Structural determination of two N-linked glycans isolated from recombinant human lactoferrin expressed in BHK cells

Dominique Legrand, Valérie Salmon, Bernadette Coddeville, Monique Benaissa, Yves Plancke, Geneviève Spik*

Laboratoire de Chimie Biologique, UMR CNRS n°111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq Cedex, France

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Abstract A full-length cDNA coding for human lactoferrin was isolated from a mammary gland library and the recombinant protein was expressed in BHK cells as described by Stowell K.M. et al. [1991, Biochem. J. 276, 349–355]. Two N-linked glycans from purified recombinant lactoferrin were released by hydrazinolysis and analyzed by 400-MHz ^{1}H-NMR spectroscopy. The identified structures corresponded to *N*-acetyllactosaminic biantennary glycans and were α-2,3-disialylated forms (80%) or α-2,3-monosialylated (20%) forms. Moreover, 70% of total glycans were α-1,6-fucosylated at the GlcNAc residue linked to asparagine. In regard to its glycan moiety, the recombinant glycoprotein is close to native lactoferrins from milk or leucocytes but shows specific structural features which should be taken into account prior to in vivo and in vitro biological studies.

Key words: Human lactoferrin; Lactotransferrin; *N*-glycan; Recombinant glycoprotein; BHK cell

1. Introduction

Human lactoferrin (or lactotransferrin) (hLf) is a 80 kDa bilobed glycoprotein of the transferrin class which is found in secretions, mainly in milk [1], and is also present in neutrophilic leucocytes [2]. No significant differences between milk and leucocyte hLfs have been reported with respect to the protein moiety [3] but the two *N*-acetyllactosaminic glycans of the molecules are different [4,5]. Milk hLf glycans differ from those of the leucocyte protein by the presence of α-1,6 and α-1,3 fucose residues and by the degree of sialylation [4,5]. Since the only specific structural features of hLfs from various tissues concern the nature of the carbohydrate chains, the question of the influence of the glycan moiety on the biological activities of hLf can be raised. In particular, it can be assumed that the clearance of hLf in the organism or its distribution on cells or tissues is mediated through carbohydrate recognition systems. The binding of milk hLf to monkey enterocytes [6], rat hepatocytes [7,8] and human macrophages [9], essentially through fucose-binding membrane lectins, has been reported. This binding probably does not occur for leucocyte hLf since the molecule is not fucosylated [5].

Expression of cloned hLf [10] and of its N-terminal half [11] in BHK cells has been previously performed to undertake structural, functional and biological studies. Recombinant hLf was shown to be *N*-glycosylated but the structure of the glycans was not determined [10].

Since the definition of the glycosylation pattern of a recombinant protein is of importance for biological studies, we have cloned hLf and produced the recombinant protein in BHK cells. We have resolved the complete structure of two glycans of the recombinant protein using 400-MHz ^{1}H-NMR spectroscopy.

2. Materials and methods

2.1. Materials

All restriction endonucleases and nucleotides were obtained from Eurogentec (Seraing, Belgium). T4 ligase and T4 polynucleotide kinase were from Stratagene (La Jolla, USA). pcDNAI/neo was obtained from Invitrogen (San Diego, USA). Dulbecco's modified essential medium (DMEM), DMEM-F12 nutrient mixture, foetal-calf serum (FCS), Ultroser-G, gentamicin and geneticin G-418 were from Gibco BRL (France). Methotrexate (MTX) was from ICN Biomedical (Aurora, USA). SP-Sepharose Fast Flow was from Pharmacia Biotech (St-Quentin-Yvelines, France) and BioGel P2 was from Bio-Rad Laboratories (Richmond, CA, USA). D_2O (99.95 atom % D) was from the Commissariat à l'Energie Atomique (Saclay, France). Hydrazine, acetic anhydride, sodium acetate, copper acetate and N+O columns sets were from Oxford GlycoSystems (Abingdon, UK). All other reagents were analytical grade or purer.

2.2. Isolation of hLf cDNA and vector construction

A human mammary gland cDNA library constructed in the λgt11 vector (Clontech, Palo Alto, USA) was screened with a synthetic oligonucleotide corresponding to nucleotides 293–317 of hLf cDNA (numbered according to [12]) as a hybridization probe and a positive clone was isolated and sequenced. Due to the presence of an *Eco*RI restriction site at position 2,413 in the coding sequence of the hLf cDNA [12], a full-length 2.3 kb cDNA was excised from λgt11 using mild *Eco*RI hydrolysis conditions (3 U enzyme/μg DNA for 2 min at 37°C) and subcloned into the *Eco*RI site of pBluescript SK (Stratagene, La Jolla, USA). The resulting plasmid was then digested with *Xba*I and *Hin*dIII and the *Xba*I/*Hin*dIII 2.3 kb fragment, made blunt-ended, was subcloned into the *Sma*I site of the expression vector pNUT (generously provided by Dr. R. Palmiter [13]) to yield pNUT-hLf.

2.3. Cell culture, transfection and expression

Baby hamster kidney cells (BHK) kindly provided by Dr. L. Montagné (Institut Pasteur, Paris, France) were maintained on DMEM with 10% (v/v) foetal-calf serum and gentamicin (50 μg/ml) (DME/FCS) at 37°C and 5% CO_2 air environment. BHK cells were cotransfected both with pcDNAI/neo and pNUT-hLf in a ratio of 1/20 by the conventional calcium phosphate technique [14]. For selection of stable transfectants, cells were first cultured in DME/FCS that contained 700 μg/ml of active G-418 and the surviving colonies were grown sequentially with increasing concentrations of methotrexate (MTX), according to [15]. Expression of recombinant hLf from cells surviving in 300 μM MTX was carried out in DME/F12 with 1% Ultroser G [15].

*Corresponding author. Fax: (33) 20 43 65 55.

Abbreviations: hLf, human lactoferrin; DMEM, Dulbecco's modified essential medium; BHK, baby hamster kidney; EPO, erythropoietin; MTX, methotrexate; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas liquid chromatography.

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2.4. Purification and analysis of recombinant protein

Recombinant hLf was purified from DME/F12U by ion exchange chromatography. Medium was first centrifuged at 3,000 × *g* for 15 min to eliminate cells in suspension and passed through a SP-Sepharose Fast Flow column equilibrated with 0.2 M sodium acetate. hLf was eluted with a salt gradient from 0 to 1 M NaCl in 0.2 M sodium acetate. The presence of recombinant hLf in eluted fractions was assessed by ELISA [16]. Positive fractions were pooled, concentrated, dialyzed and stored at −20°C. The purity of recombinant hLf was checked by SDS-PAGE.

2.5. Release and isolation of oligosaccharides

Oligosaccharides from recombinant hLf were released by hydrazinolysis using the GlycoPrep 1000 apparatus (Oxford GlycoSystems) [17]. Hydrazinolysis of 2 mg of recombinant glycoprotein was automatically processed with controlled conditions of temperature and time under an argon atmosphere. After removing excess hydrazine, N-acetylation was achieved by addition of acetic anhydride in a saturated solution of $NaHCO_3$. Sodium ions and peptide material were removed from the sample by passage through a column of Dowex AG50 × 12. The acetohydrazone glycan derivatives were converted to unreduced glycans by addition of 1 mM copper acetate for 30 min at 27°C [18]. After passage through a mixed bed column of Chelex 100/Dowex AG50 × 12, the oligosaccharides were collected, concentrated and purified on a BioGel P2 column (12 × 60 cm). Elution was carried out with distilled water. The oligosaccharide fraction was stained with the orcinol-sulfuric reagent spray on TLC plates and freeze-dried for further analysis.

2.6. Carbohydrate composition

Monosaccharide molar ratios and percentage composition of total neutral sugars, hexosamines and sialic acids were determined after methanolysis, re-*N*-acetylation [19] and GLC of the trimethylsilylated methyl glycosides [20] on a capillary CP SIL 5CB column (0.2 mm × 25 m).

2.7. 400-MHz ^{1}H-NMR Spectroscopy

For the ^{1}H-NMR spectroscopic analysis, the oligosaccharides were repeatedly exchanged in D_2O at room temperature and at pH 7 with intermediate lyophilization [21] and the 400-MHz ^{1}H-NMR spectra were recorded on a Brüker AM 400-WB spectrometer.

3. Results

3.1. Cloning, expression and purification of recombinant hLf

A human mammary gland cDNA library was screened as previously described [12], using a 25-base oligonucleotide complementary to the 5′ coding sequence of the hLf cDNA as a probe. The cDNA of a positive clone was sequenced and found to consist of the coding sequence of hLf cDNA (from nucleotide 287 to the 3′ poly-A tail [12]). As detailed in section 2, after cloning the full-length 2.3 kb cDNA into pNUT, the resulting pNUT-hLf plasmid was used together with pcDNAI-neo to transfect BHK cells. The 300 μM MTX-resistant cells clones were induced with zinc and the amount of lactoferrin in the culture media ranged approximately from 5 to 15 mg. Purification of hLf by cation-exchange chromatography led to the obtention of more than 7 mg protein/liter of culture medium. Recombinant hLf was visualized as a single protein band of 80 kDa by SDS-PAGE (data not shown).

3.2. Carbohydrate composition

The carbohydrate moiety representing 6.0% of the glycoprotein, consisted of 3.3% neutral sugars, 1.9% hexosamines and 0.8% sialic acids. These values were similar to those of milk hLf [4]. The sugars were identified as galactose, fucose, mannose, glucosamine and neuraminic acid by GLC of their trimethylsilyl derivatives. The molar ratios of these monosaccharides were similar to those estimated in the oligosaccharides released after hydrazinolysis (Table 1). Compared to milk hLf, carbohydrate compositions of recombinant hLf and of the oligosaccharides were characterized by a lower content of fucose and neuraminic acid, suggesting both incomplete fucosylation and sialylation of the glycans.

3.3. Determination of glycan primary structure

The oligosaccharides from recombinant lactoferrin were released by hydrazinolysis and submitted to 400-MHz ^{1}H-NMR spectroscopy. The procedure used allowed the release of the oligosaccharides from a small amount of lactoferrin (2 mg) with a good yield (75%). From the ^{1}H-NMR spectrum data (Table 2), the *N*-acetyllactosaminic type of the purified oligosaccharides can be asserted. The anomeric proton of the GlcNAc-1 (δ H-1α = 5.181) proved the completeness of the hydrazinolysis reaction [22]. The presence of the Man-4 and Man-4′ anomeric protons (δ = 5.116 and δ = 4.920, respectively) and also the H-2 of the three mannose residues (δ H-2 Man-3 = 4.250, δ H-2 Man-4 = 4.192, δ H-2 Man-4′ = 4.110) are in favour of a biantennary structure with two fully α-2,3-sialylated branches (approximately 80% of total glycans, calculated on the basis of NMR peak integration and carbohydrate composition) and partially α-2,3-sialylated branches (20% of total glycans) [21]. Asialo Gal-6 and Gal-6′ H-1 are observed at δ = 4.465 and δ = 4.470 ppm, respectively. The α-2,3-linkage of NeuAc is clearly verified by the chemical shift observed for the NeuAc H-3ax and H-3eq atom resonances [21] (Table 2). The spectrum also evidences the presence of α-1,6 Fuc linked to GlcNAc-1, but only for about 70% of the total glycan chains [21], in accordance with the carbohydrate composition. The identified oligosaccharides have the structures indicated in Fig. 1.

4. Discussion

Previous reports dealt with the obtention of functional recombinant hLf and of its N-terminal half in BHK cells with the

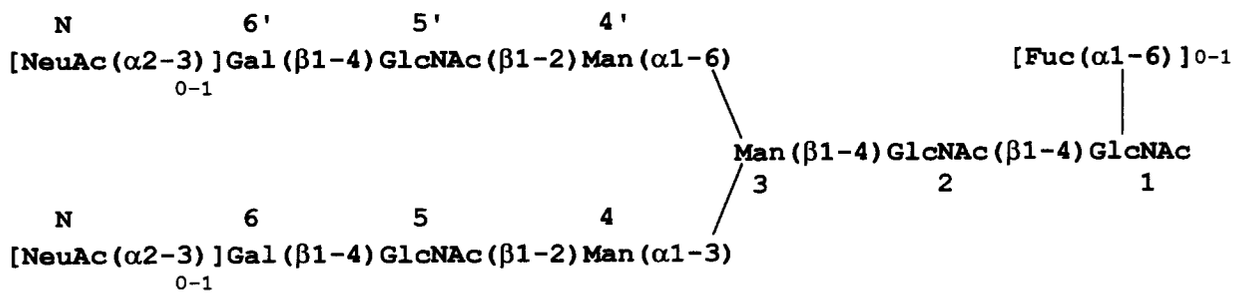


Fig. 1. Primary structures of the oligosaccharides isolated from recombinant hLf in BHK cells.

Table 1
Molar carbohydrate compositions of recombinant hLf compared to milk hLf and of the oligosaccharides released by hydrazinolysis

	Fuc	Gal	Man[a]	GlcNAc	NeuAc
Milk hLf	1.3	2.1	3.0	4.0	1.8
Recombinant hLf	0.8	1.9	3.0	3.6	1.6
Released oligo-saccharides	0.7	2.0	3.0	3.5	1.6

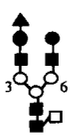
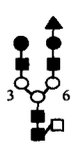
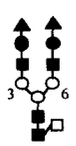
[a] Molar ratios were calculated on the basis of three Man residues.

expression vector pNUT [10,11]. In the present study, we have cloned a full-length cDNA of human lactoferrin and expressed it in BHK cells with a protein yield in the culture medium of 5–15 mg/l, similar to that previously reported [10]. On the basis of a molecular weight of 82,400 Da for hLf [23] and of the percentage carbohydrate composition of the recombinant protein (6.0%), we can assume that the recombinant hLf possesses two glycans of about 2,400 daltons, as do milk and leucocyte hLfs [4,5]. By ^{1}H-NMR spectroscopy, the two glycans of recombinant hLf have been identified as biantennary structures of the sialylated *N*-acetyllactosaminic type with one or two α-2,3-linked NeuAc residues and the partial presence of an α-1,6 fucose residue linked to GlcNAc-1.

It is thus worthy of note that, despite of fucosylation and sialylation features which are specific to the cell line used [24], the glycosylation of recombinant hLf in BHK cells occurs at the same degree of complexity as that of naturally-occurring hLfs [4,5]. A number of other glycoproteins, including serum transferrin [15,25], have been successfully produced at a high-expression level in BHK cells. The glycosylation of some recombinant proteins expressed in BHK cells, for instance human erythropoietin (EPO) [26], has been extensively studied. Human recombinant EPO contains a very complex mixture of tetra-, tri- and biantennary glycans of the *N*-acetyllactosaminic type, sometimes with *N*-acetyllactosamine repeats [26]. Interestingly, sugar chains of recombinant EPO were found to be very similar to those of human urinary EPO, except for the sialyl linkage [27]. In the particular case of recombinant human serum transferrin produced in BHK cells, electrospray mass analysis of the recombinant protein suggested the existence of a complex pattern of bi- and triantennary glycans [15]. Such a microheterogeneity was also evidenced for native human serum transferrin [28,29]. These observations indicate that, whether glycosylation of a recombinant protein depends on the host cell type, the culture environment and the method of cell culture [30], it also depends to some degree on the structure of the protein moiety.

Differences between the glycans of recombinant hLf and those of hLf from milk or from leucocytes concern the nature of the linkage between NeuAc and Gal (α-2,3 for the recombinant protein, instead of α-2,6 for the native lactoferrins), as well as the fucosylation patterns. These differences are relevant to the fact that BHK cells naturally lack the capability of α-2,6 sialylation and α-1,3 fucosylation of Gal β-1,4 GlcNAc-R motifs on *N*-glycans [24].

Table 2
^{1}H chemical shifts of structural reporter groups of constituent monosaccharides for oligosaccharides from recombinant hLf

Reporter group	Residue	Chemical shifts		
				
H-1	GlcNAc-1α	5.181	5.181	5.181
	Fuc(1-6)α,β	4.89	4.89	4.89
	GlcNAc-2	–	–	–
	Man-3	–	–	–
	Man-4	5.116	5.116	5.116
	Man-4′	4.920	4.920	4.920
	GlcNAc-5	4.58	–	4.58
	GlcNAc-5′	–	4.58	4.58
	Gal-6	4.53	4.465	4.53
	Gal-6′	4.470	4.53	4.53
H-2	Man-3	4.250	4.250	4.250
	Man-4	4.192	4.192	4.192
	Man-4′	4.11	4.11	4.11
H-3	Gal-6,6′	–	–	4.11–4.12
H-3ax	NeuAc	1.801	1.801	1.801
H-3eq	NeuAc	2.758	2.758	2.758
H-6	Fuc(1-6)α	1.210	1.210	1.210
	β	1.22	1.22	1.22
NAc	GlcNAc-1	2.04	2.04	2.04
	GlcNAc-2	2.094	2.094	2.094
	GlcNAc-5	–	2.048	2.048
	GlcNAc-5′	–	2.044	2.044
	NeuAc	2.030	2.030	2.030

Compounds are represented by schematic structures according to [21]. ■, GlcNAc; ○, Man; ●, Gal; ▲, Neu5Ac; □, Fuc. Chemical shifts (δ) are given relative to sodium 4–4-dimethyl-4-silapentane-sulphonate but were actually measured by reference to internal acetone in D_2O: δ = 2.225 ppm with an accuracy of more or less than 0.002 ppm.

It is now well admitted that glycans have effects on protein solubility and stability, immunogenicity, pharmacokinetics and biological activities [30]. In the case of lactoferrin, in vitro experiments, however, have failed to demonstrate the role of glycans in iron binding and release [31]. Nevertheless, the glycan moiety was shown to stabilize the iron-binding site of the 30 kDa N-tryptic fragment and the N-terminal domain II of milk hLf [32]. Concerning human serum transferrin, a study reported that the non-glycosylated protein bound to HeLa S3 cells with the same avidity and to the same extent as the glycosylated protein [15]. For many glycoproteins, full glycosylation equates with full biological activity [30], but there are several instances where the glycosylation status has no major impact on the biological efficacy of a protein in vitro. For example, although desialylated human EPO shows a thousand-fold reduction in specific activity in vivo compared to its native form, there is little effect in vitro. [33]. Since no drastic change in the number of sialic acid residues and in the global volume of the carbohydrate chains at the surface of the protein occurred, it is likely that the overall physicochemical properties of recombinant hLf are not altered compared to native hLfs. However, possible alterations of the in vivo biological activity, the clearance and distribution of recombinant hLf in the organism may be induced by the α-2,3 linkage of NeuAc and the lack of α-1,3 Fuc. It can be thus postulated that the masking ability and antigenicity of α-2,3 NeuAc is different from α-2,6 NeuAc and can affect the immunogenicity of the protein. Moreover, recognition systems such as the selectin ELAM-1 expressed on the vascular endothelium are able to trap α-2,3 NeuAc-containing glycoproteins [34]. Finally, the recognition of the α-1,3 Fuc residue present on milk hLf glycans by a specific lectinic receptor on rat hepatic cells has been reported [7,8]. The lack of α-1,3 fucosylation and the partial lack of α-1,6 Fuc in recombinant hLf may also influence the clearance and the distribution of the recombinant protein in the organism.

From this study, we can conclude that under our cell culture conditions, the glycosylation of recombinant hLf is close to that of homologous milk and leucocyte hLfs. Recombinant hLf glycans, however, possess specific features which have to be taken into account for the interpretation of in vitro and in vivo biological experiments.

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