L-Selectin−Carbohydrate Interactions: Relevant Modifications of the Lewis x Trisaccharide†

William J. Sanders,‡ Tamiko R. Katsumoto,§ Carolyn R. Bertozzi,§,|| Steven D. Rosen,*,§ and Laura L. Kiessling*,‡

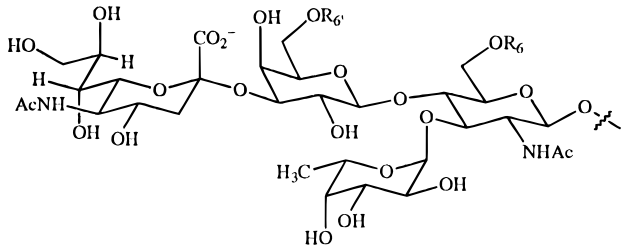
Department of Chemistry, University of Wisconsin−Madison, Madison, Wisconsin 53706, and Department of Anatomy and Program in Immunology, University of California−San Francisco, San Francisco, California 94143

Received June 10, 1996; Revised Manuscript Received September 16, 1996⊗

ABSTRACT: Protein−carbohydrate interactions are known to mediate cell−cell recognition and adhesion events. Specifically, three carbohydrate binding proteins termed selectins (E-, P-, and L-selectin) have been shown to be essential for leukocyte rolling along the vascular endothelium, the first step in the recruitment of leukocytes from the blood into inflammatory sites or into secondary lymphoid organs. Although this phenomenon is well-established, little is known about the molecular-level interactions on which it depends. All three selectins recognize sulfated and sialylated derivatives of the Lewis x [Le^x: Galβ1→4(Fucα1→3)GlcNAc] and Lewis a [Le^a: Galβ1→3(Fucα1→4)GlcNAc] trisaccharide cores with affinities in the millimolar range, and it is believed that variants of these structures are the carbohydrate determinants of selectin recognition. Recently it was shown that the mucin GlyCAM-1, a secreted physiological ligand for L-selectin, is capped with sulfated derivatives of sialyl Lewis x [sLe^x: Siaα2→3Galβ1→4(Fucα1→3)GlcNAc] and that sulfation is required for the high-affinity interaction between GlyCAM-1 and L-selectin. To elucidate the important sites of sulfation on Le^x with respect to L-selectin recognition, we have synthesized six sulfated Le^x analogs and determined their abilities to block binding of a recombinant L-selectin−Ig chimera to immobilized GlyCAM-1. Our results suggest that 6-sulfo sLe^x binds to L-selectin with higher affinity than does sLe^x or 6′-sulfo sLe^x and that sulfation of sLe^x capping groups on GlyCAM-1 at the 6-position is important for L-selectin recognition.

The targeting of leukocytes to sites of tissue injury and to secondary lymphoid organs (e.g., lymph nodes) is a critical event for immune defense. Recent discoveries have led to the identification of a family of three proteins known as the selectins that mediate leukocyte tethering and rolling along the vascular endothelium [see Rosen and Bertozzi (1994), Lasky (1995), McEver et al. (1995), and Tedder et al. (1995) for recent reviews], the initial step in leukocyte recruitment from the blood [reviewed in Springer (1995)]. The discovery that the selectins contain an *N*-terminal C-type lectin domain (a Ca^{+2}-dependent carbohydrate binding domain) (Drickamer, 1993) launched an intense search for the carbohydrate determinants of selectin recognition. Elucidation of these determinants may provide leads for small molecule inhibitors of selectin binding and potential drug candidates for the treatment of inflammatory disorders.

High-affinity physiological ligands have been identified for L-selectin (Lasky et al., 1992; Baumhueter et al., 1993; Berg et al., 1993; Briskin et al., 1993), P-selectin (Norgard et al., 1993; Sako et al., 1993), and E-selectin (Patel et al., 1994; Steegmaier et al., 1995; Stroud et al, 1996). One of the best characterized of the physiological selectin ligands



1 sLe^x: $R_6 = R_{6'} = H$
2 6–sulfo sLe^x: $R_6 = SO_3^-$, $R_{6'} = H$
3 6'–sulfo sLe^x: $R_6 = H$, $R_{6'} = SO_3^-$

FIGURE 1: Carbohydrate determinants present on the L-selectin ligand GlyCAM-1.

is the mucin GlyCAM-1 (Lasky et al., 1992), an L-selectin ligand. It has been recently shown that GlyCAM-1 is highly sulfated, exhibiting the major capping groups 6′-sulfo sialyl Lewis x [sLe^x: Siaα2→3Galβ1→4(Fucα1→3)GlcNAc; Figure 1][1] and 6-sulfo sLe^x (Figure 1), which are extended from core-2 structures (Hemmerich & Rosen, 1994; Hemmerich et al., 1994a, 1995), and that sulfation is required for high-affinity binding to L-selectin (Imai et al., 1993; Hemmerich et al., 1994b; Crommie & Rosen, 1995; Rosen & Bertozzi,

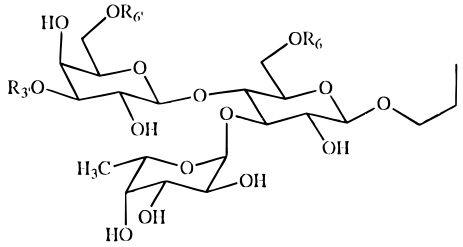
† This research was supported by grants from the NIH (GM49975 to L.L.K. and GM23547 and NS32254 to S.D.R) and the NSF (NYI. Award to L.L.K.). W.J.S. was supported by the NIH Chemistry−Biology Interface Training Program (T32-GM08505), and C.R.B. was supported by a postdoctoral fellowship from the American Cancer Society.

‡ University of Wisconsin−Madison.
§ University of California−San Francisco.
|| Present address: Department of Chemistry, University of California−Berkeley, Berkeley, CA 94720.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: sLe^x, sialyl Lewis x; Le^x, Lewis x; Le^a, Lewis a; Sia, *N*-acetylneuraminic acid; Gal, galactose; Fuc, fucose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Le^x(Glc), Lewis x modified by replacement of *N*-acetylglucosamine with glucose; ELISA, enzyme-linked immunosorbent assay; M6P, mannose-6-phosphate; HMQC, heteronuclear multiple quantum coherence; LSIMS, liquid secondary ion mass spectrometry; MS, mass spectrum; HRMS, high-resolution mass spectrum; MPM, *p*-methoxybenzyl; Bn, benzyl; PhH, benzene; Pyr·SO_3, pyridine−sulfur trioxide complex; ↑↓, reflux.

S0006-2960(96)01364-5 CCC: $12.00 © 1996 American Chemical Society



4 $R_{3'} = R_{6'} = R_6 = H$ [Le^x(Glc)]
5 $R_{3'} = R_{6'} = H$, $R_6 = SO_3Na$
6 $R_{3'} = SO_3Na$, $R_6 = R_{6'} = H$
7 $R_{3'} = R_{6'} = SO_3Na$, $R_6 = H$
8 $R_{3'} = H$, $R_6 = R_{6'} = SO_3Na$
9 $R_{3'} = R_6 = SO_3Na$, $R_{6'} = H$
10 $R_{3'} = R_{6'} = R_6 = SO_3Na$

FIGURE 2: Synthetic L-selectin ligands.

1996). The occurrence of these sLe^x-based epitopes on GlyCAM-1 combined with the sulfation requirement raises the possibility that sulfation at either the 6- or the 6′-position of sLe^x, or both, could lead to high-affinity, selective binding of L-selectin.

In an effort to determine the importance of sites of sulfation with respect to selectin recognition, several groups have made sulfated carbohydrates related to sLe^x. In one such study, Kiessling and co-workers synthesized a series of sulfated derivatives of the Lewis a trisaccharide [including the 6′-sulfo sLe^x analog 3′,6′-disulfo Le^a(Glc)] but found little or no difference in their abilities to inhibit L-selectin binding to GlyCAM-1 (Manning et al., 1995). In another study, Rosen and co-workers synthesized five sulfated derivatives of lactose and observed that the 6′,6-disulfo derivative was the most effective of these at blocking the L-selectin−GlyCAM-1 interaction (Bertozzi et al., 1995). Finally, Scudder et al., using a combination of chemical and enzymatic methods, synthesized the Siaα2→3Galβ1→4-(Fucα1→3)GlcNAc-6-*O*-SO_3β1→3 Gal pentasaccharide, which bears the 6-sulfo sLe^x determinant (Scudder et al., 1994). This synthetic pentasaccharide was found to be 4-fold more effective at blocking binding of L-selectin to a peripheral lymph node addressin (a collection of mucin-like glycoproteins that are recognized by L-selectin) than the sLe^x tetrasaccharide; however, because a tetrasaccharide and pentasaccharide were compared in this study, a direct assessment of the contribution due to the 6-sulfo group could not be made. Although these and related studies provide insights into the issue of L-selectin recognition, questions regarding the role of sulfation at various sites on sLe^x remain largely unresolved.

In the course of our continuing research program aimed at elucidation of the molecular level interactions responsible for selectin−carbohydrate recognition and development of multivalent inhibitors of these processes, we felt it necessary to determine the relative inhibitory potencies of various monovalent sulfo Le^x analogs for L-selectin. Because sLe^x demonstrates anti-inflammatory activity in acute disease models [reviewed by Rosen and Bertozzi (1994)], the identification of the important sites of sulfation has potential ramifications for the design of antiinflammatory agents. Herein we present the results of inhibition studies of six sulfated derivatives of Lewis x. These six derivatives (Figure 2) were chosen to test the effects of sulfation at the 6- and 6′-positions of sLe^x with respect to L-selectin recognition and to identify the most potent Le^x-based inhibitor. We employed an ELISA that measures the interaction of L-selectin with GlyCAM-1, one of its physiological ligands (Rosen & Bertozzi, 1994).

MATERIALS AND METHODS

General. Compounds **5**−**10** were generated by chemical synthesis from intermediates **11a** and **11b** using the conditions shown in Scheme 1. Complete synthetic protocols for their preparation will be published elsewhere. ^{1}H NMR spectra were recorded on a Varian Unity 500 Fourier transform spectrometer at 500 MHz. ^{13}C chemical shifts were obtained by analysis of HMQC (heteronuclear multiple quantum coherence) experiments performed on the Varian Unity 500 Fourier transform spectrometer. NMR chemical shifts are reported in ppm downfield from tetramethylsilane, with peak multiplicity reported as follows: s = singlet, d = doublet, t = triplet, sx = sextet, m = multiplet, app = apparent, b = broad. Mass spectra were obtained on a VG AutoSpec M (LSIMS).

*Spectral Data for **5**−**10***

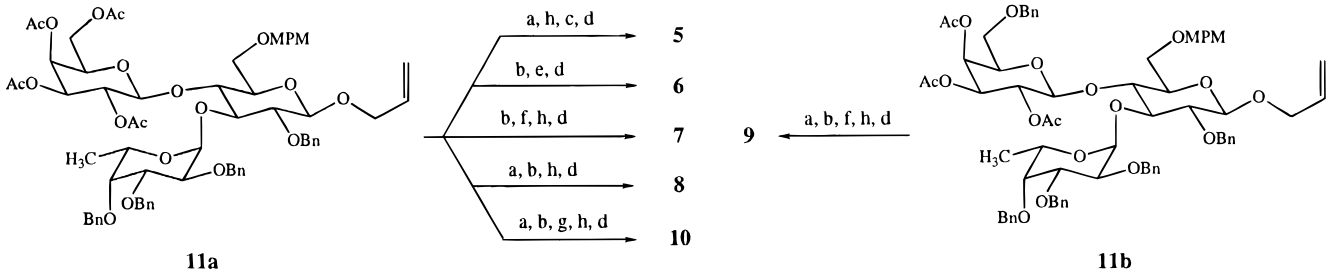
*6-Sulfo Le^x(Glc) (**5**).* ^{1}H NMR (500 MHz, D_2O, 37 °C): δ5.40 (1H, d, J = 4.0), 4.77 (1H, bq, J = 6.7), 4.49 (1H, d, J = 7.8), 4.46 (1H, d, J = 8.0), 4.34−4.29 (2H, m), 3.94−3.87 (3H, m), 3.85−3.80 (1H, m), 3.77−3.67 (6H, m), 3.64−3.56 (3H,m), 3.49 (1H, dd, J = 8.1, 9.1), 3.44 (1H, app dd, J = 7.8, 9.8), 1.58 (2H, sx, J = 7.3), 1.15 (3H, d, J = 6.7), 0.88 (3H, t, J = 7.4). ^{13}C NMR (125 MHz, D_2O, 37 °C): δ107.8, 107.2, 104.1, 82.8, 80.7, 80.1, 78.7, 78.3, 78.2, 78.0, 77.8, 76.9, 75.0, 74.2, 73.9, 72.2, 71.8, 67.2, 27.8, 20.9, 15.4. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 609.0 [M^-; calcd for $C_{21}H_{37}O_{18}S$, 609.2].

*3′-Sulfo Le^x(Glc) (**6**).* ^{1}H NMR (500 MHz, D_2O, 37 °C): δ5.44 (1H, d, J = 4.0), 4.77 (1H, bq, J = 6.8), 4.53 (1H, d, J = 7.8), 4.47 (1H, d, J = 8.1), 4.31 (1H, app dd, J = 3.3, 9.9), 4.26 (1H, app d, J = 3.3), 4.00−3.93 (2H, m), 3.90−3.70 (8H, m), 3.65−3.55 (4H, m), 3.50 (1H, dd, J = 8.3, 9.0), 1.61 (2H, sx, J = 7.2), 1.18 (3H, d, J = 6.7), 0.90 (3H, t, J = 7.4). ^{13}C NMR (125 MHz, D_2O, 37 °C): δ104.9, 104.7, 101.4, 83.1, 80.0, 78.1, 77.6, 77.6, 76.5, 75.4, 75.1, 74.8, 72.0, 70.7, 69.6, 69.6, 64.2, 62.6, 24.7, 17.8, 12.4. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 609.1 [M^-; calcd for $C_{21}H_{37}O_{18}S$, 609.2].

*3′,6′-Disulfo Le^x(Glc) (**7**).* ^{1}H NMR (500 MHz, D_2O, 40 °C): δ5.38 (1H, d, J = 4.0), 4.74 (1H, bq, J = 6.6), 4.51 (1H, d, J = 7.9), 4.42 (1H, d, J = 8.1), 4.30 (1H, app dd, J = 3.4, 9.8), 4.27 (1H, app d, J = 3.4), 4.16−4.09 (2H, m), 3.97−3.91 (2H, m), 3.87−3.76 (5H, m), 3.73−3.69 (2H, m), 3.62−3.53 (3H, m), 3.48 (1H, dd, J = 8.1, 9.1), 1.58 (2H, sx, J = 7.2), 1.14 (3H, d, J = 6.7), 0.87 (3H, t, J = 7.4). ^{13}C NMR (125 MHz, D_2O, 37 °C): δ108.0, 107.4, 104.3, 85.9, 83.4, 81.2, 80.4, 79.3, 78.2, 78.0, 77.9, 75.1, 75.1, 74.2, 73.0, 72.4, 72.3, 65.8, 28.0, 21.1, 15.4. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 711.1 [M^{2-} + Na^+; calcd for $C_{21}H_{36}O_{21}NaS_2$, 711.2].

*6′,6-Disulfo Le^x(Glc) (**8**).* ^{1}H NMR (500 MHz, D_2O, 37 °C): δ5.35 (1H, d, J = 4.1), 4.74 (1H, bq, J = 6.9), 4.48 (1H, d, J = 8.1), 4.44 (1H, d, J = 8.1), 4.35−4.25 (2H, m), 4.09 (2H, d, J = 6.9), 3.92−3.68 (9H, m), 3.62 (1H, app dd, J = 4.0, 10.0), 3.60−3.53 (1H, m), 3.50−3.41 (2H, m),

Scheme 1[a]



[a] Reaction conditions: (a) $(NH_4)_2Ce(NO_3)_6$, CH_3CN/H_2O (9:1). (b) K_2CO_3, MeOH. (c) NaOH, H_2O, MeOH, pH 12. (d) H_2, $Pd(OH)_2/C$, MeOH, H_2O, 500 psi. (e) 1.0 equiv of Bu_2SnO, PhH, ↑↓; Pyr·SO_3. (f) 1.0 equiv of $(Bu_3Sn)_2O$, PhH, ↑↓. (g) 1.5 equiv of $(Bu_3Sn)_2O$, PhH, ↑↓. (h) Pyr·SO_3, pyridine.

1.56 (2H, sx, $J = 7.3$), 1.13 (3H, d, $J = 6.8$), 0.85 (3H, t, $J = 7.4$). ^{13}C NMR (125 MHz, D_2O, 37 °C): δ107.0, 106.8, 103.8, 82.6, 79.3, 78.2, 77.9, 77.4, 77.2, 77.2, 77.1, 76.0, 74.3, 73.3, 73.1, 71.8, 71.6, 71.1, 27.3, 20.4, 14.7. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 711.1 [$M^{2-} + Na^+$; calcd for $C_{21}H_{36}O_{21}NaS_2$, 711.2].

*3′,6-Disulfo Le^x(Glc) (**9**).* ^{1}H NMR (500 MHz, D_2O, 35 °C): δ5.37 (1H, d, $J = 4.0$), 4.70 (1H, bq, $J = 6.6$), 4.54 (1H, d, $J = 7.8$), 4.42 (1H, d, $J = 8.1$), 4.31−4.23 (3H, m), 4.20 (1H, bd, $J = 3.3$), 3.90−3.86 (2H, m), 3.81−3.76 (1H, m), 3.73−3.64 (6H, m), 3.59−3.51 (3H, m), 3.45 (1H, dd, $J = 8.2, 9.1$), 1.55 (2H, sx, $J = 7.2$), 1.11 (3H, d, $J = 6.6$), 0.83 (3H, t, $J = 7.4$). ^{13}C NMR (125 MHz, D_2O, 32 °C): δ107.2, 106.2, 103.4, 85.2, 81.9, 79.5, 79.5, 77.6, 77.3, 77.2, 77.0, 77.0, 74.2, 74.2, 73.1, 71.6, 71.4, 70.9, 26.8, 19.8, 18.6. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 710.9 [$M^{2-} + Na^+$; calcd for $C_{21}H_{36}O_{21}NaS_2$, 711.2].

*3′,6′,6-Trisulfo Le^x(Glc) (**10**).* ^{1}H NMR (500 MHz, D_2O, 32 °C): δ5.34 (1H, d, $J = 3.9$), 4.71 (1H, bq, $J = 6.5$), 4.53 (1H, d, $J = 7.8$), 4.41 (1H, d, $J = 8.0$), 4.33−4.23 (4H, m), 4.11−4.05 (2H, m), 3.89−3.62 (8H, m), 3.57−3.51 (2H, m), 3.46 (1H, dd, $J = 8.1, 9.0$), 1.54 (2H, sx, $J = 7.3$), 1.10 (3H, d, $J = 6.6$), 0.82 (3H, t, $J = 7.4$). ^{13}C NMR (125 MHz, D_2O, 32 °C): δ107.4, 106.7, 103.8, 85.2, 82.7, 79.7, 78.5, 78.3, 77.7, 77.4, 77.3, 74.5, 74.5, 73.6, 72.2, 71.8, 71.7, 71.5, 27.4, 20.5, 14.9. MS (LSIMS, 3-NBA, negative ion mode): *m/z* 813.2 [$M^{3-} + 2Na^+$; calcd for $C_{21}H_{35}O_{24}Na_2S_3$, 813.2].

Preparation of GlyCAM-1 for Inhibition Binding Assay. GlyCAM-1 is synthesized by the specialized endothelial cells of high endothelial venules of murine lymph nodes. It is secreted into serum to a level of 1 μg/mL (Singer & Rosen, 1996). GlyCAM-1 was partially purified from commercially obtained mouse serum as previously described (Bertozzi et al., 1995).

L-Selectin−GlyCAM-1 Inhibition ELISA. A capture ELISA was used as previously described (Bertozzi et al., 1995). A more complete description of the development of this assay will be presented elsewhere. Briefly, 96-well plates were coated with a polyclonal antibody specific for the peptide core of GlyCAM-1. The ligand was captured from partially purified serum and then reacted with an immunoadhesion complex formed between a mouse L-selectin−Ig chimera (Watson et al., 1990), biotinylated goat anti-human Fc, and streptavidin-alkaline phosphatase. *p*-Nitrophenylphosphate was used as a colorimetric substrate for reading at 405 nm. Carbohydrate inhibitors were incubated with the L-selectin complex for 30 min at 4 °C before transfer to the plate. Complete dose−response curves were carried out for each saccharide derivative with replicate determinations for each concentration. The inhibition curves were smooth isotherms as previously observed with this assay (Bertozzi et al., 1995). Concentrations required for 50% inhibition (IC_{50}'s) were determined in each run. The mean IC_{50}'s with associated standard errors were computed based on multiple independent analyses. For all saccharide inhibitors except compounds **7** (3 runs) and **8** (2 runs), four independent dose−response curves were generated. The Student−Newman−Keuls multiple comparisons test was used to evaluate the significance of the IC_{50} differences between the inhibitors (Instat Instant Statistics, Graftpad Software, San Diego, CA).

RESULTS

The six sulfated derivatives of Lewis x in Figure 2 were generated by chemical synthesis. Our objective was to determine the effects of sulfation at each of the potentially important sites of Le^x on L-selectin recognition. To simplify the structures and synthesis of these sLe^x analogs, two conservative modifications to the core structure were made. The substitution of glucose for glucosamine in the Le^x core has been demonstrated to have little or no effect on sLe^x−selectin binding (Nelson et al., 1993). This change facilitates the synthesis of such derivatives by eliminating the need for cumbersome nitrogen protecting groups and improving the solubility of intermediates in organic solvents. The key functionality of the sialic acid residue on sLe^x necessary for selectin recognition appears to be the negatively charged carboxylate group. For example, modifications of the sialic acid residue have little effect on the L-selectin inhibitory properties of the resulting molecules (Brandley et al., 1993). Replacement of the entire sialic acid residue with a sulfate ester (Yuen et al., 1992, 1994; Brandley et al., 1993; Green et al., 1995) provides a ligand with equal or greater affinity for the selectins than sLe^x itself. Furthermore, the identification of 3′-sulfo Le^x in naturally occurring selectin ligands (Yuen et al., 1992, 1994) suggests that it may be a relevant biological determinant. To eliminate the considerable difficulties involved with sialoside synthesis and to provide simple structures from which to design new selectin inhibitors, we chose to replace the sialic acid residue with a sulfate group. Consequently, 3′-sulfo Le^x(Glc) [compound **6**, Figure 2] was employed as a sLe^x analog for these studies, a choice that is supported by its selectin inhibitory properties (vide infra).

The synthesis of compounds **5**−**10** from appropriate trisaccharide precursors is depicted in Scheme 1 [for syntheses of sulfo Le^x derivatives see Chandrasekara et al. (1992), Nicolaou et al. (1993), Jain et al. (1994), Lubineau and Lemoine (1994), Numomura et al. (1994), Singh et al.

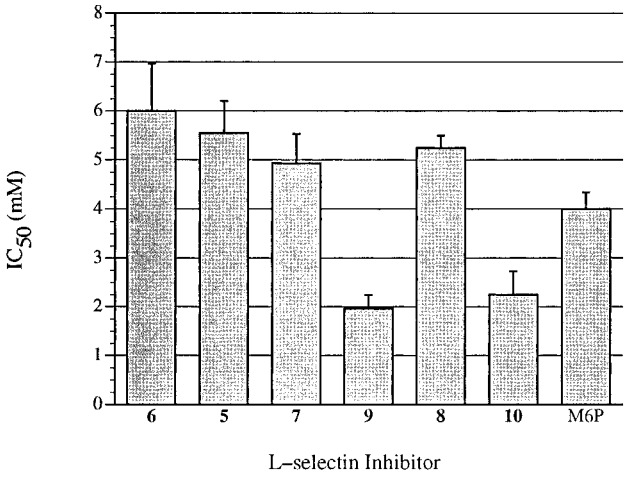


FIGURE 3: IC_{50} values for synthetic sulfo Le^x derivatives and mannose-6-phosphate. Error bars correspond to standard errors of the mean. The following differences were statistically significant on the basis of the Student−Newman−Keuls multiple comparisons test: 3′6 vs 3′ ($p < 0.01$), 3′6 vs 6 ($p < 0.01$), 3′6 vs 6′6 ($p < 0.05$), 3′6 vs 3′6′ ($p < 0.05$), 3′6′6 vs 3′ ($p < 0.01$), 3′6′6 vs 6 ($p < 0.01$), 3′6′6 vs 6′6 ($p < 0.05$), and 3′6′6 vs 3′6′ ($p < 0.05$). All other comparisons were not statistically significant ($p > 0.05$).

(1994), Hasegawa et al. (1995), and Vig et al. (1995)]. The trisaccharides **11a** and **11b** were synthesized in a sequence of nine linear steps by a route related to that reported for the synthesis of sulfated Lewis a derivatives (Manning et al., 1995). From **11a** and **11b**, all six sulfo Le^x derivatives were prepared using a combination of regioselective deprotection and sulfation conditions, culminating in hydrogenation of the benzyl protecting groups and allyl aglycone linker arm. After purification, the sulfates **5**−**10** were converted to their respective sodium salts for biological evaluation. This route provided all six of the desired compounds with high purity in 12−14 synthetic steps and 10−20% overall yield. The efficiency of this synthesis facilitated the production of significant amounts of material in a short period of time. In addition, the use of an allyl aglycone linker in trisaccharides **11a** and **11b** provides a handle for further modification and elaboration into multivalent derivatives (Brüning & Kiessling, 1996).

Compounds **5**−**10** were tested in an enzyme-linked immunosorbent assay (ELISA) for their ability to block binding of a recombinant L-selectin−Ig chimera to immobilized GlyCAM-1 (Bertozzi et al., 1995). The inhibitory potencies of the synthetic compounds were compared to that of the known L-selectin inhibitor, mannose-6-phosphate (M6P) (Imai et al., 1990). IC_{50} values, derived from multiple independent dose−response determinations, were compared. The IC_{50}'s ranged from 2 to 6 mM (Figure 3). We found that 3′-sulfo Le^x(Glc) exhibits an IC_{50} approximately one and a half times that of M6P (Figure 3). The IC_{50} of M6P has previously been determined to be close to that of sLe^x in the same assay (Bertozzi et al., 1995). Brandley et al. report that 3′-sulfo Le^x and sLe^x show distinct differences in binding to L-selectin (Brandley et al., 1993); however, they behave indistinguishably in this assay. Our results support the choice of **6** as an sLe^x equivalent, and provide further evidence that the most critical feature of the sialic acid residue in the L-selectin−sLe^x interaction is its negative charge.

On the basis of the identification of sLe^x sulfated at the 6′-position as a major GlyCAM-1 capping group, it was suggested (Hemmerich et al., 1994a) that sulfation of sLe^x at the 6′-position might be a defining modification for L-selectin recognition. This determinant has not been previously observed; moreover, it contains all the functionality shown to be necessary for the interaction of L-selectin with GlyCAM-1: sialic acid, fucose, and a sulfate group (Hemmerich et al., 1994a,b). Contrary to expectations, 3′,6′-disulfo Le^x(Glc), compound **7**, was no more effective than 3′-sulfo Le^x(Glc) at inhibiting the L-selectin−GlyCAM-1 interaction ($p > 0.05$, not significant), indicating that 6′-sulfation of the Le^x core does not confer a measurable stabilization to the selectin−saccharide interaction. This result, along with previous studies of sulfo Le^a derivatives (Manning et al., 1995), argues that monovalent 6′-sulfo sLe^x by itself is not a high-affinity recognition element for L-selectin.

Because 6-sulfo sLe^x was also identified as a GlyCAM-1 capping group (Hemmerich et al., 1995), we sought to compare the effect of 6-sulfation to that of 6′-sulfation on the Le^x template. Our 6-sulfo sLe^x analog **9** [3′,6-disulfo Le^x(Glc)] showed a 3-fold increase in inhibitory potency relative to 3′-sulfo Le^x(Glc) **6** ($p < 0.01$). The enhancement in inhibitory ability of the disulfated derivative **9** over the 3′-sulfated analog **6** is in accord with the increased activity of 6-sulfo sLe^x observed in a study of L-selectin binding to peripheral lymph node addressin (Scudder et al., 1994). Thus, the majority of the binding enhancement observed by Scudder et al. may be attributed to the 6-sulfo group. Interestingly, compound **5**, possessing a single 6-sulfo group, was as effective as 3′-sulfo Le^x(Glc) **6**. Because both sialic acid (or an anionic group at the 3′-position) and sulfation are critical for L-selectin recognition, it is reasonable that ligands that have one of these modifications can bind. Our results indicate that while monovalent ligands with a single anionic group at either the 3′- or the 6-position are inhibitors, compounds with anionic groups at both positions are better inhibitors. Together, these results indicate that 6-sulfation of sLe^x is a favorable modification, which likely contributes to the high-affinity binding of L-selectin in physiological settings.

Given that 6-sulfation in concert with 3′-sulfation enhances the interaction of Le^x derivatives with L-selectin, we investigated the ability of 6′-sulfation to substitute for 3′-sulfation. In the case of sulfated lactose ligands for L-selectin, it was shown that the combination of 6- and 6′-sulfation provided an inhibitor that was 3-fold more effective than 3′-sulfo lactose at inhibiting L-selectin (Bertozzi et al., 1995). To determine the effect of these modifications on the binding of the Le^x template, we synthesized trisaccharide **8** [6′,6-disulfo Le^x(Glc)]. In the Le^x-based system, the inhibitory ability of compound **8** was indistinguishable from that of 3′-sulfo Le^x(Glc) **6** ($p > 0.05$, not significant). This result combined with the data on 3′,6′-disulfo Le^x(Glc) **7** leads to the conclusion that 6′-sulfation of the Le^x scaffold has no effect on the interaction between Le^x and L-selectin as measured in our ELISA.

Analyses of the GlyCAM-1 *O*-glycans indicate that multiple sulfate groups may be presented within a single determinant, such as in 6′,6-disulfo sLe^x (Hemmerich et al., 1995). To test the effect of sulfation at all of the potentially relevant sites on Le^x, the inhibitory potency of **10** [3′,6′,6-trisulfo Le^x(Glc)] was determined. This compound was found to be approximately three times more effective than 3′-sulfo Le^x(Glc) **6** at inhibiting the L-selectin−GlyCAM-1

interaction ($p < 0.01$), similar to 3′,6-disulfo Le^x(Glc) **9**. Again, this data suggests that 6′-sulfation of Le^x has no effect on Le^x−L-selectin recognition in the ELISA while 6-sulfation is stabilizing.

DISCUSSION

Our data have consequences for the generation of L-selectin inhibitors. These results suggest that the L-selectin binding site may contain two positively charged residues that are important for carbohydrate recognition. One site can be occupied by an anionic group at the 3′-position of Le^x, and the other site is effectively filled by a negatively charged group at the 6-position. This conclusion is supported by the observation that 6-sulfation of Le^x(Glc) is approximately as effective as 3′-sulfation in blocking L-selectin recognition, while the combination of both is more effective by a factor of about three in both cases studied here (compounds **9** and **10**). In the case of E-selectin−carbohydrate recognition, inhibitors have been generated by simplifying the structure of sLe^x while maintaining the proper orientation of the hydroxyl and anionic groups implicated in recognition [for a recent review, see Bertozzi (1995)]. The data reported here indicate that more effective L-selectin inhibitors could be generated by the incorporation of two anionic groups occupying the analogous locations of the 3′- and 6-positions of the galactose and glucosamine residues of Le^x.

In addition to providing directions for L-selectin inhibitor design, our observation that 3′,6-disulfo Le^x(Glc) and 3′,6′,6-trisulfo Le^x(Glc) are the most potent Le^x-based ligands for L-selectin supports the hypothesis that sulfation of sLe^x at the 6-position on GlyCAM-1 is an important modification for L-selectin recognition. The contribution of the 6′-sulfation of sLe^x within L-selectin ligands merits further investigation. The present results argue that 6′-sulfo sLe^x and sLe^x have comparable equilibrium association constants for L-selectin. However, carbohydrate recognition domains for the selectins may be able to accommodate more complex structures than tetrasaccharide capping groups (Maaheimo et al., 1995). Thus, the contribution of 6′-sulfo sLe^x to L-selectin binding in the context of the branched and extended *O*-glycans in which it occurs (Hemmerich et al., 1995) may be significant. Furthermore, the ELISA that we have employed is an equilibrium assay, providing only thermodynamic information about binding interactions. L-selectin functions in the very rapid tethering and rolling of leukocytes along the vascular endothelium [reviewed by Springer (1995)], interactions that may be governed by kinetic parameters. It has previously been demonstrated that inhibitory potency of carbohydrates in equilibrium binding assays does not necessarily predict their ability to serve as ligands in L-selectin dependent tethering and rolling events (Alon et al., 1995).

In terms of the equilibrium measurements reported herein, it is clear that a 3-fold difference in binding affinity between monovalent saccharide ligands (compound **9** or **10** *vs* **6**) alone cannot account for the requirement that GlyCAM-1 be sulfated for its high-affinity binding to L-selectin. If the interaction between L-selectin and GlyCAM-1 is polyvalent, however, then the importance of small differences in monovalent binding affinities (which may not even be discriminated in the present assays) could be very significant. In fact, many lectins are known to use multivalent interactions to increase the magnitude of their functional binding affinities [for recent reviews see Lee and Lee (1995), Wong (1995), and Kiessling and Pohl (1996)]. Physiological ligands for L-selectin, such as GlyCAM-1 (Lasky et al., 1992), CD34 (Baumhueter et al., 1993), and MadCAM-1 (Berg et al., 1993; Briskin et al., 1993), are mucins, which can present multiple copies of particular carbohydrate determinants in a precise array. Moreover, it has been shown that polysaccharides or multivalent sLe^x derivatives are more effective at blocking L-selectin function than their monovalent constituents (Imai et al., 1990; Yoshida et al., 1994; Maaheimo et al., 1995; Turunen et al., 1995). In multivalent protein−carbohydrate arrays, small changes in the structure of the monovalent ligand have resulted in large changes in functional affinities. This phenomenon is exemplified in nature by viral influenza hemagglutinins. Some viral strains, such as X-31, agglutinate erythrocytes displaying Siaα2→3Gal but not those presenting Siaα2→6Gal. Despite the high selectivity of the multivalent interaction, the X-31 hemagglutinin exhibits only a 1.5-fold preference for binding to Siaα2→3Gal over the related disaccharide (Rogers et al., 1983; Sauter et al., 1989). In another example, a comparison of monovalent and multivalent ligands for concanavalin A found that multivalent ligands bound more avidly (up to 50 000-fold on a per sugar residue basis) and with higher selectivity (Mortell et al., 1996). For example, a 4-fold difference in the activities of monovalent glucose and mannose derivatives is amplified to a 160-fold difference when the potencies of the corresponding polyvalent derivatives are compared. This 160-fold difference between the polyvalent saccharide derivatives affords essentially complete recognition specificity for the mannose derivative. Similarly, sulfation of GlyCAM-1 may therefore result in a large increase in functional affinity for L-selectin.

Our results suggest that the GlyCAM-1 determinant 6-sulfo sLe^x binds to L-selectin with higher affinity than sLe^x or 6′-sulfo sLe^x. Although the binding enhancements observed in these monovalent ligands are modest, large changes in functional affinity or in the binding kinetics may result from the display of multiple copies of this determinant by physiological selectin ligands such as GlyCAM-1. We anticipate that investigating the effects of polyvalent presentation of the synthetic ligands discussed herein will further illuminate the importance of Le^x sulfation in the binding kinetics, binding affinity, and recognition specificity of cellular interactions mediated by L-selectin.

ACKNOWLEDGMENT

We thank Larry Lasky of Genentech for the L-selectin−Ig chimera and David D. Manning for helpful discussions. W.J.S. thanks J. Kurutz for his help in acquiring and interpreting HMQC NMR data.

SUPPORTING INFORMATION AVAILABLE

500 MHz ^{1}H NMR spectra and heteronuclear multiple quantum coherence (HMQC) spectra are available for compounds **5**−**10** (12 pages). Ordering information is given on any current masthead page.

REFERENCES

Alon, R., Feizi, T., Yuen, C.-T., Fuhlbrigge, R. C., & Springer, T. A. (1995) *J. Immunol. 154*, 5356−5366.

Baumhueter, S., Singer, M. S., Henzel, W., Hemmerich, S., Renz, M., Rosen, S. D., & Lasky, L. A. (1993) *Science 262*, 436−438.
Berg, E. L., McEvoy, L. M., Berlin, C., Bargatze, R. F., & Butcher, E. C. (1993) *Nature 366*, 695−698.
Bertozzi, C. R. (1995) *Chem. Biol. 2*, 703−708.
Bertozzi, C. R., Fukuda, S., & Rosen, S. D. (1995) *Biochemistry 34*, 14271−14278.
Brandley, B. K., Kiso, M., Abbas, S., Nikrad, P., Srivasatava, O., Foxall, C., Oda, Y., & Hasegawa, A. (1993) *Glycobiology 3*, 633−639.
Briskin, M. J., McEvoy, L. M., & Butcher, E. C. (1993) *Nature 363*, 461−464.
Brüning, J., & Kiessling, L. L. (1996) *Tetrahedron Lett. 37*, 2907−2910.
Chandrasekaran, E. V., Jain, R. K., & Matta, K. L. (1992) *J. Biol. Chem. 267*, 23806−23814.
Crommie, D., & Rosen, S. D. (1995) *J. Biol. Chem. 270*, 22614−22624.
Drickamer, K. (1993) *Curr. Opin. Struct. Biol. 3*, 393−400.
Green, P. J., Tamatani, T., Watanabe, T., Miyasaka, M., Hasegawa, A., Kiso, M., Yuen, C.-T., Stoll, M. S., & Feizi, T. (1992) *Biochem. Biophys. Res. Commun. 188*, 244−251.
Green, P. J., Yuen, C.-T., Childs, R. A., Chai, W., Miyasaka, M., Lemoine, R., Lubineau, A., Smith, B., Ueno, H., Nicolaou, K. C., & Feizi, T. (1995) *Glycobiology 5*, 29−38.
Hasegawa, A., Ito, A., Ishida, H., & Kiso, M. (1995) *J. Carbohydr. Chem. 14*, 353−368.
Hemmerich, S., & Rosen, S. D. (1994) *Biochemistry 33*, 4830−4835.
Hemmerich, S., Bertozzi, C. R., Leffler, H., & Rosen, S. D. (1994a) *Biochemistry 33*, 4820−4829.
Hemmerich, S., Butcher, E. C., & Rosen, S. D. (1994b) *J. Exp. Med. 180*, 2219−2226.
Hemmerich, S., Leffler, H., & Rosen, S. D. (1995) *J. Biol. Chem. 270*, 12035−12047.
Imai, Y., True, D. D., Singer, M. S., & Rosen, S. D. (1990) *J. Cell Biol. 111*, 1225−1232.
Imai, Y., Lasky, L. A., & Rosen, S. D. (1993) *Nature 361*, 555−557.
Jain, R. K., Vig, R., Rampal, R., Chandrasekaran, E. V., & Matta, K. L. (1994) *J. Am. Chem. Soc. 116*, 12123−12124.
Kiessling, L. L., & Pohl, N. L. (1996) *Chem. Biol. 3*, 71−77.
Lasky, L. A. (1995) *Annu. Rev. Biochem. 64*, 113−139.
Lasky, L. A., Singer, M. S., Dowbenko, D., Imai, Y., Henzel, W. J., Grimley, C., Fennie, C., Gillett, N., Watson, S. R., & Rosen, S. D. (1992) *Cell 69*, 927−938.
Lee, Y., & Lee, R. (1995) *Acc. Chem. Res. 28*, 321−327.
Lubineau, A., & Lemoine, R. (1994) *Tetrahedron Lett. 35*, 8795−8796.
Maaheimo, H., Renkonen, R., Turunen, J. P., Penttilä, L., & Renkonen, O. (1995) *Eur. J. Biochem. 234*, 616−625.
Manning, D. D., Bertozzi, C. R., Pohl, N. L., Rosen, S. D., & Kiessling, L. L. (1995) *J. Org. Chem. 60*, 6254−6255.
McEver, R. P., Moore, K. L., & Cummings, R. D. (1995) *J. Biol. Chem. 270*, 11025−11028.
Mortell, K. H., Weatherman, R. V., & Kiessling, L. L. (1996) *J. Am. Chem. Soc. 118*, 2297−2298.
Nelson, R. M., Dolich, S., Aruffo, A., Cecconi, O., & Bevilacqua, M. P. (1993) *J. Clin. Invest. 91*, 1157−1166.
Nicolaou, K. C., Bockovich, N. J., & Carcanague, D. R. (1993) *J. Am. Chem. Soc. 115*, 8843−8844.
Norgard, K. E., Moore, K. L., Diaz, S., Stults, N. L., Ushiyama, S., McEver, R. P., Cummings, R. D., & Varki, A. (1993) *J. Biol. Chem. 268*, 12764−12774.
Numomura, S., Iida, M., Numata, M., Sugimoto, M., & Ogawa, T. (1994) *Carbohydr. Res. 263*, C1−C6.
Patel, T. P., Goelz, S. E., Lobb, R. R., & Parekh, R. B. (1994) *Biochemistry 33*, 14815−14824.
Rogers, G. N., Paulson, J. C., Daniels, R. S., Skehel, J. J., Wilson, I. A., & Wiley, D. C. (1988) *Nature 304*, 76−78.
Rosen, S. D., & Bertozzi, C. R. (1994) *Curr. Opin. Cell. Biol. 6*, 663−673.
Rosen, S. D., & Bertozzi, C. R. (1996) *Curr. Biol. 6*, 261−264.
Sako, D., Chang, X.-J., Barone, K. M., Vachino, G., White, H. M., Shaw, G., Veldman, G. M., Bean, K. M., Ahern, T. J., Furie, B., Cumming, D. A., & Larsen, G. R. (1993) *Cell 75*, 1179−1186.
Sauter, N. K., Bednarski, M. D., Wurzburg, B. A., Hanson, J. E., Whitesides, G. M., Skehel, J. J., & Wiley, D. C. (1989) *Biochemistry 28*, 8388−8397.
Scudder, P. R., Shailubhai, D., Duffin, K. L., Streeter, P. R., & Jacob, G. S. (1994) *Glycobiology 4*, 929−933.
Singer, M. S., & Rosen, S. D. (1996) *J. Immunol. Methods* (in press).
Singh, K., Fernández-Mayoralas, A., & Martin-Lomas, M. (1994) *J. Chem. Soc., Chem. Commun.*, 775−776.
Springer, T. A. (1995) *Annu. Rev. Physiol. 57*, 827−872.
Steegmaier, M., Levinovitz, A., Isenmann, S., Borges, E., Lenter, M., Kocher, H. P., Kleuser, B., & Vestweber, D. (1995) *Nature 373*, 615−620.
Stroud, M. R., Handa, K., Salyan, M. E. K., Ito, K., Levery, S. B., & Hakomori, S.-I. (1996) *Biochemistry 35*, 770−778.
Tedder, T. F., Steeber, D. A., Chen, A., & Engel, P. (1995) *FASEB J. 9*, 866−873.
Turunen, J. P., Majuri, M.-L., Seppo, A., Tiisala, S., Paavonen, T., Miyasaka, M., Lemström, K., Penttilä, L., Renkonen, O., & Renkonen, R. (1995) *J. Exp. Med. 182*, 1133−1142.
Vig, R., Jain, R. K., & Matta, K. L. (1995) *Carbohydr. Res. 266*, 279−284.
Watson, S. R., Imai, Y., Fennie, C., Geoffroy, J. S., Rosen, S. D., & Lasky, L. A. (1990) *J. Cell Biol. 110*, 2221−2229.
Wong, S. Y. C. (1995) *Curr. Opin. Struct. Biol. 5*, 599−604.
Yoshida, T., Toyama-Sorimachi, N., Miyasaka, M., & Lee, Y. C. (1994) *Biochem. Biophys. Res. Commun. 204*, 969−974.
Yuen, C.-T., Bezouska, K., O'Brien, J., Stoll, M., Lemoine, R., Lubineau, A., Kiso, M., Hasegawa, A., Bockovich, N. J., Nicolaou, K. C., & Feizi, T. (1994) *J. Biol. Chem. 269*, 1595−1598.
Yuen, C.-T., Lawson, A. M., Chai, W., Larkin, M., Stoll, M. S., Stuart, A. C., Sullivan, F. X., Ahern, T. J., & Feizi, T. (1992) *Biochemistry 31*, 9126−9131.

BI9613640