Lactoferrin binding to the rat asialoglycoprotein receptor requires the receptor's lectin properties

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Lactoferrin binds to rat hepatic lectin 1 (RHL1), the major subunit of the asialoglycoprotein (ASGP) receptor, with high affinity, by a galactose-independent mechanism. To better understand the molecular basis of this novel interaction, we compared the binding of lactoferrin and asialo-orosomucoid (ASOR) to isolated rat hepatocytes and to purified ASGP receptors as a function of pH, Ca^{2+} and receptor acylation. Binding of ^{125}I-lactoferrin and ^{125}I-ASOR to isolated rat hepatocytes at 4 °C decreased sharply at pH < 6, following similar titration curves. Binding of ^{125}I-lactoferrin and ^{125}I-ASOR to hepatocytes was Ca^{2+}-dependent. Binding increased progressively at $\geqslant 300\ \mu$M $CaCl_2$, in the presence of 1 mM EDTA. Monensin treatment of hepatocytes, which causes hepatocytes to accumulate inactive ASGP receptors, reduced surface binding of ^{125}I-lactoferrin and ^{125}I-ASOR by 46 and 49 %, respectively, with only a 16 % loss of immunodetectable receptor protein from the cell surface. Finally, deacylation of purified ASGP receptors *in vitro* with 1 M hydroxylamine abolished receptor lectin activity as reflected by the loss of ^{125}I-ASOR binding as well as the complete loss of specific ^{125}I-lactoferrin binding. Treatment with 1 M Tris had no effect on binding of either ligand. We conclude from these data that galactose-independent lactoferrin binding to the ASGP receptor requires the receptor's carbohydrate-recognition domain to be in an active configuration. An active configuration is promoted by neutral pH and Ca^{2+}, and also requires the receptor subunits to be acylated.

Key words: acylation, calcium, carbohydrate recognition domain, hepatocytes, receptor activity.

INTRODUCTION

Lactoferrin is a member of the transferrin family of non-haem iron-binding proteins, and is present in most mammalian body fluids as well as in specific granules of neutrophils (for reviews see [1,2]). Lactoferrin in the blood is cleared from the circulation by the liver, primarily by hepatocytes but also by Kupffer cells and liver endothelia [3–6]. Lactoferrin can bind to multiple receptors on hepatocytes prior to internalization, including low-density lipoprotein receptor-related protein (LRP) [7,8], a non-LRP binding site which functions as a chylomicron remnant receptor [9,10], and the asialoglycoprotein (ASGP) receptor [11,12]. For the latter case we have found that, unlike other characterized ligands of the ASGP receptor, lactoferrin binds rat hepatic lectin 1 (RHL1) with high affinity (K_d approx. 80 nM), yet in a galactose-independent manner [12,13]. Lactoferrins contain multiple biantennate N-linked oligosaccharide chains whose composition (complex versus high-mannose types) varies as a function of species and tissue source [14,15]. Enzymic removal of sialic acids, galactose and *N*-acetylgalactosamine (GalNAc) groups from human or bovine lactoferrin has no effect on the ability of modified lactoferrins to compete with native lactoferrins for binding to rat hepatocytes [12]. In addition, molar excesses of asialo-orosomucoid (ASOR) or lactose compete with lactoferrin for binding to ASGP receptors, indicating that lactoferrin binds at or near the carbohydrate-recognition domain (CRD) on ASGP receptors [12].

The foregoing observations suggest the possibility that protein elements of lactoferrin mimic a galactose- or GalNAc-like structure sufficiently to allow high affinity binding to the CRD. One prediction of this hypothesis is that lactoferrin binding to ASGP receptors should require the same solvent conditions as that needed for ASGP receptors to exhibit their lectin properties. ASGP receptors bind galactose- or GalNAc-terminated glycoconjugates only in the presence of Ca^{2+} and at pH > 6 [16,17]. Moreover, the lectin activity of ASGP receptors is regulated by reversible palmitoylation of receptor subunits [18], such that deacylated subunits lose ligand-binding activity [19,20]. If lactoferrin's interaction with ASGP receptors requires the lectin activity of the receptor, then one might expect deacylated ASGP receptors not to bind lactoferrin. To address these predictions, we compared the binding of lactoferrin and ASOR, a galactose-bearing desialylated glycoprotein that binds ASGP receptors with high affinity, to isolated rat hepatocytes and purified ASGP receptors as a function of pH, Ca^{2+} and receptor acylation. Our findings indicate that lactoferrin and ASOR mimic each other in binding to ASGP receptors, showing similar pH and Ca^{2+} requirements for binding. Moreover, deacylated ASGP receptors lose the ability to bind ASOR and lactoferrin similarly. These results are consistent with the idea that the galactose-independent interaction of lactoferrin with ASGP receptors occurs nonetheless via the lectin capacity of these receptors.

EXPERIMENTAL

Materials

Bovine colostrum lactoferrin, BSA (fraction V), dextran sulphate (5000 Da), human orosomucoid (α_1-acid glycoprotein), and PMSF were purchased from Sigma (St. Louis, MO, U.S.A.). Collagenase (type D) was obtained from Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). 1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril (Iodogen) was purchased from Pierce (Rockford, IL, U.S.A.) and Na^{125}I was obtained from DuPont New England Nuclear (Boston, MA, U.S.A.). All other chemicals

Abbreviations used: ASGP, asialoglycoprotein; ASOR, asialo-orosomucoid; BME, basal medium Eagle; CRD, carbohydrate-recognition domain; LRP, low-density lipoprotein receptor-related protein; RHL1, rat hepatic lectin 1.

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were of reagent grade and were obtained from Fisher Scientific (Tustin, CA, U.S.A.). 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, to strip ligand bound to Ca^{2+}-independent binding sites [13]. Buffer C contained HBS, 1 mM EGTA and 0.3 % (w/v) dextran sulphate. Buffer D contained HBS and 20 mM $CaCl_2$. Basal medium Eagle (BME) was obtained from Sigma and supplemented with 2.4 g/l Hepes and 0.22 g/l $NaHCO_3$, pH 7.4. BME/BSA corresponds to BME containing 0.1 % (w/v) BSA.

Hepatocytes

Male Sprague–Dawley rats (100–350 g, Harlan Sprague, San Diego, CA, U.S.A.) were fed standard laboratory chow and water *ad libitum*. Hepatocytes were prepared by modification of a collagenase perfusion procedure [21], as described previously [13]. Cells were kept at approx. 30 °C during the filtration and differential centrifugation steps. Final cell pellets suspended in ice-cold BME/BSA were $\geqslant 85\%$ viable, single cells. Before further experiments were performed, cell suspensions ($2–4 \times 10^6$ cells/ml in BME/BSA, 10 % of the flask volume) were incubated at 37 °C for 60 min to allow recovery from the isolation procedure. Cell viability was determined microscopically by Trypan Blue exclusion.

ASOR and lactoferrin preparation

Human orosomucoid was desialylated with neuraminidase as described previously [22], and removal of terminal *N*-acetylneuraminyl groups was confirmed by lectin blotting of neuraminidase-treated orosomucoid with *Sambucus nigra* agglutinin–digoxigenin ('DIG') conjugate (DIG Glycan Differentiation Kit, Roche Molecular Biochemicals) as reported elsewhere [12]. Bovine colostrum lactoferrin (commercial preparation $> 90\%$ pure electrophoretically) at 5 mg/ml in 100 mM KCl and 10 mM potassium phosphate, pH 7.2, was further purified by ion-exchange chromatography as described previously [13]. Lactoferrin's iron content was determined spectroscopically from its A_{465}/A_{280} ratio. This was routinely 0.030–0.035, indicating approx. 75 % iron saturation [23,24]. ^{125}I-ASOR and ^{125}I-lactoferrin, prepared by the 'Iodogen' method [25], had specific radioactivities of 10–60 d.p.m./fmol. Homogeneity and structural integrity of radiolabelled and unlabelled ASOR and lactoferrin were confirmed by SDS/PAGE and autoradiography.

Anti-RHL1 immunoglobulin (Ig) purification and cell surface RHL1 protein assay

Anti-RHL1 sera were generated and analysed as described elsewhere [11,12]. The whole IgG fraction from sera (immune and non-immune) was isolated by chromatography on Protein A–agarose (Pierce) and iodinated with $Na^{125}I$ [25] to a final specific radioactivity of 70–110 d.p.m./fmol. Hepatocytes in BME/BSA were incubated with or without monensin (25 μM) for 60 min at 37 °C then chilled on ice. The cells were washed by centrifugation in cold excess BME, then incubated with ^{125}I-anti-RHL1 IgG or ^{125}I-IgG (non-immune) for 90 min at 4 °C with or without a 50-fold molar excess of unlabelled anti-RHL1 IgG or non-immune IgG. The cells were collected by centrifugation, washed twice with excess cold buffer A, then assayed for cell-associated radioactivity.

Preparation of ASGP receptors and ligand-blotting

ASGP receptors were purified from isolated rat hepatocytes by detergent extraction and affinity chromatography on ASOR–agarose as reported previously [26]. Purified preparations were concentrated by ultrafiltration to a final value of approx. 100 μg/ml. The homogeneity of ASGP receptor preparations was confirmed by SDS/PAGE. Purified ASGP receptors were incubated in buffer D containing 0.025 % (v/v) Triton X-100 in the presence or absence of 1 M NH_2OH (hydroxylamine) or 1 M Tris for 2.5 h at 4 °C. The samples were subsequently diluted 5-fold with buffer D. Treated and mock-treated ASGP receptors were absorbed onto nitrocellulose membranes using dot-blot apparatus (0.5–1.5 μg/well) and rinsed twice in the manifold with buffer D. Membranes were removed from the dot-blot manifold and incubated overnight at 4 °C in buffer D supplemented with 1 % (v/v) Tween-20, then rinsed briefly in buffer D. The membranes were then incubated with ^{125}I-ASOR (0.5 μg/ml) or ^{125}I-lactoferrin (1.5 μg/ml) in buffer A supplemented with 0.02 % (v/v) Tween-20 for 90 min at 4 °C. Membranes were washed with buffer D at 4 °C to remove unbound ^{125}I-ligand, and membrane discs were excised and assayed for associated radioactivity.

General

Protein was determined by the bicinchoninic acid protein assay procedure using BSA as a standard (Pierce). Cell suspensions were centrifuged at 400 ***g*** for 2 min at 4 °C using a Beckman GS-6R centrifuge equipped with a GH-3.8 rotor (Beckman Instruments Inc., Fullerton, CA, 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 measurements were carried out using a Shimadzu UV-160 spectrophotometer (Kyoto, Japan).

RESULTS AND DISCUSSION

pH and Ca^{2+} requirements for lactoferrin and ASOR binding to hepatocytes

C-type animal lectins bind their specific glycoconjugates in a pH- and Ca^{2+}-dependent manner such that optimal lectin activity is observed at $pH > 6$ and at Ca^{2+} concentrations > 0.1 mM [27,28]. Lectin activity of ASGP receptors requires approx. 2 Ca^{2+} ions bound per CRD [28]. As is the case with other C-type animal lectins, Ca^{2+} bound to the CRD of ASGP receptors binds the C-3 and C-4 hydroxyl groups on galactose or GalNAc sugars [29]. This active configuration of the ASGP receptor is promoted at neutral pH but is not favoured at $pH < 6$ [28]. If lactoferrin binds to RHL1 as a function of its lectin-binding activity, then lactoferrin binding should require pH conditions and Ca^{2+} concentrations that would permit galactose- and GalNAc-terminated glycoconjugates to bind ASGP receptors. We have previously shown that the interaction of lactoferrin with isolated rat hepatocytes is promoted by Ca^{2+} at $pH \geqslant 7$ [13]. In this previous report, however, the binding of lactoferrin to hepatocytes as a function of pH did not distinguish between two classes of lactoferrin binding sites, characterized subsequently by their Ca^{2+}-dependence and sensitivity to polyanions such as dextran sulphate [30]. One class of sites are ASGP receptors, which mediate the vigorous endocytosis of lactoferrin by hepatocytes and bind lactoferrin in the presence of dextran sulphate [11,12,30]. A second class of sites bind lactoferrin in the absence of Ca^{2+} in a manner sensitive to the presence of dextran sulphate; these sites

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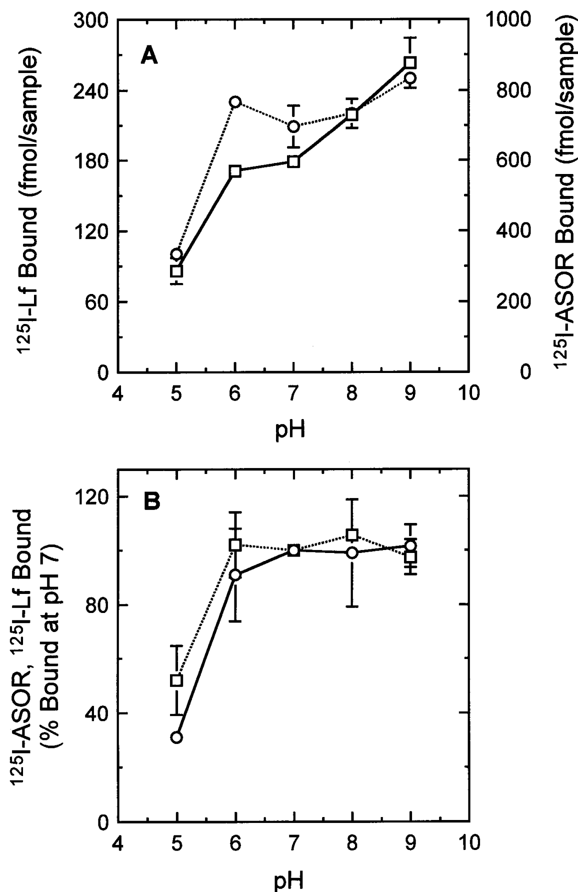

Figure 1 Comparative effect of pH on ASOR and lactoferrin binding to hepatocytes

(**A**) Hepatocytes (2×10^6 cells/ml) were incubated in isotonic saline at the designated pH with 2 μg/ml ^{125}I-lactoferrin (□) or 1 μg/ml ^{125}I-ASOR (○) for 90 min at 4 °C. The cells were pelleted by centrifugation, then washed twice by centrifugation (10 min per wash) with cold excess buffer at the same pH. Final cell pellets were resuspended in 1 ml of wash buffer, transferred to clean plastic tubes and assayed for associated radioactivity. Saline contained 150 mM NaCl, 3 mM KCl and 10 mM of one of the following buffering species: Na acetate (pH 5), Mes (pH 6), Bes (pH 7), Hepps (pH 8) or 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulphonic acid (Ampso) (pH 9). Buffers used to wash cells bound with ^{125}I-lactoferrin were supplemented with 0.3% (w/v) dextran sulphate. Results are means ± S.D. ($n = 2$). (**B**) Hepatocytes (2×10^6 cells/ml) were incubated with 2 μg/ml ^{125}I-lactoferrin (□) or 1 μg/ml ^{125}I-ASOR (○) for 90 min at 4 °C in buffer A (pH 7.4). The cells were pelleted and washed twice per centrifugation (10 min per wash) with cold saline at the designated pH. Final cell pellets were resuspended in 1 ml of wash buffer, transferred to clean plastic tubes and assayed for associated radioactivity. Buffered solutions used at the different pHs were the same as those described in (**A**). Buffers used to wash cells bound with ^{125}I-lactoferrin were supplemented with 0.3% (w/v) dextran sulphate. Results are means ± S.E.M. ($n = 2$). Lf, lactoferrin.

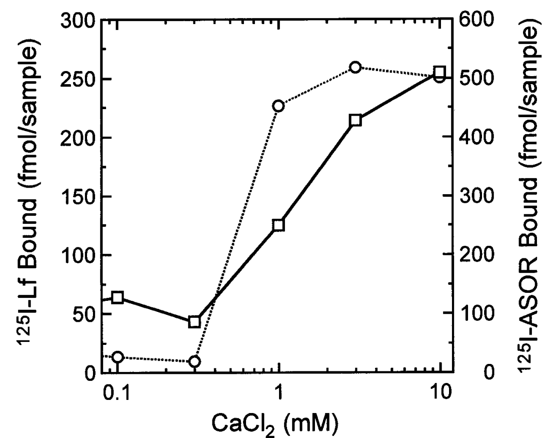

Figure 2 Comparative effect of Ca^{2+} on ASOR and lactoferrin binding to hepatocytes

Hepatocytes (2×10^6 cells/ml) were washed once with cold buffer C for 5 min, collected by centrifugation, and rinsed once with cold excess HBS. Cells were then incubated with 2 μg/ml ^{125}I-lactoferrin (□) or 1 μg/ml ^{125}I-ASOR (○) at 4 °C in HBS supplemented with the designated concentration of $CaCl_2$ and 1 mM EDTA for 90 min. Cells were pelleted by centrifugation and washed twice (10 min per wash) in the same ligand-binding buffer on ice. Buffers used to wash ^{125}I-lactoferrin-bound cells were supplemented with 0.3% (w/v) dextran sulphate. Washed cells were resuspended in 1 ml of wash buffer, transferred to clean plastic tubes and assayed for bound radioactivity. Results are means ± S.D. ($n = 2$). Error bars reflecting standard deviations are obscured by the symbols. Lf, lactoferrin.

are not endocytically active [30]. To address this matter directly, we compared the binding of ASOR and lactoferrin to isolated rat hepatocytes as a function of Ca^{2+} concentration and pH. To assess the binding of ^{125}I-lactoferrin to ASGP receptors only, cells were washed following the binding assays in the presence of dextran sulphate, which removes lactoferrin bound to non-ASGP receptor sites on these cells. We found that hepatocytes bound progressively more ^{125}I-lactoferrin and ^{125}I-ASOR at pH 5 (Figure 1A). ^{125}I-Lactoferrin binding increased somewhat between pH 7 and 9 whereas ^{125}I-ASOR binding over the same range remained relatively constant. When hepatocytes were prebound at 4 °C with ^{125}I-ASOR or ^{125}I-lactoferrin at pH 7 then washed at different pHs, we found that hepatocytes released both ligands from their surfaces significantly at pH < 6 (Figure 1B). In both experiments, ligand-binding and washes were done in the presence of 5 mM $CaCl_2$. These data indicate that lactoferrin binding to ASGP receptors on intact hepatocytes exhibited a similar pH-dependence to that for the desialylated ligand ASOR.

We also compared binding of lactoferrin and ASOR as a function of Ca^{2+} (Figure 2). Hepatocytes bound minimal amounts of ^{125}I-ASOR and ^{125}I-lactoferrin at Ca^{2+} concentrations < 0.3 mM when binding media were supplemented with 1 mM EDTA. In the absence of EDTA, hepatocytes tended to bind more ^{125}I-ASOR than ^{125}I-lactoferrin even in the absence of added Ca^{2+} (results not shown). Binding of both ligands to cells, however, increased at $CaCl_2$ concentrations > 0.3 mM. These data indicate that the Ca^{2+} requirement for lactoferrin binding to hepatocytes is similar to that needed for ASOR binding, although the Ca^{2+} concentration eliciting maximal ASOR binding was slightly lower than that observed for maximal binding of lactoferrin.

Effect of ASGP receptor inactivation on lactoferrin binding

ASGP receptors undergo reversible inactivation of their lectin properties when isolated hepatocytes are depleted of ATP or treated with agents that make acidic endosomal compartments alkaline [31,32]. More recently we have shown that overloading hepatocytes with iron, copper or zinc ions also induces cells to accumulate ASGP receptors in an inactive form [33,34]. Isolated hepatocytes treated with the Na^+/H^+ ionophore monensin accumulate a large fraction of their surface ASGP receptors, which have lost their lectin activity [32]. If ASGP receptors bind lactoferrin through their CRDs, one would predict that loss of ASGP receptor lectin activity would result in a decrease in lactoferrin binding as well. To test for this possibility, we

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Table 1 Effect of monensin on hepatocyte binding of ASOR, lactoferrin and anti-RHL1 IgG

Hepatocytes (2×10^6 cells/ml) were incubated in BME/BSA with or without 25 μM monensin for 60 min at 37 °C. The cell suspensions were chilled and collected by centrifugation. Cells were assayed for binding of ^{125}I-ASOR (1 μg/ml) and ^{125}I-Lf (2 μg/ml) in buffer A at 4 °C for 90 min. Unbound ^{125}I-ASOR and ^{125}I-lactoferrin were removed by washing cells twice (10 min per wash) in cold buffer A or buffer B, respectively. Cells were also assayed for binding of ^{125}I-anti-RHL1 IgG as described in the Experimental section. Values represent means $\pm$ S.D. ($n = 2$). Lf, lactoferrin.

		Ligand bound	
Ligand	Treatment	(fmol/sample)	(% of control)
ASOR	none	1015 ± 32	100
	monensin	521 ± 20	51
Lf	none	593 ± 22	100
	monensin	319 ± 20	54
Anti-RHL1 IgG	none	1028 ± 95	100
	monensin	859 ± 4	84

Table 2 Effect of hydroxylamine treatment on lectin and lactoferrin-binding activities of purified ASGP receptors

ASGP receptors were purified and assessed for binding of ^{125}I-ASOR (experiments 1 and 2) and ^{125}I-lactoferrin (experiment 2) by ligand-blot assays as described in the Experimental section. Ligand-blotting on untreated ASGP receptors was done in the absence or presence of a 25-fold (ASOR 25 $\times$) or 50-fold (ASOR 50 $\times$) molar excess of unlabelled ASOR. Purified ASGP receptors pretreated with 1 M NH_2OH or 1 M Tris were also assayed for ^{125}I-ASOR and ^{125}I-lactoferrin binding activity. Non-specific ^{125}I-ligand-binding activity was assessed by omission of purified ASGP receptor protein from the ligand-blot assay (no receptors). Errors reflect standard deviations of the means of triplicate and duplicate samples for experiments 1 and 2, respectively. Different ASGP receptor preparations were used for experiments 1 and 2. Lf, lactoferrin.

				Ligand bound	
Experiment	Incubation addition	Receptor treatment	Ligand	(fmol/sample)	(% of control)
1	none	none	^{125}I-ASOR	882 ± 37	100
	ASOR (25 $\times$)	none		547 ± 12	62
	ASOR (50 $\times$)	none		131 ± 68	15
	none	1 M Tris		789 ± 14	90
	none	1 M NH_2OH		274 ± 25	31
2	none	none	^{125}I-ASOR	62 ± 9	100
	ASOR (50 $\times$)	none		27 ± 3	44
	none	1 M NH_2OH		14 ± 7	23
	none	no receptors		4 ± 1	< 1
	none	none	^{125}I-Lf	5.9 ± 0.3	100
	none	1 M Tris		6.8 ± 0.8	115
	none	1 M NH_2OH		3.0 ± 0.3	51
	none	no receptors		2.8 ± 0.3	48

determined whether monensin-induced inactivation of ASGP receptors on hepatocyte surfaces is also accompanied by a loss of lactoferrin binding on treated hepatocytes (Table 1). As anticipated, ^{125}I-ASOR binding to hepatocytes decreased by 49 % following monensin treatment. Similarly, ^{125}I-lactoferrin binding to monensin-treated cells decreased by 46 %. The binding of ^{125}I-anti-RHL IgG to hepatocytes, however, was decreased by only 16 % indicating that monensin-treated cells did not greatly alter the amount of ASGP receptor protein on their surfaces. Thus, these data show that the monensin-induced loss of ^{125}I-lactoferrin binding correlated well with a loss of lectin activity of surface ASGP receptors, as reflected by the loss of ^{125}I-ASOR binding. Copper loading of hepatocytes induces a similar effect on ASGP receptor protein activity and distribution, as reflected by both ASOR and lactoferrin binding [34].

All subunits of rat and human ASGP receptors are acylated via a thioester linkage to a lone cysteine residue present near the transmembrane domain on the cytoplasmic tail of these subunits [19,20,35]. Reversible inactivation of ASGP receptor lectin activity has been associated with the deacylation of receptor subunits [19]. ASGP receptors on hepatocytes inactivated by either monensin or chloroquine treatment can be re-activated quantitatively if treated hepatocytes are permeabilized and incubated with palmitoyl-CoA (P. H. Weigel, personal communication). It is thought that ASGP receptors undergo deacylation (lectin inactivation) during recycling through the endosomal compartment followed subsequently by reacylation (lectin re-activation) before receptors return back to the cell surface [18,36]. The mechanism by which acylation of the receptor maintains the receptor's lectin activity is not clear.

To determine if ASGP receptor binding of lactoferrin requires receptor subunit acylation, we compared the ability of purified ASGP receptors to bind ASOR and lactoferrin before and after chemical deacylation with NH_2OH. ASGP receptors were purified from rat hepatocytes by ligand-affinity chromatography of hepatocyte membrane detergent extracts on ASOR–agarose [26]. Purified ASGP receptors immobilized on nitrocellulose membranes bound ^{125}I-ASOR in a Ca^{2+}-dependent manner in a standard dot-blot assay (Table 2). Inclusion of excess unlabelled ASOR progressively competed with ^{125}I-ASOR for binding to ASGP receptors under these conditions. It has been shown elsewhere that treatment of purified ASGP receptors with NH_2OH thoroughly deacylates receptor subunits concomitant with loss of receptor lectin activity [37]. Thus, not surprisingly, we found that NH_2OH treatment of purified ASGP receptors reduced ^{125}I-ASOR binding by approx. 70 %. This reduction was commensurate with that observed when the assay was done either in the presence of an excess of unlabelled ASOR or in the absence of purified ASGP receptors on the nitrocellulose membrane in the dot-blot assay (Table 2, no receptors). Purified ASGP receptors treated with 1 M Tris showed no loss of lectin activity (Table 2). Likewise, we found that NH_2OH treatment of ASGP receptors reduced ^{125}I-lactoferrin binding in this assay to levels observed when ASGP receptors were omitted from the assay. ASGP receptors treated with 1 M Tris showed no loss of ^{125}I-lactoferrin binding activity. We conclude from these data, therefore, that ASGP receptor binding of lactoferrin requires acylation of receptor subunits.

In summary, our results show that the interaction of lactoferrin with ASGP receptors correlates well with the lectin properties of the receptor, even though lactoferrin binds ASGP receptors in a galactose-independent manner. The structural conformation of the C-type lectin's CRD, necessary for high-affinity carbohydrate binding, requires Ca^{2+}, and the binding of Ca^{2+} to the CRD pocket is favoured at pH > 6.0 [38]. Lectin activity of ASGP receptors on hepatocytes also requires acylation of receptor subunits [19]. As demonstrated in the present study, the interaction of lactoferrin with ASGP receptors also exhibits these pH, Ca^{2+} and acylation requirements. One possibility is that lactoferrin does not occupy the carbohydrate-binding pocket of the CRD but binds nearby, such that conformational changes induced in the CRD by loss of Ca^{2+} or low pH also disrupt lactoferrin binding. Our data are also consistent with the possibility that lactoferrin may engage the carbohydrate-binding pocket of the CRD directly. The finding that the disaccharide lactose competes with lactoferrin and ASOR for binding to ASGP receptors [12] supports this second interpretation. At

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present, it is not clear how acylation of the cytoplasmic domain of the ASGP receptor is necessary for lectin activity of the externally exposed CRD on receptors *in situ*. It has been suggested that acylation of ASGP receptor subunits promotes a two-dimensional spacing of adjacent receptor subunits necessary for high-affinity multivalent binding of desialylated glycoconjugates [19]. Even though soluble recombinant CRDs of RHL1 exhibit native-like lectin properties [29], native ASGP receptors, either cell-associated or purified, apparently require acylation for lectin activity. Studies are currently underway in our laboratory to understand the molecular basis for the galactose-independent interaction of lactoferrin with ASGP receptors.

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