H (0) blood group determinant is present on soluble human L-selectin expressed in BHK-cells

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Abstract In the present study we show that the H (0) blood group determinant Fucα1-2Galβ1-4GlcNAcβ1-R is present on *N*-linked glycans of soluble human L-selectin recombinantly expressed in baby hamster kidney (BHK) cells. The glycans were isolated using complementary HPLC techniques and characterized by a combination of exoglycosidase digestion and mass spectrometry. The linkage of the fucose residues was determined by incubation of the glycans with specific fucosidases. The H blood determinant Fucα1-2Galβ1-4GlcNAcβ1 was detected for bi-, 2,4 branched tri- and tetraantennary structures. To our knowledge, the proposed oligosaccharide structures represent a new glycosylation motif for recombinant glycoproteins expressed on BHK cells.
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1. Introduction

L-selectin is a member of the selectin family of adhesion proteins. Selectins mediate the process of rolling, the initial step of extravasation of leukocytes from the blood stream, either into inflamed tissues [1] or into peripheral lymph nodes [2]. Rolling of leukocytes along the endothelium of blood vessels is induced by binding of selectins to surface carbohydrate ligands expressed on endothelial cells or on leukocytes [3]. While the recruitment of leukocytes to sites of inflammation is a desired action of the immune system, excessive leukocyte trafficking contributes to severe tissue destruction [4]. Many strategies have been published for blocking the interaction of selectins with their carbohydrate ligands. We are interested in the competitive prevention of cell-cell adhesion by circulating soluble forms of L-selectin (sL-selectin). During the process of rolling L-selectin is cleaved by an unknown protease and released from the cell surface [5]. The shed form of sL-selectin remains bioactive, and can inhibit L-selectin mediated adhesion in a concentration dependent manner [6]. For the structural and biochemical characterization of sL-

selectin, the cDNA was cloned and overexpressed in baby hamster kidney (BHK) cells. Recombinant sL-selectin (rsL-selectin) consists of distinct domains, including an amino-terminal C-type lectin domain, one EGF-like domain and two short consensus repeat domains. The polypeptide backbone contains seven potential *N*-glycosylation sites, but the structures of the carbohydrate moiety of natural and recombinant sL-selectin are still unknown. In the present report we describe the structural characterization of *N*-linked glycans carrying the H (0) blood group determinant which was unexpectedly found for rsL-selectin expressed in BHK cells.

2. Materials and methods

2.1. Materials
rsL-selectin from BHK cells was provided by the group of Prof. Tauber (Freie Universität Berlin, Germany). Trypsin was obtained from Serva (Heidelberg, Germany) and trifluoroacetic acid was obtained from Sigma (Deisenhofen, Germany). PNGase F, neuraminidase, β-*N*-acetylhexosaminidase and β-galactosidase were purchased from Boehringer Mannheim (Mannheim, Germany). Signal Labeling Kit was obtained from Oxford GlycoSciences (Abingdon, UK). Fucosidase II and III were purchased from Dextra (Reading, UK).

2.2. Tryptic digestion of rsL-selectin
Tryptic digestion of rsL-selectin (3 nmol) was performed overnight at 37°C in 45 μl of a buffer containing 50 mM *N*-methyl-2,2-iminodiethanol, pH 8.0, by addition of two portions of trypsin (1/100 w/w) at 0 h and 8 h. The incubation was stopped by heat inactivation of trypsin (100°C for 5 min).

2.3. Release of N-glycans by PNGase F digestion
Tryptic glycopeptides obtained from 3 nmol rsL-selectin were digested with 5 mU PNGase F from *Flavobacterium meningosepticum* in 50 μl of a buffer containing 50 mM *N*-methyl-iminodiethanol, pH 8.0, for 18 h at 37°C.

2.4. Fluorescent labeling of the glycans
Released *N*-glycans were fluorescently labeled by reductive amination with 2-aminobenzamide (2-AB) using a Signal Labeling Kit according the protocol of the manufacturer. Excess 2-AB was removed by paper chromatography as described earlier [7].

2.5. Desialylation
Fluorescently labeled *N*-glycans (about 20 nmol) were treated with 0.1 U neuraminidase from *Vibrio cholerae* in 80 μl of 50 mM sodium acetate buffer, pH 5.5, overnight at 37°C.

2.6. Separation of desialylated N-glycans
Desialylated 2-AB-labeled *N*-glycans were separated by amino-phase HPLC using an APS 2-Hypersil column (4×250 mm, 3 μm, Bischoff (Leonberg, Germany)) at room temperature at a flow rate of 1.5 ml/min. Glycans were eluted by a two-step gradient, consisting of solvent A (acetonitrile) and solvent B (15 mM sodium phosphate, pH 5.2) starting from 0% solvent B to 20% B in 10 min then to 50% B within 100 min.

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Obtained glycan fractions were rechromatographed by RP-18 HPLC using an ODS-Hypersil column (4×250 mm, Knauer (Berlin, Germany)) at room temperature at a flow rate of 1 ml/min. After injection of the sample, the column was eluted with 7 ml H_2O, followed by a linear gradient up to 25% acetonitrile within 75 min. The applied two-dimensional HPLC-based separation was described in detail recently [7].

2.7. Oligosaccharide sequence analysis

The glycan fractions were enzymatically sequenced by incubation with a mixture of β-*N*-acetylhexosaminidase and β-galactosidase, both from *Streptococcus pneunomiae* [8]. Exoglycosidase digestion was performed at an enzyme concentration of 50 mU/ml for β-galactosidase and 50 mU/ml for β-*N*-acetylhexosaminidase in 25 mM NH_4OAc, pH 5.5, overnight (37°C). Digestion products were analyzed with matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Digestion of the glycan fractions with fucosidase II or fucosidase III was performed in the same buffer at an enzyme concentration of 10 mU/ml in a final volume of 20 µl according to the protocol of the manufacturer. Digestion products were analyzed with MALDI-TOF-MS.

2.8. Mass spectrometry

MALDI-TOF-MS was carried out as described previously [9]. 6-Aza-2-thiothymine was used as a matrix as reported by Papac et al. [10].

3. Results

3.1. Isolation and mass determination of the bifucosylated N-glycans

The asparagine-linked sugar chains of rsL-selectin were enzymatically released from tryptic peptides by digestion with PNGase F and fluorescently labeled with 2-AB. Purified oligosaccharides were desialylated by incubation with neuraminidase. Desialylation was performed to reduce the structural heterogeneity of the glycans and to facilitate the analysis of side compounds.

Separation of the asialo oligosaccharides using amino-phase HPLC yielded an elution pattern with three main signals and several minor signals representing the heterogeneity of *N*-glycans (Fig. 1). All peaks were subjected to mass determination
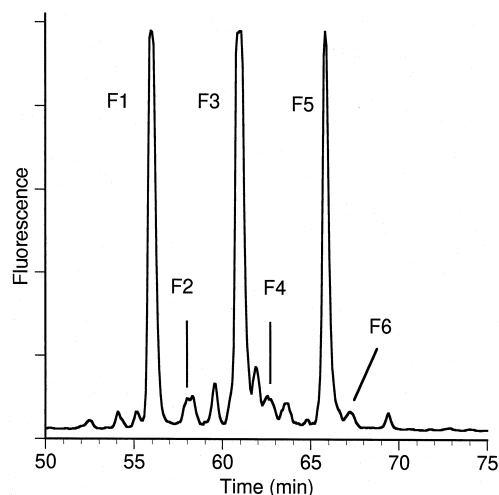

Fig. 1. Separation of desialylated 2-AB labeled *N*-glycans from rsL-selectin. The *N*-linked glycans were enzymatically released and fluorescently labeled with 2-AB. After desialylation by incubation with neuraminidase, the neutral *N*-glycans were separated using aminophase HPLC. The main signals F1, F3 and F5 represent bi-, tri- and tetraantennary structures with one fucose residue, while F2, F4 and F6 were found to contain two fucoses. These fractions were subjected to further structural characterization by exoglycosidase digestion and mass determination.

by MALDI-TOF-MS. Pseudomolecular ions of most fractions were consistent with masses of common complex-type *N*-glycans. The main signals F1, F3 and F5 consist of monofucosylated bi-, tri- and tetraantennary structures, respectively. Mass determination of minor fractions F2, F4 and F6 resulted in ions consistent with bi-, tri- and tetraantennary glycans carrying two deoxyhexose residues (Table 1). Since coeluting sugars were still present in these fractions, reversed phase chromatography was used to obtain homogeneous glycan fractions for further structure determination. Rechromatography of F2, F4 and F6 yielded several subfractions. Mass spectrometry

Table 1
Mass determination and enzymatic sequencing of bifucosylated *N*-glycans

OS fraction[a]	Ions *m/z*	$(M_{calc}+Na)^+$	Composition	Digestion with			
	before digestion			Fuc'ase II	Gal'ase/GlcNAc'ase after fuc'ase II		Gal'ase/GlcNAc'ase
F2.1	2078	2076.9			−2 Gal		−1 Gal
			d-$Hex_2Hex_5HexNAc_4$	−1 Fuc			
F2.2	2077	2076.9			−2 GlcNAc		−1 GlcNAc
F4.1	2442	2442.2			−3 Gal		−2 Gal
			d-$Hex_2Hex_6HexNAc_5$	−1 Fuc			
F4.2a	2443	2442.2			−2 GlcNAc		−1 GlcNAc
F4.2b	2442	2442.2	d-$Hex_2Hex_6HexNAc_5$	−1 Fuc	−3 Gal		−2 Gal
					−2 GlcNAc		−2 GlcNAc
F6.1	2807	2807.6	d-$Hex_2Hex_7HexNAc_6$	−1 Fuc	−4 Gal		−3 Gal
F6.2	2808	2807.6			−1 GlcNAc		−1 GlcNAc

Masses of desialylated oligosaccharides were determined before and after digestion with exoglycosidases by MALDI-TOF-MS. Applied enzymes were fucosidase II (Fuc'ase II) and a mixture of β1-4 galactosidase/*N*-acetylglucosaminidase from *S. pneumoniae* (Gal'ase/GlcNAc'ase). The number of released residues of fucose (Fuc), galactose (Gal) and *N*-acetylglucosamine (GlcNAc) is given. Monitored ions consist of the Na adducts $(M+Na)^+$ of the glycans (values are rounded). M_{calc} refers to the calculated average molecular mass (in Da) summed from the likely carbohydrate composition plus the 2-AB label.
[a]OS fraction: oligosaccharide fraction numbers refer to the glycan fractions from the amino-phase HPLC and the subsequent reversed phase HPLC.
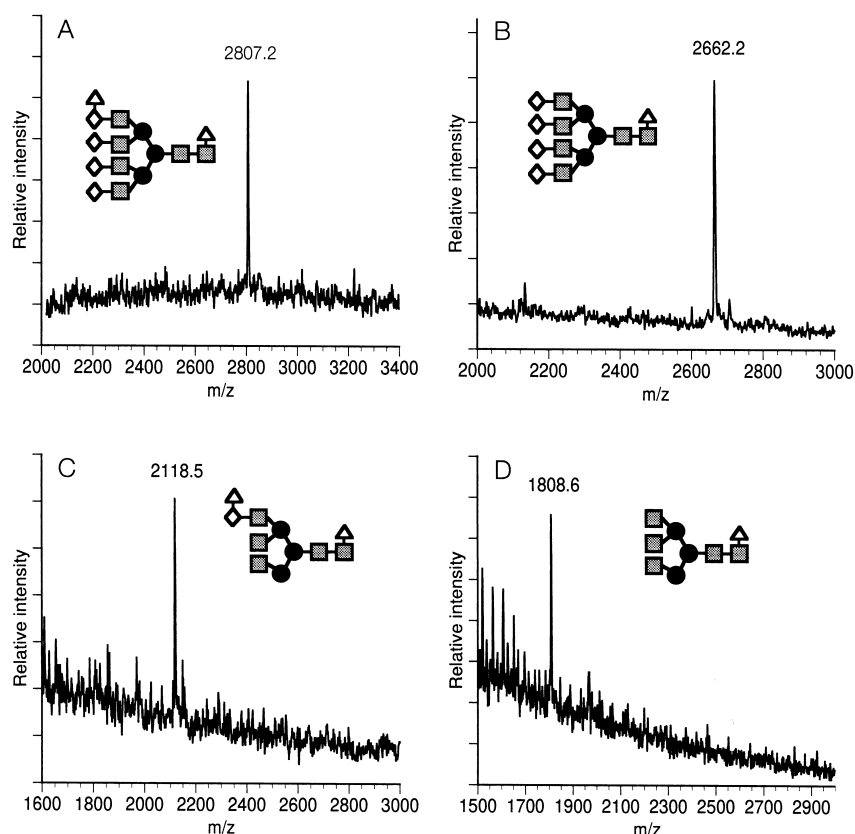
Fig. 2. MALDI-TOF-MS spectra of a tetraantennary glycan containing two fucose residues (fraction 6.1). Mass determination was performed of the undigested 2-AB labeled glycan (A) and after treatment with the following exoglycosidases: fucosidase II (B), a mixture of β1-4 galactosidase and N-acetylglucosaminidase from S. pneumoniae before (C) and after digestion with fucosidase II (D). Glycan key: ◇ = galactose, ■ = N-acetylglucosamine, ● = mannose, △ = fucose.

revealed the presence of bifucosylated sugars in designated fractions F2.1, F2.2, F4.1, F4.2, F6.1 and F6.2.

3.2. Structural characterization of the bifucosylated N-glycans

The bifucosylated sugars were digested with exoglycosidases to determine the linkage of the fucose residue to the antennae, type of antennae and branching of the N-glycans, as well as the linkage of the fucosylated antennae to the glycan core. The reaction products were detected by MALDI-TOF-MS (Fig. 2).

3.2.1. Linkage of the fucose residue to the antennae. The linkage of the fucose residues was characterized using fucosidase III, which specifically cleaves fucose α1-3/4 linked to N-acetylglucosamine, and fucosidase II, which releases fucose α1-2 linked to galactose. Treatment of the bifucosylated glycans from rsL-selectin with fucosidase III did not result in a loss of fucose residues. Surprisingly, after incubation with fucosidase II, one fucose residue was released from each fraction. Based on the specificity of the applied enzymes, we conclude that the H (0) blood group determinant Fucα1-2Gal is present in the antennae of these glycans (Table 2).

3.2.2. Type of antennae and branching of the N-glycan. The glycans obtained after digestion with fucosidase II were further characterized by incubation with a mixture of galactosidase and N-acetylglucosaminidase, both from S. pneumoniae. The applied galactosidase cleaves Galβ1-4GlcNAc/GalNAc but does not act on Galβ1-3GlcNAc/Gal-

NAc at low enzyme concentrations. Under the experimental conditions used, the β-N-acetylhexosaminidase cleaves β1-2 linked GlcNAc unless the mannose to which it is linked has an additional substituent at C-6.

Mass spectrometry revealed that all galactose residues were released from the oligosaccharides. Thus all antennae consist of the structural element Galβ1-4GlcNAc, known as a type II chain. Glycans of fractions F2.1 and F2.2 were degraded to the fucosylated pentasaccharide core. For fractions F4.1 and F4.2 all isomers were converted into the fucosylated core structure plus one HexNAc residue, suggesting the presence of a 2,4 branched triantennary oligosaccharide for the undigested glycans. Exoglycosidase treatment of fractions F6.1 and 6.2 resulted in glycan fragments consistent with the fucosylated core plus three HexNAc residues, as expected for a tetraantennary structure.

3.2.3. Linkage of the fucosylated antennae to the glycan core. To characterize the linkage of the fucosylated antennae to the glycan core we incubated all glycan fractions with a mixture of β1-4 galactosidase and N-acetylglucosaminidase, both from S. pneumoniae, without prior incubation with fucosidase. In every case, the fucosylated galactose was not released by digestion with galactosidase. Mass analysis of the obtained glycan fragments yielded ions consistent with the release of two galactose and one N-acetylglucosamine residues for fraction F4.1. Incubation of fraction F4.2 resulted in two glycans with masses corresponding to the removal of two

Table 2
Proposed structures for the *N*-linked glycans released from rsL-selectin carrying the H (0) blood group determinant

OS-fraction [a]	structure	rel. abundance (%)
F2.1	*(glycan structure)*	0.5
F2.2		0.4
F4.1	*(glycan structure)*	0.5
F4.2a		0.6
F4.2b	*(glycan structure)*	0.3
F6.1	*(glycan structure)*	0.2
F6.2		0.2

[a] OS-fraction=Oligosaccharide fraction numbers refer to the glycan fractions from the amino-phase-HPLC and the subsequent reversed phase-HPLC.

Glycan key:
☐ D-N-acetylglucosamine, ● D-mannose, ◆ D-galactose, ▲ L-fucose
—— β-glycosidic linkage, - - α-glycosidic linkage

galactose and one (F4.2a) or two *N*-acetylglucosamine residues (F4.2b). This result indicated the presence of three isomeric forms of 2,4 branched triantennary structures carrying two fucose residues in F4.1 and F4.2. However, the two potential isomers modified on the different 2′-arms of the 224 triantennary glycan cannot be distinguished by this method. Mass determination of digested fractions F6.1 and F6.2 was consistent with the release of three galactose and one *N*-acetylglucosamine residue. This indicated that the antennae linked to the 2 position of the 2,4 branched mannose in the core does not carry the fucose residue. The results of the enzymatic sequencing of the glycans are compiled in Table 1. From the mass spectrometric data of enzymatically sequenced *N*-glycans we propose the structures for these oligosaccharides shown in Table 2.

4. Discussion

The present study shows that the H (0) blood group determinant Fucα1-2Galβ1-4GlcNAcβ1-R is present in bi-, tri- and tetraantennary *N*-glycans of rsL-selectin expressed in BHK cells.

The H (0) blood group determinant Fucα1-2Galβ1-3/4GlcNAcβ1-R is the precursor of the blood group antigens A and B of the ABO system, as well as the Lewis[b] and Lewis[y] structures. The H structure is synthesized from Galβ1-3/4GlcNAcβ1-R by the action of an α1-2-fucosyltransferase (α1-2FT). At least two α1-2FTs are present in human tissues. The α1-2FT determined at the secretor locus regulates the expression of the H determinant in epithelial cells and body fluids [11]. The α1-2FT encoded at the H gene locus catalyzes the synthesis of the H determinant which is mainly exhibited on the membranes of erythrocytes [12].

The structures carrying the H determinant of rsL-selectin are common bi-, tri- and tetraantennary *N*-glycans that lack polylactosamine sequences but are α1-6 fucosylated in the core pentasaccharide. These glycan structures are abundant on many human glycoproteins in the blood plasma and on the cell surface but might be low affinity substrates of α1-2FT [13,14]. Only a few human plasma derived glycoproteins have been identified that contain minor levels of ABO blood group antigens. Some of the N-glycans on human α2-macroglobulin are modified by the H-structure [15]. Analysis of the associated proteins of factor VIII [16] and von Willebrand Factor

has revealed the presence of ABO-determinants on bi- and triantennary sugar chains [17]. It should be noted that factor VIII recombinantly expressed in BHK-cells does not contain ABO-structures while factor VIII derived from human plasma does. On human erythrocytes these determinants are predominantly linked to *N*-acetyllactosamine repeats, as demonstrated for band 3, the major ABO(H) containing glycoprotein on human erythrocytes [18]. *N*-Glycans carrying the ABO determinants have also been identified for human pancreatic elastase 1 [19], the carcinoembryonic antigen and non-specific cross-reacting antigen-2 [20,21]. In addition, blood group determinants have been determined in the *O*-linked sugar chains of some secreted human glycoproteins [22–25].

BHK and CHO cells are frequently used for the expression of recombinant glycoproteins, in particular of recombinant therapeutic agents. The glycosylation motifs synthesized by BHK and CHO cells have been extensively investigated (for review see [26]) and some glycoproteins were found to carry unique features. In general fucosylation is limited to the innermost residue of *N*-acetylglucosamine in the core of *N*-glycans, but outer chain fucosylation has been reported for recombinant factor VIII from BHK cells [16]. The antigenic Galα1-3Gal epitope has been found in human soluble CD4 variant form CHO cells [27] and in factor VIII derived from BHK cells [16]. Erythropoietin is the only secreted protein of BHK cells that possesses mannose 6-phosphate containing *N*-glycans [28]. To our knowledge the occurrence of the H determinant in a recombinant protein from BHK cells has not hitherto been reported. The presence of the H determinant might result from the activation of a silent endogenous α1-2FT, or more unlikely the activity of an α1-2FT that recognizes a peptide sequence. Unexpected activation of endogenous glycosyltransferases has important implications for the production of recombinant therapeutic agents, since particular oligosaccharides of glycoproteins are known to be immunogenic [29].

At least two immunogenic carbohydrate epitopes, Galα1-3Gal and *N*-glycolylneuraminic acid, can be synthesized by CHO and BHK cells. Factor VIII from BHK cells was found to carry about 3% of the Galα1-3Gal epitope [16]. In human sera approximately 1% of the circulating IgG is directed against this epitope [30]. However, clinical studies showed that the half-life of factor VIII was not reduced by the exposure of the Galα1-3Gal epitope [31]. Glycoconjugates containing *N*-glycolylneuraminic acid are immunogenic in human and can induce antibodies, called Hanganutziu-Deicher antibodies. Recombinantly expressed tissue type plasminogen activator, chimeric plasminogen activator, follitropin and erythropoietin (EPO) from CHO cells contain about 3% *N*-glycolylneuraminic acid in total sialic acid content [32]. In patients who received EPO from CHO cells no elevated levels of Hanganutziu-Deicher antibodies were detected and it was concluded that *N*-glycolylneuraminic acid present in recombinant EPO is minimally antigenic in humans [33]. The H blood group determinant is present in glycolipids and *N*-acetyllactosamine repeats of *N*-glycans on the erythrocytes of all humans, except humans of the rare Bombay and para-Bombay phenotypes, and is not considered to be immunogenic. Whether the H (0) blood group determinant in bi-, tri- and tetraantennary *N*-glycans of rsL-selectin contributes to immunogenicity remains open.

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