Isolation and Partial Characterization of a Lactotransferrin Receptor from Mouse Intestinal Brush Border†

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ABSTRACT: Several lines of evidence have recently suggested the occurrence of a specific lactotransferrin receptor in the small intestinal brush-border membrane in several animal species, which is thought to be involved in lactotransferrin-mediated intestinal iron absorption. We report here for the first time the isolation and partial characterization of this receptor from mouse intestinal brush border. The receptor has been purified to homogeneity by affinity chromatography on an immobilized human lactotransferrin column. The purified receptor was found to be active in that it binds iron-free and iron-saturated lactotransferrin with a K_d of 0.1 μM. Anti-receptor antibodies were prepared, and the receptor was further isolated by immunoaffinity chromatography in higher yield but in a denatured form. The purified receptor was revealed by sodium dodecyl sulfate–polyacrylamide electrophoresis to be a protein of about M_r = 130000, consisting of a single polypeptide chain. The isoelectric point was determined to be 5.8. The receptor was further shown to bear concanavalin A and phytohemagglutinin L binding glycans. Digestion by *N*-glycanase and endo-*N*-acetyl-β-D-glucosaminidase B led to a decrease of M_r = 25000, while the endo-*N*-acetyl-β-D-glucosaminidase H was uneffective, suggesting that the lactotransferrin receptor is mainly glycosylated by bi- and triantennary glycans. To gain further insight into the interaction of the receptor with lactotransferrin, namely, the number of ligand molecules bound per molecule of receptor, mouse lactotransferrin was cross-linked to its membrane-bound enterocyte receptor by use of radiolabeled sulfosuccinimidyl 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionate (SASD). A labeled complex of M_r = 200000 was obtained that, upon reduction by 2-mercaptoethanol, generated a labeled polypeptide of M_r = 130000, suggesting that one molecule of receptor binds only one molecule of mouse lactotransferrin. These results, which show that the main physicochemical properties of the lactotransferrin receptor diverge from those of transferrin receptor, are consistent with the different location and the biological significance of the two receptors.

Lactotransferrins (or lactoferrins) are iron-binding glycoproteins present in external secretions (Masson et al., 1966a; Mason & Taylor, 1978), particularly abundant in human and mouse milk (Montreuil & Mullet, 1960; Montreuil et al., 1960; Kinkade et al., 1971). Human and mouse lactotransferrins share a great deal of homologies that extend over the amino acid sequence (Metz-Boutigue et al., 1984; Pentecost & Teng, 1987) and the structure of their glycans (Spik et al., 1982b; Leclercq et al., 1987). The biological significance of human lactotransferrin has been investigated. It is thought to contribute to the inhibition of bacterial growth by a mechanism of iron deprivation (Montreuil & Mullet, 1960; Montreuil et al., 1960; Masson et al., 1966b; Bullen & Armstrong, 1979) and to the regulation of myelopoiesis (Broxmeyer et al., 1980). Stimulation by human lactotransferrin of thymidine incorporation into DNA of lymphocytic cell lines (Hashizume et al., 1983), of mitogen-stimulated peripheral blood lymphocytes (Mazurier et al., 1989), and of rat crypt cells (Nichols et al., 1987) has also been reported. Recently, a growing body of interest has been paid to its role in the intestinal iron absorption as postulated earlier (Montreuil & Mullet, 1960; Montreuil et al., 1960). Our long-term interests have been in the intestinal iron absorption and its regulation, particularly in the cellular mechanism of iron uptake from the lumen involving lactotransferrin. Uptake of lactotransferrin-bound iron by human duodenal biopsies was first demonstrated by an in vitro assay (Cox et al., 1979), and the survival of lactotransferrin in the digestive gut of human newborns was highlighted (Spik et al., 1982a). Following these findings, the presence of a lactotransferrin receptor was located in the rabbit small intestinal brush border by ligand blotting (Mazurier et al., 1985). In an attempt to fully understand the mechanism of cellular regulation of iron absorption, we have pursued our study using mouse intestine as a model. Previously, we demonstrated (Hu et al., 1988) that lactotransferrin binds to a mouse brush-border membrane receptor following a saturable and specific pattern and that the binding is dependent on pH and Ca^{2+} ions. The mouse lactotransferrin receptor shows no species specificity in that it binds equally well mouse, human, or bovine lactotransferrin. The Triton X-100 solubilized receptor retains the main binding properties of its membrane-bound counterpart (Hu et al., 1988).

In the present paper, we describe the purification of the receptor by ligand affinity chromatography on an immobilized human lactotransferrin column and by immunoaffinity chromatography on an immobilized anti-lactotransferrin receptor IgG column and the partial physicochemical characterization of the receptor. The interaction of mouse lactotransferrin with

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its receptor has also been studied by means of cross-linking to determine the number of lactotransferrin molecules bound to the receptor.

Experimental Procedures

Materials. Aprotinin, diisopropyl fluorophosphate (DFP),[1] phenylmethanesulfonyl fluoride (PMSF), pepstatin A, leupeptin, dithiothreitol (DTT), *o*-phenanthroline, Triton X-100, octyl β-D-glucoside, poly(vinylpyrrolidinone) 40, silver nitrate reagent, phytohemagglutinin L, concanavalin A, and hemocyanin were provided by Sigma (St. Louis, MO). Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril), sulfosuccinimidyl 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionate (SASD), and Reacti-Gel 6X were obtained from Pierce (Beijerland, The Netherlands). $Na^{125}I$ was from Amersham International (Amersham, Bucks, U.K.), the nitrocellulose membrane BA85 from Schleicher Schüll (Dassel, FRG), and microconcentrator Centricon 10 from Amicon (Lexington, MA). Peptide:*N*-glycosidase F (*N*-glycanase) was purchased from Genzyme Corp. (Boston, MA) and endo-*N*-acetyl-β-D-glucosaminidase H (Endo H; EC 3.2.1.96) from Boehringer (Mannheim, FRG). Endo-*N*-acetyl-β-D-glucosaminidase B (Endo B) isolated from *Basidiomyces sporotricum dimorphosporum* (Bouquelet et al., 1981) was a kind gift from Dr. S. Bouquelet (Université des Sciences et Techniques de Lille Flandres-Artois). Mouse lactotransferrin prepared according to the method of Sawatzki and Kubanek (1983) was a generous gift from Dr. Sawatzki (Milupa, Friedrichsdorf, FRG), and human lactotransferrin was purified to homogeneity from human milk by the procedure of Spik et al. (1982b). Both lactotransferrins were iron-saturated and iron-desaturated as described by Mazurier et al. (1983). Iron-free human serotransferrin was from Berhingwerke (Marburg, FRG). Molecular mass markers, isoelectric point markers, and protein A–Sepharose and Sephadex G-25 PD-10 columns were from Pharmacia Fine Chemicals (Uppsala, Sweden). All other reagents were of analytical grade.

Protein Iodination. Iodination of soluble proteins was carried out at 4 °C for 10 min by using Iodogen as a catalyst in PBS according to the manufacturer's instructions; 100 μCi was generally used to label 1 mg of protein. The unbound iodine was removed by gel filtration on a Sephadex G-25 PD-10 column equilibrated with the buffer of use; the proportion of free ^{125}I in the ^{125}I-labeled lactotransferrin fraction was never more than 5% as indicated by the amount of free ^{125}I in the trichloroacetic acid supernatant. Iodinated proteins were used within 1 week. The procedure for ^{125}I labeling of membrane proteins was identical with that for soluble proteins, unless unbound iodine was washed away by repeated membrane suspension–sedimentation.

Preparation and Solubilization of Mouse Intestinal Brush-Border Membrane Vesicles. The procedure for the preparation of mouse small intestinal brush-border membranes was that of Kessler et al. (1978). Prior to solubilization, brush-border membrane vesicles (10 mg/mL in PBS) were first treated by 1 mM DFP on ice for 15 min to inactivate proteinases and then by 10 mM EDTA and 50 mM Na_2CO_3, pH 11.5, on ice for 15 min to eliminate extrinsic proteins. Membrane proteins were then solubilized in Triton X-100 as described earlier (Hu et al., 1988).

Receptor Purification by Ligand Affinity Column. Iron-saturated human lactotransferrin (500 mg) was coupled to 50 mL of Reacti-Gel 6X according to the instructions of the manufacturer. Solubilized membranes were subjected to a centrifugation at 120000*g* for 30 min, and the resulting supernatant (about 500 mL containing 4 g of membrane proteins with 1 mg previously ^{125}I-labeled) was supplemented in 10 mM $CaCl_2$ and then mixed with 40 mL of the hLTF–Reacti-Gel preequilibrated with 10 mM Mes/NaOH, pH 5.5, 150 mM NaCl, and 0.1% Triton X-100. This mixture was incubated for 3 h at 4 °C under rotatory stirring. At the end of the incubation period, the gel suspension was packed into a 4 × 15 cm column at 4 °C and then washed with 100 mL of 10 mM Mes/NaOH, pH 5.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100 containing 1 mM PMSF. The receptor was eluted with 50 mL of 10 mM Mes/Tris, pH 9.5, 150 mM NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100 containing the mixture of proteinase inhibitors (1 mM PMSF, DTT, and *o*-phenanthroline and 50 μg/mL aprotinin, leupeptin, and pepstatin A). The pooled eluate was adjusted to pH 5.5 with 1 N HCl and then mixed with 5 mL of hLTF–Reacti-Gel. After 1 h of incubation at 4 °C under gentle rotatory agitation, the gel suspension was packed into a 2.5 × 10 cm column and then washed repeatedly in succession with 10 mL each of buffer A (10 mM Mes/NaOH, pH 5.5, 300 mM NaCl, 10 mM $CaCl_2$, 2 M urea, 0.1% Triton X-100) and buffer B (10 mM Mes/NaOH, pH 5.5, 50 mM NaCl, 10 mM $CaCl_2$, 40 mM octyl β-D-glucoside). When the radioactivity count washed out of the column dropped to less than 50 cpm/mL, the bound receptor was eluted with 10 mL of buffer C (10 mM Tris-HCl, pH 9.5, 150 mM NaCl, 5 mM EDTA, 40 mM octyl β-D-glucoside, containing the mixture of proteinase inhibitors). The elution was monitored by absorbance at 280 nm. The receptor fractions were pooled, and the pH was adjusted to 5.5 by a concentrated glycine solution. Then the receptor fractions were concentrated by microconcentrator Centricon 10.

Receptor Purification by Immunoaffinity Chromatography. A portion of the ligand affinity chromatography purified receptor (100 μg) was coupled to 100 μg of hemocyanin with 10 mM glutaraldehyde by an incubation at 37 °C for 2 h in 1 mL of 50 mM sodium phosphate and 150 mM NaCl, pH 9.0; 1 mL of buffered 200 mM glycine solution was added for another 2-h incubation to block all unreacted aldehyde groups. The solution was then mixed with 2 mL of Freund's incomplete adjuvant; 2 mL of this mixture was injected subcutaneously into a rabbit according to the procedure of Vaitukaitis et al. (1971). The remaining 2 mL was frozen and reinjected intramuscularly at days 30 and 40. Serum was collected 14 days later. An IgG fraction of this antiserum was prepared by protein A–Sepharose chromatography according to the manufacturer's directions. Purified IgG were immobilized to Reacti-Gel at a concentration of 10 mg/mL gel according to the manufacturer's directions. Triton X-100 solubilized membrane proteins were mixed with immunoabsorbant at 4 °C for 3 h under rotatory stirring, and the gel suspension was then packed into a column. The column was then washed extensively with 100 mL of PBS containing 1 M NaCl and 1% Triton X-100 followed by 20 mL of PBS containing 40 mM octyl β-D-glucoside. The receptor was eluted with 5 mL of 50 mM glycine hydrochloride, pH 2.8, containing 40 mM octyl β-D-glucoside. The receptor fractions were pooled and concentrated by microconcentrator Centricon 10.

[1] Abbreviations: PBS, phosphate-buffered saline containing 20 mM sodium phosphate and 150 mM NaCl (pH 7.4); PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropyl fluorophosphate; PVP-40, poly(vinylpyrrolidinone) 40; DTT, dithiothreitol; ASD, 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionic acid; SASD, sulfosuccinimidyl 3-[[2-(*p*-azidosalicylamido)ethyl]dithio]propionate; hLTF, human lactotransferrin; mLTF, mouse lactotransferrin; ConA, concanavalin A; PHA-L, phytohemagglutinin L.

Receptor Binding Assay. Lactotransferrin binding activity of the receptor in Triton X-100 extracts or in the purified receptor was determined by a dot-spot binding assay as described earlier (Hu et al., 1988). Briefly, samples were dotted onto a nitrocellulose membrane, and then the membrane strips were incubated with 1 μg/mL or increasing concentrations of ^{125}I-labeled iron-free or iron-saturated mLTF in 10 mM Mes/NaOH, 150 mM NaCl, 10 mM $CaCl_2$, and 0.1% Triton X-100 for a period of 1 h and then washed with the same buffer without labeled protein. Nonspecific binding was determined in the presence of 100-fold molar excess of unlabeled lactotransferrin. The membrane strips were then cut and applied to radiolabel counting in a LKB compugamma counter.

Polyacrylamide Gel Electrophoresis and Western Blotting. SDS–PAGE was carried out in accordance with the procedure of Laemmli (1970) on 5–10% or 5–15% discontinuous 1.5 mm thick gradient gels with 4% stacking gel. Sample buffer was added to the samples to a final concentration of 62.5 mM Tris-HCl, pH 6.8, 3% SDS, 10% glycerol, and 0.02% Bromophenyl Blue, with or without 0.1 M 2-mercaptoethanol, and was then boiled for 5 min. Electrophoresis was carried out at 50-mA constant current mode at 20 °C. Proteins in the gel were stained either with Coomassie Blue R250 or with silver nitrate reagent (Morrissey, 1981).

Proteins separated by SDS–PAGE were electroblotted onto a nitrocellulose membrane in accordance with the procedure of Towbin et al. (1979). Electrotransfer was carried out at 80 V at 4 °C for 16 h. The Western blots were first incubated with 5% PVP-40 in PBS containing 1 M NaCl to saturate nonspecific binding sites on the nitrocellulose strips and then exposed to 3×10^6 cpm of labeled lectins in 20 mL of the same buffer for 16 h followed by 5 × 10 min washings with 0.05% Tween 20 in PBS. After drying, the strips were autoradiographed on a film X-omat (Kodak) with an amplifier screen.

Isoelectric Focusing (IEF) of the Purified Lactotransferrin Receptor. Isoelectric focusing of the immunoaffinity chromatography purified receptor was carried out in a Pharmacia Phastsystem electrophoresis apparatus. The precast Pharmacia Phastsystem IEF 4–6.5 gel was dried at 40 °C overnight and then was allowed to swell with 300 μL of 40 mM octyl β-D-glucoside. The purified ^{125}I-labeled lactotransferrin receptor was run with the calibrated isoelectric focusing standards (pH 2.5–6.5). To allow the proteins to reach their p*I* point, an additional 10 min per run was programmed into the method. Gels were stained with Phastblue after overnight prefixation in 30% trichloroacetic acid. The position of the receptor was localized by autoradiography.

Endoglycosidase Digestion of Purified Lactotransferrin Receptor. Samples of immunoaffinity chromatography purified ^{125}I-labeled receptor (10^4 cpm) were denatured by boiling for 5 min with 0.5% SDS and 0.1 M 2-mercaptoethanol and incubated with 0.1 unit of *N*-glycanase or endo-*N*-acetyl-β-D-glucosaminidase H at 37 °C for 16 h in an appropriate buffer according to the manufacturer's recommendations and with 0.2 unit of endo-*N*-acetyl-β-D-glucosaminidase B for 16 h according to the method of Bouquelet et al. (1981). After incubation, the samples were analyzed directly by SDS–PAGE. Control samples without addition of endoglycosidase were incubated in parallel.

Cross-Linking of mLTF to Brush-Border Receptor. ^{125}I-Labeling of SASD was carried out as described by Shephard et al. (1988). Briefly, 100 μg of SASD was dissolved in 50 μL of dimethyl sulfoxide followed by addition of 200 μL of 200 mM sodium phosphate, pH 7.4. This solution was then placed into a glass test tube coated with 100 μg of Iodogen according to the manufacturer's instruction, followed by addition of 100 μCi of Na^{125}I. Reaction was terminated after 15 min by removal of the solution from the reaction tube. ^{125}I-Labeled SASD was then immediately coupled to 100 μg of mouse lactotransferrin in 100 μL of 500 mM sodium borate, pH 9.0, for 1 min at room temperature. Free iodine and unreacted ^{125}I-labeled SASD were separated from ^{125}I-labeled ASD–mLTF on a PD-10 Sephadex column equilibrated with PBS containing 10 mM octyl β-D-glucoside; 1 mg of mLTF was coupled with nonradiolabeled SASD. Prior to the incubation with the derived mLTF, 2 mg of mouse intestinal brush-border membranes was treated with 10 mM EDTA in 50 mM Na_2CO_3, pH 11.5, at 4 °C for 15 min. The membrane pellet was then washed with reaction buffer (10 mM Mes/NaOH, pH 5.5, 10 mM $CaCl_2$, 150 mM NaCl) and resuspended in 1 mL of the same buffer containing 10 mg/mL human serotransferrin in an attempt to reduce the nonspecific binding to the membrane. About 1 μg of ^{125}I-labeled ASD–mLTF was added to the membrane suspension, and the mixture was then divided into two parts, to one of which was added 25 μg of unlabeled mLTF. After 15 min of incubation at 4 °C, the membranes were pelleted at 100000*g* for 2 min in a Beckman airfuge. Immediately after the resuspension of pellets in 250 μL of reaction buffer, the membrane suspension in a quartz tube was placed into a Rayonet type RS photochemical reactor (1250 W, maximum emission at 254 nm, the SO New England Ultraviolet Co., Middletown, CT). After a 30-s photolysis, the membranes were washed twice with 10 mM EDTA and 50 mM $NaHCO_3$, pH 9, and once with distilled water. Membrane proteins were separated on 5–15% gradient SDS–PAGE under reducing or nonreducing conditions, and the gel was then subjected to autoradiography.

Results

Isolation of Lactotransferrin Receptor by Ligand Affinity Chromatography. The purification of active receptor was carried out by a three-step procedure. Purified brush-border membranes were first treated with 10 mM EDTA at a basic pH to eliminate extrinsic proteins prior to solubilization by Triton X-100. Receptor was enriched by a first chromatography on a hLTF–Reacti-Gel column. By this purification step, the volume of the receptor fraction was reduced. Final purification was achieved by a second chromatography on a hLTF–Reacti-Gel column. Nonspecifically retained proteins were eliminated by repeated washing with buffer A/0.1% Triton X-100 with a higher salt concentration to reduce ionic interactions and with buffer B/40 mM octyl β-D-glucoside/2 M urea in a lower salt concentration to reduce hydrophobic interactions. Bound receptor was eluted by passing through the column 10 mM EDTA at a basic pH. With the absorbance of octyl β-D-glucoside at UV wavelength negligible, the elution of the receptor was monitored by absorbance at 280 nm (Figure 1). By this three-step procedure, the receptor was purified to apparent homogeneity. The evaluation of the purification is summarized in Table I. About 110 μg of receptor was obtained from the starting material of 1000 mice, with a yield of about 1.2%. This low yield may be due to the relatively low ligand–receptor affinity. Purified receptor was active in regard to its capacity to bind iron-free and iron-saturated ^{125}I-labeled mLTF. Scatchard plot analysis of the binding data (Figure 2) obtained by the dot-spot binding assay reveals the affinity of the iron-saturated ^{125}I-labeled mLTF for the Triton X-100 extract (0.101 μM ± 0.065) and that for the purified receptor (0.104 μM ± 0.065) were similar, demonstrating that the purification procedure we used was not denaturing. Moreover, iron-saturated ^{125}I-labeled mLTF and

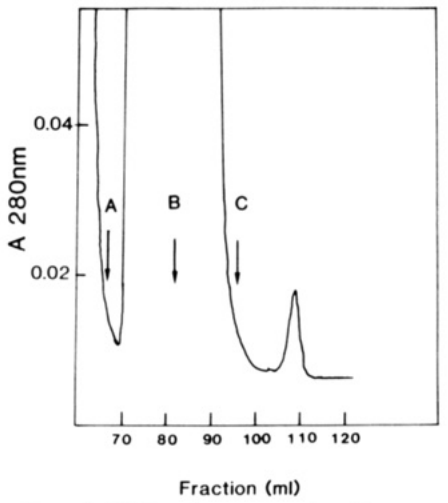

FIGURE 1: Ligand affinity chromatography of mouse enterocyte lactotransferrin receptor. The isolation procedure was outlined under Materials and Methods. Mouse brush-border membranes were solubilized with Triton X-100 and passed through a hLTF–Reacti-Gel column. Eluted proteins were again charged on a second hLTF–Reacti-Gel column. The column was extensively washed with buffer A containing Triton X-100 and with buffer B containing octyl β-D-glucoside. Final elution of receptor was realized with buffer C containing 5 mM EDTA.

Table I: Summary of Lactotransferrin Receptor Purification from Mouse Intestinal Brush-Border Vesicles

purification stage	total protein (mg)	sp act. (ng of mLTF/μg)	cumulative yield (%)
brush-border preparation	4000	1.3	100
EDTA pellets	1900	2.7	98
first hLTF–Reacti-Gel eluate	19	140	46
second hLTF–Reacti-Gel eluate	0.11	600	1.2

iron-free ^{125}I-labeled mLTF possessed close binding affinity either for the Triton X-100 extract or for the purified receptor.

Isolation of the Receptor by Immunoaffinity Chromatography. By immunoaffinity chromatography on immobilized mouse antilactotransferrin receptor IgG column, we purified the receptor to homogeneity with a recovery of about 7% of the receptor protein. The immunoaffinity chromatography purified receptor was visualized after SDS–PAGE as a single-band protein that exhibited the same mobility as the ligand affinity chromatography purified receptor. Moreover, the two receptor preparations were revealed after SDS–PAGE and electroblotting by the antiserum against the ligand affinity purified receptor. From these results, it was concluded that the receptors purified by using the two kinds of affinity chromatography possess identical physicochemical and immunological properties. However, the immunoaffinity chromatography purified receptor was found to be inactive; probably the receptor was labile to acid pH, which was the condition used to elute receptor from the immunoabsorbent column. As in our subsequent studies the receptor activity was not concerned, and the immunoaffinity chromatography was retained for receptor purification.

Characterization of the Protein Moiety of the Lactotransferrin Receptor. To estimate the molecular mass of the receptor, 2 μg of receptor preparation was submitted to electrophoresis on a 5–15% polyacrylamide gel under both reducing and nonreducing conditions. As can be seen in Figure 3, under nonreducing conditions, the receptor migrated as a single band of M_r = 130 000 (lane B). Under reducing conditions, no additional band was generated (lane C), indicating

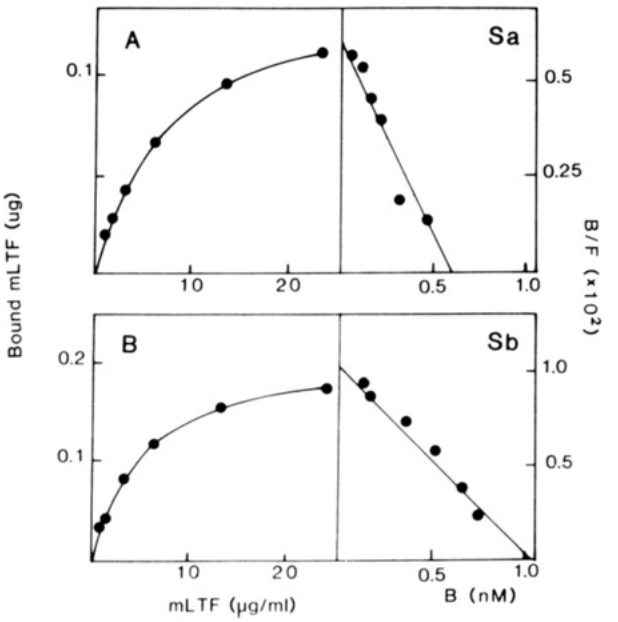

FIGURE 2: Binding assay of Triton X-100 extract (curve A) and ligand affinity isolated lactotransferrin receptor (curve B). 5-μL samples of Triton X-100 extract or receptor preparation were spotted onto nitrocellulose strips and incubated subsequently with increasing concentrations of ^{125}I-labeled mLTF. After washing, retained radioactivity was measured. Binding of ^{125}I-labeled mLTF to nitrocellulose strips of equal size but without sample was measured in parallel and extracted from the total binding. Data were analyzed by Scatchard plot analysis (Scatchard, 1949). (Inserts Sa and Sb) Scatchard plot of Triton X-100 extract and ligand affinity isolated lactotransferrin receptor, respectively.

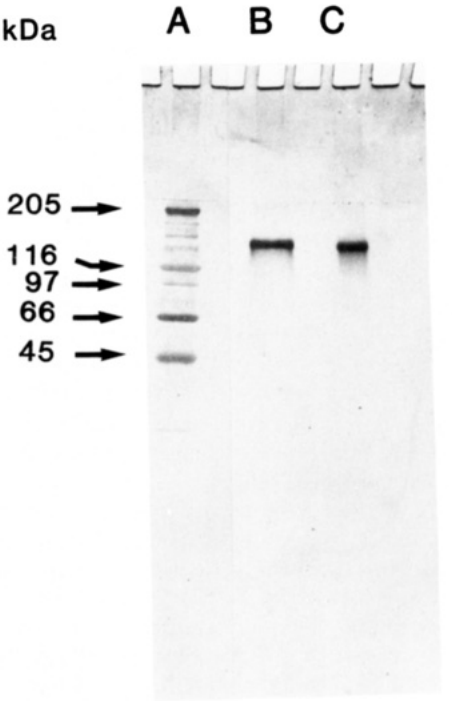

FIGURE 3: Electrophoresis analysis of isolated lactotransferrin receptor. Isolated receptor was run on a 5–15% gradient polyacrylamide gel under nonreducing (B) or reducing (C) conditions and silver-stained according to the method of Morrissey (1981). The molecular mass standard markers used were (lane A) myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), and ovalbumin (45 kDa).

that the receptor molecules consist of a single polypeptide chain.

The Phastsystem is a recently developed apparatus designed for fast and convenient electrophoresis and isoelectric focusing

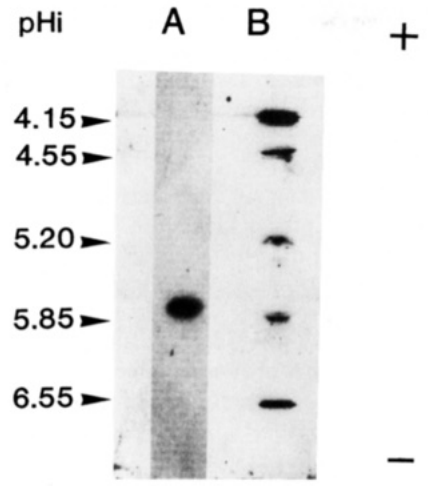

FIGURE 4: Isoelectric focusing of isolated mouse enterocyte lactotransferrin receptor. ^{125}I-Labeled receptor was isoelectrofocalized in a Phastgel IEF 4–6 gel dried and swollen in 40 mM octyl β-D-glucoside. The position of receptor was revealed by autoradiography. The p*I* standard markers used were glucose oxidase (p*I* = 4.15), soybean trypsin inhibitor (p*I* = 4.55), β-lactoglobulin A (p*I* = 5.20), bovine carbonic anhydrase B (p*I* = 5.85), and human carbonic anhydrase B (p*I* = 6.55).

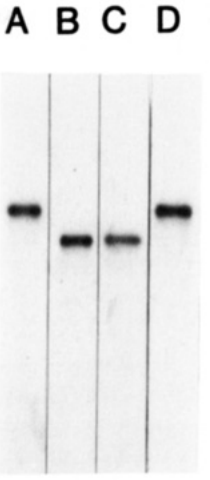

FIGURE 5: Endoglycosidase and *N*-glycanase action on mouse enterocyte lactotransferrin receptor. ^{125}I-Labeled SDS denatured lactotransferrin receptor was analyzed by SDS–PAGE before digestion (lane A) and after digestion by *N*-glycanase (lane B), endo-*N*-acetyl-β-glucosaminidase B (lane C) and endo-*N*-acetyl-β-glucosaminidase H (lane D).

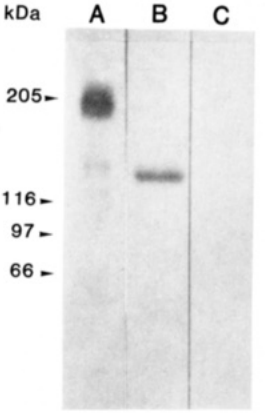

FIGURE 6: Autoradiogram of the binding pattern of mLTF cross-linking to mouse enterocyte receptor. Assays were undertaken as outlined under Materials and Methods in which mouse brush-border membranes were incubated with 1 μg/mL ^{125}I-labeled ASD–mLTF, without hLTF (lanes A and B) or with 100 μg/mL of hLTF (lane C), and then exposed to UV light for 30 s. The resulting material was analyzed by 5–10% gradient SDS–PAGE under nonreducing (lanes A and C) or reducing (lane B) conditions.

experiments. However, the precast gels are suitable only for soluble proteins. We therefore modified the precast gels by including the nonionic detergent octyl β-D-glucoside in the gel to extend the usage of the precast gels to membrane proteins. As the detergent interferes with the staining, an overnight prefixation of the gel in a large volume of 30% trichloroacetic acid after isoelectric focusing is necessary to reduce the background staining. As shown in Figure 4, the receptor was focused as a single band of p*I* = 5.8 (lane A), as deduced from the calibration standards migration (lane B).

Characterization of the Glycan Moiety of the Lactotransferrin Receptor. The glycoprotein nature of the receptor was determined by its reaction with lectins. Purified receptor and membrane proteins were incubated with ^{125}I-labeled ConA and PHA-L after SDS–PAGE and Western blotting onto nitrocellulose sheets. In these conditions, the receptor was recognized by both lectins. According to the specificity of these two lectins (Kornfeld & Ferris, 1975; Debray et al., 1981; Cummings & Cornfeld, 1982), the receptor seems to possess at least one biantennary glycan of the *N*-acetyllactosaminic or oligomannosidic type and one tri- or tetraantennary glycan of the *N*-acetyllactosaminic type. To gain further insight into the glycan moiety of the receptor, we treated the purified receptor with endoglycosidases. As can be seen in Figure 5, the digestion by endo-*N*-acetyl-β-D-glucosaminidase H was without any effect on the electrophoretic migration (lane D), indicating most probably the absence of almost all oligomannosidic type glycans in the lactotransferrin receptor. In contrast, digestion by *N*-glycanase (lane B), which is known to split all N-linked oligosaccharides examined to date including bi-, tri-, and tetraantennary glycans fucosylated or not (Tarentino et al., 1985), and digestion by endo-*N*-acetyl-β-D-glucosaminidase B (lane C), which is able to cleave monoasialo and asialo biantennary glycans and to a lesser extent asialo triantennary glycan (Bouquelet et al., 1981), resulted in a decrease of 25 kDa in apparent molecular mass. These results underline the importance of the contribution of bi- and/or triantennary *N*-acetyllactosaminic type glycans to the M_r of the lactotransferrin receptor. The exact number of glycans could not be deduced from the obtained results; however, taking into account an average of 2.2 kDa for a biantennary glycan, this number, if only biantennary glycans are present, should be about 12.

Number of mLTF Bound to the Receptor. We used a heterobifunctional, iodinable, photoreactive cross-linking agent, SASD, in an attempt to cross-link mLTF to its membrane-bound enterocyte receptor to define the number of lactotransferrin molecules bound to the receptor. SASD was first ^{125}I-labeled at the 2-(*p*-azidosalicylamido) moiety by the Iodogen method. The product, ^{125}I-labeled SASD, was then incubated with mLTF. To couple the mLTF to its receptor, ^{125}I-labeled ASD–mLTF was incubated with the mouse intestinal brush-border membrane preparation. Following this incubation, cross-linking of the bound mLTF to its receptor, through photolysis of the azide bond, was initiated by exposing the membrane to UV light. The membranes were then washed and prepared for SDS–PAGE. The autoradiogram (Figure 6) shows that under reducing conditions a sharp band is seen at M_r = 130 000 (lane B), which represents the receptor, whereas under nonreducing conditions a broad band at M_r = 200 000 is seen (lane A), which is unlikely to be the polymers of lactotransferrin (control data not shown) and theoretically represents the mLTF–receptor complex. This band is not seen

when a 100-fold excess of unlabeled mLTF (lane C) is included with ^{125}I-labeled ASD–mLTF in the incubation medium, suggesting that the binding of ^{125}I-labeled ASD–mLTF to its receptor is specific and therefore susceptible to competitive inhibition. As can be seen in lane A, we also detect a minor band of approximately 150 000 molecular mass. This may represent a membrane protein adjacent to the receptor. No other band having a molecular mass higher than 200 000 is detectable in lane A, indicating that the receptor can bind only one molecule of mLTF.

Discussion

The cellular mechanism of intestinal iron absorption remains still unknown, particularly that of the uptake of iron across the enterocyte brush-border membrane. Several iron binders have been described in the apical membrane (O'Donnel & Cox, 1980; Stremmel et al., 1987). Both these iron binders and the fatty acids (Simpson & Peters, 1987) in brush-border membranes have been thought to act as the iron carrier across this membrane. A transferrin-mediated intestinal iron absorption has been previously postulated on the basis of a tied-off loop experiment (Huebers et al., 1983). However, recent observations have suggested that such a mechanism seems unlikely to be at work in enterocytes. The main challenges are about the origin of the so-called "mucosal" transferrin (Schümann et al., 1986; Idzerda et al., 1986) and the occurrence of the transferrin receptor on the brush border (Parmley et al., 1985; Banerjee et al., 1986). Moreover, several lines of evidence have suggested the iron transporter role of lactotransferrin as it was postulated by Montreuil et al. in 1960. In fact, the bioavailability of human milk iron is unusually high, and the incidence of iron deficiency in breast-fed infants is extremely low (Saarinen et al., 1977), coincident with the much higher lactotransferrin content in human milk as compared with cow's milk. Subsequent evidence in human or animal newborns has shown that lactotransferrin does resist to a large extent the gastrointestinal proteolytic degradation and survives in human and animal newborns (Spik et al., 1982a; Lönnerdal 1985). Recently, Kawakami et al. (1988) have provided evidence showing that in mice the lactotransferrin-bound iron has higher bioavailability than ferrous iron salts. The observation that the mucosa surface is covered with lactotransferrin (Masson et al., 1966a; Mason & Taylor, 1978; Isobe et al., 1979; Tedeschi et al., 1987) and that lactotransferrin-bound iron can be absorbed by human duodenum biopsies in a specific and saturable pattern (Cox et al., 1979) has extended the early notion of the iron nutritional role of lactotransferrin to intestinal iron absorption in general. In addition to these physiological observations, a specific receptor has been evidenced on the small intestinal brush border of several animal species such as rabbits, monkeys, and mice (Mazurier et al., 1985; Davidson & Lönnerdal, 1988; Hu et al., 1988). This receptor could be one of several possible candidates that participate in the internalization of iron into the mucosa cells. The mucosal surface lactotransferrin has been shown to derive from goblet cells (Lorimier, 1987). This locally secreted lactotransferrin could be responsible for the basic level of iron absorption. The pancreatic or bile lactotransferrin content, which has been shown to increase in iron deficiency (van Vugt et al., 1975), could take over the increased need for iron.

An important approach in the elucidation of the mechanism of action of a ligand is the identification, isolation, and characterization of the specific cellular receptor with which the ligand initially interacts. The first lactotransferrin receptor that was isolated corresponds to the lactotransferrin receptor purified by anti-ligand affinity chromatography from the Triton X-100 soluble extract of mitogen-stimulated blood peripheral lymphocytes (Mazurier et al., 1989). This receptor was visualized as two protein bands of 100 000 and 110 000 molecular mass. The present study reports the isolation and some basic properties of the mouse enterocyte lactotransferrin receptor. Our work demonstrated that the mouse enterocyte lactotransferrin receptor binds to iron-free or iron-saturated mouse lactotransferrin with similar affinity. The receptor is a highly glycosylated, single-chain polypeptide of M_r = 130 000 and pI = 5.8. The receptor was found to be recognized by ConA and PHA-L and susceptible to treatment with *N*-glycanase and endo-*N*-acetyl-β-D-glucosaminidase B, suggesting the presence of glycans of the bi- and/or triantennary *N*-acetyllactosaminic type.

Although there exist species variations, lactotransferrin receptors seem to be a class of proteins different from transferrin receptor, at least when the subunits and the molecular mass are concerned. Transferrin receptor is an integral membrane glycoprotein having a molecular mass of 180 000. The receptor consists of two 90-kDa subunits linked by two disulfide bridges (Seligman et al., 1979; Wada et al., 1979; Schneider et al., 1982). The human receptor is reported to have three N-linked oligosaccharides (Omary & Trowbridge, 1981b; Schneider et al., 1982), a covalently bound fatty acid (Omary & Trowbridge, 1981a,b), and a phosphoserine residue on the cytoplasmic domain near the NH_2 terminus (Rothenberger et al., 1987). The presence of these two last components has not yet been assessed in the mouse lactotransferrin intestinal brush-border receptor.

It is worth noting that in spite of a pronounced structural similarity between the two molecules, the function of lactotransferrin markedly deviates from that of serotransferrin on several accounts. In fact, the functional specificity of the two glycoproteins is underlined by the differences that exist in the distribution of their receptors on target cells. Transferrin receptors are available on a large variety of eukaryotic cells (Hamilton et al., 1979; Newman et al., 1982), while lactotransferrin seems to interact with specific cells such as macrophages (Van Snick & Masson, 1976; Birgens et al., 1983; Goavec et al., 1985), monocytes (Markowetz et al., 1979), lymphocytes (Hashizume et al., 1983; Mazurier et al., 1989), and enterocytes (Mazurier et al., 1985; Davidson & Lönnerdal, 1988; Hu et al., 1988).

The cellular mechanism by which the lactotransferrin receptor allows the intake of lactotransferrin-bound iron by the enterocytes is now under investigation. A clear understanding of the events triggered by lactotransferrin binding would undoubtedly lead to a better knowledge of intestinal iron absorption and its regulation.

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