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N-Glycan Analysis of Recombinant L-Selectin Reveals Sulfated GalNAc and GalNAc−GalNAc Motifs

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The leukocytic adhesion receptor L-selectin plays a crucial role in the first step of the adhesion cascade, enabling leukocytes to migrate into surrounding tissues during inflammation and immune surveillance. We analyzed the site-specific N-glycosylation of the lectin and EGF-like domain of L-selectin using recombinant variants ("LEHis"). The three glycosylation sites of LEHis were mutated to obtain singly glycosylated variants that were expressed in HEK293F cells. α1-Acid glycoprotein (AGP), expressed in the same system, was used to distinguish between cell type- and protein-specific glycosylation. Using mass spectrometry and exoglycosidase digestions, we established that LEHis was mostly bearing multifucosylated diantennary N-glycans with a major fraction terminating with GalNAc residues replacing the more common Gal. We could also show that parts of the GalNAc residues were sulfated. Furthermore, we identified novel diantennary glycan structures terminating with the motif GalNAc−GalNAc or SO_4−GalNAc−GalNAc, which have not been described for N-glycans yet. Interestingly, none of these specific features were found in the N-glycan profile of AGP. This indicates that protein intrinsic information of L-selectin leads to decoration with specific N-glycans, which in turn may be related to L-selectin function.

Keywords: L-selectin • N-glycans • mass spectrometry • sulfate • GalNAc

Introduction

L-selectin is a type I transmembrane receptor that is constitutively expressed on the surface of leukocytes. As a member of the selectin family of adhesion molecules, L-selectin mediates the first step of the adhesion cascade, the capture and rolling of leukocytes on endothelial cells, in concert with its ligands and other adhesion molecules (for review, see ref 1). This process is necessary for leukocytes to migrate out of the bloodstream and enter surrounding tissues to perform their tasks of detecting and eliminating pathogens during immune response and inflammation. Subsequent to leukocyte activation, L-selectin is rapidly shed from the cell surface. Soluble L-selectin is present in the blood serum at concentrations of ∼1.6 μg/mL and retains its ligand-binding activity.[2] It was hypothesized that soluble L-selectin acts as an immunomodulatory component by competing with membrane-bound L-selectin for ligands.[2]

L-selectin is composed of a N-terminal C-type lectin domain, followed by an EGF-like domain, two short consensus repeats (SCR), a transmembrane domain and a short cytoplasmic tail.[3] The lectin and the EGF-like domain are crucial for the adhesive function mediating the interaction with ligands. The extracellular portion of L-selectin contains seven potential N-glycosylation sites, two in the lectin domain, one in the EGF-like domain and four in the SCRs.[4,5] It is commonly accepted that protein glycosylation can be protein- as well as cell-type specific.[6] Moreover, glycosylation has the potential to modify protein stability, biological function, serum half-live and clearance and may be responsible for the antigenicity of glycoproteins.[7,8] It has been shown previously that N-glycan sialylation of recombinant L-selectin expressed in BHK cells was elevated in comparison to the one expressed in K562 cells. This led to differently charged isoforms which could be related to the ligand-binding properties of L-selectin indicating that glycosylation may modulate L-selectin binding function.[9]

In the present study, we analyzed the site-specific glycosylation of the functionally important lectin and EGF-like domain of L-selectin. With respect to the low concentration of soluble L-selectin in blood serum, we chose a recombinant approach using a human cell line for expression in order to obtain sufficient material for analysis. We constructed a soluble form of L-selectin comprising the lectin and EGF-like domain fused

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to a His-tag ("LEHis"). By site-directed mutagenesis we generated mutant LEHis variants bearing single intact glycosylation sites and expressed these variants in HEK293F cells. This approach allowed us to analyze the site-specific N-glycosylation profile of each respective N-glycosylation site within the lectin- and the EGF-like domain. In order to discriminate between cell-type specific and protein specific N-glycosylation, we compared the N-glycan profiles of LEHis with the profile of α1-acid glycoprotein (AGP), which was expressed in the same system.

Materials and Methods

All chemicals were purchased from Sigma-Aldrich, MO unless stated otherwise.

Cloning of Expression Constructs. We constructed a soluble form of human L-selectin comprising the lectin and EGF-like domain fused to a C-terminal His-tag ("LEHis"). To this end, the cDNA sequence of L-selectin spanning the signal peptide sequence, the lectin and the EGF domain were excised with *Bam*HI and *Eco*RV (all restriction enzymes: Fermentas, Lithuania) from a pET22b+ vector, containing the respective sequence and a His-tag. The fragment was subcloned into the vector pBluescript KSII (+) (Stratagene, CA). The insert was then cut with *Bam*HI and *Sal*I and inserted into the vector pcDNA3 (Invitrogen, CA). Site-directed mutagenesis of the codons for asparagine 22, 66, and 139 of the N-glycosylation consensus sequences was performed via PCR using the following mismatch-primers: primer N22Q: 5′-gccgagaccagtacacagatttagttgcc-3′; primer N66Q: 5′-ggtgggaacccagaaatctcttactg-3′; primer N139Q: 5′-gtagaaatcatcaatcagtacacctgcaactg-3′. Thereby, codons for asparagine were changed to codons for glutamine. All combinations of mutants were constructed by digestion and ligation of internal DNA fragments with the use of unique restriction sites. The constructs were named by a three-digits-code for the three glycosylation sites: "1" for an intact site (resulting in glycosylation at that site) and "0" for a mutant site.

Full length α1-acid glycoprotein (AGP) was constructed as a fusion protein with the Fc-part of human IgG. The sequence of IgG-Fc was amplified from pIG1 vector[10] via PCR with the extending primer 5′-ttttctagactggtgccccgcggcagcgcgcaggtaagtgggagggagggtgtctgct-3′, containing a splice acceptor site and the reverse primer 5′-tttaccggttttacccggagacagggagag-3′. The product was inserted into pcDNA6 V5HisA (Invitrogen, CA) via the XbaI and AgeI restriction sites, thereby gaining an additional C-terminal His-tag. The sequence of AGP was amplified via PCR from a cDNA-containing vector with the primers 5′-ttaggatccaccatggcgctgtcctggg-3′ and 5′-ttttctaagagccggattccccctcctcctg-3′. The PCR product was cloned into pCRIIbluntTOPO (Invitrogen, CA), cut, and inserted via *Bam*HI and *Xba*I into the IgG-containing pcDNA6 V5HisA.

Glycoprotein Production and Purification. LEHis constructs and AGP were transiently expressed in the Freestyle 293 expression system (Invitrogen, CA). HEK293F cells were cultured in glass 1 L-Erlenmeyer flasks in an orbital shaker at 96 rpm (5 cm diameter orbit) at 8% CO_2. The cell density for transfection was 1×10^6 cells/ml. Transfection complexes were formed by diluting each 1 μg plasmid per 1×10^6 cells and 3.5 μg polyethyleneimine (PEI) (Sigma-Aldrich, MO, # 40872−7, 0.45 mg/mL solution in H_2O, pH neutralized with HCl) in 1/30 of the culture volume in Opti-MEM (Invitrogen, CA). The DNA and PEI dilutions were combined, vortexed and incubated 15 min at room temperature. After incubation, the transfection solution was added to the cells. Three days after transfection, the supernatant was separated from the cells by centrifugation for 5 min at 500 g and filtered through 0.22 μm filters.

Recombinant LEHis variants were purified from the cell culture supernatant using a specific biotinylated ssDNA aptamer (5′-biotin-gcggtaaccagtacaaggtgctaaagctaatggcgc-3′)[11] which was coupled to Streptavidin-Sepharose (GE Healthcare, WI). The column was equilibrated with TBSCa buffer (20 mM Tris pH 7.0, 150 mM NaCl, 2 mM $CaCl_2$) and eluted with 20 mM EDTA in 20 mM Tris pH 7.0, 150 mM NaCl. Protein-containing fractions were concentrated using ultrafiltration units (Amicon Ultra-15, Millipore, MA).

Recombinant AGP, expressed as described above, was purified from the cell culture supernatant in a first step via Protein A Sepharose (GE Healthcare, WI). Column-bound material was washed with Dulbecco's PBS (phosphate buffered saline without Ca^{2+} and Mg^{2+} (PAA Laboratories GmbH, Austria) and eluted with 100 mM glycin-HCl buffer pH 2.5. The eluates were immediately neutralized by addition of 1 M Tris-HCl pH 9.5. The protein was further purified using a Ni-NTA column (Qiagen, Hilden, Germany) that has been equilibrated with 300 mM NaCl and 10 mM imidazole in Dulbecco's PBS. Bound protein was eluted with 250 mM imidazole in Dulbecco's PBS. Eluates were concentrated using ultrafiltration units (Amicon Ultra-15, Millipore, MA). The buffer was exchanged to PBS by concentrating and refilling the ultrafiltration units 4 times.

SDS-PAGE. The purity of recombinant proteins was analyzed by 12% polyacrylamide gelelectrophoresis under reducing conditions and bands were visualized with Coomassie Blue dye.

Monosaccharide Analysis. LEHis variants and AGP (30 μg) were hydrolyzed in 2 N TFA for 4 h at 100 °C. After evaporation of the samples under reduced atmosphere, the internal standard, 2-deoxyribose was added. This mixture was analyzed by HPAEC-PAD on a PA-1 column using a Dionex ICS-3000. Neutral monosaccharides were separated by isocratic elution with 2.25 mM NaOH. Postcolumn addition of 200 mM NaOH allowed amperometric detection.

Release and Isolation of N-Linked Glycans. One-hundred micrograms of each glycoprotein was denatured in the presence of 1% sodium dodecyl sulfate (w/v) and 10% 2-mercaptoethanol for 5 min at 95 °C. The glycoprotein solution was then diluted 1/10 and digested with 100 mU PNGase F (EC 3.5.1.52; Roche Applied Science, IN) for 16 h at 37 °C in the presence of 1% NP-40. Detergents were removed using Calbiosorb beads (Calbiochem, San Diego, CA) and N-glycans were isolated and desalted on graphitized carbon columns (Alltech, Deerfield, IL).[12]

2AB-Labeling of N-Glycans and HPLC Profiling. Dried N-glycans were incubated with 0.35 M 2-aminobenzamide/1 M sodium cyanoborohydride in acetic acid-dimethyl sulfoxide (3:7, v/v) (8 μL) for 2 h at 65 °C.[13,14] The excess of 2AB-reagent was removed by paper chromatography (Whatman, Maidstone, United Kingdom) pretreated with 0.5% acetic acid for 24 h. The mobile phase, consisting of *n*-butanol/ethanol/H_2O (4/1/1), was run twice. N-glycans, eluted from the paper strip with 3 × 500 μL H_2O, were finally lyophilized.

Charged N-glycan profiling was performed by HPLC at a temperature of 50 °C on an Asahipak NH2 P-50 E4 5 μ (250 × 4.6 mm, Shodex, NY) column using a Dionex Ultimate 3000 HPLC system, equipped with a Dionex fluorescence detector RF2000 ($\lambda_{exc.max}$ = 330 nm, $\lambda_{em.max}$ = 420 nm). Elutions were performed at a flow rate of 0.8 mL/min using two solvent systems: solvent A, 2% acetic acid, 1% tetrahydrofurane in acetonitrile; solvent B, 5% acetic acid, 1% tetrahydrofurane and

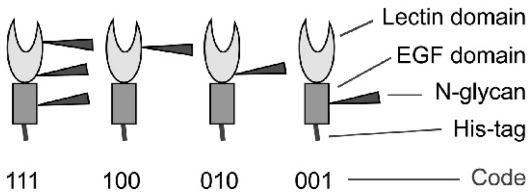

Figure 1. Schematic representation of recombinant L-selectin constructs ("LEHis"). For simple identification, LEHis variants were named by a three-digits-code representing the three N-glycosylation sites, which reside in the lectin and EGF-like domains. "1" stands for an intact site and "0" stands for a mutated site which consequently lacks N-glycosylation.

3% triethylamine in H_2O. The linear gradients were comprised of 30−95% B in 82 min, 95% B for 5 min before returning to the initial conditions in 1 min.

Exoglycosidase Digestions. N-glycans, dissolved in 100 mM sodium acetate, pH 5.0, were digested for 18 h at 37 °C using exoglycosidases at the following concentrations: 100 mU/mL *Arthrobacter ureafaciens* neuraminidase (EC 3.2.1.18, Roche Applied Science, IN); 1.5 U/mL bovine testes β-galactosidase (GKX-5013, Prozyme, CA); 15 U/mL jack bean β-*N*-acetylhexosaminidase (GKX-5003, Prozyme, CA); 4 U/mL β-*N*-acetylhexosaminidase, recombinant from *Streptococcus pneumoniae*, expressed in *E. coli* (GKX-80050, Prozyme, CA); 50 U/mL almond meal α(1−3,4) fucosidase (GKX-5019, Prozyme, CA), 0.2 U/mL α(1−2,3,4,6) fucosidase from bovine kidney (GKX-5006, Prozyme, CA). For sulfatase digestions, N-glycans were dissolved in 30 mM ammonium acetate pH 5.5 and then digested with 1 mg/mL sulfatase from *Patella vulgata* type V (Sigma-Aldrich, MO). After inhibition at 95 °C for 5 min, samples were desalted on Toptips (SunChrom, Germany) and lyophilized.

Permethylation. Permethylation was carried out according to standard protocols[15,16] with slight modifications. After the reaction, chloroform was added and the chloroform phase was washed with water until the pH of the water phase has become neutral. The chloroform phase was finally evaporated under reduced atmosphere and the sample was dissolved in 75% aqueous acetonitrile for MALDI-TOF measurements.

Mass Spectrometry. MALDI-TOF mass spectra were recorded on an Ultraflex III mass spectrometer (Bruker Daltonics) equipped with a Smartbeam laser and a LIFT-MS/MS facility. Spectra were recorded in reflector positive- or negative-ionization mode. Calibration was performed either on a glucose ladder for the positive-ion mode or on a peptide standard mixture (Pepmix, Bruker Daltonics, Bremen, Germany). Half of a microliter of desialylated or permethylated N-glycans were mixed on the ground steel target in a ratio 1:1 with the matrix consisting of D-arabinosazone (5 mg/mL) dissolved in 70% aqueous ethanol for the positive-ion mode. In the negative-ion mode, saturated 6-aza-2-thiothymine dissolved in 20 mM ammonium citrate/acetonitrile (1/1) was used as the matrix.

Results

We constructed soluble derivatives of L-selectin comprising the lectin and the EGF domain fused to a His-tag ("LEHis") (Figure 1). These constructs include the ligand binding site and the first three N-glycosylation sites of L-selectin. To examine the site-specific glycosylation profile of each of these three glycosylation sites, the N-glycosylation consensus sequences were deleted by site-directed mutagenesis. We used three variants of mutant LEHis which have only one remaining N-glycan on either the first, second or third glycosylation site. For easy identification, the mutant proteins were named by a three-digits-code, where a "1" stands for an intact N-glycosylation site and "0" stands for a deleted N-glycosylation site. These mutant LEHis variants were expressed in HEK293F cells and purified from the cell culture supernatant by aptamer affinity chromatography as described.[11] The N-glycan profiles were analyzed and compared to the profiles of fully glycosylated LEHis (111) and α1-acid glycoprotein (AGP), which was produced in the same expression system.

Table 1. Molar Ratios of the Monosaccharides Constituting LEHis and AGP as Determined by HPAEC-PAD[a]

	Man	$GlcNH_2$	Gal	Fuc	$GalNH_2$
100	3.0	4.7	0.5	1.7	2.3
010	3.0	5.1	0.8	2.5	2.0
001	3.0	5.3	1.0	2.9	1.7
111	3.0	4.7	1.1	2.0	1.2
AGP	3.0	4.9	2.1	0.9	0.0

[a] Man is taken as 3.0. As the monosaccharides were not re-N-acetylated after the TFA hydrolysis, GlcNAc and GalNAc were measured as $GlcNH_2$ and $GalNH_2$, respectively.

Monosaccharide Analysis. Monosaccharide analysis revealed a neutral carbohydrate content of 6% for LEHis 100, 8% for LEHis 010, 8% for LEHis 001, 20% for LEHis 111 and 20% by mass for AGP, respectively. The N-glycans of all LEHis variants were found to be more fucosylated than those of AGP (Table 1), presenting on average two Fuc residues per structure compared to one, respectively. Although L-selectin and AGP are not O-glycosylated, GalNAc was present in all LEHis variants in the range of 1.2−2.3 per N-glycan structure. The glycans of all LEHis variants were also less galactosylated than those of AGP, which could indicate that GalNAc residues are partially replacing Gal residues in N-glycans of LEHis.

MALDI-TOF-MS. For mass spectrometric analysis by MALDI-TOF-MS, N-glycans were released by PNGase F digestion and part of the N-glycan pool was permethylated. Relative quantification of signal intensities of permethylated N-glycans was performed as it was demonstrated earlier to give reliable results when compared to chromatographic methods.[16] An aliquot of each sample was also sequentially digested with exoglycosidases and analyzed by MALDI-TOF mass spectrometry. The data was interpreted with the assistance of the GlycoWorkbench software[17] as well as the Glyco-Peakfinder tool.[18]

Mass spectra of the permethylated N-glycans measured in the positive-ion mode are presented in Figure 2 and in Table 2. The N-glycan structures of the LEHis variants were more fucosylated and less sialylated than the glycans of AGP. On average, LEHis presented 1−3 Fuc and 0−1 Neu5Ac per structure whereas AGP was bearing 1 Fuc and 0−4 Neu5Ac. The N-glycans of LEHis 100 showed less heterogeneity than the N-glycans of LEHis 010 and 001 (Figure 2D). All the N-glycans present in the three LEHis variants, namely 100, 010 and 001, were also detected in fully glycosylated LEHis (111). Structures which were observed as traces only, namely at m/z 2990.5, 2994.5 and 3164.5, are not presented in Table 2. The MS compositional data was in accordance with the data from the monosaccharide analysis.

MALDI-TOF/TOF Sequencing in the Positive-Ion Mode and Exoglycosidase Digestions (see Supporting Information Tables S1 and S2). MALDI-TOF/TOF sequencing of desialylated and/or 2AB-labeled glycans was performed in combination with exoglycosidase digestions to unravel the N-glycan struc-

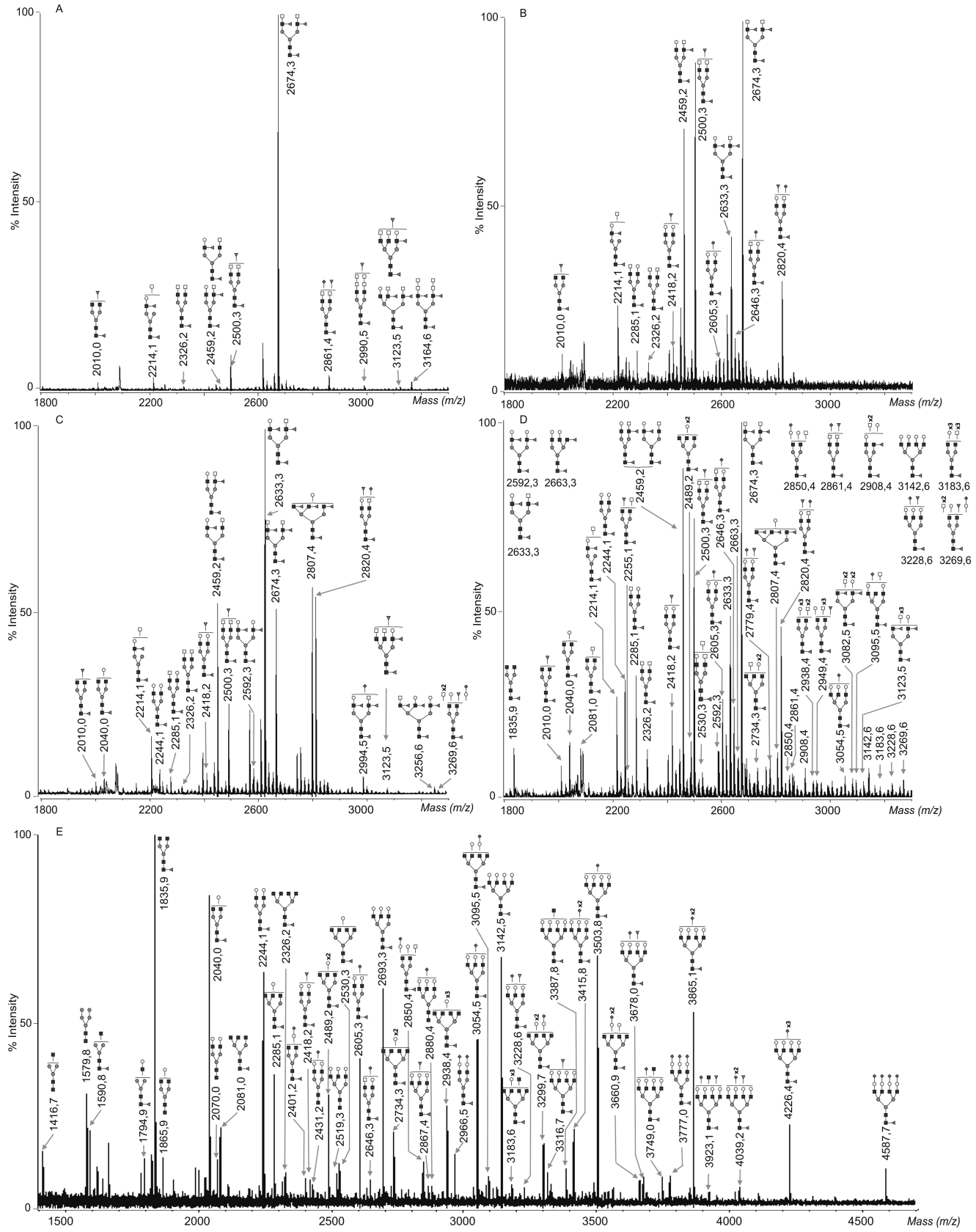

Figure 2. MALDI-TOF spectra of permethylated N-glycan pools measured in the positive-ion mode. All molecular ions are present in sodiated form $[M + Na]^+$. (A) LEHis 100; (B) LEHis 010; (C) LEHis 001; (D) LEHis 111; (E) AGP. Green circle, Man; yellow circle, Gal; yellow square, GalNAc; blue square, GlcNAc; red triangle, Fuc; purple diamond, Neu5Ac. N-acetyl hexosamine is represented by a white square when our data and knowledge of biosynthetic pathways did not allow differentiating between GlcNAc and GalNAc. Structures outside the bracket have not been unequivocally assigned. Based on MALDI-TOF/TOF analyses and exoglycosidase digestions, it was nevertheless established that Fuc outside the bracket were linked to one of the antennary GlcNAc.

Table 2. Relative Intensities of the Permethylated N-Glycans from LEHis Variants and AGP Measured by MALDI-TOF-MS in the Positive-Ion Mode[a]

m/z	Composition	100	010	001	111	AGP
1416.7	Hex3HexNAc3					0.9
1579.8	Hex5HexNAc2					2.2
1590.8	dHex1Hex3HexNAc3					1.6
1794.9	dHex1Hex4HexNAc3					1.1
1835.9	dHex1Hex3HexNAc4				1.6	9.8
1865.9	Hex4HexNAc4					1.2
2010.0	dHex2Hex3HexNAc4	1.6	2.5	0.8	1.0	
2040.0	dHex1Hex4HexNAc4			1.2	1.9	8.3
2070.0	Hex5HexNAc4					1.2
2081.0	dHex1Hex3HexNAc5				2.8	2.0
2214.1	dHex2Hex4HexNAc4	2.8	6.4	4.0	3.5	
2244.1	dHex1Hex5HexNAc4			1.6	4.3	6.5
2255.1	dHex2Hex3HexNAc5				0.7	
2285.1	dHex1Hex4HexNAc5		2.5	1.4	4.9	3.4
2326.2	dHex1Hex3HexNAc6	1.2	2.2	0.5	1.7	0.8
2401.2	dHex1Hex4HexNAc4SA1					0.9
2418.2	dHex2Hex5HexNAc4		3.1	1.4	3.0	0.8
2431.2	Hex5HexNAc4SA1					0.6
2459.2	dHex2Hex4HexNAc5	1.0	17.1	12.9	12.0	
2489.2	dHex1Hex5HexNAc5				2.0	3.0
2500.3	dHex2Hex3HexNAc6	7.3	21.9	6.0	10.3	
2519.3	Hex6HexNAc5					0.9
2530.3	dHex1Hex4HexNAc6				1.0	1.2
2592.3	dHex3Hex5HexNAc4			1.3	1.7	
2605.3	dHex1Hex5HexNAc4SA1		1.8		3.5	3.6
2633.3	dHex3Hex4HexNAc5		9.3	26.2	6.7	
2646.3	dHex1Hex4HexNAc5SA1		3.5		3.4	0.7
2663.3	dHex2Hex5HexNAc5				1.0	
2674.3	dHex3Hex3HexNAc6	79.0	22.4	12.8	14.2	
2693.3	dHex1Hex6HexNAc5					5.7
2734.3	dHex1Hex5HexNAc6				1.2	1.8
2779.4	dHex2Hex5HexNAc4SA1					1.4
2807.4	dHex4Hex4HexNAc5			13.9	1.4	
2820.4	dHex2Hex4HexNAc5SA1		7.3	13.8	6.5	
2850.4	dHex1Hex5HexNAc5SA1				0.8	1.0
2861.4	dHex2Hex3HexNAc6SA1	3.2			1.0	
2867.4	dHex2Hex6HexNAc5					0.6
2880.4	Hex6HexNAc5SA1					0.4
2908.4	dHex2Hex5HexNAc6				0.6	
2938.4	dHex1Hex6HexNAc6				0.2	2.7
2949.4	dHex2Hex4HexNAc7				0.7	
2966.5	dHex1Hex5HexNAc4SA2					1.4
2990.5	dHex2Hex3HexNAc8	1.1				
2994.5	dHex3Hex4HexNAc5SA1			1.2		
3054.5	dHex1Hex6HexNAc5SA1				0.7	4.4
3082.5	dHex3Hex5HexNAc6				0.6	
3095.5	dHex1Hex5HexNAc6SA1				0.6	0.4
3123.5	dHex3Hex4HexNAc7	0.7		0.2	0.5	
3142.5	dHex1Hex7HexNAc6				1.0	6.5
3164.6	dHex3Hex3HexNAc8	2.1				
3183.6	dHex1Hex6HexNAc7				0.4	0.5
3228.6	dHex2Hex6HexNAc5SA1				0.5	0.3
3256.6	dHex4Hex5HexNAc6			0.5		
3269.6	dHex2Hex5HexNAc6SA1			0.3	0.7	
3299.7	dHex1Hex6HexNAc6SA1					1.7
3316.7	dHex2Hex7HexNAc6					1.0
3387.8	dHex1Hex7HexNAc7					1.0
3415.8	dHex1Hex6HexNAc5SA2					2.1
3503.8	dHex1Hex7HexNAc6SA1					6.6
3660.9	dHex1Hex6HexNAc6SA2					0.8
3678.0	dHex2Hex7HexNAc6SA1					0.7
3749.0	dHex1Hex7HexNAc7SA1					0.8
3777.0	dHex1Hex6HexNAc5SA3					0.4
3865.1	dHex1Hex7HexNAc6SA2					4.9
3923.1	dHex2Hex7HexNAc7SA1					0.2
4039.2	dHex2Hex7HexNAc6SA2					0.4
4226.4	dHex1Hex7HexNAc6SA3					2.1
4587.7	dHex1Hex7HexNAc6SA4					0.9

[a] Singly charged sodiated molecular ions $[M + Na]^+$ were observed.

tures of the singly glycosylated LEHis variants. For all samples, digestions were performed in a first step with neuraminidase next with β-galactosidase then with $\alpha(1-3,4)$ fucosidase followed by recombinant β-*N*-acetylhexosaminidase from *Streptococcus pneumoniae* and finally by jack bean β-*N*-acetylhexosaminidase. Additionally we used $\alpha(1-2,3,4,6)$ fucosidase and sulfatase.

Six peaks of the empirical composition $dHex_2Hex_4HexNAc_4$, $dHex_2Hex_5HexNAc_4$, $dHex_2Hex_4HexNAc_5$, $dHex_3Hex_4HexNAc_5$, $dHex_4Hex_4HexNAc_5$ and $dHex_3Hex_4HexNAc_7$ were found to be resistant to β-galactosidase digestion but shifted after $\alpha(1-3,4)$ fucosidase digestion, which indicated the presence of a Lewis^X (Le^X) antenna. Using $\alpha(1-2,3,4,6)$ fucosidase, it was established that all remaining fucosylated structures were core-fucosylated (data not shown). This was confirmed by the presence of the diagnostic fragment ions $2ABdHex_1HexNAc_1$ at m/z 509.9 and $2ABdHex_1HexNAc_2$ at m/z 712.9. The use of $\alpha(1-3,4)$ fucosidase resulted in the removal of antennary fucoses, leaving only core-fucose on the N-glycan pools. All the HexNAc content was not cleaved using β-*N*-acetylhexosaminidase from *Streptococcus pneumoniae* but was cleaved using jack bean β-*N*-acetylhexosaminidase (namely for $dHex_1Hex_3HexNAc_5$, $dHex_2Hex_3HexNAc_5$, $dHex_1Hex_4HexNAc_5$, $dHex_1Hex_3HexNAc_6$, $dHex_2Hex_4HexNAc_5$, $dHex_2Hex_3HexNAc_6$, $dHex_3Hex_4HexNAc_5$, $dHex_3Hex_3HexNAc_6$, $dHex_2Hex_3HexNAc_8$, $dHex_3Hex_4HexNAc_7$ and $dHex_3Hex_3HexNAc_8$). β-*N*-acetylhexosaminidase from *Streptococcus pneumoniae* cleaves GlcNAc residues only but not GalNAc residues, whereas the enzyme from jack bean cleaves both. This corroborates the results of the monosaccharide analysis that had revealed the presence of GalNAc residues. In addition, a fragment ion $HexNAc_2$ was observed at m/z 428.9 in the MALDI-TOF/TOF spectra of desialylated 2AB derivatives. Considering our data from MS, monosaccharide analysis and exoglycosidase digestions together, the presence of GalNAc−GlcNAc antennae can be inferred. The signal at m/z 533.6 ($dHex_1Hex_1HexNAc_1$) and the absence of signal at m/z 330.6 ($dHex_1Hex_1$) confirmed the presence of fucose at an antennary HexNAc. These structures were assigned to be GalNAc-Le^X motifs in accordance with the MALDI-TOF/TOF data ($dHex_1HexNAc_2$, m/z 574.7) and the N-glycan structures which have been reported on HEK293 cells.[19,20]

In contrast to LEHis 010 and 001, LEHis 100 presented two N-glycan structures of higher masses, namely at m/z 2990.5 and 3164.6 (Figure 2A). With the aid of exoglycosidase digestions, we identified two diantennary N-glycan structures bearing the unusual motif GalNAc-HexNAc-GlcNAc (Supporting Information Table S1), which were further subjected to MALDI-TOF/TOF analyses (Figure 3). Signals at m/z 631.7 ($HexNAc_3$) and at m/z 793.6 corroborate the presence of GalNAc−HexNAc−GlcNAc antennae. As GalNAc−GlcNAc antennae but no GlcNAc−GlcNAc antennae were found in the samples, we assigned such antennae to be of the GalNAc−GalNAc−GlcNAc type. A minor peak at m/z 834.8 was also detected, indicating the presence of a minor compound bearing asymmetrically distributed antennae, one of them bearing three GalNAc. Fucosylation at the antennary GlcNAc was confirmed by signals at m/z 777.7 ($dHex_1HexNAc_3$) and at m/z 533.6 ($dHex_1Hex_1HexNAc_1$). A similar spectrum was obtained for $dHex_2Hex_3HexNAc_8$. Therefore, it was concluded that these structures were fucosylated at the core and at an antennary GlcNAc.

In contrast, exoglycosidase digestions of N-glycans from AGP indicated the presence of core-fucosylated and galactosylated structures only, while GalNAc residues were absent. This was corroborated in the MALDI-TOF/TOF spectrum by the presence of diagnostic fragments at m/z 387.9 ($dHex_1HexNAc_1$) and at m/z 549.9 ($Hex_2HexNAc_1$) (data not shown). Galactosylated

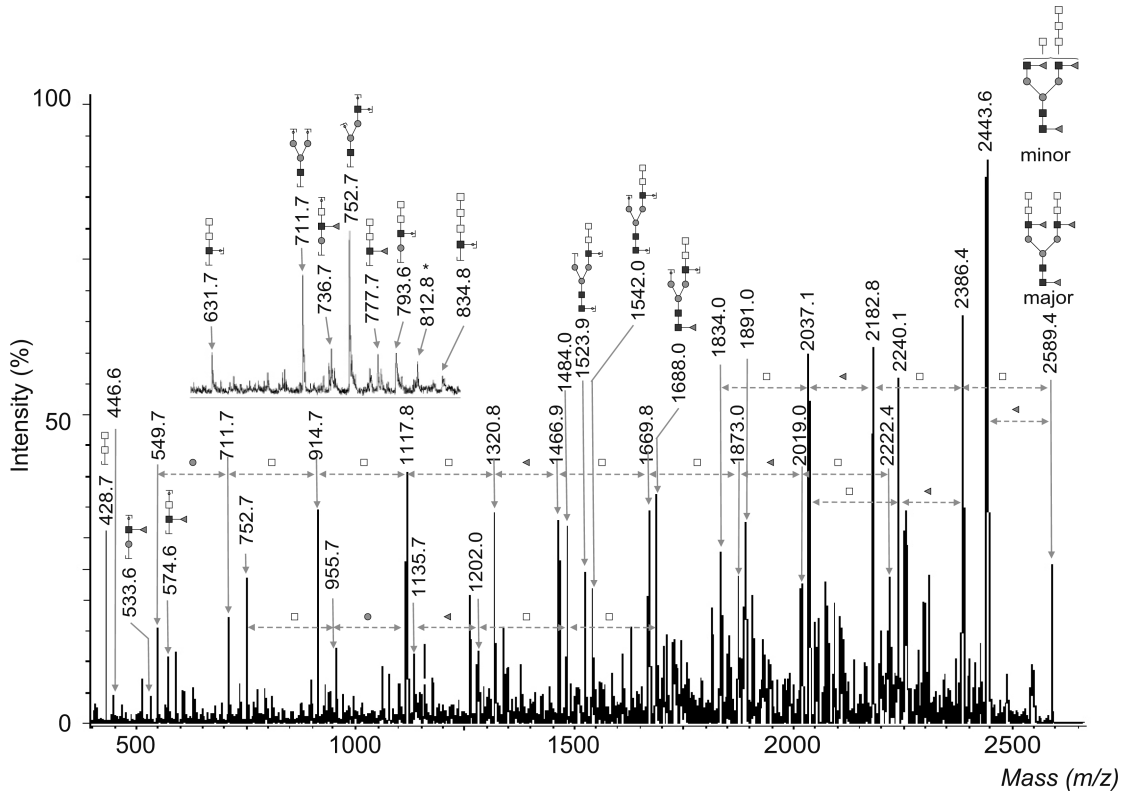

Figure 3. MALDI-TOF/TOF mass spectrum of the $[M + Na]^+$ molecular ion at *m/z* 2589.4 of the composition $dHex_3Hex_3HexNAc_8$ derived from the native N-glycans of LEHis 100. Fragments are assigned using GlycoWorkbench according to the recommendations of the Consortium for Functional Glycomics. Registered fragments may be formed by different fragmentation pathways, only one of which is depicted in the figure. Green circle, Man; yellow circle, Gal; yellow square, GalNAc; blue square, GlcNAc; red triangle, Fuc. *N*-Acetylhexosamine is represented by a white square when our data did not allow differentiating between GlcNAc and GalNAc. * Not assigned.

di, tri, and tetraantennary N-glycans were carrying 0−4 Neu5Ac residues (Figure 2D).

Characterization of Sulfated N-Glycans. In the negative-ion mode, a total of 9 permethylated structures were observed for LEHis variants (Figure 4 and Table 3), whereas in the spectrum of AGP glycans, these structures were absent (data not shown). Sulfatase digestion allowed verifying the presence of sulfate but not phosphate groups. Using exoglycosidase digestions, we found the sulfate group always attached to a terminal GalNAc residue, except in LEHis 111 for the structure $S_1dHex_1Hex_4HexNAc_4$, in which the sulfate was linked to a Gal residue (Supporting Information Table S2). Only one structure in LEHis 111, namely $S_1dHex_1Hex_4HexNAc_5SA_1$, representing 1.9% of the total sulfated glycan pool, was sialylated (Figure 4D). For all LEHis variants, the major sulfated structure was $S_1dHex_2Hex_3HexNAc_6$. In order to determine the proportion of sulfated N-glycans in each pool, we run charged 2AB-HPLC profiles of each desialylated pool. The degree of sulfation was about 50% for LEHis 100 and 010, 30% for LEHis 001 and 111 (data not shown). The monosulfated forms of $dHex_2Hex_3HexNAc_8$ and $dHex_3Hex_3HexNAc_8$, which were annotated as diantennary glycans bearing the unusual motif GalNAc−GalNAc−GlcNAc, were also detected among LEHis 100 N-glycans in the negative-ion mode, but with relatively higher signal intensity than in the positive-ion mode (3.2% and 14.5%, respectively). Traces of these compounds were also found in LEHis 111 (Figure 4D). To our knowledge, this is the first time that neutral and sulfated GalNAc−GalNAc−GlcNAc antennae are described on N-glycans.

Discussion

The use of recombinant mutant singly glycosylated LEHis variants allowed us to selectively investigate the N-glycans on each of the three N-glycosylation sites of L-selectin's lectin and EGF domain. In order to determine how the expression host system and the intrinsic information of the protein sequence have an influence on the resulting N-glycan structures, we compared the N-glycan profiles of the LEHis variants with those of AGP. All proteins were expressed in the same human cell line, namely HEK293F.

Based on mass spectrometric data in combination with exoglycosidase digestions, we show that the N-glycan structures of L-selectin were mainly of the diantennary-type terminating with either Gal or GalNAc residues. All structures were core-fucosylated and a major fraction contained additional antennary Fuc. Only a few triantennary structures were observed while tetraantennary structures were solely detected in the glycan pool of LEHis 111. As a few N-glycan structures were found in LEHis 111 only, it seems that the presence of the other N-glycosylation sites has modified the accessibility of glycosyltransferases to the N-glycan moieties. This could be an indication that the absence of N-glycosylation sites has an influence on the 3-D structure of LEHis. For comparison, N-glycans from AGP showed markedly higher antennarity and less fucosylation. The overall sialylation of the LEHis variants was much lower than that of AGP.

It was established in the present study that a majority of the N-glycans of LEHis contain GalNAc residues whereas no

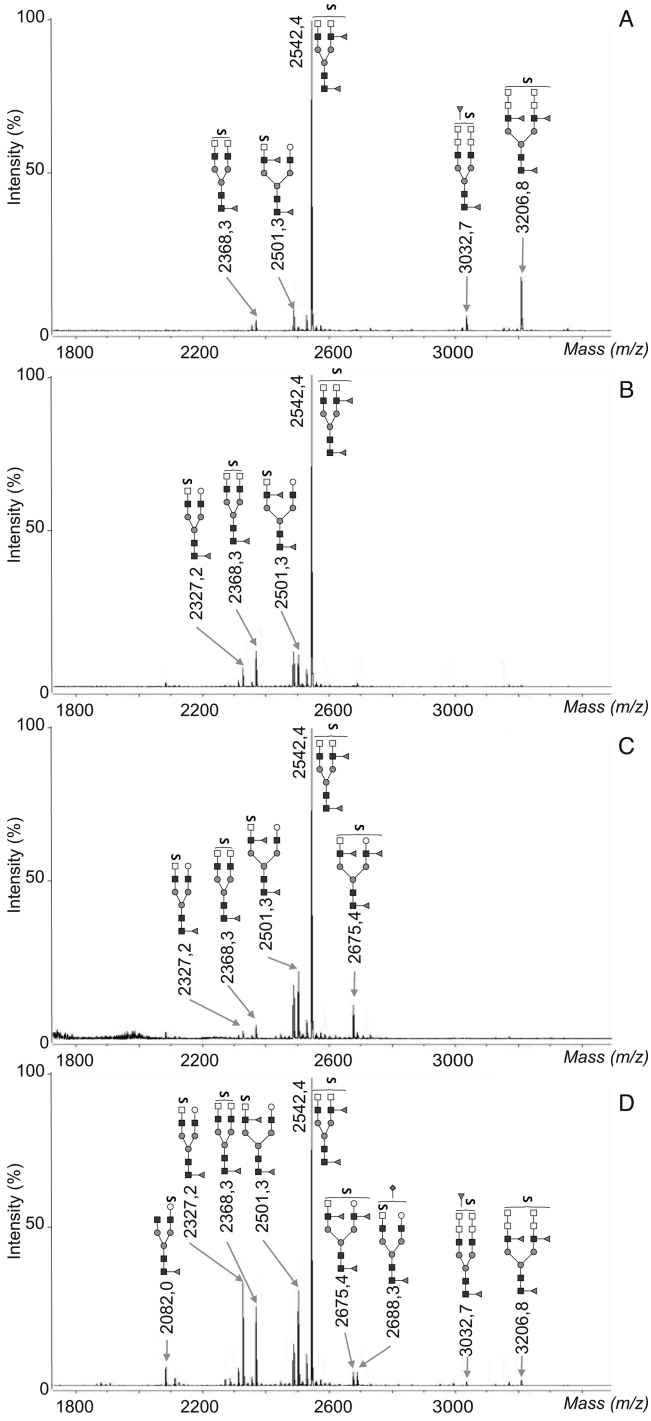

Figure 4. MALDI-TOF spectra of permethylated N-glycan pools measured in the negative-ion mode. All molecular ions are present in $[M - H]^-$ form. (A) LEHis 100; (B) LEHis 010; (C) LEHis 001; (D) LEHis 111. Green circle, Man; yellow circle, Gal; yellow square, GalNAc; blue square, GlcNAc; red triangle, Fuc; purple diamond, Neu5Ac; S, sulfate.

GalNAc residues were detected in the N-glycan pool of AGP. Therefore, it can be inferred that the incorporation of GalNAc is determined by the protein sequence. In an earlier study, traces (3%) of GalNAc were found in the N-glycan pool of the complete extracellular part of L-selectin expressed in BHK cells.[21] There are only few reports on recombinant as well as natural glycoprotein N-glycans bearing GalNAc residues. Among these are Bowes melanoma tissue plasminogen activator

Table 3. Relative Intensities of the Permethylated N-Glycans from LEHis Variants Measured by MALDI-TOF-MS in the Negative-Ion Mode

m/z	Composition	100	010	001	111
2082.0	S1dHex1Hex4HexNAc4				2.4
2327.2	S1dHex1Hex4HexNAc5		9.5	1.7	15.1
2368.3	S1dHex1Hex3HexNAc6	2.2	19.6	2.7	11.4
2501.3	S1dHex2Hex4HexNAc5	0.8	17.2	14.4	13.9
2542.4	S1dHex2Hex3HexNAc6	79.8	53.7	74.2	52.1
2675.4	S1dHex3Hex4HexNAc5			7.0	1.8
2688.3	S1dHex1Hex4HexNAc5SA1				1.9
3032.7	S1dHex2Hex3HexNAc8	3.2			0.6
3206.8	S1dHex3Hex3HexNAc8	14.0			0.8

Singly charged molecular ions $[M - H]^-$ were observed.

(tPA),[22] human protein C from HEK293 cells,[19] bovine lactotransferrin,[23] human urokinase,[24] human kallidinokinase,[25] bee venom phospholipase A_2,[26] bovine CD36[27] and the glycoprotein hormones LH and TSH.[28] For the alpha-subunit of the pituitary glycoprotein hormones, the sequence for recognition by the GalNAc transferase was shown to be a pattern of basic amino acid residues (underlined) positioned toward the N-terminus of the N-glycosylation consensus sequence (bold): ...AYPTPLRSKKTMLVQK**NVT**SE...[29] The sequence of L-selectin presents a similar basic amino acid pattern of 7 residues located N-terminal of the first N-glycosylation consensus sequence: ...NWQRARRFCRD**NYT**DL..., which may equally serve as a recognition pattern for the responsible GalNAc transferase.

It has been shown that GalNAcβ(1−4)[Fucα(1−3)]GlcNAcβ(1−2), also called the "PC-293-determinant" or "GalNAc Le^X",[19,30] has the potential to inhibit E-selectin mediated cell adhesion,[31] probably by adopting a conformation similar to sialyl Le^X. Sialyl Le^X is regarded as the prototypic ligand for all three selectins, E-, P, and L-selectin.[32] This raises the hypothesis that the GalNAc Le^X structures found in this study could mediate binding of L-selectin to itself and thereby competitively modulate the affinity for its ligands. It seems also possible that the N-glycans of L-selectin mediate binding to the endothelial selectins as it has been shown that L-selectin could bind to E-selectin, depending on the glycosylation characteristics of the respective cell line.[33,34] Intriguingly, we described novel structures terminating with two consecutive GalNAc residues on each antenna of the diantennary N-glycans of LEHis 100 and 111. This is, to our knowledge, the first report of such N-glycan structures and it would be of importance to investigate their functions.

In addition to the presence of GalNAc, we establish that antennae terminating with GalNAc residues were modified with sulfates. This sulfation was only observed in the N-glycan pool of LEHis, whereas no sulfation was observed in the glycan pool of AGP. The ability of HEK293 cells to form sulfated GalNAc residues on N-glycans has been reported on tissue factor pathway inhibitor.[35]

The present study is, according to our knowledge, the first report of L-selectin bearing sulfated N-glycans. Previously, the N-glycan pool of recombinant soluble L-selectin from BHK cells did not show any sulfated glycans,[21,36] likely because BHK cells do not express the respective sulfotransferases.[37,38] In the present study, sulfation was exclusively found on LEHis and not on AGP. Thus, the sulfation appears to be protein-specific and it seems likely that native L-selectin could also bear sulfated N-glycans. The N-glycosylation of soluble L-selectin from human serum has not been unraveled yet, probably because

of the difficulty to obtain enough highly purified material, but we are currently working on this task.

Diantennary sulfated GalNAc termini are typical N-glycans of the glycoprotein hormones LH and TSH.[28] There are only a few other glycoproteins reported which bear N-glycans with SO_4−GalNAc termini. Among these are bovine pro-opiomelanocortin,[39,40] recombinant tissue factor pathway inhibitor,[35] Tamm-Horsfall glycoprotein[41] and secreted carbonic anhydrase VI.[42] It has been shown that sulfated GalNAc termini can be bound by the macrophage mannose receptor (Man/S4GGnM receptor).[43,44] Interestingly, the macrophage mannose receptor is not only expressed on macrophages but also on lymphatic vessels, where it was shown to be involved in the control of lymphocyte exit from lymphatic tissues.[45] In another study, it was demonstrated that the macrophage mannose receptor mediates lymphocyte binding to lymphatic endothelium possibly by forming a receptor−ligand pair with L-selectin.[46] The authors could show direct binding of an L-selectin-IgM chimera to the mannose receptor in depletion experiments. Considering the results of the present study, we suggest that this binding could be mediated via the SO_4−GalNAc terminating antennae of L-selectin's N-glycans. Alternatively, the sulfate residues could also function in an analogous way to the sulfate residues of the L- and P-selectin ligand PSGL-1 and mediate homotypic interaction with L-selectin itself or binding of L- to P-selectin. This interaction could lead to secondary rolling of leukocytes on other leukocytes or to competitive regulation of the functionally active soluble L-selectin in the blood serum. Future studies are needed to test these hypotheses.

Abbreviations: 2AB, 2-aminobenzamide; AGP, α-1 acid glycoprotein; BHK, baby hamster kidney; dHex, deoxyhexose; Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HEK, human embryonic kidney; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPAEC-PAD, high pH anion-exchange chromatography equipped with pulsed amperometric detection; LEHis, truncated derivative of L-selectin comprising the lectin and EGF domain fused to a His-tag; Le^X, Lewis X; LH, luteinizing hormone; MALDI, matrix-assisted laser-desorption ionization; MS, mass spectrometry; Neu5Ac, *N*-acetylneuraminic acid; PBS, phosphate buffered saline; PNGase F, peptide N-glycosidase F; PSGL-1, P-selectin glycoprotein ligand 1; S, sulfate; SA, *N*-acetylneuraminic acid; TFA, trifluoroacetic acid; TOF, time-of-flight; TSH, thyroid-stimulating hormone.

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Supporting Information Available: Table S1. Exoglycosidase digestions of neutral N-glycans from LEHis variants and AGP. Sulfated structures were also sporadically observed in the spectrum recorded in the positive-ion mode but they are not represented here. Assignments of sulfated structures were done in the negative-ion mode where ionization is more efficient, therefore rendering assignments unambiguous. G, bovine testes β-galactosidase; F, almond meal α(1−3,4) fucosidase, H^1, β-*N*-acetylhexosaminidase, recombinant gene from *Streptococcus pneumoniae*, expressed in *E. coli*; H, jack bean β-*N*-acetylhexosaminidase. X denotes the presence of a peak in the MALDI-TOF spectra of the desialylated N-glycans. Note that some low abundant structures observed in Figure 2 were not observed here. Table S2. Exoglycosidase digestions of sulfated N-glycans from LEHis variants. G, bovine testes β-galactosidase; F, almond meal α(1−3,4) fucosidase, H^1, β-*N*-acetylhexosaminidase, recombinant gene from *Streptococcus pneumoniae*, expressed in *E. coli*; H, jack bean β-*N*-acetylhexosaminidase. X denotes the presence of a peak in the MALDI-TOF spectra of the desialylated N-glycans. This material is available free of charge via the Internet at http://pubs.acs.org.

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