Endocytosis and Degradation of Bovine Apo- and Holo-lactoferrin by Isolated Rat Hepatocytes Are Mediated by Recycling Calcium-Dependent Binding Sites†

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ABSTRACT: We characterized endocytosis of iron-saturated (holo) and iron-depleted (apo) ^{125}I-labeled bovine lactoferrin (Lf) by isolated rat hepatocytes. Hepatocytes ingested both Lf forms—determined by EGTA/dextran sulfate removal of surface-bound Lf—at maximal endocytic rates of 1.85 and 1.52 fmol cell^{-1} min^{-1} for ^{125}I-apo-Lf and ^{125}I-holo-Lf, respectively. First-order endocytic rate constants (37 °C) for ^{125}I-apo-Lf and ^{125}I-holo-Lf were 0.276 and 0.292 min^{-1}, respectively. Regardless of Lf's iron content, hyperosmotic media (~500 mmol/kg) inhibited Lf uptake by ~90%, indicating endocytosis of both Lf forms was primarily clathrin-dependent. Endocytosis of both Lf forms was not altered significantly in the presence of excess iron chelator desferrioxamine or rat holo-transferrin, or by cycloheximide treatment. Fluorescein isothiocyanate- and cyclohexanedione-modified Lf competed fully with native Lf for binding and endocytosis, indicating that, unlike human Lf, modification of lysine or arginine residues does not block the interaction of bovine Lf with cells. After binding Lf at 4 °C, cells at 37 °C internalized ~90% of Lf bound to Ca^{2+}-dependent sites but not Lf bound to Ca^{2+}-independent sites. Following uptake, hepatocytes released acid-soluble (degraded) products of ^{125}I-Lf biphasically at 37 °C, an initial rapid phase within the first 20 min—more pronounced with ^{125}I-holo-Lf—followed by a sustained linear release of 298 and 355 molecule equiv cell^{-1} min^{-1} for ^{125}I-apo-Lf and ^{125}I-holo-Lf, respectively. At 4 °C, both digitonin-permeabilized and intact cells bound ~1.1×10^6 ^{125}I-Lf molecules to Ca^{2+}-dependent sites per cell, indicating that hepatocytes do not contain a sizeable intracellular pool of these sites. Moreover, cells retained >70% of Ca^{2+}-dependent sites on the surface during sustained Lf endocytosis. Thus, these Lf binding sites recycle during endocytosis at an estimated 4–5 min/circuit.

Lactoferrin (Lf)[1] belongs to the transferrin family of nonheme iron-transport glycoproteins. While Lf is located predominantly in nonblood fluids, it is present at low steady-state concentrations (~20 nM) in mammalian blood, originating in specific granules of polymorphonuclear leukocytes. Blood Lf helps retrieve iron during turnover of senescent red blood cells (Courtoy et al., 1984; Moguilevsky et al., 1984; Retegui et al., 1984), acts as a negative regulator of myelopoiesis (Broxmeyer et al., 1980; Hangoc et al., 1987; Pelus et al., 1979; Zucali et al., 1989), and may provide nutrient iron to mitogen-stimulated T-cells (Mazurier et al., 1989; Rochard et al., 1989; Zimecki et al., 1991). The liver regulates Lf's steady-state concentration by rapidly clearing it from the circulation (Bennett & Kokocinski, 1979; Imber & Pizzo, 1983), and parenchymal and nonparenchymal cells internalize Lf although hepatocytes accumulate >90% of the circulating Lf (Courtoy et al., 1984; Imber & Pizzo, 1983; Regoeczi et al., 1985; Ziere et al., 1992). Elevation of plasma Lf may have several deleterious effects including neutropenia (Boxer et al., 1982), inhibition of hepatic uptake of β very-low-density lipoprotein-containing chylomicron remnants (Huettinger et al., 1988; Willnow et al., 1992), and inhibition of granulocyte macrophage colony stimulating factor production following trauma (Peterson et al., 1988). Thus, clearance of plasma Lf constitutes an important hepatic homeostatic function and may represent a significant iron-scavenging pathway. Hepatic uptake of Lf has been studied in whole animals, but endocytosis of Lf by isolated liver cells has not been examined.

We showed previously that isolated rat hepatocytes bind bovine apo- and holo-Lf to similar extents by the same binding sites (McAbee & Esbensen, 1991). These cells exhibit two classes of Lf binding sites: one binds Lf in a Ca^{2+}-dependent manner (K_d ~ 20 nM); the other binds Lf independent of divalent cations (K_d ~ 1 μM). Washing cells with EGTA removes Lf bound to the Ca^{2+}-dependent sites but not the Ca^{2+}-independent sites. On the other hand, the polyanion dextran sulfate strips Lf bound to the Ca^{2+}-independent sites but has no effect on Lf bound to Ca^{2+}-dependent sites. Lf binding is not inhibited by ligands specific for hepatocyte galactosyl and transferrin receptors or by cationic proteins such as lysozyme or cytochrome *c*. Hepatocytes exhibit upward of 1 million high-affinity, Ca^{2+}-dependent sites per cell surface, making them a prominent membrane component on these cells.

Unlike Lf which binds to hepatocytes regardless of its iron content, transferrin binds its receptor at neutral pH only in the diferric form (Dautry-Varsat et al., 1983). Transferrins undergo reversible configurational changes when an Fe^{3+} occupies or vacates the metal binding site (Anderson et al., 1987, 1989, 1990; Baker et al., 1987, 1991; Kilar & Simon, 1985; Norris et al., 1989; Shimazaki et al., 1992). Such a

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[1] Abbreviations: apo-Lf, iron-depleted lactoferrin; BME, basal medium Eagle's; BSA, bovine serum albumin; DHCH-Lf, cyclohexanedione-modified Lf; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FITC, fluorescein isothiocyanate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HBS, HEPES-buffered saline; HBSS, Hanks' buffered salt solution; holo-Lf, iron-saturated lactoferrin; K_d, dissociation constant; *k*, rate constant; Lf, lactoferrin.

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conformational change apparently alters the affinity between transferrin and its receptor—a key step in the recycling of transferrin during its delivery of iron to cells (Klausner et al., 1983). In this paper, we examined endocytosis of bovine Lf by isolated rat hepatocytes as a function of Lf's iron content. We found that hepatocytes take up Lf regardless of its iron content by similar rates and extents. Uptake of both forms of Lf was clathrin-dependent and mediated almost exclusively by the high-affinity, Ca^{2+}-dependent binding sites. Unlike human Lf, modification of bovine Lf's arginine residues with cyclohexanedione did not block its ability to compete with native Lf for binding and endocytosis by hepatocytes. We also show that the Ca^{2+}-dependent sites recycle during endocytosis while internalized Lf is degraded by cells.

EXPERIMENTAL PROCEDURES

Materials. Acrylamide, bis(acrylamide), bovine colostrum Lf, BSA (fraction V), deferoxamine mesylate (desferrioxamine), dextran sulfate (5000 Da), FITC (monomer I), HEPES, and rat diferric transferrin were purchased from Sigma (St. Louis, MO). Collagenase (types A and D) was obtained from Boehringer Mannheim (Indianapolis, IN). HEPES was also obtained from Fisher Biochemicals (Chicago, IL). $Na^{125}I$ (~17 Ci/mg of iodine) was from ICN Biomedicals, Inc. (Irvine, CA), or Amersham (Arlington Heights, IL). Digitonin was obtained from Eastman Kodak (Rochester, NY). Urea was from Serva (Heidelberg, FRG). Ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine were from BioRad (Richmond, CA). All other chemicals were reagent grade. HBSS and BME were obtained from GIBCo (Grand Island, NY) and Sigma. HBS contained 150 mM NaCl, 3 mM KCl, and 10 mM HEPES, pH 7.4. BME was 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.

Hepatocytes. Male Sprague-Dawley rats (150–350 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; Oka & Weigel, 1987). Cells were kept at ~30 °C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME–BSA were ≥85% viable and single cells. Prior to experiments, cell suspensions [(2–4) × 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. 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/10 mM KH_2PO_4, pH 7.2, was further purified by ion-exchange chromatography as described previously (McAbee & Esbensen, 1991). Apo-Lf was prepared by dialysis of Lf against citric acid (Masson & Heremans, 1968). Holo-Lf was prepared by dialysis of Lf against sodium carbonate/sodium citrate followed by incubation with $FeCl_3$ (Schryvers, 1988). Purified apo-Lf and holo-Lf were dialyzed against HBS, filter-sterilized (0.2 μm), and stored at –20 °C prior to use. Lf's iron content was determined spectroscopically (Brown & Parry, 1974; Hashizume et al., 1987) from its A_{465nm}:A_{280nm} ratios, which were ~0.002 and ~0.04, respectively, for routine apo-Lf and holo-Lf preparations. ^{125}I-Apo- and ^{125}I-holo-Lf, prepared by the "Iodogen" method (Fraker & Speck, 1978), had specific activities of 6–65 dpm/fmol. Homogeneity of radiolabeled and unlabeled apo- and holo-Lf used for all binding studies was confirmed by SDS–polyacrylamide gel electrophoresis (McAbee & Esbensen, 1991).

Differential Electrophoresis of Apo- and Holo-Lf. Apo-Lf and holo-Lf were analyzed for differential electrophoretic migration using a modification of the procedure of Makey and Seal (1976). Resolving gels consisted of 6% acrylamide/bis(acrylamide) (19:1 molar ratio) in 0.05 M Tris, 0.1 M boric acid, pH 9.0, 0.05 M EDTA, and 6 M urea. A loading gel positioned atop the resolving gel consisted of 3% acrylamide/bis(acrylamide) (29:1 molar ratio) in 0.23 M Tris-HCl, pH 8.8. Electrode buffer consisted of 0.05 M Tris, 0.1 M boric acid, pH 9.0, and 0.05 M EDTA. Lf samples in 0.05 M Tris, 0.1 M boric acid, pH 9.0, 0.05 M EDTA, and 10% glycerol were electrophoresed on a Mini-Protean II gel system (BioRad) at constant voltage for ~1200 V-h and then stained with Coomassie Blue. Electrophoresed ^{125}I-Lf samples were detected by autoradiography following exposure to Kodak X-Omat film at –70 °C.

FITC-Lf Preparation. Purified bovine Lf (10 mg/mL in 0.1 M $NaHCO_3$, pH 9.5) was incubated with 0.25 mM FITC (monomer I) with stirring at 22 °C for 1 h. The reaction was stopped by applying the protein–FITC mixture to a GF-5 desalting column (Pierce Chemical Co.) equilibrated in 0.1 M $NaHCO_3$, pH 9.5. Lf-containing fractions were dialyzed against HBS and analyzed spectroscopically at 496 nm (FITC) and 280 nm (protein). FITC incorporation into Lf—calculated as described elsewhere (Jobbagy & Kiraly, 1966)—was 2 mol of FITC per mole of Lf. FITC-Lf was filter-sterilized (0.2 μm) and stored at –20 °C prior to use.

DHCH-Lf Preparation. Purified bovine Lf was derivatized with cyclohexanedione as described elsewhere (Huettinger et al., 1992). Briefly, ~20 mg of bovine Lf in 3 mL of 0.15 M NaCl was supplemented with 1 mL of 0.4 M borate buffer, pH 9.0, containing 1.2 mM 1,2-cyclohexanedione and incubated 4 h at 37 °C. Under these conditions, argininyl residues are converted stably to N^7,N^8-(1,2-dihydroxycyclohex-1,2-ylene)-L-arginine (DHCH-arginine; Smith, 1966). The modified Lf (DHCH-Lf) was dialyzed against 20 mM borate, pH 8.0, and stored at –20 °C prior to use. DHCH-Lf was analyzed for the extent of argininyl modification by electrophoresis of native and modified Lf on a vertical 5% acrylamide/bis(acrylamide) slab gel (19:1 molar ratio) buffered with 0.77 mM Tris–hippurate, pH 8.8 (Ziere et al., 1992). Protein bands were detected by Coomassie Blue or silver staining (Merril et al., 1980) and quantitatively analyzed using an LKB UltraScan laser densitometer and LKB GelScan XL software (LKB Pharmacia). Native Lf migrated as a single band under these conditions, whereas DHCH-Lf migrated as two bands, one (~30% of total) comigrating with native Lf and the other (~70% of total) significantly more acidic than native Lf. DHCH-Lf was also analyzed for the loss of argininyl residues by complete acid hydrolysis (6 N HCl, 24 h, 110 °C *in vacuo*) and subsequent fractionation on a Bio-Sil ODS 5S (BioRad) reverse-phase HPLC column (Notre Dame Biocore Facility). By this analysis, 78.4% of the argininyl residues in DHCH-Lf had been modified by cyclohexanedione treatment compared to untreated control bovine Lf.

Lf Binding, Endocytosis, and Degradation Assays. For Lf binding assays, cells [(2–4) × 10^6 cells/mL] were routinely incubated with ^{125}I-Lf at 4 °C for 90 min in HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Cells were washed free of unbound ^{125}I-Lf by centrifugation in excess binding buffer twice, 10 min per wash. In some instances, wash buffer was also supplemented with 0.1% (w/v) dextran sulfate to strip ^{125}I-Lf bound to Ca^{2+}-independent Lf binding sites. After being washed, cells were resuspended in 0.5–1.0 mL of wash buffer, transferred to clean plastic tubes, and assayed for

radioactivity and protein. To measure hepatocyte endocytosis of Lf, cells [(2–4) × 10^6 cells/mL of BME–BSA] were incubated with Lf at 37 °C as described in the figure legends. Uptake was stopped rapidly by diluting the cells into a 5–10-fold excess volume of ice-cold HBSS or HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. To assay total cell-associated (surface and intracellular) Lf, cells were washed by centrifugation twice in 2–4 mL of cold HBSS or in HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. To assay internalized Lf only, cells were washed by centrifugation twice, 10 min each wash, in 2–4 mL of 0.1% (w/v) dextran sulfate and 5 mM EGTA in either HBSS or HBS at 4 °C. After cells were washed, they were resuspended in 0.5–1.0 mL of wash buffer, transferred to clean plastic tubes, and assayed for radioactivity and protein. To measure degraded ^{125}I-Lf released from hepatocytes following endocytosis at 37 °C, samples of cell-free incubation media (clarified by centrifugation) were added to a 2× volume of ice-cold 10% phosphotungstic acid in 2 N HCl, mixed and kept on ice ≥15 min, and then pelleted by centrifugation (4 min, 4 °C, 12000*g*). Supernatants were recovered and assayed for acid-soluble (degraded) radioactivity. In some experiments, pellets were also assayed for acid-precipitable radioactivity. Routinely, 95–99% of intact ^{125}I-Lf is precipitated under these conditions.

General. Protein was determined by the bicinchoninic acid protein assay procedure (Smith et al., 1985) using BSA as standard (Pierce Chemical Co.). Hepatocytes contain 1.1 mg of protein/10^6 cells. 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). Osmolality was determined using a Wescor 5500 vapor pressure osmometer (Wescor, Inc., Logan, UT). Spectral analysis of Lf was performed using a Beckman DU-64 spectrophotometer.

RESULTS

Effect of Desferrioxamine on Hepatocyte Binding and Endocytosis of Lf. A critical question regarding iron-transport proteins in general is whether or not they require bound metal for endocytosis by cells. For example, serum transferrin binds cells at neutral pH only in the diferric form (Dautry-Varsat et al., 1983; Klausner et al., 1983). We found previously that hepatocytes bound Lf regardless of Lf's iron content (McAbee & Esbensen, 1991). It was possible, however, that apo-Lf readily absorbed some Fe^{3+} present in the reaction mixture during its incubation with cells. If Lf binding to hepatocytes is iron-dependent, then absorption of free Fe^{3+} by apo-Lf would promote its binding to cells. To eliminate this possibility, we examined the effect of desferrioxamine, an iron chelator, on the binding of ^{125}I-apo-Lf and ^{125}I-holo-Lf to hepatocytes at 4 °C (Figure 1). In the absence of desferrioxamine, hepatocytes bound similar amounts ^{125}I-apo-Lf and ^{125}I-holo-Lf at 4 °C (Figure 1). We found that despite the presence of desferrioxamine in the incubation medium up to 1 mM (≤50 000-fold molar excess over ^{125}I-Lf present), hepatocytes bound a constant amount of ^{125}I-apo-Lf and ^{125}I-holo-Lf. While ^{125}I-apo-Lf binding to cells at 4 °C was essentially unaltered by 1 mM desferrioxamine, hepatocytes at 37 °C internalized 28% less ^{125}I-apo-Lf than untreated cells (Table I). This minor difference reflected reduced uptake rather than reduced surface binding since treated and untreated cells bound similar amounts of ^{125}I-apo-Lf on their surfaces following 37 °C incubation. Desferrioxamine reduced ^{125}I-holo-Lf endocytosis by hepatocytes only very slightly. We conclude that desferrioxamine added to the incubations to chelate any free Fe^{3+} present in the incubation does not substantially alter apo- or holo-Lf binding and uptake by hepatocytes.

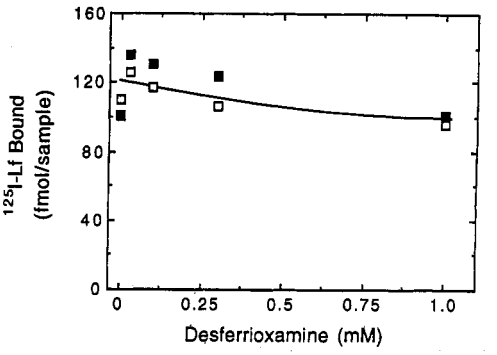

FIGURE 1: Effect of desferrioxamine on binding of ^{125}I-Lf to hepatocytes. Cells (10^6/0.5 mL of HBSS) were incubated with 1 μg/mL (13 nM) ^{125}I-apo-Lf (□) or ^{125}I-holo-Lf (■) at 4 °C with the designated concentrations of desferrioxamine, washed in the presence of dextran sulfate, and assayed for cell-associated radioactivity as described under Experimental Procedures. Symbols represent the mean of duplicate samples that differed by ≤5%.

To confirm that apo-Lf retained its iron-free status following incubation with hepatocytes, we examined ^{125}I-apo-Lf and ^{125}I-holo-Lf stripped from hepatocytes for iron content using a modified procedure of Makey and Seal (1976) which differentiates electrophoretically the metal-induced conformation changes in transferrin structure (Anderson et al., 1987, 1989, 1990; Baker et al., 1987, 1991; Kilar & Simon, 1985; Norris et al., 1989; Shimazaki et al., 1992). Briefly, hepatocytes were incubated with ^{125}I-apo-Lf or ^{125}I-holo-Lf at 4 °C in the presence of desferrioxamine. Bound ^{125}I-Lf was stripped off cells by EGTA/dextran sulfate wash, concentrated, and analyzed electrophoretically (Figure 2). Holo-Lf migrated substantially faster than apo-Lf due to its more compact structure. ^{125}I-Lf recovered from hepatocytes showed separation patterns similar to unlabeled proteins although the bands were more diffuse. Notably, ^{125}I-apo-Lf showed a slower migration pattern (Figure 2, lane 3), suggesting that most, if not all, apo-Lf had not bound free Fe^{3+}. Taken together, these results confirm that hepatocytes bind apo-Lf as well as holo-Lf and show that binding to cells at 4 °C did not induce significant changes in the iron content of the two Lf forms.

Kinetics of Lf Binding and Endocytosis. Even though isolated hepatocytes bind apo-Lf and holo-Lf similarly at 4 °C, cells may not handle the two Lf forms identically under endocytically-permissive temperatures. We examined the kinetics of hepatocyte accumulation of apo-Lf and holo-Lf at 37 °C (Figure 3). In the continuous presence of ^{125}I-Lf (0.66 μM), hepatocytes bound ^{125}I-apo-Lf and ^{125}I-holo-Lf at 37 °C with similar nonlinear kinetics (Figure 3A), and ≥50% of the total bound ligand was intracellular at all incubation times. First-order rate analysis revealed that hepatocytes accumulated Lf biphasically, and the rates of ^{125}I-apo-Lf and ^{125}I-holo-Lf uptake were virtually identical (Figure 3B). As seen in Table II, the continuous uptake of ^{125}I-apo-Lf and ^{125}I-holo-Lf was not blocked by a 100-fold molar excess of diferric rat transferrin nor by treatment with cycloheximide used to block *de novo* protein synthesis. Under these conditions, cycloheximide-treated hepatocytes bound and internalized ~2 million ^{125}I-Lf molecules cell^{-1} h^{-1} (>76% of control untreated cells), indicating that Lf uptake was largely independent of protein synthesis.

Table I: Effect of Desferrioxamine on Apo- and Holo-Lf Binding and Uptake[a]

		^{125}I-Lf bound (pmol/sample)			
		apo-Lf		holo-Lf	
temp (°C)	[DFO] (mM)	total	internal (%)[b]	total	internal (%)[b]
4	0	8.55 ± 0.10	0.36 ± 0.01 (4.7)	6.10 ± 0.35	0.25 ± 0.02 (4.9)
	1	7.59 ± 0.03	0.32 ± 0.01 (3.9)	5.67 ± 0.04	0.23 ± 0.05 (3.5)
37	0	30.64 ± 0.01	23.93 ± 0.03 (78.1)	27.23 ± 0.64	21.18 ± 0.43 (77.9)
	1	23.88 ± 0.46	17.31 ± 0.06 (72.4)	23.79 ± 0.11	20.45 ± 0.13 (86.1)

[a] Cells (2.7×10^6 cells/mL of BME–BSA) with or without desferrioxamine (DFO) were incubated with 10 μg/mL ^{125}I-apo-Lf or ^{125}I-holo-Lf at the designated temperature for 1 h. The cells were chilled, and samples (2.7×10^6 cells) were assayed for cell-associated and internalized radioactivity as described under Experimental Procedures. Values represent the mean of duplicate samples ± the standard deviation. [b] Values in parentheses represent the percent of total bound ^{125}I-Lf that was intracellular.

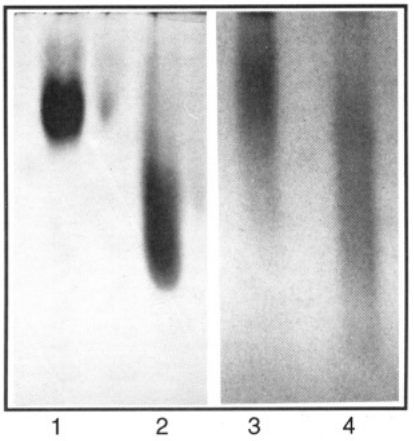

FIGURE 2: Differential native gel electrophoresis of apo-Lf and holo-Lf. Hepatocytes (4×10^6 cells/mL) were incubated with 10 μg/mL ^{125}I-apo-Lf or ^{125}I-holo-Lf at 4 °C for 90 min and then washed in the presence of 0.1% (w/v) dextran sulfate. Bound ^{125}I-Lf was stripped off cells with cold HBS containing 5 mM EGTA and 0.1% (w/v) dextran sulfate; stripped material was concentrated by ultrafiltration (10 000-Da cutoff). Unlabeled Lf (lanes 1 and 2) and ^{125}I-Lf recovered from hepatocytes (lanes 3 and 4) were analyzed by electrophoresis in Tris/borate/EDTA/urea acrylamide gels as described under Experimental Procedures. Apo-Lf, lanes 1 and 3; holo-Lf, lanes 2 and 4.

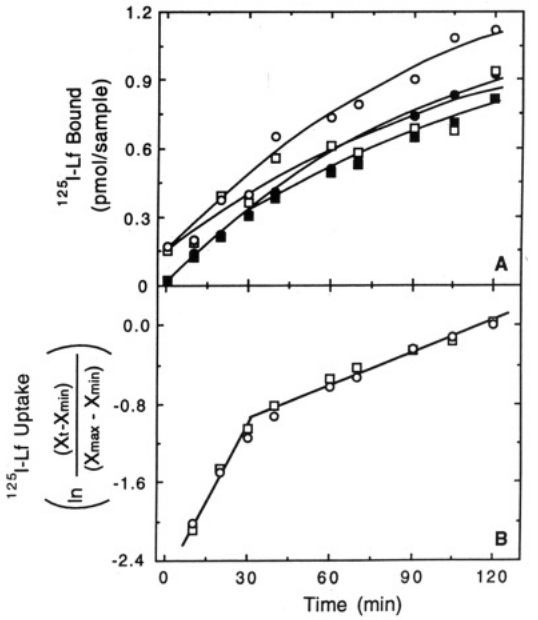

FIGURE 3: Kinetics of apo-Lf and holo-Lf binding and uptake. (A) Cells (2×10^6 cells/mL of BME–BSA) were incubated with 50 μg/mL ^{125}I-apo-Lf (□, ■) or ^{125}I-holo-Lf (○, ●) at 37 °C in the presence of 1 mM desferrioxamine (apo-Lf only). At the designated times, cells were assessed for cell-associated (□, ○) and intracellular radioactivity (■, ●) and cellular protein as described under Experimental Procedures. (B) First-order rate constants for Lf uptake were determined using the equation $\ln[(X_t - X_{min})/(X_{max} - X_{min})] = k_{in}t$, where X_t is Lf uptake at time t, X_{min} is Lf uptake at $t = 0$, X_{max} is maximal Lf uptake during the time course, and k_{in} is the first-order rate constant. Isotherms were determined by linear regression analysis. Half-times for Lf uptake were calculated using the equation $\ln 0.5 = k_{in}t_{1/2}$. For the initial phase, $r = 0.98$, $k_{in} = 0.039$ min^{-1}, and $t_{1/2} = 17.8$ min; for the latter phase, $r = 0.98$, $k_{in} = 0.009$ min^{-1}, and $t_{1/2} = 77$ min.

Isolated rat hepatocytes internalize macromolecules by clathrin-dependent and -independent pathways (England et al., 1986; Oka et al., 1989; Thirion & Wattiaux, 1988), the former of which can be selectively inhibited by hyperosmolality (Daukas & Zigmond, 1985; Heuser & Anderson, 1989; Oka et al., 1989). We examined, therefore, if uptakes of apo- and holo-Lf by hepatocytes were differentially dependent upon an intact clathrin-coated pit endocytic pathway by titrating Lf endocytosis as a function of media hyperosmolality. Hepatocytes were incubated with either ^{125}I-apo-Lf or ^{125}I-holo-Lf at 37 °C for 60 min in BME–BSA supplemented with various amounts of sucrose to increase media osmolality and then assayed for total cell-associated and intracellular radioactivity (Figure 4). The amounts of ^{125}I-Lf bound (surface and intracellular) and internalized by cells decreased in parallel as media osmolality increased. At osmolalities >500 mmol/kg, ^{125}I-apo-Lf and ^{125}I-holo-Lf uptakes were almost completely blocked. The dose–response curves for ^{125}I-apo-Lf and ^{125}I-holo-Lf uptake were very similar, indicating that Lf uptake was clathrin-dependent regardless of Lf's iron content. Notably, hepatocytes bound fairly constant amounts of ^{125}I-Lf to their surfaces regardless of the media osmolality (Figure 4, dashed lines), and cells bound equivalent amounts of ^{125}I-Lf when incubated either at 4 °C in normal medium or at 37 °C in hyperosmotic medium (≥500 mmol/kg). These data indicated that hyperosmotic conditions *per se* did not alter Lf binding but rather blocked ligand internalization. We conclude from these data (Figures 3 and 4, Table II) that hepatocytes do not distinguish between the iron-free and iron-bound forms of Lf for continuous binding and uptake at 37 °C.

Determination of Endocytically-Competent Lf Binding Sites. Because hepatocytes bind Lf *via* low- (Ca^{2+}-independent) and high-affinity (Ca^{2+}-dependent) sites, we determined if one or both populations of Lf binding sites mediated endocytosis. We incubated cells at 4 °C with ^{125}I-apo-Lf (Figure 5A,B) or ^{125}I-holo-Lf (Figure 5C,D) so as to occupy both high- and low-affinity sites on the cell surface. Cells were washed in the presence of either EGTA or Ca^{2+} and dextran sulfate, thereby generating two populations of cells: (i) those with ^{125}I-Lf bound to low-affinity sites only (EGTA-washed; Figure 5A,C); (ii) those with ^{125}I-Lf bound to high-affinity sites only (dextran sulfate-washed; Figure 5B,D). When these cells were incubated at 37 °C, we found that regardless of Lf's iron content, low-affinity Ca^{2+}-independent

Table II: Effect of Various Agents on ^{125}I-Lf Endocytosis[a]

	^{125}I-Lf bound (pmol/sample)			
	apo-Lf		holo-Lf	
treatment	total	internal (%)[b]	total	internal (%)[b]
none	10.52 ± 0.24	10.25 ± 0.02 (98.1)	10.70 ± 0.09	10.16 ± 0.40 (95.3)
holo-Tf	9.83 ± 0.03	9.11 ± 0.21 (92.9)	11.11 ± 0.53	9.93 ± 0.21 (89.2)
cycloheximide	8.81 ± 0.21	7.77 ± 0.14 (88.6)	9.04 ± 0.38	8.09 ± 0.27 (90.0)

[a] Cells (2.4 × 10^6 cells/mL of BME–BSA) were incubated with 10 μg/mL (0.13 μM) ^{125}I-apo-Lf or ^{125}I-holo-Lf at 37 °C for 1 h with or without rat holo-transferrin (13 μM) or cycloheximide (20 μM). The cells were chilled, washed in the presence of 0.1% (w/v) dextran sulfate, and assayed for cell-associated and internalized radioactivity as described under Experimental Procedures. Each sample contained 2.4 × 10^6 cells, and values represent the mean of duplicates ± the standard deviation. [b] Values in parentheses represent the percent of total bound ^{125}I-Lf that was intracellular.

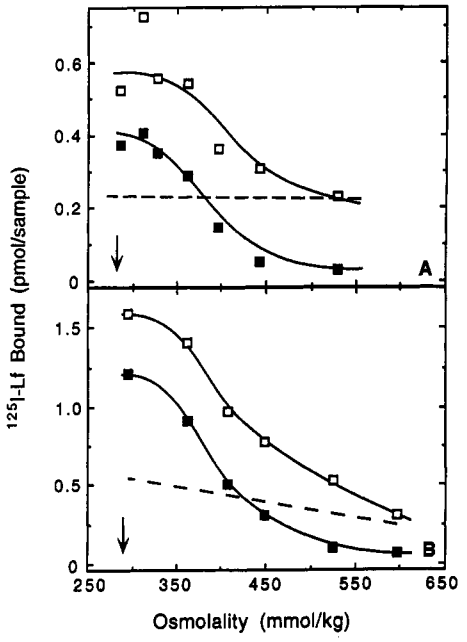

FIGURE 4: Effect of hyperosmolality on apo-Lf and holo-Lf endocytosis. Cells (2 × 10^6 cells/mL) were preincubated in BME–BSA supplemented with 0–0.3 M sucrose for 10 min at 37 °C and then with 1 μg/mL ^{125}I-apo-Lf (A) or ^{125}I-holo-Lf (B) for another 30 min at 37 °C. The cells (2 × 10^6/sample) were assayed for total cell-associated (□) and intracellular radioactivity (■) as described under Experimental Procedures. The amount of surface-bound ^{125}I-Lf (dashed lines) was determined by subtracting the intracellular radioactivity from the total cell-associated radioactivity, and isotherms were generated by linear regression analysis. The osmolality of normal BME–BSA is ~280 mmol/kg (arrows). Symbols represent the mean of duplicate samples (10^6 cells/sample) that differed by ≤15%. Experiments using the two types of Lf were performed with different cell preparations.

sites (EGTA-washed cells, Figure 5A,C) mediated very little Lf uptake. Rather, these cells released most of their prebound Lf into the incubation medium within 10 min after warm-up in an acid-precipitable form (data not shown), suggesting that this Lf was sloughed intact from the cell surface. In contrast, cells with Lf bound to high-affinity, Ca^{2+}-dependent sites (dextran sulfate-washed cells, Figure 5B,D) retained most of the prebound ^{125}I-Lf when incubated at 37 °C, and ≥85% of bound ^{125}I-Lf was internalized shortly after warm-up. It is possible that some ^{125}I-Lf released from the low-affinity sites following warm-up entered cells by binding to unoccupied high-affinity sites, thereby accounting for the low level of Lf uptake observed for the EGTA-washed cells. These data provide strong evidence that the high-affinity Ca^{2+}-dependent Lf binding sites mediate endocytosis via *bona fide* Lf receptors. The low-affinity Lf binding sites may represent nonspecific anionic surface molecules that bind Lf electrostatically but mediate its uptake poorly or not at all.

To determine the maximal endocytic rates exhibited by Ca^{2+}-dependent Lf binding sites, we examined Lf uptake by Ca^{2+}-dependent Lf binding sites as a function of Lf concentration and iron content (Figure 6). Binding and uptake of ^{125}I-Lf by hepatocytes exhibited saturation kinetics at Lf concentrations >40 μg/mL (Figure 6A). Maximal binding calculated from double-reciprocal analysis of these data (Figure 6B, inset) was 111 μg of ^{125}I-apo-Lf/mL and 91 μg of ^{125}I-holo-Lf/mL. Under these conditions, >90% of the Lf bound by the hepatocytes was internalized (Figure 6B) regardless of the Lf concentration tested. Maximal endocytic rates were 1.85 fmol of ^{125}I-apo-Lf cell^{-1} min^{-1} (r = 1.00) and 1.52 fmol of ^{125}I-holo-Lf cell^{-1} min^{-1} (r = 0.99). These values correspond to 5200 molecules of apo-Lf cell^{-1} s^{-1} and 4200 molecules of holo-Lf cell^{-1} s^{-1}.

Endocytosis and Degradation of Lf Prebound to Hepatocytes. To better understand the dynamics of Lf uptake and processing by hepatocytes, we characterized the uptake of a single wave of apo-Lf and holo-Lf at 37 °C. Cells were incubated at 4 °C with ^{125}I-Lf, washed with dextran sulfate to remove Lf from low-affinity sites, and then assayed for uptake at various times following incubation at 37 °C (Figure 7). Regardless of Lf's iron content, cells internalized ≥90% of prebound ^{125}I-Lf rapidly within 5 min after warm-up (Figure 7A), accumulating ~20% more ^{125}I-holo-Lf than ^{125}I-apo-Lf. First-order endocytic rate constants for the two Lf forms were not significantly different (^{125}I-apo-Lf, k_{in} = 0.276 min^{-1}; ^{125}I-holo-Lf, k_{in} = 0.292 min^{-1}; $t_{0.05,14}$; Figure 7B).

Radiolabeled Lf injected intravenously is cleared by the liver, and degradation products are released over time (Bennett & Kokocinski, 1979; Karle et al., 1979); human Lf internalized by rat liver is delivered to lysosomes and hydrolyzed (Ziere et al., 1992). We examined the kinetics and extent of Lf degradation by hepatocytes as a function of Lf's iron content. Hepatocytes were prebound with ^{125}I-Lf at 4 °C, washed in the presence of dextran sulfate, and then incubated at 37 °C (Figure 8). Cells released ~15% of prebound ^{125}I-Lf in an acid-precipitable form within the first 10 min at 37 °C, which did not increase significantly thereafter. Regardless of Lf's iron content, hepatocytes released degraded Lf products in a biphasic manner with an early rapid rate of release during the first 30 min of incubation followed by a prolonged slower rate of release through the rest of the time course. The long-term kinetics of release, however, were very similar for the two Lf forms: 300 and 350 molecules cell^{-1} min^{-1} for, respectively, ^{125}I-apo-Lf and ^{125}I-holo-Lf. Differential degradation was due to the rapid initial burst during which cells released more degraded ^{125}I-holo-Lf than degraded ^{125}I-apo-Lf. By 3.5 h of incubation at 37 °C, cells had released ~30% of prebound ^{125}I-holo-Lf but only 20% of prebound ^{125}I-apo-Lf in the form of acid-soluble radioactivity. Similar kinetics were obtained regardless of the amount of Lf prebound to the cells (data not shown).

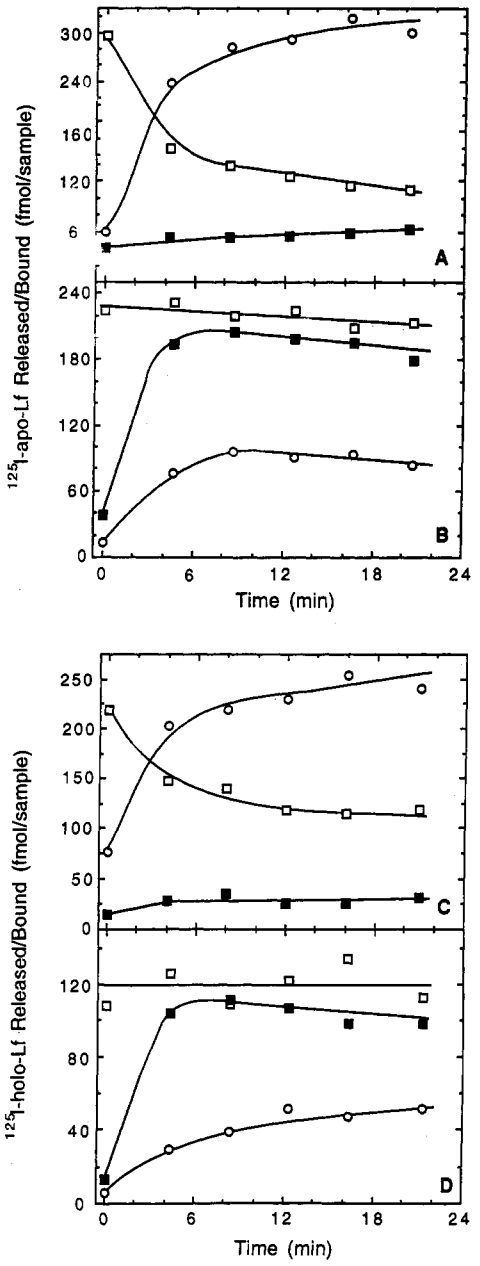

FIGURE 5: Analysis of high- and low-affinity Lf binding sites for endocytosis. Cells (2×10^6 cells/mL) were incubated with 5 μg/mL ^{125}I-apo-Lf (A, B) or ^{125}I-holo-Lf (C, D) for 90 min at 4 °C, and then washed twice, 10 min per wash, at 4 °C with HBS supplemented with either 5 mM EGTA (A, C) or 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.1% (w/v) dextran sulfate (B, D). All cells were rinsed once in excess fresh cold BME–BSA (BME contains 1 mM $CaCl_2$) resuspended at the original density in fresh cold BME–BSA and then incubated at 37 °C. At the designated times, cells were collected by centrifugation and assayed for total cell-associated (□) and internalized (■) radioactivity as described under Experimental Procedures; supernatants were assayed for released radioactivity (○). Symbols represent means of duplicate samples that differed by ≤10%. Experiments using the two types of Lf were performed using different cell preparations.

When we examined the degradation of internalized Lf as a function of the amount of Lf prebound to the cells, we found that cells released more acid-soluble radioactivity derived from ^{125}I-holo-Lf than from ^{125}I-apo-Lf (Figure 9). This difference was due largely to the higher initial release of degradation products from ^{125}I-holo-Lf compared to ^{125}I-apo-Lf; the long-term degradation kinetics for the two Lf forms were similar (data not shown). In addition, the extent of ^{125}I-Lf degradation increased linearly with the amount of ^{125}I-Lf prebound to the cells (≤500 000 molecules bound/cell) but was substantially greater for holo-Lf than for apo-Lf.

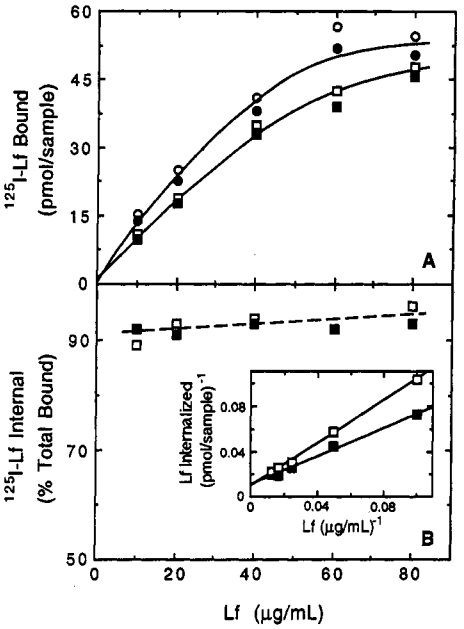

FIGURE 6: Endocytosis of Lf as a function of Lf concentration. (A) Cells (3.6×10^6 cells/mL of BME–BSA) in the presence of 1 mM desferrioxamine were incubated at 37 °C with the designated concentration of ^{125}I-apo-Lf (□, ■) or ^{125}I-holo-Lf (○, ●) for 60 min and then chilled on ice and assayed for cell-associated (□, ○) and intracellular radioactivity (■, ●) as described under Experimental Procedures. Symbols represent means of duplicate samples (3.6×10^6 cells/sample) that differed by ≤3%. (B) The percent of total cell-associated radioactivity that was intracellular determined in (A) was calculated according to the equation ([intracellular Lf]/[total cell-associated Lf]) × 100 for ^{125}I-apo-Lf (□) and ^{125}I-holo-Lf (■). Maximal endocytic rates were derived from double-reciprocal (inset) and regression analyses of these data. For apo-Lf (□), $m = 0.937$, $b^{-1} = 111.1$ μg/mL, and $r = 1.000$. For holo-Lf (■), $m = 0.620$, $b^{-1} = 90.9$ μg/mL, and $r = 0.997$.

Effect of Arginine and Lysine Modification on Lf Binding and Endocytosis. Human phytohemagglutinin-activated lymphocytes bind human Lf via a 105-kDa cell-surface glycoprotein with high affinity (Mazurier et al., 1989). FITC derivatization of human Lf (fluorescein:protein molar ratio 1:1) diminished Lf binding to these cells, suggesting that one or more lysines are at or near the lymphocyte binding domain of human Lf or affect significantly the protein's conformation (Legrand et al., 1991). Other studies have suggested that Lf can bind hepatocytes by way of the cell-surface proteoglycan heparan sulfate (Hu et al., 1993), the hepatocyte chylomicron remnant receptor (Huettinger et al., 1992; Ziere et al., 1992), and the low-density lipoprotein receptor-related protein (Willnow et al., 1992). In three of these reports, cyclohexanedione modification of human Lf's arginine side chains reduced derivatized Lf's ability to compete with native Lf for clearance by the liver or binding to hepatocytes (Hu et al., 1993; Huettinger et al., 1992; Ziere et al., 1992), suggesting that arginine side chains comprise at least a part of the hepatocyte recognition domain of Lf. Because low-affinity Lf binding sites may depend upon the cationic nature of Lf for binding but do not mediate its endocytosis, we determined if Lf modified either in its lysines or in its arginines blocked the binding and uptake of native Lf by hepatocytes by way of the Ca^{2+}-dependent high-affinity Lf binding site.

Hepatocytes were incubated at 4 or 37 °C with ^{125}I-Lf with or without a 100-fold molar excess of native Lf, FITC-Lf (fluorescein:protein molar ratio 2:1), or DHCH-Lf (~80% of arginines modified). As seen in Table III, excess native Lf

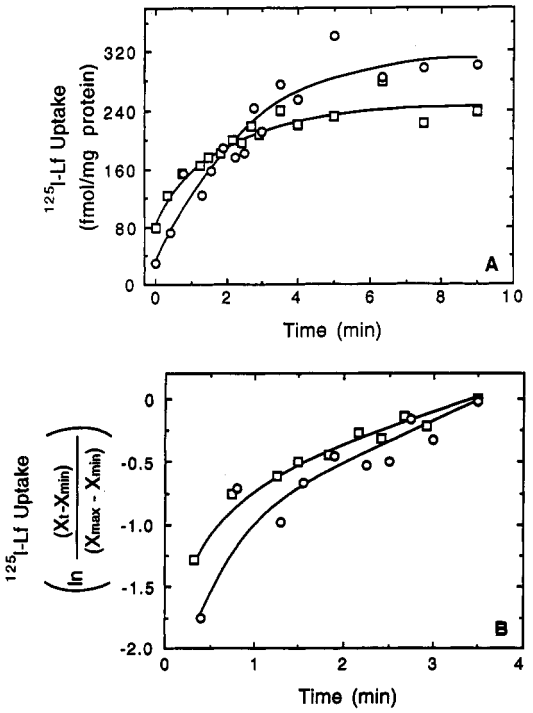

FIGURE 7: Kinetics of uptake of apo-Lf and holo-Lf prebound to cells at 4 °C. (A) Cells (2.7×10^6 cells/mL of BME–BSA) were incubated with 5 μg/mL ^{125}I-apo-Lf (□) or ^{125}I-holo-Lf (○) in the presence of 1 mM desferrioxamine for 90 min on ice and then washed in the presence of 0.1% (w/v) dextran sulfate to remove unbound ligand. After the cells were sampled for total cell-associated radioactivity, they were incubated at 37 °C. At the designated times, cells were assayed for internalized radioactivity and cell protein as described under Experimental Procedures. The amount of Lf bound to cells after the 4 °C incubation prior to warm-up was 263 ± 22 fmol of ^{125}I-apo-Lf/mg of protein and 247 ± 1 fmol of ^{125}I-holo-Lf/mg of protein (± standard deviation). (B) First-order rate constants and half-times for uptake of ^{125}I-apo-Lf (□) or ^{125}I-holo-Lf (○) were determined as described in the legend to Figure 3. Samples assayed during the 37 °C incubation at ≥0.75 min were used for calculation of rate constants by regression analysis. Slopes generated were analyzed for statistical difference using a Student's t-test and found not to be significantly different ($t_{0.05,14}$). For apo-Lf, k_{in} = 0.276 min^{-1}, r = 0.97, and $t_{1/2}$ = 2.5 min. For holo-Lf, k_{in} = 0.292 min^{-1}, r = 0.88, and $t_{1/2}$ = 2.4 min.

reduced ^{125}I-Lf binding to both low- and high-affinity sites at 4 °C by 40–50% as assessed by washing cells without dextran sulfate. To determine Lf's competitive ability for binding Ca^{2+}-dependent high-affinity binding sites alone, cells were washed with dextran sulfate to remove Lf bound to low-affinity sites. Under these conditions, Lf reduced ^{125}I-Lf binding to the high-affinity sites by ~90%. Using these different washing conditions to distinguish between the two binding site populations, we found that excess FITC-Lf reduced total ^{125}I-Lf binding to cells by ~25% but binding of ^{125}I-Lf to the high-affinity sites alone by 82%. In addition, native and FITC-Lf competed equally well with ^{125}I-Lf for binding and uptake by cells at 37 °C, reducing ^{125}I-Lf uptake by ~90%. Because hepatocytes internalize Lf by the high-affinity Ca^{2+}-dependent sites only, these data provide strong evidence that FITC modification of bovine Lf lysine residues did not alter its ability to be recognized and endocytosed by hepatocytes.

We found that a 100-fold molar excess of DHCH-Lf reduced the total ^{125}I-Lf bound to cells by 77% but reduced ^{125}I-Lf bound to the high-affinity sites alone by 85% (Table III). Similarly, both excess native Lf and DHCH-Lf reduced endocytosis of ^{125}I-Lf by ~90%. In this respect, we found essentially no difference in the ability of native and DHCH-Lf to compete with ^{125}I-Lf for binding to either Lf binding site

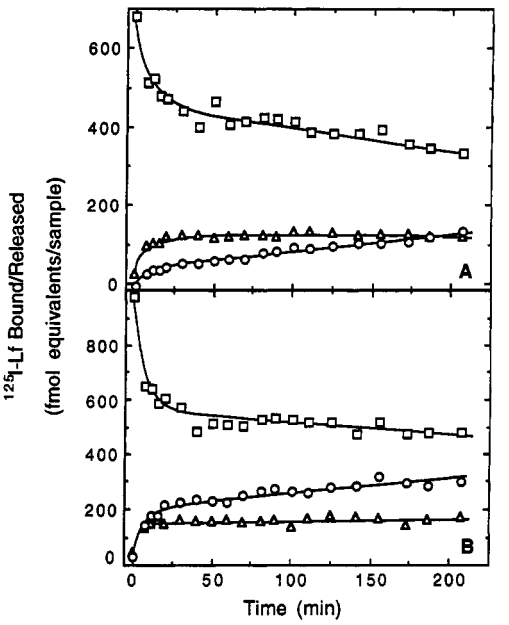

FIGURE 8: Kinetics of release of degraded products of ^{125}I-Lf. Cells (2×10^6 cells/mL of HBS, 5 mM $CaCl_2$, and 5 mM $MgCl_2$) were incubated in the presence of 1 mM desferrioxamine with 30 μg/mL ^{125}I-apo-Lf (A) or ^{125}I-holo-Lf (B) for 90 min at 4 °C. Cells were washed in the presence of 0.1% (w/v) dextran sulfate, resuspended in fresh BME–BSA, warmed to 37 °C, and assayed at various times for total cell-associated radioactivity (□) and for acid-precipitable (△) and acid-soluble (○) products released from the cells as described under Experimental Procedures. Samples contained 10^6 cells in 0.5 mL of medium.

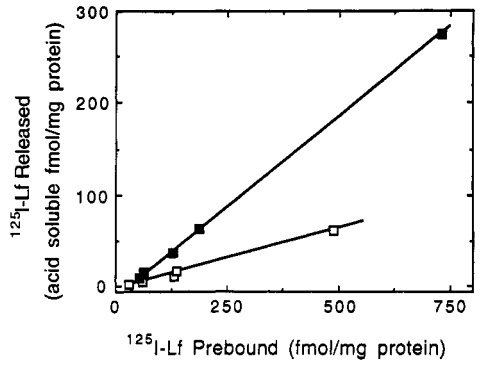

FIGURE 9: Extent of ^{125}I-Lf degradation as a function of ^{125}I-Lf bound to cells. Cells (1.5×10^6 cells/mL in HBS, 5 mM $CaCl_2$, and 5 mM $MgCl_2$) were incubated with 1–100 μg/mL ^{125}I-apo-Lf (□) or ^{125}I-holo-Lf (■) for 90 min at 4 °C, washed in the presence of 0.1% (w/v) dextran sulfate, and suspended in fresh medium and assayed for cell-associated radioactivity as described under Experimental Procedures. The cells were incubated at 37 °C for 80 min, chilled, and assayed for cell-associated radioactivity, acid-soluble degradation products released into the medium, and cell protein as described under Experimental Procedures. Symbols represent the mean of duplicate samples that differed by ≤10%.

population. We concluded from these results that arginine side chains probably do not participate in bovine Lf binding to the hepatocyte Ca^{2+}-dependent Lf binding sites.

Evidence for Recycling of Lf Binding Sites. Receptor recycling is a feature common to many endocytic receptor systems (Dautry-Varsat et al., 1983; Goldstein et al., 1983; Kaplan & Ward, 1990; Knutson, 1992; McGary et al., 1989; Stahl et al., 1984; Vonzastrow & Kobilka, 1992; Weigel & Oka, 1984). Data presented in Figures 3 and 6 indicated that hepatocytes accumulated levels of apo-Lf or holo-Lf that exceeded the number of high-affinity Lf binding sites present on the surfaces of these cells (McAbee & Esbensen, 1991).

Table III: Effect of Lysine and Arginine Modification of Lf on ^{125}I-Lf Binding and Uptake[a]

		^{125}I-Lf bound, 4 °C (fmol/10^6 cells)		^{125}I-Lf bound, 37 °C (fmol/10^6 cells)	
expt	competitor	(–) dextran sulfate	(+) dextran sulfate	total	internal
1	none	631 ± 40	309 ± 21	1752 ± 115	1096 ± 55
	native Lf	353 ± 241	21 ± 11	311 ± 34	105 ± 9
	FITC-Lf	486 ± 9	56 ± 4	277 ± 34	130 ± 37
2	none	1116 ± 165	356 ± 117	3125 ± 263	1915 ± 218
	native Lf	379 ± 19	50 ± 5	634 ± 46	200 ± 11
	DHCH-Lf	258 ± 30	54 ± 13	461 ± 12	242 ± 39

[a] Cells (2 × 10^6 cells/mL of HBS, 5 mM $CaCl_2$, and 5 mM $MgCl_2$) were incubated with 5 μg/mL ^{125}I-apo-Lf with or without 500 μg/mL apo-Lf (native Lf), FITC-Lf, or DHCH-Lf at either 4 °C for 90 min or 37 °C for 30 min. The cells were then washed and assayed for cell-associated or internalized radioactivity as described under Experimental Procedures. Cells incubated at 4 °C were washed in the presence or absence of dextran sulfate. Cells incubated at 37 °C assayed for cell-associated (total) radioactivity were washed in the presence of dextran sulfate. Experiments 1 and 2 were performed using different hepatocyte preparations. Values are means of duplicate samples ± the standard deviation.

This suggested that high-affinity Lf binding sites either recycled during endocytosis or were recruited from an intracellular pool, mediated Lf uptake, and then degraded along with the Lf brought into the cell. To address this issue, it is important to determine the total number of high-affinity Lf binding sites, both surface and intracellular. To do this, we compared ^{125}I-Lf binding to intact hepatocytes vs hepatocytes perforated with the steroid detergent digitonin. Digitonin permeabilization allows detection of intracellular pools of galactosyl receptors in rat hepatocytes (McAbee & Weigel, 1988; Weigel et al., 1983) and hyaluronate receptors in rat hepatocytes (Frost et al., 1990) and liver endothelial cells (McGary et al., 1989). Digitonin perforations are large enough to allow transmembrane diffusion of protein subunits ≤200 kDa and permeabilize all intracellular compartments along the endocytic pathway (Weigel et al., 1983). First, we determined whether or not high-affinity Lf binding activity on the surfaces of hepatocytes was sensitive to digitonin treatment. Hepatocytes prebound with ^{125}I-Lf at 4 °C were washed in HBSS containing 0.1% (w/v) dextran sulfate and then incubated with or without 0.055% (w/v) digitonin. Hepatocytes bound (6.93 ± 0.55) × 10^5 and (5.83 ± 0.59) × 10^5 ^{125}I-Lf molecules, respectively, when washed without or with digitonin (± standard deviation, n = 8). As these values were significantly different (Student's t-test, $t_{0.05,12}$), this indicated that digitonin dissociated or solubilized only a small percentage (≤16%) of the ^{125}I-Lf/Lf receptor complexes present on the cell surface. Second, we found that intact and permeable hepatocytes bound similar amounts of ^{125}I-Lf to high-affinity, Ca^{2+}-dependent sites. Intact and digitonin-permeabilized hepatocytes (2 × 10^6 cells/mL) were incubated with ^{125}I-Lf (30 μg/mL) at 4 °C, washed with dextran sulfate, and assayed for bound radioactivity. Intact and permeable hepatocytes bound (1.09 ± 0.07) × 10^6 (n = 4) and (1.15 ± 0.2) × 10^6 (n = 3) ^{125}I-Lf molecules, respectively (± standard deviation). These values were not significantly different by Student's t-test ($t_{0.05,3}$). Notably, when dextran sulfate was omitted from the wash buffer, permeable cells routinely bound 5–10 times more ^{125}I-Lf than intact cells, reflecting a large increase in nonspecific binding of Lf to intracellular components (data not shown). When a similar binding experiment was performed on hepatocytes incubated previously with Lf (25 μg/mL) at 37 °C for 1 h, we found that intact cells bound (4.17 ± 0.30) × 10^5 ^{125}I-Lf molecules/cell, but permeable cells bound (5.76 ± 0.86) × 10^5 ^{125}I-Lf molecules per cell; these values were significantly different by Student's t-test ($t_{0.05,4}$). Thus, the cellular distribution of high-affinity Lf binding sites between the cell surface and one or more intracellular compartments may be altered when the cells are subjected to a substantial endocytic load. These data also provided evidence that high-affinity Lf binding sites were

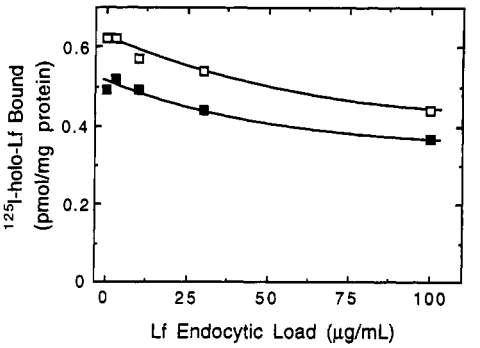

FIGURE 10: Effect of endocytic load on the surface number of high-affinity Lf binding sites. Cells (3 × 10^6 cells/mL) were incubated at 37 °C for 1 h with BME–BSA supplemented with the designated concentrations of Lf in the absence (□) or presence (■) of 20 μM cycloheximide, which blocks protein synthesis in isolated hepatocytes by >98% (Weigel & Oka, 1983). The cells were chilled on ice and washed with HBSS/5 mM EGTA to strip Lf from high-affinity Lf binding sites from the cell surface. After the cells were rinsed once with HBSS at 4 °C, they were incubated with 5 μg/mL ^{125}I-holo-Lf for 90 min at 4 °C, washed in the presence of 0.1% (w/v) dextran sulfate, and then assayed for cell-associated radioactivity and cell protein as described under Experimental Procedures. Symbols represent the mean of duplicates that differed by ≤10%.

detectable intracellularly in digitonin-permeabilized cells. From these data we concluded that hepatocytes express most of their high-affinity, Ca^{2+}-dependent Lf binding activity on the cell surface.

One would predict that during sustained Lf endocytosis, Lf binding sites—if not recycled—would be lost when ligand/receptor complexes were internalized and degraded. This would decrease the number of surface Lf binding sites because the cells lack a sizeable intracellular pool from which to recruit receptors to the cell surface. To test for this possibility, we incubated hepatocytes with different amounts of unlabeled Lf at 37 °C for 1 h and then assayed subsequently at 4 °C for the number of high-affinity Lf binding sites remaining on the cell surface (Figure 10). On the basis of the results shown in Figure 6, we expected that under these conditions the number of Lf molecules accumulated in 1 h by hepatocytes at 37 °C should exceed ≤15-fold the number of cellular Lf binding sites. We found that regardless of the endocytic load (≤100 μg of Lf/mL), cells retained ≥70% of their surface high-affinity Lf binding sites whether or not protein synthesis was blocked (Figure 10). We conclude, therefore, that hepatocytes recycle high-affinity Lf binding sites during endocytosis, maintaining a fairly constant number of these sites on their surfaces. Assuming cells possess 1.2 × 10^6 high-affinity Lf binding sites/cell, the cycling time per binding site is 3.9 min for apo-Lf (1.85 fmol cell^{-1} h^{-1}; Figure 6) and 4.8 min for holo-Lf (1.52 fmol cell^{-1} h^{-1}; Figure 6).

Table IV: Comparison of Maximal Endocytic Rates for Various Receptors

ligand	cell type and receptor	max endocytic rate (molecules per cell per second)	binding sites per cell	max turnover rate (molecules per receptor per hour)
asialoorosomucoid	rat hepatocytes, galactosyl receptor	650[a]	500000	4.7
hyaluronic acid	rat liver endothelia, hyaluronate receptor	220[b]	220000	4.1
mannosyl-BSA	alveolar macrophages, mannosyl receptor	550[c]	360000	5.5
apo-Lf	rat hepatocytes, Ca^{2+}-dependent binding site	5200[d]	1.2×10^6	15.6
holo-Lf	rat hepatocytes, Ca^{2+}-dependent binding site	4200[d]	1.2×10^6	12.6

[a] Weigel & Oka (1982). [b] McGary et al. (1989). [c] Stahl et al. (1984). [d] This report.

DISCUSSION

In this report, we determined the salient features of endocytosis of bovine Lf by isolated rat hepatocytes and the effect Lf's iron content had on its uptake and degradation. Our primary findings are as follows: (1) Isolated rat hepatocytes vigorously bind and ingest bovine ^{125}I-Lf *via* a clathrin-dependent endocytic pathway regardless of Lf's iron content. In general, cells accumulated apo-Lf and holo-Lf at nearly identical rates and to similar extents, and cells degraded internalized Lf regardless of its iron content. (2) Of the two categories of Lf binding sites on hepatocyte surfaces (McAbee & Esbensen, 1991), only the high-affinity Ca^{2+}-dependent binding sites mediated Lf uptake. (3) Derivatization of Lf's lysines with FITC or Lf's arginines with cyclohexanedione had no affect on Lf binding and uptake by hepatocytes. This suggests that hepatocytes bind and internalize bovine Lf independent of its cationic nature and may reflect a difference with the way human and bovine Lfs interact with cells. (4) Rat hepatocytes express ~95% of their high-affinity Lf binding sites on their surfaces. (5) High-affinity Lf binding sites recycle during Lf endocytosis with each binding site making a complete circuit within 4–5 min.

Several lines of evidence indicate that hepatocytes accumulate Lf by a high-capacity, clathrin-dependent endocytic pathway. First, hepatocytes recycle Lf binding sites during endocytosis. Hepatocytes possess ~1 million high-affinity Ca^{2+}-dependent Lf binding sites per cell (McAbee & Esbensen, 1991), most of which are at the cell surface even during sustained endocytosis (Figure 10). Nevertheless, each cell accumulated >15 million molecules of Lf per hour regardless of Lf's iron content (Figure 6). In addition, sustained uptake of Lf by hepatocytes was not dependent on *de novo* protein synthesis (Table II). Taken together, these data provide very strong evidence that Lf binding sites recycle during endocytosis. That Lf binding to cells decreases at pH ≤6 (McAbee & Esbensen, 1991) is consistent with this recycling scenario as the low pH found in sorting endosomal compartments (Collins et al., 1989) may mediate dissociation of internalized Lf receptor–ligand complexes.

Second, the kinetics of Lf endocytosis are commensurate with other known endocytic receptors. Hepatocytes exhibited maximal endocytic rates roughly 2–3 times greater than those for other receptor systems after normalization for the number of ligand binding sites per cell (Table IV). We consistently observe maximal endocytic rates of 2000–5000 molecules of Lf per cell per second. The first-order endocytic rate constants for apo-Lf and holo-Lf uptake were, respectively, 0.276 and 0.292 min^{-1} (37 °C). By comparison, the first-order rate constant for asialoorosomucoid uptake by isolated rat hepatocytes is 0.23 min^{-1} (35 °C; Weigel & Oka, 1982). The lack of a significant number of intracellular Lf receptors in the absence of Lf endocytosis is similar to the hepatocyte distribution of low-density lipoprotein receptors (Pathak et al., 1990) but dramatically different from that of hepatocyte galactosyl receptors which are roughly equally distributed between the cell-surface and intracellular compartments (McAbee et al., 1991; Weigel et al., 1983). The steady-state amount of recycling intracellular Lf receptors may increase somewhat under heavy endocytic loads as suggested by the results shown in Figure 10.

Third, Lf uptake by hepatocytes was clathrin-dependent. Retention of plasma membrane proteins by clathrin-coated pits requires the presence of an appropriate pit-directing signal present on its cytoplasmic domain (Chen et al., 1990; Jing et al., 1990; Johnson et al., 1990; Ktistakis et al., 1990) which may engage clathrin AP-2 components (Beltzer & Spiess, 1991; Pearse & Robinson, 1990). The large majority of generic cell-surface components lack this specific signal and, thereby, are excluded from coated pits (Hubbard, 1989). Inhibition of ligand endocytosis by hyperosmotic conditions, intracellular K^+ depletion, or acidification of the cytosol (Daukas & Zigmond, 1985; Heuser & Anderson, 1989; Larkin et al., 1983; Madshus et al., 1987; Oka et al., 1989; Sandvig et al., 1987, 1988) is diagnostic of a clathrin-dependent internalization event. Because hyperosmolality blocked Lf uptake (Figure 4), it is likely, therefore, that the Ca^{2+}-dependent Lf binding sites are *bona fide* endocytic receptors that readily engage clathrin-coated pits and mediate Lf uptake in a highly specific manner. Taken together, these results indicate that hepatocyte Ca^{2+}-dependent Lf receptors mediate Lf uptake in a manner that resembles biochemically in every way a class II endocytic receptor-mediated process (Kaplan, 1981).

The biphasic kinetics of Lf degradation and release were surprising and are difficult to interpret. Degraded products of desialylated glycoproteins internalized by the hepatocyte galactosyl receptor are released only in substantial quantity by 15–20 min following uptake (Weigel, 1980; Weigel & Oka, 1982) although degradation and release of some material occur immediately at a low rate following internalization (Clarke et al., 1987). We found no substantial difference in the long-term rates of degradation of the two Lf forms (Figure 8), but hepatocytes preferentially degraded holo-Lf to a greater extent than apo-Lf (Figures 8 and 9) apparently due to the release of a small burst of degraded Lf very shortly after internalization—more pronounced for holo-Lf (Figure 8). Because the iron-dependent differences in Lf degradation occurred early after internalization, the initial intracellular processing of Lf may vary depending on whether or not the metal binding sites of Lf are occupied. For instance, structural differences between the two Lf forms may alter the protein's accessibility to lysosomal hydrolases, thereby changing the kinetics of its degradation. Alternatively, hepatocytes may preferentially route holo-Lf along an intracellular pathway that delivers a greater fraction of the ligand to an early degradative compartment. Notably, differential uptake and degradation of asialoglycoproteins by the galactosyl receptor in rat hepatocytes occur by separate parallel pathways (Clarke et

al., 1987; McAbee et al., 1991; Weigel & Oka, 1984). We are currently investigating the basis of this unique and differential degradation of Lf by hepatocytes.

The molecular nature of Lf's interaction with liver cells remains obscure. Results from other studies have suggested that the association of Lf with liver cells may depend in part upon the cationic nature of Lf. Human or bovine Lf can reduce ^{59}Fe-transferrin and ^{59}Fe-asialotransferrin uptake by rat liver apparently by competing with these molecules for binding to cell-surface heparan sulfate proteoglycan, which binds transferrin and various cationic proteins such as Lf with moderately low affinity (Hu et al., 1993). Excess human Lf also reduces liver clearance of chylomicron remnants from rat circulation (Huettinger et al., 1988; Ziere et al., 1992). It has been suggested that human Lf competes with apolipoprotein E for binding to the rat hepatic chylomicron remnant receptor because of the presence of a cationic cluster in human Lf (Lf^{25-31}, Arg-X-X-Arg-Lys-X-Arg) that mimics the apolipoprotein E cell recognition structure (apolipoprotein $E^{142-147}$, Arg-X-X-Arg-Lys-Arg; Huettinger et al., 1992). The N-terminal halves of human and bovine Lf (338 amino acids) each contain ~50 lysine and arginine residues—in roughly equal numbers—with sequence identity of lysine and arginine conserved at 23 positions and charge homology conserved at 31 positions between the 2 proteins (Goodman & Schanbacher, 1991). In contrast to bovine Lf, modification of human Lf's arginines with cyclohexanedione reduces Lf's ability to be cleared from the rat circulation (Huettinger et al., 1992) and to block chylomicron remnant uptake by rat liver (Huettinger et al., 1992; van Dijk et al., 1992). Bovine Lf can bind to the low-density lipoprotein receptor-related protein (Herz et al., 1988)—the putative apolipoprotein E receptor—on nitrocellulose blots of rat liver membrane fractions (Willnow et al., 1992) even though bovine Lf lacks the Arg-X-X-Arg-Lys-X-Arg motif. Our results presented here and elsewhere (McAbee & Esbensen, 1991) suggest, however, that the Ca^{2+}-dependent interaction of bovine Lf with high-affinity Ca^{2+}-dependent sites on hepatocytes is independent of the cationic nature of Lf. Thus, the endocytically-competent Lf receptor remains to be identified conclusively. Work is underway to identify and characterize the hepatic Ca^{2+}-dependent Lf receptor.

Our findings also suggest that the interaction of Lf with rat liver cells may show significant species differences between human and bovine Lf forms. (i) Human Lf binds to isolated rat hepatocytes at 4 °C with much lower affinity (K_d ~ 10 μM; Ziere et al., 1992) than does bovine Lf (K_d ~ 20 nM; McAbee & Esbensen, 1991). (ii) Binding of human Lf to rat hepatocytes is not enhanced by Ca^{2+} (Ziere et al., 1992), whereas Ca^{2+} is required for the binding of bovine Lf to the high-affinity sites but not the low-affinity sites (McAbee & Esbensen, 1991). (iii) Ca^{2+} (>100 μM) promotes the formation of human Lf tetramers (Bennett et al., 1981) but has no polymerizing effect on bovine Lf (McAbee & Esbensen, 1991). (iv) Arginine modification of human Lf with cyclohexanedione alters its ability to interact with rat liver (Ziere et al., 1992), but similar treatment of bovine Lf has no effect on its ability to compete with native Lf for binding and uptake by isolated hepatocytes (Table III). We found that human Lf competes with bovine Lf for binding to Ca^{2+}-dependent sites on isolated rat hepatocytes but equivalent extents of competition required ≥10 times more human Lf than bovine Lf (unpublished observation). Therefore, while human Lf can bind to Ca^{2+}-dependent sites, its affinity for these sites is considerably less than that of bovine Lf. In addition, the human intestinal mucosal Lf receptor binds human but not bovine Lf (Kawakami & Lonnerdal, 1991). Taken together, these data strongly suggest that species-related differences in ligand/receptor interactions exist for Lf.

FITC-derivatized human Lf lost its ability to bind to the Lf receptor expressed on phytohemagglutinin-stimulated lymphocytes (Legrand et al., 1991). In this study, ~90% of the FITC (FITC:protein molar ratio 1:1) was conjugated to one of three N-terminal half-lysine residues (Lys^{263}, Lys^{280}, Lys^{282}). These investigators concluded that one or more of these N-terminal lysines are at or near the lymphocyte binding domain of human Lf. The lysine residues in human Lf modified by FITC are conserved in bovine Lf (Goodman & Schanbacher, 1991; Pierce et al., 1991). Regardless, bovine Lf modified with FITC (FITC:protein ratio, 2:1) competed fully with native Lf for binding to Ca^{2+}-dependent sites on hepatocytes at 4 °C and endocytosis at 37 °C (Table III). Notably, FITC conjugation partially reduced Lf's ability to compete with native Lf for binding Ca^{2+}-independent sites (Table III), consistent with the notion that Lf's cationic nature may help mediate its interaction to these low-affinity sites. It is possible that the lysines modified by FITC in human Lf were not modified in bovine Lf. Alternatively, the hepatocyte Lf receptor may recognize a different domain on the Lf molecule than does the lymphocyte Lf receptor, such that the difference in binding reflects significant differences in receptor structure and specificity. Without distinguishing between these possibilities, we conclude nonetheless that conditions used to modify human Lf which abolished its ability to bind to lymphocytes had no apparent effect on bovine Lf uptake by high-affinity Ca^{2+}-dependent sites on hepatocytes.

Our working hypothesis is that Ca^{2+}-dependent Lf binding sites comprise *bona fide* endocytic Lf receptors whereas lower-affinity dextran sulfate-sensitive binding sites may include cell-surface heparan sulfate (Hu et al., 1993) or the chylomicron remnant receptor (Huettinger et al., 1988, 1992; van Dijk et al., 1992; Ziere et al., 1992). Ca^{2+}-dependent sites exhibit a K_d for Lf (20 nM) similar to the steady-state Lf concentration in blood. This type of high-affinity interaction is required if the Ca^{2+}-dependent sites maintain physiologic Lf levels in blood and reflects a degree of biological specificity consistent with a receptor-mediated event. Lower-affinity sites (K_d > 1 μM) probably do not perform a meaningful Lf binding/uptake function *in situ* because (i) Lf concentrations high enough to elicit significant binding to these sites (≥K_d) may not be achieved in the circulation except under contrived conditions and (ii) the low-affinity sites do not stably bind Lf at physiological temperatures (Figure 6), and it is questionable whether they internalize bound Lf (Figure 7). In the presence of Lf concentrations sufficient to maintain their occupancy at 37 °C, low-affinity sites may retain Lf long enough for it to be internalized at a slow rate. This may explain why human Lf injected into rats binds readily to the liver but a considerable fraction is released by the polyanion fucoidin (Regoeczi et al., 1985; Ziere et al., 1992). Importantly, our findings indicate that the hepatocyte Lf endocytic pathway is well suited to function as an iron-scavenging pathway due to its high capacity and endocytic vigor. Currently, we are investigating the intracellular processing of Lf protein and its bound iron.

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