Removal of lactoferrin from plasma is mediated by binding to low density lipoprotein receptor-related protein/α_2-macroglobulin receptor and transport to endosomes

Melinda Meilinger**, Markus Haumer, Kati A. Szakmary, Ferdinand Steinböck, Barbara Scheiber, Hans Goldenberg, Manfred Huettinger*

Department of Medical Chemistry, University of Vienna, Waehringerstr. 10, A-1090 Vienna, Austria

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Abstract **LDL receptor related protein (LRP) is a ubiquitously expressed cell surface receptor that binds, at least in vitro, a plethora of ligands among them α_2-macroglobulin and lactoferrin (Lf). The function of LRP in internalisation and distribution of ligands within cellular metabolism is still unclear. We here investigated by combined ligand- and immunoblotting the participation of LRP/α_2MR and its associated protein (RAP) in receptor mediated endocytosis of Lf into rat liver. We found LRP highly enriched in sucrose density gradient fractions around density 1.10 g/ml, previously characterised as endosomal fractions. RAP was concentrated in distinct fractions around density 1.14 g/ml. This separation of RAP from LRP/α_2MR is physiologically meaningful as RAP avidly binds to LRP/α_2MR and by that shuts off all ligand binding function. In endosomal fractions we found one single binding protein for ^{125}I-labelled Lf. With a specific anti LRP/α_2MR antibody and ligand blotting with ^{125}I-labelled RAP this endosomal Lf binding site was verified to be LRP/α_2MR. Endosomes did not bind labelled Lf when prepared from rats that received an intravenous injection of Lf (20 mg per animal) 20 min prior to preparation. Surprisingly we immunodetected Lf in these endosomes at a position around 600 kDa, comigrating with LRP/α_2MR. We determined Lf binding to be optimal at pH 5.8, what led us to suggest the existence of a very stable LF–LRP/α_2MR complex in endosomes. These data support the idea of effective binding of Lf at pH as found in inflamed tissue environment where Lf is reported to be involved in leukocyte mediated inflammation regulation.**

Key words: Endocytosis; Inflammation; Lactoferrin

1. Introduction

Numerous studies suggest that the liver can play a role in the clearance of lactoferrin (Lf) from the circulation. In contrast to milk derived Lf, which is produced by synthesis in the lactating breast glands, blood born Lf is produced in leukocytes and set free into the blood after stimulation of leukocytes to release the content of their granules. Whether uptake into liver is mediated in part by a sui generis receptor remains a matter of dispute. It has been reported that binding of Lf to cells is mediated via several mechanisms, but internalisation is likely to be mediated by a receptor mediated process [1–5]. A candidate receptor for this might be LRP/α_2MR. The current opinion on the performance of the multifunctional LRP/α_2MR was outlined lately in several reviews [6–8]. It is believed that the clustering of cysteine-rich type A binding repeats, resembling those found in LDL receptor, is the molecular principle for the ability to bind a variety of ligands so far thought to be unrelated. The physiological importance of recognition of the hitherto identified or proposed ligands – activated α_2-macroglobulin (α_2M*), apolipoprotein E (apoE), lipoprotein lipase (LPL), plasminogen activators and complexes with their inhibitor (PA and PA/PAI-1), lipoprotein(a), pseudomonas exotoxin A, human rhinovirus, Lf and the so-called receptor associated protein (RAP) – by a single receptor entity is one crucial question to be answered [9–27]. Within the description of miscellaneous ligands of LRP/α_2MR also in vitro binding of Lf was demonstrated [1]. Together with our first description of the potency of Lf to inhibit chylomicron remnant uptake in vivo [4] these data prompted us to more directly investigate a possible physiologic importance of the binding of Lf to LRP/α_2MR. In a first step we here confirm the identity of LRP/α_2MR with the sole Lf-binding protein found in endosomes by ligand- and immunoblotting. Direct evidence for the participation of LRP/α_2MR will emerge from the evidence of Lf–LRP/α_2MR complexes in liver endosomes and emphasise that plasma Lf is cleared by receptor mediated internalisation. We will demonstrate that binding is optimal at pH 5.8 which explains the finding of intact receptor ligand complexes in the acidic endosomal compartment and supports the idea of highly effective binding at pH as found in inflamed tissue environment. Numerous reports exist about Lf's action after its release from activated leukocytes in inflamed tissues ([28] and references therein). This process also elevates blood levels of Lf considerably that led us to suggest a role of the Lf–LRP/α_2MR system in inflammation.

2. Experimental procedures

2.1. Materials

We obtained chemicals from Sigma and Na(^{125}I) for protein iodination from NEN. Suramin was kindly provided by Bayer (Leverkusen, Germany). Human α_2M was from Boehringer (Mannheim, Germany) and was activated (α_2M*) with methylamine according to Barret [29]. Polyclonal rabbit anti LRP/α_2MR antibody, recognising the 515 kDa ligand binding and 85 kDa transmembrane portion of LRP/α_2MR as well as the 39 kDa RAP, was kindly provided by J. Gliemann (University Aarhus, Denmark). Recombinant glutathione S-transferase (GST) and rat 39-kDa fusion protein (designated GST-RAP) were produced in DH5α bacteria transformed with a GST-RAP expression plasmid (kindly provided by J. Herz, UTHSC at Dallas) according to Herz. et al. [21]. Full binding activity of the isolated product was achieved after extensive dialysis in chaotropic buffers (1 × 1000 ml 5 M urea, 18 h, 10 × 20 ml from 4 M to 0.1 M urea 1 h, 1 × 1000 ml TBS, 18 h, 4°C).

*Corresponding author. Fax: (43) (1) 310 7210.

***Present address:* St. Emeric Hospital, Budapest, Hungary.

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Ligands were radiolabelled using IODO-BEADS (Pierce, Rockford, IL) according to the manufacturer's instructions. Typically 100 μg of protein in 100 μl TBS (Tris-buffered saline, 50 mM Tris, 150 mM NaCl, pH 7.4) were labelled with 100 μCi of Na(^{125}I) using 1 bead to a specific activity of 3–4 × 10^6 cpm/μg and extensively dialysed against TBS/0.1 mM EDTA.

2.2. Animals and tissue fractionation

Male Sprague–Dawley rats (weight approx. 200 g) were received from Institut für Versuchstierkunde, University of Vienna. Endosomes were isolated from rat liver homogenates essentially as described [30]. Ligands (Lf: 7 mg per animal, α_2M*: 2 mg per animal) were injected as described [4] and were circulating in anaesthetised animals for 20 min.

2.3. Electrophoresis and blotting

Typically 80 μg of protein (non-reduced and non-boiled) per lane were loaded onto 7.5% linear or 4–15% SDS-PAGs as indicated. After electrophoresis the samples were transferred to nitrocellulose membranes at 12°C. Individual strips were incubated in 1 ml of incubation buffer (Tris-buffered saline (TBS) 50 mM Tris, 150 mM NaCl, pH 7.4, 2 mM $CaCl_2$, 0.01% Triton X-100, Pierce Rockford, IL) after blocking with 5 mg non-fat dry milk per ml (2 h each). Immunodetection was done essentially as described [31] using HRP-conjugated second antibodies (Bio-Rad, Vienna) and the ECL reagent (Amersham, Vienna) according to the specifications of the manufacturer for visualisation. For quantification of bound radioactive ligands densitometric scanning of autoradiographs was performed with a scanner system (IMAGE-MASTER, Pharmacia, Sweden).

3. Results

3.1. LRP/α_2MR but not its associated protein (RAP) is enriched in endosomes

A light particle fraction obtained from differential centrifugation was subjected to zonal sucrose density gradient centrifugation. Fractions in the range of 1.06 to 1.15 g/ml, as characterised by marker protein enrichment in a previous paper [30], were separated by electrophoresis and blotted onto nitrocellulose membranes. By immunodetection we observed the highest enrichment of LRP/α_2MR in endosomal fractions with a density of 1.10 g/ml (Fig. 1). Interestingly, we could not detect RAP

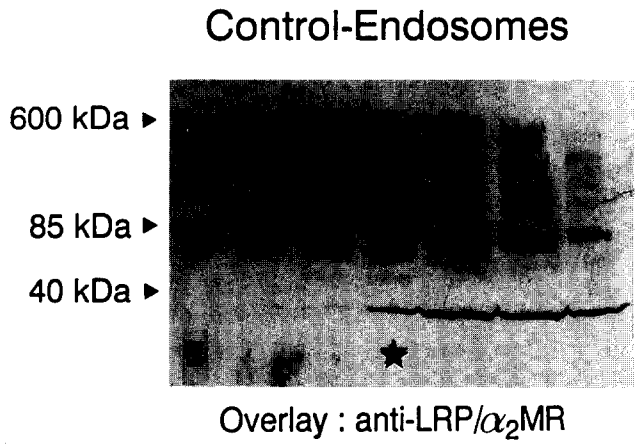

Fig. 1. Immunodetection of LRP and RAP in sucrose density gradient fractions. Endosomal fractions were prepared from the livers by differential and sucrose gradient centrifugation. The fractions from density 1.06 (left lane) to 1.16 g/ml (rightmost lane) were separated by 4–15% gradient SDS-PAGE and blotted onto nitrocellulose membranes (*denotes peak endosomal fraction with density 1.10 g/ml). Arrows give the approximate positions of 600 kDa (LRP/α_2MR and the tetramer form of α_2M), 85 kDa (transmembrane part of LRP/α_2MR) and 40 kDa (receptor associated protein RAP)

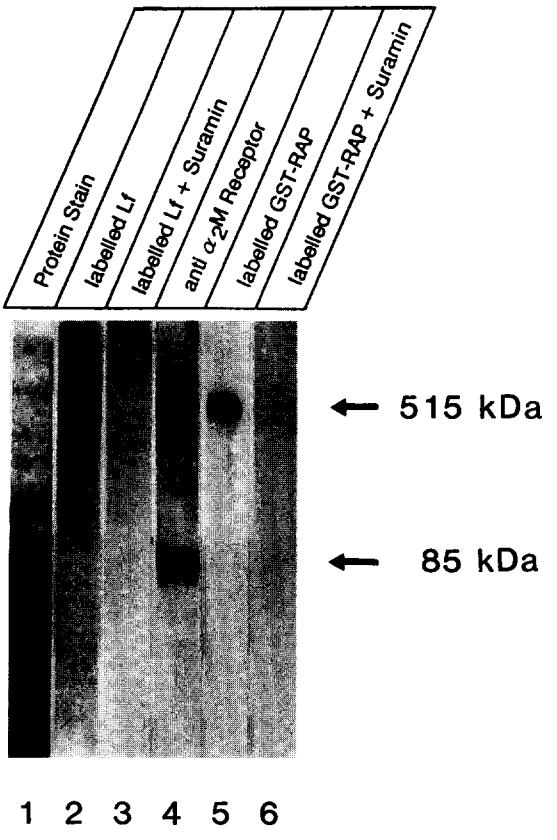

Fig. 2. Identification of Lf-BP by ligand- and immunoblotting . Rat liver endosomal fractions (80 μg protein per lane of the 1.10 g/ml peak endosomal fraction as described in Fig. 1) were separated by 7.5% SDS-PAGE, blotted onto nitrocellulose membranes and stained with Ponceau red (strip 1). Replicate strips were subjected to ligand- and immunoblot analysis. Strips show the results of ligand blotting with ^{125}I-labelled Lf (strip 2 and 3, 0.9 × 10^6 cpm/ml) and ^{125}I-labelled GST-RAP (strip 5 and 6, each 0.7 × 10^6 cpm/ml) in the absence and presence (strip 3 and 6) of 5 mg Suramin per ml. Strip 4 shows the result of immunoblot analysis with a polyclonal rabbit antibody, raised against purified LRP/α_2MR, as first antibody, followed by HRP labelled second antibody and subsequent visualisation by ECL. One typical set of results out of three identical experiments is shown.

in these fractions, although the primary antibody is able to recognise RAP with high sensitivity. We localised the so-called receptor associated protein RAP clearly separated from the receptor in gradient fractions around 1.13 g/ml which contain Golgi-derived material as we described earlier [30]. Also lysosomal localisation can be excluded, as fractions >1.19 g/ml were virtually free of RAP.

3.2. Identity of Lf-binding protein (Lf-BP) with LRP/α_2MR in endosomes

Numerous types of binding sites for Lf have been postulated on the cell surface. Their involvement in endocytosis has not been investigated in detail. We therefore tested by ligand blotting with radiolabelled Lf how many binding sites are internalised and thus can be found in purified endosomes. In Fig. 2 a Ponceau-stained sample of an endosomal fraction (strip 1) is shown which was subjected to ligand blotting (strip 2) with [^{125}I]Lf resulting in a single prominent band migrating at approx. 600 kDa. This signal comigrates with the one resulting

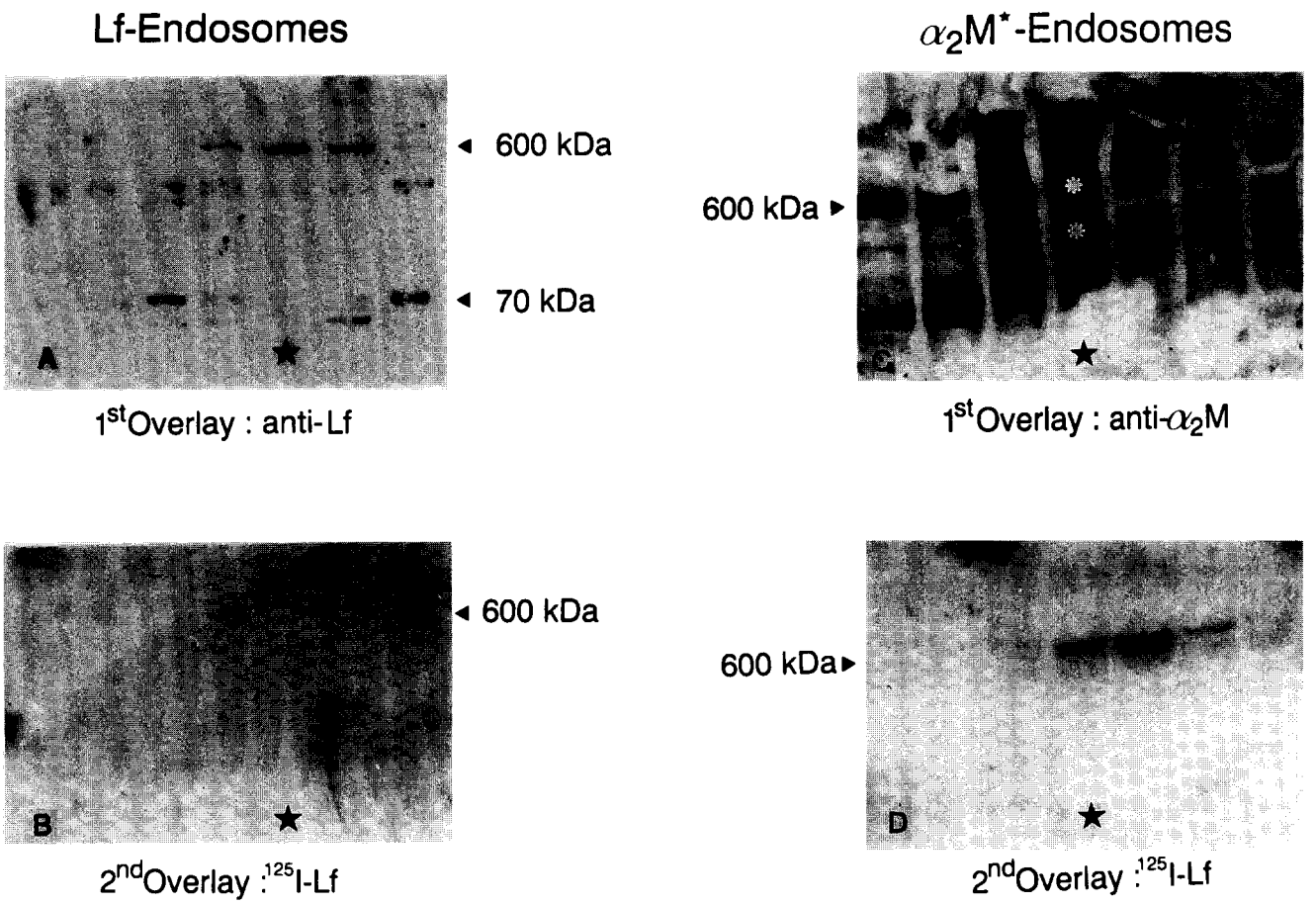

Fig. 3. Immunodetection of internalised ligands and determination of binding function in endosomes. Rats were injected with unlabelled Lf (Fig. 3A and B, Lf-Endosomes) and α_2M (Fig. 3C and D, α_2M-Endosomes) and subcellular fractions prepared as described. The unlabelled ligands recovered in these fractions (free Lf at 70 kDa and α_2M at 720 kDa and fragments, marked by white star) were analysed as described in Fig. 2 by incubation with specific rabbit primary antibodies followed by HRP-labelled anti-rabbit IgG and visualised by ECL (2 min exposure). This membranes were subjected to a second overlay (B and D) with radiolabelled Lf to determine unsaturated binding capacity after autoradiographic visualisation (16 h exposure) of bound radioactivity. Arrows give the approximate positions of 600 kDa (LRP/α_2MR and α_2M), 85 kDa (transmembrane part of LRP/α_2MR), 70 kDa (free Lf). One representative set of results from two identical experiments is shown.

from developing this preparation with a polyclonal antibody raised against purified α_2MR (strip 4). The exact comigration was also verified by alignment of lanes split into two halves and developed by ligand- and immunodetection respectively (not shown). The migration behaviour of the Lf-BP was also indistinguishable from the RAP-BP described to be the LRP/α_2MR. Ligand blotting with [^{125}I]GST-RAP again showed an identically migrating reaction product at approx. 600 kDa (strip 5). Finally, the binding of Lf as well as of GST-RAP to LRP/α_2MR was abolished by 5 mg Suramin per ml (strips 3 and 6), which prevents internalisation of Lf and chylomicron remnants in tissue culture, as demonstrated earlier [4]. We thus demonstrate the existence of one sole protein capable of binding Lf and RAP in endosomes, which is, with high likelihood, LRP/α_2MR.

3.3. *Lf injected into animals is recovered in the endocytotic compartment of the liver complexed to LRP/α_2MR*

We have previously shown that coinjection of excess Lf with labelled chylomicron remnants led to exclusion of remnants from the endocytotic compartment. Labelled Lf was accumulated by hepatocytes into vesicles of a density around 1.10 g/ml most likely endosomes [4]. To bring further insight whether LRP/α_2MR is involved in the transport of Lf from plasma into cells, we injected unlabelled Lf into rats 20 min prior to removal of livers and subsequent subcellular fractionation. Fractions from density 1.06 to 1.18 g/ml were separated by SDS-PAGE and transferred to nitrocellulose and Lf immunodetected using a polyclonal antibody. Unexpectedly, immunopositive material at approx. 70 kDa (free Lf, arrow Fig. 3A) was not enriched in the peak endosomal fraction (lane marked with asterisk). In this fraction, however, a strong signal at 600 kDa was detected. At that position a complex of Lf bound to LRP/α_2MR would be found. This slow migrating form of Lf was detected exclusively in endosomal fractions of rats injected with Lf but did not occur in the plasma. If this was indeed Lf tightly bound to the receptor we reasoned that in this fractions the binding of Lf should be diminished due to saturation of the receptors. We therefore subjected the same nitrocellulose membrane to a second overlay with ^{125}I-labelled Lf (Fig. 3B) and could not detect any binding. As a control we prepared matching fractions from rats preinjected with α_2M* (Fig. 3C and D). Activated α_2M was substantially enriched in the fraction of a density of 1.10 g/ml. Under these conditions the 720 kDa protein α_2M was fragmented to typical subunit fragments, mainly 360 and 80 kDa

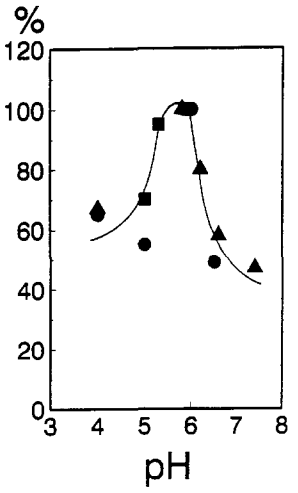

Fig. 4. Influence of pH on the binding of Lf to LRP/α_2MR. Endosomal fractions were prepared as described in Fig. 2 and were subjected to ligand blotting. Strips were individually incubated with 1–2 × 10^6 cpm of labelled Lf per ml as described in section 2, in appropriate buffers from pH 4.0 to 7.4. Washes were performed at the pH of incubation. Autoradiographs of strips were quantitated by densitometric scanning. The maximal value obtained was set to 100% (symbols denote values obtained from three independent experiments).

(white asterisks Fig. 3C) as described by Barret et al. [29]. We subjected the nitrocellulose membrane to a second overlay with ^{125}I-labelled Lf (Fig. 3D) and clearly detect binding. Thus, LRP/α_2MR present in Lf-loaded endosomal preparations was saturated with Lf. On the other hand, in fractions prepared from α_2M*-endosomes, the huge amount of α_2M*, as immunodetected, did not block access of [^{125}I]Lf to the receptor and binding of [^{125}I]Lf was clearly visible on the autoradiograph.

3.4. Binding of Lf to LRP/α_2MR is maximal at pH 5.8

Dissociation of ligands from receptors in endosomes is mediated by pH induced conformation changes. To characterise the interaction of Lf with the receptor in endosomes we studied its pH dependency. For α_2M*, the maximal binding was reported at pH 7.8 [35]. The affinity of [^{125}I]Lf to LRP/α_2MR was determined by incubating nitrocellulose strips at pH ranging from 3.5 to 7.4 for one hour, followed by extensive washing at the same pH. Autoradiographs were scanned densitometrically. The results are shown in Fig. 4 as the percentage of maximal binding detected at pH 5.8 calculated from three independent experiments. We consistently found a maximal binding around pH 5.8 whereas around pH 7.4 and 4.0 binding was reduced.

4. Discussion

LRP is an ubiquitously expressed cell surface receptor that binds, at least in vitro, a plethora of ligands. The function of LRP/α_2MR in internalisation and distribution of these ligands is still unclear. It was demonstrated by Herz et al. [32] by tracing with antibodies that this receptor can carry out receptor mediated endocytosis. The so called receptor associated protein (RAP) copurified with the receptor upon affinity purification with LRP/α_2MR and inhibited most, if not all, ligand binding and was hypothesised to be a regulatory unit for the receptor. We here present data that confirm the localisation of LRP/α_2MR in endosomes but show that RAP is enriched in a compartment of distinct density. This suggests that, as the fractions were prepared from intact organelles, RAP is normally separated from LRP/α_2MR. RAP has been recently found to inhibit chylomicron uptake in animals where overexpression induced a high plasma level of the normally intracellular protein [33]. Our findings give a first hint for the compartment where RAP is separated when it is not needed to shut off receptor function. RAP was also discussed as the immunogenic stimulus causing Heyman nephritis. Under what metabolic circumstances, if any, RAP is presented to antibody producing cells in complex with gp330, another member of the LDL receptor family, remains to be elucidated [34].

In our first report about competition of chylomicron remnants with Lf for uptake into hepatocytes in vivo, this was considered to arise from an indiscriminate random sequence homology. The subsequent discovery of various proteins binding to LRP/α_2MR led us to investigate more intensively a physiologic significance of the affinity of Lf to LRP/α_2MR. We here present experiments that strongly advocate for a factual ligand role of Lf to LRP/α_2MR. Our experiments demonstrate the clearance of Lf from the bloodstream into liver endosomes by a solitary receptor. We found after intravenous injection of Lf a high molecular weight form of LF at about 600 kDa, presumably a Lf/Lf-receptor complex in liver endosomes. This complex, accordingly, immunostained with antibodies against LRP/α_2MR. Such in vivo Lf-loaded endosomes were evidently saturated as we saw no further capacity to bind Lf in ligand blots. In control experiments, endosomes prepared after injection of α_2M under otherwise identical conditions bound labelled Lf. Evidently α_2M was at least partially dissociated from LRP/α_2MR in endosomes as we found fragments of the molecule at lower molecular weights as described [29]. This finding parallels our previous results that showed independent binding of Lf and α_2M* to LRP/α_2MR.

Usually the low pH in endosomes induces a conformation shift that brings forward the release of ligands from the receptor. An explanation why this was not operative for the Lf-LRP/α_2MR complex came from our experiment showing optimal binding of Lf at pH 5.8. We cannot at this moment clearly deduce from the experiments that Lf is functioning as a non dissociable ligand in vivo. If so, this would anticipate that the receptor is not resorted to the cell surface as was found for non-dissociating receptor antibody complexes. We are now in the process of investigating the intracellular localisation of LRP/α_2MR and Lf after prolonged endocytosis of Lf.

The extension of our understanding of the physiologic performance of this receptor brought upon by a new ligand is to this end speculative. The demonstration of LRP/α_2MR as the sole demonstrable protein mediating endocytosis of Lf from plasma into liver in the intact organism supported the idea of physiological importance. The detection of extremely stable Lf-LRP/α_2MR complexes in the intrinsic environment, the endosome, is a strong argument that this reaction occurs in vivo and has the potency of regulating receptor levels at the cell surface.

Lf in plasma is derived from leukocytes activated at sites of tissue remodelling during the acute inflammatory response. This process elevates plasma Lf concentrations tenfold and even more at the site of the acute response. Other ligands of

LRP/α_2MR like α_2M, PA/PAI-1and apoE are also involved in these metabolic situations [36–40] pointing towards a concerted action of their receptor in this condition. As an iron complexing glycoprotein, Lf might sequester free iron in the extracellular space delivering it via LRP/α_2MR mediated endocytosis into hepatocytes and other cells expressing LRP/α_2MR, like macrophages. Our finding that Lf is most avidly bound around pH 6 implicates that the Lf-LRP/α_2MR interaction provides a powerful system in a florid inflammatory environment. It is therefore attractive to speculate that Lf is a member of the LRP/α_2MR ligand family that is involved in tissue remodelling at sites of inflammation or of physiologic processes. As a common feature they are, after having fulfilled a particular function, bound and internalised by LRP/α_2MR thus signalling the status of the extracellular events.

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