6′-Sulfated Sialyl Lewis x Is a Major Capping Group of GlyCAM-1†

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Received December 14, 1993; Revised Manuscript Received February 10, 1994®

ABSTRACT: The binding of L-selectin to the HEV-derived ligand GlyCAM-1 bears a strict requirement for oligosaccharide sulfation. In the companion study [Hemmerich, S., Bertozzi, C. R., Leffler, H., & Rosen, S. D. (1994) *Biochemistry 33*, 4820–4829], we identified the major sulfated mono- and disaccharides of GlyCAM-1 as Gal-6-SO_4, GlcNAc-6-SO_4, (SO_4-6)Galβ1→4GlcNAc, and Galβ1→ 4(SO_4-6)GlcNAc. Sialic acid and fucose are also critical to the recognition determinants on GlyCAM-1. However, the hydrolysis conditions employed in the previous study resulted in cleavage of these moieties, precluding their positional assignment. Here, we employ lectins of defined specificity in conjunction with specific exoglycosidases to identify a major GlyCAM-1 capping structure that includes all three critical elements. The complementary reactivity of *Maackia amurensis* agglutinin with fully sialylated, undersulfated GlyCAM-1 and *Sambucus nigra* agglutinin/*Trichosanthes japonica* agglutinin with desialylated but normally sulfated GlyCAM-1 indicates the presence of terminal 6′-sulfated sialyllactosamine. α(1→3/4)Fucosidase removes fucose almost quantitatively from asialo-GlyCAM-1 while substantially enhancing its binding to *Lycopersican esculentum* agglutinin (specific for β1→4–linked GlcNAc), indicating the presence of Fuc in an α1→3 linkage to GlcNAc. The strict requirement for desialylation to achieve defucosylation indicates a proximal location of Fuc with respect to terminal sialic acid. The nature of the capping group was further defined by studying the effects of sulfation, sialylation, and fucosylation on the ability of exo-β(1→4)galactosidase to release [^{3}H]Gal from GlyCAM-1. These results, in concert with the findings of the companion study, indicate that 6′-sulfated sialyl Lewis x, i.e., Siaα2→3(SO_4-6)Galβ1→4(Fucα1→3)GlcNAc, is a major capping group of GlyCAM-1.

L-Selectin is a member of the selectin family of adhesion proteins [reviewed by Lasky (1992) and Bevilacqua and Nelson (1993)] that mediates the initial adhesive interaction of lymphocytes with HEV[1] of lymph nodes during the process of lymphocyte recirculation (Gallatin et al., 1983). All of the selectins recognize carbohydrate-based ligands on target cells, but the carbohydrate structures on biological selectin ligands have yet to be determined. As reviewed in the companion paper (Hemmerich et al., 1994), two HEV-associated ligands for L-selectin have been identified as mucin-like glycoproteins, known as Sgp50/GlyCAM-1 and Sgp90/CD34 (Imai et al., 1991; Lasky et al., 1992; Baumhueter et al., 1993). Previous work has established that the carbohydrate chains of both ligands are modified by sulfation, sialylation, and fucosylation, all of which appear to be required for their avid interaction with L-selectin. The biological ligands for L-selectin may possess functional determinants that are related to sialyl Lewis x, since various sLe^x-type structures or structural isomers thereof exhibit binding activity, albeit very weak, for L-selectin (Imai et al., 1992; Foxall et al., 1992; Berg et al., 1992). Moreover, a monoclonal antibody that recognizes a complex form of sLe^x binds to human tonsillar HEV ligands for L-selectin (Sawada et al., 1993).

In an effort to understand the molecular nature of L-selectin recognition, we have undertaken the structural characterization of the O-linked carbohydrate chains on GlyCAM-1. In the companion study (Hemmerich et al., 1994), we subjected metabolically labeled GlyCAM-1 to controlled acid hydrolysis and identified the primary monosulfated fragments as Gal-6-SO_4, GlcNAc-6-SO_4, (SO_4-6)Galβ1→4GlcNAc, and Galβ1→4(SO_4-6)GlcNAc. In the present study, we define the sialylation and the fucosylation associated with these structures and present evidence that 6′-sulfated sialyl Lewis x is a major capping group of GlyCAM-1.

EXPERIMENTAL PROCEDURES

Metabolic Labeling of Murine Lymph Nodes and Immunoprecipitation of GlyCAM-1. Murine peripheral and mesenteric lymph nodes were labeled in organ culture with $Na_2{}^{35}SO_4$, D-[6-^{3}H]galactose, or D-[2-^{3}H]mannose (DuPont-New England Nuclear, Boston, MA), and GlyCAM-1 was immunoprecipitated from conditioned media using CAM02 Ab as described (Hemmerich et al., 1994). D-[2-^{3}H]Mannose is exclusively incorporated into fucose moieties of GlyCAM-1 (Hemmerich et al., 1994). To generate undersulfated GlyCAM-1, the metabolic labeling was carried out under the same conditions in the presence of 10 mM $NaClO_3$ (chlorate) as described (Hemmerich et al., 1994). While GlyCAM-1 incorporated about the same [^{3}H]galactose activity in the presence or absence of chlorate (2.8×10^6 cpm from five mice and 0.5 mCi [^{3}H]Gal input), the incorporation of [^{3}H]Man was elevated 2-fold in the presence of chlorate (9.3×10^5 vs 5.1×10^5 cpm in the control, from the lymph nodes of five

† S.H. was the recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (No. He 1807/1-1), 1990–1992. This research was supported by grants to S.D.R. from the NIH (GM23547) and Genentech Inc.

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® Abstract published in *Advance ACS Abstracts,* April 1, 1994.

[1] Abbreviations: 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; HEV, high endothelial venule; Lewis x, Galβ1→4(Fucα1→3)GlcNAc; NDV, Newcastle disease virus; Neu5Ac, *N*-acetylneuraminic acid; PBS, Dulbecco's phosphate-buffered saline; sialyl Lewis x or sLe^x, Neu5Acα2→3Galβ1→4(Fucα1→3)GlcNAc; Sia, sialic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline. All sugars, except fucose, are in the D-configuration.

0006-2960/94/0433-4830$04.50/0

mice with an input of 0.5 mCi [^{3}H]Man). As expected, the incorporation of $^{35}SO_4$ was suppressed by 90% with chlorate (0.16×10^6 vs 1.75×10^6 cpm in the control, 0.5 mCi input), consistent with previous findings (Imai et al., 1993).

Isolation of O-Linked Chains (Glycans) from [^{3}H]Fuc-Labeled GlyCAM-1. O-Linked chains were liberated from immunoprecipitated [^{3}H]Fuc-labeled GlyCAM-1 by treatment with $NaBH_4$ in NaOH as described previously (Imai & Rosen, 1993) and redissolved in 100 μL of H_2O.

Exoglycosidase Digestions of GlyCAM-1. Prior to enzymatic digestion, GlyCAM-1 preparations (200 μL) were desalted by passage over a Sephadex G-25 column (250 × 10 mm) equilibrated in 0.5 M NH_4HCO_3/5% 2-propanol. The radioactivity in the void volume was pooled, lyophilized, and redissolved in 50 mM NaOAc (pH 5.3), 100 mM NaCl, 0.1% bovine serum albumin (BSA), 0.1% NaN_3, and 0.25% Triton X-100. For desialylation, 10-μL aliquots were treated with 10 μL of either *Arthrobacter ureafaciens* sialidase (Calbiochem, La Jolla, CA; 25 milliunits) or Newcastle disease virus sialidase (Oxford Glycosystems, Rosedale, NY; 5 milliunits) in 50 mM NaOAc (pH 5.3) and 100 mM NaCl for 18 h at 37 °C. In parallel controls, samples were incubated under the same conditions without enzyme.

Defucosylation was achieved by treating 10-μL aliquots of either intact GlyCAM-1 (^{3}H-labeled in Gal or Fuc) or [^{3}H]-Fuc-labeled O-linked chains with 100 milliunits of α(1→2)-fucosidase from *Corynebacterium* or with 5 microunits of α(1→3/4)fucosidase from *Streptomyces* sp. 142 in 50 mM sodium cacodylate (pH 6.0), 0.05% BSA, 0.05% NaN_3, and 0.125% Triton X-100 (final volume, 100 μL). These reactions were carried out in both the presence and absence of 25 milliunits of *Arthrobacter ureafaciens* sialidase with incubation for 48 h at 37 °C. The fucosidases were purchased from Takara Shuzo Co. (Berkeley, CA).

Degalactosylation was achieved by treating 10-μL aliquots of [^{3}H]Gal-labeled GlyCAM-1 with 5 milliunits of β(1→4)-galactosidase (from *Diplococcus pneumoniae*; Boehringer Mannheim, Germany) with or without 5 microunits of *Streptomyces* α(1→3/4)fucosidase in 50 mM sodium-cacodylate (pH 6.0), 0.05% BSA, 0.05% NaN_3, and 0.125% Triton X-100 (final volume, 100 μL). These reactions were carried out in the presence or absence of 25 milliunits of *Arthrobacter ureafaciens* sialidase with incubation for 48 h at 37 °C.

Degalactosylation/Defucosylation of Lacto-N-fucopentaose (LNF-III). Tritiated LNF-III alditol was provided by Dr. Rolf Nuck, Freie Universität Berlin, F.R.G. A portion of this material (2000 cpm) was digested with *Diplococcus* β(1→4)galactosidase alone (5 milliunits), *Steptomyces* α-(1→3/4)fucosidase alone (5 microunits), or both enzymes together in 50 mM sodium cacodylate (pH 6.0), 0.05% BSA, 0.05% NaN_3, and 0.125% Triton X-100 (final volume, 100 μL) for 48 h at 37 °C. The digests were applied to a Microsorb aminopropyl HPLC column (10 × 250 mm; Rainin) and eluted with a decreasing gradient of acetonitrile in water (80% to 40% acetonitrile over a 120-min period; flow rate = 1 mL/min). Fractions (1 mL) were collected, mixed with 10 mL of Ultima Gold scintillation cocktail (Packard, Downers Grove, IL), and counted in a liquid scintillation counter.

Gel Filtration. Exoglycosidase digests of intact GlyCAM-1 (100 μL) were supplemented with 10 μL of Blue Dextran (10 mg/mL in water; Pharmacia, Uppsala, Sweden) and loaded onto Sephadex G-25 columns (8 × 270 mm) equilibrated in Dulbecco's phosphate-buffered saline (PBS) with 0.02% Triton X-100 at 22 °C. Columns were eluted with the same buffer, and 0.5-mL fractions were collected and mixed with 10 mL of scintillation cocktail for counting. Protein-bound radioactivity eluted in the void volume (4 mL) as defined by Blue Dextran. Liberated free [^{3}H]Gal or [^{3}H]Fuc eluted 3.5 mL (7 fractions) later. For gel filtration of O-linked glycans, [^{3}H]Fuc-labeled chains (100 μL) were mixed with 50 μL of 2.5% hemoglobin (Sigma) in 0.1 M pyridine–acetate, pH 5.4, and loaded onto a column of Biogel P4 (200–400 mesh, 112 × 1 cm, bed volume = 88 mL; Bio-Rad Laboratories, Richmond, CA) 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, and radioactivity in the fractions (0.85 mL) was determined by liquid scintillation counting.

Lectin Panels. Wheat germ agglutinin–agarose (7 mg of WGA/mL of gel), *Sambucus nigra* agglutinin–agarose (3 mg of SNA/mL of gel), and *Lycopersicum esculentum* agglutinin–agarose (2 mg of LEA/mL of gel) were purchased from Vector, Burlingame, CA. *Trichosanthes japonica* agglutinin–agarose (2 mg of TJA-1/mL of gel) was provided by Dr. K. Yamashita, Sasaki Institute, Tokyo, Japan, to Dr. L. Lasky of Genentech Inc., South San Francisco, CA. Recombinant *Aleuria aurantia* agglutinin (AAA) was the kind gift of Dr. A. Kobata, University of Tokyo. *Maackia amurensis* agglutinin (MAA) was obtained from Sigma. *Limax flavus* agglutinin was obtained from Calbiochem Inc. (San Diego, CA). The latter three lectins were immobilized on CNBr-activated Sepharose 4B (Sigma) at a concentration of 2 mg of lectin/mL of gel using the protocol recommended by the manufacturer.

For lectin binding studies, aliquots of [^{3}H]Gal-labeled GlyCAM-1, subjected to the indicated treatments, were diluted to about 10^5 cpm/mL in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl) supplemented with 2 mM $CaCl_2$, 0.02% BSA, 0.02% NaN_3, and 0.2% Triton X-100. Aliquots (40 μL) were agitated with 10 μL of lectin gel for 2 h at 4 °C. The gel matrix was centrifuged, washed four times with 0.5 mL of 0.25% Triton X-100 in PBS (4 °C), and eluted at 25 °C (1 h) with 0.5 mL of specific mono- or disaccharide (100 mM) in PBS containing 0.25% Triton X-100. Eluates were mixed with 10 mL of scintillation cocktail and counted. An immunoaffinity matrix directed at the C-terminal peptide sequence of GlyCAM-1 was generated by binding rabbit polyclonal antibody (CAM05) directed to a C-terminal peptide from GlyCAM-1 (Lasky et al., 1992) to protein A–agarose (2 mg of protein A/mL of gel; Zymed, Hayward, CA). This gel was eluted with 0.1 M glycine hydrochloride pH 3.0, 0.2 M NaCl, and 0.25% Triton X-100. Total input counts were determined by counting 40 μL of ligand solution. Binding was expressed as a percentage of total input counts.

RESULTS

In order to define the relationship of sialic acid to the sulfated mono- and disaccharides of GlyCAM-1 (Hemmerich et al., 1994), we examined the binding of the ligand, labeled with [6-^{3}H]galactose, to a panel of lectins with defined carbohydrate specificities (Table 1). Undersulfated GlyCAM-1 was generated by organ culture of murine lymph nodes in sodium chlorate, and desialylation was accomplished by treatment with a broad-spectrum sialidase from *Arthrobacter ureafaciens* or with Newcastle disease virus (NDV) sialidase with specificity for Siaα2→3 (Paulson et al., 1982).

As shown in Table 1, desialylation of normally sulfated GlyCAM-1 with *Arthrobacter* sialidase exposed a cryptic binding site for both TJA-1 and SNA, which have specificity

Table 1: Binding of GlyCAM-1 to Lectins of Defined Specificity[a,b]

presence of		cpm precipitated (%)					
Sia	SO_4	TJA-1	SNA	MAA	WGA	AAA	CAM05 Ab
+	+	8.2	11	4.2	87	62	59
+	–	1.6	3.6	29	83	81	89
–	+	63	35	0.2	14	64	73
–	–	3.1	0.2	0.4	24	70	86

[a] Normal or undersulfated [^{3}H]Gal-labeled GlyCAM, with or without prior desialylation (*Arthrobacter ureafaciens* sialidase), was incubated with the indicated immobilized lectins as described in Experimental Procedures. The TJA-1 and MAA data are averages of duplicates (deviations were <5% of the average). The incomplete precipitation of CAM02 Ab-purified GlyCAM-1 by CAM05 Ab reflects the fact that CAM02 (which is an independent peptide antibody) binds with higher affinity. [b] The carbohydrate specificities of the lectins used are as follows: Wheat germ agglutinin (WGA) recognized sialic acid (Sia) and terminal GlcNAc (Osawa, 1982); eluant, GlcNAc. *Aleuria aurantia* agglutinin (AAA), α1→2-, α1→3-, and α1→6-linked Fuc (Yamashita et al., 1985); eluant, fucose. *Trichosanthes japonica* agglutinin (TJA-1), (SO_4-6)Galβ1→4GlcNAc and Siaα2→6Galβ1→4GlcNAc (Yamashita et al., 1992); eluant, lactose. *Sambucus nigra* agglutinin (SNA), (SO_4-6)Galβ1→4GlcNAc, Siaα2→6GalNAc, and Siaα2→6Galβ1→4GlcNAc (Shibuya et al., 1987a,b; Yamashita et al., 1992); eluant, lactose. *Maackia amurensis* agglutinin (MAA), Siaα2→3Galβ1→4GlcNAc (Knibbs et al., 1991; Wang & Cummings, 1988); eluant, lactose. *Lycopersicon esculentum* agglutinin (LEA), repeats of β1→4GlcNAc in the form of GlcNAc homooligomers or multiple *N*-acetyllactosamine units (Merkle & Cummings, 1987; Nachbar & Oppenheim, 1980); eluant, lactose. *Limax flavus* agglutinin (Limax), Sia (Miller, 1987); eluant, Neu5Ac.

Table 2: Binding of GlyCAM-1 to Lectins of Defined Specificity[a]

presence of		cpm precipitated (%)		
Siaα2→3	SO_4	TJA-1	MAA	Limax
+	+	5.0	22	35
+	–	1.3	58	44
–	+	54	6.7	1.8
–	–	12	8.7	2.9

[a] Normal or undersulfated [^{3}H]Gal-labeled GlyCAM, with or without prior desialylation (α2→3-specific NDV sialidase) was incubated with immobilized MAA, TJA-1, or Limax, as described in Experimental Procedures.

for (SO_4-6)Galβ1→4GlcNAc. This site depended on sulfation, since the undersulfated material, with or without desialylation, failed to bind to these two lectins. In contrast to these results with TJA-1/SNA, undersulfation of GlyCAM-1 substantially enhanced its reactivity for MAA, which has specificity for Siaα2→3Galβ1→4GlcNAc. This binding was dependent on sialylation, since desialylated/undersulfated GlyCAM-1 exhibited negligible binding to MAA. Desialylation substantially reduced the binding of GlyCAM-1 to WGA, reflecting the interaction of this lectin with sialic acid. The residual binding to WGA following sialidase treatment is likely to reflect the interaction of this lectin with GlcNAc moieties. The sialylation or sulfation status of GlyCAM-1 did not appreciably affect its binding to AAA, a fucose-specific lectin. Three additional independent lectin panels similar to the one presented in Table 1 yielded qualitatively identical results (not shown).

Table 2 presents a similar lectin binding panel in which [^{3}H]Gal-labeled GlyCAM-1 was desialylated with the linkage-specific sialidase from NDV. The almost complete desialylation of GlyCAM-1 by this enzyme, as indicated by >90% loss of reactivity with *Limax* agglutinin, suggests that Siaα2→3 is the predominant linkage in GlyCAM-1. As with the broad-spectrum *Arthrobacter* sialidase, desialylation of GlyCAM-1 with the NDV enzyme significantly enhanced binding to TJA-1. With respect to MAA, the binding of normal GlyCAM-1 and the potentiated binding of undersulfated GlyCAM-1 were

Table 3: Treatment of GlyCAM-1 with Specific Fucosidases[a]

presence of		release of [^{3}H]Fuc (%) from GlyCAM-1 by	
Sia	SO_4	α(1→2)fucosidase	α(1→3/4)fucosidase
+	+	ND	0
+	–	ND	0
–	+	–	70
–	–	0	85

[a] The percent release of [^{3}H]fucose from [^{3}H]Man-labeled GlyCAM-1 by linkage-specific fucosidases was calculated as described in the caption of Figure 1a. Normal or undersulfated GlyCAM-1, with or without desialylation by *Arthrobacter* sialidase, was treated with the indicated fucosidase as described in Experimental Procedures. ND indicates not determined.

substantially reduced by NDV sialidase (Table 2). In light of the presence of (SO_4-6)Galβ1→4GlcNAc in GlyCAM-1 (Hemmerich *et al.*, 1994), the binding of desialylated GlyCAM-1 to SNA/TJA-1 and the binding of undersulfated GlyCAM-1 to MAA suggest that

$$\text{Sia}\alpha2\rightarrow3(SO_4\text{-}6)\text{Gal}\beta1\rightarrow4\text{GlcNAc} \quad (1)$$

is a component of a GlyCAM-1 capping structure.

Since fucose has been implicated in the ligand activity of GlyCAM-1, we investigated the linkages of fucose on its oligosaccharide chains. We have previously shown that GlyCAM-1 contains fucose (Imai et al., 1991), none of which appears to be modified with sulfate (Hemmerich et al., 1994). For the linkage analysis, normal or undersulfated ligand was metabolically labeled with [^{3}H]Gal or [^{3}H]Man. The [^{3}H]-Man is incorporated exclusively into fucosyl residues of GlyCAM-1 (Hemmerich et al., 1994). The labeled ligand was digested, with or without desialylation (*Arthrobacter ureafaciens* sialidase), with linkage-specific exofucosidases. The release of free [^{3}H]Fuc was then determined by gel filtration. *Streptomyces* α(1→3/4)fucosidase released 70% of the fucose from desialylated [^{3}H]Fuc-labeled GlyCAM-1, while fully sialylated GlyCAM-1 was refractory to this enzyme (Figure 1a and Table 3). Desulfation enhanced the removal of fucose to 85%. In contrast to these results, there was no detectable release of fucose from desialylated GlyCAM-1 by the α(1→2)fucosidase (*Corynebacterium*), whether or not the ligand was fully sulfated (Table 3).

These experiments were repeated with purified O-linked chains obtained from GlyCAM-1 by alkaline borohydride treatment. Desialylation of chains allowed 89% of [^{3}H]Fuc to be released by α(1→3/4)fucosidase (Figure 1b), regardless of the sulfation state. As with the intact glycoprotein, there was no detectable release of counts by the α(1→2)fucosidase (Figure 1b). These findings indicate that all of the fucose within GlyCAM-1 is linked α1→3 and/or α1→4. The observation that no counts were released without desialylation is reminiscent of the observation that sLe^x-type structures are almost completely resistant to α(1→3/4)fucosidase unless the sialic acid is first removed (Maemura & Fukuda, 1992; Sano et al., 1992). Our results imply that all of the fucosylated chains of GlyCAM-1 must bear proximal and terminal sialic acid moieties.

To further identify the sugars to which fucose might be linked, we investigated the contribution of fucose to the binding of GlyCAM-1 by LEA. This lectin recognizes β1→4-linked GlcNAc in the form of GlcNAc homooligomers or as multiple *N*-acetyllactosamine units (Table 1). Desialylated [^{3}H]Gal-labeled GlyCAM-1 was treated with *Streptomyces* α(1→3/4)fucosidase and tested for binding to AAA (a broad-spectrum fucose-specific lectin) and to LEA. As shown in Table 4, binding to AAA was greatly reduced, confirming the sub-

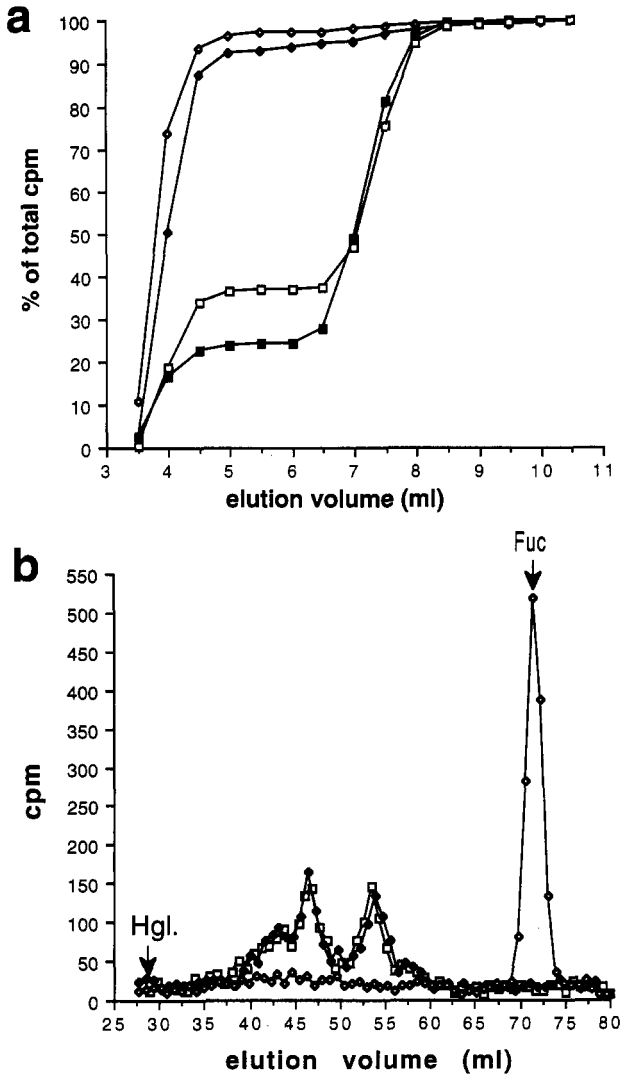

FIGURE 1: Defucosylation of GlyCAM-1. Panel a: Parallel samples of normal (◇, □) or undersulfated (◆, ■) GlyCAM-1, metabolically labeled with [^{3}H]Fuc, were digested with *Streptomyces* $\alpha(1\rightarrow3/4)$fucosidase alone (◇, ◆) or combined with *Arthrobacter ureafaciens* sialidase (□, ■). Digests were subjected to gel filtration on Sephadex G-25 (0.5-mL fractions; void volume, 4–4.5 mL). The data are plotted as a running sum (integration) of the percentage of total radioactivity (relative to the starting sample) vs elution volume. For calculation of percentage of [^{3}H]Fuc cpm released, all radioactivity before 6 mL was considered glycoprotein-associated, while all counts eluting after 6 mL represented free [^{3}H]Fuc. Panel b: O-Linked glycans were released from [^{3}H]Fuc-labeled GlyCAM-1 by β-elimination. Aliquots of the glycan preparation were then digested with *Arthrobacter* sialidase alone (□), with sialidase and $\alpha(1\rightarrow2)$-fucosidase (◆), or with sialidase and $\alpha(1\rightarrow3/4)$fucosidase (◇). The digests were subjected to gel filtration through Bio-Gel P4. The void volume, defined by hemoglobin (Hgl), was 28.4 mL. The elution volume of free Fuc was determined with a [^{3}H]Fuc standard. The counts in each 0.85-mL fraction are plotted vs elution volume. For calculation of percentage of [^{3}H]Fuc cpm released, all radioactivity eluting before 65 mL was considered glycan-associated fucose, while all radioactivity eluting after 65 mL was free [^{3}H]Fuc.

stantial removal of fucose. In contrast, binding to LEA was enhanced almost 10-fold, indicating the exposure of $\beta1\rightarrow$4GlcNAc moieties. The same qualitative effects on AAA and LEA binding were seen when desialylated/undersulfated GlyCAM-1 was subjected to this fucosidase treatment (not shown). These results are consistent with the presence of terminal fucose in an $\alpha1\rightarrow3$ linkage to $\beta1\rightarrow$4-linked GlcNAc.

We next wished to determine the position of fucose relative to the penultimate galactose in Structure **1** defined above. The presence of Fuc$\alpha1\rightarrow3$ in Le^x-type structures has been

Table 4: Binding of Defucosylated GlyCAM-1 to AAA and LEA[a]

	binding of desialylated GlyCAM-1 (%) to:	
treatment	AAA	LEA
none	60	5.2
$\alpha(1\rightarrow3/4)$fucosidase	23	48

[a] [^{3}H]Gal-labeled GlyCAM-1, desialyated with *Arthrobacter ureafaciens* sialidase, with or without treatment with *Streptomyces* $\alpha(1\rightarrow3/4)$fucosidase, was tested for binding to immobilized AAA and LEA as described in Experimental Procedures. The LEA data are averages of duplicates (deviations were <8% of the average).

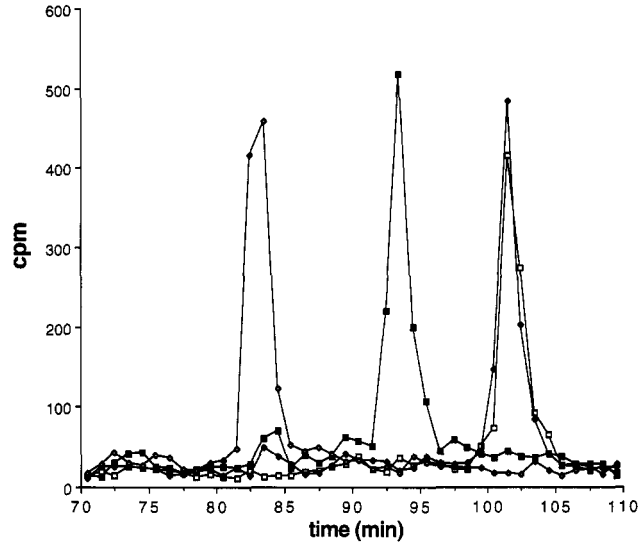

FIGURE 2: Defucosylation requirement for removal of galactose from LNF-III. Parallel samples of LNF-III alditol (Gal$\beta1\rightarrow$4(Fuc$\alpha1\rightarrow$3)-GlcNAc$\beta1\rightarrow$3Gal$\beta1\rightarrow$4Glc-ol) labeled with ^{3}H at the reducing end were digested with $\beta(1\rightarrow4)$galactosidase alone (◆), $\alpha(1\rightarrow3/4)$-fucosidase alone (■), or a combination of $\beta(1\rightarrow4)$galactosidase and $\alpha(1\rightarrow3/4)$fucosidase (◇). Digests and an untreated control sample (□) were fractionated by HPLC on an aminopropyl column. Thus, the peak eluting at 102 min is LNF-III alditol, the peak at 93 min is Gal$\beta1\rightarrow$4GlcNAc$\beta1\rightarrow$3Gal$\beta1\rightarrow$4Glc-ol, and the peak at 83 min is GlcNAc$\beta1\rightarrow$3Gal$\beta1\rightarrow$4Glc-ol.

reported to hinder the activity of *Diplococcus pneumoniae* $\beta(1\rightarrow4)$galactosidase (Maemura & Fukuda, 1992). Using LNF-III [^{3}H]alditol (Gal$\beta1\rightarrow$4(Fuc$\alpha1\rightarrow$3)GlcNAc$\beta1\rightarrow$3Gal$\beta1\rightarrow$4[1-^{3}H]Glc-ol) as a test substrate, we confirmed this result (Figure 2). Thus, $\beta(1\rightarrow4)$galactosidase alone did not digest LNF-III alditol, but a mixture of *Streptomyces* $\alpha(1\rightarrow3/4)$fucosidase and $\beta(1\rightarrow4)$galactosidase produced GlcNAc$\beta1\rightarrow$3Gal$\beta1\rightarrow$4Glc-ol.

We determined the relevance of this observation to GlyCAM-1 as follows. [^{3}H]Gal-labeled GlyCAM-1 (normal or undersulfated) was digested with or without concurrent desialylation with (1) *Diplococcus* $\beta(1\rightarrow4)$galactosidase alone or (2) $\beta(1\rightarrow4)$galactosidase and *Streptomyces* $\alpha(1\rightarrow3/4)$-fucosidase. The release of free [^{3}H]Gal under the various conditions is shown in Figure 3 and tabulated in Table 5. A number of conclusions emerge from this analysis:

(1) At least 39% of the galactose in GlyCAM-1 is in a penultimate position relative to sialic acid and is in a $\beta1\rightarrow4$ linkage, presumably to GlcNAc.[2] This result follows from the observation that undersulfated/desialylated/defucosylated material released 39% of the [^{3}H]Gal counts upon treatment

[2] The failure of exo-$\beta(1\rightarrow4)$galactosidase to release a higher percentage of the [^{3}H]Gal is attributable in part to the presence of Gal$\beta(1\rightarrow3)$-GalNAc within GlyCAM-1 (S. Hemmerich, unpublished observations). The possibility of internal linkages of Gal (e.g., within internal *N*-acetyllactosamine units) is under investigation.

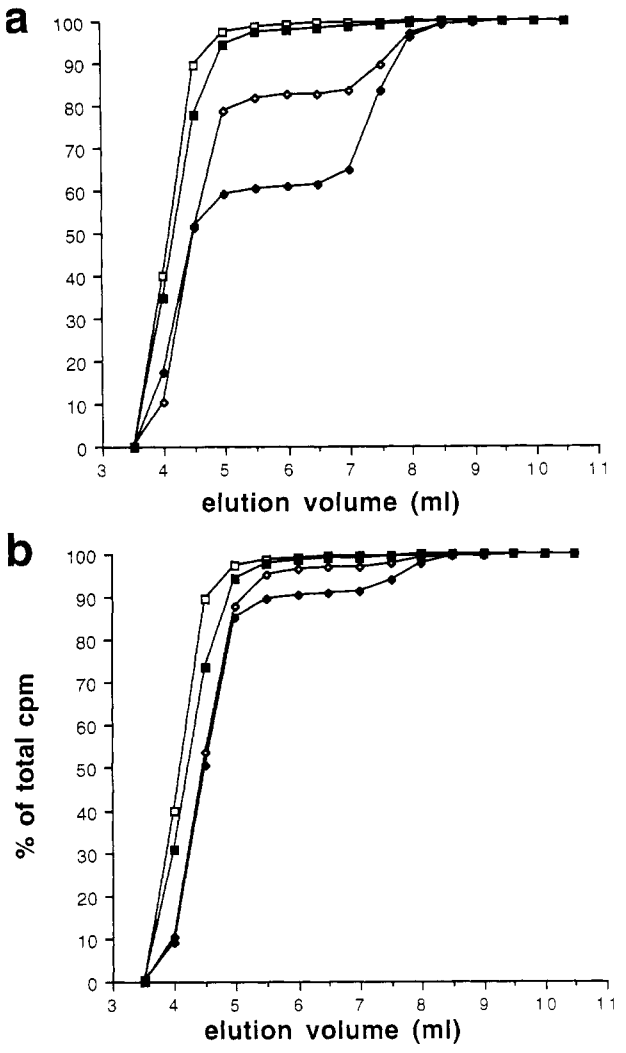

FIGURE 3: Exo-β-galactosidase digestion of [^{3}H]Gal-labeled GlyCAM-1. Parallel samples (40 000 cpm each) of undersulfated (panel a) and normal (panel b) [^{3}H]Gal-GlyCAM-1 were digested with β(1→4)galactosidase in combination with the following enzymes: *Arthrobacter ureafaciens* sialidase (◇), α(1→3/4)fucosidase (■), or *Arthrobacter ureafaciens* sialidase plus α(1→3/4)fucosidase (◆). Digests and a control sample without any enzyme (□) were analyzed by gel filtration on the column described in Figure 1. Integration analysis was carried out as described in the caption to Figure 1a.

Table 5: Release of Gal from GlyCAM-1 by exo-β(1→4)galactosidase[a]

presence of		release of [^{3}H]Gal from GlyCAM-1 (%) by	
Sia	SO_4	exo-β(1→4) galactosidase	exo-β(1→4)galactosidase + exo-α(1→3/4)fucosidase
+	+	1.2	1.3
+	–	2.0	2.0
–	+	3.2	9.1
–	–	17	39

[a] Normal or undersulfated [^{3}H]Gal-labeled GlyCAM-1, with or without desialylation by *Arthrobacter* sialidase, was treated with *Diplococcus* exo-β(1→4)galactosidase alone or combined with *Streptomyces* exo-α(1→3/4)fucosidase. The percent release of [^{3}H]Gal was determined as described in the caption of Figure 3.

with *Diplococcus* exo-β(1→4)galactosidase, an enzyme with strict specificity for Galβ(1→4) (Glasgow et al., 1977). In a repeat experiment, a maximum of 35% of the galactose was released.

(2) Almost all of the Gal that is susceptible to the exo-β-(1→4)galactosidase must normally be sialylated, as judged by treating undersulfated GlyCAM-1 with a combination of β-galactosidase plus α(1→3/4)fucosidase. Without desialylation, only 2% of the [^{3}H]Gal counts were released, compared to 39% released with sialic acid removed. In the context of a sLe^x-type structure, this conclusion is completely compatible with the previous demonstration (Table 3; Figure 1) that α(1→3/4)fucosidase did not release [^{3}H]fucose unless GlyCAM-1 (normal or undersulfated) was also desialylated.

(3) Since β-galactosidase alone released 17% of the [^{3}H]-Gal from undersulfated/desialylated GlyCAM-1, and β-galactosidase combined with fucosidase released 39% of the [^{3}H]Gal, it is estimated that 57% of the GlcNAc residues closest to the nonreducing termini are substituted with fucose. In an independent analysis (12% and 35% release of free [^{3}H]-Gal, respectively), this estimate was 66%. These calculations rely on the observation that β(1→4)galactosidase can cleave galactose from a Lewis x structure only if fucose is first removed [Maemura and Fukuda (1992) and Figure 2].

(4) The presence of sulfation on GlyCAM-1 greatly hindered the release of [^{3}H]Gal by treatment with a combination of exo-β(1→4)galactosidase and α(1→3/4)fucosidase (Table 5). In the preceding study, we documented that sulfation completely blocks the cleavage of galactose from (SO_4-6)-Galβ1→4GlcNAc by a β-galactosidase. From the activity of *Diplococcus* β-galactosidase in releasing [^{3}H]Gal from defucosylated/*sulfated* GlyCAM-1 vs defucosylated/*undersulfated* GlyCAM-1 (9.1% vs 39%) (Figure 3; Table 5), we estimate that 77% of the penultimate Galβ1→4 residues are substituted with sulfate. In an independent analysis, the respective determinations were 10% vs 35%, giving an estimate of 72%. These findings provide further evidence that the galactose residues in β1→4 linkages are substituted with sulfate. Since the sialic acid occurs in an α2→3 linkage to Gal (Tables 1 and 2), these results provide further support for the presence of 6-sulfation on the majority (~70%) of the penultimate Galβ1→4 residues.

DISCUSSION

In our companion study (Hemmerich et al., 1994), the linkages of sialic acid and fucose within GlyCAM-1 could not be assigned because of the hydrolysis conditions used to obtain sulfated fragments. In the present study, we have examined the structural relationship of sialic acid and fucose to the sulfated *N*-acetyllactosamine core. To define the position of sialylation relative to sulfation, we took advantage of two lectin groups with complementary specificity for *N*-acetyllactosamine substituted on galactose with either 6-sulfation or 3-sialylation. The finding that desialylation or undersulfation of GlyCAM-1 exposed cryptic binding sites for TJA-1/SNA or for MAA, respectively, argues that structure **1** is part of a capping group for GlyCAM-1. The observation that the TJA-1/SNA site was exposed with NDV sialidase provides independent evidence that the sialic acid must be in an α2→3 linkage. The pronounced effect of both sulfation and sialylation on the release of galactose by β(1→4)galactosidase also suggests the dual substitution of subterminal galactose with 3-sialylation and 6-sulfation. Finally, the lectin and glycosidase analyses presented herein are consistent with the findings (Hemmerich et al., 1994) that (SO_4-6)Galβ1→4GlcNAc and Gal-6-SO_4 are major constituents of GlyCAM-1.

Our analysis of the structural relationship of fucose to structure **1** was guided by the knowledge that sLe^x-related structures have been detected in lymph node HEV ligands (Sawada et al., 1993) and that Fucα1→3 is essential to the ligand activity of sLe^x (Imai et al., 1992). The application

of specific fucosidases established that the linkage of at least 85% of fucose is Fucα1→3/4. sLe^x shows a requirement for desialylation before fucose can be released by the *Streptomyces* α(1→3/4)fucosidase (Maemura & Fukuda, 1992; Sano et al., 1992). GlyCAM-1 exhibits a similar behavior, indicating the proximal location of the susceptible fucose moieties to the terminal sialic acid. Moreover, the proximal location of fucose relative to the penultimate Galβ(1→4) is strongly suggested by our finding that galactose release from GlyCAM-1 by *Diplococcus* β(1→4)galactosidase depends on removal of fucose. Finally, we found that treatment of GlyCAM-1 with α(1→3/4)fucosidase dramatically enhances binding to LEA, a lectin with specificity for β1→4-linked *N*-acetylglucosamine.

Taken together, the present results indicate the occurrence of terminal fucose in an α1→3 linkage to GlcNAc in structure **1**. The resulting structure, 6′-sulfated sialyl Lewis x, is hereafter designated structure **2**:

$$\text{Sia}\alpha2\rightarrow3(SO_4\text{-}6)\text{Gal}\beta1\rightarrow4(\text{Fuc}\alpha1\rightarrow3)\text{GlcNAc} \quad (2)$$

Our quantitative analysis indicates that structure **2** represents a major capping group of GlyCAM-1. At least 39% of total galactose within GlyCAM-1 is in the form of β(1→4)-Gal, which is in a subterminal position relative to Sia. The NDV sialidase results indicate that the predominant and probably exclusive linkage of Sia is α2→3. About 70–80% of the subterminal β(1→4)Gal residues appear to be sulfated. The lectin data also strongly argue that the majority of these Gal residues are simultaneously modified with sialic acid and sulfate. Finally, we estimate that about 60–70% of the GlcNAc residues in the capping structures are substituted by Fucα(1→3).

Structure **2** does not accommodate the occurrence of $\text{Gal}\beta1\rightarrow4(SO_4\text{-}6)\text{GlcNAc}$ within GlyCAM-1, which was revealed in the companion study (Hemmerich et al., 1994). We do not know at present whether the GlcNAc in the capping structure is modified by 6-sulfation or whether more internal *N*-acetyllactosamine units bear this modification. A likely possibility is that α(1→3)fucosylation and 6-sulfation represent mutually exclusive substitutions on the GlcNAc residues. This notion is supported by the observation that undersulfated GlyCAM-1 incorporates twice as much fucose as normal GlyCAM-1, while incorporation of [^{3}H]Gal label was found to be sulfation independent.

Structure **2** represents a proposal for a major capping group of the O-linked chains of GlyCAM-1. We are not aware of any previously described examples of structure **2**. However, $\text{Sia}\alpha2\rightarrow3(SO_4\text{-}6)\text{Gal}\beta1\rightarrow4\text{GlcNAc}$ has been described as a minor substituent in a recombinant form of tissue plasminogen activator (Pfeiffer et al., 1992). Confirmation of structure **2** will require the application of physical techniques and methylation analysis. Chemical quantities of GlyCAM-1, as opposed to the radiochemical levels used here, will be necessary for these studies.

A critical question for future investigation is whether the previously reported weak affinity of sLe^x-related structures for L-selectin (Imai et al., 1992) can be substantially enhanced by 6-sulfation of Gal. The fact that the avidity of GlyCAM-1 for L-selectin is dramatically reduced by desulfation makes this possibility extremely attractive (Imai et al., 1993). We are currently pursuing the synthesis of 6′-sulfated sLe^x in order to test this proposal.

ACKNOWLEDGMENT

We thank Carolyn Bertozzi for her assistance with HPLC analysis and for many helpful discussions. TJA-1 was the kind gift of Dr. K. Yamashita of Sasaki Institute, Tokyo, received through Dr. Larry Lasky of Genentech Inc. LNF-III was a kind gift of Dr. Rolf Nuck, Freie Universität Berlin.

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