Production and characterization of recombinant human lactoferrin

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Chapter 4

Characterization of the recombinant N- and C-lobe of human lactoferrin

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Abstract
Human lactoferrin (hLF), an iron-binding N-linked glycoprotein of Mr 77,000, consists of two highly homologous lobes, the N- and C-lobe. Functional activities of hLF reside in binding of metals like iron and ligands like bacterial lipopolysaccharide (LPS) or heparin. These properties were localized by using the recombinant N- and C-lobe expressed in human 293(S) cells. SDS-PAGE studies of untreated and enzymically deglycosylated recombinant lobes confirmed that the major iron-binding associated conformational change occurs in the N-lobe and that N-linked glycosylation is not essential in maintaining the stability of the iron-saturated N-lobe. Ligand binding assays revealed that lipid A and heparin as well as chelated iron appeared to bind only to the N-lobe, whereas chelated copper bound to both lobes.

Introduction
Human lactoferrin (hLF[1]) is a metal-binding glycoprotein of Mr 77,000 that belongs to the transferrin family [1]. Human LF is found in secretions such as milk, tears, saliva as well as in the secondary granules of neutrophils [2]. Based on extensive in-vitro and in-vivo studies, showing LF to have antibacterial, antifungal, antiviral and anti-inflammatory activities, the molecule is postulated to be involved in the innate host defense against infection and excessive inflammation [2]. Many of the biological actions of LF are mediated by the sequestration of iron or by a positively charged domain located in the N-terminus which is involved in binding to negatively charged ligands such as the lipid A moiety of lipopolysaccharide (LPS) [3], DNA [4] and heparin [5].
The amino acid sequence of human lactoferrin (hLF) has been determined by protein and DNA sequencing [6, 7]. Human LF consists of a single polypeptide chain of 692 amino acids which is folded into two globular lobes (designated the N- and C-lobe) connected by an α-helix [1]. The N- and C-lobe share an internal amino acid homology of about 40% [6] suggesting that hLF evolved by gene duplication from an ancestral gene half its size. Each lobe consist of two domains (I and II), connected by a hinge region, creating a deep iron-binding cleft that can bind a single ferric ion with high affinity while simultaneously incorporating a bicarbonate ion [1]. Upon binding of iron, domain I of the N- and C-lobe rotates relative to domain II by ~54° and ~20°, respectively, resulting in a more globular closed and stable conformation of hLF [8]. Besides the high affinity metal binding in the iron-binding cleft, LF can also bind metals at much lower affinity [9] which occurs at least in part via surface-exposed histidyl residues [10]. Human LF contains three possible N-glycosylation sites, Asn^{138} in the N-lobe and Asn^{479} as well as Asn^{624} in the C-lobe which are utilized in ~94%, 100% and ~9% of the molecules, respectively [11].

[1] *The abbreviations used are: LF, lactoferrin; hLF, human LF; natural hLF, hLF purified from human milk; iron-saturated hLF and Fe-hLF, natural hLF that has completely been saturated with iron in vitro; rN-lobe, recombinant N-lobe; rC-lobe, recombinant C-lobe.*

The N-lobe of hLF has been cloned and expressed in BHK cells [12] and site-directed mutagenesis of this lobe has been employed [8] to obtain new insights into the structure and iron-binding properties of hLF. In contrast to the N-lobe, the expression of the C-lobe of hLF failed in BHK cells [13]. Recently, we reported the expression of the recombinant N- and C-lobe (rN- and rC-lobe) in human kidney 293(S) cells [14]. Herein, we describe the characterization of the expressed rN- and rC-lobe and the use of these lobes in studies of metal-binding, ligand interaction and N-linked glycosylation of hLF.

Materials and methods

Purification and saturation with iron of natural human lactoferrin
Natural hLF was purified from fresh human milk by cation-exchange chromatography on S Sepharose [15]. Natural hLF was saturated with iron for 4%; complete saturation of natural hLF with iron was achieved as described [15].

Expression and purification of the recombinant N- and C-lobe of hLF
Human kidney 293(S) cells [ATCC CRL 1573] were transfected with expression vectors based on regulatory elements from the bovine αS_1-casein gene and cDNA sequences encoding either for the hLF N-lobe (pCMV/rN, residues Gly^{1} to Lys^{334}) or the C-lobe, including the connecting α-helix (pCMV/rC, residues Ser^{335} to Lys^{692}) [14]. Supernatants of (subcloned) 293(S) cells were tested for expression of the rN- or rC-lobe with a hLF-specific ELISA using natural hLF as standard [11] and the recombinant lobes were purified as described [14].

Binding of natural hLF, iron-saturated hLF and the recombinant lobes to Fe- and Cu-Chelating Sepharose
Immobilized metal-Chelating Sepharoses were prepared by incubating Chelating Sepharose (GE Healthcare, Uppsala, Sweden) with an excess of iron(II)sulphate or copper(II)chloride. The Sepharoses were then washed, suspended (8 µl Sepharose/0.5 ml) and incubated with 100 ng (100 µl, 1 µg/ml) of purified natural hLF, iron-saturated hLF, rN-lobe or the rC-lobe in 10 mM sodium phosphate pH 7.0, 0.1% w/v Tween-20, 0.02% w/v sodium azide and 0.14, 0.56 or 1.8 M sodium chloride. After incubation for 16 h at 20°C, the suspensions were centrifuged and non-bound hLF, rN- or rC-lobe in the supernatants was determined with the ELISA specific for hLF. The specificity of the interaction of the lactoferrin variants with chelated metal was demonstrated by control experiments in which non-charged Chelating Sepharose was tested (result not shown).

Results

Expression of the recombinant N- and C-lobe of human lactoferrin in 293(S) cells
Subcloning of pCMV/rN or pCMV/rC transfected 293(S) cells resulted in stable lines expressing the recombinant N- or C-lobe, designated as the rN- and rC-lobe, respectively, up to 0.5 µg/ml. N-terminal protein sequencing of purified rN- and rC-lobe showed sequences predicted by the DNA sequence (Table 1), which indicated that the bovine αS_1-casein signal sequence [14] was correctly and completely removed in 293(S) cells to yield the mature rN- and rC-lobe. Analysis of purified rN-lobe by analytical cation-exchange chromatography on Mono S revealed one major protein peak at 0.78 M NaCl (Figure 1), which is about 0.1 M higher than the elution of natural hLF [15]. The purified rC-lobe did not bind to Mono S (not shown). The binding of natural hLF and rN-lobe, but not of the rC-lobe to the negatively charged molecules heparin and lipid A indicated that this interaction is mediated by the N-lobe of hLF (Figure 2). These results confirm that the cationic nature of hLF is determined by its N-lobe.

Table 1 N-terminal sequence analysis of the recombinant N- and C-lobe

rN-lobe	N-terminal sequence [a]	GRRRRSVQWXAVSQPEATKXFQ
	cDNA sequence [b]	GRRRRSVQWCAVSQPEATKCFQ
		↓
rC-lobe	N-terminal sequence	SEEEVAARRARVVWXAVGEQELRK [c]
	cDNA sequence	SEEEVAARRARVVWCAVGEQELRK

[a] N-terminal sequences were determined with the automatic Edman degradation procedure (Applied Biosystems gasphase sequencer, model 473A). Sequencing results are provided with the standard one-letter code for amino acids.

[b] Predicted sequences based on the cDNA sequence of hLF [11].

[c] The α-helix, connecting the N- and C-lobe, is indicated underlined; the arrow indicates the first residue of the C-terminal half [1].

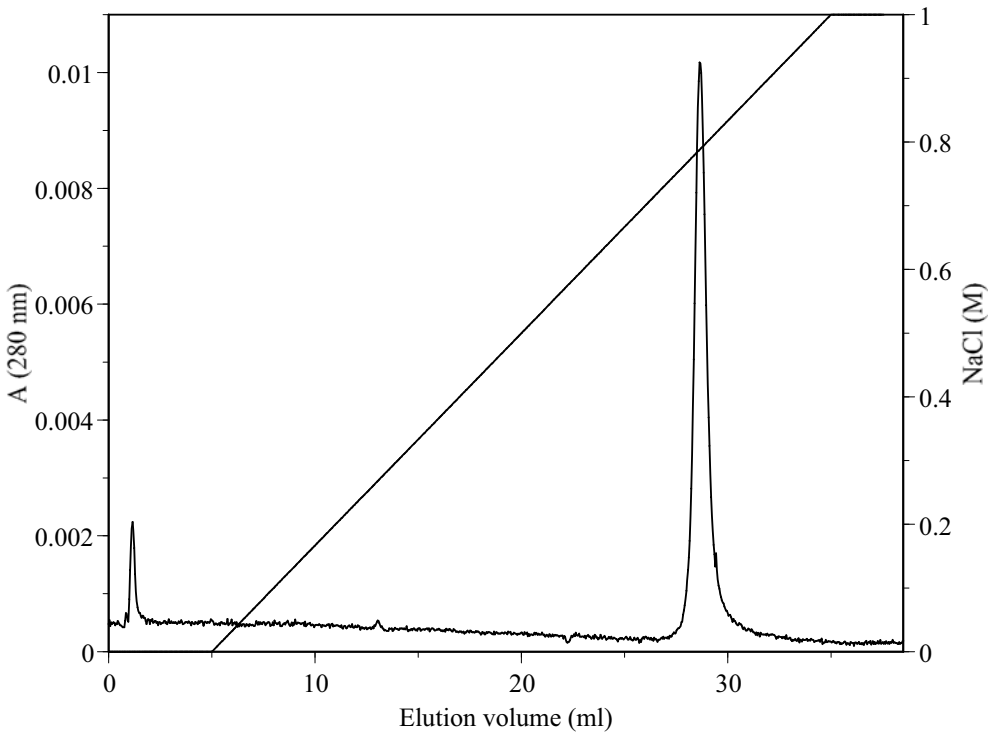

Figure 1 Mono S profile of the recombinant N-lobe
Ten µg of S Sepharose purified rN-lobe was applied to a Mono S HR 5/5 column (GE Healthcare, Uppsala, Sweden) in 20 mM sodium phosphate, pH 7.5 (buffer A). Bound protein was eluted with a linear salt gradient from 0 to 1.0 M NaCl in 30 ml buffer A at a flow rate of 1.0 ml/min. Eluted protein was detected by absorbance measurement at 280 nm.

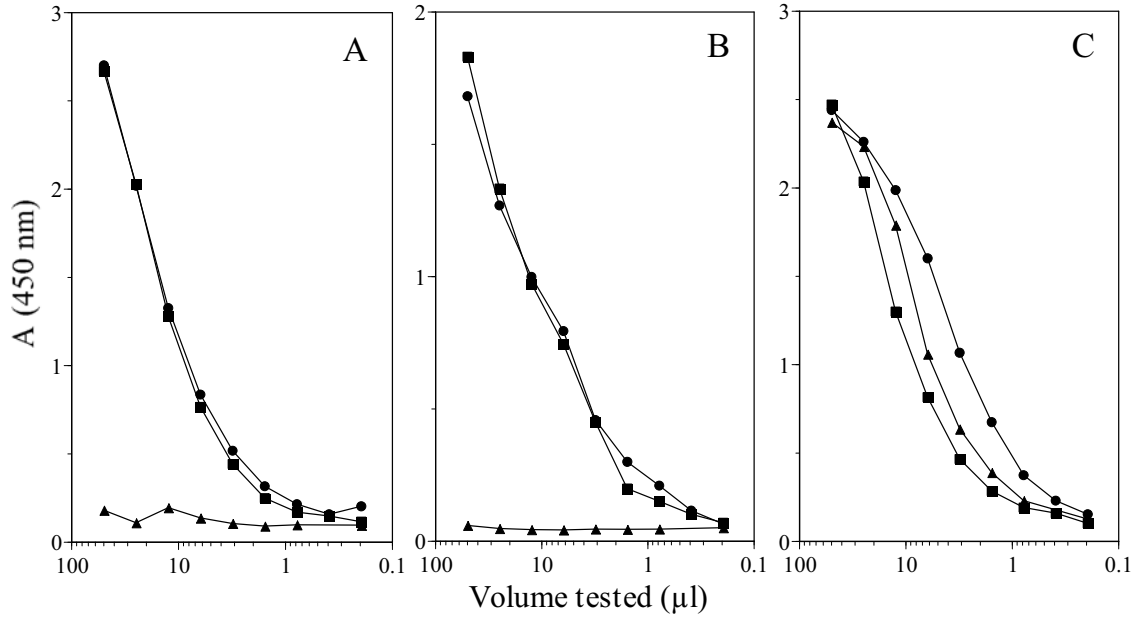

Figure 2 Binding of rN- and rC-lobe to solid-phase heparin and lipid A
Serial dilutions of natural hLF (■, 0.1 µg/ml), purified rN-lobe (●, 0.3 µg/ml) and rC-lobe (▲, 0.2 µg/ml) were incubated with solid-phase heparin (panel A), lipid A (panel B) and affinity purified rabbit anti-hLF (panel C) as described [20]. After incubation plates were washed and bound antigen was detected by incubation with peroxidase-conjugated bovine anti-hLF. The A_{450} values measured as described [20] are plotted against the experimental volume (µl) tested.

SDS-PAGE analysis of the recombinant N- and C-lobe of human lactoferrin
On non-reduced SDS-PAGE, boiled samples of purified rN-lobe migrated as a doublet of protein bands of Mr 44,000 and 40,000 (Figure 3A, lane 2). The rC-lobe migrated as two doublets of protein bands (Figure 3A, lane 3). The most prominent band within each doublet was of Mr 43,000 and 38,000. Immunoblotting analysis revealed that all protein bands observed on non-reduced SDS-PAGE were recognized by polyclonal anti-hLF antibodies as well as conformational specific monoclonal antibodies directed either against the N- or C-lobe of hLF [14]. On reduced SDS-PAGE, the rN-lobe migrated as a doublet of protein bands of Mr 45,000 and 42,000, at a ratio of 97:3 (Figure 3B, lane 2), whereas the rC-lobe migrated as a doublet of protein bands of Mr 47,000 and 43,000 at a ratio of 70:30 (Figure 3B, lane 3).
Previously, we showed that N-glycosylation heterogeneity at Asn^{138} and Asn^{624} account for the minute, major and minor protein bands of hLF on SDS-PAGE [11]. SDS-PAGE analysis of untreated- and enzymically deglycosylated recombinant lobes revealed that the deglycosylated rN-lobe migrated as a protein band of Mr 40,000 with the same mobility as the band representing 3% of the untreated rN-lobe (Figure 4, lanes 3 and 4). This suggest that the protein of Mr 40,000 in untreated rN-lobe preparations, designated N_0, represented unglycosylated N-lobe and that the rN-lobe protein band of Mr 44,000 represented rN-lobe bearing one glycan attached to Asn^{138} (designated N_1, Figure 4, lane 3). The deglycosylated rC-lobe showed a protein band of Mr 35,000 (designated C_0, Figure 4, lane 5) on non-reduced SDS-PAGE. Considering the two possible N-linked glycosylation sites in the C-lobe (Asn^{479} and Asn^{624}), we hypothesized that the protein bands of Mr 38,000 and 43,000, designated C_1 and C_2, bear one and two glycans, respectively. Figure 5 depicts a model for the distribution of N-glycans in the recombinant N- and C-lobe, inferred from the results presented in Figure 4.

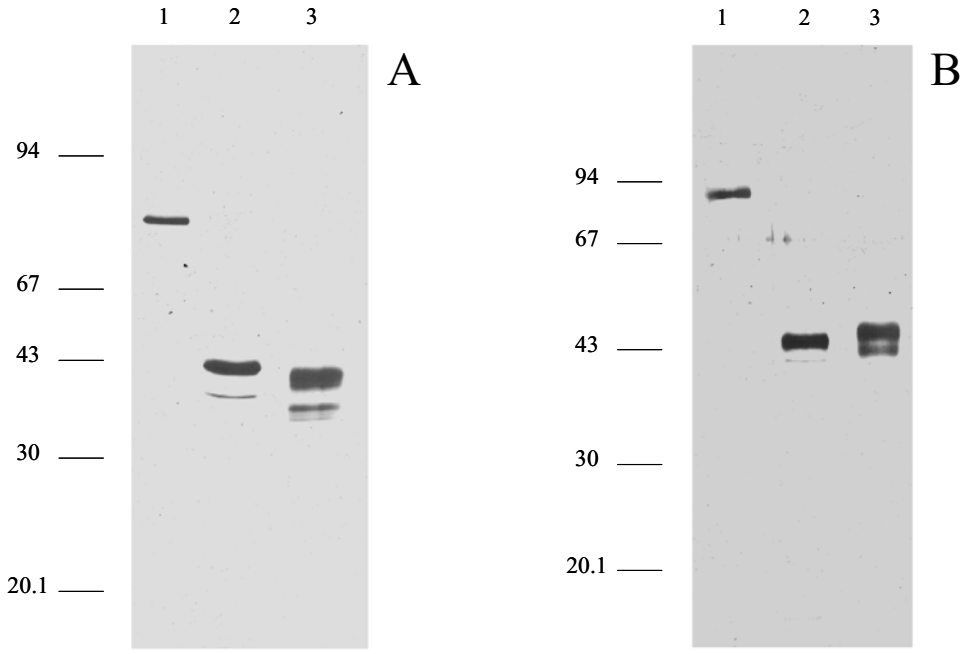

Figure 3 Non-reduced and reduced SDS-PAGE of purified rN- and rC-lobe
Samples were diluted in non-reducing (A) or reducing (B) sample buffer, boiled and applied to 12.5% (w/v) SDS-PAGE as described [15]. Lane 1: natural hLF, lane 2: rN-lobe and lane 3: rC-lobe. Proteins, about 350 ng per lane, were visualized by silver staining. Left-hand numbers (10^{-3} x Mr) indicate the migration of the protein standards.

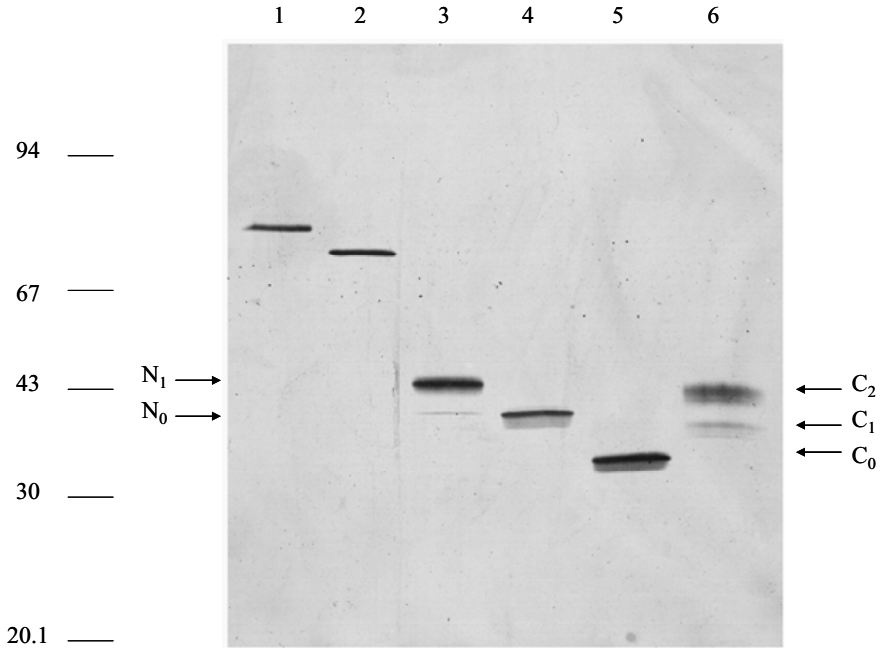

Figure 4 SDS-PAGE of enzymically deglycosylated rN- and rC-lobe
Human lactoferrin and recombinant lobes were deglycosylated with N-glycosidase F [11] and analyzed on non-reduced, boiled 12.5% (w/v) SDS-PAGE. Untreated (lane 1) and deglycosylated (lane 2) natural hLF; untreated (lane 3) and deglycosylated (lane 4) rN-lobe; deglycosylated (lane 5) and untreated (lane 6) rC-lobe. Proteins, about 500 ng per lane, were visualized by silver staining. Left-hand numbers (10^{-3} x Mr) indicate the migration of the protein standards.

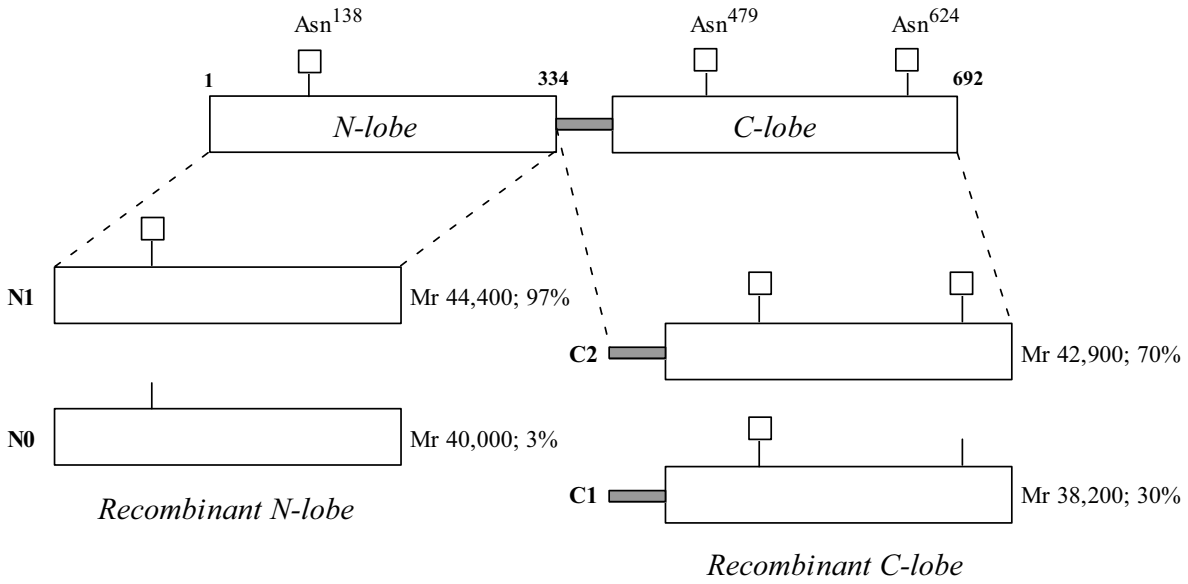

Figure 5 Proposed model for the distribution of N-glycans in the rN- and rC-lobe of human lactoferrin
Short vertical lines mark the potential N-linked glycosylation sites (Asn^{138}, Asn^{479} and Asn^{624}) in natural hLF [11] and the recombinant lobes and a box on top of that indicates that the site is actually used. The α-helix connecting the N- and C-lobe is in grey. The Mr values and percentages indicated are based on densitometric analysis (IplabGel, Signal Analytics, Vienna, VA, USA) of at least four SDS-PAGE gels.

The N-lobe of hLF determines the conformational change on SDS-PAGE.
The binding of metal ions to hLF is coincided by a change in the conformation of this molecule [8] and SDS-PAGE under non-reducing conditions of freshly diluted, non-boiled samples can be used to assess the degree of saturation with iron of hLF [16]. Under these conditions the migration of iron-saturated hLF is faster than that of unsaturated hLF. Boiling of the protein in the presence of SDS causes denaturation of the iron-saturated molecule with release of iron [16]. Iron-saturated hLF as well as the purified recombinant N- and C-lobe were analyzed on non-reduced SDS-PAGE after boiling of the samples or not. The faster migration of iron-saturated hLF (Fe-hLF, Figure 6, lane 1) when compared to unsaturated hLF (Apo-hLF, Figure 6, lane 2) reflects the conformational change that occurred in hLF upon the incorporation of iron. The difference in Mr between these two conformations of natural hLF is approximately 8,000. Non-boiled and boiled rN-lobe showed major bands of Mr 36,000 (Fe-N_1, Figure 6, lane 3) and 44,000 (Apo-N_1, Figure 6, lane 4), respectively, indicating that the rN-lobe was fully saturated with iron, which was released upon boiling. The unglycosylated rN-lobe, N_0, was also found in the iron-saturated conformation (Fe-N_0, Figure 6, lane 3). No differences in the mobility of bands representing the purified rC-lobe were observed after boiling of the samples or not, (Figure 6, compare lanes 5 and 6). The addition of freshly prepared FeNTA-solution to the rC-lobe to increase the degree of iron-saturation prior to SDS-PAGE did also not reveal a differential mobility between boiled and non-boiled samples (not shown). These results suggest that the major conformational change of hLF observed on SDS-PAGE upon incorporation of iron occurs in the N-lobe. This conformational change is resistant to SDS and accounts for the possibility to assess the degree of iron-saturation of natural hLF [17] and rN-lobe preparations by non-reduced, non-boiled SDS-PAGE.

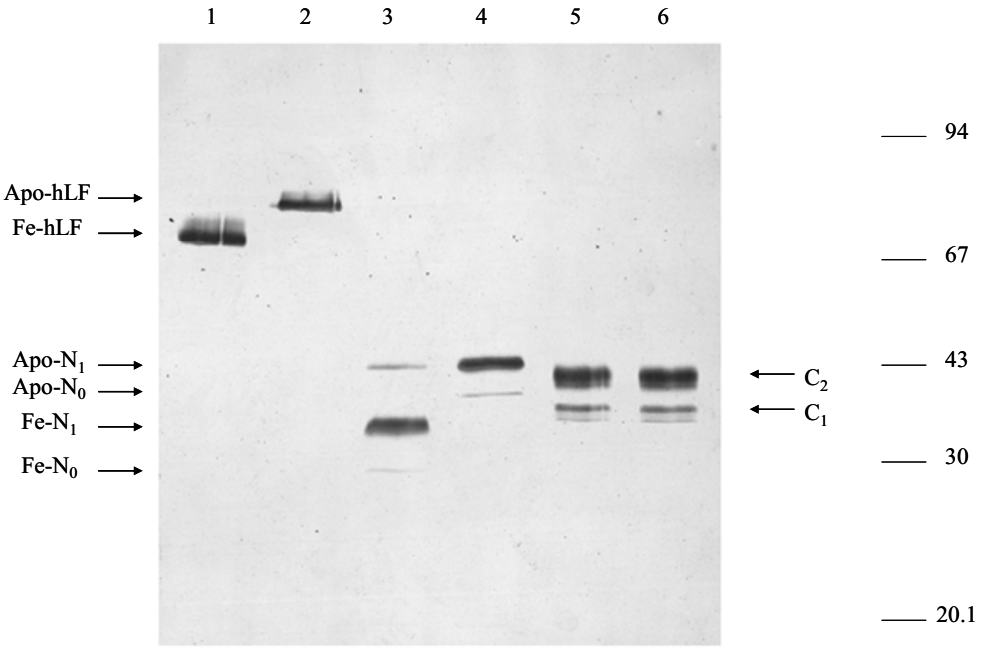

Figure 6 Iron-saturation of the rN- and rC-lobe
Purified natural hLF and recombinant lobes were freshly diluted in non-reducing SDS sample buffer and applied to 12.5% (w/v) non-reducing SDS-PAGE either directly or after boiling for 5 min. Non-boiled (lane 1) and boiled sample (lane 2) of iron-saturated natural hLF; non-boiled (lane 3) and boiled (lane 4) sample of the rN-lobe; non-boiled (lane 5) and boiled (lane 6) sample of the rC-lobe. Proteins, 300 ng per lane, were visualized by silver staining. Right-hand numbers (10^{-3} x Mr) indicate the migration of the protein standards.

The N-lobe of human lactoferrin determines binding to Fe-Chelating Sepharose.
Lactoferrin can bind to metals through surface-exposed residues [9, 10, 18]. To determine whether surface exposed residues in the N- or C-lobe play a role in the interaction with chelated metals, the binding of natural hLF, iron-saturated hLF and the recombinant lobes to Fe- and Cu-Chelating Sepharose were studied. At low salt concentrations i.e. 0.14 M NaCl, all added rN-lobe and approximately 75% of natural and iron-saturated hLF bound to Fe-Chelating Sepharose, whereas only about 5% of the rC-lobe bound (Figure 7, open bars). At 0.56 M NaCl, all hLF variants had lost their ability to bind to Fe-Chelating Sepharose, whereas with Cu-Chelating Sepharose (and even at 1.8 M NaCl; not shown) each hLF variant had retained the capacity to bind (Figure 7, black and grey bars, respectively). These results indicate that the interaction of hLF with chelated iron is different from the interaction with chelated copper. The interaction with chelated iron is determined by the N-lobe and ionic in nature, whereas chelated copper can coordinate to likely surface-exposed histidyl residues in both lobes.

Discussion

This paper describes the characterization of the recombinant N- and C-lobe of hLF expressed in human kidney 293(S) cells. Recombinant production of the N- and C-lobe enables studies of the relation between the structure of hLF and its function. Characterization of the structure and iron-binding properties of the recombinant N-lobe and mutants, produced in BHK cells, has been described [8, 19], but expression of the C-lobe in these cells had failed presumably due to rapid degradation of translated C-lobe within the cell [13]. We produced the N- and C-lobe of hLF in human 293(S) cells and obtained recombinant lobes of high purity and devoid of proteolysis (Figure 3, Table 1). Successful expression of

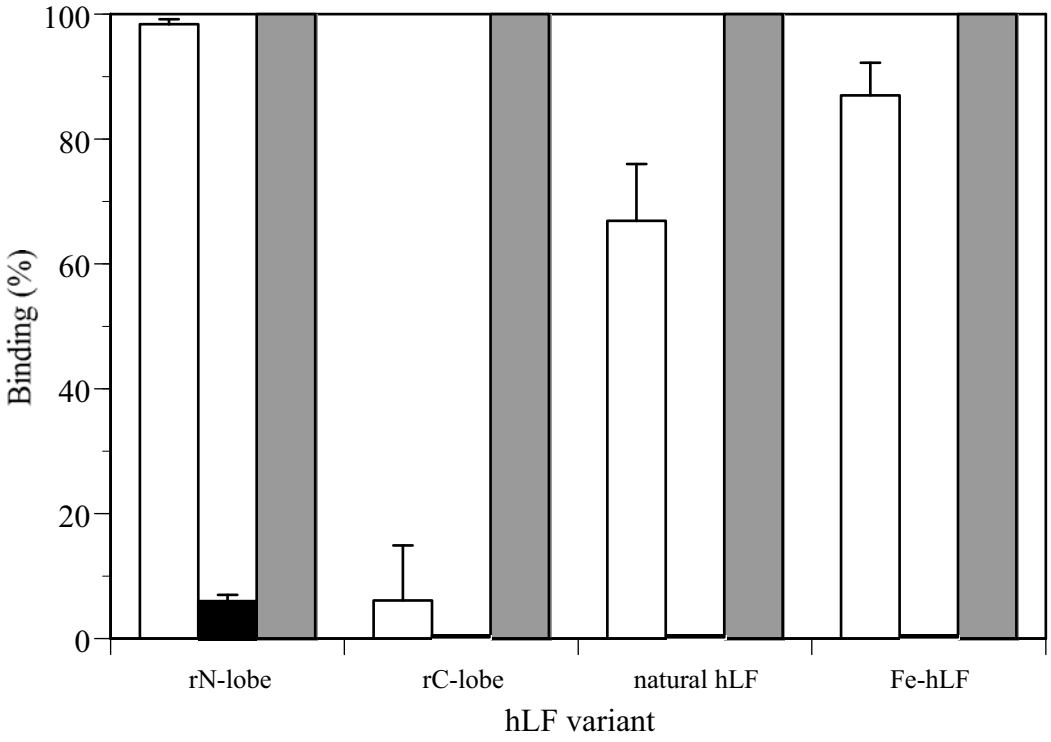

Figure 7 Interaction of hLF variants with immobilized metals
Binding of natural hLF, iron-saturated hLF, recombinant N- and C-lobe to Fe- and Cu-Chelating Sepharose was investigated as described in the Materials and Methods section. Open and black bars represent the results with Fe-Chelating Sepharose when incubated in 0.14 and 0.56 M NaCl, respectively. Grey bars show the result with Cu-Chelating Sepharose at 0.56 M NaCl. The percentage of protein bound to the metal-Chelating Sepharose is indicated on the ordinate. Results are given as mean ± SD of at least 2 experiments.

the C-lobe in human 293(S) cells may relate to culture conditions, this particular cell type or to the fact that we included the entire connecting α-helix to the C-lobe whereas Sheth et al. (1994) did not.
Recently, it was demonstrated that basic clusters in the N-terminus of hLF determine the electrostatic interaction of this protein with LPS, heparin, human lysozyme, DNA as well as certain receptors [5, 20-22]. Cation-exchange chromatography (Figure 1) and binding experiments with the purified recombinant lobes to the negatively charged ligands heparin and lipid A (Figure 2) confirmed that the cationic properties of hLF localize to its N-lobe.
SDS-PAGE analysis of both untreated and deglycosylated purified recombinant lobes (Figure 4) provided proof that the N_1 and N_0 bands of the rN-lobe as well as C_2 and C_1 bands of the rC-lobe result from N-glycosylation heterogeneity. Based on our model for the distribution of the N-glycans in natural hLF [11] and the observations in Figure 4, a model is proposed for the distribution of N-glycans in the recombinant lobes (Figure 5). Similar to natural hLF [11], a minor portion of the rN-lobe (about 3%) is not glycosylated at Asn^{138} (N_0, Figure 4), whereas 97% of the molecules contain a glycan at Asn^{138} (N_1, Figure 4). The rC-lobe contains at least one glycan, likely attached to Asn^{479} (C_1, Figure 4), because this site is always utilized in natural hLF [11]. C_2 presumably represents the rC-lobe with two glycans, attached to Asn^{479} and Asn^{624}. This implies that Asn^{624} is glycosylated in 70% of the rC-lobe molecules, whereas this site is only used in 9% of natural hLF [11]. Apparently, lack of the entire N-lobe causes an increase in glycosylation at Asn^{624}, confirming our original hypothesis that glycosylation at Asn^{624} in natural hLF is limited due to conformational and/or primary sequence constraints [11].
Heterogeneity in the glycan structures may explain for the doublets of rC-lobe derived C_1 and C_2 on non-reduced SDS-PAGE (Figure 3A, 4 and 6) although we can not exclude that variation in disulphide

arrangement had occurred as the C-lobe contains 10 disulfide bonds arranged in a complex way [6]. Alternative disulfide bonding, either during intracellular folding or induced on SDS-PAGE, may occur as a result of lack of stabilizing interactions from the N-lobe. Such interactions between the two lobes do exist in natural hLF [8, 23, 24].

The rN-lobe (N_1 and N_0) was saturated with iron (Figure 6, lanes 3 and 4) similarly to what has been described for full length hLF and N-lobe expressed in 293(S) [15] and BHK cells, respectively [12, 19]. Apparently, the rN-lobe was able to retain the iron bound to it even in the presence of SDS. An equal shift in Mr occurred in iron-saturated hLF and rN-lobe upon the release of iron by boiling (about Mr 8,000; Figure 6), suggesting that the major conformational change upon binding of iron occurs in the N-lobe. This confirms crystallographic studies of hLF, which showed that the major conformational change occurs in the N-lobe [8].

Ying and coworkers reported that the iron-saturated and iron-free 39 kDa N-terminal tryptic fragment migrated equally on SDS-PAGE and concluded that the conformational change observed with natural hLF required an intact hLF molecule [25]. It is obvious from our work that this conclusion, which was based on iron-binding studies with a non-intact N-lobe fragment, is not valid as the conformational change of the intact N-lobe upon binding of iron is detected by SDS-PAGE analysis (Figure 6). Unglycosylated rN-lobe was also completely saturated with iron (FeN_0, Figure 6, lane 3), indicating that the N-linked glycan of the N-lobe is of less importance for the stability of the iron-saturated conformation.

X-ray scattering studies of hLF revealed that structural changes also occur in the C-lobe upon uptake and release of iron [26], which was confirmed by crystallography [8]. This structural change in the C-lobe was not observed in this study as boiled and non-boiled purified rC-lobe migrated on SDS-PAGE with equal Mr (Figure 6, lanes 5 and 6). We can, however, not exclude that conformational changes had occurred in the rC-lobe, but these may not be resistant to SDS or too minor to be determined on SDS-PAGE. Moreover, at this stage we do not have data to support that the rC-lobe is able to bind iron in the iron-binding cleft, similar to observed for the rN-lobe.

Next to specific high-affinity binding of iron in the iron-binding cleft, the low affinity binding of iron or other metals to surface exposed residues may also play a role in hLF physiology [9, 18, 27]. Low-affinity metal binding may account for hLF increasing hydroxyl radical formation [18], polymerization of the molecule under low ionic strength conditions [27] and for the formation of a complex with ceruloplasmin [28]. Our results show that surface-exposed residues in the N-lobe determine the low affinity and ionic binding to chelated iron (Figure 7). The slightly stronger binding of the rN-lobe to both Fe-Chelating Sepharose and Mono S when compared to natural hLF suggests that N-terminal arginines, which determine binding of the protein to Mono S [15], are also involved in binding to chelated iron. This hypothesis was confirmed by the lower affinity of limitedly trypsinized hLF, which lacks N-terminal arginines [22], for Fe-Chelating Sepharose (not shown). Involvement in the binding of chelated iron adds another feature to the N-terminal domain of LF.

In conclusion, the successful expression and initial characterization of the recombinant N- and C-lobe of hLF has further clarified the role of these lobes in the metal-binding, ligand interaction and N-linked glycosylation of hLF.

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