Heterogeneity in utilization of N-glycosylation sites Asn^{624} and Asn^{138} in human lactoferrin: a study with glycosylation-site mutants

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Human lactoferrin (hLF) is a glycoprotein involved in the host defence against infection and excessive inflammation. Our objective was to determine to what extent each of the three sequons for N-linked glycosylation in hLF is actually used. Human kidney-derived 293(S) cell lines expressing recombinant hLF (rhLF) or glycosylation-site mutants were produced. The mutations involved replacement of asparagine residues with glutamine at one or more sequons for N-glycosylation (Asn^{138}, Asn^{479} and Asn^{624}). Comparative SDS/PAGE analyses of rhLF, mutated rhLF and human-milk-derived (natural) hLF led us to propose that glycosylation of hLF occurs at two sites (at Asn^{138} and Asn^{479}) in approx. 85% of all hLF molecules. Glycosylation at a single site (Asn^{479}) or at all three sites occurs in approx. 5% and 9% of hLF respectively. The extent of glycosylation at Asn^{624} was increased to approx. 29% and 40% of Asn^{479} and Asn$^{138/479}$ mutant molecules respectively, which indicates that glycosylation at Asn^{624} in natural hLF might be limited by glycosylation at Asn^{479}. The presence in supernatant of unglycosylated hLF (approx. 60% of the total) after mutations of Asn^{138} and Asn^{479} suggests that glycosylation of hLF is not an absolute requirement for its secretion. The pronounced degradation of unglycosylated hLF in supernatant after mutation at all three glycosylation sites (Asn$^{138/479/624}$ mutant) but not after mutation at both Asn^{138} and Asn^{479} suggests that an altered conformation rather than the lack of glycosylation has rendered the Asn$^{138/479/624}$ mutant susceptible to intra- and/or extra-cellular degradation.

INTRODUCTION

Lactoferrin (LF) is a metal-binding glycoprotein of M_r 80000 [1] found in milk, tears, saliva and bronchial, intestinal, vaginal and other secretions. It is also present in the secondary granules of neutrophils [2]. On the basis of many reports on antimicrobial and anti-inflammatory activity *in vitro*, LF is thought to be involved in the host defence against infection and excessive inflammation, most notably at the mucosal surface. Antimicrobial effects of LF *in vitro* are mediated through sequestration of iron required for microbial growth [3] and through its binding to microbial cell-wall components [4]. Anti-inflammatory activities of LF include inhibition of complement activation [5], inhibition of the production of hydroxyl radicals [6], inhibition of cytokine production [7] and binding of bacterial lipopolysaccharide [8].

Human LF (hLF) consists of a single polypeptide chain of 692 amino acids [9]. It has a 2-fold internal homology with 40% amino acid identity between the N- and C-terminal halves. The bilobal structure of hLF has recently been confirmed by crystallography [1,10]. The so-called N-and C-lobes have very similar tertiary structures and each consists of two subdomains. Each lobe can bind one Fe^{3+} ion while simultaneously incorporating a bicarbonate ion.

Many eukaryotic proteins, including hLF [11], are modified by N-linked glycosylation, an enzymically catalysed process in which oligosaccharides are covalently linked to asparagine residues in the sequon Asn-Xaa-Thr/Ser [12]. However, approx. one-third of these sequons are not glycosylated or are incompletely so [13,14]. Certain sequons might not be glycosylated because the protein structure precludes them from being accessible to the glycosylation enzymes [12,15,16].

Human LF contains three possible N-glycosylation sites, Asn^{138} in the N-lobe and Asn^{479} and Asn^{624} in the C-lobe [9]. Spik et al. [17] have shown that hLF contains two glycan chains of the sialyl N-acetyl-lactosaminic type and have suggested that N-glycosylation at Asn^{624} does not occur. We have recently shown that N-glycosylation heterogeneity in the C-lobe of hLF accounts for the doublet of protein bands, designated the minor and major bands, on SDS/PAGE [18]. We speculated that the major band represents hLF glycosylated at Asn^{138} and Asn^{479}, whereas the minor band is glycosylated at Asn^{138}, Asn^{479} and Asn^{624}. However, neither our results nor those of Spik et al. provided proof as to which N-glycosylation sites are preferentially being used.

In the present paper we describe the mutagenesis of single glycosylation sites and combinations of them in hLF. Analysis of these mutants revealed that Asn^{138} and Asn^{479} are preferentially glycosylated. Glycosylation at Asn^{624} is limited owing to glycosylation at Asn^{479} and/or primary sequence constraints.

MATERIALS AND METHODS

Reagents

The Transformer® site-directed mutagenesis kit was purchased from Clontech (Palo Alto, CA, U.S.A.). All restriction endonucleases, T4 DNA ligase, lipofectamine and cell culture reagents were from Gibco (Paisley, Scotland, U.K.). The expression vector pRc/CMV was obtained from Invitrogen (San Diego,

Abbreviations used: Asn$^{138/479}$ mutant, rhLF with Asn → Gln mutations at positions 138 and 479; Asn$^{138/479/624}$ mutant, rhLF with Asn → Gln mutations at positions 138, 479 and 624; hLF, human lactoferrin; natural hLF, hLF from human milk; rhLF, recombinant hLF.

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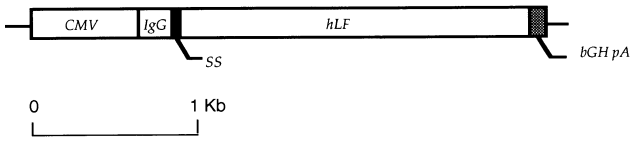

Figure 1 Schematic representation of the expression vector pCMV/hLF (for rhLF) and derivatives (for rhLF glycosylation-site mutants)

The labelled elements are: *CMV*, cytomegalovirus immediate early gene promoter/enhancer element; *IgG*, human IgG splice acceptor site; *SS*, bovine αS_1 casein signal sequence; *hLF*, hLF cDNA [9] with $Thr^{130} \rightarrow Ile$ and $Cys^{404} \rightarrow Gly$ changes (for rhLF) or glycosylation-site mutants: the same cDNA with $Asn^{138} \rightarrow Gln$ and/or $Asn^{479} \rightarrow Gln$ and/or $Asn^{624} \rightarrow Gln$ mutation(s); *bGH pA*, bovine growth hormone polyadenylation sequence.

CA, U.S.A.). Mutagenic primers, the T7 DNA sequencing kit and S Sepharose were obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Human LF was purified from fresh human milk (hereafter designated 'natural hLF') [18]. Polyclonal rabbit anti-hLF antiserum was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). N-glycosidase F and n-octylglucoside were obtained from Boehringer-Mannheim (Mannheim, Germany).

Construction of the expression vector for recombinant hLF (rhLF)

Human LF cDNA was obtained from $p\alpha S_1$/hLF [18]. This hLF cDNA [9] contains two unique differences from other published hLF cDNA sequences. Therefore to obtain the most frequently occurring cDNA sequence for hLF [19–21], the codons ACA (Thr^{130}) and TGC (Cys^{404}) were mutated by the method of Kunkel [22] into ATA (Ile) and GGC (Gly) respectively. After mutagenesis, hLF cDNA was ligated back into $p\alpha S_1$/hLF. A fragment containing the 5′ IgG splice acceptor site, the bovine αS_1 casein signal sequence and hLF cDNA was excised by *Hin*dIII–*Sal*I digestion and subcloned in pKUN [23]. The fragment was subsequently excised with *Hin*dIII/*Xba*I and cloned in pRc/CMV, which contains a neomycin resistance gene. This expression vector was designated pCMV/hLF (Figure 1).

We have previously shown that the bovine αS_1 casein signal sequence is correctly and completely removed in 293(S) cells to yield mature rhLF [18].

Site-directed mutagenesis of the hLF N-glycosylation sites

Site-directed mutagenesis of the hLF N-glycosylation sites on the expression vector pCMV/hLF was performed by the method of Deng and Nickoloff [24] with the Transformer® site-directed mutagenesis kit. A selection primer was designed that changed a unique *Xba*I site (from the polylinker of pRc/CMV) into an *Nru*I site. Mutagenic primers for N-glycosylation sites were used that changed AAT (Asn^{138} and Asn^{624}) and/or AAC (Asn^{479}) into CAG (Gln). The asparagine residues were replaced with glutamine as this blocks glycosylation and supposedly does not lead to an altered conformation of a mutant protein [25].

Mutagenesis was performed in accordance with the protocols of Clontech. After mutagenesis, clones were screened by *Nru*I digestion for the selection mutation. Clones positive for this selection mutation were screened for the desired mutation by dideoxy sequence analysis.

Transfection and cell culture conditions

Human kidney 293(S) cells (ATCC CRL 1573) were cultured in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) fetal calf serum, 50 units/ml penicillin and 50 μg/ml streptomycin as described [18]. Cells were transfected with the various expression vectors using lipofectamine in accordance with the instructions of Gibco. After transfections, 400 μg/ml Geneticin (G418) was included in the culture medium to select for stable transfectants. Subcloning of cells expressing rhLF (as determined with ELISA for hLF), by repeated limiting dilutions, resulted in several lines expressing rhLF. Clones expressing rhLF at the highest level were used for further experiments.

Purification of hLF

rhLF and natural hLF were purified from culture medium and human milk, respectively, by cation-exchange chromatography on S Sepharose as described [18]. Human LF eluted from the S Sepharose was diluted, applied on a Mono S HR 5/5 column (Pharmacia) and eluted with a linear salt gradient of 0–1.0 M NaCl as described [18]. Human LF eluting from Mono S at 0.7 M NaCl was subjected to SDS/PAGE analysis [18].

Deglycosylation of natural hLF

To 100 μg of hLF (in 20 μl), SDS was added to 1% (w/v) final concentration. This sample was boiled for 2 min. Deglycosylation buffer (9 vol.; 180 μl) of 20 mM sodium phosphate, pH 7.2/10 mM sodium azide/50 mM EDTA/0.5% (w/v) n-octylglucoside was added and the mixture boiled for another 2 min. N-glycosidase F (4 units) was added to the mixture and incubated for 16 h at 37 °C.

SDS/PAGE analysis

SDS/PAGE analysis (7.5% w/v) of hLF was performed after boiling for 5 min in non-reducing SDS sample buffer [62.5 mM Tris/HCl, pH 6.8, containing 4% (w/v) SDS, 8.7% (w/v) glycerol and 0.005% (w/v) Bromophenol Blue] to achieve denaturation and concomitant desaturation of hLF [18]. Proteins were revealed by staining with Coomassie Brilliant Blue and hLF protein bands were quantified by densitometry with IPlabGel software of Signal Analytics (Vienna, VA, U.S.A.).

ELISA for hLF

A quantitative hLF-specific ELISA was performed as follows: PBS containing 1 μg/ml affinity-purified rabbit anti-hLF [27] was incubated for 16 h at 20 °C in microtitre plates (Polysorp; Nunc, Roskilde, Denmark). Plates were then washed with PBS, 0.02% (w/v) Tween-20 and incubated with 2-fold serial dilutions of the test samples and natural hLF standard in PTG buffer (PBS containing 0.2% (w/v) gelatin and 0.02% (v/v) Tween-20). After 1 h, plates were washed and PTG containing 1% (v/v) normal bovine serum and 0.4 μg/ml peroxidase-conjugated bovine anti-hLF [18] was added to each well. Plates were incubated for a further 1 h, washed, and substrate solution [0.01% 3,3′,5,5′-tetramethylbenzidine (TMB), 0.003% (v/v) H_2O_2 in 0.11 M sodium acetate, pH 5.5] was added. Substrate conversion was stopped by the addition of 2 M H_2SO_4 and A_{450} was measured with a 340 ATCC microplate reader (SLT-Labinstruments, Austria). All incubations were performed with 100 μl volumes. This assay can detect as little as 50 pg of hLF per 100 μl.

RESULTS

Expression of rhLF and rhLF glycosylation-site mutants

Cell lines expressing rhLF or rhLF glycosylation-site mutants were made by transfection of 293(S) cells with hLF cDNA-based

Table 1 Ratio of intracellular to total rhLF produced by 293(S) cells

Cloned 293(S) cells producing either rhLF or glycosylation-site mutants were washed with PBS and inoculated into fresh culture medium (2×10^5 cells in 5.0 ml). After 96 h, 5.0 ml supernatants and cells were harvested. Cells were counted, washed and resuspended in 250 μl of PBS. The cells were lysed by repeated freezing and thawing (three times) and diluted 2-fold with 5 M NaCl, and the lysate was cleared by centrifugation at 14000 ***g*** for 10 min at 4 °C [28]. The concentration of rhLF in supernatants and cell lysates was analysed by ELISA. Results (means ± S.D. for four separate cultures) were expressed as ng of rhLF produced per 96 h per 10^5 cells. Abbreviation: n.d., not detectable (below the detection limit of the ELISA for hLF).

	rhLF (ng/96 h per 10^5 cells)		
Variant	Secreted	Cell lysate	Intracellular/ total (%)
rhLF	76.6 ± 6.0	3.7 ± 0.3	4.6 ± 0.6
Asn → Gln at 138	13.3 ± 1.0	1.0 ± 0.1	6.9 ± 0.8
Asn → Gln at 479	43.0 ± 7.6	2.6 ± 0.1	5.7 ± 1.0
Asn → Gln at 624	138 ± 17	5.6 ± 0.2	4.0 ± 0.5
Asn → Gln at 138 and 479	71.3 ± 4.3	3.1 ± 0.2	4.2 ± 0.4
Asn → Gln at 138, 479 and 624	n.d.	0.06 ± 0.01	–

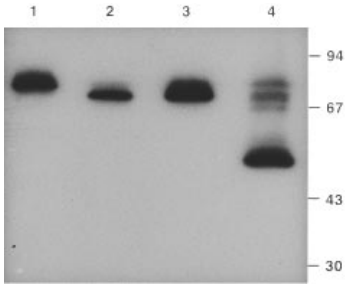

Figure 2 SDS/PAGE and immunoblotting analysis of secreted rhLF glycosylation-site mutants

Supernatant of 293(S) cells expressing glycosylation-site mutants as well as purified natural hLF and enzymically deglycosylated hLF were incubated with purified rabbit anti-hLF antibodies coupled to Sepharose. After washing, hLF was dissociated into non-reducing sample buffer by boiling for 5 min and then subjected to SDS/PAGE (12.5% gel). Proteins from the gel were transferred on to nitrocellulose [26] and incubated with peroxidase-labelled bovine anti-hLF antibodies. After being washed, hLF protein bands were revealed by chemiluminescence detection (ECL, Amersham International). Lane 1, 15 ng of natural hLF; lane 2, 15 ng of enzymically deglycosylated hLF; lane 3, 15 ng of $Asn^{138/479}$ mutant; lane 4, $Asn^{138/479/624}$ mutant precipitated from 20 ml of supernatant obtained after 6 days of culture. Numbers at the right-hand side ($M_r \times 10^{-3}$) indicate the migration of protein standards.

expression vectors (Figure 1). Table 1 shows that the expression levels of the various 293(S) cells expressing (mutated) rhLF varied considerably. However, except for the mutant with all N-glycosylation sites being mutated (hereafter designated the $Asn^{138/479/624}$ mutant), the ratio of intracellular to total rhLF was approx. 5% for all rhLF species. This suggests that differences in integration site and copy number of hLF cDNA between the cell lines explain at least in part the extensive variation in the expression of rhLF mutants.

Secretion of the $Asn^{138/479/624}$ mutant, under the conditions described in Table 1, was below the detection limit of the ELISA for hLF. The same holds for all other $Asn^{138/479/624}$ clones obtained from three different transfections. SDS/PAGE and immunoblotting analysis of the $Asn^{138/479/624}$ mutant immunoprecipitated from large volumes of supernatant showed faint bands of M_r 77000, 71000 and 67000 as well as a pronounced band of M_r 50000 (Figure 2, lane 4). The band of M_r 71000

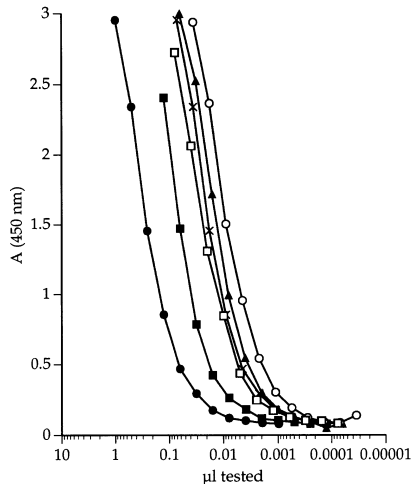

Figure 3 ELISA for rhLF and glycosylation-site mutants

Serial dilutions of purified natural hLF (●, 5 μg/ml), rhLF (○, 154 μg/ml) and rhLF mutated at Asn^{138} (×, 77 μg/ml), at Asn^{479} (■, 21 μg/ml), at Asn^{624} (▲, 102 μg/ml) or at $Asn^{138/479}$ (□, 57 μg/ml) were incubated with affinity-purified rabbit anti-hLF coated on microtitre plates as described in the Materials and methods section. Bound hLF was detected by subsequent incubation with peroxidase-conjugated bovine anti-hLF. The A_{450} values measured after the conversion of substrate was stopped with H_2SO_4 are indicated on the ordinate. The experimental volume tested (μl) is indicated on the abscissa.

represents unglycosylated rhLF (compare with enzymically deglycosylated hLF; Figure 2, lane 2), whereas that of M_r 77000 most probably results from very-low-grade contamination of immunoprecipitates with hLF and its sensitive detection on the blot. The notion that contamination with natural hLF has occurred during immunoprecipitation is supported by the absence of a protein band of M_r 77000 in preparations of purified rhLF with Asn → Gln mutations at positions 138 and 479 ($Asn^{138/479}$ mutant) (see below). The hLF degradation products of M_r 67000 and 50000 were not observed in the supernatant of cells expressing the $Asn^{138/479}$ mutant, although approx. 60% of total hLF represents unglycosylated hLF of M_r 71000 (Figure 2, lane 3). These observations indicate that the lack of N-glycosylation and/or an aberrant conformation resulting from the introduction of three mutations has rendered the $Asn^{138/479/624}$ mutant susceptible to intracellular and/or extracellular degradation, which limits the amount of intact unglycosylated rhLF accumulating in the supernatant.

rhLF and glycosylation-site mutants were purified by cation-exchange chromatography. Absorbance measurements at 280 and 465 nm as well as non-boiled non-reduced SDS/PAGE [18] revealed that (mutated) rhLF was completely saturated with iron. Purified rhLF, mutated rhLF and natural hLF showed identical slopes in the ELISA for hLF (Figure 3). This indicates that (mutated) rhLF is immunologically identical with natural hLF and that quantification of rhLF by reference to natural hLF is justified (see also [18]).

SDS/PAGE analysis of natural hLF and rhLF glycosylation-site mutants

We have previously shown that glycosylation heterogeneity of natural hLF accounts for the minor (M_r 80000; approx. 10% of the molecules) and major band (M_r 77000; approx. 90% of the molecules) of hLF on SDS/PAGE [18]. In the present study we also noticed a third band (M_r 74000) designated the minute band

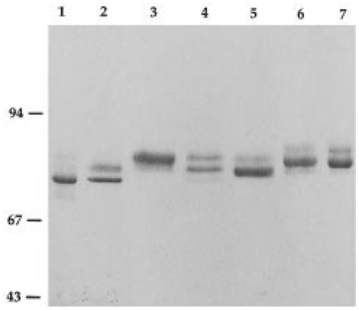

Figure 4 SDS/PAGE analysis of rhLF glycosylation-site mutants

Samples of purified hLF (5 μg) were diluted in non-reducing sample buffer and subjected to SDS/PAGE (7.5% gel) after boiling for 5 min. Lane 1, enzymically deglycosylated natural hLF; lane 2, rhLF mutated at $Asn^{138/479}$; lane 3, rhLF mutated at Asn^{624}; lane 4, rhLF mutated at Asn^{479}; lane 5, rhLF mutated at Asn^{138}; lane 6, rhLF; lane 7, natural hLF. Numbers at the left-hand side ($M_r \times 10^{-3}$) indicate the migration of protein standards.

Table 2 Densitometric analysis of protein bands in natural hLF, rhLF and glycosylation-site mutants

Values shown are the relative amounts of total hLF, expressed as percentages. Means ± S.D. were calculated from densitometric analyses of at least four different SDS/PAGE gels.

	Relative amount (%) of total hLF			
M_r of hLF protein band ...	80000	77000	74000	71000
Natural hLF	9±3	85±7	5±6*	–
rhLF	7±1	88±9	5±8*	–
Asn → Gln at 138	–	10±6	89±5	–
Asn → Gln at 479	–	29±9	57±9	11±4
Asn → Gln at 624	–	94±9	4±6*	–
Asn → Gln at 138 and 479	–	–	42±8	57±7

* The relatively high S.D. on densitometry of protein bands of M_r 74000 is related to differences in resolution from the more pronounced bands of M_r 77000 resulting in quantification problems.

as it comprises only approx. 5% of total natural hLF (Figure 4, lane 7; Table 2). On the basis of the differences in M_r between minor, major and minute hLF, the M_r of 71000 of enzymically deglycosylated hLF (Figure 4, lane 1) and the M_r of 2200 for a biantennary glycan [17], we hypothesized that the minor, major and minute bands represent hLF bearing three (to Asn^{138}, Asn^{479} and Asn^{624}), two (most probably to Asn^{138} and Asn^{479}) and one *N*-glycan chains respectively. In support of this hypothesis is the observation (with SDS/PAGE analysis of natural hLF and rhLF proteolysed with trypsin) that N-glycosylation heterogeneity resides in the C-lobe of hLF [18]. Obviously, definite proof for this model by studies with glycosylation-site mutants can be obtained only from an expression system in which rhLF bears the same glycosylation profile as natural hLF. This indeed seems to be so with the 293(S) cells: the ratio of minor to major to minute band in rhLF (Figure 4, lane 6; Table 2) was assessed as being 7:88:5 respectively, which is the same as that in natural hLF (Figure 4, lane 7; Table 2).

To prove that the minor band of M_r 80000 in natural hLF and rhLF represents hLF bearing three glycans at Asn^{138}, Asn^{479} and Asn^{624}, we analysed the mutants in which single glycosylation sites had been mutated (Figure 4, lanes 3–5). The highest M_r observed with any of these mutants was 77000, which matches a maximum content of two glycans. SDS/PAGE analysis of purified glycosylation mutants treated with N-glycosidase F showed a single protein band of M_r 71000, which confirms that protein bands of M_r 77000 and 74000 represent hLF molecules bearing two and one glycans respectively (P. H. C. van Berkel, unpublished work).

Mutation at Asn^{624} resulted in bands of M_r 77000 and 74000 at a ratio of 94:4 (Figure 4, lane 3; Table 2). This result indicates that sites Asn^{138} and Asn^{479} are glycosylated in 94% of the Asn^{624} mutant molecules, and that glycosylation at both sites accounts for the major band in rhLF (Figure 4, lane 6). Thus apparently only glycosylation at Asn^{624}, which occurs in 7% of rhLF (minor hLF), was blocked after the mutation at this site.

Mutation at Asn^{138} yielded bands of M_r 77000 and 74000 at a ratio of 10:89 (Figure 4, lane 5; Table 2). This ratio corresponds to the ratio of minor to major band in natural hLF and rhLF (compare lane 5 in Figure 4 with lanes 6 and 7; see also Table 2). This result indicates that glycosylation at Asn^{479} and Asn^{624} occurred in 100% and 10% of the Asn^{138} mutant molecules respectively and that glycosylation at Asn^{138} occurs in all rhLF molecules except in minute hLF (5% of hLF).

Unglycosylated hLF was present only in the Asn^{479} mutant (11% of total; Figure 4, lane 4; Table 2), but not in rhLF, nor after mutation at Asn^{138} or Asn^{624} (Figure 4, lanes 5 and 3). This indicates that glycosylation at Asn^{479} occurs always, whereas that at Asn^{138} occurs in approx. 95% of hLF molecules. This conclusion implies that protein of M_r 74000 after mutation of Asn^{624} (4%; Figure 4, lane 3), after mutation at Asn^{138} (89% of mutant hLF; Figure 4, lane 5) as well as in rhLF (i.e. minute hLF; Figure 4, lane 6) is glycosylated only at Asn^{479}. In addition the band of M_r 77000 after mutation at Asn^{479} (29% of mutant

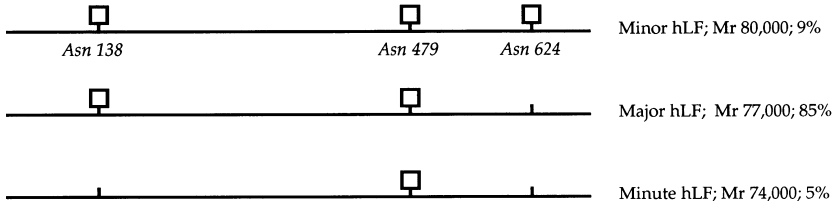

Figure 5 Proposed distribution of N-glycans in natural hLF

The horizontal lines represent minor (top), major (middle) and minute (bottom) hLF species with three, two and one N-glycan chains respectively. Short vertical lines mark the potential glycosylation sites and a box on top of that indicates that the site is actually used. The M_r values and percentages indicated on the right are estimates based on SDS/PAGE analyses (Figure 4) and densitometric analyses (Table 2).

hLF; Figure 4, lane 4) represents hLF glycosylated at both Asn^{138} and Asn^{624}.

Mutation at both Asn^{138} and Asn^{479} showed bands of M_r 74000 and 71000 that represent hLF glycosylated only at Asn^{624} (42% of mutant) and unglycosylated hLF (57% of molecules) respectively. Glycosylation at Asn^{624} had occurred in 42% and 29% of the $Asn^{138/479}$ and Asn^{479} mutant molecules respectively, (Figure 4, lanes 2 and 4), whereas in the Asn^{138} mutant and rhLF approx. 10% of the molecules were glycosylated at Asn^{624} (Figure 4, lanes 5 and 6). This result indicates that the absence of glycosylation at Asn^{479} increased the degree of glycosylation at Asn^{624}, whereas mutation at Asn^{138} or Asn^{624} did not affect glycosylation at other sites.

Figure 5 depicts a model for the distribution of *N*-glycans in natural hLF, inferred from the results presented in Figure 4 and Table 2.

DISCUSSION

We employed mutagenesis of N-glycosylation sites in hLF to determine the extent to which each of the three sequons for N-glycosylation is used. All observations on the glycosylation-site mutants expressed by 293(S) cells led us to propose a model for the distribution of *N*-glycans in natural hLF (Figure 5). N-glycosylation heterogeneity results in the three differently glycosylated natural hLF variants here designated minor hLF (glycosylated at Asn^{138}, Asn^{479} and Asn^{624}), major hLF (glycosylated at Asn^{138} and Asn^{479}) and minute hLF (glycosylated only at Asn^{479}), which are expressed at a ratio of 9:85:5. Obviously the validity of this model requires that rhLF bears the same glycosylation profile as hLF from the human mammary gland. The comparison of rhLF [expressed in 293(S) cells] with natural hLF (Figure 4, lanes 6 and 7; Table 2) demonstrates that this is the case. Differences in culturing conditions of 293(S) cells expressing rhLF or glycosylation-site mutants did not affect their glycosylation profiles on SDS/PAGE. Other investigators have shown that aberrant glycosylation of rhLF occurs in a non-human cell line [29], in fungi [30] and in yeast [22].

The information to predict the efficiency of glycosylation at a particular sequon is limited. It is known that proline residues at or near a sequon can preclude glycosylation [12]. Therefore the cluster of prolines ($Pro^{142,\ 143\ and\ 145}$) after the first sequon might be responsible for the lack of glycosylation at Asn^{138} in approx. 5% of hLF molecules (minute hLF). It has been proposed that glycosylation at one sequon might affect glycosylation at another site [12,16]. The results with the Asn^{479} and $Asn^{138/479}$ mutants (Figure 4, lanes 4 and 2) indicates that mutation at Asn^{479} increases the degree of glycosylation at Asn^{624}. This suggests that glycosylation at Asn^{479} in the Asn^{138} mutant as well as in natural hLF decreases the glycosylation efficiency at Asn^{624}. In addition we consider that residues at and near the third sequon cause its relatively inefficient glycosylation. First, glycosylation at Asn of an Asn-Xaa-Ser sequon (third sequon in hLF) has been shown to occur less efficiently than at an Asn-Xaa-Thr sequon (first and second sequons in hLF) in several proteins [31]. Secondly, the folding of a protein (e.g. the kinetics of folding and/or the formation of disulphide bonds) might render a sequon inaccessible to the addition of the dolichol-linked precursor [32,33]. In this respect, it is to be noted that charged amino acids precede (Arg^{623}) and follow (Asp^{627}) only in the third sequon and a cysteine residue is present at position 628.

We have recently described a 293(S)-derived cell line that expresses rhLF from a vector containing the hLF cDNA isolated by Rey et al. [9,18]. This hLF cDNA contains two unique differences with respect to other published hLF cDNA sequences, i.e. a threonine and a cysteine residue is present at positions 130 and 404 instead of isoleucine and glycine respectively. SDS/PAGE analysis of this rhLF (referred to below as $rhLF^{cys}$) revealed a minor band to major band ratio of approx. 4:6, whereas this ratio is 1:9 in natural hLF [18]. Another rhLF variant containing only the $Iso^{130} \rightarrow Thr$ amino acid change showed a glycosylation profile identical with that of rhLF and natural hLF (P. H. C. van Berkel, unpublished work). Thus the $Gly^{404} \rightarrow Cys$ amino acid change, which results in an odd number of cysteine residues in hLF, apparently caused an increase in the amount of minor hLF, i.e. an increase in glycosylation at Asn^{624}. This result demonstrates that alterations in the primary sequence that might cause differences in folding (e.g. in disulphide bonding) can affect the efficiency of glycosylation of a sequon 220 amino acids downstream. It is to be noted that $rhLF^{cys}$ either from 293(S) cells [18] or the milk of transgenic mice ([34]; J. H. Nuijens and P. H. C. van Berkel, unpublished work) was indistinguishable from rhLF or natural hLF with respect to iron binding and release, binding to chromatographic media and several different molecules (e.g. DNA, lipopolysaccharide, human lysozyme, heparin and lectins) and its reactivity towards a number of different monoclonal and polyclonal antibodies.

Besides glycosylation heterogeneity at Asn^{624} and Asn^{138} described herein, Spik and co-workers have described microheterogeneity in hLF glycan chains [11,17,35]. For example, *N*-glycan chains of hLF from milk contain a variable number of sialic acid residues, and the external *N*-acetylglucosamine can be fucosylated. In addition, hLF isolated from human polymorphonuclear leucocytes lacks fucose residues [35]. Site-specific differences in the glycan structure of a number of proteins have been described [36,37]. However, no results are yet available on the composition of *N*-glycan chains attached to the three glycosylation sites in hLF from milk. This is currently under investigation in our laboratory.

Protein glycosylation is involved in intermolecular interactions (e.g. intracellular routing, secretion, receptor recognition) and in stabilizing and controlling the conformation of the protein (e.g. protecting the protein from intracellular or extracellular degradation) (for a detailed review see [12]). Studies with enzymically (partially or completely) deglycosylated hLF did not reveal any role of glycosylation in binding of hLF to human intestinal receptors [38] or to bacterial receptors [39], nor in its clearance from the mouse circulation [40]. However, one study showed that deglycosylation impaired the binding of iron by hLF fragments [41]. We have recently shown [18] that glycosylated and unglycosylated rhLF, obtained by expressing rhLF in the absence or presence of the N-glycosylation inhibitor tunicamycin, can both bind iron, and have identical affinities for human lysozyme and bacterial lipopolysaccharide. However, unglycosylated rhLF is much more susceptible to tryptic proteolysis [18]. The presence, in the supernatant, of unglycosylated hLF of M_r 71000 with the $Asn^{138/479/624}$ mutant (Figure 2, lane 4), and particularly with the $Asn^{138/479}$ mutant (Figure 2, lane 3; Figure 4, lane 2) as well as on expression of unglycosylated rhLF in the presence of tunicamycin [18], suggests that N-glycosylation is not an absolute requirement for secretion of hLF. With the $Asn^{138/479/624}$ mutant we observed that approx. 80% of hLF in supernatant was cleaved into a protein of M_r 50000 (Figure 2, lane 4), whereas hLF degradation products were not observed with the $Asn^{138/479}$ mutant (Figure 2, lane 3) nor on expression of unglycosylated rhLF in the presence of tunicamycin. This suggests that an altered conformation after the introduction of three mutations rather than the absence of glycosylation has rendered the $Asn^{138/479/624}$ mutant susceptible to at least extracellular degradation. The antigenic determinants for rabbit and bovine polyclonal anti-hLF are equally accessible

in natural hLF and the $Asn^{138/479}$ mutant (Figure 3), which indicates that the conformation of the latter is identical with, or very similar to, natural hLF.

In conclusion, our results indicate that hLF consists of three differently glycosylated hLF variants due to glycosylation heterogeneity at Asn^{624} and Asn^{138}.

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