Identification and Isolation of a 45-kDa Calcium-Dependent Lactoferrin Receptor from Rat Hepatocytes†

David J. Bennatt and Douglas D. McAbee*

Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556

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

ABSTRACT: Isolated rat hepatocyes bind, internalize, and degrade bovine lactoferrin (Lf) via high-affinity Ca^{2+}-dependent sites [$<10^6$ sites/cell; McAbee et al., (1993) *Biochemistry 32*, 13749−13760]. In this study, we identified a 45-kDa Ca^{2+}-dependent Lf binding protein on rat hepatocytes by three independent approaches. First, dithiobis(sulfosuccimidylproprionate) (DTSSP) cross-linked ^{125}I-Lf to a 45-kDa adduct in a Ca^{2+}-dependent manner on intact cells. The ^{125}I-labeled cross-linked complexes were absent when either surface-bound ^{125}I-Lf was stripped prior to cross-linking or an excess of unlabeled Lf was included in the DTSSP reaction. Second, ^{125}I-Lf bound to a 45-kDa hepatocyte polypeptide in a Ca^{2+}-dependent fashion following incubation with SDS−PAGE fractioned hepatocyte membrane proteins absorbed on nitrocellulose. Third, when Triton X-100 extracts of hepatocyte membrane ghosts were chromatographed on Lf-agarose, a 45-kDa polypeptide (p45) was eluted by EGTA. Column fractions enriched in p45—but not those depleted of p45—possessed soluble Lf receptor activity as determined by competition binding assay. Monospecific polyclonal anti-p45 IgG detected p45 in crude hepatocyte ghost homogenates and blocked vigorously ^{125}I-Lf binding and endocytosis to intact rat hepatocytes. We conclude, therefore, that p45 constitutes the Ca^{2+}-dependent Lf receptor on isolated rat hepatocytes.

Lactoferrin (Lf)[1] is a member of the transferrin family of non-heme iron-binding proteins and is present in virtually all exocrine fluids [for reviews, see Brock (1995); Levay and Viljoen (1995); Lonnerdal and Iyer (1995)]. Blood Lf is released from granulocytes upon exocytosis of specific granules. The liver regulates Lf's steady-state concentration (∼20 nM) by clearing it from the blood (Bennett & Kokocinski, 1979; Imber & Pizzo, 1983), most of it accumulating in hepatocytes (DeBanne et al., 1985; Imber & Pizzo, 1983; Ziere et al., 1992). Clearance of plasma Lf constitutes an important hepatic homeostatic function and may represent a significant iron-scavenging pathway (Ismail & Brock, 1993; Regoeczi et al., 1994).

The molecular basis for hepatic metabolism of blood Lf has received considerable scrutiny over the last several years. Human Lf injected into the rat circulation can be internalized by hepatic parenchymal and non-parenchymal by the chylomicron remnant receptor (Huettinger et al., 1992; Meilinger et al., 1995; Ziere et al., 1992). Other studies have shown that rat hepatocytes bind human Lf via cell surface proteoglycans (Hu et al., 1993; Regoeczi et al., 1985). We found that isolated rat hepatocytes bind bovine Lf by two classes of binding sites: one is high-affinity ($K_d \sim 20$ nM) and Ca^{2+}-dependent; the other is low-affinity ($K_d \sim 1\ \mu$M), Ca^{2+}-independent, and sensitive to the polyanion dextran sulfate (McAbee & Esbensen, 1991; McAbee et al., 1993). Only the high-affinity, Ca^{2+}-dependent sites are endocytically competent (McAbee et al., 1993), mediating the uptake of both Lf protein and Lf-bound iron (McAbee, 1995). In contrast, human Lf binds to isolated rat hepatocytes at 4 °C with lower affinity ($K_d \sim 10\ \mu$M) than does bovine Lf, and binding is not enhanced by Ca^{2+} but is blocked by polyanions (Ziere et al., 1992). In light of these differences and because the steady-state blood Lf concentrations are well below the reported K_d values for human Lf interaction with hepatocytes, the chylomicron remnant receptor probably does not constitute the Ca^{2+}-dependent Lf receptors on rat hepatocytes.

In this study, we employed three independent techniques to identify the rat hepatocyte polypeptide(s) responsible for Ca^{2+}-dependent binding and endocytosis of bovine Lf: (i) cross-linking of bovine Lf to hepatocyte cell surface proteins using the homobifunctional reagent DTSSP; (ii) blotting of bovine Lf onto renatured nitrocellulose-absorbed replicas of hepatocyte proteins separated by SDS−PAGE; and (iii) affinity chromatography of solubilized hepatocyte membrane proteins on Lf-agarose. By these approaches, we identified and purified a 45-kDa polypeptide (p45) from isolated rat hepatocyes that binds bovine Lf specifically and in a Ca^{2+}-dependent manner. p45 exhibited potent soluble Lf receptor activity, and antibodies raised against p45 blocked the binding and endocytosis of bovine Lf by intact rat hepatocytes. These results indicate that p45 functions as the Ca^{2+}-dependent hepatocyte Lf receptor.

EXPERIMENTAL PROCEDURES

Materials. Acrylamide, bis(acrylamide), bovine colostrum Lf, BSA, dextran sulfate (5000 Da), ferritin (rat liver type VI), and glutaraldehyde (stock 25% w/v) were purchased

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

* To whom correspondence should be addressed: Department of Chemistry & Biochemistry, California State University, Long Beach, CA 90840. Telephone: (562) 985-4941. FAX: (562) 985-8557.

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

[1] Abbreviations: BME, basal medium Eagle's; BSA, bovine serum albumin; DTSSP, dithiobis(sulfosuccimidylproprionate); EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; HBS, HEPES-buffered saline; HEPES, *N*-(2-hydroxylethyl)piperazine-*N*′-2-ethanesulfonic acid; Lf, lactoferrin; LRP, low-density lipoprotein receptor-related protein/α_2-macroglobulin receptor; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

S0006-2960(96)03078-4 CCC: $14.00

from Sigma (St. Louis, MO). Iodogen, DTSSP, Reacti-gel, NHS-LC-Biotin, and protein A agarose were obtained from Pierce (Rockford, IL). Collagenase (type D) was from Boehringer Mannheim (Indianapolis, IN). Digitonin and PMSF were obtained from Eastman Kodak (Rochester, NY). $Na^{125}I$ (17 Ci/mg of iodine) was from ICN Biomedicals, Inc. (Irvine, CA) or DuPont New England Nuclear (Boston, MA). Ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were from BioRad (Richmond, CA). All other chemicals were reagent grade and obtained from Fisher Biochemicals (Itasca, IL) or Sigma. Centricon microconcentrators were purchased from Amicon (Beverly, MA). BME was obtained from Sigma and supplemented with 2.4 g/L HEPES, pH 7.4, and 0.22 g/L $NaHCO_3$. BME-BSA is BME containing 0.1% (w/v) BSA. HBS contains 150 mM NaCl, 3 mM KCl, and 10 mM HEPES, pH 7.4. Buffer A contains HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Buffer B contains buffer A supplemented with 0.2% (w/v) dextran sulfate. Buffer C contains buffer A with 1% (v/v) Triton X-100. Buffer D contains HBS supplemented with 5 mM EGTA. Buffer E contains 150 mM NaCl, 5 mM $CaCl_2$, 10 mM sodium borate, pH 9.0, and 0.2% (v/v) Tween-20. Buffer F contains 150 mM NaCl, 5 mM EGTA, 10 mM sodium borate, pH 9.0, and 0.2% (v/v) Tween-20. PBS contains 0.9% (w/v) NaCl and 10 mM Na_2HPO_4, pH 8.0. TBS-Tween contains 0.5 M NaCl, 0.2% Tween-20, and 10 mM Tris, pH 8.0.

Hepatocytes. 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 (Seglen, 1973) as described previously (McAbee & Esbensen, 1991). 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. For the soluble Lf receptor activity assay (see below), isolated intact hepatocytes (2×10^7 cells/mL) were fixed with 0.06% (v/v) glutaraldehyde in buffer A for 1 h at 4 °C. Excess or unreacted glutaraldehyde was quenched by washing cells for 30 min on ice with 5−10-fold excess volumes of buffer A supplemented with 0.1 M glycine. Cells were washed twice with excess cold buffer A, resuspended in buffer A (5×10^6 cells/mL), and stored at −20 °C prior to use. Fixed hepatocytes prepared in this manner are resistant to detergent and mechanical disruption and bind ^{125}I-Lf specifically in a Ca^{2+}-dependent manner ≤4 months in storage.

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 as described previously (McAbee & Esbensen, 1991). Lf used in experiments was 30−70% iron-saturated as determined by 460:280 nm absorbance ratio (Hashizume et al., 1987). Purified Lf was dialyzed against HBS, filter-sterilized (0.2 μm), and stored at −20 °C before use. ^{125}I-Lf—prepared in Iodogen-coated tubes—had specific activities of 100−300 dpm/fmol for cross-linking studies and 6−65 dpm/fmol for cell-binding studies. Homogeneity of radiolabeled and unlabeled Lf was confirmed periodically by SDS−PAGE and autoradiography.

Cell-Binding Assays. Isolated rat hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf ≤ 90 min in buffer A as described in the figure legends. Unbound ^{125}I-Lf was removed from cells by centrifugation in excess buffer B. Dextran sulfate present in buffer B strips Lf bound to Ca^{2+}-independent, low-affinity Lf binding sites but not from the high-affinity, Ca^{2+}-dependent sites (McAbee & Esbensen, 1991; McAbee et al., 1993). Washed cells were resuspended in 0.5 mL of buffer B, transferred to clean tubes, and assayed for radioactivity. The presence of soluble Lf receptor activity in various detergent extracts of rat hepatocytes was determined by a ligand-competition assay (Ray & Weigel, 1985) modified for use with Lf. Briefly, various Triton X-100-containing fractions of crude or purified hepatocyte membrane proteins (≤0.2 mL) were mixed with 1 μg of ^{125}I-Lf in buffer A supplemented with 0.1% Trition X-100 for 30 min on ice (final volume 0.8 mL). The ^{125}I-Lf−extract mixture was supplemented with 10^6 glutaraldehyde-fixed hepatocytes (prepared as described above) in 0.2 mL of buffer A and incubated an additional 30 min at 4 °C. Fixed cells were washed free of unbound ligand by centrifugation in buffer B as described above, resuspended into buffer B, transferred to clean tubes, and assayed for bound radioactivity.

Cross-Linking of Lf to Hepatocytes. Hepatocytes (2×10^7) were incubated with 20 μg of ^{125}I-Lf/mL buffer A for 90 min at 4 °C. Cells were washed twice in cold buffer B for 10 min to remove unbound ^{125}I-Lf and ^{125}I-Lf bound to Ca^{2+}-independent sites. DTSSP (stock solution ∼50 mg/mL in buffer A) was added to give a final concentration of ≤2 mM, and the cell suspension was incubated for 60 min at 4 °C. The cells were washed twice in excess cold buffer D supplemented with 0.1% glycine to strip non-cross-linked ^{125}I-Lf from hepatocytes and to block unreacted DTSSP, then permeabilized with 0.06% (w/v) digitonin to generate hepatocyte "ghosts" in the presence of 1 mM PMSF. Digitonin at this concentration solubilizes ≤16% of the high affinity, Ca^{2+}-dependent sites on hepatocytes (McAbee et al., 1993) and allows transmembrane diffusion of cytoplasmic polypeptides ≤200 kDa (Weigel et al., 1983). Cells were permeabilized for 30 min on ice and washed twice by centifugation (500*g*, 4 °C) in HBS supplemented with 1 mM PMSF. Hepatocyte ghosts were solubilized in HBS and 1% (v/v) Triton X-100 for 30 min at 4 °C followed by sedimentation of detergent-insoluble material (15000*g*, 30 min, 4 °C); supernatants were clarified by filtration (0.2 μm). Triton X-100 extracts were subjected to SDS−PAGE and autoradiography.

Ligand Blotting. Hepatocytes were solubilized in Triton-X-100 as described above, and detergent extract was subjected to SDS−PAGE. Electrophoresed proteins were transferred to nitrocelluose and renatured by incubation in buffer E containing 5% BSA overnight at 4 °C, a modification of a ligand blotting technique described elsewhere (Zeng et al., 1996). Nitrocellulose was incubated with 1 μg of ^{125}I-Lf/mL in either buffer E or buffer F for 60 min at 22 °C as described in the figure legends, washed twice with an excess of the same buffer, air-dried, and exposed to X-ray film.

Lf-Agarose Affinity Chromatography. Lf was cross-linked to Reactigel (Pierce) or CNBr-activated agarose at a density of 5−10 mg/mL gel as per manufacturer's instructions. Digitonin-permeabilized hepatocytes (2×10^7/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). Protein samples were 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 (4 mL resin). The column was washed with ~10 bed volumes of buffer B containing 0.1% Triton X-100 and eluted with buffer D containing 0.1% Triton X-100. Flow-through, wash, and eluted fractions were analyzed for soluble Lf receptor activity and subjected to SDS−PAGE as described in the figure legends.

Cell-Surface Biotinylation. NHS-LC-biotin, a water soluble membrane-impermeant biotinylation reagent, was mixed with hepatocytes (8×10^6 cells/mL) in PBS (final concentration 0.2 mg/mL) for 30 min at 4 °C. Cells were washed twice in ice-cold PBS supplemented with 0.1 M glycine to quench unreacted NHS-LC-biotin. Biotinylated cells were prepared for Lf-agarose affinity chromatography as described above.

Electrophoresis and Autoradiography. 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 7.5% acrylamide discontinuous Tris-HCl slab gels 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 BioRad prestained electrophoresis standards. Dried gels containing ^{125}I-labeled polypeptides were exposed to Kodak X-Omat-AR film at −70 °C for 1−2 weeks and developed using a Kodak X-Omat Clinic 1 processor.

p45 Purification and Polyclonal Antibody Production. p45 present in EGTA-eluted fractions from Lf-agarose was further purified by preparative SDS−PAGE. Electrophoretically-purified p45 in acrylamide gel pieces was electro-eluted into dialysis retainers (BioRad) and further concentrated by ultrafiltration (10 000 Da cutoff). Typically, we isolated 150−250 μg of p45 per hepatocytes from one rat liver using this procedure. New Zealand white rabbits were inoculated with purified p45 (~200 μg/inoculum) in Freunds complete and incomplete adjuvant. Whole IgG was purified from antisera by chromatography on protein A-agarose according to manufacturer's protocols (Pierce). Rabbit antisera against bovine Lf was prepared by inoculating animals with 200 μg of purified Lf. IgG from pre-immune and immune sera was purified by Na_2SO_4 precipitation and DEAE chromatography (Johnstone & Thorpe, 1982). Antisera detected 1 ng of Lf at 1:10 000 dilution by dot-blot assay.

General. Protein was determined by the bicinchoninic acid protein assay procedure using BSA as standard (Pierce Chemical Co.). 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 Lf were done using a Beckman DU-64 spectrophotometer.

RESULTS

Detection of Ca^{2+}-Dependent Lf-Binding Proteins on Isolated Rat Hepatocytes. In order to identify candidate membrane proteins on isolated hepatocytes that interact with Lf in a Ca^{2+}-dependent manner, we employed the homobifunctional cross-linker DTSSP that has been used to identify ligand-specific receptors on intact cells (Waugh et al., 1989). DTSSP (Figure 1A) is a membrane-impermeant reagent that contains a reducible disulfide bond within its linker arm and forms amide bonds with primary amines on adjacent proteins positioned within 12 Å of each other. We prebound hepatocytes with ^{125}I-Lf at 4 °C and washed the cells in the presence of Ca^{2+} and dextran sulfate to remove ^{125}I-Lf bound to Ca^{2+}-independent low-affinity sites (McAbee et al., 1993). Following incubation of these cells with DTSSP, we stripped non-covalently bound ^{125}I-Lf from the cells by EGTA/dextran sulfate wash (McAbee et al., 1993) and extracted the membrane proteins with Triton X-100. The ^{125}I−Lf adduct complexes were detected by SDS−PAGE and autoradiography. We anticipated that ^{125}I-Lf adduct cross-linked complexes would migrate with an apparent molecular weight corresponding to the combined molecular weights of Lf (~80 kDa) and the cross-linked adduct. Autoradiography detected ^{125}I-Lf-containing bands at M_r of 80, 125, and 260 (Figure 1B, lanes 5 and 6). The 80 kDa ^{125}I-polypeptide associated with DTSSP-treated cells (Figure 1B, lanes 5 and 6) comigrated with ^{125}I-Lf monomer (Figure 1B, lane 9), indicating that it is either residual non-cross-linked ^{125}I-Lf associated with the cells or a small amount of free ^{125}I-Lf generated by reduction of the DTSSP disulfide bonds during sample preparation. The 125- and 260-kDa bands were reduced or absent when ^{125}I-Lf binding was done in the presence of a 50-fold molar excess of unlabeled Lf (Figure 1B, lanes 1, 2, 7, 8) or when DTSSP was omitted from the incubation (Figure 1B, lanes 1−4). We reported previously that low amounts of bovine Lf dimers (160 kDa) and trimers (240 kDa) are artifactually generated during sample preparation for SDS−PAGE and detected when gels are overloaded (McAbee & Esbensen, 1991). While these multimers were detected when ^{125}I-Lf was incubated alone with DTSSP (Figure 1B, lane 9), these species did not comigrate with the DTSSP-dependent 125- and 260-kDa bands. Reduction of the DTSSP-cross-linked samples with β-mercaptoethanol prior to SDS−PAGE resulted in the loss of all 125-kDa species and diminishment of the 260-kDa species with concurrent enrichment of 80 kDa band (data not shown).

We also found that the DTSSP-dependent generation of the 125-kDa species was Ca^{2+}-dependent. Removal of surface-bound ^{125}I-Lf with EGTA prior to incubation with DTSSP abolished the appearance of the 125-kDa ^{125}I-Lf−adduct complex (Figure 1C, lanes 1−4). When cross-linking was done in the presence of Ca^{2+}, we found that the amount of the 125-kDa species generated increased as the DTSSP concentration increased (Figure 1C, lanes 5−8). The appearance of the 260-kDa species was dependent neither on Ca^{2+} nor cross-linker (Figure 1C, compare lanes 5−8) although its amount was reduced when cells were washed with EGTA prior to cross-linking (Figure 1C, compare lanes 1−4 with lanes 5−8). The nature of the 260-kDa cross-linked species is unclear. These data indicate that the formation of the 125-kDa ^{125}I-Lf−adduct complex was Ca^{2+}-

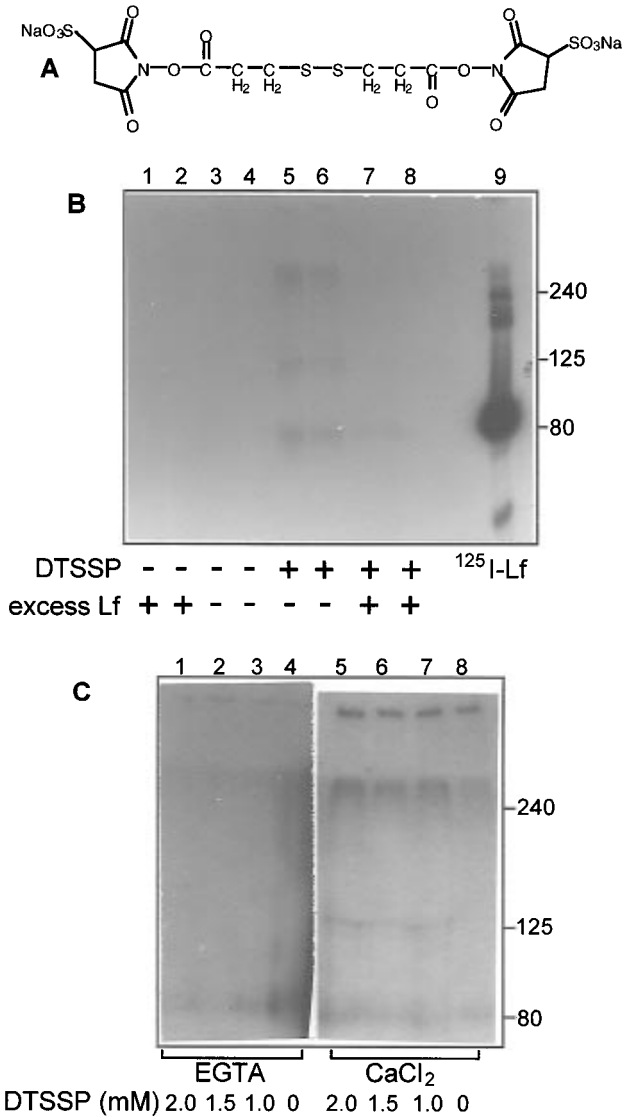

FIGURE 1: DTSSP cross-linking of Lf to hepatocyte polypeptides. (A) Schematic structure of DTSSP. (B) Isolated rat hepatocytes (2×10^7 cells/mL) were incubated with 20 μg ^{125}I-Lf/mL of buffer A on ice for 1 h in the presence (lanes 1, 2, 7, 8) or absence (lanes 3−6) of a 50-fold molar excess of unlabeled Lf. Cells were washed in buffer B and then incubated with (lanes 5−8) or without (lanes 1−4) 2 mM DTSSP on ice for 1 h as described in Experimental Procedures. Cells were stripped of non-cross-linked ^{125}I-Lf by washing with buffer D and permeabilized with 0.06% digitonin. Washed hepatocyte ghosts were solubilized with Triton X-100-containing buffer, and membrane proteins were analyzed by SDS−PAGE and autoradiography as described in Experimental Procedures. In other experiments, ^{125}I-Lf (20 μg/mL) alone in buffer A was incubated with 2 mM DTSSP on ice for 1 h; unreacted DTSSP was quenched by addition of 1 M glycine. Treated ^{125}I-Lf was subjected to SDS−PAGE and autoradiography (lane 9). (C) Isolated rat hepatocytes (2×10^7 cells/mL) were incubated with 20 μg of ^{125}I-Lf/mL of buffer A on ice for 1 h after which cells were washed at 4 °C in buffer D (EGTA; lanes 1−4) or buffer B ($CaCl_2$; lanes 5−8). Cells were incubated with DTSSP at the designated concentrations for 1 h at 4 °C, then washed, quenched, and processed for SDS−PAGE and autoradiography as described in panel B and Experimental Procedures. High molecular weight ^{125}I-labeled material accumulated at the top of the stacking gel, but its appearance was not DTSSP-dependent (compare lanes 5 and 8).

and cross-linker-dependent. Because monomeric Lf migrates at 80 kDa on SDS−PAGE, these findings suggest that the adduct cross-linked to ^{125}I-Lf to form the 125 kDa species was ∼45-kDa.

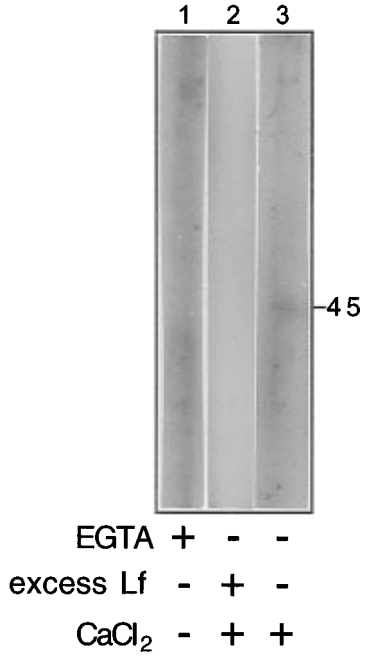

FIGURE 2: Lf binding to nitrocellulose-immobilized hepatocyte membrane proteins. Isolated rat hepatocytes (2×10^7 cells/mL) were permeabilized with 0.06% digitonin at 4 °C, washed in HBS to rid cytosolic contents, and then solubilized in HBS containing 1% Triton X-100. Detergent-solubilized material was fractioned by SDS−PAGE and then transferred to nitrocellulose. Papers were incubated in buffer E supplemented with 5% BSA overnight at 4 °C. Renatured blots were incubated at 22 °C for 60 min with ^{125}I-Lf (1 μg/mL) in buffer E in the presence (lane 2) or absence (lane 3) of a 50-fold molar excess of unlabeled Lf or in buffer F (lane 1). Strips were washed in binding buffer, air-dried, and subjected to autoradiography as described in Experimental Procedures. Laser scanning densitometry of lane 3 revealed a major peak at M_r 45 000 with an Ab_{max} = 2.18 AU (area = 0.331) and a minor peak at around M_r 35 000 with an Ab_{max} = 2.18 AU (area = 0.096). The background absorbance in this lane was 2.09 AU; no other significant peaks were detected.

In the next series of experiments, we probed for the presence of hepatocyte Lf-binding polypeptides by ligand blotting. Human and bovine Lfs can bind the hepatic LRP absorbed onto nitrocellulose following denaturing electrophoresis of partially-purified hepatocyte membrane fractions (Meilinger et al., 1995; Willnow et al., 1992). These reports, however, did not examine whole hepatocyte membrane extracts nor the affects of Ca^{2+} and denaturing conditions on Lf blotting. To address this, we generated polypeptide replicas of Triton X-100-solubilized hepatocyte membrane proteins separated by SDS−PAGE onto nitrocellulose. Residual SDS bound to absorbed proteins was removed by thorough washing in Tween-20-containing buffer, which fully restores ligand-binding activity to the RHL-1 subunit of the asialoglycoprotein receptor following denaturation by SDS−PAGE (Zeng et al., 1996). We found that ^{125}I-Lf bound to a polypeptide with an apparent mass of ∼45 kDa in the presence of Ca^{2+} (Figure 2, lane 3) but was abolished when EGTA was substituted for Ca^{2+} during the ^{125}I-Lf binding step (Figure 2, lane 1). Similar negative results were obtained if nitrocellulose replicas were washed with EGTA following ^{125}I-Lf binding or when renaturation with Tween-20 prior to ^{125}I-Lf incubation was omitted (data not shown). The binding was competitive as demonstrated by the loss of ^{125}I-Lf bound in the presence of a 50-fold molar excess of unlabeled Lf (Figure 2, lane 2). These results indicated that

Lf binds specifically in a Ca^{2+}-dependent manner to a protein present in hepatocyte membrane extract with mass of ~45 kDa, consistent with results we obtained from cross-linking experiments.

Affinity Chromatographic Purification of Lf Binding Proteins. Ligand-affinity chromatography is commonly used to identify and isolate membrane receptors that recognize and bind specifically those ligands. Successful application of this technique to identify Lf receptors, however, is aggravated by Lf's cationic nature, which greatly increases non-specific binding of proteins to Lf-agarose. We chose, therefore, to identify Lf-binding proteins in hepatocyte membrane preparations using Lf-agarose affinity chromatography under conditions that reduce non-specific binding of Lf to hepatocytes. Briefly, hepatocyte ghosts were washed extensively in Ca^{2+}-free buffers prior to solubilization with Triton X-100 at pH 7.5. The detergent extract was passed sequentially over ethanolamine-agarose and Lf-agarose in the presence of Ca^{2+}. The Lf-agarose was washed with ~10 bed volumes in the presence of Ca^{2+} and 0.2% dextran sulfate to remove anionic polypeptides bound non-specifically to Lf-agarose prior to column elution with EGTA. Eluted fractions were supplemented with $CaCl_2$ and analyzed for protein content by SDS−PAGE (Figure 3A) and assayed for soluble Lf receptor activity (Figure 4). SDS−PAGE of eluted fractions revealed a major polypeptide of 45 kDa and a minor band at 90 kDa (Figure 3A, lanes 3 and 4). Inclusion of 5% β-mercaptoethanol with electrophoresed samples decreased substantially the 90-kDa band with concomitant enrichment of the 45-kDa species (Figure 5), suggesting that the 90-kDa species was an oxidized form of the 45-kDa polypeptide (p45).

To determine if p45 was a membrane protein, we used the reagent NHS-LC-biotin, a membrane impermeant reagent, to tag hepatocyte surface proteins with biotin prior to Triton X-100 solubilization. When assayed for ^{125}I-Lf binding at 4 °C to the Ca^{2+}-dependent sites, untreated hepatocytes bound 594 ± $28_{(n=2)}$ fmol ^{125}I-Lf/10^6 cells whereas biotinylated cells bound 833 ± $80_{(n=2)}$ fmol ^{125}I-Lf/10^6 cells. p45 isolated from non-biotinylated cells was not detected with strepatavidin−alkaline phosphatase conjugates by dot blot, indicating that it is not naturally biotinylated (data not shown). When p45 was prepared from surface-biotinylated cells, it and the 90-kDa species were readily detected on blots using a streptavidin−alkaline phosphatase probe (Figure 3B, lanes 2−6). These data confirm that p45 is a hepatocyte surface membrane protein.

We also assayed for soluble Lf receptor activity in crude detergent extract and various Lf-agarose column fractions using a modification of an assay reported elsewhere (Ray & Weigel, 1985). Briefly, detergent extract or column fractions were incubated with ^{125}I-Lf so that soluble Lf receptors could form complexes with ^{125}I-Lf. Glutaraldehyde-fixed hepatocytes were incubated with the mixture to bind non-complexed ^{125}I-Lf and then assayed for cell-associated radioactivity. In this way, the amount of ^{125}I-Lf bound to fixed cells was inversely proportionate to the amount of soluble Lf receptor activity present in detergent extract or column fractions. Elution fractions enriched in p45 exhibited potent soluble Lf receptor activity (Figure 4). Importantly, Lf-agarose flow-through depleted of p45 showed very little soluble Lf-receptor activity, indicating that the soluble Lf-receptor activity present in whole detergent extracts of

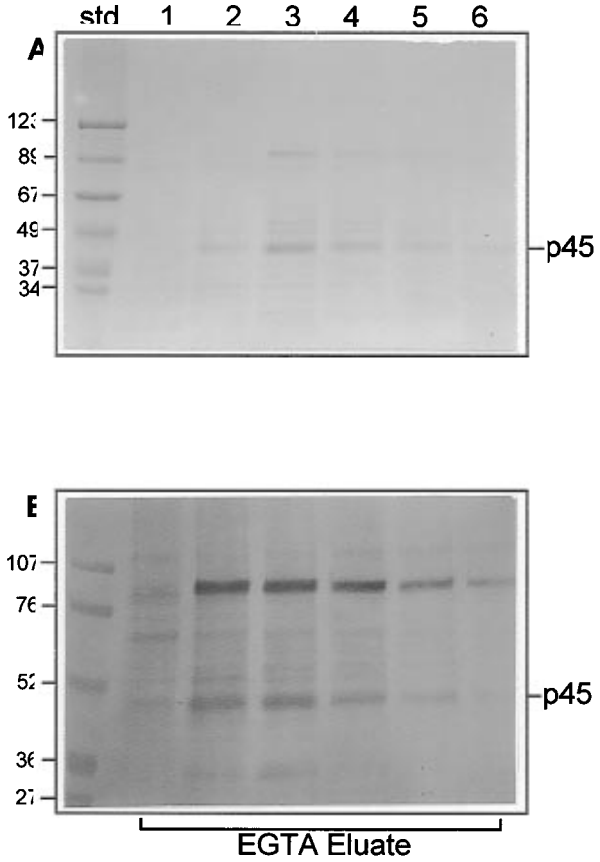

FIGURE 3: Ligand affinity chromatography of hepatocyte membrane proteins on Lf-agarose. (A) Detergent-solubilized membrane proteins from digitonin-permeabilized hepatocyte ghosts were prepared and chromatographed sequentially on ethanolamine-agarose and Lf-agarose as described in Experimental Procedures. Proteins bound to Lf-agarose were eluted with 5 mM EGTA (lanes 2−6), fractioned by SDS−PAGE, and detected by Coomassie Blue staining. (B) Triton X-100 extracts of surface-biotinylated hepatocyte ghosts—prepared as described in Experimental Procedures—were subjected to ligand affinity chromatography on Lf-agarose as in panel A. Lf-agarose bound proteins eluted with EGTA were transferred to nitrocellulose paper, and biotinylated proteins were detected by streptavidin-alkaline phosphatase conjugate (1:1000 dilution), 5-bromo-4-chloro-3-indoylyl phosphate, and nitroblue tetrazolium alkaline phosphatase substrate according to the manufacturer's instructions (BioRad). M_r of prestained electrophoresis molecular weight standards (std) are indicated.

hepatocyte membranes copurified with p45. In other experiments, we found that p45 was not detected by anti-Lf sera confirming that it was not a Lf fragment eluting off the Lf-agarose in the presence of EGTA (data not shown).

Blocking of Lf Interaction with Hepatocytes with Anti-p45 Antibodies. The preceding findings suggested that p45 functions as the hepatocyte Ca^{2+}-dependent Lf receptor. If true, then antibodies directed against p45 could bind to the extracellular domain of p45 present on intact cells and prevent Lf binding. Antisera raised against electrophoretically-purified p45 recognized on Western blots a single 45-kDa band present in whole detergent extracts of hepatocyte ghosts (data not shown), indicating that the antibody was monospecific for p45. Antisera recognized p45 and the 90-kDa species on Western blot analysis of EGTA-containing fractions eluted from Lf-agarose. Because p45 excised from preparative SDS−polyacrylamide gels was used for inoculations, these data confirmed that the 90-kDa reactive species was a dimerized form of p45. We also observed that

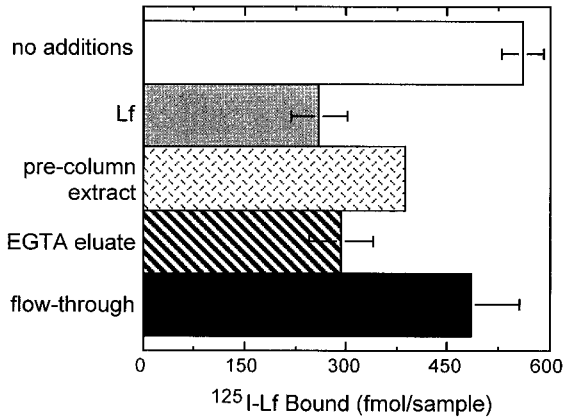

FIGURE 4: Soluble Lf receptor activity assay on Lf-agarose fractions. Glutaraldehyde-fixed hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf for 90 min at 4 °C in buffer A in the absence (no additions) or presence of a 50-fold molar excess of unlabeled Lf (Lf), precolumn Triton X-100 extract of hepatocyte membranes (10% total volume; precolumn extract), EGTA-eluted p45-containing fraction (10% total volume; EGTA eluate), or postcolumn Triton X-100 extract of hepatocyte membranes unbound by Lf-agarose (10% total volume; flow-through). Cells were washed free of unbound ^{125}I-Lf and assayed for cell-associated radioactivity as described in Experimental Procedures. Values are means of duplicate samples; error bars = standard deviation.

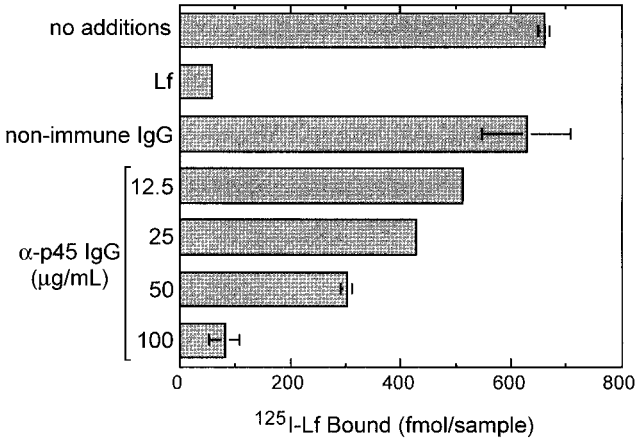

FIGURE 5: Competitive binding of anti-p45 IgG with Lf to intact hepatocytes. Isolated rat hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf at 4 °C for 90 min in buffer A without (no additions) or with a 100-fold molar excess of unlabeled Lf (Lf), rabbit non-immune IgG (100 μg/mL; non-immune IgG), or with anti-p45 IgG (α-p45) at the designated concentrations. Cells were washed and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent means of duplicate samples; error bars = standard deviations.

β-mercaptoethanol-reduced p45 migrated slightly slower than non-reduced p45, suggesting that p45 may contain intrachain disulfide bonds.

We tested the ability of non-immune and anti-p45 whole IgG to block the binding of ^{125}I-Lf at 4 °C to the Ca^{2+}-dependent sites on hepatocytes (Figure 5). We found that increasing amounts of anti-p45 IgG progressively reduced ^{125}I-Lf binding at 4 °C up to 71%, a degree of binding competition observed with a 100-fold molar excess of unlabeled Lf. An equivalent amount of non-immune IgG had essentially no effect on ^{125}I-Lf binding (Figure 5). To confirm that p45 mediates Lf endocytosis, we examined the ability of anti-p45 antibodies to block the binding and uptake of ^{125}I-Lf at 37 °C. We found that titration of anti-p45 serum progressively reduced total bound (surface and intracellular)

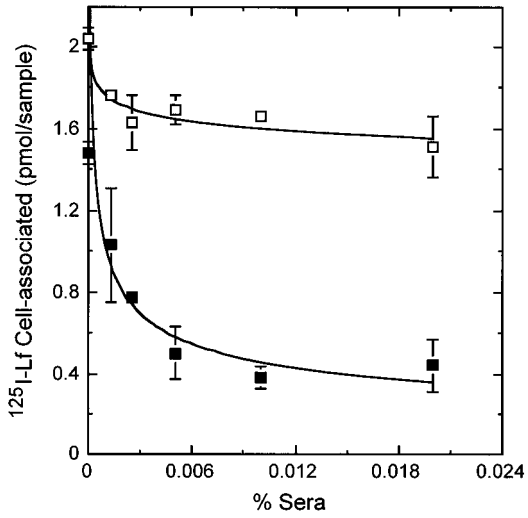

FIGURE 6: Efficacy of anti-p45 serum to block Lf endocytosis by isolated hepatocytes. Hepatocytes (2×10^6 cells/mL) were incubated with ^{125}I-Lf (2 μg/mL) in BME-BSA with or without pre-immune serum (□) or anti-p45 serum (■) at the designated concentrations (0−0.02%) at 37 °C for 45 min. The cells were chilled, washed twice in cold buffer B, 10 min per wash, and assayed for cell-associated radioactivity as described in Experimental Procedures. Values represent the means of duplicate samples; error bars reflect standard deviations from the means. Isotherms were drawn according to the following equations: non-immune serum (□): $y = 1.32x^{-0.042}$; anti-p45 serum (■): $y = 0.091x^{-0.35}$.

^{125}I-Lf by up to 75% as compared to pre-immune sera (Figure 6). Thus, antibodies specific for p45 blocked the binding and endocytosis of Lf by isolated rat hepatocytes.

Rat hepatocytes bind ferritin via a receptor with a mass of 30−45 kDa, and human Lf partially blocks binding of ferritin to this receptor immobilized on agarose (Mack et al., 1983). A human ferritin receptor with mass of 53 kDa has also been described (Adams et al., 1988). Because of these observations, we examined the ability of rat ferritin to compete with ^{125}I-Lf for binding to hepatocytes. In the absence of ferritin, cells bound 951 ± $68_{(n=3)}$ fmol ^{125}I-Lf/10^6 cells, whereas in the presence of a 50-fold molar excess of ferritin bound 873 ± $46_{(n=3)}$ fmol ^{125}I-Lf/10^6 cells; inclusion of a 50-fold molar excess of unlabeled Lf reduced ^{125}I-Lf binding to cells by 40% (572 ± $29_{(n=3)}$ fmol ^{125}I-Lf/10^6 cells). We conclude from these results that p45 is not the hepatic ferritin receptor previously identified. Rather, we interpret all the above findings to indicate that p45 is the Ca^{2+}-dependent hepatocyte Lf receptor.

DISCUSSION

We identified and isolated a 45-kDa hepatocyte membrane polypeptide that binds Lf in the presence of Ca^{2+}. Several lines of evidence suggest that p45 is the rat hepatocyte Ca^{2+}-dependent Lf receptor. (i) ^{125}I-Lf was cross-linked specifically to an adduct on intact cells with a molecular weight of ∼45 kDa only in the presence of Ca^{2+} (Figure 1). Moreover, ^{125}I-Lf bound to a polypeptide of ∼45 kDa in a Lf- and Ca^{2+}-dependent manner on nitrocellulose replicas of electrophoretically-separated whole hepatocyte membrane proteins (Figure 2). (ii) Chromatography of Triton X-100-extracted hepatocyte membrane proteins on Lf-agarose identified a cell surface 45-kDa polypeptide that eluted from Lf-agarose with the Ca^{2+} chelator EGTA (Figure 3). Specific, Ca^{2+}-dependent soluble Lf-receptor activity was present

Table 1: Summary of Lactoferrin Receptors' Characteristics

cells (species)	M_r (kDa)	Lf species	Lf affinity (K_d)	site density	Ca^{2+}-dependent	ref
T-lymphocytes (human, PHA-stimulated)	107, 115	human	83 nM	2×10^6 sites/cell	no	Mazurier et al., 1989
platelets (human)	105	human	?	?	no	Leveugle et al., 1993
intestinal mucosa (murine)	130	murine	0.3 μM	0.5×10^{12} sites/mg of protein	Ca^{2+}-stimulated	Hu et al., 1990; Hu et al., 1988
intestinal mucosa (human)	110 (37 kDa trimer)	human	0.3 μM	4×10^{14} sites/mg of protein	?	Kawakami & Lonnerdal, 1991
N. meningitidis	105	human	?	?	no	Schryvers & Morris, 1988
Aortic endothelial cells (bovine)	35	bovine	0.1 nM	3×10^4 sites/cell	no	Schmidt et al., 1994, 1992
hepatocytes (rat)	45	bovine	20 nM	1×10^6 sites/cell	yes	McAbee & Esbensen, 1991; this report

in Triton X-100 extracts that contained p45 and in p45-enriched fractions eluted from Lf-agarose by EGTA but was absent in detergent extracts depleted of p45 (Figure 4). Notably, the amount of p45 we routinely isolate from 10^9 hepatocytes (150−250 μg) is commensurate with the number of high-affinity, Ca^{2+}-dependent Lf binding sites present on isolated rat hepatocytes ($\sim 10^6$ sites/cell) (McAbee & Esbensen, 1991). (iii) Monospecific anti-p45 IgG blocked the binding and internalization of ^{125}I-Lf via Ca^{2+}-dependent sites on intact hepatocytes (Figure 6).

To date, LRP and heparan sulfate proteoglycan have been implicated as Lf receptors in hepatocytes. LRP is a heterotrimer composed of 420, 85, and 39 kDa subunits (Strickland et al., 1990). The large subunit binds α_2-macroglobulin (Meilinger et al., 1995), apo-E-bearing lipoproteins, plasminogen activator−inhibitor complexes (Willnow et al., 1992), and human and bovine Lf (Huettinger et al., 1992; Meilinger et al., 1995; Willnow et al., 1992) apparently by domains whose occupancy is regulated in part by the 85- and 39-kDa subunits (Willnow et al., 1992). Our results, however, did not implicate LRP in bovine Lf's interaction with hepatocytes. In DTSSP cross-linking studies, only the 125-kDa complex was observed in a DTSSP- and Ca^{2+}-dependent manner (Figure 1B,C). In addition, we did not detect ^{125}I-Lf binding to a high molecular weight species corresponding to LRP on ligand blots (Figure 2), nor did we find LRP-like species present in EGTA-eluted fractions of Lf-agarose (Figure 3). Human Lf binds LRP optimally at pH 5.8 (Meilinger et al., 1995) without Ca^{2+} (Ziere et al., 1992), so it is possible that ^{125}I-Lf blotting done at pH 9.0 (Figure 2) and Lf-agarose elutions done with EGTA at pH 7.5 (Figure 3) would reduce or preclude LRP's interaction with bovine Lf. Regardless, because LRP's binding to Lf is Ca^{2+}-independent, of relatively low affinity, and sensitive to polyanions, LRP cannot be the Ca^{2+}-dependent Lf receptor on hepatocytes. Heparan sulfate proteoglycan derived from rat hepatocytes binds Lf-agarose (Hu et al., 1993). A consensus glycoaminoglycan-binding domain has been mapped to a highly basic region of the N1 domain of human Lf (Mann et al., 1994; Wu et al., 1995). The interaction of heparan sulfate proteoglycan with Lf is blocked by polyanions (e.g., dextran sulfate) and other cationic proteins (e.g., lactoperoxidase; DeBanne et al., 1985; Hu et al., 1993), consistent with our findings that Ca^{2+}-independent binding of bovine Lf to hepatocytes is sensitive to dextran sulfate (McAbee & Esbensen, 1991; McAbee et al., 1993). We have also found that a tryptic fragment containing the C-lobe of bovine Lf (N-terminus at Ala^{339} of the mature protein), which lacks a glycosaminoglycan-binding consensus sequence, binds to the Ca^{2+}-dependent Lf binding sites on hepatocytes (Sitaram & McAbee, 1997). Also, we find that transferrin and Lf donate ^{59}Fe to hepatocytes by different delivery pathways (McAbee, 1995), consistent with the notion that heparan sulfate proteoglycan binding transferrin at the cell surface is not responsible for the binding and endocytosis of ^{59}Fe-Lf. From these sundry observations, we conclude that neither LRP nor cell surface heparan sulfate proteoglycan functions as the hepatocyte Ca^{2+}-dependent Lf receptor.

Lf-binding proteins have been identified in phytohemagglutinin-stimulated T-lymphocytes (Mazurier et al., 1989), platelets (Leveugle et al., 1993), various bacterial cells (Schryvers & Lee, 1993), intestinal mucosae (Hu et al., 1988; Kawakami & Lonnerdal, 1991), and endothelial cells that bind advanced glycation endproducts (Schmidt et al., 1994). Significant differences exist between these various Lf-binding proteins in terms of molecular weight, subunit composition, divalent cation binding requirements, and affinities and species specificities for Lf (Table 1). p45 reported here differs in molecular weight and endocytic activity from all Lf-binding proteins reported previously. We show in a companion study that p45 shares amino acid sequence homology and immunocrossreactivity with the RHL-1 subunit of the Gal/GalNAc receptor (Bennatt et al., 1997). Both human and bovine Lf bind to p45/RHL-1, and the interaction of Lfs with p45/RHL-1 appears to be independent of the presence of Gal on Lf's glycan chains. This finding supports the possibility that at least one of the subunits of the Gal/GalNAc receptor in rat hepatocytes may help clear Lf as well as Gal/GalNAc-terminated glycoconjugates from the circulation.

ACKNOWLEDGMENT

We thank Yuan Yuan Ling for her help in preparation of hepatocytes and Dr. Mort Fuchs for the use of his spectrophotometer. We also thank Dr. David Hyde for his helpful discussions during the course of this study. This work was supported by a grant from the National Institutes of Health (DK-43355).

REFERENCES

Adams, P., Powell, L., & Halliday, J. (1988) *Hepatology 8*, 719−721.

Bennatt, D. J., Ling, Y. Y., & McAbee, D. D. (1997) *Biochemistry 36*, 8367−8376.

Bennett, R. M., & Kokocinski, T. (1979) *Clin. Sci. 57*, 453−460.

Brock, J. (1995) *Immunol. Today 16*, 417−419.

DeBanne, M. T., Regoeczi, E., Sweeney, G. D., & Krestynski, F. (1985) *Am. J. Physiol. 248*, G463−G469.

Hashizume, S., Kuroda, K., & Murakami, H. (1987) *Methods Enzymol. 147*, 302−314.

Hu, W.-L., Mazurier, J., Sawatzki, G., Montreuil, J., & Spik, G. (1988) *Biochem. J. 249*, 435−441.

Hu, W. L., Regoeczi, E., Chindemi, P. A., & Bolyos, M. (1993) *Am. J. Physiol. 264*, G112−G117.
Huettinger, M., Retzek, H., Hermann, M., & Goldenberg, H. (1992) *J. Biol. Chem. 267*, 18551−18557.
Imber, M. J., & Pizzo, S. V. (1983) *Biochem. J. 212*, 249−257.
Ismail, M., & Brock, J. H. (1993) *J. Biol. Chem. 268*, 21618−21625.
Johnstone, A., & Thorpe, R. (1982) in *Immunocytochemistry in Practice*, pp 41−76, Blackwell Scientific Publications, Oxford.
Kawakami, H., & Lonnerdal, B. (1991) *Am. J. Physiol. 261*, G841−G846.
Levay, P. F., & Viljoen, M. (1995) *Haematologica 80*, 252−267.
Leveugle, B., Mazurier, J., Legrand, D., Mazurier, C., Montreuil, J., & Spik, G. (1993) *Eur. J. Biochem. 213*, 1205−1211.
Lonnerdal, B., & Iyer, S. (1995) *Annu. Rev. Nutr. 15*, 93−110.
Mack, U., Powell, L., & Halliday, J. (1983) *J. Biol. Chem. 258*, 4672−4675.
Mann, D. M., Romm, E., & Migliorini, M. (1994) *J. Biol. Chem. 269*, 23661−23667.
Mazurier, J., LeGrand, D., Hu, W. L., Montreuil, J., & Spik, G. (1989) *Eur. J. Biochem. 179*, 481−487.
McAbee, D. D. (1995) *Biochem. J. 311*, 603−609.
McAbee, D. D., & Esbensen, K. (1991) *J. Biol. Chem. 266*, 23624−23631.
McAbee, D. D., Nowatzke, W., Oehler, C., Sitaram, M., Sbaschnig, E., Opferman, J. T., Carr, J., & Esbensen, K. (1993) *Biochemistry 32*, 13749−13760.
Meilinger, M., Haumer, M., Szakmary, K. A., Steinbock, F., Scheiber, B., Goldenberg, H., & Huettinger, M. (1995) *FEBS Lett. 360*, 70−74.
Ray, D. A., & Weigel, P. H. (1985) *Anal. Biochem. 145*, 37−46.
Regoeczi, E., Chindemi, P. A., Debanne, M. T., & Prieels, J. P. (1985) *Am. J. Physiol. 248*, G8−G14.
Regoeczi, E., Chindemi, P. A., & Hu, W. L. (1994) *Hepatology 19*, 1476−1482.
Schmidt, A. M., Mora, R., Cao, R., Yan, S. D., Brett, J., Ramakrishnan, R., Tsang, T. C., Simionescu, M., & Stern, D. (1994) *J. Biol. Chem. 269*, 9882−9888.
Schryvers, A. B., & Lee, B. C. (1993) *J. Microbiol. Methods 18*, 255−266.
Seglen, P. O. (1973) *Exp. Cell Res. 82*, 391−398.
Sitaram, M. P., & McAbee, D. D. (1997) *Biochem. J. 323*, 815−822.
Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M., & Argraves, W. S. (1990) *J. Biol. Chem. 265*, 17401−17404.
Waugh, S. M., DiBella, E. E., & Pilch, P. F. (1989) *Biochemistry 28*, 3448−3455.
Weigel, P. H., Ray, D. A., & Oka, J. A. (1983) *Anal. Biochem. 133*, 437−449.
Willnow, T. E., Goldstein, J. L., Orth, K., Brown, M. S., & Herz, J. (1992) *J. Biol. Chem. 267*, 26172−26180.
Wu, H. F., Monroe, D. M., & Church, F. C. (1995) *Arch. Biochem. Biophys. 317*, 85−92.
Zeng, F. Y., Oka, J. A., & Weigel, P. H. (1996) *Glycobiology 6*, 247−255.
Ziere, G. J., Vandijk, M. C. M., Bijsterbosch, M. K., & Vanberkel, T. J. C. (1992) *J. Biol. Chem. 267*, 11229−11235.

BI963078U