Isolated rat hepatocytes differentially bind and internalize bovine lactoferrin N- and C-lobes

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Isolated rat hepatocytes bind and internalize bovine lactoferrin (Lf) and its bound iron in a Ca^{2+}-dependent manner. In this study, we determined if one or both halves of Lf (N- and C-lobes) were responsible for the interaction of Lf with hepatocytes. We isolated three tryptic fragments of bovine Lf. Cleavage at Arg^{284}–Ser^{285} generated two fragments: N-terminal pp36 that contained 80% of Lf N-lobe and C-terminal pp51. A second cleavage at Arg^{338}–Ala^{339} generated a smaller fragment (pp44) that contained all of the C-lobe with no N-lobe elements. Hepatocytes bound Lf and pp51 in a Ca^{2+}-dependent manner with the same affinity (K_d approx. 75 nM) and to nearly identical extents (approx. 10^6 sites per cell). Lf and pp51 competed with each other for binding to cells over a similar titration range. Hepatocytes internalized Lf at a faster rate than pp51 ($k_{in} = 0.28$ and 0.19 min^{-1} respectively), but cells degraded pp51 at approx. twice the rate of native Lf. pp44 competed with ^{125}I-labelled Lf for binding to Ca^{2+}-dependent binding sites on hepatocytes as well as native Lf or pp51. In contrast, hepatocytes bound pp36 ($K_d = 90$ nM, $\leqslant 5 \times 10^6$ sites per cell) but did not internalize or degrade it appreciably. Moreover, pp36 binding to cells was not Ca^{2+}-dependent, and pp36 competed poorly with native Lf and pp51 for binding to cells. We conclude from these findings that the Lf determinants responsible for binding to the Ca^{2+}-dependent receptor on hepatocytes is present within the C-lobe of Lf.

INTRODUCTION

Transferrins constitute a class of structurally related non-haem iron-binding proteins that includes serum transferrin, ovotransferrin, melanotransferrin and lactoferrin (Lf). Each of these proteins consists of a single polypeptide chain of 680–738 amino acids arranged as a bi-lobed molecule [1]. With the exception of melanotransferrin, which binds Fe^{3+} only by its N-lobe [2], the N- and C-lobes of serum transferrin, ovotransferrin and Lf each reversibly bind one Fe^{3+} along with a requisite counter-ion [1]. There is a striking conservation in the primary, secondary and tertiary structures among transferrins and between the N- and C-lobes of these proteins. Nevertheless, the metal-binding sites in the two lobes differ with respect to their local environment [3–5] and bind and release metal ions with non-identical characteristics [6,7]. N- and C-lobes also exhibit different glycosylation patterns [8] and possess unique binding regions for microbial membranes [9], glycosaminoglycans [10,11], and advanced glycation end products [12]. Moreover, the participation of N- and C-lobes in binding to cell-surface receptors differs between various transferrins [13–16].

Lf interacts with many cells, including mitogen-stimulated T-lymphocytes [17], monocytes and macrophages [18–20], intestinal mucosal cells [21,22], bacterial cells [23], endothelia [24] and hepatocytes [25,26]. Several Lf-binding proteins have been identified [12,17,22,23], which differ significantly in molecular mass, subunit composition and cell-type distribution. In addition, T-lymphocytes, monocytes and hepatocytes bind large amounts of Lf with high affinity (K_d approx. 20–100 nM), but T-lymphocytes and monocytes internalize bound Lf minimally compared with hepatocytes, which can take up $\leqslant$ 5000 molecules of $Lf \cdot cell^{-1} \cdot s^{-1}$ [18,19,27]. Because several cellular Lf-binding proteins exist that elicit different responses upon Lf binding, there may be unique regions on the Lf molecule that are responsible for its interaction with these different cells. The T-lymphocyte-binding region of human Lf, for instance, has been mapped strictly to the N1 region of the protein [28], whereas the binding determinants for certain *Neisseria* species are present in both the N- and C-lobes of human Lf [16].

Blood Lf is cleared from the circulation by the liver [29]. We have shown previously that isolated rat hepatocytes bind and internalize bovine Lf protein and Lf-bound Fe^{3+} via a class of high-affinity Ca^{2+}-dependent binding sites that recycle during Lf endocytosis [25,27,30]. We are interested in understanding the molecular basis of Lf interaction with hepatocytes, in particular in identifying region(s) of the Lf molecule that are necessary for its interaction with these cells. In this study, we addressed this issue by generating large tryptic fragments of bovine Lf that approximate the N- and C-lobes of Lf and analysing their ability to interact with isolated rat hepatocytes. We found that the tryptic C-lobe of Lf is bound and internalized by isolated rat hepatocytes in a manner similar to that of the native protein. Tryptic N-lobe fragments, on the other hand, bind to cells with high affinity, but in a Ca^{2+}-independent manner. Additionally, isolated rat hepatocytes internalize N-lobe poorly compared with C-lobe and the native molecule.

EXPERIMENTAL

Materials

Bovine colostrum Lf, BSA (fraction V), dextran sulphate (5000 Da), bovine pancreatic trypsin (type XI; EC.3.4.21.4; 6300 units/mg of protein), soya bean trypsin inhibitor–agarose and PMSF were purchased from Sigma (St. Louis, MO, U.S.A.). Collagenase (Type D) was from Boehringer Mannheim (Indianapolis, IN, U.S.A.). 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril (Iodogen) was from Pierce Chemical Co. (Rockford, IL, U.S.A.) and $Na^{125}I$ was from ICN Biomedicals Inc. (Irvine, CA, U.S.A.). All other chemicals were reagent grade and obtained

Abbreviations used: BME, Basal Medium Eagle's; HBS, Hepes-buffered saline; Lf, lactoferrin.

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from Fisher Biochemicals (Itasca, IL, U.S.A.). Hepes-buffered saline (HBS) contained 150 mM NaCl, 3 mM KCl and 10 mM Hepes, pH 7.4. Buffer A contained HBS supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. Buffer B contained Buffer A supplemented with 0.3 % (w/v) dextran sulphate. Buffer C contained HBS, 5 mM EGTA and 0.3 % (w/v) dextran sulphate. Basal Medium Eagle's (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.

Hepatocytes

Male Sprague–Dawley rats (150–350 g; Harlan Labs, Indianapolis, IN, U.S.A.) were fed standard laboratory chow and water *ad libitum*. Hepatocytes were prepared by a modification of a collagenase perfusion procedure as described previously [25]. Cells were kept at approx. 30 °C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME/BSA were approx. 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. Viability was determined microscopically by Trypan Blue exclusion.

Lactoferrin 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 [25]. The iron content of Lf, determined spectroscopically from its A_{465}/A_{280} ratio, was routinely 0.03–0.035, indicating approx. 75 % iron saturation [31]. ^{125}I-labelled Lf, prepared by the 'Iodogen' method (Pierce), had specific radioactivities of 3–65 d.p.m./fmol. The homogeneity of radiolabelled and unlabelled Lf used for all binding studies was confirmed by SDS/PAGE and autoradiography.

Lf tryptic fragment generation and purification

Tryptic fragments of bovine Lf were generated by using modifications of a previously reported procedure [16]. Unless indicated otherwise, Lf (10 mg/ml) in 0.025 M $CaCl_2$ and 0.1 M Tris/HCl, pH 8.2, was incubated with bovine pancreatic trypsin for 45 min at 37 °C at a Lf/trypsin ratio of 385:1 (w/w). Free trypsin was removed from the mixture by chromatography on soya bean trypsin inhibitor–agarose at 4 °C; residual trypsin was inactivated by periodic additions of PMSF (0.1 mM). Tryptic fragments were fractioned on a BioGel-P60 column (2.5 cm × 100 cm) equilibrated in 10 % (v/v) acetic acid. Fractions were analysed for protein content by SDS/PAGE, and homogeneous fractions were pooled. In some cases, fractions enriched for particular fragments were pooled, concentrated and rechromatographed to yield homogeneous fragment preparations. N-terminal amino acid sequencing of individual fragments was done at the University of Notre Dame Biosciences Core Facility using a Beckman L-3000 protein sequencer and a Spherogel Micro-PTH HPLC column (3 μm bore). Selected tryptic fragments were iodinated (20–100 d.p.m./fmol) and used within 3 weeks of labelling.

Binding, endocytosis and degradation assays

For binding assays, $(1–2) \times 10^6$ hepatocytes were incubated in 0.5–1.0 ml of Buffer A with ^{125}I-ligand at 4 °C for 90 min. In some cases, unlabelled ligand was included in the incubation mixture as indicated in Figure legends. Cells were pelleted by centrifugation (400 ***g***, 2 min, 4 °C) and washed by centrifugation in excess cold Buffer B twice, 10 min per wash, to remove unbound ligand and ligand bound to dextran sulphate-sensitive sites [27]. Washed cells were resuspended in Buffer B, transferred to clean plastic tubes and assayed for cell-associated radioactivity and protein. For endocytosis assays, $(1–2) \times 10^6$ hepatocytes/ml of BME/BSA were incubated with designated amounts of ^{125}I-ligand as described in the Figure legends. Uptake was stopped by rapidly diluting the cells into a 3–6-fold excess volume of cold Buffer A. To determine total bound (surface and intracellular) ^{125}I-ligand, cells were washed twice at 4 °C, 10 min per wash, with Buffer B. To determine internalized ^{125}I-ligand, cells were washed twice at 4 °C in Buffer C, 10 min per wash. Washed cells were resuspended in 1.0 ml of Buffer B, transferred to clean plastic tubes and assayed for cell-associated radioactivity and protein. In some experiments, we determined the extent of ^{125}I-ligand degradation by cells by measuring the amount of acid-soluble radioactivity released into conditioned medium at 37 °C as reported previously [27]. Briefly, conditioned medium from cells incubated with ^{125}I-ligand were collected and clarified by centrifugation (400 ***g***, 2 min, 4 °C). Non-degraded ^{125}I-labelled polypeptides in the conditioned media were precipitated by the addition of a 2 volumes of cold 10 % (w/v) phosphotungstic acid in 2 M HCl. After incubation for ⩾ 15 min on ice, precipitated material was sedimented (4 °C, 4 min, 1000 ***g***); acid-precipitated pellets and acid-soluble supernatants were assayed for radioactivity.

SDS/PAGE and autoradiography

Protein samples were heated in 0.0625 M Tris/HCl (pH 6.8)/2 % (w/v) SDS/10 % (v/v) glycerol/2 % (v/v) β-mercaptoethanol/0.01 % (w/v) Bromophenol Blue for 5 min at 100 °C, then fractioned by electrophoresis on 10 % discontinuous acrylamide-bis (30:0.8, w/w) slab gels using a Mini-Protean II slab gel apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Polypeptides were visualized by fixation staining in 0.25 % (w/v) Coomassie Brilliant Blue R250/45 % (v/v) methanol/10 % (v/v) acetic acid. The molecular masses of various polypeptides were determined relative to Bio-Rad prestained electrophoresis standards. Dried gels containing ^{125}I-polypeptides were exposed to Kodak X-Omat-AR film at −70 °C and developed using a Kodak X-Omat Clinic 1 Processor.

General

Protein was determined by the bicinchoninic acid protein assay procedure using BSA as standard (Pierce). Hepatocytes contained 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 Company, Wilmington, DE, U.S.A.). ^{125}I-radioactivity was determined using a Packard Cobra Auto-Gamma Counting System (Model 5002; Packard Instrument Co., Downers Grove, IL, U.S.A.). Spectroscopic analysis of protein samples was performed using a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.).

RESULTS

Generation and analysis of tryptic fragments of bovine Lf

Half-molecules corresponding to the N- and C-lobes of human transferrin, human Lf and ovotransferrin can be generated by limited proteolysis with trypsin or thermolysin [13,32,33]. We initially examined the fragmentation pattern elicited by limited trypsin treatment of bovine Lf to determine the conditions under

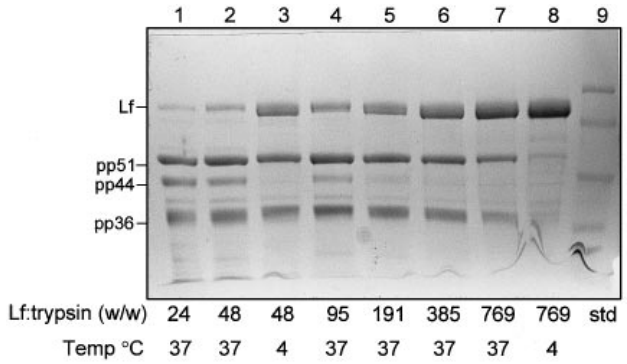

Figure 1 Electrophoretic analysis of tryptic digests of bovine Lf

Bovine Lf (20 μg) was incubated with various amounts of trypsin at the designated Lf/trypsin (w/w) ratios at either 4 or 37 °C (lanes 1–8) as described in the Experimental section. After 20 min, the samples were placed into denaturing electrophoresis sample buffer preheated at 100 °C, boiled for 5 min, and then subjected to SDS/PAGE as described in the Experimental section. Approximately 10 μg of protein was loaded per well. Lane 9 contained the following molecular-mass standards (std; top to bottom): 105, 82, 49, 33 and 27 kDa.

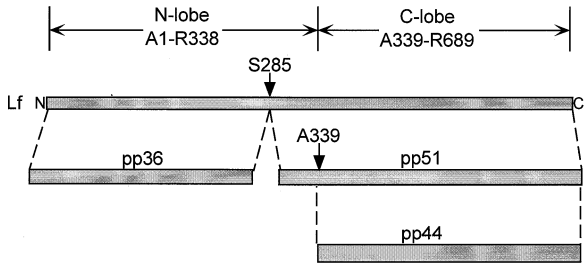

Figure 2 Tryptic fragmentation pattern of bovine Lf

Schematic diagram shows the relative positions of the tryptic cut sites on the N-sides of Ser^{285} (S285) and Ala^{339} (A339) that gave rise to pp51, pp36 and pp44. The segments of the Lf primary structure that correspond to the N- and C-lobes were taken from the report by Goodman and Schanbacher [34].

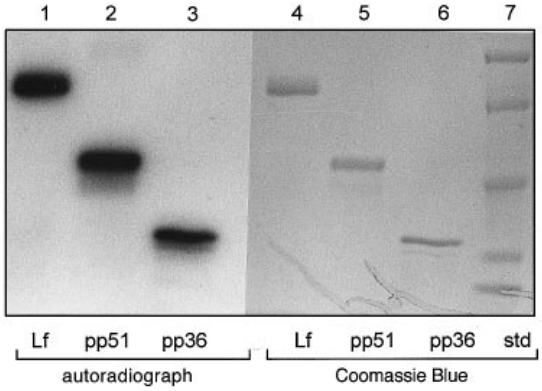

Figure 3 Electrophoresis and autoradiography of ^{125}I-Lf, ^{125}I-pp51 and ^{125}I-pp36

Native bovine Lf (lanes 1 and 4) and Lf tryptic fragments pp51 (lanes 2 and 5) and pp36 (lanes 3 and 6) were radiolabelled and subjected to SDS/PAGE and autoradiography as described in the Experimental section. Lane 7 contained the following molecular mass standards (std; top to bottom): 105, 82, 49, 33 and 27 kDa.

Table 1 N-terminal amino acid sequences of major Lf tryptic fragments

pp51, pp44 and pp36 tryptic fragments of bovine Lf were purified and subjected to N-terminal amino acid analysis as described in the Experimental section and compared with the bovine Lf amino acid sequence published elsewhere [34].

Tryptic fragment	N-terminal sequence	Matching Lf sequence	Maximal sequence size	Estimated molecular mass (kDa)
pp51	SFQLFGSP	$Ser^{285} \rightarrow Pro^{292}$	$Ser^{285} \rightarrow Arg^{689}$	44.5
pp44	ARYTR	$Ala^{339} \rightarrow Arg^{343}$	$Ala^{339} \rightarrow Arg^{689}$	40.0
pp36	APRKNVRW	$Ala^{1} \rightarrow Trp^{8}$	$Ala^{1} \rightarrow Arg^{284}$	31.7

which large polypeptides could be generated with minimal production of small fragments. Lf/trypsin ratios of $\leqslant 100$ resulted in the generation of three large fragments of molecular mass 51 kDa (pp51), 44 kDa (pp44) and 36 kDa (pp36) (Figure 1, lanes 1, 2 and 4). At higher Lf/trypsin ratios, pp51 and pp36 were generated predominantly (Figure 1, lanes 5–7) and pp44 was only produced in significant amounts following prolonged treatment ($\geqslant 3$ h, 37 °C). By contrast, optimal fragment generation from human Lf requires $\geqslant 4$ h at 37 °C at Lf/trypsin ratios of < 20 using trypsins with similar specific activities (M. P. Sitaram and D. D. McAbee, unpublished work; [16,32]). It appears therefore that bovine Lf is more susceptible to trypsin than is human Lf under these conditions. We observed a similar fragment generation pattern when Lf tryptic digests were analysed over time, in that pp51 and pp36 were produced before pp44 (results not shown). These findings suggested that pp51 and pp36 resulted from a primary tryptic cut of Lf, and pp44 was derived from a secondary tryptic cut of pp51 (Figure 2). These fragments were separated by gel-filtration chromatography in the presence of 10% acetic acid, and column fractions enriched in pp51, pp44 or pp36 were combined and rechromatographed to obtain fragment samples that were $\geqslant 98\%$ electrophoretically homogeneous, as determined by laser scanning densitometry (Figure 3).

To determine the region of the Lf molecule that corresponded to these major fragments, we determined the N-terminal amino acid sequence for pp51, pp44 and pp36 (Table 1). pp51 produced multiple peaks from each degradation cycle, but the major sequence, SFQLFGSP, matched the mature bovine Lf peptide $Ser^{285} \rightarrow Pro^{292}$. Thus the longest peptide represented by pp51 would be $Ser^{285} \rightarrow Arg^{689}$, a fragment with a predicted molecular mass of 44.5 kDa that contains all of the C-lobe, approx. 20% of the carboxyl end of the N-lobe, and the three-turn α-helix that connects the C- and N-lobes [1]. The N-terminus of pp44, ARYTR, corresponds to $Ala^{339} \rightarrow Arg^{343}$ of the mature protein, and analysis of pp36 yielded the sequence APRKNVRW, which corresponds to $Ala^{1} \rightarrow Trp^{8}$ of mature bovine Lf. pp44 contains the entire C-lobe, but unlike pp51 lacks N-lobe elements as well as the α-helical linker; its projected molecular mass is 40 kDa. Because the tryptic cut at Ala^{339} splits the molecule into equal halves, we predict that pp36 extends maximally between Ala^{1} and Arg^{284} (Figure 2), giving it a projected molecular mass of 31.7 kDa. The slightly higher-than-predicted molecular mass of the two fragments, as determined by SDS/PAGE, could

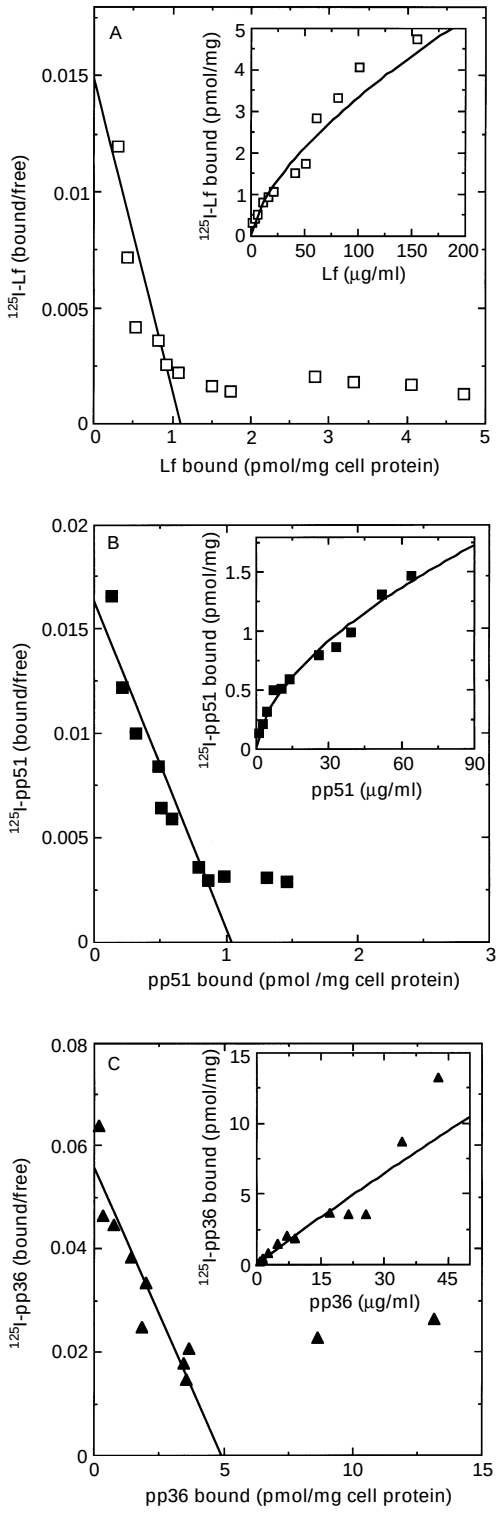

Figure 4 Equilibrium binding of Lf, pp51 and pp36 to hepatocytes

Hepatocytes (10^6/0.5 ml) were incubated with the indicated concentrations of ^{125}I-Lf (**A**), ^{125}I-pp51 (**B**) or ^{125}I-pp36 (**C**) for 90 min at 4 °C. The cells were pelleted by centrifugation and the amount of unbound ^{125}I-ligand in the supernatants was determined. The cells were washed and assayed for total bound radioactivity and protein as described in the Experimental section (insets). Best-fit isotherms were calculated according to the following equations: Lf (**A**, inset), $y = 0.162x^{0.656}$; pp51 (**B**, inset), $y = 0.126x^{0.581}$; pp36 (**C**, inset), $y = 0.251x^{0.955}$. From these data, equilibrium binding constants were determined [36]. Isotherms were calculated by linear regression analysis.

Table 2 Equilibrium binding constants for Lf, pp51 and pp36

Binding of ^{125}I-Lf, ^{125}I-pp51 and ^{125}I-pp36 to isolated rat hepatocytes at 4 °C was performed as described in the Experimental section and in the legend to Figure 4. Equilibrium binding constants were determined [36] from two separate experiments performed using different isolated rat hepatocyte preparations.

Ligand	Dissociation constant (nM)	$10^{-6} \times$ Maximal binding (molecules per cell)
Native Lf	75 ± 14	1.05 ± 0.21
pp51	78 ± 17	1.20 ± 0.14
pp36	90 ± 21	4.95 ± 0.64

be attributed to the presence of one or more N-linked glycan chain present in these fragments [35]. Because the C-terminal sequences of pp51, pp44 and pp36 were not determined, we cannot exclude the possibility that these fragments may have undergone minor tryptic truncation at the C-terminus. Nonetheless, these data indicate that, based on their size and sequence, pp51 and pp36 correspond respectively to the C-and N-lobe fragments produced by trypsinization of human Lf [16,32].

Comparative binding, endocytosis and degradation of Lf, pp51 and pp36

Isolated rat hepatocytes express $\leqslant 10^6$ Ca^{2+}-dependent high-affinity (K_d approx. 20 nM) binding sites for bovine Lf [25], most of which are on the cell surface [27]. Because pp51 and pp36 together contained the entire Lf sequence, we examined the interaction of ^{125}I-labelled pp51 and ^{125}I-labelled pp36 with isolated hepatocytes at 4 °C and compared their binding behaviour with that of full-length ^{125}I-labelled Lf (Figure 4). We found that cells bound all three ligands in a saturating manner (Figures 4A–4C insets) by using sets of high-affinity sites. Equilibrium binding constants calculated from two separate binding experiments indicated that cells bound all three ligands with similar affinities (K_d = 75–90 nM), but cells bound approx. 5 times more ^{125}I-pp36 at saturation than they did ^{125}I-Lf or ^{125}I-pp51 (Table 2). Binding constants exhibited by ^{125}I-Lf and ^{125}I-pp51 were not significantly different. Scatchard analysis of binding data also revealed a set of non-saturable Lf binding sites on hepatocytes that probably reflected non-specific association of Lf and tryptic fragments with cells; binding constants for these sites were not determined.

We then examined the ability of isolated rat hepatocytes to bind and internalize Lf tryptic fragments. In this experiment, cells were incubated in the continuous presence of ^{125}I-Lf, ^{125}I-pp51 or ^{125}I-pp36 at 37 °C and assayed for total bound (Figure 5A) and EGTA/dextran sulphate strip-resistant (Figure 5B) ^{125}I-ligand. We found that the amount of ^{125}I-Lf and ^{125}I-pp51 bound by cells increased linearly over time at essentially identical rates [39 fmol of ^{125}I-Lf$\cdot$min$^{-1}\cdot$mg^{-1}, $r = 0.95$ ($n = 6$); 36 fmol of ^{125}I-pp51$\cdot$min$^{-1}\cdot$mg^{-1}, $r = 0.995$ ($n = 6$)], and 77–80% of the total bound ^{125}I-Lf or ^{125}I-pp51 was intracellular regardless of the length of incubation (Figure 5B). In contrast, cells bound ^{125}I-pp36 at an approx. 4-fold lower rate [11 fmol$\cdot$min$^{-1}\cdot$mg^{-1}, $r = 0.905$ ($n = 6$)], and $\geqslant 95\%$ of cell-associated ^{125}I-pp36 was not removed from cells following washing with EGTA and dextran sulphate regardless of the incubation time. In other experiments, we determined the internalization rate constants for uptake of Lf, pp51 and pp36. To do this, hepatocytes were prebound at

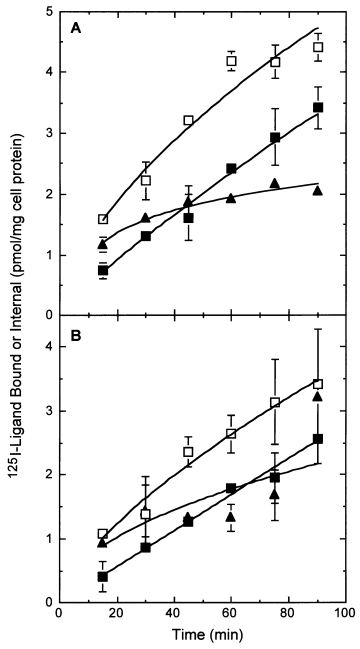

Figure 5 Kinetics of binding and endocytosis of Lf, pp51 and pp36 by hepatocytes

Hepatocytes (10^6 cells/ml) were incubated with 26 pmol/ml ^{125}I-Lf (□), ^{125}I-pp51 (■), or ^{125}I-pp36 (▲) at 37 °C. At the designated times, cells were assayed for bound (**A**) and intracellular (**B**) radioactivity as described in the Experimental section. Symbols represent means of duplicate samples (error bars = S.D.). Isotherms were calculated according to the following equations: bound Lf (**A**), $y = 0.302x^{0.612}$; internal Lf (**B**), $y = 0.157x^{0.689}$; bound pp51 (**A**), $y = 0.071x^{0.854}$; internal pp51 (**B**), $y = 0.028x^{1.000}$; bound pp36 (**A**), $y = 0.498x^{0.332}$; internal pp36 (**B**), $y = 0.228x^{0.500}$.

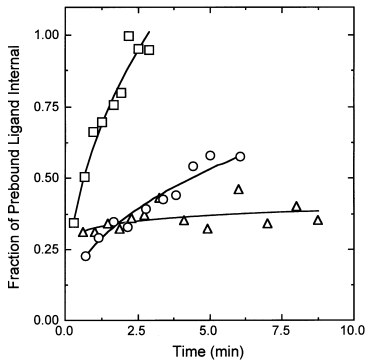

Figure 6 Kinetics of internalization of Lf, pp51 and pp36 prebound to hepatocytes

Hepatocytes (4×10^6 cells/ml) were incubated with 65 pmol/ml ^{125}I-Lf (□), ^{125}I-pp51 (○), or ^{125}I-pp36 (△) for 90 min at 4 °C. Cells were washed free of unbound ligand with cold Buffer B, then resuspended in BME/BSA warmed to 37 °C. At the designated times, cells were chilled rapidly in Buffer C and assayed for associated radioactivity and protein as described in the Experimental section. At $t = 0$, cells had bound 332 ± 5 fmol of ^{125}I-Lf, 280 ± 20 fmol of ^{125}I-pp51 and 640 ± 50 fmol of ^{125}I-pp36 per mg of cell protein ($n = 2$). Symbols represent the fraction of ^{125}I-ligand bound at various times relative to the amount of ^{125}I-ligand prebound to cells at 4 °C. Best-fit isotherms were calculated according to the following equations: Lf, $y = 0.623x^{0.456}$ $_{(r=0.99)}$; pp51, $y = 0.263x^{0.436}$ $_{(r=0.98)}$; pp36, $y = 0.326x^{0.078}$ $_{(r=0.52)}$.

4 °C with ^{125}I-Lf, ^{125}I-pp51 or ^{125}I-pp36 at 4 °C, and then washed free of unbound ligand. The cells were switched to 37 °C and the amount of ^{125}I-ligand resistant to EGTA/dextran sulphate stripping was monitored as a function of time (Figure 6). Cells internalized $> 90\%$ of surface-bound ^{125}I-Lf within 3 min of warm-up, which is consistent with the rapid internalization of Lf by hepatocytes that we reported previously [27]. Similarly, cells internalized $> 50\%$ of ^{125}I-pp51 within 5 min at 37 °C. In contrast, the fraction of prebound ^{125}I-pp36 not released by stripping buffer remained relatively constant throughout the time course [0.36 ± 0.05 ($n = 13$)]. From these data, internalization rate constants were determined using the equation $\ln[(X_t - X_{min})/(X_{max} - X_{min})] = k_{in}t$, where X_t is radioactivity bound at time t, X_{min} is radioactivity bound at the first time point, X_{max} is maximal radioactivity bound during the time course and k_{in} is the first-order rate constant. We found that the rate constants for uptake of Lf and pp51 were similar (Lf, $k_{in} = 0.277$ min^{-1}, $r = 0.92$, $t_{1/2} = 2.5$ min; pp51, $k_{in} = 0.194$ min^{-1}, $r = 0.976$, $t_{1/2} = 3.5$ min) and correlated well with previously published internalization rate constants reported for apo- and holo-Lf [27] and [^{59}Fe]Lf [30]. On the other hand, the half-time for uptake of prebound pp36 was approximately an order of magnitude slower ($k_{in} = 0.018$ min^{-1}, $r = 0.405$ and $t_{1/2} = 38.5$ min), indicating that cells did not internalize pp36 at appreciable rates even though they bound it with relatively high affinity.

That the fraction of non-strippable pp36 was relatively large at very early time points in the previous experiment suggested the possibility that surface-bound pp36 was resistant to stripping conditions (EGTA and dextran sulphate) that routinely remove

Table 3 Dependence of pp36 binding to hepatocytes on Ca^{2+} and dextran sulphate

Hepatocytes (10^6/ml) were incubated with 26 pmol of ^{125}I-pp36 in Buffer A for 90 min at 4 °C. Cells were pelleted by centrifugation, then washed twice at 4 °C, 10 min per wash, in the designated buffer. Washed cells were analysed for bound radioactivity as described in the Experimental section. Values are means ± S.D. of duplicate samples. Experiments 1 and 2 were performed using different batches of isolated rat hepatocytes and with different pp36 preparations. Buffer C Hi-Salt: 0.5 M NaCl/3 mM KCl/10 mM Hepes (pH 7.4)/5 mM EGTA/0.3% (w/v) dextran sulphate; Buffer 9, 150 mM NaCl/3 mM KCl/10 mM sodium borate (pH 9.0)/5 mM EGTA/0.3% (w/v) dextran sulphate; Buffer 9 Hi-Salt, Buffer 9 supplemented with NaCl to give 0.5 M NaCl; Buffer 5, 150 mM NaCl/3 mM KCl/10 mM sodium acetate (pH 5.0)/5 mM EGTA/0.3% (w/v) dextran sulphate; Buffer 5 Hi-Salt: Buffer 5 supplemented with NaCl to give 0.5 M NaCl.

Experiment	Wash buffer	^{125}I-pp36 bound (fmol/sample)
1	Buffer A	1267 ± 35
	Buffer B	721 ± 78
	Buffer C	573 ± 46
	Buffer C Hi-Salt	419 ± 67
	Buffer 9	616 ± 34
	Buffer 9 Hi-Salt	499 ± 40
2	Buffer B	162 ± 17
	Buffer C	158 ± 1
	Buffer 5	1041 ± 94
	Buffer 5 Hi-Salt	805 ± 40

$\leqslant 85\%$ of surface-bound Lf [25,27]. When we examined the retention of pp36 bound to hepatocytes following various wash conditions (Table 3), we found that dextran sulphate in the presence of Ca^{2+} removed $> 40\%$ of surface-bound ^{125}I-pp36 from cells. This is reminiscent of the ability of dextran sulphate/Ca^{2+} buffers to remove a substantial portion of intact Lf from isolated rat hepatocytes [25]. A slightly greater amount of ^{125}I-pp36 was stripped off when cells were washed with an EGTA/dextran sulphate buffer, indicating that the residual ^{125}I-pp36

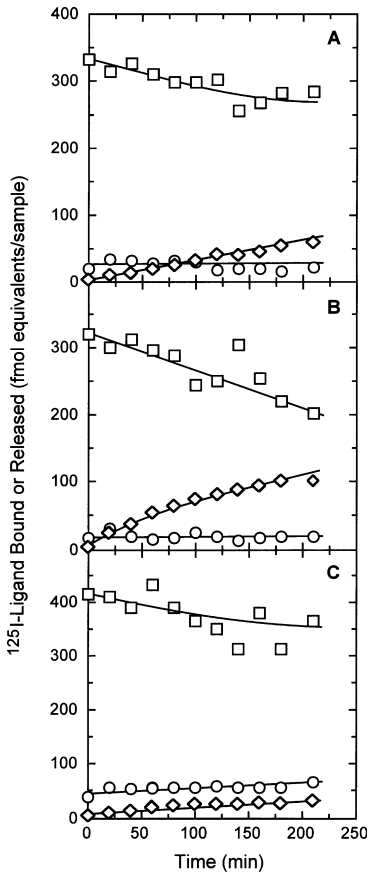

Figure 7 Endocytosis and degradation of Lf, pp51 and pp36

Cells (4×10^6/ml) were incubated with 131 pmol/ml ^{125}I-Lf (**A**), ^{125}I-pp51 (**B**) or ^{125}I-pp36 (**C**) for 90 min at 4 °C. Cells were washed free of unbound ^{125}I-ligand with cold Buffer B, resuspended in BME/BSA and incubated at 37 °C. At the designated times, cells were chilled rapidly and pelleted by centrifugation. Acid-precipitable (○) and acid-soluble (◇) radioactivity in cell-free conditioned media and cell-associated (□) radioactivity were determined as described in the Experimental section.

binding to cells was Ca^{2+}-independent. Increasing the ionic strength or pH of the stripping buffer resulted in only a small enhancement of ^{125}I-pp36 removal from cells. Lf binding to cells is reduced at pH < 7 [25], but washing cells with stripping buffer at pH 5.0 enhanced binding of ^{125}I-pp36 by cells (Table 3, Experiment 1). In similar experiments, we found that Buffer C, containing EGTA and dextran sulphate, removed > 90 % and 80 % respectively of ^{125}I-Lf and ^{125}I-pp51 from hepatocyte surfaces. These data confirmed therefore that a substantial fraction of pp36 binding to hepatocytes was Ca^{2+}-independent and insensitive to the polyanion dextran sulphate, whereas Lf and pp51 binding were Ca^{2+}-dependent.

The foregoing results indicated that hepatocytes readily bound and internalized Lf and pp51 in a similar fashion, whereas endocytosis of cell-associated pp36 was questionable. We have shown that hepatocytes degrade apo-and holo-Lf following endocytosis, a process that can be monitored by the release of acid-soluble ^{125}I-labelled by-products into conditioned medium [27]. We reasoned therefore that if hepatocytes internalized pp36, they would release pp36-derived acid-soluble byproducts following lysosomal degradation. To test this, hepatocytes were prebound with ^{125}I-Lf, ^{125}I-pp51 or ^{125}I-pp36 at 4 °C, washed free of unbound ligand, warmed to 37 °C and assayed for cell-associated and released radioactivity over time (Figure 7). Cells prebound with ^{125}I-Lf or ^{125}I-pp51 lost ^{125}I-ligand and generated acid-soluble radioactivity at nearly identical rates [Lf, loss of binding = 0.28 fmol/min per sample ($r = 0.82$), acid-soluble release = 0.27 fmol/min per sample ($r = 0.99$); pp51, loss of binding = 0.49 fmol/min per sample ($r = 0.85$), acid-soluble release = 0.46 fmol/min per sample ($r = 0.96$)]. We found that the fraction of bound ligand eventually released as acid-soluble radioactivity varied. Cells degraded and released ⩽ 31 % of prebound ^{125}I-pp51 (Figure 7B), whereas approx. 17 % of prebound ^{125}I-Lf was released as acid-soluble byproducts (Figure 7A). Cells also released a small fraction of prebound ^{125}I-Lf or ^{125}I-pp51 in acid-precipitable form (approx. 6 %). In contrast, cells released ⩽ 7 % of prebound ^{125}I-pp36 in the form of acid-soluble radioactivity (0.12 fmol/min per sample ($r = 0.93$)) but approx. 13 % in the form of acid-precipitable polypeptides which accounted for the majority of ^{125}I-pp36 released from the cells during the time course (Figure 7C). We conclude from these data that internalized pp51 is degraded more readily than the native Lf molecule, possibly because it is sterically more accessible to hydrolases than the intact protein. pp36, on the other hand, is either (i) not internalized by the cells or (ii) internalized but not delivered to a degradative compartment.

Competition binding of Lf, pp51, pp44 and pp36

The previous results indicated that Lf and pp51 exhibited similar biochemical features in their interaction with hepatocytes in contrast with those describing the interaction of pp36 with cells. These results suggested that the Ca^{2+}-dependent binding domain of Lf resides within the C-lobe structure contained within pp51, not in the bulk of the N-lobe domain present within pp36. If pp51 and pp36 bind to similar sites, then one would predict that these fragments would compete with each other for binding to cells. To explore this possibility, we examined the binding of ^{125}I-Lf, ^{125}I-pp51 or ^{125}I-pp36 to hepatocytes at 4 °C in the presence or absence of molar excesses of unlabelled Lf, pp51 or pp36 (Table 4). We found that the amounts of ^{125}I-Lf bound to cells progressively diminished by 90 % and 80 % respectively when unlabelled Lf or pp51 was added at a 100-fold molar excess; a 10-fold molar excess of these unlabelled ligands reduced ^{125}I-Lf binding by almost 70 % (Table 4). Similarly, ^{125}I-pp51 binding was progressively reduced by 70–80 % when cells were co-incubated with Lf or pp51 (Table 4). In contrast, we found that pp36, but not Lf or pp51, competed with ^{125}I-pp36 for binding to hepatocytes. Furthermore, pp36 only competed with ^{125}I-Lf or ^{125}I-pp51 for binding to cells when present at a 100-fold molar excess over labelled ligand (Table 4). It has been shown by others that proteolytic fragments corresponding to the N- and C-lobes of lactoferrin and ovotransferrin can reassociate [13,37]. In the case of ovotransferrins, isolated half-molecules do not bind appreciably to transferrin receptors until they reassociate [13]. Similarly, lactoferrin C-lobe binds to mitogen-activated T-cells only when it reassociates with N-lobe [15]. Notably, we did not observe an increase in ^{125}I-pp51 or ^{125}I-pp36 binding when labelled half-molecules were co-incubated with equimolar amounts of opposite halves (pp36 and pp51 respectively; Table 4). We conclude therefore that either pp51 and pp36 did not readily reassociate under these conditions or N- and C-lobe reassociation, if it occurred, did not enhance fragment binding to cells.

The N-terminal segment of pp51 contains elements of the N-lobe in addition to the helical peptide that links the N- and C-lobes together [1]. It is possible therefore that N-lobe elements within pp51 may be responsible for its Ca^{2+}-dependent interaction

Table 4 Comparative competition binding between Lf, pp51 and pp36

Hepatocytes (2×10^6 cells/ml) were incubated with 26 pmol/ml ^{125}I-Lf, ^{125}I-pp51 or ^{125}I-pp36 in the presence or absence of unlabelled Lf, pp51 or pp36 at the designated molar ratios for 90 min at 4 °C, after which cells were assayed for bound radioactivity as described in the Experimental section. ^{125}I-labelled and unlabelled ligands were incubated for 16 h at 4 °C in Buffer A before incubation with cells. Values are means ± S.D. of duplicate samples.

Ligand	Competitor	Competitor molar excess	^{125}I-Ligand bound (fmol/sample)
^{125}I-Lf	Lf	None	304 ± 3
		1	277 ± 7
		10	97 ± 3
		100	32 ± 1
	pp51	1	225 ± 4
		10	100 ± 3
		100	60 ± 1
	pp36	1	225 ± 7
		10	238 ± 11
		100	87 ± 6
^{125}I-pp51	pp51	None	145 ± 23
		1	100 ± 7
		10	86 ± 3
		100	31 ± 4
	Lf	1	110 ± 13
		10	79 ± 3
		100	42 ± 1
	pp36	1	134 ± 3
		10	134 ± 6
		100	71 ± 4
^{125}I-pp36	pp36	None	337 ± 6
		1	247 ± 10
		10	177 ± 7
		100	100 ± 4
	Lf	1	277 ± 7
		10	265 ± 9
		100	278 ± 7
	pp51	1	253 ± 1
		10	252 ± 6
		100	245 ± 3

Table 5 Competition of Lf, pp51 and pp44 with ^{125}I-Lf for binding to hepatocytes

Hepatocytes (2×10^6 cells/ml) were incubated with 26 pmol/ml ^{125}I-Lf in the absence or presence of a 50-fold molar excess of Lf, pp51 or pp44 for 90 min at 4 °C. Cells were washed and assayed for bound radioactivity as described in the Experimental section. Values are means ± S.D. of duplicate samples.

Competitor	^{125}I-Lf bound (fmol/sample)
None	34.4 ± 4.2
Lf	14 ± 4.2
pp51	11.1 ± 3.8
pp44	14.1 ± 0.6

with hepatocytes. To address this possibility, we examined the ability of pp44 Lf fragment, which contains only the C-lobe proper (Figure 3), to compete with ^{125}I-Lf for binding to cells. We found that a 50-fold molar excess of pp44 reduced ^{125}I-Lf binding approx. 60 %, which is comparable with the competition elicited by excess pp51 and Lf (Table 5). We conclude therefore that the domain of Lf responsible for its interaction with Ca^{2+}-dependent Lf binding sites on hepatocytes is present predominantly within the C-lobe.

DISCUSSION

The purpose of this study was to determine the region(s) of the Lf molecule responsible for its Ca^{2+}-dependent interaction with isolated rat hepatocytes. To accomplish this, we isolated multiple tryptic fragments of bovine Lf that corresponded approximately to Lf N- (pp36) and C- (pp44, pp51) lobes and analysed their Ca^{2+}-dependent interaction with hepatocytes. By using several criteria, we conclude that the C-lobe is responsible for the Ca^{2+}-dependent interaction of Lf with isolated rat hepatocytes. Hepatocytes bound Lf and pp51 in a Ca^{2+}-dependent manner with similar affinities and to similar extents. Lf and pp51 competed with each other for binding to cells over similar titration ranges and were internalized and degraded by hepatocytes at similar rates. Moreover, a tryptic fragment containing only the C-lobe (pp44) competed with ^{125}I-Lf for binding to Ca^{2+}-dependent binding sites on hepatocytes as well as native Lf or pp51. In contrast, pp36 bound hepatocytes with moderately high affinity, and it was not endocytosed or degraded appreciably by cells. Furthermore, pp36 binding to cells was Ca^{2+}-independent and it competed poorly with native Lf and pp51 for binding.

No consensus receptor-binding domain common to all transferrins has been identified. Studies using thermolysin-generated fragments of human transferrin showed that the C-lobe mimicked native transferrin in that it bound to transferrin receptors on HuH-7 hepatoma cells and K562 cells, and donated its iron to HuH-7 cells [14]. Notably, while transferrin C-lobe alone bound to transferrin receptors, its binding affinity for the receptor increased significantly when co-incubated with cognate N-lobe [14]. Other studies have shown that thermolysin-generated N-lobe of transferrin can bind and donate its iron to isolated rat hepatocytes but by using a site independent of the transferrin receptor [33]. In contrast, the interaction of ovotransferrin with transferrin receptors on chick reticulocytes apparently requires both N- and C-lobes because the interaction of either lobe with the transferrin receptor depends on half-molecule reassociation. In particular, monoclonal antibodies that disrupt reassociation of ovotransferrin half-molecules block the interaction of the half-molecules with cells but do not block the binding of native ovotransferrin to transferrin receptors [13]. Findings presented here and elsewhere indicate that Lf may use either or both of its lobes for binding to cells. Phytohaemagglutinin-stimulated human T-lymphocytes bind Lf via an approx. 105 kDa binding protein [17]; such binding is reduced competitively by a tryptic N-lobe fragment (of approx. 32 kDa) of human Lf [15]. Notably, T-lymphocytes bound tryptic C-lobe only when it was re-associated with N-lobe [15]. More recently, a highly cationic N-terminal 6 kDa proteolytic fragment of mature human Lf ($Arg^4 \rightarrow Glu^{52}$) partially blocked the binding of intact Lf to these cells [28]. On the other hand, C-lobe tryptic fragments (and to a lesser extent N-lobe fragments) of human Lf blocked the binding of intact Lf to membranes of *Neisseria meningitidis*, *Neisseria gonorrhoeae* and *Moraxella catarrhalis*, all of which express an approx. 100 kDa receptor for human Lf [16]. Other studies have shown that rat hepatocytes bind human Lf in a Ca^{2+}-independent manner at relatively low affinity via the chylomicron remnant receptor [26,38]. It has been suggested that an apo-E-like binding determinant present in the N-lobe is responsible for this interaction [38], but the interaction of human Lf half-molecules with these cells has not been reported. Additionally, removal of N-terminal cationic regions of human Lf with aminopeptidase-M enhances rather than diminishes its binding to rat hepatocytes

[39,40]. Elsewhere it has been shown that intact or fragments of Lf mediate the interaction of advanced glycation end products with their receptor on endothelial cells [24]; it appears that portions of Lf C-lobe may interact with this receptor [12]. These findings, in conjunction with our results presented here, suggest that the domain used by Lf to interact with cells may be cell- (and possibly species-)specific.

It is noteworthy that whereas the pp51 binding constants were the same as that for native Lf (Figure 4, Table 2), pp51 internalization and degradation rates differed from those of the native protein (Figures 6 and 7). It is possible that even though Lf interacts with hepatocytes via its C-lobe, the N-lobe may help stabilize its association with its receptor during events leading up to or concomitant with internalization of receptor–Lf complexes. After internalizing pp51, cells degraded pp51 at a higher rate than native Lf. It is likely that the portion of the C-lobe that normally faces its N-lobe counterpart is directly accessible to hydrolytic attack, thereby accounting for the increased degradative rate observed. It has been suggested that the strong association that exists between Lf half-molecules may be responsible in part for its relative resistance to proteolytic degradation in the gut of the suckling infant [41].

At present, the membrane molecule(s) that mediate the interaction of pp36 with hepatocytes remain unidentified. pp36 may interact with apo-E binding sites on hepatocytes. Human Lf competes with apo-E-bearing molecules for binding to hepatocytes [38]. This interaction is Ca^{2+}-independent, and the number of pp36 binding sites on hepatocytes we report here correlates with the number of human Lf binding sites observed on isolated rat hepatocytes [26]. Alternatively, pp36 (pI approx. 9.0) may interact non-specifically with generic anionic molecules on the cell surface or, more specifically, with cell-surface proteoglycans. Lf possesses glycosaminoglycan-binding sequences within the N-lobe and can interact with high affinity with heparin, heparan sulphate, dermatan sulphate and chondroitin sulphate [10,11]. At present, we are examining whether or not the pp36 interaction with isolated rat hepatocytes is altered by soluble glycosaminoglycans or by treatment of hepatocytes with glycosaminoglycan-specific hydrolases.

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