_2O (24 h, 37 °C). After desulfation, the residues were concentrated, redissolved in water, and loaded onto a DEAE-Sepharose column (80 × 5 mm, bed volume = 1.6 mL) equilibrated in 2 mM pyridine–acetate (pH 5.0). The column was eluted with 2 mM pyridine–acetate (pH 5.0), and uncharged material was recovered as the flow-through fraction. The unbound products were lyophilized and redissolved in water for further analysis.

β-Galactosidase Digestions. Radiolabeled oligosaccharide fragments were reacted with jack bean exo-β-galactosidase (0.25 unit/mL; Sigma) in 20 mM NaH_2PO_4 (buffered to pH 3.5 with acetic acid) in a final volume of 50 μl for 18 h at 37 °C. The digests were then subjected to analysis by HPAEC.

Partial Hydrolysis of ($^{35}SO_4$)-Labeled Peak III. The material from $^{35}SO_4$-labeled peak III was reacted with 0.1 M H_2SO_4 in a final volume of 10 μl (overlaid with mineral oil) for 1 h at 100 °C. After neutralization with 2 M NH_4OH, the hydrolysate was analyzed by HPAEC.

High pH Anion-Exchange Chromatography (HPAEC). Peaks I, II, and III and their respective products obtained from desulfation, enzymatic digestion, and hydrolysis were analyzed by HPAEC using a Carbopac PA1 column (Dionex, Sunnyvale, CA, 250 × 4 mm). Each sample was injected in 25 μL of water. Elution conditions (flow rate, 1 mL/min) were as follows: for analysis of total hydrolysates and desulfated peak II, 20 mM NaOH, isocratic; for desulfated peak III and its β-galactosidase digest, 100 mM NaOH, isocratic; for sulfated peak II, 150 mM NaOH for 4 min, followed by a linear gradient of 0–250 mM NaOAc in 150 mM NaOH over 6 min and then a linear gradient of 250–850 mM NaOAc in 150 mM NaOH over 20 min (program 1); for sulfated peak I, peak III, β-galactosidase digest, and partial hydrolysate of peak III, 50 mM NaOAc in 150 mM NaOH for 5 min, followed by a linear gradient of 50–850 mM NaOAc in 150 mM NaOH over 30 min (program 2). Standards were used at 0.4 mM and analyzed with pulsed amperometric detection. Radiolabeled products were detected by collecting 0.5-min fractions followed by liquid scintillation analysis as described above.

Carbohydrate Standards Employed in HPLC Analysis. Gal-6S, GlcNAc-3S, GlcNAc, Galβ1→3GlcNAc, Galβ1→4GlcNAc (*N*-acetyllactosamine, LacNAc), and Galβ1→6GlcNAc were from Sigma. Gal-4S and GlcNAc-6S were

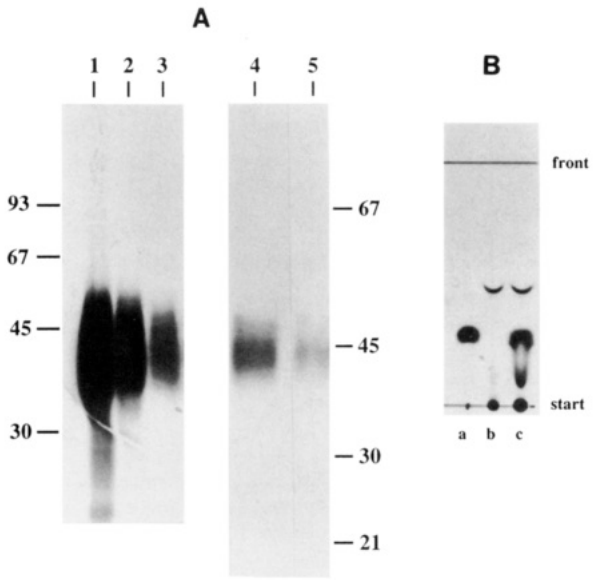

FIGURE 1: Panel A: SDS–PAGE analysis of GlyCAM-1 from mouse lymph nodes. Mouse lymph nodes were labeled in organ culture with (**1**) $^{35}SO_4$, (**2**) [^{3}H]Gal, (**3**) [^{3}H]GlcN, (**4**) [^{3}H]Man, or (**5**) [^{3}H]-Fuc. GlyCAM-1 was then immunoprecipitated from the conditioned medium and subjected to SDS–PAGE followed by fluorography. The migrations of protein standards for samples 1, 2, and 3 are indicated on the left, while those for samples 4 and 5 are indicated on the right. Panel B: Thin-layer chromatography of GlyCAM-1 mild acid hydrolysate. A sample of $^{35}SO_4$-labeled GlyCAM-1 was subjected to mild acid hydrolysis. Aliquots of the hydrolysate were analyzed by thin-layer chromatography (*n*-BuOH/*N*,*N*-dimethylformamide/1 M sodium borate, pH 9, 50:20:25) with fluorography: (a) $Na_2{}^{35}SO_4$; (b) hydrolysate; (c) hydrolysate combined with $Na_2{}^{35}SO_4$. The spots at the origin in lanes b and c represent unhydrolyzed, protein-bound material.

from V-Labs (Covington, LA). Gal-3S was obtained by hydrolysis of bovine sulfatides (Matreya Inc., Pleasant Gap, PA) in 0.1 M H_2SO_4 (30 min, 100 °C). Gal-2S was synthesized from 1,3,4,6-tetra-*O*-acetyl-α-D-galactopyranose (Helferich & Zimer, 1962) according to a previously described procedure (Peat et al., 1968). The product was characterized by ^{1}H NMR spectroscopy and by FAB mass spectrometry. Neutral monosaccharide standards (an equimolar mixture of Fuc, GalN, GlcN, Gal, Glc, and Man) were from Dionex.

RESULTS

GlyCAM-1 was metabolically labeled with [^{35}S]sulfate, [6-^{3}H]galactose, [6-^{3}H]glucosamine, [5,6-^{3}H]fucose, or [2-^{3}H]-mannose in lymph node organ culture and purified from conditioned medium with an antibody directed to the core protein (Lasky et al., 1992). With all five of the radioactive labels employed, SDS–PAGE analysis of the isolated material showed that >95% of the radioactivity was concentrated in a diffuse band centered at 45 kDa (Figure 1A).

We first wished to approximate the extent of sulfation on the O-linked chains of GlyCAM-1. O-Linked chains were isolated from normal or undersulfated [^{3}H]Gal-labeled GlyCAM-1 (prepared with chlorate during organ culture).[2] After desialylation, the chains were analyzed by DEAE chromatography. For normally sulfated GlyCAM-1, 68% of the incorporated [^{3}H]Gal label was found in negatively charged chains, i.e., retained on the column and requiring elevated salt for elution (Figure 2). For undersulfated GlyCAM-1, the percentage of [^{3}H]Gal label in negatively charged chains

[2] Chlorate had no effect on the overall incorporation of [^{3}H]Gal into GlyCAM-1 (Hemmerich & Rosen, 1994)

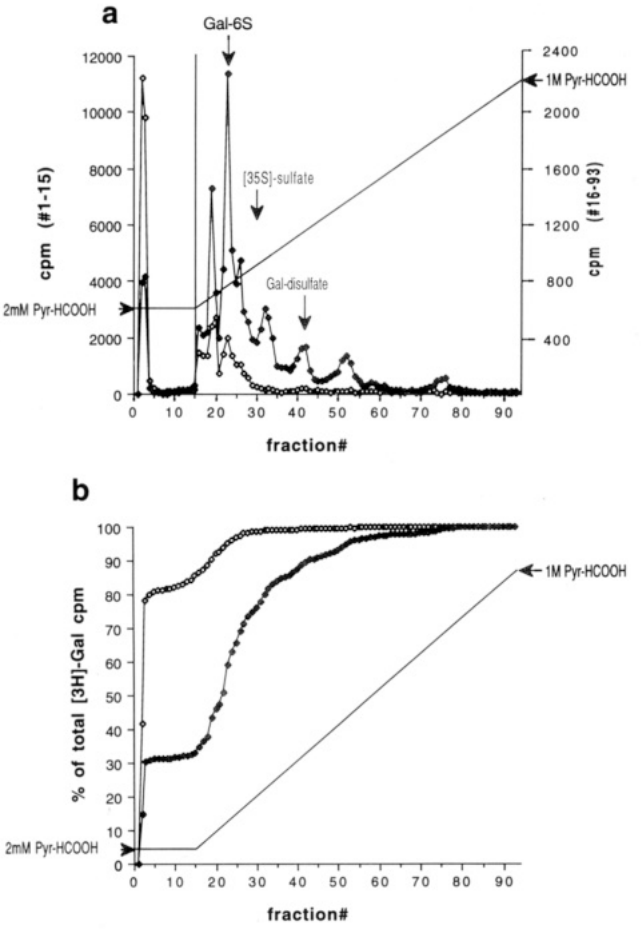

FIGURE 2: Anion-exchange chromatography of O-linked chains from normal and undersulfated GlyCAM-1. O-Linked chains were isolated from normal and undersulfated GlyCAM-1 labeled with [^{3}H]Gal. After desialylation with *Arthrobacter* sialidase, the chains were subjected to DEAE-Sepharose anion-exchange chromatography (80 × 5 mm column, formate form). (a) [^{3}H]Gal counts in individual fractions. Different scales were used for fractions 1–15 and fractions 16–93 in order to present the data clearly in a single plot. (b) Integration of the peaks from the elution profile in panel a. Data points represent the summation of [^{3}H]Gal counts in the fractions as a percentage of total [^{3}H]Gal counts: (◆) chains from normal [^{3}H]Gal-GlyCAM; (◇) chains from undersulfated GlyCAM-1 prepared by culture in the presence of chlorate. The diagonal lines indicate the concentration of eluting electrolyte (13.5 mL of 2 mM pyridineformate, pH 3, followed by 70 mL of a 2–1000 mM gradient; fraction volume, 0.9 mL). The elution positions of standards are indicated with arrows. The percentage of label in negatively charged chains was obtained by determining the fraction of total counts that required >2 mM pyridine-formate for elution.

was reduced to 20% (Figure 2). Quantitatively similar results were obtained when the label was [^{3}H]GlcN (79% vs 31%). Thus, it is estimated that at least 48% of the O-linked chains carry sulfate. This value represents a lower limit estimate, since chlorate treatment is at best 90% effective in preventing sulfation (Imai et al., 1993).

In order to define the nature of the sulfated monosaccharides on GlyCAM-1, we subjected the intact molecule, labeled with $Na_2{}^{35}SO_4$, [^{3}H]Gal, [^{3}H]GlcN, [^{3}H]Man, or [^{3}H]Fuc, to controlled acid hydrolysis. As shown by thin-layer chromatography (Figure 1B), the hydrolysate obtained from $^{35}SO_4$-labeled material contained only trace levels of free sulfate. Thus, sulfate esters remained intact under these hydrolysis conditions. Since we wanted to focus our analysis on low molecular weight fragments, we subjected the hydrolysates to gel filtration with Bio-Gel P4. The P4-included material contained most of the radioactivity (85% of $^{35}SO_4$ cpm, 80%

of [^{3}H]GlcN cpm, 75% of [^{3}H]Gal cpm, and 100% of [^{3}H]-Man and [^{3}H]Fuc cpm) and was further fractionated by anion-exchange chromatography on DEAE-Sepharose. A gradient elution with pyridine–acetate allowed separation of the fragments into unbound material (flow through), singly charged material (50–150 mM pyridine–acetate), and multicharged material (>200 mM pyridine–acetate).

With $^{35}SO_4$ as the label, 12% of the P4-included counts were unbound (possibly representing complex zwitterionic fragments), 48% of the P4-included counts were singly charged, eluting as a sharp peak at 100 mM pyridine–acetate, while the multiply charged material comprised 40% of the counts and eluted as a poorly resolved series of peaks between 200 and 400 mM pyridine–acetate (Figure 3a). With [^{3}H]Gal or [^{3}H]GlcN as the label, all of the counts were found in the unbound and monocharged fractions, with no multiply charged species in evidence (Figure 3b,c). Since we were interested in carbohydrate-associated sulfation, we did not further investigate the multiply charged material with its restricted labeling pattern.

With [^{3}H]Man or [^{3}H]Fuc as the labels, all radioactivity was in the DEAE unbound fraction. We further examined this material by high-pH anion-exchange chromatography (HPAEC) and found it to represent free L-fucose, whether it was derived from [^{3}H]Man- or [^{3}H]Fuc-labeled GlyCAM-1 (Figure 4). We conclude from these observations that GlyCAM-1 does not contain mannose.

The singly charged carbohydrate fragments from the DEAE columns, which had been labeled in parallel with $^{35}SO_4$, [^{3}H]-Gal, or [^{3}H]GlcN (Figure 3), were subjected to gel filtration through Bio-Gel P4. Four well-resolved peaks were obtained (Figure 5). Peak I contained both the $^{35}SO_4$ and [^{3}H]Gal labels. Peak II contained both the $^{35}SO_4$ and [^{3}H]GlcN labels. Peak III contained all three labels, while peak IV contained only the [^{3}H]GlcN label. Due to metabolic interconversions, the tritium in [6-^{3}H]GlcN can occur in GlcNAc, GalNAc, and sialic acid residues (Varki, 1991). When [^{3}H]GlcN-labeled GlyCAM-1 was treated with sialidase, the released counts comigrated with peak IV on the P4 column. On this basis, peak IV was identified as sialic acid, although the exact form of sialic acid (among the extended set of naturally occurring variants) was not defined (Norgard et al., 1993).

To identify the constituent monosaccharides, peaks I, II, and III were subjected to strong acidic conditions (6 N HCl, 100 °C, 4 h) which hydrolyze all glycosidic bonds, sulfate esters, and *N*-acetyl bonds (Cummings et al., 1989). After evaporation to remove acid, the redissolved fractions were analyzed by HPAEC on a Dionex Carbopac I column and compared with authentic standards. All of the counts derived from [^{3}H]Gal-labeled peak I coeluted with Gal (Figure 6a). All of the counts derived from [^{3}H]GlcN-labeled peak II coeluted with GlcN, while none of the radioactivity coeluted with GalN (Figure 6b). All of the counts derived from [^{3}H]-Gal-labeled peak III coeluted with galactose (Figure 6c). Finally, all of the counts derived from [^{3}H]GlcN-labeled peak III coeluted with GlcN and none coeluted with GalN (Figure 6c). These results, together with the observed peak profiles on the P4 column (Figure 5), indicate that peak I contains Gal-sulfate, peak II contains GlcN(Ac)-sulfate, and peak III contains a monosulfated form of Gal→GlcN(Ac) or GlcN-(Ac)→Gal.

For further characterization of peaks II and III, these fractions were subjected to solvolysis (50 mM HCl in MeOH, 5% H_2O, 37 °C, 24 h) to remove sulfate. About 70% of both [^{3}H]Gal-labeled peak III and [^{3}H]GlcN-labeled peak II did

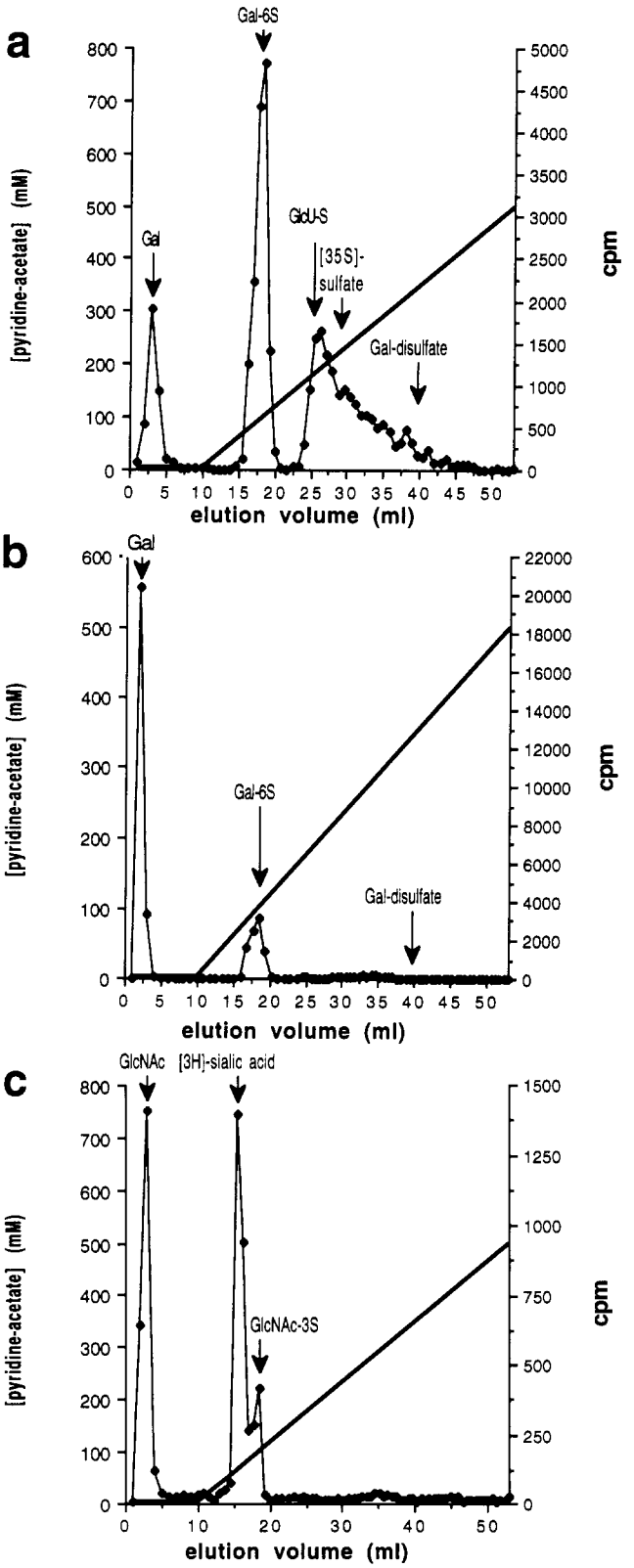

FIGURE 3: Anion-exchange chromatography of hydrolysis products of GlyCAM-1. The P4-included fragments generated by mild acid hydrolysis of GlyCAM-1, metabolically labeled with different precursors, were subjected to DEAE-Sepharose anion-exchange chromatography: (a) $^{35}SO_4$; (b) [^{3}H]Gal; (c) [^{3}H]GlcN. The diagonal lines indicate the concentration of eluting electrolyte (2–1000 mM pyridine–acetate, pH 5). The elution positions of monosaccharide standards and $^{35}SO_4$ are indicated with arrows. The arrow labeled [^{3}H]sialic acid denotes the elution position of sialic acid released from [^{3}H]GlcN-labeled GlyCAM-1 by *Arthrobacter* sialidase treatment. At the pH of the elution buffer, Gal-6S and sialic acid are singly charged whereas galactose disulfate and GlcU-S are doubly charged.

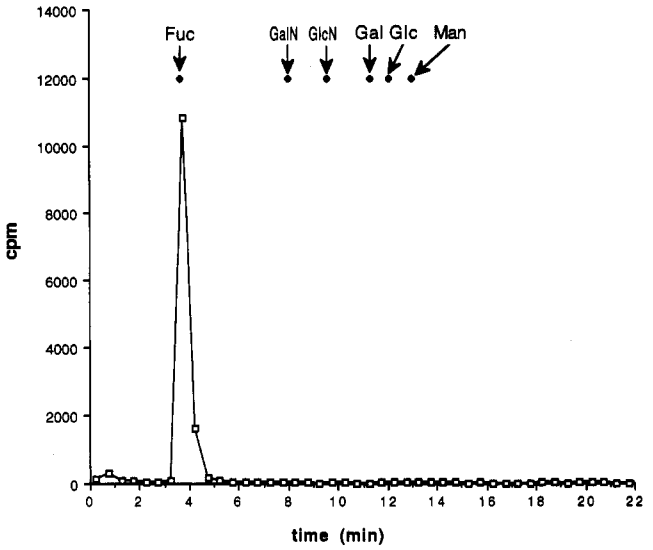

FIGURE 4: HPAEC analysis of hydrolysis products of [^{3}H]Man-labeled GlyCAM-1. A sample of GlyCAM-1 labeled with [^{3}H]Man was hydrolyzed in 0.2 M H_2SO_4 at 100 °C for 30 min, and the hydrolysate was subjected to gel filtration on Bio-Gel P4. All [^{3}H]-Man counts were P4-included and unbound by DEAE-Sepharose. This material was further analyzed by HPAEC (20 mM NaOH isocratic). The elution time of the solvent front is defined as zero. The elution times of the monosaccharide standards are indicated with arrows.

not bind to the DEAE column after this treatment. By HPAEC analysis, the unbound fraction derived from the hydrolysis of [^{3}H]GlcN-labeled peak II coeluted with GlcNAc rather than GlcN, establishing that peak II contains GlcNAc–O–SO_3 and not GlcN–SO_3 (Figure 7a). By a similar HPAEC analysis, the unbound product derived from the hydrolysis of [^{3}H]Gal-labeled peak III coeluted with Galβ1→4GlcNAc but not with Galβ1→3GlcNAc or Galβ1→6GlcNAc (Figure 7b). Digestion of this unbound material with exo-β-galactosidase (jack bean) converted it quantitatively into [^{3}H]Gal (Figure 7c). These results establish that peak III is a monosulfated form (or forms) of *N*-acetyllactosamine (Galβ1→4GlcNAc).

To determine the linkage positions of the sulfate esters in peaks I, II, and III, these fractions were compared to standards by HPAEC analysis (Carbopac I, NaOAc gradient in 150 mM NaOH). All of the counts in both [^{3}H]Gal-labeled peak I and $^{35}SO_4$-labeled peak I coeluted with Gal-6S, which was clearly resolved from Gal-2S, Gal-3S, and Gal-4S (Figure 8a). The majority of the counts (95%) in [^{3}H]GlcN-labeled peak II coeluted with GlcNAc-6S, which was well resolved from GlcNAc-3S (Figure 8b). From these results and the preceding analysis, peaks I and II were identified as Gal-6S and GlcNAc-6S, respectively.

Chemical hydrolysis of $^{35}SO_4$-labeled peak III (0.1 M H_2SO_4, 60 min, 100 °C) gave rise to both Gal-6S and GlcNAc-6S as well as some free $^{35}SO_4$ (Figure 8d). These results, together with the assignment of the *N*-acetyllactosamine core structure described above, reveal that peak III is a mixture of Galβ1→4(SO$_4$-6)GlcNAc and (SO$_4$-6)Galβ1→4GlcNAc. In support of this conclusion, peak III was fractionated by HPAEC into two major peaks (designated IIIa and IIIb) comprising about 90% of the applied counts. The peak profiles

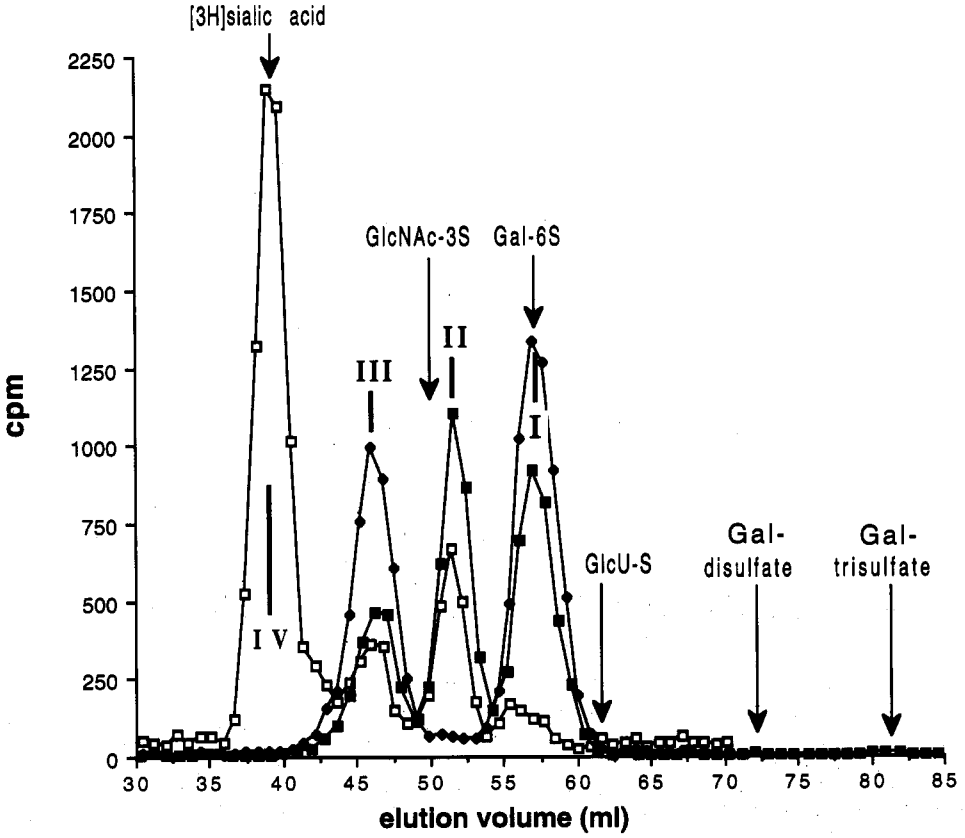

FIGURE 5: Gel filtration analysis of singly charged components of hydrolyzed GlyCAM-1. The P4-included fractions of the mild acid hydrolysates of GlyCAM-1, metabolically labeled with different precursors, were subjected to DEAE-Sepharose anion-exchange chromatography as in Figure 3, and singly charged fractions were collected between 50 and 150 mM pyridine–acetate. These pools were subjected to rechromatography on a Bio-Gel P4 gel filtration column: (■) labeled with $^{35}SO_4$; (◆) labeled with [^{3}H]Gal; (□) labeled with [^{3}H]GlcN. The counts from the [^{3}H]GlcN-labeled material were multiplied by 4 in this plot. The void volume was defined as the first appearance of hemoglobin in the eluate. The elution volume is given relative to the void volume. The elution positions of carbohydrate standards are indicated with arrows. No counts were eluted between 0 and 30 mL.

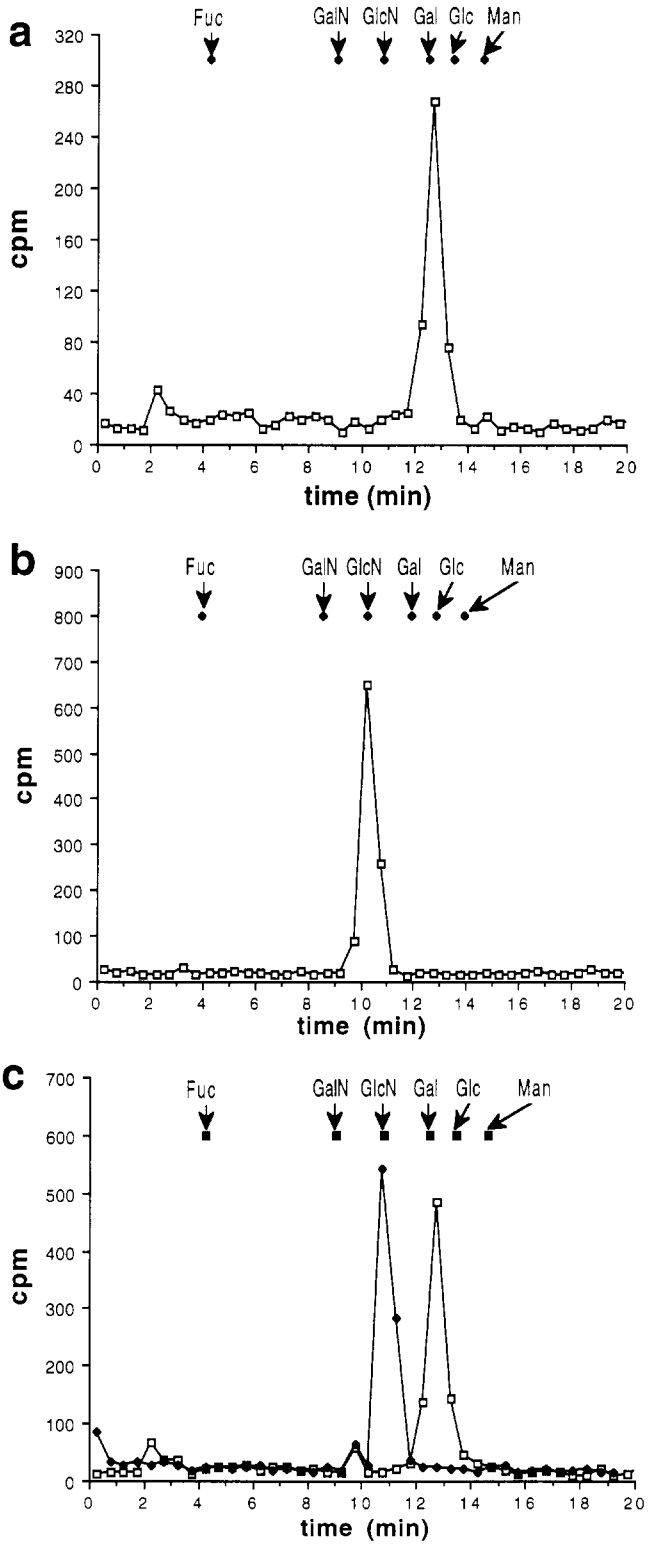

FIGURE 6: Monosaccharide compositions of peaks I, II, and III by HPAEC analysis. Singly charged fractions obtained from GlyCAM-1 mild acid hydrolysates (labeled with different precursors) were refractionated by P4 gel filtration as in Figure 5. Peaks I, II, and III were subjected to exhaustive acid hydrolysis (6 M HCl, 4 h, 100 °C) and analyzed by HPAEC (20 mM NaOH isocratic). (a) [^{3}H]Gal-labeled peak I; (b) [^{3}H]GlcN-labeled peak II; (c) [^{3}H]Gal-labeled peak III (□) and [^{3}H]GlcN-labeled peak III (◆). The elution time is defined as in the caption to Figure 4. The elution times of the monosaccharide standards are indicated with arrows.

were identical for material labeled with $^{35}SO_4$ (Figure 8c,d), [^{3}H]Gal, or [^{3}H]GlcN (data not shown). Treatment with jack bean exo-β-galactosidase converted $^{35}SO_4$-labeled peak

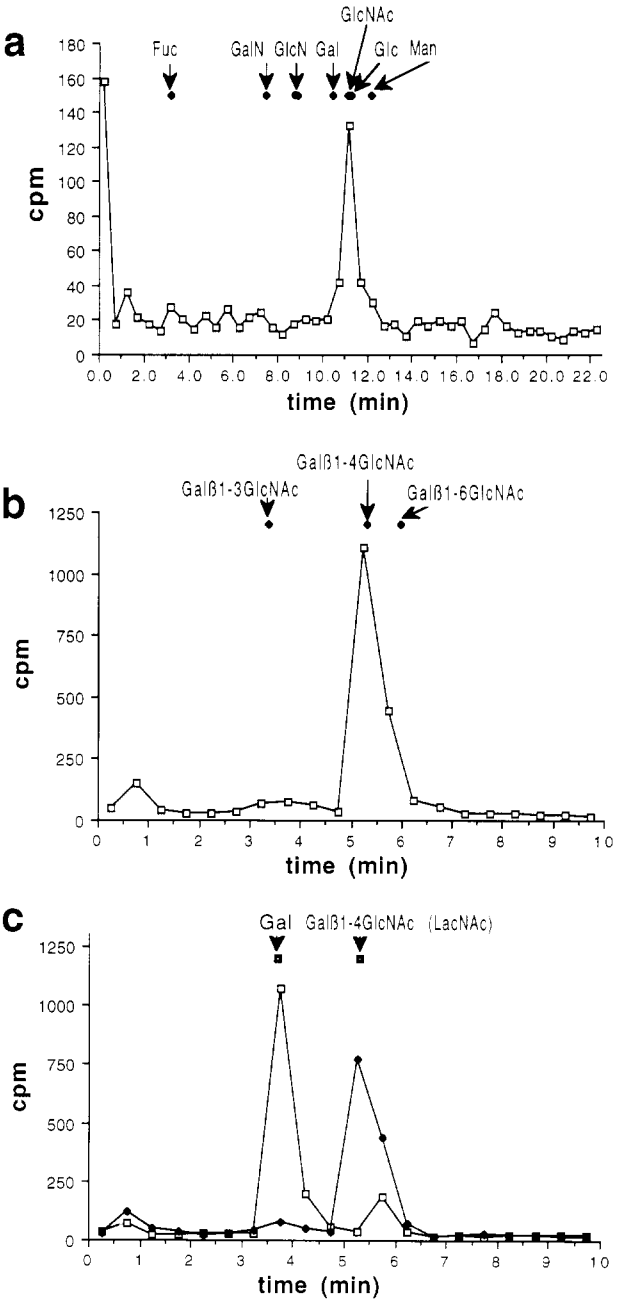

FIGURE 7: HPAEC analysis of desulfated peaks II and III. [^{3}H]-GlcN-labeled peak II and [^{3}H]Gal-labeled peak III were desulfated by treatment with methanolic HCl and then analyzed by HPAEC: (a) [^{3}H]GlcN-labeled peak II eluting with 20 mM NaOH, isocratic; (b) [^{3}H]Gal-labeled peak III eluting with 100 mM NaOH, isocratic; (c) desulfated [^{3}H]Gal-labeled peak III digested with jack bean exo-β-galactosidase and analyzed by HPAEC (100 mM NaOH, isocratic) (□) compared to an undigested control sample (◆). The elution times of the carbohydrate standards are indicated with arrows.

IIIa into a species that comigrated with GlcNAc-6S, identifying peak IIIa as Galβ1→4(SO_4-6)GlcNAc (Figure 8c). Peak IIIb was identified as (SO_4-6)Galβ1→4GlcNAc on the basis of its resistance to cleavage by jack bean exo-β-galactosidase (Figure 8c), which is predicted to be inactive toward sulfate-substituted galactosides (Green & Baenziger, 1988; Spiro & Bhoyroo, 1988).

With these structural assignments to the individual peaks of Figure 5, integration of these peaks revealed that 40% of the $^{35}SO_4$ counts were in Gal-6S, 37% were in GlcNAc-6S, 13% were in Galβ1→4(SO_4-6)GlcNAc, and 10% were in (SO_4-6)Galβ1→4GlcNAc. Thus, about half of the carbohydrate-associated $^{35}SO_4$ label (P4 included) was esterified to the 6-OH

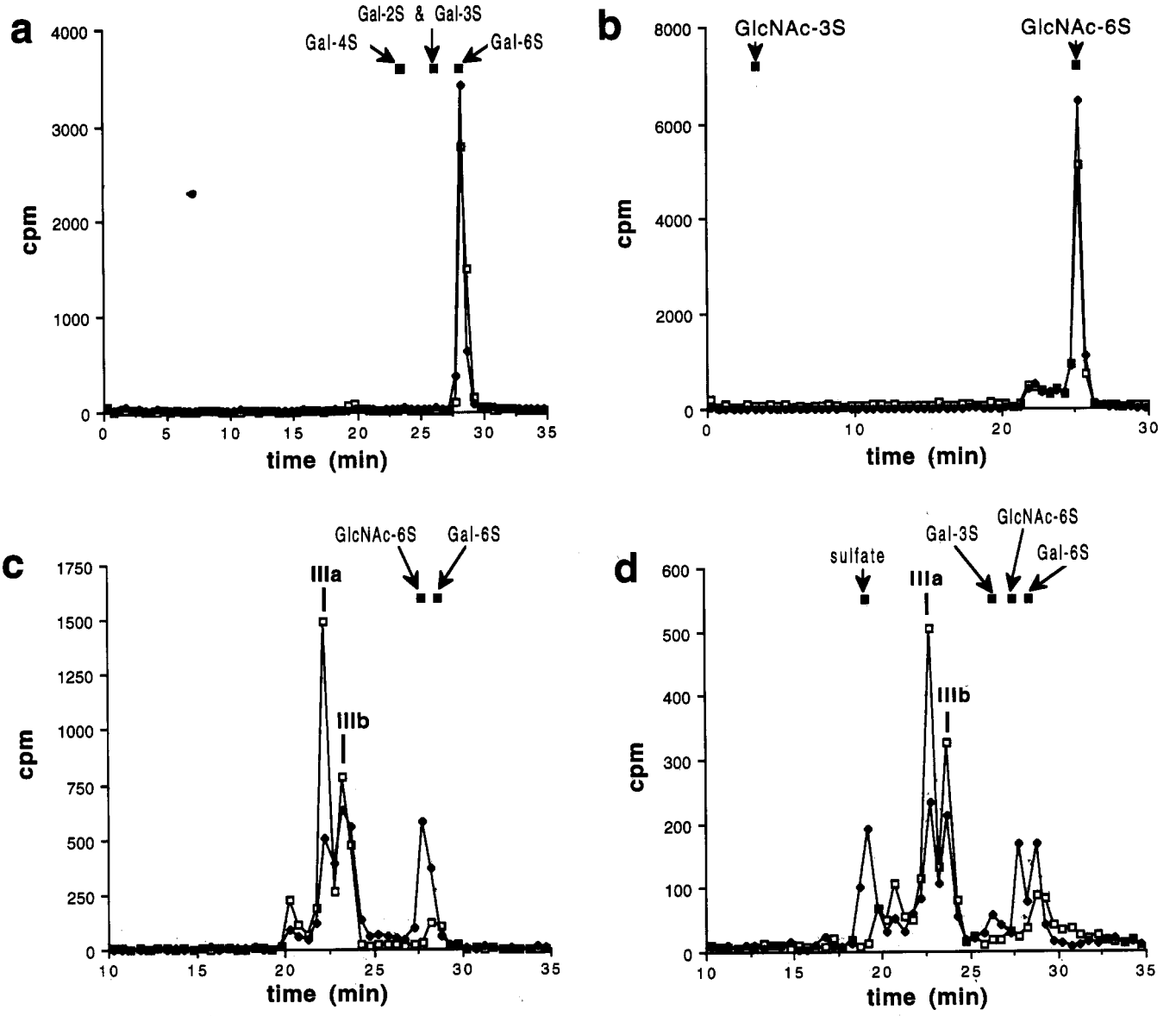

FIGURE 8: HPAEC analysis of sulfated peaks I, II, and III. (a) HPAEC profile of peak I labeled with either $^{35}SO_4$ (□) or [^{3}H]Gal (◆), elution program 2 (cf. Experimental Section). (b) HPAEC profile of peak II labeled with either $^{35}SO_4$ (□) or [^{3}H]GlcN (◆), elution program 1. (c) HPAEC profiles of $^{35}SO_4$-labeled peak III (□) and a jack bean exo-β-galactosidase-digested sample (◆), elution program 2. (d) HPAEC profiles of $^{35}SO_4$-labeled peak III (□) and a hydrolyzed sample (0.1 M H_2SO_4, 1 h, 100 °C) (◆), elution program 2. The elution times of the carbohydrate and sulfate standards are indicated with arrows.

of galactose, and the other half, to the 6-OH of GlcNAc.

DISCUSSION

The presence of sulfate on GlyCAM-1 has been shown to be essential for its avid interaction with L-selectin. We establish here that sulfation represents a quantitatively significant modification of the O-linked chains of GlyCAM-1. At least one-half of the chains are modified with sulfate esters. The present study also identifies the nature of the sulfate modifications of GlyCAM-1. Since conventional analysis of the GlyCAM-1 carbohydrates has so far been hampered by the limited quantities of available ligand, we employed radioactive tracer techniques that have been used widely in the sequencing of glycoprotein oligosaccharides (Cummings et al., 1989; Varki, 1991; Maemura & Fukuda, 1992; Shilatifard et al., 1993). The analysis relied on the use of hydrolysis conditions that released sulfated oligosaccharides without the significant cleavage of sulfate esters.

In light of the previously demonstrated ligand activity of fucoidin and egg jelly coat fucan (fucose 4-sulfate rich polysaccharides) for L-selectin (Stoolman & Rosen, 1983; Imai et al., 1990), we initially suspected that sulfated fucose might be present in GlyCAM-1. Prior work has shown that mild acid hydrolysis (0.15 M H_2SO_4, 30 min, 100 °C) liberates sulfated fucose (fucose 3-sulfate and fucose 3,4-disulfate) from a sea cucumber chondroitin sulfate (Viera et al., 1991). However, application of these hydrolysis conditions to GlyCAM-1, labeled with either [^{3}H]Man or [^{3}H]Fuc, yielded counts only as neutral fucose, arguing against sulfation of fucose residues in this ligand. The exclusive incorporation of all mannose label into GlyCAM-1 as fucose indicates the complete absence of mannose in GlyCAM-1. This result further substantiates the conclusion that the oligosaccharide chains of GlyCAM-1 are all O-linked (Imai et al., 1991).

Our analysis identified Gal-6-SO_4, GlcNAc-6-SO_4, Galβ1→4(SO_4-6)GlcNAc and (SO_4-6)Galβ1→4GlcNAc as components of GlyCAM-1. Sulfation on the 6-OH of galactose and the 6-OH of GlcNAc occur in approximately equal amounts. The results provide no evidence for the presence of galactose 3-sulfate on GlyCAM-1, although various Gal-3S-containing

carbohydrates exhibit ligand activity for L-selectin (see above). GlcNAc-6S has been identified in a wide assortment of glycoconjugates which includes the glycosaminoglycan chains of keratan sulfate (Rodén, 1980), viral envelope glycoproteins of type I human immunodeficiency virus (Shilatifard et al., 1993), porcine zona pellucida glycoproteins (Hokke et al., 1993), glycoproteins of bovine large vessel endothelial cells (Roux et al., 1988), bovine and human thyroglobulin (Spiro & Bhoyroo, 1988), and a rhizobial nodulation factor (Roche et al., 1991). Gal-6S has been found in keratan sulfates (Rodén, 1980), human tracheobronchial mucins (Mawhinney et al., 1986), rat salivary mucins together with GlcNAc-6S (Slomiany et al., 1988), and recombinant human tissue plasminogen activator (Pfeiffer et al., 1992). In keratan sulfate and plasminogen activator, Gal-6S occurs on *N*-acetyllactosamine units, as is the case for the structures identified in the present study.

The functional requirement for sialic acid, as well as for sulfate, must be included in any model for the recognition determinants on GlyCAM-1. One possibility is that individual O-linked chains of this mucin-like ligand contain either sialic acid-based or sulfate-based determinants. Neighboring chains on the protein backbone would be recognized by separate lectin domains of an oligomeric array of L-selectin molecules. Alternatively, the pairing of the two types of chain might form a combined epitope for single lectin domains (Norgard et al., 1993). In both cases, removal of either sialic acid or sulfate would greatly reduce the overall avidity of the interaction between the ligand and L-selectin.

Another possibility is that sulfation and sialylation (as well as fucosylation) on individual O-linked chains comprise the recognition site for L-selectin. Consistent with this possibility, we previously observed that all $^{35}SO_4$-labeled O-linked chains, released from GlyCAM-1 by β-elimination, are able to bind to a sialic acid-specific lectin (*Limax* agglutinin) (Imai & Rosen, 1993). Thus, sulfation does not occur without sialylation on individual chains. In the companion study (Hemmerich & Rosen, 1994), we directly analyze a major capping group of GlyCAM-1 and provide evidence for sialylation and fucosylation of a core based on (SO_4-6)-Galβ1→4GlcNAc.

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