Isolated Rat Hepatocytes Bind Lactoferrins by the RHL-1 Subunit of the Asialoglycoprotein Receptor in a Galactose-Independent Manner[†]

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Received December 16, 1996; Revised Manuscript Received April 10, 1997[⊗]

ABSTRACT: Isolated rat hepatocytes bind and internalize the iron-binding protein lactoferrin (Lf) by a set of high-affinity, recycling, Ca^{2+}-dependent binding sites. We have purified a 45-kDa membrane protein (p45) from rat hepatocytes that exhibits Ca^{2+}-dependent receptor activity. In this study, we found p45 to be identical to the major subunit (RHL-1) of the rat asialoglycoprotein receptor. Two tryptic fragments of p45 showed 100% identity with RHL-1 internal sequences ($Leu^{121} \rightarrow Lys^{126}$ and $Phe^{198} \rightarrow Lys^{220}$), and monospecific antisera against p45 and RHL-1 cross-reacted equally well with each protein. Molar excesses of anti-p45 IgG, anti-RHL-1 IgG, asialoorosomucoid, and asialofetuin competitively blocked the binding of ^{125}I-Lf to isolated rat hepatocytes at 4 °C. Similarly, either excess anti-p45 or Lf blocked the binding of ^{125}I-asialoorosomucoid to cells at 4 °C. We did not detect the minor subunits of the rat asialoglycoprotein receptor (RHL-2/3) in p45 preparations from Triton X-100 extracts of hepatocytes and ^{125}I-Lf bound to purified RHL-1 but not to RHL-2/3 immobilized on nitrocellulose. Nonetheless, anti-RHL-2/3 IgG reduced the binding of ^{125}I-Lf to hepatocytes at 4 °C. Exoglycosidases were used to remove terminally-exposed *N*-acetylneuraminyl, α- and *β*-galactosyl, and *N*-acetylhexosaminyl sugars from human and bovine Lf glycans, and lectin blotting confirmed that glycosidase-treated Lfs lacked detectable terminal galactosyl sugars. Unexpectedly, these deglycosylated Lfs exhibited no loss in their ability to compete with unmodified Lfs for binding to isolated hepatocytes. In addition, molar excess of *β*-lactose but not sucrose competitively blocked the binding of ^{125}I-Lf to cells, indicating that Lf bound at or very near the carbohydrate-recognition domain of RHL-1. We conclude that RHL-1 is the Ca^{2+}-dependent Lf receptor on hepatocytes and that it binds Lf at its carbohydrate-recognition domain yet in a galactose-independent manner.

Lactoferrin (Lf)[1] is a 78-kDa non-heme iron-binding glycoprotein that reversibly binds two metal atoms per protein molecule (Baker et al., 1987). It is present in virtually all exocrine fluids and in neutrophil specific granules (Levay & Viljoen, 1995). Lfs exhibit diverse metal-dependent and -independent functions and can reside in multiple extracellular and intracellular compartments (Brock, 1995; Lonnerdal & Iyer, 1995). Following its release from neutrophils, Lf is removed from the blood by the liver and catabolized while its bound iron is recovered and retained in parenchymal and non-parenchymal cells (Bennett & Kokocinski, 1979; Courtoy et al., 1984; Regoeczi et al., 1985, 1994).

We reported previously that isolated rat hepatocytes bind and internalize bovine Lf by Ca^{2+}-dependent high-affinity sites that recycle during Lf endocytosis (McAbee et al., 1993). By these sites, hepatocytes internalize apo- and holo-Lf at a high rate and degrade Lf protein but retain Lf-delivered iron (McAbee, 1995). We also found that loading primary cultures of rat hepatocytes with iron reversibly increases the number of Ca^{2+}-dependent Lf binding sites by a mechanism that requires translation but not transcription (McAbee & Ling, 1997). The identification and characterization of the hepatocyte Ca^{2+}-dependent Lf receptor, therefore, is crucial for an understanding of hepatic metabolism of Lf. To this end, we isolated a 45-kDa membrane protein (p45) from rat hepatocytes which binds bovine Lf specifically and in a Ca^{2+}-dependent manner (Bennatt & McAbee, 1997). Anti-p45 antibodies block the binding and endocytosis of Lf by isolated hepatocytes, indicating that p45 functions as the Ca^{2+}-dependent hepatic Lf receptor. In this study, we provide evidence that p45 is identical with the RHL-1 subunit of the ASGP-R. p45 shares amino acid sequence homology and immunocrossreactivity with RHL-1. Also, Lf and desialylated glycoproteins competed with each other for binding to hepatocytes, and antibodies against RHL-1 and p45 blocked the interaction of p45 with cells. Finally, we show that removal of terminal NeuNAc and Gal by exoglycosidase treatment of bovine and human Lfs did not alter Lf's ability to bind to p45/RHL-1 on hepatocytes, suggesting the unexpected and novel conclusion that Lfs bind to this ASGP-R subunit in a Gal-independent manner.

EXPERIMENTAL PROCEDURES

Materials and Buffers. Acrylamide, AF, bis(acrylamide), BSA, neuraminidase (*C. perfringens*, Type X), α-galactosi-

[†] This work was supported by National Institutes of Health Grant DK-43355.

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[⊗] Abstract published in *Advance ACS Abstracts,* July 1, 1997.

[1] Abbreviations: AF, asialofetuin; ASGP-R, asialoglycoprotein receptor; ASOR, asialoorosomucoid; BME, basal medium Eagle's; BSA, bovine serum albumin; EGTA: ethylene glycol bis(*β*-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, HBS: HEPES-buffered saline; Lf, lactoferrin; NeuNAc, *N*-acetylneuraminic acid; RCA, *Ricinis communis* agglutinin; RHL, rat hepatic lectin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SNA, *Sambucus nigra* agglutinin; TBS, Tris-buffered saline; WGA, *Triticum vulgaris* agglutinin.

S0006-2960(96)03079-6 CCC: $14.00

dase (EC 3.2.1.22, green coffee bean), β-*N*-acetylglucosaminidase (EC 3.2.1.30, jack bean), dextran sulfate (5000 Da), EGTA, HEPES, Lfs (bovine colostrum, bovine milk, human milk), Nonidet-P40, Tween-20, and Triton X-100 were purchased from Sigma (St. Louis, MO). Iodogen was obtained from Pierce (Rockford, IL). β-Galactosidase (EC 3.2.1.23, bovine testis), RCA digoxigenin-labeled lectin, WGA digoxigenin-labeled lectin, SNA digoxigenin-labeled lectin, anti-digoxigenin IgG−alkaline phosphatase conjugate, alkaline phosphatase substrate reagent kit, and collagenase (Type D) were from Boehringer Mannheim (Indianapolis, IN). $Na^{125}I$ (17 Ci/mg of iodine) was from DuPont NEN (Boston, MA). PMSF, sodium deoxycholate, and digitonin were obtained from Eastman Kodak (Rochester, NY). Ammonium persulfate, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, goat-anti-rabbit alkaline phosphatase conjugate and substrate developer were from BioRad (Richmond, CA). ASOR—desialylated with neuraminidase as described elsewhere (Schachter et al., 1970)—anti-RHL-1, and anti-RHL-2/3 sera were generous gifts of Dr. P. H. Weigel (University of Oklahoma Health Sciences Center). Centricon microconcentrators were purchased from Amicon (Beverly, MA). BME, obtained from Sigma, was supplemented with 2.4 g/L HEPES, pH 7.4, and 0.22 g/L $NaCO_3$. BME-BSA is BME containing 0.1% (w/v) BSA. HBS contained 150 mM NaCl, 3 mM KCl, and 10 mM HEPES, pH 7.4. Buffer A contained HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Buffer B contained buffer A supplemented with 0.2% (w/v) dextran sulfate. Buffer C contained buffer A with 1% (v/v) Triton X-100. Buffer D contained HBS supplemented with 5 mM EGTA. TBS-Tween contained 10 mM Tris-HCl, pH 7.8, 0.5 M NaCl, and 0.2% (v/v) Tween-20. HBS-BSA contains HBS supplemented with 0.1% (w/v) BSA.

Hepatocyte Preparation. Male Sprague−Dawley rats (100−350 g; Harlan Labs, Indianapolis, IN) were fed standard laboratory chow and water *ad libitum*. Hepatocytes were prepared by a modification of a collagenase perfusion procedure (McAbee & Esbensen, 1991; Seglen, 1973). Cells were kept at approximately 30 ° C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME-BSA were ≥85% viable and single cells. Before experiments, cell suspensions ($2-4 \times 10^6$ cells/mL in BME-BSA, 10% of the flask volume) were incubated at 37 °C for 60 min to allow recovery from the isolation procedure. Cell viability was determined microscopically by trypan blue exclusion.

Lf Preparation. Bovine colostrum Lf (commercial preparation >90% pure electrophoretically) at 5 mg/mL in 100 mM KCl and 10 mM KH_2PO_4, pH 7.2, was further purified by ion-exchange chromatography on diethylaminoethane-cellulose (McAbee & Esbensen, 1991). Commercial preparations of bovine milk Lf and human milk Lf (>95% electrophoretically homogeneous) were dissolved at 5 mg/mL in HBS. Lfs were dialyzed against HBS, filter-sterilized (0.2 μm), and stored at −20 °C before use. Lfs were 30−70% iron-saturated as determined by 460:280 nm absorbance ratio (Hashizume et al., 1987). ^{125}I-bovine Lf—prepared in Iodogen-coated tubes—had specific activities of 6−101 dpm/fmol. Homogeneity of radiolabeled and unlabeled Lfs was confirmed by SDS−PAGE.

Lf-Hepatocyte Binding and Endocytosis Assays. Hepatocytes (2×10^6 cells/mL) were incubated with 1−2 μg of ^{125}I-Lf/mL on ice for 90 min in buffer A or with 5 μg of ^{125}I-Lf /mL at 37 °C ≤ 60 min in BME-BSA. Determination of non-specific ^{125}I-Lf binding was performed by including a 50−100-fold molar excess of unlabeled Lf in the binding mixture. Various competitive ligands, sera, or IgG from pre-immune, non-immune, or immune rabbits were included in the reaction mixtures as indicated in the figure legends. Washed cells were resuspended into buffer B, transferred to clean tubes, and assayed for cell-associated radioactivity.

p45 Purification and NH_2-Amino Acid Analysis. Lf-agarose and ethanolamine-agarose were prepared using CNBr-activitated resin as described previously (Bennatt & McAbee, 1997). Digitonin-permeabilized hepatocytes (20×10^6/mL, $4-10 \times 10^8$ cells total) were solubilized in buffer C for 30 min at 4 °C. Detergent-insoluble material was sedimented (15000*g*, 4 °C, 20 min), and the supernatants were clarified by filtration (0.2 μm). Cell extract was dosed periodically with PMSF (final concentration 0.1 mM) throughout the preparation. Membrane extract was pre-adsorbed on ethanolamine-agarose and then chromatographed on Lf-agarose. The Lf-agarose was washed with 10 column volumes of buffer B and eluted with buffer D. Pre-column membrane extract and purified p45 were stored at −20 °C prior to use. For amino acid sequencing, purified p45 was subjected to SDS−PAGE and transferred via electroblotting onto polyvinylidene difluoride membrane (Schleicher & Schuell, Keene, NH). The membrane was washed extensively in H_2O, stained in 0.2% Ponceau-S for 1 min, and washed in H_2O. Tryptic fragmentation and NH_2-terminal amino acid sequencing of isolated fragments was performed at the Worcester Foundation for Experimental Biology Microchemistry Facility (Worcester, MA).

RHL Purification by AF-Agarose Chromatography. RHL subunits were purified by AF-agarose affinity chromatography (Ray & Weigel, 1985). Briefly, AF-agarose was prepared at a density of 5 mg of AF/mL of resin. Digitonin-permeabilized hepatocytes (2×10^7/mL, $4-10 \times 10^8$ cells total) were solubilized in 20 mM $CaCl_2$, 0.4 M KCl, 1% Trition X-100, and 10 mM Tris-HCl, pH 7.8, and applied to the AF-agarose column. The column was washed with 5−8 bed volumes of 50 mM $CaCl_2$, 0.5 M KCl, 0.5% Triton X-100, and 10 mM Tris-HCl, pH 7.8, and AF-agarose bound polypeptides were eluted with 0.4 M KCl, 0.5% Triton X-100, and 40 mM ammonium acetate, pH 5.5. Eluted fractions were stored at −20 °C prior to use.

Exoglycosidase Treatment of Lfs. Lfs (~5 mg/mL) were treated with 0.5 U of neuraminidase in 50 mM KCl and 10 mM KH_2PO_4, pH 6.0, supplemented with 1 mM $CaCl_2$, for 16 h at 37 °C to remove terminal NeuNAc-(α2,6). Neuraminidase-treated Lfs were incubated with 0.1 U of β-galactosidase in 0.1 M sodium citrate phosphate, pH 4.3, for 16 h at 37 °C to remove terminal Gal from Gal-(β1,3)-GlcNAc, Gal-(β1,4)-GlcNAc, and Gal-(β1,4)-GalNAc linkages. β-galactosidase-treated bovine colostrum Lf was also treated with 0.5 U of α-galactosidase in 50 mM KCl and 10 mM KH_2PO_4, pH 6.0, for 16 h at 22 °C to remove terminal Gal-(α1,*n*)-*x*, and subsequently with 0.5 U of β-*N*-acetylglucosaminidase in 0.1 M sodium citrate phosphate, pH 5.0, for 16 h at 22 °C to remove terminal GalNac-(β1,*n*)-*x* and GlcNac-(β1,*n*)-*x* sugars. Following each glycosidase treatment, Lfs were repurified by chromatography on diethylaminoethane-cellulose in 0.1 M KCl and 10 mM NaH_2PO_4, pH 7.2, and concentrated by ultrafiltration (Centricon 10 000 Da cutoff). Mock-treated Lfs were subjected to the same

procedures without added glycosidase. The presence of glycan-terminal NeuNAc-(α2,6)-*x*, Gal-(β1,*n*)-*x*, and GlcNAc-(β1,*n*)-*x* were determined by blotting of electrophoresed Lfs with digoxigenin-labeled lectins specific for these sugars.

Electrophoresis and Western, Lectin, and ^{125}I-Lf Ligand Blotting. Samples for electrophoresis were denatured in 63 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.01% (w/v) Bromophenol Blue, heated for 5 min at 60 °C and electrophoresed at 200 V on a 7.5% acrylamide discontinuous Tris-HCl slab gel containing 0.1% SDS using a Mini-Protean II slab gel apparatus (BioRad). Polypeptides were visualized by fixation staining in 0.25% (w/v) Coomassie Brilliant Blue R250, 45% (v/v) methanol, and 10% (v/v) acetic acid. M_r of various polypeptides were determined relative to Sigma or BioRad prestained electrophoresis standards. In some cases, polypeptides fractioned by SDS−PAGE were electroblotted onto nylon-supported nitrocellulose (BioRad) in 0.15 M glycine, 20 mM Tris, and 20% (v/v) methanol at 200 mA for 45 min. Western blotting was performed using a modified procedure of Burnette (1981). Blocking and antibody incubation buffers contained 0.5 M NaCl, 20 mM Tris-HCl, pH 7.8, and 5% (w/v) BSA. Nitrocellulose wash buffer contained 0.5 M NaCl, 20 mM Tris, pH 7.8, 0.1% (w/v) Nonidet-P40, 0.1% (w/v) SDS, and 0.25% (w/v) sodium deoxycholate. Nitrocellulose-bound rabbit IgG were detected with goat anti-rabbit IgG−alkaline phosphatase conjugate and an AP conjugate substrate kit (BioRad). Carbohydrates were detected using a DIG glycan differentiation kit (Boehringer Mannheim) according to the manufacture's instructions. Digoxigenin-labeled lectins used included SNA to detect NeuNAc-(α2,6)-*x*, RCA to detect Gal-(β1,*n*)-*x*, and WGA to detect GlcNAc-(β1,*n*)-*x*. For ligand blotting studies, purified RHL was electrophoresed on 4−15% acrylamide slab gels under non-reducing conditions followed by electrophoretic transfer to nitrocellulose. Lanes containing molecular weight standards and RHL were stained with 0.1% (w/v) amidoschwartz 10B (Serva, New York, NY) in 7% (v/v) acetic acid. Lanes with immobilized RHL were incubated overnight at 4 °C in HBS supplemented with 20 mM $CaCl_2$, 5% (w/v) BSA, and 0.2% (v/v) Tween-20. Nitrocellulose was then incubated with ^{125}I-Lf (5 μg/mL; 101 dpm/fmol) in HBS, 5 mM $CaCl_2$, 0.2% (v/v) Tween-20, 5% (w/v) BSA for 2 h at room temperature after which the papers were washed three times, 10 min per wash, in 25 mL of HBS, 5 mM $CaCl_2$, , 0.02% (v/v) Tween-20. ^{125}I-Lf was detected by exposure of papers to a phosphor screen and developed on a Molecular Dynamics Storm 840 image analyzer (Sunnyvale, CA).

General. Protein was determined by the bicinchoninic acid protein assay procedure using BSA as standard (Pierce). Centrifugation of cell suspensions was at 400*g* for 2 min at 4 °C (Sorvall RT6000B centrifuge; DuPont Co., Wilmington, DE). ^{125}I radioactivity was determined using a Packard Cobra Auto-Gamma counting system (Model 5002; Packard Instrument Co., Downers Grove, IL). Spectroscopic measurements of Lfs were done using a Beckman DU-64 spectrophotometer (Fullerton, CA).

RESULTS

Identification of p45 as RHL-1. In the companion study to this report, we purified a 45-kDa membrane polypeptide (p45) from rat hepatocytes that mediated the Ca^{2+}-dependent interaction of bovine Lf with hepatocytes (Bennatt & McAbee, 1997). To determine the novelty of p45, we electrophoretically purified p45 by SDS−PAGE followed by limited trypsinization and automated NH_2-terminal sequencing of multiple tryptic fragments. Analysis of two fragments gave the sequences LLLHVK and FVQQHMGPLNTWIGLTDQNGPWK, which upon Blast search of the NCBI data base showed 100% identity with Leu^{121} → Lys^{126} and Phe^{198} → Lys^{220}, respectively, of the RHL-1 subunit of the ASGP-R (Leung et al., 1985). RHL-1 is a 42 kDa type II membrane protein that is the major component of the rat ASGP-R which consists of three subunits (RHL 1, RHL-2, RHL-3) isolated from rat liver by ligand affinity chromatography (Drickamer et al., 1984). ASGP-Rs bind specifically to Gal- and GalNAc-terminated glycoconjugates (Ashwell & Harford, 1982; Hardy et al., 1985). Our p45 sequences coincided with portions of the domain positioned between the "neck" and carbohydrate-recognition domains of RHL-1's extracellular region (Iobst & Drickamer, 1996; Ruiz & Drickamer, 1996).

If RHL-1 functions as the Ca^{2+}-dependent Lf receptor, then ligands specific for RHL-1 should compete with Lf for binding to intact hepatocytes. We assayed hepatocytes for their ability to bind ^{125}I-Lf at 4 °C in the presence or absence of molar excess of ASOR, a desialylated form of orosomucoid possessing multiple Gal-terminated, triantennary Asn-linked *N*-acetyllactosaminyl complex glycan chains (Pricer & Ashwell, 1971). We found that a 50−150-fold molar excess of ASOR reduced ^{125}I-Lf binding to the Ca^{2+}-dependent Lf binding sites >90%, comparable to the reduction observed with a 50-fold molar excess of unlabeled Lf (87%) (Figure 1A). A 50-fold molar excess of AF−another high-affinity ligand for the ASGP-R−also reduced ^{125}I-Lf binding to cells by >60% (Figure 1B). In other experiments, we found that a 50-fold molar excess of Lf reduced ^{125}I-ASOR binding to hepatocytes by ∼40% (Figure 1C). It is likely that the differences in competitive binding activity of Lf as compared to ASOR reflect Lf's ∼20−50-fold higher K_d values for binding isolated rat hepatocytes (Hardy et al., 1985; McAbee & Esbensen, 1991).

We then examined p45 and RHL subunits for immunocrossreactivity. p45 was purified by Lf-agarose affinity chromatography as described previously (Bennatt & McAbee, 1997). Routinely, 4−8 times more RHL-1 was purified by ligand-affinity chromatography than RHL-2 and RHL-3, a ratio that generally reflects the RHL subunit content of rat hepatocytes (Halberg et al., 1987; Herzig & Weigel, 1990; Sawyer et al., 1988). p45 and RHL subunits were then probed for immunoreactivity using mono-specific antibodies against p45, RHL-1 (carbohydrate-recognition domain), and RHL-2/3. We found that both anti-p45 and anti-RHL-1 antibodies each detected p45 and RHL-1 in an identical manner (Figure 2B, compare lanes 1 and 2 with 3 and 4). In each case, a dominant 45-kDa polypeptide was detected as well as a band at ∼90 kDa; we have shown elsewhere that the 90-kDa form is a disulfide-bonded dimer of the 45-kDa species that is generated during SDS−PAGE (Bennatt & McAbee, 1997). Antibodies specific for RHL-2/3 detected these proteins in the fractions eluted from AF-agarose (Figure 2B, lane 6), but RHL-2/3 were not detected in fractions eluted from Lf-agarose (Figure 2B, lane 5). Taken together, these data suggest that Lf readily binds RHL-1 but may not bind to RHL-2/3. To address this more directly, we examined

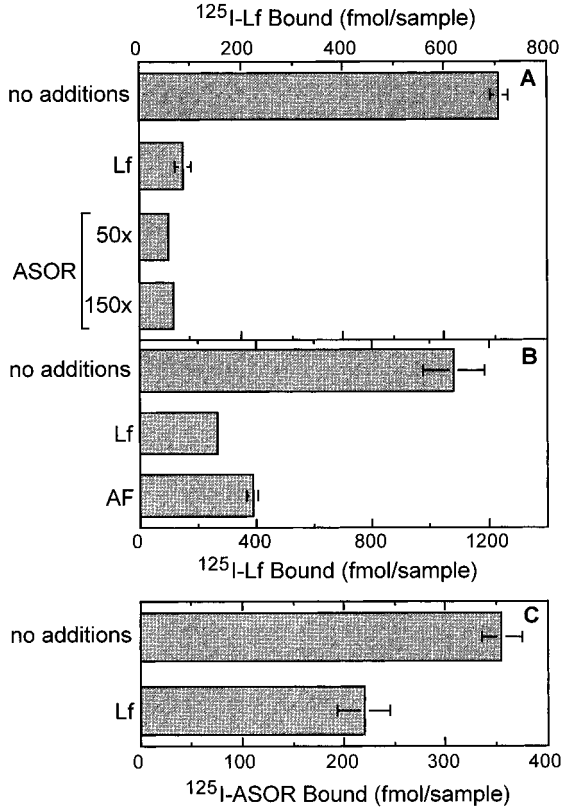

FIGURE 1: Competition binding of Lf, ASOR, and AF to isolated rat hepatocytes. Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (2 μg/mL) or ^{125}I-ASOR (1 μg/mL) for 90 min at 4 °C in buffer A in the absence (no additions) or presence of a 50-fold molar excess of unlabeled Lf (Lf, a 50- and 150-fold molar excess of ASOR (A), or a 50-fold molar excess of AF (B). Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in. Values are means of duplicate samples; error bars reflect standard deviation from the mean. Experiments shown were performed with different preparations of hepatocytes.

the ability of ^{125}I-Lf to bind to purified RHL subunits immobilized on nitrocellulose. Affinity-purified RHL was subjected to SDS−PAGE, transferred to nitrocellulose, and blocked with BSA in the presence of Ca^{2+} and Tween-20 overnight. Such treatment has been shown to renature the ligand-binding activity of RHL subunits following denaturing electrophoresis (Zeng et al., 1996). We found that immobilized RHL-1, but not RHL-2/3, bound ^{125}I-Lf (Figure 2C, lane 2), consistent with the conclusion that RHL-1 alone interacts with Lf.

To provide further confirmation that p45 is identical to RHL-1 and responsible for Lf binding to hepatocytes, we examined the ability of antibodies against p45, RHL-1, and RHL-2/3 to block the binding of ^{125}I-Lf and ^{125}I-ASOR to isolated rat hepatocytes. We found that 12.5 molar excess of anti-p45 IgG, but not non-immune IgG, reduced ^{125}I-ASOR binding by 43% (Figure 3A). Likewise, a 12.5-fold molar excess of anti-p45 IgG and anti-RHL-1 IgG reduced ^{125}I-Lf binding to cells by 62 and 54%, respectively (Figure 3B). Unexpectedly, we found that excess anti-RHL-2/3 also reduced ^{125}I-Lf binding to cells by 62% (Figure 3B), suggesting that RHL-2/3 subunits are in sufficient proximity to p45/RHL-1 on hepatocyte surfaces that antibodies against these proteins blocked Lf's interaction with p45/RHL-1. We conclude from all the above data that p45 and RHL-1 polypeptides are identical and that RHL-1 of the ASGP-R constitutes the Ca^{2+}-dependent Lf receptor on rat hepatocytes.

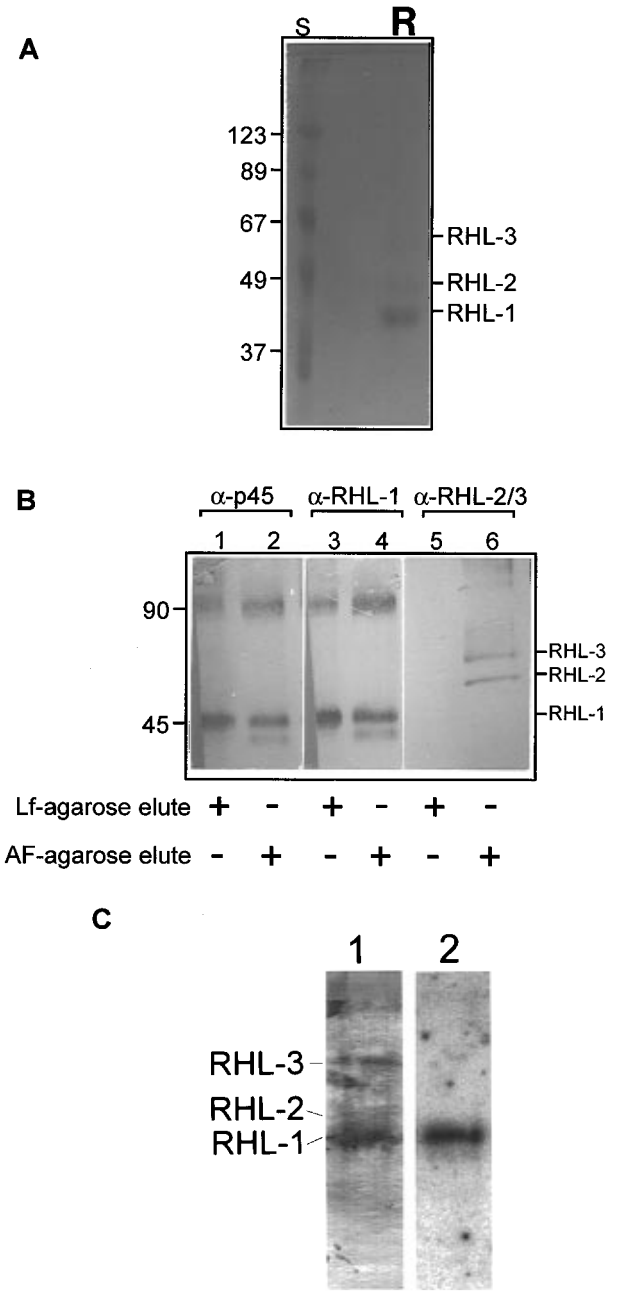

FIGURE 2: Purification of RHL subunits and immunocrossreactivity of p45, RHL-1 and RHL-2/3. (A) ASGP-R subunits were purified from isolated rat hepatocytes by ligand affinity chromatography on AF-agarose as described in Experimental Procedures. AF-agarose-bound material was eluted at pH 5.5 in the absence of Ca^{2+} and analyzed for polypeptide composition by SDS−PAGE (lane R). Lane S shows migration of molecular weight markers. (B) p45 and ASGP-R were purified as described in Experimental Procedures and subjected to SDS−PAGE. Fractioned p45 (Lf-agarose elute, lanes 1, 3, and 5) and ASGP-R (AF-agarose elute, lanes 2, 4, and 6) were transferred to nitrocellulose and probed with 1:200 dilutions of rabbit antisera against p45 (α-p45, lanes 1 and 2), RHL-1 (α-RHL-1; lanes 3 and 4), or RHL-2/3 (α-RHL-2/3; lanes 5 and 6). Bound IgG was detected using a goat anti-rabbit IgG−alkaline phosphatase conjugate (1:3000 dilution) and AP substrate kit (BioRad). (C) Affinity-purified ASGP-R were subjected to SDS−PAGE, transferred to nitrocellulose, and incubated with ^{125}I-Lf as described in Experimental Procedures. Bound ^{125}I-Lf was detected by a phosphor-imaging system. Lane 1, Amidoschwartz-stained ASGP-R; lane 2, ASGP-R incubated with ^{125}I-Lf.

Role of Lf's Glycan Structure in its Interaction with p45/RHL-1. The ability of fucoidin to block hepatic uptake of Lf suggested that blood Lf was cleared from the circulation

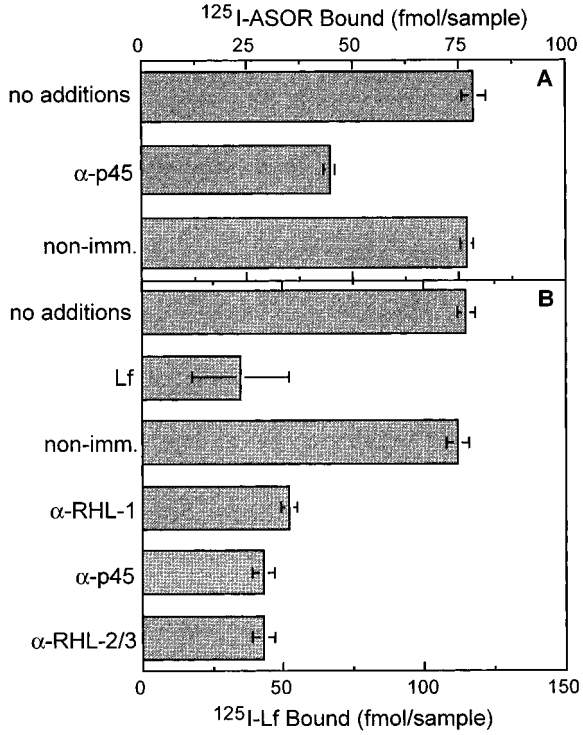

FIGURE 3: Ligand competition binding activity of anti-p45, anti-RHL-1, and anti-RHL-2/3 IgG. IgG was purified from non-immune rabbit sera and rabbit antisera raised against p45, RHL-1, or RHL-2/3 by affinity chromatography on protein A-agarose. (A) Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-ASOR (1 μg/mL) for 90 min at 4 °C in buffer A in the absence (no additions) or presence of 50 μg of either non-immune IgG (non-imm.) or anti-p45 IgG (α-p45). Cells were washed free of unbound ^{125}I-ASOR in cold buffer A and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent means of duplicate samples; error bars reflect standard deviations from the means. (B) Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (2 μg/mL) for 60 min at 4 °C in buffer A in the absence (no additions) or presence of 100 μg of unlabeled Lf (Lf), 20 μg of non-immune IgG (non-imm.), 20 μg of anti-RHL-1 IgG (α-RHL-1), 20 μg of anti-p45 IgG (α-p45), or 20 μg of anti-RHL-2/3 IgG (α-RHL-2/3). Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in Experimental Procedures. Values are means of duplicate samples; error bars reflect standard deviations from the means.

by its interaction with fucosyl receptors on liver parenchymal cells (Prieels et al., 1978). It was subsequently found, however, that fucoidin and Lf form tightly-bound complexes that interferes with Lf clearance and that defucosylated Lfs competed fully with native Lfs for clearance from rat and mouse circulation (Imber & Pizzo, 1983; Moguilevsky et al., 1984). Furthermore, it was learned that granulocyte-derived Lf—unlike milk Lf—is not fucosylated (Derisbourg et al., 1990), and modification of Lf protein by carbamylation reduced its clearance from the blood (Moguilevsky et al., 1984). In addition, ligands specific for various mammalian hepatic lectin systems, when co-injected with Lf, did not reduce Lf clearance from the circulation (Imber & Pizzo, 1983). In view of these findings, we investigated what role Lf's glycan chains played in its interaction with p45/RHL-1, a protein whose carbohydrate recognition properties have been well documented (Halberg et al., 1987; Iobst & Drickamer, 1996; Weis & Drickamer, 1996). Initially, we examined the ability of various Lfs possessing different glycan structures to compete with bovine colostral Lf for binding to isolated hepatocytes in a Ca^{2+}-dependent manner.

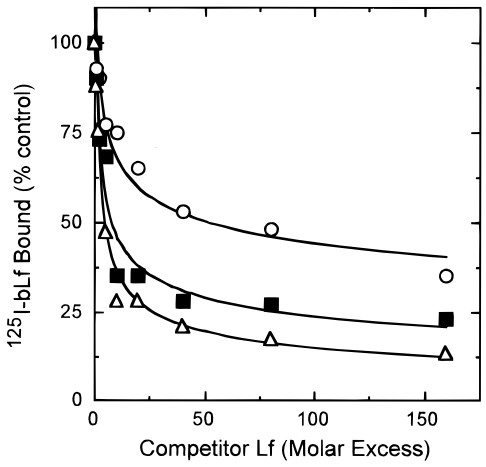

FIGURE 4: Competition binding activity assay for human Lf, bovine milk Lf, and bovine colostrum Lf. Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (bovine colostrum; 2 μg/mL) in buffer A for 90 min at 4 °C in the absence or presence of a molar excess of unlabeled bovine colostrum Lf (Δ), bovine milk Lf (■), or human milk Lf (○). Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent the percent of ^{125}I-Lf bound by cells in the absence of competitor Lf (% control).

Bovine Lfs contain four Asn-linked oligosaccharides in which at least two glycans are polymannosyl oligosaccharides and up to two glycans are *N*-acetyllactosaminyl oligosaccharides, the ratio of polymannosyl to *N*-acetyllactosaminyl-glycans depending on the cow's lactational stage (Coddeville et al., 1992). Lf from human milk possesses two glycan chains—one per each lobe of the protein—both of which are *N*-acetyllactosaminyl oligosaccharides (Spik et al., 1994, 1982). Notably, bovine and human Lfs are heterogeneous with regard to capping of *N*-acetyllactosaminyl glycans in that one arm of most biantennary structures terminate invariably in NeuNAc-(α2,6)−Gal-(β1,4) while the other arm terminates predominately in either NeuNAc-(α2,6)−Gal-(β1,4), Gal-(β1,4)−GlcNAc-(β1,2), or Gal-(α1,3)−Gal-(β1,4) (Coddeville et al., 1992; Spik et al., 1988). We found that Lfs from bovine and human milk competed with bovine colostral ^{125}I-Lf for binding to isolated rat hepatocytes at 4 °C (Figure 4). In this particular experiment, a 150-fold molar excess of human Lf reduced ^{125}I-Lf by >65% whereas a similar excess of bovine milk Lf reduced ^{125}I-Lf binding by ∼75%. By comparison, unlabeled bovine colostral Lf reduced ^{125}I-Lf binding by >80%. These data suggest that human and bovine Lfs bind to the same Lf receptors, but the affinities of these Lfs for cells may differ. These data also indicate that the presence of high-mannose oligosaccharides on bovine colostral Lf is not crucial for its interaction with hepatocytes because human milk Lf, which lacks high-mannose glycans, competed vigorously with bovine colostral ^{125}I-Lf for binding to cells.

In the next series of experiments, we modified the glycan structures of bovine colostral and human milk Lfs using various glycosidase treatments and examined the ability of modified Lfs to compete with native bovine colostral ^{125}I-Lf for binding to cells. Initially, bovine Lf was treated with endoglycosidase-F at 37 °C for ≤96 h, which removed only high-mannosyl oligosaccharides (as determined by lectin blotting using digoxigenin-labeled *Galanthus nivalis* agglutinin, which recognizes terminal mannose residues); such treatment did not alter Lfs ability to compete with native Lf

for binding to hepatocytes (data not shown). Human and bovine Lfs were then treated sequentially with neuraminidase and β-galactosidase to remove NeuNAc-(α2,6) and Gal-(β1,4) from the terminal positions of Lfs' *N*-acetyllactosaminyl oligosaccharides. We found that glycosidase-treated Lfs migrated slightly faster on SDS−PAGE gels than did mock-treated Lfs (Figure 5A, lanes 1 and 2), indicative of a loss of sugar moieties. That these sugars were actually removed from the Lfs was confirmed by lectin blotting using digoxigenin-labeled SNA lectin, which specifically recognizes terminal NeuNAc-(α2,6) (Figure 5A, lanes 3−4; Figure 5B, lanes 1−3) and digoxigenin-labeled RCA lectin, which specifically recognizes terminal Gal-(β1,4) (Figure 5A, lanes 5−6; Figure 5B, lanes 4−6). Importantly, Gal-(β1,4) on glycosidase-treated human Lf (Figure 5A) and bovine Lf (Figure 5B) was not detected by lectin blotting despite overloading the gel (5 μg of protein per lane). If Lfs bind p45/RHL-1 via their terminal β-Gal moieties, then the degalactosylated Lfs should not compete with native ^{125}I-Lf for binding to cells. We were surprised to find, however, that a 50-fold molar excess of degalactosylated human or bovine Lf reduced the binding of ^{125}I-Lf to cells by the same extent ($\geq$75%) as did mock-treated Lfs (Figure 5C).

Up to 25% of bovine Lfs possess biantennary glycans in which one of the antennae terminates in either Gal-(α1,3)−Gal-(β1,4) or NeuNAc-(α2,6)−GalNAc-(β1,4). In the latter instance, the penultimate GalNAc-(β1,4) would be exposed following neuraminidase treatment and potentially could mediate the interaction of degalactosylated Lfs with p45/RHL-1. It has also been reported that thioglycosides of α-Gal in neoglycoproteins are bound by the ASGP-R (Lee, 1989). To address these possibilities, bovine Lf was treated with neuraminidase, α-galactosidase, β-galactosidase, and *N*-acetylhexosaminidase and examined for the presence of terminal α-NeuNAc, β-Gal, and β-GlcNAc by lectin blotting (Figure 6A). Glycosidase-treated Lf lost all detectable terminal α-NeuNAc (Figure 6A, lane 4) and β-Gal (Figure 6A, lane 6) as compared to mock-treated Lf (Figure 6A, lanes 3 and 5). The amount of β-GlcNAc present on glycosidase-treated Lf was reduced slightly as compared to mock-treated Lf (Figure 6A, compare lanes 7 and 8), which was likely due to the resistance of inner core β-GlcNAc to β-*N*-acetylhexosaminidase treatment. We have found in other analyses that all Asn-linked oligosaccharides released from Lf by hydrazinolysis were fully susceptible to mannosidase treatment (data not shown), indicating that *N*-acetylhexosaminidase had indeed removed all terminal β-GalNAc and β-GlcNAc from Lf. We found that $\leq$80-fold molar excess of glycosidase- and mock-treated Lf competed vigorously with ^{125}I-Lf for binding to cells at 4 °C (Figure 6B). The removal of terminal Gal or GalNAc from a fraction of Lf molecules should result in a partial loss of competition binding activity in these Lf preparations, but we observed no differences in the competition isotherms between the two forms of Lf. In other experiments, we have found that RHL-1 can be purified on affinity columns derivatized with neuraminidase- and β-galactosidase-treated Lf (data not shown). We conclude from all these data, therefore, that p45/RHL-1 binds to bovine and human Lfs in a Ca^{2+}-dependent manner but by a mechanism independent of the presence of terminal Gal or GalNAc moieties on Lfs' glycan structures.

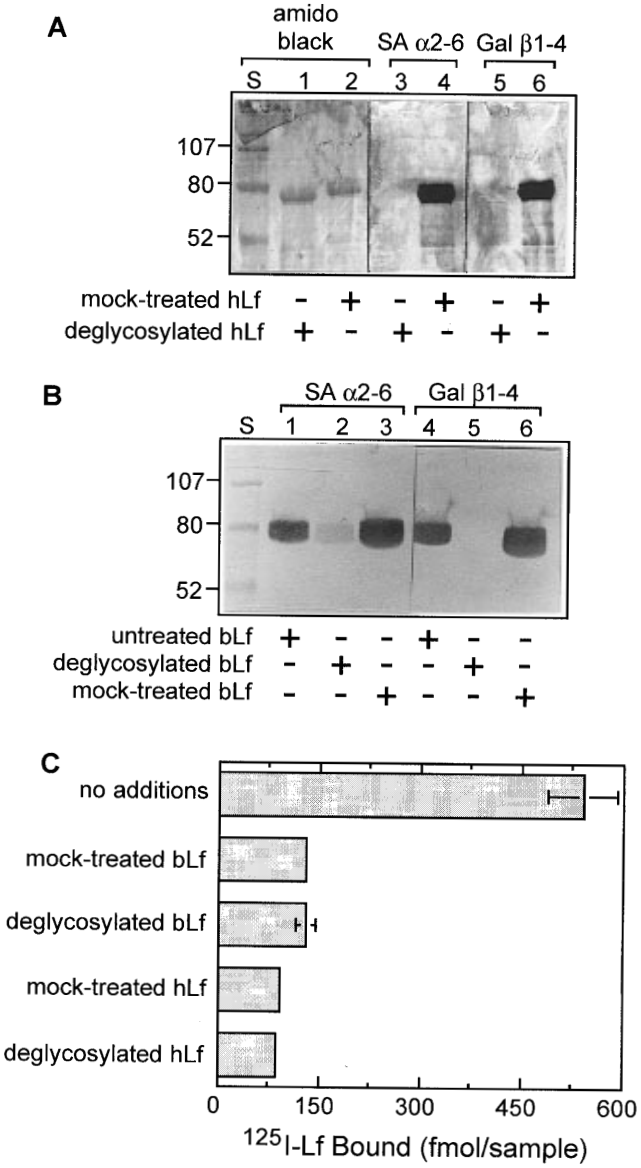

FIGURE 5: Deglycosylation and Ligand Competition Binding Activity Assay of Human and Bovine Lfs. (A) Human milk Lf was incubated at 37 °C sequentially in the absence (mock-treated Lf) or presence (deglycosylated Lf) of neuramindase and β-galactosidase as described in Experimental Procedures. Purified Lfs (5 μg/lane) were subjected to SDS−PAGE, electrophoretically-transferred to nitrocellulose, then either stained with amido black (lanes 1 and 2) or probed with digoxigenin-labeled SNA to detect terminal NeuNAc (SA α2−6; lanes 3 and 4) or digoxigenin-labeled RCA to detect terminal β-Gal (Gal β1−4; lanes 5 and 6). Digoxigenin-labeled lectins were detected with anti-digoxigenin IgG (Boehringer Mannheim). (B) Bovine colostrum Lf was deglycosylated sequentially with neuraminidase and β-galactosidase as in panel A. Equivalent amounts of untreated bovine colostrum Lf (untreated), deglycosylated Lf, and Lf incubated at 37 °C without glycosidases (mock-treated) were fractioned by SDS−PAGE and transferred to nitrocellulose. Lfs were probed with digoxigenin-labeled SNA and RCA lectins as described in panel A. (C) Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (2 μg/mL) for 90 min at 4 °C in buffer A in the absence (no additions) or presence of a 50-fold molar excess of either mock-treated or deglycosylated bovine or human Lf. Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in Experimental Procedures. Values are means of duplicate samples; error bars reflect standard deviations of means.

Although relatively large bulky protein ligands blocked Lf binding to ASGP-R, it is not clear from these data if Lf bound to the carbohydrate-recognition domain or some other

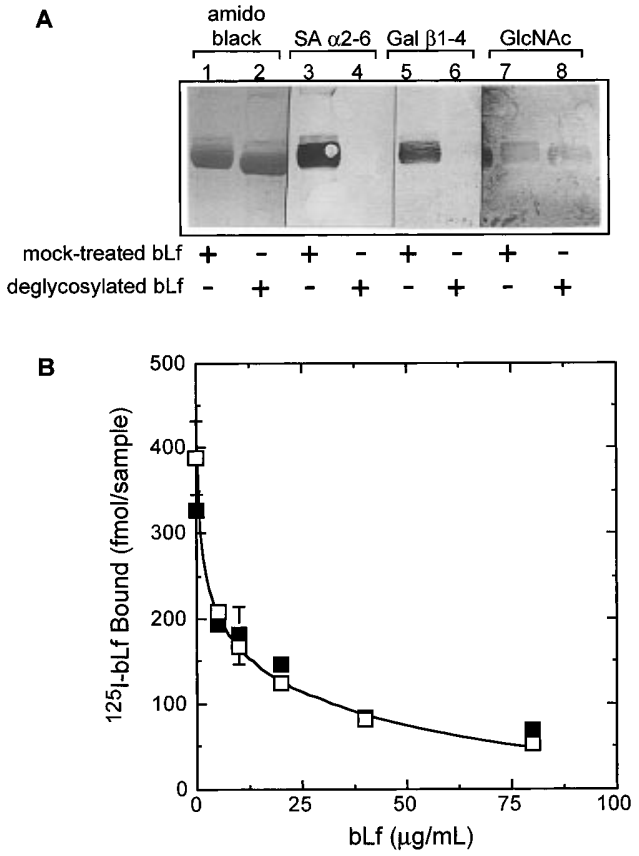

FIGURE 6: Deglycosylation of bovine colostrum Lf and titration of its ligand competition binding activity. (A) Bovine colostrum Lf was treated with neuraminidase, α-galactosidase, β-galactosidase, and *N*-acetylhexosaminidase as described in Experimental Procedures. Deglycosylated Lf and Lf treated similarly but without glycosidases (mock-treated) were subjected to SDS−PAGE and transferred to nitrocellulose. Fractioned Lfs were either stained with amido black (lanes 1 and 2) or probed with digoxigenin-labeled SNA to detect terminal NeuNAc (SA α2−6; lanes 3 and 4), digoxigenin-labeled RCA to detect terminal β-Gal (Gal β1−4; lanes 5 and 6), or digoxigenin-labeled WGA to detect β-GlcNAc (GlcNAc; lanes 7 and 8). Digoxigenin-labeled lectins were detected with anti-digoxigenin IgG (Boehringer Mannheim). (B) Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (bovine; 2 μg/mL) for 90 min at 4 °C in buffer A supplemented with the designated concentration of either mock-treated (□) or deglycosylated (■) Lfs analyzed in panel A. Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent means of duplicate samples; error bars reflect standard deviations of means.

site(s) on ASGP-R. Gal-terminated oligosaccharides alone—branched or monovalent—bind to ASGP-R via the receptor's carbohydrate-recognition domain and thereby block the interaction of Gal-terminated glycoproteins with hepatocytes (Lee et al., 1983; Weis & Drickamer, 1996). To address this issue, we examined the ability of β-lactose to block the interaction of ^{125}I-Lf and ^{125}I-ASOR to isolated rat hepatocytes. We found that, at large molar excesses, β-lactose progressively blocked hepatocyte interaction of ^{125}I-Lf by ≤77% (Figure 7A) and of ^{125}I-ASOR by ≤62% (Figure 7B). An equivalent amount of sucrose had little or no effect on the interaction of these ligands with the cells. Comparison of the β-lactose titration isotherms suggests that it was a more potent competitor for Lf binding than for ASOR binding. These findings strongly suggest that Lf interacts with the carbohydrate-recognition domain of ASGP-R.

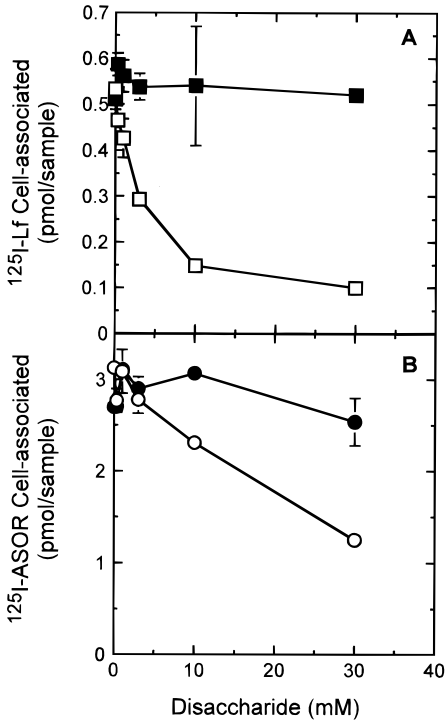

FIGURE 7: Effect of β-lactose and sucrose on Lf and ASOR Binding to hepatocytes. Hepatocytes (2×10^6/mL) were incubated at 4 °C with ^{125}I-Lf (2 μg/mL; panel A) or ^{125}I-ASOR (1 μg/mL; panel B) in the absence or presence or β-lactose (□, ○) or sucrose (■, ●) at the designated concentrations for 90 min. Cells were washed in cold buffer B and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent means of duplicate samples; error bars reflect standard deviations of means.

DISCUSSION

Isolated rat hepatocytes bind and internalize Lf by a set of high-affinity, Ca^{2+}-dependent recycling binding sites (McAbee & Esbensen, 1991; McAbee et al., 1993). In a companion study to this paper (Bennatt & McAbee, 1997), we identified and isolated a 45-kDa Ca^{2+}-dependent Lf-binding membrane protein (p45) from rat hepatocytes that functions as the Ca^{2+}-dependent hepatic Lf receptor. Two lines of evidence presented in this report argue strongly that p45 is identical to the RHL-1 subunit of the ASGP-R. First, two tryptic peptides of p45 (6 and 23 amino acids) showed 100% identity with corresponding sequences of RHL-1. Second, affinity-purified RHL-1 and p45 exhibited identical migration patterns on SDS−PAGE gels, and monospecific antibodies to p45 cross-reacted with RHL-1 protein, and anti-RHL-1 antibodies recognized p45 protein (Figure 2B). While homooligomers of RHL-1 and RHL-2/3 can function as independent receptors for galactosylated ligands (Geffen et al., 1989; Halberg et al., 1987), it appears that a portion of RHL-1 and most of RHL-2/3 exist as heterooligomers on the cell surface (Herzig & Weigel, 1989, 1990; Oka et al., 1990; Sawyer et al., 1988). Data presented here and in the companion study (Bennatt & McAbee, 1997), however, suggest that Lfs bind to RHL-1 but not to RHL-2/3. First, we found that ^{125}I-Lf cross-linked to hepatocytes by dithiobis-(sulfosuccimidylproprionate) was detected by autoradiography at M_r of ~125 000; no higher molecular weight bands corresponding to complexes of Lf with RHL-2/3 were detected in a cross-linker- and Ca^{2+}-dependent fashion (Bennatt & McAbee, 1997). In contrast, ^{125}I-ASOR cross-linked to intact hepatocytes with several *N*-hydroxysuccin-

imidyl-based cross-linkers with ~12-Å linker arms similar to dithiobis(sulfosuccimidylproprionate) form adducts with RHL-1, RHL-2, and RHL-3 in roughly equimolar quantities (Herzig & Weigel, 1990). Second, RHL-2/3 does not copurify with p45/RHL-1 on Lf-agarose(Bennatt & McAbee, 1997) (Figure 2B), whereas all three RHL subunits are purified on AF-agarose (Figure 2A, lane R; Figure 2B, lanes 4 and 6). This suggests that Lf binding to RHL-1 may displace RHL-2/3 from RHL heterooligomers. Third, ^{125}I-Lf binds to RHL-1 immobilized onto nitrocellulose whereas it interacts poorly or not at all with immobilized RHL-2/3 (Figure 2C). Studies examining the lactoperoxidase-mediated ^{125}I-labeling of RHL subunits on intact cells suggest that RHL-2/3 subunits are more topologically prominent on the cell surface than RHL-1 (Lee & Lee, 1987; Sawyer et al., 1988). As such, our finding that anti-RHL-2/3 antibodies blocked the binding of ^{125}I-Lf to hepatocytes (Figure 3B) is consistent with the notion that interaction of anti-RHL-2/3 antibodies may sterically hinder the interaction of Lf with RHL-1. At present, we are examining the interaction of Lf with Swiss 3T3 fibroblasts expressing the human RHL homologs H1 and/or H2 (Fuhrer et al., 1994) to determine if one or both ASGP-R subunits are required for Lf binding and endocytosis.

Even though RHL-1 functions as the Ca^{2+}-dependent Lf receptor on isolated hepatocytes, several lines of evidence argue that Lf binds to RHL-1 in a Gal-independent manner. (i) Lf's glycan structure predicts it to be a low-affinity ligand for the ASGP-R. The number of Gal residues per glycan cluster and the extent of glycan branching are major determinants of the binding affinity of ligands to the hepatic Gal/GalNAc receptor. Tetraantennary and triantennary *N*-acetyllactosamine type glycans bind to Gal/GalNAc receptors on isolated hepatocytes with affinities 10^3-fold greater than biantennary *N*-acetyllactosamine type glycans and 10^6-fold greater than monoantennary chains (Lee et al., 1983). Lf's biantennary complex-type oligosaccharides—in which only one arm terminates in Gal—predicts that Lf should bind to the ASGP-R via its terminal Gal sugars with affinity constants in the milli- or micromolar range, yet bovine colostral Lf binds to isolated rat hepatocytes with high-affinity (K_d = 20−75 nM; Sitaram & McAbee, 1997; McAbee & Esbensen, 1991). Examination of bovine Lf's three-dimensional structure reveals that Asn-linked glycosylation sites (Asn^{386}, Asn^{476}) that attach at least one *N*-acetyllactosamine type glycan are at opposite faces of the C-lobe of the Lf molecule, a linear C_α to C_α distance of 46.7 Å (Haridas et al., 1994). Even though Lf's glycan chains exhibit considerable conformational flexibility (Dauchez et al., 1992), it is unlikely that Gal- or GalNAc-bearing *N*-acetyllactosaminyl antennae present on Asn^{368} and Asn^{476} could attain a geometry suited to bind to multiple adjacent p45/RHL-1 subunits (15−25 Å; Hardy et al., 1985). Even if this occurred, juxtaposition of two terminal Gal residues would yield at best a bivalent interaction, a density of Gal sugars that typically produces ligand affinity constants in the micromolar range (Hardy et al., 1985; Lee et al., 1983). (ii) Hepatocytes bind degalactosylated and native Lfs equally well. If Lf binds p45/RHL-1 via terminal Gal sugars, then enzymatic removal of even a fraction of Lfs' Gal sugars should at the very least reduce the ability of the modified protein to compete with native Lfs for binding to hepatocytes. Our evidence indicates, however, that Lfs which lacked all detectable terminal Gal moieties competed as well as unmodified Lfs for binding to cells (Figures 5 and 6). (iii) Nonglycosylated recombinant Lf polypeptides inhibit binding of native Lf to hepatocytes. We have expressed two truncated variants of bovine Lf in *Escherichia coli*, one lacking the first 70 NH_2-terminal amino acids and the other lacking the first 270 N-terminal amino acids. *E. coli* extracts enriched in either of these variant proteins, but not extract from mock-transformed cells, specifically block the interaction of native Lf to the Ca^{2+}-dependent sites on hepatocytes (Sitaram et al., 1996). We conclude, therefore, that Lfs bind to p45/RHL-1 by a mechanism that is independent of Gal- or GalNAc-terminated glycans. To our knowledge, this is the first report showing that the major subunit of the ASGP-R binds to a protein ligand with high affinity in a carbohydrate-independent manner.

Previous reports suggest the ASGP-R is not involved in clearance of plasma Lf based on the observations that ASOR and Lf do not block removal of each other from murine circulation (Imber & Pizzo, 1983; Moguilevsky et al., 1984). While ASOR and Lf compete for binding to isolated rat hepatocytes, the two ligands appear to bind ASGP-Rs by different mechanisms. First, ASOR's interaction with the ASGP-R is mediated by Gal sugars that terminate its glycan structures (Pricer & Ashwell, 1971) whereas Lf binds at or near the carbohydrate-recognition domain of RHL-1 yet in a Gal-independent manner (Figures 5−7). This is consistent with the earlier report that Lf's protein, but not its carbohydrate, is responsible for its binding to hepatocytes (Moguilevsky et al., 1984). Second, high-affinity binding of ASOR to hepatocytes is mediated by heterooligomers of RHL-1 and RHL-2/3 (Herzig & Weigel, 1990; Sawyer et al., 1988) although ASOR can bind to H1 homooligomers alone with high affinity (Bider et al., 1995). Lf, on the other hand, binds readily to RHL-1 subunits but poorly or not at all to RHL-2/3 (Figure 2B,C) (Bennatt & McAbee, 1997). Third, hepatocytes possess a large intracellular pool of ASGP-R (Ashwell & Harford, 1982; Weigel & Oka, 1983) that bind ^{125}I-ASOR or ^{125}I-anti-ASGP-R IgG when cells are permeabilized with digitonin (McAbee et al., 1991; McAbee & Weigel, 1988; Weigel & Oka, 1983; McAbee & Ling, 1997). In contrast, digitonin-permeabilized rat hepatocytes in suspension or primary cultures do not bind significantly more ^{125}I-Lf than do intact cells (McAbee et al., 1993; McAbee & Ling, 1997), suggesting that intracellular ASGP-Rs can bind ligands by their lectin activity but not to ligands by a Gal-independent mechanism. Some evidence suggests that the intracellular ASGP-R subunit composition is similar to that on the cell surface (Herzig & Weigel, 1990; Oka et al., 1990), but others have reported biochemical and subunit compositional differences between plasma membrane and intracellular ASGP-R as well as differences in subunit expression (Andersson et al., 1988; Enrich et al., 1992). It remains to be determined conclusively, therefore, that the lack of ASOR competition for Lf clearance reflects the removal of these two ligands from the circulation by entirely different receptor systems.

At least two implications arise from our findings. First, ASGP-Rs may be multifunctional, binding different ligands by carbohydrate-dependent and -independent mechanisms. One precedent for this is the cation-independent mannose 6-phosphate/insulin-like growth factor II receptor that delivers populations of acid hydrolases bearing mannose 6-phos-

phate tags to lysosomes as well as mediating endocytosis of insulin-like growth factor II—a nonglycosylated polypeptide—at the plasma membrane (Kornfeld, 1992). Notably, the ligand-binding domains for these two classes of proteins are unique on this receptor (Dahms et al., 1994; Garmroudi et al., 1996). It has been suggested that the primary homeostatic function of mammalian ASGP-R is to remove "deleterious" ligands from the circulation (Ashwell & Morell, 1974), particularly Gal- or GalNAc-terminated glycoconjugates (Weigel, 1994), but the identity of endogenous ligands for ASGP-R is unclear. ASGP-R mediate uptake of tissue plasminogen activator by a carbohydrate-dependent interaction (Smedsrod et al., 1988), and isoforms of polymeric immunoglobulin A derived from human myelomas bind to ASGP-R with moderately-high affinity by way of O-linked Gal-terminated oligosaccarides present near the hinge region of the immunoglobulin molecule (Daniels et al., 1989; Stockert et al., 1982). It remains to be determined if proteins other than Lfs can interact with ASGP-Rs in a Gal- or GalNAc-independent manner. A second implication is that Lf clearance from blood and non-blood fluids may be mediated in part by RHL-1-like receptors present on cells of the reticuloendothelial system. For example, rat peritoneal macrophages possess a 42-kDa Gal/GalNAc receptor (Kelm & Schauer, 1988) that has high homology with RHL-1 (Ii et al., 1990) and is endocytically competent when expressed in COS cells (Ozaki et al., 1992). Notably, Lf has been shown to interact with a 42-kDa polypeptide present on promonocytic U937 cells (Britigan et al., 1996), but it is not known if this polypeptide shares identity with the macrophage 42-kDa ASGP-R. Non-parenchymal liver cells internalize Lf (Courtoy et al., 1984), and Kupffer cells exhibit two known Gal-specific lectins, one a 61-kDa polypeptide that binds fucosyl- or Gal-terminated glycoconjugates and shows partial homology with RHL-1 (Hoyle & Hill, 1991) and the other a 30-kDa Gal-particle receptor that is a peripheral membrane protein that shows no immunological cross-reactivity to RHL-1 but is homologous to C-reactive protein (Kempka et al., 1990). Under normal conditions, the steady-state concentration of Lf in plasma is ~20 nM (Bennett & Kokocinski, 1979; Levay & Viljoen, 1995), suggesting that the Lf receptors responsible for Lf clearance must have comparable affinity constants for Lf. Some have reported that hepatic uptake of human Lf from rat circulation is accomplished by the low density lipoprotein receptor-related protein (Huettinger et al., 1992; Meilinger et al., 1995; Willnow et al., 1992). Other reports implicate cell surface proteoglycan (Ji & Mahley, 1994; Regoeczi et al., 1994) and a chylomicron remnant receptor (Ziere et al., 1996; 1992) in clearance of plasma Lf. None of these components, however, binds Lf with a sufficiently high affinity to maintain low steady-state plasma Lf concentrations. It is possible that homeostasis of plasma Lf is accomplished by multiple receptor systems. Based on our findings here, examination of monocyte/macrophage RHL-1-like receptors for binding and endocytosis of Lf is warranted. Also, studies on plasma Lf content in H2 knockout mice, which are deficient in H1 and ASGP-R functional expression (Ishibashi et al., 1994), should confirm whether or not hepatocyte ASGP-Rs are involved in regulation of hepatic clearance of plasma Lf.

ACKNOWLEDGMENT

We thank Kay Stewart and her staff at Freimann Life Sciences Center for their help in antibody preparation and Dr. John Leszyk at the Worchester Foundation Microchemistry Facility for his assistance in sequencing p45 tryptic fragments. We are grateful to Dr. Ted Baker (Massey University, Palmerston North, New Zealand) for the atomic coordinates of bovine lactoferrin. Finally, we thank Dr. Roger Bretthauer, Dr. David Hyde, and Dr. Paul Weigel for their helpful discussions during the preparation of this manuscript.

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BI963079M