The N-terminal Arg^2, Arg^3 and Arg^4 of human lactoferrin interact with sulphated molecules but not with the receptor present on Jurkat human lymphoblastic T-cells

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We previously characterized a 105 kDa receptor for human lactoferrin (hLf) on Jurkat human lymphoblastic T-cells. To delineate the role of the basic cluster Arg^2-Arg^3-Arg^4-Arg^5 of hLf in the interaction with Jurkat cells, we isolated N-terminally deleted hLf species of molecular mass 80 kDa lacking two, three or four N-terminal residues (hLf^{-2N}, hLf^{-3N} and hLf^{-4N}) from native hLf that had been treated with trypsin. Native hLf bound to 102 000 sites on Jurkat cells with a dissociation constant (K_d) of 70 nM. Consecutive removal of N-terminal arginine residues from hLf progressively increased the binding affinity but decreased the number of binding sites on the cells. A recombinant hLF mutant lacking the first five N-terminal residues (rhLf^{-5N}) bound to 17 000 sites with a K_d of 12 nM. The binding parameters of bovine lactoferrin (Lf) and native hLf did not significantly differ, whereas the binding parameters of murine Lf (8000 sites; K_d 30 nM) resembled those of rhLf^{-5N}. Culture of Jurkat cells in the presence of chlorate, which inhibits sulphation, decreased the number of binding sites for both native hLf and hLf^{-3N} but not for rhLf^{-5N}, indicating that the hLf-binding sites include sulphated molecules. We propose that the interaction of hLf with a large number of binding sites (approx. 80 000 per cell) on Jurkat cells is dependent on Arg^2-Arg^3-Arg^4, but not on Arg^5. Interaction with approx. 20 000 binding sites per cell, presumably the hLf receptor, does not require the first N-terminal basic cluster of hLf. Moreover, the affinity of hLf for the latter binding site is enhanced approx. 6-fold after removal of the first basic cluster. Thus N-terminal proteolysis of hLf *in vivo* might serve to modulate the nature of its binding to cells and thereby its effects on cellular physiology.

INTRODUCTION

Lactoferrin (Lf), also called lactotransferrin, is a metal-binding glycoprotein found in most external secretions, such as milk [1], tears and saliva [2], as well as in the specific granules of polymorphonuclear leucocytes [3]. On the basis of the many reports on antimicrobial and anti-inflammatory activities *in vitro*, Lf is thought to be involved in the host defence against infection and severe inflammation. Some biological activities of human Lf (hLf) are linked to its ability to chelate iron strongly, whereas others relate to the interactions of hLf with host cells (intestinal cells [4], mammary gland epithelial cells [5], hepatocytes [6,7], monocytes [8], activated lymphocytes [9] and platelets [10]) or its binding to bacterial lipopolysaccharides [11,12], proteoglycans [13,14], DNA [15] and human lysozyme [16,17]. The highly positively charged N-terminus of hLf might be involved in most of these interactions [7,11–15,17].

The amino acid sequences of many lactoferrins, including hLf, bovine Lf (bLf) and murine Lf (mLf) have been determined by protein and DNA sequencing [18–21]. The N-terminal portion of hLf contains a unique cluster of four consecutive arginine residues as well as a second cluster of basic amino acid residues that is also present in other Lf species [18–21]. Mann et al. [13] suggested that the heparin-binding site in hLf represents a 'cationic cradle' formed by the juxtaposition of the first (Arg^2-Arg^3-Arg^4-Arg^5) and second (Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}) basic clusters. Residues Arg^4 and Arg^5 of the cationic cradle were shown to be essential in the binding of hLf to heparin [13]. We have previously reported a specific hLf receptor of 105 kDa on activated lymphocytes [9] that binds hLf via the loops involving residues 28–34 and 39–42 [22]. The binding of hLf to lymphocytes promotes their differentiation [23]. Moreover, when presented as a sole iron source to cells, hLf was shown to promote growth of T-lymphocytes [9]. In other conditions, no effect or inhibitory effects on lymphocyte growth were reported [24,25]. Evidence for a similar 105 kDa receptor has also been found on the Jurkat T-cell line [26] and platelets [10].

The interaction of hLf with cells might involve multiple classes of binding sites [27]. It was postulated that the rapid hepatic clearance of hLf from the rat circulation involves at least two classes of hLf-binding sites, proteoglycans and the chylomicron remnant receptor and/or the LDL-receptor-related protein [7,27,28]. The Arg-Lys-rich sequence (Arg^{25}-Asn^{26}-Met^{27}-Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}) in hLf, which resembles the receptor recognition structure of apolipoprotein-E2, presumably mediates binding and internalization into the hepatocytes by the chylomicron remnant receptor and/or by LDL-receptor-related protein, whereas the Arg^2-Arg^3-Arg^4-Arg^5 stretch might have an important role in the massive low-affinity interaction of hLf with the large number of cell-associated chondroitin sulphate-type

Abbreviations used: bLf, bovine lactoferrin; GAG, glycosaminoglycan; hLf, human lactoferrin; hLf^{-2N}, hLf^{-3N}, hLf^{-4N}, hLf lacking the first two, three or four N-terminal residues respectively; Lf, lactoferrin; mLf, murine lactoferrin; rhLf, recombinant hLf; rhLf^{-5N}, recombinant hLf lacking the first five N-terminal residues.

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proteoglycans [7,27,28]. The contribution of each Arg residue of the first basic cluster to binding of hLf to hepatocytes or other cells has yet not been elucidated.

Here we describe the preparation of N-terminally deleted hLf species lacking two to five N-terminal residues by tryptic proteolysis or recombinant DNA technology. Cell binding experiments were performed with the distinct hLf species, in the presence or the absence of sodium chlorate, which inhibits sulphation [29], as well as with mLf and bLf. The results allow us to discern the role of the first basic cluster in binding to cell-associated proteoglycans and the hLf lymphocyte receptor.

MATERIAL AND METHODS

Reagents

Soybean trypsin inhibitor (type I-S), bovine pancreatic trypsin (type III-S), human serum transferrin and sodium chlorate were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Restriction endonucleases and oligonucleotides were obtained from Eurogentec (Seraing, Belgium). T4 ligase, calf alkaline phosphatase and pBluescript SK were from Stratagene (La Jolla, CA, U.S.A.). Sequanase DNA sequencing kit was from United States Biochemical Corporation (Cleveland, OH, U.S.A.). Baculogold kit containing linearized AcNPV DNA and agarose were from Pharmingen (San Diego, CA, U.S.A.). Plasmid pVL1392 and cell line *Spodoptera frugiperda* (Sf9) were kindly provided by Dr. C. Auriault (Institut Pasteur, Lille, France). Carrier-free $Na^{125}I$ was from ICN Pharmaceuticals (Orsay, France) and Iodo-Beads were from Pierce (Rockford, IL, U.S.A.). Sephadex G25 PD-10 columns were purchased from Pharmacia–LKB Biotechnology (Uppsala, Sweden). RPMI 1640 medium and fetal calf serum were from Techgen International (Les Ulis, France) and D. Dutscher (Brumath, France) respectively. SF900II insect cell medium and gentamycin were from Gibco BRL (Cergy-Pontoise, France). All other chemicals were of analytical grade.

Lactoferrins

Native hLf was purified from fresh human milk from a single donor by ion-exchange chromatography as previously described [30]. bLf was kindly provided by Biopole (Brussels, Belgium). mLf was isolated from mouse milk as follows: mouse milk was diluted 1:1 in 10 mM sodium phosphate/0.15 M NaCl (pH 7.5) (PBS) containing 0.8 M NaCl. Diluted milk was centrifuged at 40000 ***g*** for 1 h at 4 °C to obtain a whey fraction; this was diluted in 20 mM sodium phosphate, pH 7.5 (buffer A), and applied to an S-Sepharose column (Pharmacia, Uppsala, Sweden). The column was washed with buffer A containing 0.1 M NaCl, then eluted with buffer A containing 0.5 M NaCl. The eluate was diluted and rerun on a Mono S HR 5/5 column (Pharmacia) in buffer A. Bound protein was eluted with a linear salt gradient of 0–0.5 M NaCl in 15 ml of buffer A at a flow rate of 0.5 ml/min. mLf eluting at 0.28 M NaCl was used for further experiments. Non-modified recombinant hLf (rhLf) was prepared as described in [31]. SDS/PAGE of the purified protein preparations showed no other protein bands than those characteristic of each of the lactoferrins.

Production of an N-terminally deleted hLf mutant lacking amino acids 1-5 (rhLf^{-5N})

A full-length 2.3 kbp cDNA encoding for hLf was obtained from a human mammary-gland cDNA library (Clontech, Palo Alto, CA, U.S.A.), as described [32]. The Sculptor *in vitro* mutagenesis system kit (Amersham International, Little Chalfont, Bucks., U.K.) was used to delete the sequence 5′-GGCCGTAGGAG-AAGG-3′ coding for Gly^1-Arg^2-Arg^3-Arg^4-Arg^5 of hLf [19]. For that purpose a mutagenic oligonucleotide, 5′-CTGTGTCTGG-CTAGTGTTCAGTGGTG-3′, was synthesized. The mutagenesis template was the phage M13mp11, which contains a 310 bp *Eco*RI–*Acc*I fragment of the coding sequence (nt 295–606 [19]) cloned into pBluescript SK [32]. After mutagenesis, the deletion was confirmed by DNA sequence analysis and the mutated *Eco*RI–*Acc*I fragment was ligated back into pBluescript SK with the 3′ complementary part of the full-length cDNA of hLf [32]. Finally, the mutated cDNA was subcloned into pVL1392, yielding the pVL1392–rhLf^{-5N} construct. Production of rhLf^{-5N} in recombinant baculovirus-infected Sf9 insect cell culture was performed as described [31]. The recombinant protein was purified from the cell culture medium on an SP-Sepharose Fast Flow column equilibrated with 0.2 M sodium acetate, pH 7.8, and eluted with a linear salt gradient from 0 to 1 M NaCl. N-terminal amino acid sequencing of rhLf^{-5N} was performed by the automatic Edman degradation procedure (Applied BioSystems 477 Protein Sequencer). Purity of rhLf^{-5N} was confirmed by this analysis and assessed by SDS/PAGE.

SDS/PAGE analysis

SDS/PAGE of non-reduced and reduced hLf was performed as described [17]. Proteins were stained with Coomassie Brilliant Blue and hLf protein bands were quantified by densitometry with IPlabGel software of Signal Analytics (Vienna, VA, U.S.A.).

Radiolabelling of proteins

The different lactoferrin variants were labelled with ^{125}I by using Iodo-Beads as a catalyst. Two Iodo-Beads were washed twice with 1 ml of PBS in a 1.5 ml polypropylene tube and preincubated at 20 °C with 0.2 mCi of radioactive iodine in 100 μl of PBS. Protein (100 μg) in 100 μl of PBS was then added and incubated for 10 min at 4 °C. The volume was adjusted to 500 μl with PBS and free iodine was removed by gel filtration on a PD-10 column equilibrated in serum-free RPMI 1640. The specific radioactivity of the radioiodinated protein was estimated by measuring the absorbance at 280 nm and the radioactivity on a Compugamma LKB-Wallac (Turku, Finland) γ-radiation counter.

Cell culture

Jurkat cells were routinely grown at 37 °C in a humidified air/CO_2 (19:1) atmosphere in RPMI 1640 medium, pH 7.4, containing 5 μg/ml gentamycin, 2 mM L-glutamine, 20 mM Hepes and 10% (v/v) heat-inactivated fetal calf serum. Cells were kept in the logarithmic growth phase and diluted to a cell density of 4×10^5/ml. After 24 h, cell viability was checked with Trypan Blue stain. Cells were then washed twice in ice-cold serum-free RPMI 1640 and harvested by centrifugation at 200 ***g*** for 10 min at 4 °C.

Treatment of Jurkat cells with sodium chlorate

Jurkat cells were diluted to a cell density of 4×10^5/ml in fresh RPMI 1640 medium containing 10% (v/v) fetal calf serum and 5 mg/ml gentamycin, with or without 30 mM sodium chlorate. After 24 h, cells incubated in the presence of sodium chlorate were counted and cell viability was assessed with Trypan Blue. Cells were then harvested and washed as described above.

Cell binding experiments

Equilibrium binding experiments were performed in serum-free RPMI 1640 containing 0.4 % (w/v) human serum transferrin to prevent non-specific binding of radioiodinated protein to cells or to plastic. Aliquots (100 μl) containing 5×10^5 cells were added to 1.5 ml polypropylene tubes and incubated with serial dilutions of ^{125}I-labelled protein (concentrations ranging from 0 to 80 nM). Incubation of cells with proteins was performed for 1 h at 4 °C in the presence of 0.01 % (w/v) sodium azide to prevent ligand internalization. Cells were washed three times by centrifugation at 180 ***g*** for 7 min with 1 ml of RPMI, resuspended in 0.5 ml of PBS and bound radioactivity was measured. Non-specific binding measured in the presence of a 100-fold molar excess of unlabelled hLf was typically around 25 % of the total binding and was subtracted from the total binding to obtain the specific binding. All binding experiments were performed in duplicate on two or three separate occasions. Binding parameters (K_d and number of binding sites per cell; means ± S.E.M.) were calculated by Scatchard plot analysis [33] with the Enzfitter program software 1.05 (BioSoft).

RESULTS

Preparation of N-terminally deleted hLf variants

We have previously noted that several preparations of milk-derived hLf from various commercial suppliers contained hLf species lacking three (Gly^1-Arg^2-Arg^3) or two (Gly^1-Arg^2) N-terminal residues that eluted from a Mono S cation-exchange column at 0.5 and 0.6 M NaCl respectively [17]. We speculated that these N-terminally deleted hLf species would be useful in determining the role of the consecutive N-terminal arginine residues in hLf function. To obtain large amounts of N-terminally deleted hLf, we studied limited proteolysis of hLf by trypsin. Tryptic proteolysis of native hLf was stopped after various incubation periods by the addition of excess soybean trypsin inhibitor, and N-terminal integrity was assessed by analytical Mono S chromatography [17] and N-terminal protein sequencing. Table 1 shows that after 1 min of incubation with trypsin, all hLf molecules had been N-terminally cleaved: 20 % and 80 % of the hLf molecules lacked residues Gly^1-Arg^2-Arg^3 or Gly^1-Arg^2 respectively. After 3 h, 2 %, 49 % and 42 % of the hLf molecules lacked residues Gly^1-Arg^2-Arg^3-Arg^4 (designated hLf^{-4N}), Gly^1-Arg^2-Arg^3 (designated hLf^{-3N}) and Gly^1-Arg^2 (designated hLf^{-2N}) respectively.

Table 1 Relative amounts of N-terminally deleted hLf variants obtained after limited tryptic proteolysis of native hLf

Native hLf was incubated with trypsin as described. Digestion was stopped by the addition of a 12-fold molar excess of soybean trypsin inhibitor and N-terminal integrity was assessed by analytical Mono S chromatography [17]. Digested hLf was applied to Mono S in buffer A. Bound protein was eluted with a linear salt gradient of 0–1.0 M NaCl in 30 ml of buffer A at a flow rate of 1.0 ml/min. Eluted protein was detected by absorbance measurement at 280 nm. N-terminal protein sequence analysis of hLf eluted at 0.7, 0.6, 0.5 and 0.4 M NaCl indicated these to represent native hLf, hLf^{-2N}, hLf^{-3N} and hLf^{-4N} respectively. Relative amounts (percentages) were calculated by integrating peak areas.

	Relative amount (%) of total hLf			
Time (min)	hLf^{-4N}	hLf^{-3N}	hLf^{-2N}	Intact hLf
0	0	0	0	100
1	0	20	80	0
5	0	22	78	0
25	1	28	70	0
180	2	49	42	0

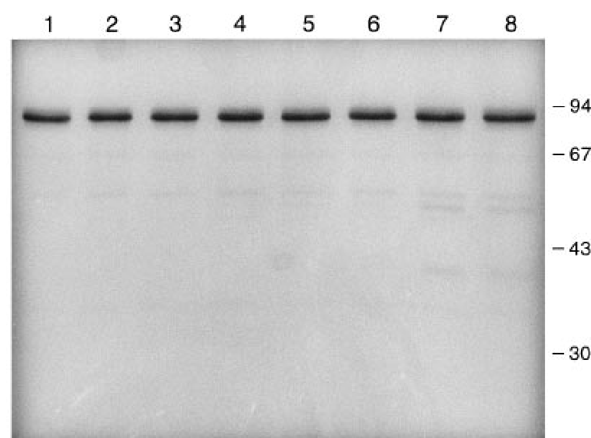

Figure 1 Reduced SDS/PAGE analysis of purified N-terminally deleted hLf variants

Native hLf was incubated with trypsin at an 8:1 molar ratio in PBS at 37 °C. At several time points, samples were taken in which further proteolysis was stopped by the addition of a 12-fold molar excess of soybean trypsin inhibitor. The samples were then applied to Mono S chromatography. SDS/PAGE analysis [12.5% (w/v) gel] of hLf treated with trypsin for 1, 5, 25 and 180 min that was eluted from Mono S at 0.6 M NaCl (hLf^{-2N}) is shown in lanes 1, 3, 5 and 7 respectively, whereas that of hLf eluted at 0.5 M NaCl (hLf^{-3N}) is shown in lanes 2, 4, 6 and 8 respectively. All lanes contained 5 μg of protein. The positions of protein molecular mass standards are indicated at the right, in kDa. Similar results were obtained with non-reduced SDS/PAGE analysis of the samples (results not shown).

Tryptic cleavage of hLf at Lys^{283} results in the major N- and C-terminal fragments of molecular masses 39 and 51 kDa [34]. Non-reduced SDS/PAGE analysis of non-reduced samples of hLf^{-2N} and hLf^{-3N} isolated after digestion for 1 and 5 min revealed no 39 or 51 kDa fragments, whereas such cleavage had occurred in approx. 1 % and 5 % of the N-terminally deleted hLf variants after 25 and 180 min respectively (Figure 1). These results indicate that tryptic proteolysis at Arg^2 and Arg^3 occurs before cleavage at Lys^{283}.

Preparation of rhLf^{-5N}

We observed that cleavage at Arg^5 did not occur on limited treatment of hLf with trypsin. Therefore we produced Sf9 cells secreting rhLf^{-5N}. Cell culture medium containing rhLf^{-5N} at levels up to 8 mg/ml was loaded on an SP-Sepharose Fast Flow column and rhLf^{-5N} was eluted as a single peak at 0.4 M NaCl. The protein migrated as a single 78 kDa band on SDS/PAGE. The N-terminus of rhLf^{-5N} (Ser-Val-Gln-Trp-Cys-Ala-Val) was confirmed by N-terminal protein sequencing.

Binding of native Lf and N-terminally deleted hLf species to Jurkat cells

To delineate the role of Arg^2-Arg^3-Arg^4-Arg^5 (first basic cluster) of hLf in the binding to Jurkat human lymphoblastic T-cells, we studied the binding of ^{125}I-labelled native hLf and N-terminally deleted hLf species at concentrations ranging from 0 to 80 nM. Figure 2 shows that the binding of all hLf species was concentration-dependent and saturable with a single class of binding sites. Moreover, binding of all proteins was inhibited by approx. 75 % in the presence of a 100-fold molar excess of unlabelled hLf, suggesting that the binding was reversible and

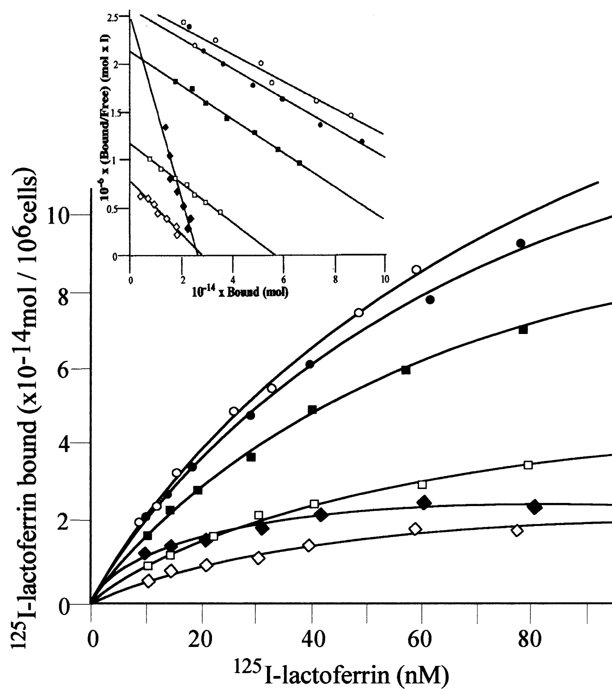

Figure 2 Binding of N-terminally deleted hLf variants to Jurkat cells

Curves show the specific binding of hLf (●); rhLf (○); hLf^{-2N} (■); hLf^{-3N} (□); hLf^{-4N} (◇) and $rhLf^{-5N}$ (◆). Values are means of two or three separate experiments conducted in duplicate. Errors were less than 15% of the mean. The inset shows a Scatchard analysis [33] of the data.

Table 2 Dissociation constants (K_d) and numbers of binding sites per Jurkat cell for N-terminally deleted hLf variants

Values are means ± S.E.M. for two or three separate experiments conducted in duplicate.

Protein	K_d (nM)	Number of sites per cell
hLf	69 ± 8	102 000 ± 19 500
rhLf	81 ± 9	117 100 ± 12 300
hLf^{-2N}	65 ± 18	75 000 ± 11 000
hLf^{-3N}	57 ± 7	35 600 ± 8500
hLf^{-4N}	41 ± 9	17 500 ± 8000
hLf^{-5N}	12 ± 5	16 500 ± 5000

Table 3 Dissociation constants (K_d) and numbers of binding sites per Jurkat cell for hLf, bLf and mLf

Values are means ± S.E.M. for two or three separate experiments conducted in duplicate.

Protein	K_d (nM)	Number of sites per cell
hLf	53 ± 15	106 700 ± 5800
bLf	65 ± 7	97 900 ± 12 000
mLf	31 ± 7	7800 ± 2500

specific. Scatchard analysis revealed that, in the range of hLf concentrations used, the affinity of N-terminally deleted hLf was significantly increased when compared with N-terminally intact hLf (Table 2). K_d shifted from 69 or 81 nM for hLf or rhLf to 65, 57 and 41 nM for hLf^{-2N}, hLf^{-3N} and hLf^{-4N} respectively. The highest affinity (K_d 12.4 nM) was observed with $rhLf^{-5N}$. In addition we found that the number of binding sites per cell decreased from 102 000 for N-terminally intact hLf to 17 000 for both hLf^{-4N} and $rhLf^{-5N}$ (Table 2). hLf^{-2N} and hLf^{-3N} bound to approx. 75 000 and 36 000 binding sites respectively. These results suggest that the binding of hLf to approx. 80 000 binding sites on Jurkat cells depends on the presence of Gly^1-Arg^2-Arg^3-Arg^4.

Table 4 Dissociation constants (K_d) and numbers of binding sites per cell of native hLf and N-terminally deleted hLf variants with Jurkat cells cultured with sodium chlorate

Values are means ± S.E.M. for two or three separate experiments conducted in duplicate.

Protein	Sodium chlorate	K_d (nM)	Number of sites per cell
hLf	−	71 ± 5	102 000 ± 8600
hLf	+	62 ± 11	62 450 ± 9600
hLf^{-3N}	+	57 ± 16	21 100 ± 3400
hLf^{-5N}	+	28 ± 6	20 700 ± 1800

To assess the species specificity of Lf-Jurkat cell interactions, we compared the binding of hLf with that of bLf and mLf. Table 3 shows that the binding parameters of bLf did not significantly differ from those of hLf; K_d and the number of binding sites were approx. 60 nM and 100 000 sites per cell for both Lf species. In contrast, mLf bound to about 8000 binding sites per cell (Table 3) with a K_d of 30 nM, which is close to the values obtained with hLf^{-4N} and $rhLf^{-5N}$ (Table 2).

Effect of sodium chlorate treatment on the binding of hLf species to Jurkat cells

To evaluate to what extent the binding of hLf to Jurkat cells is determined by sulphated glycosaminoglycans (GAGs) such as heparan sulphate, dermatan sulphate or chondroitin sulphate, we pretreated the cells with sodium chlorate. Chlorate inhibits ATP sulphurylase and hence the production of phosphoadenosine phosphosulphate, the active sulphate donor for sulphotransferases. Chlorate has been shown to inhibit sulphation of carbohydrate residues on intact cells without interfering with cell growth or protein synthesis [29]. Culturing Jurkat cells in the presence of sodium chlorate altered neither their growth rate nor their morphology. Table 4 shows that chlorate treatment of Jurkat cells decreased the number of binding sites for native hLf from 102 000 to 62 000 per cell, whereas the K_d was decreased slightly from 71 to 62 nM. Depletion of cell-associated sulphate groups resulted in approx. 21 000 binding sites for hLf^{-3N} and $rhLf^{-5N}$ with K_d values of 57 and 28 nM respectively (Table 4). This number of binding sites is very close to that found in untreated cells for both hLf^{-4N} and $rhLf^{-5N}$ (approx. 17 000 sites per cell; Table 2). The chlorate treatment decreased the number of binding sites for hLf^{-3N} from 36 000 to 21 000 (Table 4), but did not affect the number of sites for $rhLf^{-5N}$. This suggests that hLf^{-3N}, but not $rhLf^{-5N}$, is still able to interact with sulphated groups exposed on the cell surface of untreated Jurkat cells.

DISCUSSION

In the present paper we show the role of Arg^2-Arg^3-Arg^4-Arg^5 of hLf in the binding of this protein to the lymphoblastic cell line Jurkat.

We have previously reported the purification and partial characterization of an hLf-specific receptor expressed in

phytohaemagglutinin-stimulated human lymphocytes [9]. Recently we identified a similar lactoferrin receptor on Jurkat cells [26]. First attempts to delineate the binding site of hLf for the lymphocyte receptor suggested that residues 28–34 and 39–42 were involved in hLf–receptor interactions [22]. Quantitative studies of structure–affinity relationships and comparative molecular field analysis of residues 4–52 confirmed the existence of two large regions involved in hLf–Jurkat cell interactions [35]. A striking structural feature of the first 50 residues of hLf, when compared with lactoferrin and transferrin of other species, is the basic cluster Arg^2-Arg^3-Arg^4-Arg^5. To our knowledge, no systematic study has as yet been made of the role of consecutive Arg residues in the first basic cluster in hLf–receptor interactions.

Here we describe limited tryptic proteolysis of native hLf yielding N-terminally deleted hLf variants lacking Gly^1-Arg^2, Gly^1-Arg^2-Arg^3 or Gly^1-Arg^2-Arg^3-Arg^4. N-terminally deleted hLf variants were previously noted in purified hLf from milk from various suppliers [17]. Tryptic cleavage at Arg^3 has been observed on preparing the 39 kDa N-terminal and 51 kDa C-terminal tryptic fragments of hLf (residues 4–283 and 284–692 respectively) [34]. The results in Table 1 suggest that cleavage at Arg^2 occurs before cleavage at Arg^3. Subsequent cleavage of the peptide bond after Arg^4, yielding hLf^{-4N}, occurs at a much slower rate. SDS/PAGE analysis showed that, under the mild hydrolysis conditions used, proteolysis had occurred almost solely at the N-terminus. Tryptic proteolysis at Lys^{283} was noted in less than 5 % of total hLf molecules after 3 h of digestion, whereas all molecules lacked at least two N-terminal residues. These results clearly demonstrate that tryptic proteolysis of the N-terminus occurs before cleavage at the subsequent major cleavage site at Lys^{283}. We were not able to remove the entire first basic cluster by limited tryptic proteolysis of hLf. Therefore an rhLf mutant lacking residues 1–5 has been produced in Baculovirus-infected insect Sf-9 cells.

The binding parameters of native hLf to Jurkat cells were close to those previously described [26], whereas subsequent removal of the N-terminal arginine residues progressively decreased the number of the binding sites per cell, while slightly increasing the binding affinity. These results indicate that Arg^2, Arg^3 and, to a smaller extent, Arg^4 of hLf contribute to the binding of hLf to approx. 80 % of the total number of binding sites. Removal of the Gly^1-Arg^2-Arg^3-Arg^4-Arg^5 portion from hLf increased the affinity of hLf approx. 6-fold for a residual number of 20000 binding sites (Table 2), presumably representing the Lf-specific receptor. Comparison of the K_d of hLf^{-4N} with that of $rhLf^{-5N}$ suggests that Arg^5 sterically hinders interaction of the hLf lymphocytic receptor with the second basic cluster, Arg^{28}-Lys^{29}-Val^{30}-Arg^{31}, a region that we previously identified as part of the hLf receptor-binding site [22]. The X-ray crystallographic data of hLf does indeed indicate that Arg^5 is linked to the protein core through a hydrogen bond (results not shown). Arg^5 is thus probably involved more in the structural integrity of hLf than in the interactions of hLf with other molecules. This might also explain why Arg^5 is not readily released from hLf after treatment with trypsin.

A recent study on fibroblast growth factor activity with chlorate treatment of Jurkat cells, which inhibited sulphation by up to 90 %, revealed cell-surface-associated molecules as low-affinity binding sites [36]. Treatment of Jurkat cells with chlorate resulted in a 40 % decrease in the total number of binding sites for native hLf, suggesting that at least half of the sites that interact with the first N-terminal basic cluster of hLf include sulphate-containing molecules. Both hLf and bLf are able to interact with anionic cell-surface molecules such as proteoglycans or GAGs [13,14,27]. Moreover, hLf can be purified on heparin–Sepharose [37] and the binding of this protein to sulphated GAGs has been characterized [13,14]. A recent study on the binding of synthetic peptides comprising the first 33 residues of hLf to GAGs suggested that Gly^1-Arg^2-Arg^3-Arg^4-Arg^5-Ser^6 acts synergistically with the second basic cluster, Arg^{28}-Lys^{29}-Val^{30}-Arg^{31} [13], through the formation of a 'cationic cradle'. Wu et al. [14] proposed that a structural motif formed by residue Arg^5 only together with residues Arg^{25}, Arg^{28}, Arg^{29} and Arg^{31} represents the GAG-binding domain. Our results suggest that Arg^2, Arg^3 and Arg^4 but not Arg^5 contribute to the binding of hLf to proteoglycans on Jurkat cells.

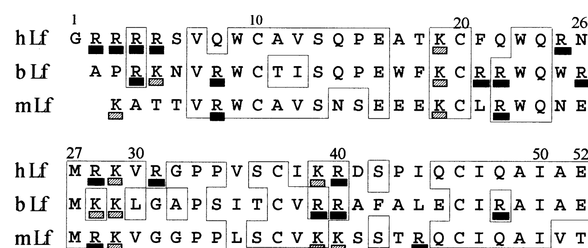

Figure 3 N-terminal protein sequence alignment of hLf, bLf and mLf

Identical amino acids between hLf [18,19], bLf [20] and mLf [21] are boxed. Arg and Lys residues are underlined with black and hatched blocks respectively. The numbering of the sequence is in accordance with [19].

The N-terminal cluster of four consecutive arginine residues is unique for hLf (Figure 3) [18]. Nevertheless the binding of bLf and hLf to Jurkat cells was comparable. mLf displayed relatively high-affinity binding to a much smaller number of binding sites (approx. 8000 per cell), which resembles the binding parameters of hLf^{-4N} and $rhLf^{-5N}$. This suggests that, in contrast with hLf and bLf, mLf does not interact with the sulphated molecules on the Jurkat cells but only with the lymphocyte receptor. The N-terminus of mLf contains a single basic residue (lysine) at position 1 (Figure 3) and lacks a pair of basic residues at the position homologous with Arg^4 and Arg^5 in hLf. The similar binding of both Lf species to Jurkat cells indicates that other basic residues in the N-terminus of bLf contribute to proteoglycan binding. In terms of basic residues, both hLf and bLf contain nine basic amino acids at different positions between residues 1–37, whereas mLf contains only six basic residues. Moreover, mLf possesses four negatively charged Glu residues in the 1–37 region instead of one in hLf and bLf.

In conclusion, our results indicate that Arg^2-Arg^3-Arg^4 of hLf participates in the binding of the protein to lymphocytes. Arg^2-Arg^3-Arg^4, but not Arg^5, interacts with approx. 80000 binding sites, which include sulphated cell-surface molecules. Only approx. 20000 binding sites are likely to correspond to the hLf lymphocyte receptor that we have previously characterized [9]. Binding of hLf to these binding sites does not require the presence of the first basic cluster. In view of these results one could expect that native hLf has no preferential binding to its receptor but interacts mainly with sulphated molecules on T-lymphocytes. Cell surface proteoglycans often bind biologically active molecules such as growth factors, then sequester and present them to specific receptors or modulate their activity. It was previously shown that proteoglycans modulate Lf uptake by the liver by modifying the internalization of lactoferrin into rat hepatocytes via the lipoprotein remnant receptor [27]. One could hypothesize that native hLf is bound by sulphated molecules for further presentation to the hLf lymphocyte receptor. In that process, hLf bound by sulphated molecules might be N-terminally

degraded by membrane proteases or by extracellular proteases present in secretions or released during inflammation, thus leading to preferential binding of the protein to its receptor. As a matter of fact, our proteolysis experiments *in vitro* indicate that degradation of the N-terminus of hLf can readily be achieved *in vivo*. This hypothesis has to be investigated further. The quantitative preparation of N-terminally deleted hLf variants, described here, offers the opportunity to gain further insight into the biological role of different binding sites expressed at the cell surface.

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