Lactoferrin receptor mediates apo- but not holo-lactoferrin internalization via clathrin-mediated endocytosis in trophoblasts

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LfR [Lf (lactoferrin) receptor] is expressed in most mammalian tissues, including placental trophoblasts, and is presumed to mediate the internalization of Lf. However, the physiological significance of trophoblast LfR is not understood. Using the CT (cytotrophoblast) cell model BeWo, we demonstrated that transfection with LfR siRNA (small interfering RNA) significantly decreased apo- but not holo-Lf uptake compared with mock-transfected controls and that apo- but not holo-Lf significantly increased MMP (matrix metalloproteinase)-2 activity. As Lf function is related to the presence (holo-Lf) or absence (apo-Lf) of iron within the Lf molecule, our results suggest that apo-Lf may play a role in cellular invasion. Moreover, we detected LfR (∼105 kDa) in association with the plasma membrane, and ligand blotting confirmed that Lf binds to a LfR of ∼105 kDa. Apo-Lf treatment significantly increased LfR abundance at the plasma membrane and internalization probably occurs via clathrin-mediated endocytosis through early and recyc-ling endosomes, as LfR was co-localized with EEA1 (early endosome antigen 1) and TIR (transferrin receptor) using confocal microscopy, and hypertonic medium (0.4 M sucrose) significantly inhibited apo-Lf internalization. In summary, our data demonstrate that apo- but not holo-Lf is internalized by LfR and suggest that, following internalization via LfR, apo-Lf plays a role in CT invasiveness by inducing MMP-2 activity. Moreover, LfR facilitates apo-Lf uptake specifically through clathrin-mediated endocytosis into early endosomes and potentially into a recycling pathway. Taken together, our data provide a new dimension in understanding ligand-dependant function that may be directly related to the ability of LfR to selectively internalize apo- but not holo-Lf.

Key words: BeWo cell, endocytosis, lactoferrin, lactoferrin receptor, matrix metalloproteinase-2 (MMP-2).

INTRODUCTION

The presence of a LfR [Lf (lactoferrin) receptor] was first identified in human small intestine [1]. It presumably facilitates the uptake of its protein ligand Lf, which is present in milk and mucosal secretions [2]. Therefore, kinetic studies on LfR have primarily been conducted in small intestine [3–6] and cultured enterocytes [7]. We have previously cloned the LfR gene (GenBank® accession number NM_017625) [3] and determined that the intestinal LfR is an ∼110 kDa trimeric (∼37 kDa subunits), glycosylated protein [7]. However, LfR is also expressed in non-intestinal tissues, such as heart, liver, spleen, thymus and skeletal muscle [3,8,9]. Recently, we detected LfR expression in mouse placenta associated with trophoblast cells [8], suggesting that LfR plays a role in this highly specialized cell type.

Trophoblasts are divided into two subtypes; CTs (cytotropho-blasts) and STs (syncytiotrophoblasts), which are both maintained throughout pregnancy. The primary function of CTs is invasion into the uterine epithelium to secure successful implantation [10], whereas STs’ further implantation progression by establishing the nutrient and gas exchange barrier (maternal–fetal interface) and secreting hormones such as progesterone which are essential for successful pregnancy maintenance [11]. The invasive capacity of CTs relies on the secretion of MMPs (matrix metalloproteinases), which function to degrade the surrounding ECM (extracellular matrix). Several disorders of pregnancy, including pre-eclampsia and spontaneous abortion, are believed to relate to ineffective CT proliferation, migration and invasion into the uterine endothelium [10].

Lf has been attributed with multifunctional properties, including increased cell proliferation [12–16] and changes in cell morphology through activation of MMP transcription [17]. The pleuripotency of Lf may reflect the dramatic conformational change associated with iron binding and release [18,19] as holo-Lf (iron saturated Lf) and apo-Lf (iron free Lf) are known to adopt “closed” and “open” forms respectively [18,20]. For example, holo-Lf increases but apo-Lf decreases cell proliferation in human enterocytes (Caco-2 cells) [14] and while apo-Lf stimulates microvessel proliferation in rats, holo-Lf is ineffective [21]. Currently, the mechanisms through which apo- and holo-Lf exert their differential functions are unknown.

Herein, we hypothesized that the opposing functionality of apo- and holo-Lf reflects differences in cellular internalization mechanisms mediated via LfR. In order to address this, we utilized the human placental CT cell line BeWo and determined: (1) the effects of apo- and holo-Lf on MMP-2 activity, the major MMP in trophoblasts; (2) the ability of the LfR to facilitate apo- and holo-Lf uptake and internalization; and (3) the subsequent intracellular compartmentalization of LfR following Lf uptake. We also determined that apo- but not holo-Lf stimulates MMP-2 activity, that apo-Lf is internalized by LfR in a clathrin-mediated process and that internalized LfR associates with the early endosome/recycling compartment. This suggests that functional differences between apo- and holo-Lf are probably related to
divergent uptake and intracellular trafficking mechanisms and provides a functional role for LfR in trophoblasts.

**MATERIALS AND METHODS**

**Cell culture**

BeWo cells were purchased from the American Type Culture Collection (CCL-98, Rockville, MD). Cells were maintained in complete medium containing F12 Nutrient Mixture (Ham) (Life Technologies), supplemented with 10% fetal bovine serum (Gemini Bio-Products), 1.5 g/l sodium bicarbonate, 10 μg/ml streptomycin and 10 units/ml penicillin (Sigma) at 37°C in a humidified atmosphere with 5% CO₂ until 90% confluent. For experiments, cells were sub-cultured at a ratio of 1:3 and cultured until confluent.

**Purification of human Lf**

Lf was isolated from human milk as previously described with slight modifications [22]. Casein was precipitated from pooled human milk with 0.06 M CaCl₂; pH 4.6, for 1 h at room temperature (25°C) with constant stirring and centrifuged at 8600 g for 20 min at 4°C. The whey fraction was collected, centrifuged at 8600 g for 20 min at 4°C and then mixed with heparin-Septarose Fast Flow resin (GE Health-Amersham Biosciences) overnight at 4°C. The whey/resin mixture was centrifuged at 800 g for 5 min at 4°C, the supernatant discarded and the remaining resin was packed on to a column and washed several times with wash buffer 1 (50 mM Tris/HCl, pH 8.0). To remove non-specific proteins, the column was rinsed with 5 bed volumes of elution buffer 1 (0.05 M Tris/HCl, 0.3 M NaCl, pH 8.0). Next, Lf was eluted with 5 bed volumes of elution buffer 2 (0.05 M Tris/HCl, 1.0 M NaCl, pH 8.0) and fractions were collected. Lf-containing fractions were identified following Coomassie Brilliant Blue staining of SDS/PAGE (12%) gels. Samples were pooled and concentrated in YM-50 centrifugation tubes (Millipore Centriplus, Fisher Scientific) at 3000 g for 90 min at 4°C. The protein concentration of purified Lf was determined spectrophotometrically (A₃₅₀) as previously established [23].

Apo-Lf was prepared as previously described [24]. Holo-Lf was prepared with FeSO₄ (Lf/Fe; 1:2 molar ratio) in sodium bicarbonate (Fe/HCO₃; 1:1 molar ratio) for 1 h at room temperature (25°C). Unincorporated Fe was removed with YM-50 microcentrifuge tubes at 12000 g at 4°C and stored at -20°C. Fe saturation was determined spectrophotometrically by the A₆₀₀/A₃₅₀ ratio (> 22) [25].

**MMP activity assay (zymography)**

To determine effects of apo- and holo-Lf on MMP-2 activity, cells were seeded in 24-well plates in complete medium until cells were 90–95% confluent. Cells were rinsed twice with 1 × PBS and then incubated with apo- or holo-Lf (50 μg/ml) in F12 medium plus 2% FBS (fetal bovine serum) for 4 h. Conditioned medium was collected after 24 h and concentrated to one third of the original volume using YM-50 microcentrifugation tubes (Millipore Centriplus) at 5000 g for 20 min at 4°C. Protein concentration of conditioned media was determined using the Bradford assay (Bio-Rad) and proteins (20 μg) were electrophoresed by SDS/PAGE (7.5% gel containing 1 mg/ml gelatin). Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 in PBS for 15 min to remove SDS and incubated in wash buffer 2 (50 mM Tris/HCl, pH 7.6, 10 mM NaCl, 50 mM CaCl₂) overnight at 37°C. Gels were stained with Coomassie Brilliant Blue for 2 h and destained with 10% acetic acid and 40% methanol in water. Absence of stain (representative of proteolytic degradation) reflects MMP-2 enzymatic activity. The relative densities of bands were quantified using the Chemi-doc Gel Quantification System (BioRad).

**Uptake of ¹²⁵I-Lf**

Apo- and holo-Lf were labelled with ¹²⁵I (GE Health-Amersham Biosciences) by the iodogen method [26]. Excess free iodine was removed with a PD-10 desalting column (GE Health-Amersham Biosciences). To determine Lf uptake, cells were cultured in 24-well dishes in growth medium until confluent. Cells were incubated with ¹²⁵I-labelled apo- or holo-Lf (10000–100000 c.p.m.) in SFM (serum-free medium) for 1 h at 37°C. Medium was collected, cells were rinsed once with rinse buffer (0.5 M NaCl, pH 3.0) and pooled with the medium. Cells were washed three times with PBS, solubilized with 0.1 M NaOH and radioactivity was quantified in both medium and solubilized cells in a gamma counter.

**Affinity purification and validation of LfR antibody**

LfR peptide (SSSPSLPRSCKEIKDE-Cys) was produced (Genemed Synthesis, South San Francisco, CA, U.S.A.) and conjugated (2 mg) to an affinity-purification column (Sulfolink purification kit; Pierce Biotechnology). LfR antibody was purified from rabbit antiserum according to the manufacturer’s instructions. To demonstrate antibody specificity, affinity-purified LfR antibody (1 μg/ml) was pre-incubated with LfR peptide (1 mg/ml) in 5% (w/v) non-fat milk in PBS-T (PBS, 0.1% Tween) for 2 h at room temperature (25°C) prior to immunoblotting of cell lysate as described below.

**Ligand binding and immunoblotting**

Confluent cells were lysed in homogenization buffer [20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 × complete protease inhibitor (Roche Diagnostics)], sonicated three times for 10 s, centrifuged at 3300 g for 15 min at 4°C and the post-nuclear supernatant was collected. Protein concentration of the supernatant was determined by the Bradford method and cell lysates (50–100 μg protein) were resolved by electrophoresis. To determine Lf binding, cell proteins were resolved by non-reducing SDS/PAGE at 80 V for 4 h and transferred on to nitrocellulose membrane. Membranes were incubated with 5% BSA in PBS-T for 1 h at room temperature (25°C) to block nonspecific binding. Membranes were incubated overnight with ¹²⁵I-labelled apo-Lf, blots were rinsed twice with PBS-T and bound ligand was visualized by autoradiography.

For immunoblotting, cell extracts were electrophoresed by SDS/PAGE (10% gel, 200 V for 1 h) under reducing conditions and membranes were incubated with LfR antibody for 1 h. Membranes were washed in PBS-T and primary antibody was detected with donkey anti-rabbit IgG-HRP (lgG-horseradish peroxidase; GE Health-Amersham Biosciences) in 5% (w/v) nonfat milk in PBS-T. Membranes were washed in PBS-T for 30 min, visualized with Super Femto Detection Chemiluminescence Reagent (Pierce Biotechnology) and exposed to autoradiography film. Relative band density was quantified using the Chemi-doc Gel Quantification System (Bio-Rad).

**Transient transfection with LfR siRNA (small interfering RNA)**

Cells were seeded on to 24-well or 6-well plates and cultured until 50% confluent, then transfected with 80 pmol of LfR siRNA (Ambion) in Lipofectamine™ 2000 (Invitrogen) for 24 h according to the manufacturer’s instructions. Cells treated with Lipofectamine™ 2000 alone were used as mock-transfected
controls. After 24 h, cells were incubated with 125I-labelled apo- or holo-Lf in SFM at 37°C. Lf uptake was measured after 30 min as described above. To verify LfR suppression, transfected cells were scraped into 25 μl of homogenization buffer (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1× protease inhibitor) and immunoblotted as described above.

**Biotin-labelling of cell surface proteins**

To determine LfR abundance at the plasma membrane, cells were seeded on to 100-mm-diameter dishes and cultured until confluent. Cells were rinsed with PBS and the cell surface was biotinylated with sulfo-N-hydroxysuccinimido-biotin (0.5 mg/ml; EZ-Link Sulfo-NHS-Biotin; Pierce) at 4°C for 1 h. Cells were rinsed with ice-cold 0.2 M glycine buffer, pH 5.0, and then with cold PBS to remove unbound biotin. Cells were lysed with lysis buffer [50 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, 1× protease inhibitor (Roche Diagnostics)], sonicated (three 10 s bursts) and centrifuged at 100 000 g for 1 h. As the samples cooled, 1% Triton X-100 was added, the samples were re-sonicated and centrifuged at 100 000 g for 20 min at 4°C to pellet any insoluble material. The supernatant was collected and incubated with 300 μl of slurry (1:1) of immobilized avidin beads (UltraLink Immunobilized Monomeric Avidin; Pierce) for 1 h at room temperature (25°C) with constant rotation. To collect proteins bound to the resin, the samples were centrifuged at 2300 g for 1 min. The supernatant was discarded and the pellets were rinsed with PBS and centrifuged for 1 min at 2300 g. To elute biotinylated proteins, an equal volume of Laemmli sample buffer (Bio-Rad) containing 2-mercaptoethanol (5%) and incubated at 60°C for 5 min. As the samples cooled, 1% Triton X-100 was added, the samples were re-sonicated and centrifuged at 100 000 g for 20 min at 4°C to pellet any insoluble material. The supernatant was collected and incubated with 300 μl of slurry (1:1) of immobilized avidin beads (UltraLink Immunobilized Monomeric Avidin; Pierce) for 1 h at room temperature (25°C) with constant rotation. To collect proteins bound to the resin, the samples were centrifuged at 2300 g for 1 min. The supernatant was discarded and the pellets were rinsed with PBS and centrifuged for 1 min at 2300 g. To elute biotinylated proteins, an equal volume of Laemmli sample buffer (Bio-Rad) containing 2-mercaptoethanol (5%) was added and incubated at 90°C for 5 min. The samples were centrifuged at 2300 g for 1 min and biotinylated proteins were resolved by SDS/PAGE and immunoblotted for LfR as described above.

**Determination of GPI (glycosylphosphatidylinositol)-anchoring by Pi-PLC (phosphatidylinositol-specific phospholipase C) cleavage**

To determine if LfR is a GPI-anchored protein, cells were seeded on to 24-well plates and pretreated with Pi-PLC (1 unit/ml; Molecular Probes). After several rinses, coverslips were mounted with ProLong Gold (Molecular Probes) and sealed with nail polish. Immunofluorescence imaging was performed using an Olympus BX50WI microscope with UPlanApo objective (1.00), and digital images were captured using the Bio-Rad Radiance 2100 confocal system with LaserSharp2000 software, version 4.1 (Bio-Rad).

**Confocal microscopy**

To determine the subcellular localization of LfR, cells were seeded on to glass coverslips, cultured for 3–4 days in growth medium and incubated with apo-Lf (where indicated, 200 μg/ml) in SFM for up to 1 h at 37°C. After treatment, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min and permeabilized with 0.2% Triton X-100 in PBS for 5 min. To eliminate non-specific binding, cells were incubated in blocking buffer (5% goat serum, 1% BSA and 0.2% Triton X-100) for 1 h. Cells were rinsed with PBS and LfR was detected with Alexa 488-conjugated-anti-rabbit IgG (1:500, Molecular Probes) in PBS containing 0.2% goat serum, 1% BSA and 0.2% Triton X-100 for 1 h. To determine if LfR mediates apo- and/or holo-Lf uptake, cells were seeded on to 12- or 24-well plates until confluent and pretreated with cycloheximide (Cyhex, 10 μg/ml, Sigma) in SFM for 6 h at 37°C. Cells were either collected for SDS/PAGE analysis or incubated with 125I-labelled apo-Lf in the presence of Cyhex at 37°C and Lf uptake was measured over 7 h.

**Inhibition of endocytosis**

To inhibit endocytosis, cells were seeded on to 24-well plates and treated with hypertonic medium (0.4 M sucrose). Cells were pre-treated in hypertonic medium for 30 min and incubated with 125I-labelled apo-Lf in SFM at 37°C in the presence of 0.4 M sucrose and uptake was measured after 60 min. Lf uptake was measured as described above.

**Cycloheximide studies**

To determine if de novo LfR protein synthesis is required for Lf uptake, cells were seeded on to 12- or 24-well plates until confluent and pretreated with cycloheximide (Cyhex, 10 μg/ml, Sigma) in SFM for 6 h at 37°C. Cells were either collected for SDS/PAGE analysis or incubated with 125I-labelled apo-Lf in the presence of Cyhex at 37°C and Lf uptake was measured over 7 h.

**Statistical analysis**

Results are presented as means ± S.D. of triplicate samples from 2–3 separate experiments. Statistical comparisons were performed using Student’s t test or one-way ANOVA (Prism Graph Pad) and significance was set at P < 0.05.

**RESULTS**

**Active form of MMP-2 is increased by apo- but not holo-Lf**

Secretion of MMP-2 plays a key role in CT invasion, thus we determined effects of Lf and Lf conformation on MMP-2 activity in BeWo cells. Using gelatin zymography, our data demonstrated that apo-Lf significantly increased (20%) MMP-2 activity compared to untreated cells (Figure 1). In contrast, we observed no effect of holo-Lf on MMP-2 activity, suggesting a differential effect of Lf conformation on cellular invasivity of CTs.

**LfR facilitates uptake of apo- but not holo-Lf**

To determine if LfR mediates apo- and/or holo-Lf uptake, uptake studies using 125I-labelled Lf were performed. Our data...
Figure 1 Apo-Lf increases MMP-2 activity in BeWo cells

Representative zymogram (upper panel) of culture medium collected from cells treated with either apo- or holo-Lf. Equal amounts of protein (20 μg) were electrophoresed through polyacrylamide gels containing gelatin and stained with Coomassie blue. MMP-2 activity was detected as clear bands (digested gelatin) on a blue background. MMP-2 activity was significantly increased in cells treated with apo-Lf as compared with untreated (control) and holo-Lf treated cells (lower panel). Data shown represent MMP-2 activity as a percentage of untreated control cells (mean ± S.D.) for two independent experiments (n = 4). * P < 0.05.

Figure 2 Uptake of apo-Lf is significantly greater than that of holo-Lf in BeWo cells

Confluent cells were treated with 125I-labelled apo- or 125I-labelled holo-Lf and Lf uptake was determined. Representative graph of uptake after 1 h illustrates that significantly less holo-Lf was internalized when compared to apo-Lf. Data shown represent mean Lf uptake (Lf uptake/mg protein) ± S.D. from two independent experiments (n = 4). * P < 0.001.

Figure 3 Validation of LfR antibody by immunoblotting and confirmation of apo-Lf binding in BeWo cells

Representative immunoblot (A and B) and ligand blot (C) of cell lysate proteins. Proteins (50 μg) were resolved by SDS/PAGE and immunoblotted with LfR antibody (A, LfR Ab, 1 μg/ml) or pre-absorbed with excess Lf antigen (B, Lf Ab+peptide) for 1 h. The LfR antibody detected specific proteins at ~105 and ~35 kDa (A) which were both completely eliminated following pre-absorption with LfR peptide (B). (C) Cell lysate (100 μg) was resolved under non-reducing and non-denaturing conditions, and incubated with 125I-labelled apo-Lf. Detection of ligand binding to proteins at 105 and 75 kDa was observed.

Figure 4 Cell surface expression of LfR in BeWo cells

Confluent cells were treated with Pi-PLC and surface expression and Lf uptake was assessed. (A) A representative LfR immunoblot of biotinylated cell surface proteins documents the presence of LfR on the plasma membrane after Pi-PLC treatment. (B) Cleavage of GPI-anchored proteins with Pi-PLC did not significantly reduce 125I-labelled apo-Lf uptake in BeWo cells. Data represent mean Lf uptake/mg protein ± S.D. for two independent experiments (n = 4). (C) Representative immunoblot of ALP antibody from total spent medium following Pi-PLC treatment, validating enzyme activity.

Pi-PLC increased ALP abundance in spent medium compared to untreated cells (Figure 4C).

In order to determine a role for LfR in Lf uptake, LfR expression was attenuated in cells transfected with LfR siRNA (Figure 5A). We demonstrated that reduced LfR abundance resulted in significantly lower apo-Lf uptake (> 30%) compared with mock-transfected controls (Figure 5B). In contrast, holo-Lf uptake was not significantly reduced (< 10%), suggesting that...
internalization of holo-Lf in BeWo cells is not predominantly LfR-mediated. Due to our finding of limited LfR-mediated holo-Lf uptake and lack of an effect of holo-Lf on MMP-2 activity, we subsequently focused on effects of apo-Lf on the intracellular trafficking of LfR.

**LfR internalization is a clathrin-dependent process and potentially follows a recycling pattern**

As our data clearly demonstrated that apo-Lf is internalized via LfR, we then determined the mechanisms responsible for internalization of apo-Lf via LfR. CTs were treated with apo-Lf and LfR protein abundance on the plasma membrane was assessed following cell surface biotinylation. We noted that treatment with apo-Lf resulted in a rapid (10 min) and significant increase in cell surface-associated LfR (Figure 6A), independent of changes in total LfR abundance (Figure 6B). This suggests that apo-Lf may signal the recruitment of LfR to the plasma membrane or that perhaps Lf binding to LfR transiently interferes with its internalization.

Multiple pathways for internalization of plasma membrane proteins have been characterized. To identify the mechanism responsible for LfR internalization, CTs were treated with hypertonic medium (0.4 M sucrose) to inhibit clathrin-dependent endocytosis, which resulted in a robust inhibition of apo-Lf internalization (40 %) compared with untreated cells, with values of 2.4 ± 0.25 and 5.7 ± 0.42 pmol/mg protein ± S.D. for treated and control cells respectively; n = 5–8 per group, **P < 0.001. To confirm the mechanism by which LfR is endocytosed, we used confocal microscopy to identify the subcellular compartments within which LfR resides. As indicated in Figures 7(A) and 7(B), LfR and clathrin co-localized and were largely associated with the plasma membrane, as illustrated by yellow pixilation in the merged images (Figure 7C). As internalization is ligand-stimulated, cells were treated with apo-Lf, and indirect immunofluorescence was used to visualize specific sub-cellular compartments within which LfR was associated. Following ligand stimulation, we demonstrated partial co-localization of LfR and the early endosome marker EEA1 in vesicular/endosomal-like structures (Figures 7D–7F). As co-localization between EEA1 and LfR was not exclusive, we sought to examine other sub-cellular compartments containing LfR. Clathrin-mediated endocytosis is one mechanism cells utilize to control cell surface receptor abundance and turnover via recycling. To investigate whether LfR trafficking from the plasma membrane potentially includes endocytic recycling, we used the indirect immunofluorescence of TIR as a marker for recycling endosomes. We showed partial co-localization of LfR and TIR within distinct internalized vesicles in cells following ligand stimulation (Figures 7G–7I). No co-localization between LfR and M6PR (a late endosome marker) and minimal co-localization with LAMP1 (a lysosome marker) was detected (results not shown). These results indicate that, in response to apo-Lf stimulation, LfR is internalized via clathrin-mediated endocytosis and consequently enters a distinct intracellular endocytic pathway.

Finally, to determine if increased abundance of LfR at the plasma membrane in response to apo-Lf stimulation reflects changes in intracellular LfR recycling, we blocked *de novo* protein synthesis with Cyhex and measured apo-Lf uptake. Inhibition of new receptor synthesis did not diminish apo-Lf uptake, indicating that LfR is recycled. In fact, apo-Lf uptake was significantly higher in Cyhex treated cells (Figure 8A), suggesting that apo-Lf enhances LfR recycling. Moreover, our data indicated that CT-derived LfR is a relatively long-lived protein (Figure 8B), as abundant LfR remained detectable after 7 h of Cyhex treatment.
Figure 7 LfR co-localizes with specific endosomal subcellular compartments in BeWo cells

Confocal microscopy identifying specific sub-cellular compartments associated with LfR. Cells were fixed with paraformaldehyde and imaged for LfR (green) and sub-cellular markers (red): clathrin (A and C), EEA1 (D and F) and TIR (G and I). Clathrin (A) and LfR (B) were co-localized (yellow, C) and stained in a plasma membrane-like pattern in cells. Following apo-Lf treatment, partial co-localization with the early endosomal marker EEA1 (D) and LfR (E) was detected (yellow, F) and treatment of cells with apo-Lf clearly resulted in vesiculization of LfR. Furthermore, co-localization with the recycling endosome marker TIR (G) and LfR (H) was detected (yellow, I) following treatment of cells with apo-Lf.

Figure 8 Apo-Lf uptake in BeWo cells occurs despite the elimination of de novo protein synthesis

Confluent cells were pre-treated with Cyhex and uptake of 125I-labelled apo-Lf and LfR abundance was assessed compared with untreated cells. (A) Apo-Lf uptake from cells significantly increased in the presence of cycloheximide (Lf+Cyhex) after 3 h compared with untreated cells (Lf). Data represent means±S.D. for two independent experiments (n=3–4), and *P<0.05 compared with untreated cells, as analysed by Student’s t test. (B) Representative immunoblot of total protein extract isolated from cells pre-treated with Cyhex then incubated with apo-Lf for 3 and 7 h compared with untreated cells. The immunoblot demonstrates that Cyhex did not affect LfR abundance. β-Actin was used as a loading control.

DISCUSSION

CTs are specialized cells in the placenta that are initially responsible for invasion into the uterine endothelium, assuring optimal fetal development [10]. Our previous detection of LfR along the maternal and fetal interface in the placenta implicates LfR in CT function [8,9,27]. The aim of the present study was to explore the function of Lf in the placenta as mediated via LfRs using a CT cell model. While primarily regarded as a component of milk [2], Lf is also found in decidua (9–95 μg/g), amniotic fluid (2–37 μg/g) and chorion (2–26 μg/g) [28], and is secreted directly from uterine epithelia [29,30]. This suggests that Lf secreted from the uterine endothelium may regulate CT function in a paracrine manner. Results from this study documented an enhancing effect of apo- but not holo-Lf on MMP-2 activity in CTS, suggesting a regulatory role for apo-Lf in CT invasivity as this process is dependent upon the secretion and activation of MMPs. There is precedence for effects of Lf on MMP regulation as over-expression of Lf activates MMP-1 expression through binding to AP-1 promoter elements and through stimulation of the p38 MAPK (mitogen activated protein kinase)-dependent pathway in mouse fibroblasts and human COS-1 cells [17]. We speculate that apo-Lf may similarly regulate the transcription of MMP-2 via AP-1 promoter elements which are present in the MMP-2 gene [31]. In support of the concept of Lf acting as a transcription factor, a nuclear localization signal within the N-terminal region of the Lf molecule (amino acids 1–5) has been previously identified [32], and Lf binds to DNA [33,34]. Alternatively, the induction of MMP-2 via apo-Lf could be through the p38 MAPK pathway as p38 activation is required for the up-regulation of MMP-2 protein expression [31]. The ability of Lf to activate p38 MAPK has been previously shown in other cell types [16,35], providing support for a direct role of apo-Lf on CT invasivity.

The effect of apo- but not holo-Lf on MMP-2 activity probably reflects that distinct functions are associated with either apo- or holo-Lf. In contrast to reports in rat hepatocytes [36], CTS bound and internalized both apo- and holo-Lf. However, in CTS, apo-Lf was internalized more avidly than holo-Lf, suggesting preferential uptake mechanisms specific to apo-Lf which may reflect the requirement of this molecule for CT-specific functions such as invasivity, while precise Lf-downward functions in hepatocytes remain unknown. Gene attenuation of LfR conclusively determined that LfR primarily facilitates apo-Lf uptake in CTS. Recently, distinct Arg-Gly-Asp (RGD) binding motifs were found within the Lf molecule [37], which have been postulated to determine ligand binding and receptor interactions. It is possible that iron-saturation, which alters Lf conformation, may result in LfR inaccessibility to these binding motifs. While this is not to imply that holo-Lf binding and uptake does not elicit cellular events, it does not appear that these effects are mediated via LfR internalization in CTS. Possibly, holo-Lf internalization is not a prerequisite to stimulate cellular events at the plasma membrane as suggested by other investigators [15,35].

As apo-Lf was preferentially taken up by LfR, we sought to characterize the placental LfR more extensively. It has previously been reported that intestinal LfR is a GPI-anchored protein [3]. Contrary to this observation, we determined that, in CTS, the LfR receptor is not a GPI-anchored protein and is in fact internalized via clathrin-mediated endocytosis following ligand binding. Our observations in trophoblasts are consistent with previous reports in other cell types such as hepatocytes, where inhibition of clathrin-mediated endocytosis by hypertonic medium [38] significantly and that de novo protein synthesis may play a minor role in sustaining total and surface receptor abundance.
reduced Lf internalization. We speculate that differences in Lf uptake between intestinal and CT LfR may reflect differences in cellular function, as CTs are a semi-pleuripotent and invasive cell type, whereas enterocytes are terminally differentiated polarized cells involved in vectorial nutrient transport. Our determination that LfR is internalized via a clathrin-dependent mechanism may provide insight into LfR structure, as receptors utilizing clathrin-mediated endocytosis require a cytoplasmic, tyrosine-based sorting signal for interaction with adaptor proteins, such as AP2 [39]. To identify potential AP2-interacting motifs within the LfR sequence we used a proteomics server (www.elm.eu.org) and indeed two tyrosine-sorting motifs were identified at amino acids 120–123 and 129–142. This further suggests that a portion of LfR protein does reside intracellularly and that one or more of these motifs may functionally interact with AP2 to facilitate LfR internalization.

LfRs have been identified in various cell-types and reports have determined a molecular mass of ~110 kDa [8]. Recently, Tsuji et al. [40] examined the structure of human LfR in greater detail and determined that it is an 105.5 kDa oligomeric protein made up of three 35.5 kDa subunits, similar to what we observed in trophoblasts. Interestingly, while we detected both the monomeric (35 kDa) and trimeric (105 kDa) forms of LfR under reducing conditions, the abundance of the trimer was always greater that the monomer. For some reason, we were unable to extensively reduce the trimeric form to monomers even when using dithiothreitol and iodoacetate acid (V. Lopez, unpublished observations). Interestingly, we did not detect the dimeric form of LfR under reducing conditions, which is similar to the findings of Tsuji et al. [40]. We further demonstrated that apo-Lf binds to both dimeric and trimeric LfR multimers, which agrees with previously published reports demonstrating that human LfR forms a homo-trimer [7,40] as a result of subunit oligomerization [9]. We speculate that the trimeric form is an unusually stabilized form, mediated by several intracellular disulfide bonds and possibly also hydrophobic interactions, whereas the dimeric form is less stable and can be reduced to the monomeric form. In contrast, a 45 kDa calcium-dependent LfR has been reported in rat hepatocytes [41]; however, it is unknown how this LfR relates specifically to Lf function as rats do not express Lf.

It is well established that cell signalling and cellular homeostasis are altered by increasing or decreasing cell surface receptor abundance [42]. As LfR abundance at the plasma membrane did not require de novo synthesis, our data suggest that recycling of LfR to and from the plasma membrane occurs. Endocytic recycling requires internalization of cell surface receptors and their ligands within clathrin coated pits [43], with the interaction between transferrin and TIR being the classic example of this process [44]. Receptor recycling is one strategy by which cells maintain a mobilizable receptor pool to quickly traffic to the cell surface in response to physiological stimuli, to allow cells to redistribute cell-surface molecules to specific cell locations or compartments undergoing a physiological activity [45]. We speculate that Lf may either stimulate the recruitment of LfR to the plasma membrane, or that perhaps Lf binding to the receptor results in transient accumulation of LfR prior to internalization via clathrin-mediated mechanisms. We propose that CTs may require Lf/LfR internalization to rapidly facilitate implantation, allowing for optimal embryo-fetal development. Further studies are needed to address this issue.

Collectively, the results from this study demonstrated a specific role for apo-Lf but not holo-Lf in regulating MMP-2 activity and thus potentially endothelial invasion in CTs. Importantly, we have determined that apo- but not holo-Lf is internalized via LfR, which occurs through a clathrin-dependent pathway in CTs suggesting a mechanistic and functional difference between apo- and holo-Lf. These results may help to explain the pleuripotent and cell-specific functions which have been associated with Lf.

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