Molecular Cloning and Functional Expression of a Human Intestinal Lactoferrin Receptor‡

Yasushi A. Suzuki, Kouichirou Shin,§ and Bo Lönnerdal*

Department of Nutrition, University of California, Davis, One Shields Avenue, Davis, California 95616

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ABSTRACT: Lactoferrin (Lf), a major iron-binding protein in human milk, has been suggested to have multiple biological roles such as facilitating iron absorption, modulating the immune system, embryonic development, and cell proliferation. Our previous binding studies suggested the presence of a specific receptor for Lf (LfR) in the small intestine of newborn infants, which may facilitate iron absorption. We here report the cloning and the functional expression of the human intestinal LfR and the evidence of its involvement in iron metabolism. The entire coding region of the LfR cDNA was cloned by PCR based on amino acid sequences of the purified native LfR (nLfR). The recombinant LfR (rLfR) was then expressed in a baculovirus−insect cell system and purified by immobilized human Lf (hLf) affinity chromatography where binding of hLf to the rLfR was partially Ca^{2+} dependent. The apparent molecular mass was 136 kDa under nonreducing conditions and 34 kDa under reducing conditions. ^{125}I-hLf bound to the rLfR with an apparent K_d of ∼360 nM. These biochemical properties of the rLfR are similar to those of the nLfR. RT-PCR revealed that the gene was expressed at high levels in fetal small intestine and in adult heart and at lower levels in Caco-2 cells. PI-PLC treatment of Caco-2 cells indicated that the LfR is GPI anchored. In Caco-2 cells transfected with the LfR gene, ^{125}I-hLf binding and ^{59}Fe-hLf uptake were increased by 1.7 and 3.4 times, respectively, compared to those in mock-transfected cells. Our findings demonstrate the presence of a unique receptor-mediated mechanism for nutrient uptake by the newborn.

Iron deficiency is the most common nutrient deficiency among infants and children in developing countries (*1*). On the other hand, absorbing excess iron has been linked to cardiac disease (*2*, *3*) and hepatic failure (*4*) because of the toxicity of iron. Thus, controlling iron absorption is important, especially for infants and young children. The bioavailability of iron from human milk is higher than from cow's milk or infant formula (*5*), which most likely is explained by the effect of components in human milk (*6*). Lactoferrin (Lf) is an 80 kDa, single-chain glycoprotein, binds iron tightly, and is abundant in milk of some species such as humans, rhesus monkeys, and mice (*7*). We have previously shown that Lf resists proteolysis during passage through the infant's digestive system (*8*) and that Lf binds to enterocytes and brush-border membranes in a saturable manner, suggesting the presence of a specific receptor for Lf (LfR) in the infant intestine (*9*, *10*). We have therefore hypothesized that the LfR regulates absorption of Lf-bound iron by intestinal cells. Lf is also found in various biologic fluids, including circulating neutrophil granules and pancreatic and bronchial secretions (*11*), and has been shown to be involved in a variety of biological functions such as immune function (*7*), embryonic development (*12*), and cell proliferation (*13*). LfRs have also been reported in lymphocytes (*14*) and liver (*15*, *16*), but little is known about its tissue distribution. Thus, it is possible that LfRs may also be involved in different functions in different cell types. We therefore find it important to determine the characteristics of the LfR, to explore the mechanism of Lf transport mediated by the LfR, and to clarify the biological significance of this mechanism.

We have previously identified and purified the LfR from brush-border membranes from fetal and infant small intestine (*17*). Human Lf (hLf) showed specific binding to the LfR with an apparent K_d of ∼1 μM. Bovine Lf and serum transferrin did not compete with hLf for binding. Binding was also pH dependent, with optimum binding occurring at pH 6.5−7. The LfR existed as a 110−120 kDa complex consisting of ∼38 kDa subunits. Deglycosylation of the protein decreased the molecular weight of the subunit to ∼34 kDa, indicating that the receptor is glycosylated, with the molecular mass of the glycan being ∼4 kDa. The purified receptor maintained its ability to bind hLf. Although biochemical properties of the native LfR (nLfR) were characterized to some extent, it is still necessary to elucidate the detailed molecular structure of the LfR, the mechanism of its interaction with hLf, and the physiological role of the LfR.

In this paper, we isolated the nLfR from fetal intestine and determined the N-terminal and an internal amino acid sequence of the nLfR. We searched for homologues on the expressed sequence tag (EST) database. ESTs are sequences of cDNA fragments prepared from different tissue sources (*18*), which now represent more than half of all human genes

‡ The sequence reported in this paper has been deposited in the GenBank database (Accession Number AF271386).

* To whom correspondence and reprint requests should be addressed. E-mail: bllonnerdal@ucdavis.edu.

§ Present address: Nutritional Science Laboratory, Morinaga Milk Industry Co. Ltd., 5-1-83 Higashihara, Zama, Kanagawa 228-8583, Japan.

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(*19*), thereby giving us valuable genetic information. We designed gene-specific primers based on the human EST homologues to the determined peptides and cloned a LfR cDNA by PCR. We then expressed a recombinant LfR (rLfR) in a baculovirus system, isolated the rLfR, and showed direct binding of the rLfR to hLf. We also investigated uptake of hLf-bound iron mediated by the LfR as one of the possible functions of the LfR.

EXPERIMENTAL PROCEDURES

Isolation and Amino Acid Analysis of a Human Fetal Intestinal LfR. Frozen human fetal intestinal tissue from 19−21 weeks of gestation was obtained from Anatomic Gift Foundation (White Oak, GA). The mucosa was scraped, and brush-border membrane vesicles (BBMV) were isolated by a method described previously (*20*). BBMV were solubilized with Triton X-100 (Sigma, St. Louis, MO), and the native LfR (nLfR) was isolated from the solubilized BBMV by affinity chromatography using immobilized iron-saturated hLf as described previously (*17*). The purified nLfR (2 μg) was subjected to analysis of the N-terminal and an internal amino acid sequence at the Protein Structure Laboratory (University of California, Davis, CA).

Homology Search and Construction of a Gene-Specific Primer. A homology search for the N-terminal amino acid sequence, WXXDEANTYXKE, and the internal amino acid sequence, ASYYSPYGQ, was performed on both the Swall protein and human EST database using the FASTA3 program at the European Bioinformatics Institute (http://www.ebi-.ac.uk/fasta3). A sense primer was designed as 5′-ACCCAAGGAAAGTGCAGCTGAGA-3′, based on the human EST homologous to the N-terminal sequence (GenBank R06009), which is located upstream from the potential open reading frame of a mouse homologue (GenBank NM010584). An antisense primer was designed downstream from the potential stop codon, 5′-GTTCCCTCCCACAAAACTCTCAACGA-3′, based on the human EST homologous to the internal amino acid sequence and containing a poly(A) region (GenBank AW029086).

Cloning of the LfR cDNA. PCR was performed on single-stranded cDNA from human fetal intestine (Invitrogen, Carlsbad, CA) as a template. PCR amplification was performed with 2 μL of cDNA in the presence of 0.2 μM sense and antisense primers, 0.2 mM dNTP, and 1 μL of Advantage cDNA polymerase mix (Clontech, Palo Alto, CA) in a total volume of 50 μL. The conditions for PCR were as follows: initial denaturation at 94 °C for 1 min, 20 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, extension at 72 °C for 3 min, and final extension at 72 °C for 10 min. The PCR product was cloned into the plasmid vector pCR-XL-TOPO (Invitrogen) and sequenced for both strands of the entire region by using automated DNA sequencing on an ABI Prism 377 DNA sequencer (PE Biosystems, Foster City, CA) at Davis Sequencing Inc. (Davis, CA).

Generation of a Recombinant Baculovirus. The Bac-to-Bac expression system (Life Technologies Inc., Rockville, MD) was used for expression of the rLfR. The LfR cDNA was excised from the pCR-TOPO-XL vector at *Spe*I and *Xho*I restriction sites and ligated into the donor plasmid pFastBac via the same restriction sites. The recombinant baculovirus containing LfR cDNA was generated according to the manufacturer's instructions.

Expression of the rLfR. Tricoplusia ni (T. ni) cells were routinely maintained at 28 °C in 250 mL Erlenmeyer flasks containing 100 mL of a serum-free medium (ESF921; Expression System, Woodland, CA) and subcultured every 3 or 4 days. For the expression of the LfR, *T. ni* cells were seeded at 0.5×10^{10} cells/mL and infected by the recombinant baculovirus at a multiplicity of infection of 0.1. At 3 days postinfection, the culture was centrifuged at 1000*g* for 10 min at 4 °C to separate supernatant and cells.

Isolation of the rLfR and Peptide Sequence Analysis. The supernatant of infected *T. ni* cells was further centrifuged at 20000*g* for 10 min and filtered though a Nylon filter cartridge with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI). The filtered supernatant (200 mL) was loaded onto 1 mL of hLf-immobilized gel. The gel was washed thoroughly with either 0.5 M NaCl and 25 mM Tris-HCl buffer (pH 7.4) or 5 mM $CaCl_2$, 0.5 M NaCl, and 25 mM Tris-HCl buffer (pH 7.4). The rLfR was eluted with buffer A, B, or C: (A) 0.15 M NaCl and 0.2 M sodium acetate buffer (pH 3.7), (B) 0.15 M NaCl, 5 mM EGTA, and 25 mM Tris-HCl buffer (pH 7.4), or (C) 0.15 M NaCl, 5 mM EGTA, and 0.2 M sodium acetate buffer (pH 3.7). The eluted fractions, in case the pH was lower than 6.5, were immediately adjusted to pH 7.0 with 1.5 M Tris-HCl buffer, pH 8.8. SDS−PAGE was performed in 10% polyacrylamide gels containing 1% SDS under both nonreducing and reducing conditions. Gels were stained with Coomassie Brilliant Blue R solution. Two internal amino acid sequences were determined from the purified protein as described above.

Binding of ^{125}I-hLf to the rLfR. Purified rLfR (0, 20, 40, and 50 ng) in 20 mM $NaHCO_3$, pH 9.6, was coated onto Maxisorp surface LockWell modules (Nalge Nunc International, Naperville, IL). Wells were washed four times with dissociation buffer (25 mM Tris, pH 7.4, containing 0.15 M NaCl, 5 mM EGTA, and 0.05% Tween-20). After incubation with 1% BSA in dissociation buffer for 1 h at room temperature, wells were washed four times with binding buffer (25 mM Tris, pH 7.4, containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05% Tween-20). Different concentrations of ^{125}I-hLf, labeled by the iodogen method, were prepared in binding buffer and added to the wells in the presence or absence of a 100-fold excess of unlabeled hLf for 2 h at 4 °C. Wells were then washed four times with binding buffer and snapped off to separate into individual wells. Radioactivity in wells was quantified in a γ counter (Gamma 8500, Beckman, Fullerton, CA). Total binding of ^{125}I-hLf to the rLfR (TR) was determined by subtracting total radioactive counts in the rLfR-uncoated well (TW) from those in the rLfR-coated well (TRW), and nonspecific binding of ^{125}I-hLf to the rLfR (NR) was obtained by subtracting TW from TRW with excess unlabeled hLf. Specific binding (SR) was determined by subtracting the NR from the TR, and units were converted to picomoles of hLf per picomole of rLfR subunit. The apparent dissociation constant (K_d) and the number of binding sites (n) were determined by nonlinear regression analysis using the following model equation (for one binding site): $C = nL/(K_d + L)$, where C is the receptor/ligand complex number and L is the free ligand concentration.

RT-PCR. Caco-2 cells were grown for 3 weeks in minimum essential medium (MEM; Life Technologies Inc.) supplemented with 10% fetal bovine serum (FBS), 10 μg/mL streptomycin, and 10 units/mL penicillin (Sigma). Total RNA was extracted from 3 million cells by using the SV total RNA isolation system (Promega) according to the manufacturer's instructions. Total RNA from human adult duodenum and ileum was generously provided by C. Halsted of the University of California (Davis, CA). First-strand cDNA was synthesized from 5 μg of total RNA isolated from various human organs (Clontech) by using the cDNA cycle kit (Invitrogen) according to the manufacturer's instructions. PCR was carried out using 1 μL of the synthesized cDNA solution and under the same conditions as described above. The PCR product was then subjected to agarose gel analysis. A picture of the gel stained with ethidium bromide was taken under a UV lamp, and the intensity of each band was analyzed using the Chemi-Doc system (Bio-Rad).

Phosphatidylinositol-Specific Phospholipase C (PI-PLC) Treatment. Caco-2 cells were grown in the normal medium (MEM, 10% FBS, streptomycin/penicillin) for 3 weeks in a T-75 flask (Corning Inc., Corning, NY) to let them differentiate. Cells were washed with PBS and 5 million cells were collected into each test tube. Cells were incubated at 37 °C for 3 h either with or without phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma) in a total volume of 1 mL. After incubation, cells were centrifuged at 1000*g* for 5 min, and 500 μL of supernatant was filtered through a 0.22 μm PVDF membrane. These preparations were applied either to Western blot or to an immobilized hLf affinity column. The LfR was purified, and the peak height detected by a UV sensor was compared between the PI-PLC treated and control (untreated) sample.

Transfection of the LfR into Caco-2 Cells. The LfR cDNA was excised from the pCR-TOPO-XL vector at *Hin*dIII and *Xho*I restriction sites and ligated into the expression vector pcDNA3.1 (Invitrogen) via the same restriction sites. This plasmid was named pcDNA-LfR. Caco-2 cells were seeded to become 50−70% confluent at day 2−3, and the linearized pcDNA3.1 or pcDNA-LfR by *Bgl*II was transfected using lipofectamine plus reagent (Life Technologies Inc.) according to the manufacturer's instructions. Transfected cells were grown in a selection medium (MEM, 10% FBS, 400 μg/mL Geneticin) and split every week. After three passages, untransfected cells had all died. Cells were kept growing in the selection medium and split every other week. Total RNA was extracted from transfected cells, and RT-PCR was carried out as described above.

Binding of ^{125}I-hLf to Transfected Cells. LfR-transfected and mock-transfected Caco-2 cells were seeded on 24 well plates (0.5 million/well; Corning Corster Corp., Cambridge, MA) and grown for 4 weeks. Cells were washed with MEM, and various amounts of ^{125}I-hLf were added onto the cells in the presence or absence of a 100-fold excess of unlabeled hLf. Cells were incubated at 4 °C for 2 h and washed three times with MEM. Cells were then dissolved in 1 M NaOH, and radioactivity was determined in the γ counter. The apparent K_d and the number of binding sites (n) were determined as described above.

Uptake and Transport of ^{59}Fe-hLf into Transfected Cells. LfR-transfected and mock-transfected cells were seeded on inserts of a Transwell plate (12 well size, 1 million/well; Corning Costar Corp., Cambridge, MA) and cultured for 4 weeks. Transepithelial electrical resistance of the insert was measured to ensure integrity of the cell barrier. Apo-human transferrin (20 μM) was added in the basolateral medium. Then, 3 μM ^{59}Fe-hLf (6 μM as Fe) or 6 μM ^{59}Fe-NTA was prepared in MEM, and 500 μL of each solution was added onto the apical chamber and incubated at 37 °C for 3 h. After incubation, both the apical and basolateral sides of the membrane were washed three times with MEM. Radioactivity of the medium on the basolateral side and that of cells were determined in the γ counter.

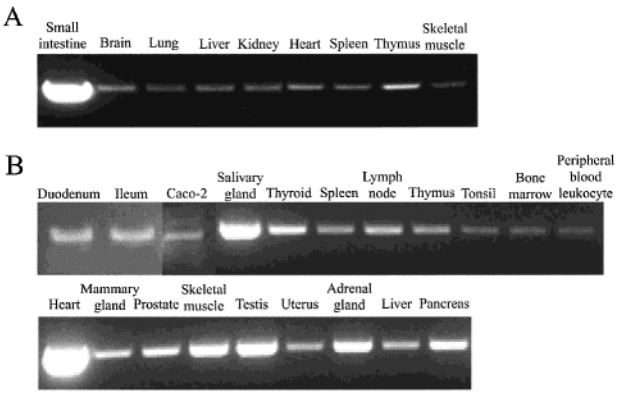

FIGURE 1: Agarose gel electrophoresis. PCR was performed with 30 cycles on cDNA synthesized from 5 μg of total RNA in each human tissue. (A) Human fetal tissues. (B) Human adult tissues and Caco-2 cells.

Statistical comparison of multiple means was performed initially by one-way ANOVA (5%) followed by post hoc analysis with the Newman−Keuls test.

Western Blot. Antiserum (P247AS) was produced in rabbits against a mixture of three chemically synthesized peptides, CTVGDRWSSQQGSKAD, DFGDAQKTASYYS-PC, and SSSPSLPRSCKEIKDE, which correspond to parts of the deduced LfR amino acid sequence (Genemed Synthesis Inc., South San Francisco, CA). For Western blots, proteins separated by SDS−PAGE were transferred to a PVDF membrane (Bio-Rad). The membrane was blocked with 5% skim milk in PBS for 1 h at room temperature, washed three times with 0.1% Tween-20 in PBS, and incubated with a 1:1000 dilution of P247AS in the blocking buffer containing 0.05% Tween-20 for 1 h at room temperature. After another wash, the membrane was incubated with a 1:20000 dilution of HRP-conjugated anti-rabbit IgG antibody in the blocking buffer containing 0.1% Tween- 20 for 1 h at room temperature. After the final wash (four times in Tween-PBS), the LfR was detected with West Femto substrate (Pierce, Rockford, IL). The intensity of each band was analyzed using the Chemi-Doc system (Bio-Rad).

RESULTS

Cloning of the LfR cDNA. To initiate the cloning of the LfR, a PCR approach was taken (see Experimental Procedures), which resulted in the generation of an ~1 kb PCR product (Figure 1A, small intestine). The nucleotide sequence of the LfR cDNA and the deduced protein sequence are shown in Figure 2. The cDNA sequence of the cloned protein predicts an open reading frame of 939 nucleotides, encoding a polypeptide of 313 residues. The deduced protein sequence

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acccaaggaaagtgcagctgagactcagacaagattacaatgaaccaa          48
                                              M  N  Q
ctcagcttcctgctgtttctcatagcgaccaccagaggatggagtaca          96
 L  S  F  L  L  F  L  I  A  T  T  R  G  W  S  T
gatgaggctaatacttacttcaaggaatggacctgttcttcgtctcca         144
 D  E  A  N  T  Y  F  K  E  W  T  C  S  S  S  P
tctctgcccagaagctgcaaggaaatcaaagacgaatgtcctagtgca         192
 S  L  P  R  S  C  K  E  I  K  D  E  C  P  S  A
tttgatggcctgtattttctccgcactgagaatggtgttatctaccag         240
 F  D  G  L  Y  F  L  R  T  E  N  G  V  I  Y  Q
accttctgtgacatgacctctgggggtggcggctggaccctggtggcc         288
 T  F  C  D  M  T  S  G  G  G  G  W  T  L  V  A
agcgtgcatgagaatgacatgcgtgggaagtgcacggtgggcgatcgc         336
 S  V  H  E  N  D  M  R  G  K  C  T  V  G  D  R
tggtccagtcagcagggcagcaaagcagactacccagaggggacggc         384
 W  S  S  Q  Q  G  S  K  A  D  Y  P  E  G  D  G
aactgggccaactacaacacctttggatctgcagaggcggccacgagc         432
 N  W  A  N  Y  N  T  F  G  S  A  E  A  A  T  S
gatgactacaagaaccctggctactacgacatccaggccaaggacctg         480
 D  D  Y  K  N  P  G  Y  Y  D  I  Q  A  K  D  L
ggcatctggcacgtgcccaataagtcccccatgcagcactggagaaac         528
 G  I  W  H  V  P  N  K  S  P  M  Q  H  W  R  N
agctccctgctgaggtaccgcacggacactggcttcctccagacactg         576
 S  S  L  L  R  Y  R  T  D  T  G  F  L  Q  T  L
ggacataatctgtttggcatctaccagaaatatccagtgaaatatgga         624
 G  H  N  L  F  G  I  Y  Q  K  Y  P  V  K  Y  G
gaaggaaagtgttggactgacaacggcccggtgatccctgtggtctat         672
 E  G  K  C  W  T  D  N  G  P  V  I  P  V  V  Y
gattttggcgacgcccagaaaacagcatcttattactcaccctatggc         720
 D  F  G  D  A  Q  K  T  A  S  Y  Y  S  P  Y  G
cagcgggaattcactgcgggatttgttcagttcagggtatttaataac         768
 Q  R  E  F  T  A  G  F  V  Q  F  R  V  F  N  N
gagagagcagccaacgccttgtgtgctggaatgagggtcaccggatgt         816
 E  R  A  A  N  A  L  C  A  G  M  R  V  T  G  C
aacactgagcaccactgcattggtggaggaggatactttccagaggcc         864
 N  T  E  H  H  C  I  G  G  G  G  Y  F  P  E  A
agtccccagcagtgtggagatttttctggttttgattggagtggatat         912
 S  P  Q  Q  C  G  D  F  S  G  F  D  W  S  G  Y
ggaactcatgttggttacagcagcagccgtgagataactgaggcagct         960
 G  T  H  V  G  Y  S  S  S  R  E  I  T  E  A  A
gtgcttctattctatcgttgagagttttgtgggagggaac                1000
 V  L  L  F  Y  R  -
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FIGURE 2: Nucleotide sequence of the LfR cDNA and the deduced amino acid sequence. The analyzed amino acid sequences from the nLfR are highlighted by gray shading, and those from the rLfR are boxed.

of the LfR contains an amino acid sequence, ^{17}WSTDEANTYFKE^{28}, which is similar to the N-terminal sequence from the purified nLfR, which in turn is identical to the EST from which the sense primer was designed. The N-terminal end of the nLfR is a tryptophan, which corresponds to residue 17 in the cDNA coding sequence. This finding suggests the presence of a 16-residue cleavable signal sequence; such a signal peptide was also predicted by analysis with the peptide sequencing program. There was also a sequence identical to the internal peptide sequence, ASYYSPYGQ (gray shading in Figure 2). The calculated mass of a mature LfR consisting of 297 amino acids after removal of a 16 amino acid putative signal peptide is 33.2 kDa, which is identical to the nLfR after deglycosylation (*17*). The deduced amino acid sequence of the LfR does not contain a transmembrane region, but there is a putative glycophosphatidylinositol (GPI) linkage domain present on the deduced protein (Figure 8), suggesting that the LfR may be GPI anchored.

Functional Expression and Characterization of the rLfR. To investigate whether the LfR cDNA encodes a functional receptor, we attempted expression of the cloned LfR cDNA in a baculovirus−insect cell system and applied the cell culture supernatant to affinity chromatography for purification of the rLfR. The chromatogram exhibited a single, sharp peak during elution with the low pH buffer after extensive washing (Figure 3A), suggesting that the protein in this fraction specifically bound to hLf. The fraction applied onto SDS−PAGE revealed a single band with the size of 136 kDa under nonreducing conditions and 34 kDa under reducing conditions, respectively (Figure 4), indicating that

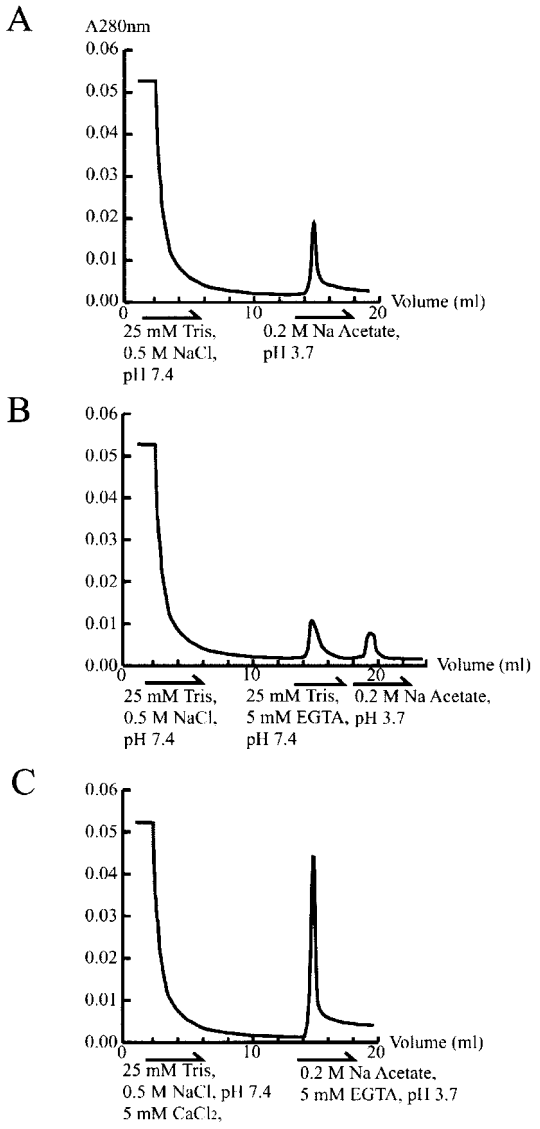

FIGURE 3: Isolation of the rLfR by immobilized hLf affinity chromatography. *T. ni* cell culture medium (200 mL) at day 2 after infection by LfR recombinant baculovirus was applied to the hLf affinity column. Chromatograms: Wash of the column with 25 mM Tris and 0.5 M NaCl, pH 7.4 (A, B), or with 25 mM Tris, 0.5 M NaCl, and 5 mM $CaCl_2$, pH 7.4 (C). (A) Elution with 0.2 M sodium acetate and 0.15 M NaCl, pH 3.7. (B) Elution with 25 mM Tris, 5 mM EGTA, and 0.15 M NaCl, pH 7.4, and then with 0.2 M sodium acetate and 0.15 M NaCl, pH 3.7. (C) Elution with 0.2 M sodium acetate, 0.15 M NaCl, and 5 mM EGTA, pH 3.7.

the rLfR was completely purified by the one-step affinity chromatography. The 136 kDa band under nonreducing conditions corresponds to four identical 34 kDa subunits observed under reducing conditions. The apparent size of the subunit was virtually identical to the calculated mass of 33.2 kDa and the mass of the nLfR. In some cases under different purification conditions, two bands, 136 and 102 kDa, were revealed on SDS−PAGE under nonreducing conditions (data not shown), but the single band with the size of 34 kDa was observed from the same sample under reducing conditions. Two bands appear to represent a tetramer and a trimer of 34 kDa subunits, indicating that there is a variance in oligomer formations and that oligomer appearance depends on conditions during purification. Two internal amino acid sequences, TDEANTY and LGIWHVPNK, were determined from the 34 kDa fragments, both

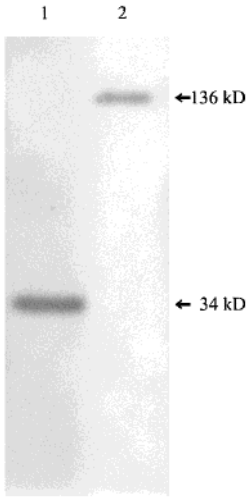

FIGURE 4: SDS−PAGE analysis of the eluted fraction from immobilized hLf affinity chromatography. Lanes: 1, the eluted peak under reducing conditions; 2, the eluted peak under nonreducing conditions.

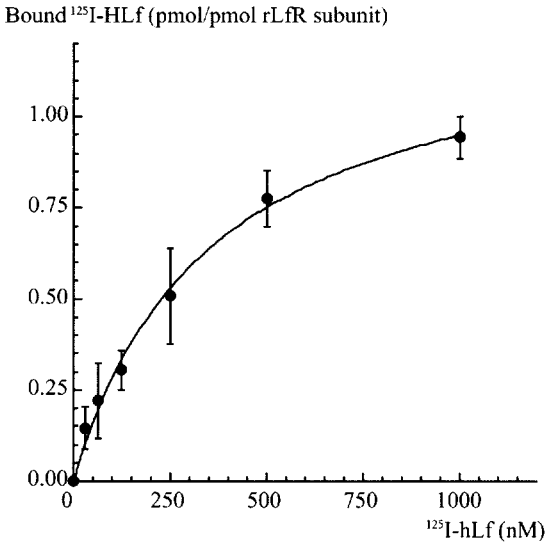

FIGURE 5: Specific binding of hLf to the rLfR. Different concentrations of ^{125}I -hLf were added to LockWells coated with 0, 20, 40, and 50 ng of purified rLfR and incubated for 2 h at 4 °C. Wells were washed four times with 25 mM Tris, pH 7.4, containing 0.15 M NaCl, 5 mM $CaCl_2$, and 0.05% Tween-20, and the radioactivity in the wells was determined in the γ counter. Units of all specific binding data (see Experimental Procedures for details) were converted to pmol of bound hLf/pmol of rLfR subunit. Each point represents mean of three experiments ± SE. The K_d was 360 ± 50 nM.

of which were identical to the parts of the deduced amino acid sequence (boxed in Figure 2). Three different elution buffers were used for the elution of the rLfR (Figure 3). First, a buffer at pH 3.7, the same pH as used for the nLfR elution, effectively eluted the rLfR (Figure 3A), suggesting that binding properties of the nLfR and the rLfR to hLf are similar. Second, the effect of a Ca^{2+} chelator, EGTA, at pH 7.4 on the rLfR elution was assessed. According to the elution peak in the chromatogram (Figure 3B) and SDS−PAGE (data not shown), EGTA at pH 7.4 eluted the rLfR, but to less extent. After elution by EGTA at pH 7.4, the buffer at pH 3.7 eluted more rLfR, suggesting that the binding between hLf and rLfR is at least partially Ca^{2+} dependent. Third, $CaCl_2$ was added to the washing buffer to minimize a possible release of the rLfR from the column during the washing process, and the rLfR was eluted with the combination of EGTA and low pH (Figure 3C). The yield of rLfR was more than twice as high as in the first chromatogram, supporting our hypothesis that Ca^{2+} is at least partially required for the receptor−hLf interaction.

Binding of ^{125}I-hLf to the rLfR. Approximately 0.3% of ^{125}I-hLf bound nonspecifically to the plate, which was not reversible by addition of excess amounts of unlabeled hLf. Thus, total binding to the rLfR (TR) was determined by subtracting the nonspecific binding to the plate (TW) from the total radioactive count (TRW). Excess unlabeled hLf was able to replace ^{125}I-hLf up to ∼95%, suggesting that ∼5% was nonspecificlly bound to the rLfR. Saturation binding was observed in all cases (except for those samples in which rLfR was omitted) with maximal binding increasing as a function of the amount of rLfR absorbed onto the plates. The specific binding (SR) curve for ^{125}I-hLf to the purified rLfR is shown in Figure 5. Nonlinear regression analysis revealed that the apparent K_d was 360 ± 50 nM, which is somewhat higher affinity than the number we obtained previously for ^{125}I-hLf binding to human infant intestinal BBMV.

Expression of the LfR in Other Tissues. The LfR was expressed in all 9 human fetal tissues, 19 human adult tissues, and Caco-2 cells (Figure 1). Among these tissues, expression was especially high in adult heart and fetal small intestine, high in adult testis, salivary gland, skeletal muscle, pancreas, salivary gland, and thyroid, moderate in fetal thymus, adult prostate, lymph node, and thymus, and low in the rest of the tissues. In addition, adult duodenum, ileum, and Caco-2 cells expressed the LfR mRNA even though the expression level was much lower than that in fetal small intestine.

PI-PLC Treatment of Caco-2 Cells. The peak height of the eluted fraction for the PI-PLC treated sample from the immobilized Lf affinity column was 2.8 times higher than that for the control sample (Figure 6A). According to Western blot analysis (Figure 6B), the relative band intesity of the PI-PLC treated sample was 4.6 times higher than that of the control sample. These results indicate that PI-PLC treatment releases proteins bound to hLf, including the LfR, and, therefore, that the LfR is GPI anchored.

Transfection of the LfR to Caco-2 Cells. RT-PCR revealed that the LfR gene was successfully transfected into Caco-2 cells, transcribed to mRNA, and translated to protein (Figure 7A,B). The LfR protein expression in LfR-transfected cells was 3.2 times higher than that in mock-transfected cells. In the binding studies, excess unlabeled hLf replaced ^{125}I-hLf up to 75%, indicating that ∼25% was nonspecifically bound to the cell membrane. The specific ^{125}I-hLf binding to LfR-transfected Caco-2 cells was 74% higher than that to mock-transfected Caco-2 cells, while the apparent K_d values were in the identical range for both LfR-transfected and mock-transfected cells, indicating that the LfR was overexpressed on the LfR-transfected Caco-2 cell membrane in a functional form (Figure 7C). We thus used LfR-transfected Caco-2 cells and mock-transfected Caco-2 cells for a ^{59}Fe-hLf transport study (Figure 7D,E). ^{59}Fe uptake from hLf into LfR-transfected cells was 3.4 times higher than that into mock-transfected cells and also higher than that for ^{59}Fe itself into LfR-transfected cells, whereas there were no significant

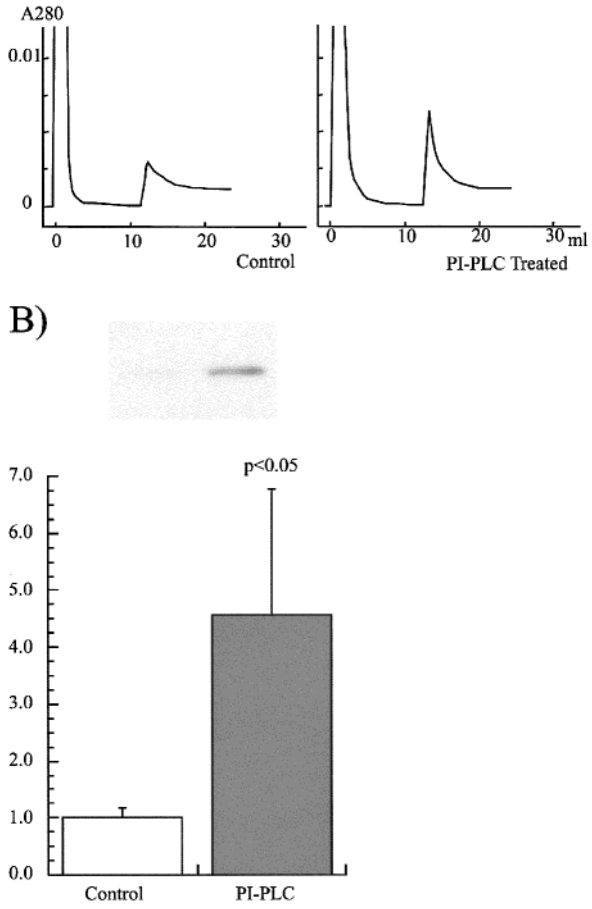

FIGURE 6: PI-PLC treatment of Caco-2 cells. Fully differentiated cells (5 million) were treated with or without PI-PLC, and cells were centrifuged down. (A) 0.5 mL of supernatant was applied to an immobilized hLf affinity column. The LfR was purified by the method described in Figure 3C. (B) 20 μL of supernatant was subjected to Western blot analysis. A representative picture is shown on the top of the graph. Each bar represents the mean of the relative band intensity from four experiments ± SE. Means were compared by Student's *t*-test.

differences in the amount of transported ^{59}Fe into the basolateral side within 3 h.

DISCUSSION

The present study, for first time, demonstrates the cloning, primary structure, functional expression, and characteristics of a LfR in human fetal intestine. We purified the nLfR and cloned the LfR cDNA, both from human fetal intestine. There are, however, a variety of tissues from which human ESTs that are homologous to the LfR cDNA have been sequenced (data not shown). Therefore, we speculated that a similar LfR is expressed in tissues other than intestine, such as liver, spleen, or heart. RT-PCR revealed that the LfR was expressed in a variety of human tissues although expression levels varied considerably among tissues (Figure 1), suggesting that the presence of the LfR is essential for most tissues to capture hLf, which may have multiple functions in different types of cells.

The deduced amino acid sequence of the LfR cDNA is homologous to the mouse intestinal lectin-like protein, intelectin, with 81% identity (*21*) and cortical granule lectin (CGL) in *Xenopus* oocytes with 67% identity (*22*). Alignment of these three proteins is shown in Figure 8. The N-terminal region is not conserved, but the rest of the protein is highly conserved. Therefore, the LfR may also have functions similar to intelectin or CGL.

The function of mouse intelectin is not yet known, but it is speculated to be involved in antibacterial systems because it appears to be specifically localized in Paneth cells (*21*). Paneth cells, which are located at the base of the crypts in the small intestine, synthesize and secrete antimicrobial proteins (*23*, *24*). Lf is also known to have antimicrobial activity by means of several different mechanisms (*25*−*28*). It is therefore possible that the LfR is expressed on Paneth cells and modulates the antibiotic effect of Lf.

The role of CGL in *Xenopus* oocytes has been proposed to be to prevent the oocyte from polyspermy after fertilization (*29*, *30*). CGL is also found in the blastula-stage embryo (*31*) and thought to be involved in early embryogenesis (*22*, *32*). Lf is also important in the developing embryo (*12*). It is, thus, possible that the LfR is expressed in the human oocyte or blastula and mediates a function of Lf during fertilization and early embryogenesis or vice versa: Lf mediates a function of the LfR. Native CGL is also known to form an oligomer (*29*). The size of the oligomer is 539 kDa, consisting of 12 monomers (*33*). The nLfR appears to form a trimer and the rLfR appears to form a tetramer according to their apparent masses determined by SDS−PAGE, indicating that the LfR is capable of forming oligomers. This may occur in human embryogenesis if the LfR is present in the human oocyte.

CGL has Ca^{2+}-dependent lectin activity, which is important for its function (*30*). In the present study, partial Ca^{2+} dependency for Lf-LfR binding was observed in the immobilized hLf affinity chromatography (Figure 3B), suggesting that the LfR may also have Ca^{2+}-dependent lectin activity, which contributes to the interaction with hLf, a glycoprotein. The rLfR did not react in the agglutination assay toward rabbit, sheep, and goat erythrocytes, regardless of the presence of Ca^{2+}, but did bind to an immobilized galactose column, showing lectin activity (data not shown).

Human intelectin has been recently characterized as a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cells (*34*). The gene of this protein is identical to the hLfR that we present in this paper. Tsuji et al. found that the recombinant protein revealed affinities to *d*-pentoses and a *d*-galactofuranosyl residue in the presence of Ca^{2+}. Calcium dependency for its activities, tendency to form homooligomers, and no agglutination activities, which they found for the recombinant protein, are similar to what we have found.

A Ca^{2+}-dependent LfR for bovine Lf (bLf), which is identical to the rat asialoglycoprotein receptor (ASGP-R), has been characterized from rat liver (*35*). The binding domain of the rat ASGP-R for bLf was determined to be the major subunit (RHL-1) of the ASGP-R. Even though the primary structure of either RHL-1 or human ASGP-R has no significant similarity to the LfR (data not shown), it is interesting that both proteins bind Lf when Ca^{2+} is present. Although having Ca^{2+}-dependent lectin activity, RHL-1 binds bLf in a carbohydrate-independent manner, indicating that the lectin activity is not necessary for the binding between RHL-1 and bLf (*36*). This feature is similar to our finding that the binding between rLfR and hLf does not require lectin activity but partially requires Ca^{2+}. These results are also

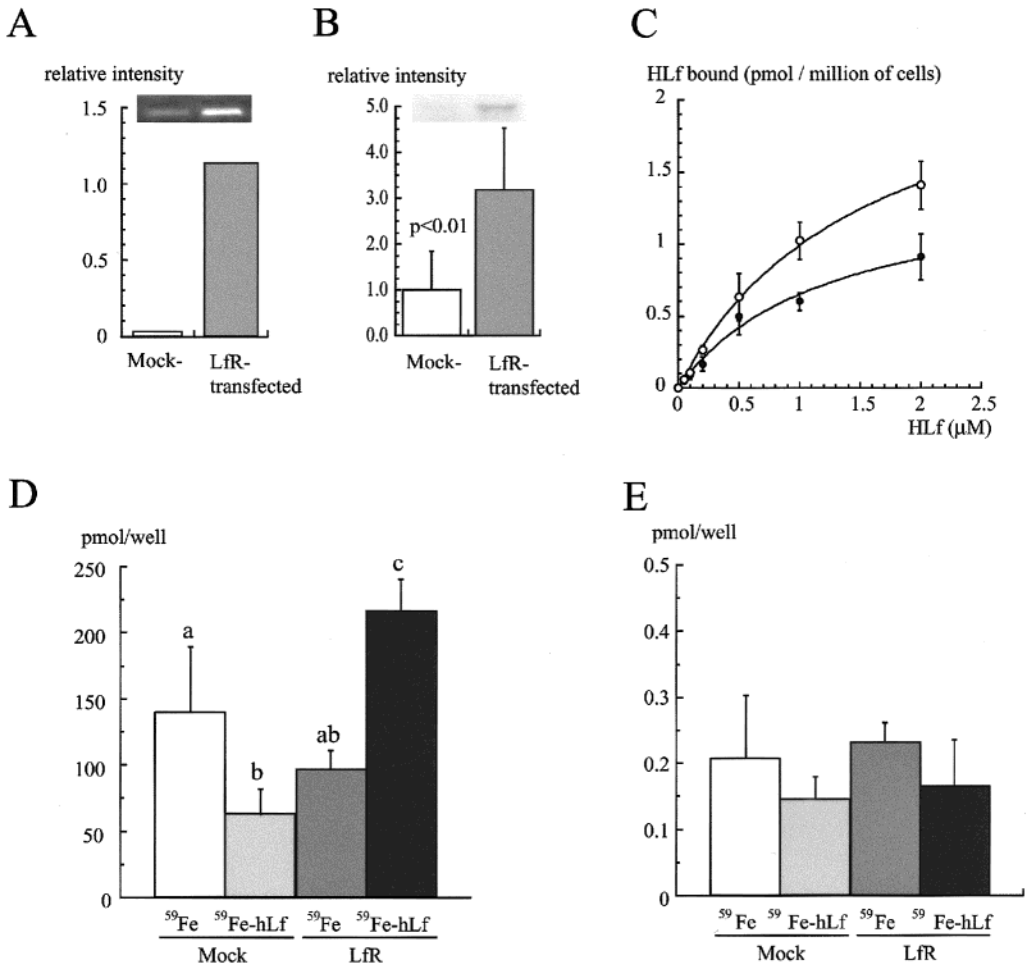

FIGURE 7: Transfection of the LfR into Caco-2 cells. (A) RT-PCR for the LfR: Total RNA was extracted from either LfR-transfected or mock-transfected Caco-2 cells (3 million cells). 1 μg of total RNA was used for RT-PCR. The intensity of the PCR product for the LfR was standardized by using that for G3PDH. (B) Western blot for the LfR: The cell lysate from either LfR-transfected or mock-transfected Caco-2 cells (50000 cells) was subjected to Western blot analysis. A representative picture is shown on the top of the graph. Each bar represents the mean of the relative band intensity from six experiments ± SE. Means were compared by Student's *t*-test. (C) ^{125}I-hLf binding to LfR-transfected (○) and to mock-transfected (●) Caco-2 cells. Various amounts of ^{125}I-hLf were added onto the cells in the absence or presence of a 100-fold excess of hLf (nonlabeled). Cells were incubated at 4 °C for 2 h and washed three times with MEM. Cells were then dissolved in 1 M NaOH, and radioactivity was determined in the γ counter. Each point represents the mean of three experiments ± SE. K_d and the number of binding sites for LfR-transfected cells were 1.55 ± 0.21 μM and 2.53 ± 0.19 pmol/million of cells, respectively, and those for mock-transfected cells were 1.23 ± 0.34 μM and 1.45 ± 0.21 pmol/million of cells, respectively. (D) Uptake of ^{59}Fe and ^{59}Fe-hLf and (E) transport of ^{59}Fe and ^{59}Fe-hLf into transfected Caco-2 cells. 3 μM ^{59}Fe-hLf (6 μM as Fe) or 6 μM ^{59}Fe-NTA was added to the apical chamber of Caco-2 cells and incubated at 37 °C for 3 h. After incubation, apical chambers were washed three times. (D) Radioactivity of the cells and (E) that of the medium in the basolateral side were determined in the γ counter. Statistical comparison of multiple means was performed initially by one-way ANOVA (5%) followed by post hoc analysis with the Newman−Keuls test. A different lower-case letter indicates a significant difference at $P < 0.05$. Each value represents the mean of three experiments ± SE.

consistent with our previous binding studies of hLf to human infant small intestinal BBMVs in which deglycosylated Lf bound to the BBMVs in a manner identical to that of native Lf (*37*).

We determined the apparent K_d for the hLf binding to the rLfR expressed in the baculovirus system (Figure 5). The apparent K_d for the rLfR is 0.36 μM, which is slightly lower than ~1 μM which was obtained from binding of hLf to fetal intestinal BBMV (*17*), possibly due to differences in the extent of glycosylation or to the effect of Ca^{2+}. Deglycosylation of the nLfR indicated that ~4 kDa of glycans were attached (*17*). Deglycosylation of the rLfR, however, did not result in any significant shift from nondeglycosylated rLfR in migration distance on SDS−PAGE (data not shown). The nLfR is therefore likely to be more glycosylated than the rLfR, which may cause a slightly lower affinity for the binding of hLf to the nLfR. It is also possible that the presence of Ca^{2+} in the binding buffer for the studies on rLfR causes higher affinity since Ca^{2+} is partially required for the binding.

The deduced amino acid sequence of the LfR does not contain a transmembrane region. Indeed, the rLfR was isolated from the water-soluble fraction of the insect cell culture medium. The nLfR, however, was isolated from the Triton X-100 soluble fraction of BBMV (*17*). Recently, a glycophosphatidylinositol (GPI) anchored homologue to CGL was purified from the *Xenopus* blastula (*38*). It is therefore possible that the nLfR in human infant small intestine is GPI anchored. A GPI anchor can be considered as an alternative to a hydrophobic transmembrane polypeptide anchor and may also be involved in functions such as transmembrane signaling (*39*) and endocytosis (*40*). A protein destined to be GPI anchored is translated with a cleavable N-terminal signal peptide that directs the growing polypeptide to the

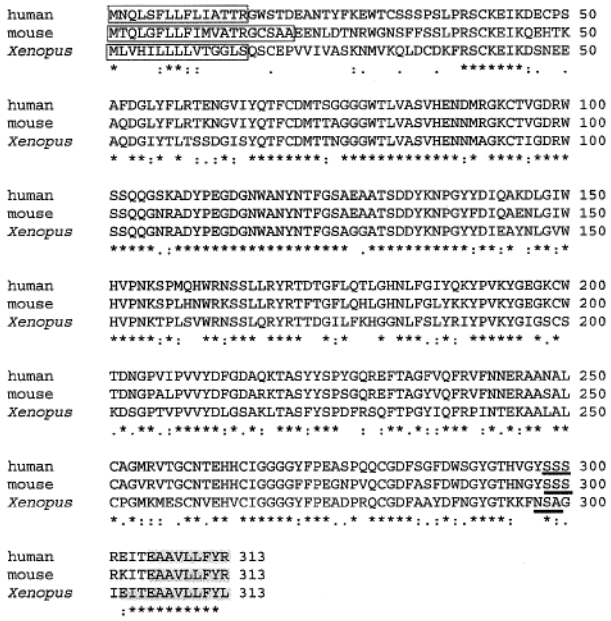

FIGURE 8: Alignment of the amino acid residues of the human LfR, mouse intelectin, and CGL in *Xenopus* oocyte. Identical or conserved residues in the three sequences are indicated by asterisks. Conserved substitutions are indicated with two dots. Semiconserved substitutions are indicated with one dot. Putative N-terminal signals are boxed. C-Terminal hydrophobic areas which would be GPI anchor additional signals are highlighted by gray shading. The potential cleavage site for a GPI anchor is indicated by an underline.

endoplasmic reticulum (ER) for cotranslational insertion into the ER membrane. The C-terminal region of such proteins is another signal sequence, which directs the addition of the preformed GPI anchor (*41*). The C-terminal signal sequence should consist of a predominantly hydrophobic region of 8−20 amino acids (*42*). This C-terminal spacer region is cleaved, and the GPI anchor is attached to the newly formed C-terminal site by a transamidation reaction (*43*, *44*). The GPI attachment site can be predicted in proprotein sequences by an automatic detection program for GPI-anchored proteins available on the World Wide Web. The deduced amino acid sequence of the LfR was annotated by this program and is shown in Figure 8. There is an N-terminal signal and a C-terminal hydrophobic region. There is a potential cleavage site at residue 298, suggesting that the LfR may be GPI -anchored. Our attempt to cleave GPI-anchored proteins by PI-PLC treatment of Caco-2 cells successfully demonstrates that the LfR on Caco-2 cells is GPI anchored. These findings are likely to explain how the LfR is located on the apical membrane in human infant intestine.

In the transfection study, the relative expression level of protein was much lower than that of mRNA, and the amount of hLf bound was considerably lower than what we had anticipated on the basis of the LfR mRNA and LfR protein quantitations for these two cell types. These results suggest that the LfR mRNA overexpressed in Caco-2 cells is not readily translated to functional receptor and/or that the translated LfR protein is not readily localized to the cell membrane. However, we cannot exclude the possibility that hLf binds with similar affinity to some component on mock-transfected cells other than the LfR.

Bacterial LfRs have been previously characterized (*45*−*47*). There is no significant homology between the human LfR we have cloned and these bacterial LfRs. However, the bacterial LfR is thought to be responsible for iron acquisition by bacteria, and our ^{59}Fe transport experiment with LfR-transfected Caco-2 cells showed that the human LfR mediates Lf-bound ^{59}Fe uptake into cells. It will therefore be interesting to compare the binding specificity of both types of LfRs and the mechanisms giving them the capacity to take up iron.

In conclusion, the cloning and functional expression of the LfR performed in this study provide a valuable source for future studies to elucidate the mechanisms behind the LfR-mediated function of hLf in human infant intestine and, possibly, in other tissues.

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