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Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in Caco-2 cells

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Abstract

Lactoferrin (Lf) is a major iron-binding and multi-functional protein in exocrine fluids such as breast milk and mucosal secretions. The functions of Lf appear dependent upon the iron-saturation of the Lf protein and are postulated to be mediated through Lf internalization by a Lf receptor (LfR). However, mechanisms by which LfR mediates Lf internalization in enterocytes are unknown. We now demonstrate that a LfR previously cloned from the small intestine mediates Lf endocytosis in a human enterocyte model (Caco-2 cells). LfR was detected at the plasma membrane by cell surface biotinylation; both apo-Lf and holo-Lf uptake were significantly inhibited in cells transfected with LfR siRNA. Treatments of hypertonic sucrose and clathrin siRNA and co-immunoprecipitation of LfR with clathrin adaptor AP2 indicate that LfR regulates Lf endocytosis via clathrin-mediated endocytosis. Although both iron-free Lf (apo-Lf) and iron-saturated Lf (holo-Lf) enter Caco-2 cells via a similar mechanism and no significant differences were observed in the binding and uptake of apo- and holo-Lf in Caco-2 cells, apo-Lf but not holo-Lf stimulates proliferation of Caco-2 cells. Interestingly, apo-Lf stimulated extracellular signal-regulated mitogen-activated protein kinase (ERK) cascade to a significantly greater extent than holo-Lf and the apo-Lf induced proliferation was significantly inhibited by an ERK cascade inhibitor (U0126) and clathrin siRNA. Taken together, our data suggest that LfR is a major pathway through which Lf is taken up by enterocytes, which occurs independently of iron saturation through clathrin-mediated endocytosis. The differential effects of apo- and holo-Lf are not due to differences in cellular internalization mechanisms.

Keywords

Caco-2 cells; lactoferrin; lactoferrin receptor; endocytosis

Introduction

Lactoferrin (Lf) is an iron-binding protein present in external secretions such as tears, nasal fluids, saliva, pancreatic, gastrointestinal and reproductive tissue secretions. It is particularly abundant (1–2 g/L) in human milk (Lonnerdal and Iyer 1995) and has been shown to be relatively resistant to proteolysis in the gastrointestinal tract during infancy (Brines and Brock 1983; Davidson and Lonnerdal 1987; Britton and Koldovsky 1989). As a result it has been suggested that Lf is an essential modulator of intestinal epithelium development in

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early infancy (Nichols, McKee et al. 1987). Buccigrossi et al. reported a dose-dependent relationship between Lf concentration and a positive effect on proliferation and differentiation of intestinal epithelial cells (Caco-2 cells). High concentrations of Lf in early lactation stimulate cellular proliferation, whereas low concentrations of Lf in late lactation stimulate differentiation rather than proliferation (Buccigrossi, de Marco et al. 2007). It has also been demonstrated that Lf is capable of stimulating intestine-associated immune functions and may thereby play an important role in immunocompetence during early infancy (Kuhara, Yamauchi et al. 2006). Moreover, it has been demonstrated that exogenous Lf can be transported through intestinal enterocytes and enter the systemic circulation. Transepithelial transport of Lf has been documented in milk-fed preterm infants (Hutchens, Henry et al. 1991), in a human enterocyte model (Mikogami, Heyman et al. 1994) and in animal models (Kitagawa, Yoshizawa et al. 2003; Fischer, Debbabi et al. 2007). Thus, Lf in human milk may play important physiological functions not only in the intestine but systemically in newborn infants, including positively affecting cellular proliferation, immune competence, anti-inflammatory activity and host defense (Mazurier, Legrand et al. 1989; Lonnerdal and Iyer 1995; Brock 2002; Ward, Paz et al. 2005). However, how Lf is internalized by enterocytes, which is critical for Lf to exert its multiple functions, remains unknown.

The multiple biological activities of Lf have been suggested to depend on its target cells and on the presence of specific receptors (LfRs) at their surfaces (Mazurier, Legrand et al. 1989; Suzuki, Shin et al. 2001; Suzuki, Lopez et al. 2005). Cell-type specific receptors have been identified in a variety of epithelial and immune cells (Leveugle, Mazurier et al. 1993; Ziere, Bijsterbosch et al. 1993; Bennatt and McAbee 1997; Baveye, Elass et al. 2000). We previously cloned the intestinal lactoferrin receptor (LfR) (Suzuki, Shin et al. 2001) and verified that Lf binding was significantly increased in Caco-2 cells transfected to over-express LfR. This suggests that LfR is involved in Lf internalization and therefore mediates the multiple physiological functions of Lf. Additionally, low density lipoprotein receptor-related protein (LRP) has been demonstrated to be involved in Lf endocytosis by osteoblastic cells (Grey, Banovic et al. 2004). The endocytosis mechanism through which the receptor medicates Lf internalization in enterocytes is unknown. Thus, in the present study we explored the role of LfR and LRP in Lf endocytosis by enterocytes.

In addition to multiple receptors of Lf, evidence implicates iron saturation in the facilitation of Lf function (Oguchi, Walker et al. 1995). Previous studies indicated that iron-saturated Lf (holo-Lf) enhances proliferation of intestinal epithelial cells (Oguchi, Walker et al. 1995), whereas proliferation is suppressed by iron-unsaturated Lf (apo-Lf). Differential effects might result from the ability of LfR or LRP to bind and internalize apo- and holo-Lf differently (Lopez, Kelleher et al. 2008) or ultimately elicit different downstream effects. In this study, we used Caco-2 cells as a cell model of enterocytes (Sambuy, De Angelis et al. 2005) and determined that while Caco-2 cells express LRP in addition to LfR (Ashida, Sasaki et al. 2004), LRP does not play a role in Lf internalization in these cells. Moreover, we determined that LfR is a major pathway through which Lf is taken up by enterocytes, which occurs independently of iron saturation and through clathrin-mediated endocytosis.

Material and methods

Cell culture

Human colon adenocarcinoma Caco-2 cells (American Type Culture Collection, HTB-37, Rockville, MD) were maintained in a humidified incubator at 37°C under an atmosphere of 5% CO_2 in Minimum Essential Medium (MEM, Life Technologies Inc., Gaithersburg, MD) supplemented with fetal bovine serum (FBS, 10%), streptomycin (10 μg/mL), and penicillin

(100 units/mL, Sigma, St. Louis, MO). Cells between passages 20–55 were harvested at 90% confluence and the medium was changed every other day.

Generation of human anti-LfR antibody

Antiserum was produced in rabbits against a synthesized peptide CTVGDRWSSQQGSKAD of LfR (Genemed Biotechnologies Inc., San Antonio, TX). The purification and verification of LfR antibody was described previously (Lopez, Kelleher et al. 2008).

Preparation of human Lf

Human Lf was purified from pooled milk collected from healthy lactating donors as previously described (Davidson and Lonnerdal 1986; Davidson, Litov et al. 1990) with slight modifications (Lopez, Kelleher et al. 2008). Purified apo- and holo-Lf were then labeled with ^{125}I (Amersham, Buckinghamshire, UK) using the Iodogen (Pierce, Rockford, IL) method (Fraker and Speck 1978).

Immunoblotting

Cells were lysed in homogenization buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 x Roche complete protease inhibitor cocktail), sonicated briefly and centrifuged at 500 × g for 10 min at 4°C. Protein concentration was measured with the Bradford assay. Proteins (50 μg/lane) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. The membrane was incubated in blocking buffer (5% skim milk in PBST: 0.1% Tween-20 in PBS) for 45 min at room temperature, washed three times with PBST and then probed with primary antibodies (rabbit anti-LfR, 1 μg/mL; rabbit anti-LRP1, 1:2000, Epitomics, Burlingame, CA; mouse anti-heavy chain clathrin, 1 μg/mL, Abcam, Cambridge, MA; mouse anti-adaptin α, 1μg/mL, BD Bioscience, San Jose, CA; chicken anti-adaptin μ2, 1 μg/mL, Genetex, San Antonio, TX; rabbit anti-phosphorylated extracellular signal-regulated kinase (ERK1/2) and mouse anti-total ERK1/2, 1:2000, Cell Signaling Technology, Davers, MA) in blocking buffer for 45 min at room temperature. After three washes with PBST, primary antibodies were detected with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (1:20,000, Amersham Pharmacia Biotech, Piscataway, NJ), HRP-conjugated goat anti-mouse IgG (1:25,000, Dako Corporation, Carpinteria, CA) or HRP-conjugated rabbit anti-chicken IgY (1: 20,000, Pierce) in blocking buffer for 45 min. Bands were visualized with West Femto substrate (Pierce, Rockford, IL) after a final wash (30 min wash with PBST). To evaluate the equivalence of protein loading, blots were incubated in Restore Western Blot Stripping buffer (Pierce, Rockford, IL) for 15 min at room temperature, blocked for 1 h, and then reprobed with mouse anti-β-actin (1:5000, Sigma, St. Louis. MO). Density of bands was quantified by the Chemi-doc Gel Quantification System (Bio-Rad Laboratories, Hercules, CA).

Receptor-associated protein, hypertonic sucrose solution, cytochalasin D and filipin treatments

Caco-2 cells (cultured for 16 d, D16) were pre-treated with receptor-associated protein (RAP, 50 nM in SFM, Calbiochem, La Jolla, CA) or hypertonic sucrose solution (0.45 M sucrose in SFM) or cytochalasin D (10 μM in SFM, Sigma Chemical Co.) or filipin (5 μg/mL in SFM, Sigma Chemical Co.) for 1 to 2 h at 37°C and then incubated with RAP, or hypertonic sucrose, or cytochalasin D solution containing ^{125}I-labeled apo- or holo-Lf (100,000 cpm per well) for 10 min at 37°C. After incubation, cells were rinsed with ice-cold NaCl wash buffer (0.15 M, pH 3.0) once and ice-cold PBS three times. After cells were solubilized by NaOH (1 M), cellular radioactivity was quantified in a gamma counter (Packard Minaxi-γ; Meriden, CT). Prior to this experiment, we verified that our NaCl wash

buffer did not remove bound lactoferrin (data not shown). Non-specific binding was assessed by incubation with unlabeled Lf (100-fold molar excess). Cells were lysed and protein concentration was measured as described above.

RNA interference

Subconfluent Caco-2 cells (~40–50% confluence) were transiently transfected with LfR small interfering RNA (siRNA) (1.0 μg oligonucleotide/well, Ambion, Austin, TX) for 48 h or clathrin siRNA (0.8 μg/well, Ambion) for 24 h using Lipofectamine 2000 (2 μL/well, Invitrogen, CA) according to the manufacturer's instructions. The target sequences were as follows: LfR, sense: 5′-CGAAUGUCCUAGUGCAUUU-3′, antisense: 5′-AAAUGCACUAGGACAUUCG-3′; clathrin; clathrin, sense: 5′-GCUAGCAAAGUAAUUGCAC-3′, antisense: 5′-GUGCAAUUACUUUGCUAGC-3′. Effects of LfR- and clathrin-suppression on ^{125}I-Lf uptake were evaluated as described above and the effect of clathrin-suppression on Lf-induced cell proliferation was assessed as described below.

Indirect Immunofluorescence

Caco-2 cells were seeded and grown on glass coverslips using 24-well plates overnight. After cells were treated with apo- or holo-Lf (3μM) in SFM for 10 min at 37°C, cells were rinsed with PBS, fixed with phosphate-buffered paraformaldehyde (4%, 0.4 mL/well) for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 10 min. Cells were then blocked with blocking buffer (5% heat-inactivated rabbit serum in PBS, 0.5 mL/well) for 20 min. After the blocking buffer was removed, cells were rinsed with PBS and LfR was probed with rabbit anti-LfR, (1 μg/mL) and then Alexa 488-conjugated-anti-rabbit IgG (1 μg/ml; Molecular Probes) in blocking buffer. Co-localization of LfR with clathrin (1 μg/ml, Abcam) was detected with Alexa Fluor-633-conjugated-anti-mouse IgG (1 μg/mL; Molecular Probes, Eugene, OR). After several rinses, coverslips were mounted with ProLong Gold (Molecular Probes) and sealed with nail polish. Immunofluorescence imaging was used confocal laser scanning microscope (FV1000, Olympus America Inc., Melville, NY) with 100 × magnification under an oil-immersion lens and analyzed with complete integrated image analysis software systems (Olympus).

Detection of LfR at the plasma membrane by cell surface biotinylation

D16 Caco-2 cells were pre-treated with or without apo- or holo-Lf (3 μM) in SFM for 1 h at 37°C to internalize Lf and then washed twice with ice-cold PBS. To assess the abundance of LfR on the cell membrane, cell surface proteins were biotinylated with Sulfo-NHSS biotin (Pierce, 0.5 mg/mL) at 4°C for 1 h. After two washes with glycine (50 mM, pH 5.0) and three washes with ice-cold PBS, cells were lysed by ice-cold homogenization buffer (50 mM Tris-HCl pH 7.4, 2 mM EDTA, 2 mM EGTA, with 1x Roche complete protease inhibitor cocktail) and sonicated for 30 s on ice. The membrane fractions were isolated by centrifugation at 100,000 × g for 20 min at 4°C and then re-suspended in precipitation buffer (homogenization buffer plus 100 mM NaCl). Subsequently, SDS was added to 0.2% followed by incubation at 60°C for 5 min. After incubation, Triton X-100 was added to membrane fractions to 1% and they were then briefly sonicated on ice followed by precipitation of insoluble material by ultracentrifugation (100,000 × g, 20 min). To pull down biotinylated plasma membrane proteins, the supernatant was incubated with slurry of BSA-blocked Ultralink-neutravidin beads (50 μL 1:1, Pierce) and rotated for 1 h at room temperature. After four washes with 1% Triton in PBS, the biotinylated membrane protein from each well was eluted by boiling with Laemmli sample loading buffer (50 μL, Bio-Rad) containing β–mercaptoethanol (5%) and immunoblotted. Cells without biotin treatment were used as negative control to evaluate nonspecific binding of proteins to the Ultralink-neutravidin beads.

Effects of apo- or holo-Lf on total LfR abundance in Caco-2 cells

D16 Caco-2 cells were incubated with apo- or holo-Lf (3 μM) in SFM for 1 h at 37°C. Total cell homogenate was obtained and subjected to immunoblotting as described above.

Immunoprecipitation

Co-immunoprecipitation was performed to evaluate the association between LfR and AP2. D 16 Caco-2 cells were incubated with or without apo- or holo-Lf for 15 min and then were solubilized in Ripa buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1.0% Triton X-100, 1% deoxycholate, 5 mM EDTA, 1 mM sodium orthovanadate, 1mM PMSF, 1mM NaF, 1x Roche complete protease inhibitor cocktail). Cell lysates were centrifuged at 13,000 × g for 15 min at 4°C and 500 μg of total cell protein (supernatant) was immunoprecipitated with adaptin α antibody (2 μg, BD Biosciences, San Jose, CA) or adaptin μ2 antibody (2 μg, GeneTex, San Antonio, TX) conjugated to protein A/G plus agarose (25 μL) using the Crosslink Immunoprecipitation Kit (Pierce) and immunoprecipitated according to the manufacturer's recommendation. The Immunoprecipitation Kit contains crosslinker to irreversibly attach the antibody to the agrose beads, minimizing the co-elution of heavy and light IgG with the immunoprecipitated protein. The protein was eluted with elution buffer and resuspended in Laemmli sample loading buffer (30 μL, Bio-Rad) containing β–mercaptoethanol (5%), boiled 5 min and subjected to immunoblotting. To evaluate the specificity of the immunoprecipitation, total cell protein (500 μg) was immunoprecipitated with control mouse IgG or chicken IgY (2 μg, Santa Cruz Biotechnology, Santa Cruz, CA) conjugated to Protein G-agarose beads as described above.

Effects of apo- and holo-Lf on cellular proliferation

To determine effects of apo- and holo-Lf on cellular proliferation of intestinal epithelial cells, Caco-2 cells were cultured until ~50% confluence. Cells were maintained in serum free medium (SFM) containing apo- or holo-Lf (400 μg/mL) for 12 h, and effects of Lf on cell proliferation were evaluated using the ELISA BrdU Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. BrdU was normalized to total cell protein and protein concentration was measured by the Bradford protein assay.

Binding and kinetics of internalization of ^{125}I-labeled apo- and holo-Lf by Caco-2 cells

Purified apo- and holo-Lf were labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) using the Iodogen (Pierce, Rockford, IL) method (Fraker and Speck 1978). Human transferrin (Sigma) was labeled with ^{125}I using the same method and was used as a positive control for clathrin-mediated endocytosis. D16 Caco-2 cells were rinsed three times with ice-cold PBS and then incubated in SFM containing different concentrations of ^{125}I-labeled apo- or holo-Lf (1–10 μM) for 90 min at 4°C or incubated in SFM containing ^{125}I-labeled apo- or holo-Lf (100,000 cpm per well) for different times (15– 90 min) at 37°C. Cells were rinsed with ice-cold NaCl (0.15 M, pH 3.0) once to remove loosely bound Lf, then with ice-cold PBS three times and digested with NaOH (1 M). Cells were solubilized and cell-associated radioactivity was quantified in a gamma counter.

Effects of apo- or holo-Lf on extracellular signal-regulated mitogen-activated protein kinase (ERK) signaling transduction

Subconfluent Caco-2 cells (~50% confluence) were treated with apo- or holo-Lf (400 μg/mL) in SFM for 5, 10 and 20 min at 37°C or pre-incubated with the MEK inhibitor U0126 (50 μM in DMSO; Promega, San Luis Obispo, CA) for 30 min at 37°C prior to incubation with apo- or holo-Lf. In parallel, cells were treated with the same volume of DMSO and no effect was found (data not shown). Cells were homogenized and subjected to immunoblotting as described above. Membranes were first immunoblotted with anti-

phosphorylated ERK1/2, then stripped as described above and re-probed with anti-total ERK1/2.

Statistical analysis

Data represent mean ± standard deviation from 2–3 independent experiments. Comparisons between treatment and control were conducted using an unpaired Student's t test or one-way ANOVA (Prism Graph Pad, Berkeley, CA). $P<0.05$ was considered to be statistically significant.

Results

LRP and LfR are both expressed in Caco-2 cells

To determine if Caco-2 cells express LRP and LfR, immunoblotting was performed. As shown in Figure 1, Caco-2 cells endogenously express both LRP (Fig. 1A) and LfR (Fig. 1B). Similar to our previous report in placental trophoblasts (Lopez, Kelleher et al. 2008), we detected two isoforms of LfR in Caco-2 cells that immunoreacted with LfR antibody with molecular masses of 35 kDa and 105 kDa (Fig. 1B), reflecting the monomer (35 kDa) and homotrimeric (105 kDa) forms of LfR (Tsuji, Yamashita et al. 2007).

LfR but not LRP mediates Lf endocytosis in Caco-2 cells

Next, we determined whether LRP or LfR mediate Lf endocytosis in Caco-2 cells. To do so, effects of receptor-associated protein (RAP) and LfR siRNA on Lf uptake were evaluated. RAP, a chaperone for endocytic receptors, has been demonstrated to block binding of ligands to LRP (Moestrup and Gliemann 1991), and was used to determine the role of LRP in Lf uptake by Caco-2 cells. Figure 2A shows that uptake of both apo-Lf and holo-Lf was not inhibited by RAP, suggesting that LRP is not involved in Lf endocytosis by Caco-2 cells.

Whether LfR plays a role in Lf endocytosis was examined by RNA interference. We attenuated LfR expression by transient transfection with LfR siRNA, which reduced LfR protein abundance by ~70% (Fig. 2B). Interestingly, although the uptake of apo- and holo-Lf was reduced to a similar extent in LfR-suppressed cells, Lf uptake was only reduced by ~40% (Fig. 2C). This directly documents that LfR plays a role in apo- and holo-Lf uptake.

LfR is located on the plasma membrane and is internalized by apo- and holo-Lf

To investigate whether the monomer or homotrimer of LfR is associated with the plasma membrane, we biotinylated cell surface proteins and captured them with Ultralink-neutravidin beads. As indicated in Figure 3A, only the 105 kDa homotrimer was associated with the plasma membrane. To assess the response of plasma membrane-associated LfR to ligand stimulation, Caco-2 cells were treated with apo- or holo-Lf then cell surface LfR was captured following biotinylation. Our results clearly illustrated that the abundance of LfR at the plasma membrane was significantly decreased by both apo- and holo-Lf exposure (Fig. 3A). Secondly, the reduction in cell surface-associated LfR was due to internalization and not due to LfR degradation as total abundance of LfR was unaffected by ligand stimulation (Fig. 3B).

LfR-bound Lf enters Caco-2 cells through a clathrin-mediated endocytosis pathway

We next aimed to determine the cellular mechanism through which Lf is taken up by LfR in Caco-2 cells. We first treated cells with hypertonic sucrose solution and determined that both apo- and holo-Lf uptake was significantly inhibited by ~80% in comparison with untreated cells, which was similar to the results for a positive control, transferrin (Fig. 4A),

suggesting that clathrin-dependent endocytosis is a major pathway by which apo- and holo-Lf are internalized by Caco-2 cells. We obtained a similar inhibition rate for both apo- and holo-Lf uptake when phenylarsine oxide, another inhibitor of clathrin-dependent endocytosis (Ivanov, Nusrat et al. 2004), was used to treat Caco-2 cells (data not shown). We also tested whether Lf is internalized via macropinocytosis, micropinocytosis and caveolae endocytosis (Lamaze and Schmid 1995;Swanson and Watts 1995;Kruth, Jones et al. 2005;Swanson 2008). As shown in Figure 4B and 4C, uptake of apo- and holo-Lf was not inhibited by cytochalasin D (Cyt D) which specifically inhibits both macropinocytosis and micropinocytosis by blocking F-actin polymerization (Riezman, Woodman et al. 1997) or filipin which disrupts lipid raft structure and function and thus blocks caveolae endocytosis and micropinocytosis (Kruth, Jones et al. 2005), indicating that neither apo-Lf nor holo-Lf enter Caco-2 cells via macropinocytosis, micropinocytosis and caveolae endocytosis. In addition, similar results were obtained when amiloride, a specific inhibitor of macropinocytosis (West, Bretscher et al. 1989) was used (data not shown).

To verify a role for clathrin-mediated endocytosis in the internalization of Lf, we suppressed clathrin expression using siRNA in Caco-2 cells by ~ 95% (Fig. 5A) and detected significantly lower uptake (~20 % of mock-transfected controls) of both apo- and holo-Lf. This provides direct evidence of a major role for clathrin in Lf endocytosis. Confocal microscopy also showed that LfR was co-localized with clathrin after both apo-and holo-Lf were internalized (Fig. 5B). To confirm that Lf is taken up by Caco-2 cells through LfR-facilitated clathrin/AP2-dependent endocytosis, co-immunoprecipitation of LfR and AP2 was performed. As shown in Figure 5C, adaptin α, a subunit of AP2, was successfully pulled down by the corresponding antibody and adaptin α co-immunoprecipitated with LfR (105 kDa) after cells were briefly incubated with apo- or holo-Lf at 37°C. To further confirm the interaction between AP2 and LfR, we also conducted the same experiment using adaptin μ2, another subunit of AP2. Again, adaptin μ2 was co-immunoprecipitated with LfR (105 kDa, Fig. 5D), demonstrating that LfR specifically associates with AP2.

Apo-Lf but not holo-Lf stimulates proliferation of Caco-2 cells

Since our results showed that both apo- and holo-Lf enter Caco-2 cells via a similar mechanism, we queried if there are functional differences in response to apo- and holo-Lf in enterocytes. Thus, Caco-2 cells were treated with apo- or holo-Lf and effects on cellular proliferation were then examined. We used the BrdU method to evaluate effects of Lf on cell proliferation. Apo-Lf significantly stimulated proliferation of Caco-2 cells, whereas holo-Lf did not (Fig. 6), indicating that effects of Lf on cellular proliferation in Caco-2 cells relate directly to the iron saturation of Lf.

Apo- and holo-Lf have similar binding affinity and uptake kinetics

To determine if the different effects of apo- and holo-Lf on cellular proliferation result from differences in Lf binding affinity and/or uptake, binding assays and uptake experiments were conducted. Apo- and holo-Lf were found to have a similar binding affinity (Fig. 7A). The association constants (K_{ass}) for the binding of apo-Lf and holo-Lf to LfR were similar, $0.07 \times 10^6\ M^{-1}$ and $0.06 \times 10^6\ M^{-1}$, respectively. In addition, apo- and holo-Lf were internalized to a similar extent (Fig. 7B). These findings suggest that the functional differences between apo- and holo-Lf do not result from differences in binding or uptake of Lf.

Apo- and holo-Lf activate extracellular signal-regulated mitogen-activated protein kinase cascade (ERK) signaling transduction and ERK activation plays a role in apo-Lf-induced proliferation

Since the different effects of apo- and holo-Lf on proliferation do not result from differences in binding and endocytotic internalization; we assessed whether there are differences in

signaling transduction initiated by apo- or holo-Lf. Subconfluent Caco-2 cells were treated with apo- or holo-Lf for 5, 10 or 20 min, and phosphorylation of ERK1/2 was then assessed by immunoblotting. Both apo- and holo-Lf strongly and rapidly activated ERK1 (the upper band) and ERK 2 (the lower band) as shown by phosphorylation of ERK1/2 detected by a phosphorylation site (Thr^{202}/Tyr^{204}) specific antibody (Fig. 8A). However, apo-Lf stimulated phosphorylation of ERK1/2 to a significantly greater extent (30–50%) relative to holo-Lf (Fig. 8B). To verify that ERK1/2 is involved in apo-Lf-induced proliferation, Caco-2 cells were pre-treated with U0126 (a MEK inhibitor) and proliferation was assessed following apo-Lf treatment. Our data demonstrated that U0126 specifically inhibited apo-Lf-induced proliferation (Fig. 8C), indicating that apo-Lf stimulated proliferation via activation of the ERK1/2 signaling cascade. In addition, apo-Lf –induced proliferation was dramatically decreased by clathrin siRNA, demonstrating that clathrin-dependent endocytosis is a mechanism for signal attenuation of apo-Lf-initiated ERK activation (Fig. 8D). Taken together, our data suggest that the differences between apo- and holo-Lf with respect to cellular functionality do not result from ligand/receptor kinetics or internalization but alternatively may result from different down-stream signaling-mediated events.

Discussion

Our study clearly demonstrates that LfR and not LRP mediate Lf endocytosis in Caco-2 cells. Previous reports had suggested that both LfR (Suzuki, Shin et al. 2001) and LRP (Grey, Banovic et al. 2004) may mediate Lf endocytosis. Our results demonstrated that LfR but not LRP plays a role in Lf internalization in Caco-2 cells. Consistent with our previous report in placental trophoblasts (Lopez, Kelleher et al. 2008), LfR exists as a 35 kDa, glycosylated monomer (Kawakami and Lonnerdal 1991; Tsuji, Yamashita et al. 2007), which forms a homotrimer in Caco-2 cells. Our data further illustrated that the homotrimer is unusually stable under denaturing conditions which we speculate reflects the unique tertiary structure of the LfR homotrimer which contains unexposed, intramolecular disulfide bonds. Importantly, only the homotrimer LfR is localized at the plasma membrane of Caco-2 cells and participates in the endocytosis of Lf into Caco-2 cells. Critically, we verified the dependence upon LfR for uptake of both apo- and holo-Lf. Despite the fact the LRP does not play a role, our inability to abrogate Lf uptake more than 40% provides evidence that LfR may not be exclusivley responsible for Lf uptake in Caco-2 cells. Therefore, we tested if Lf is internalized via macropinocytosis, a pathway with little or no guidance from receptors characterized by plasma membrane ruffling (Swanson and Watts 1995; Swanson 2008). We were unable to document reduced Lf uptake following pre-treatment with cytochalasin D and amiloride; thus further experiments are needed to elucidate other mechanisms contributing to Lf endocytosis by Caco-2 cells.

Following binding of Lf to the LfR, the Lf-LfR complex is internalized. We have previously demonstrated that Lf binds to enterocytes and intestinal brush-border membranes in a saturable manner (Davidson and Lonnerdal 1988; Gislason, Iyer et al. 1994; Gislason, Douglas et al. 1995), suggesting that Lf is internalized by enterocytes through a receptor-mediated mechanism. In the current study, we confirmed that LfR mediates Lf endocytosis in Caco-2 cells. The mechanisms responsible for Lf internalization into the enterocyte were therefore investigated. We hypothesized that LfR internalizes Lf through a clathrin-mediated endocytotic pathway. Hypertonicity selectively inhibits dissociation of clathrin and accumulation of microcages, thereby inhibiting clathrin-mediated endocytosis (Lamaze and Schmid 1995). In the present study, apo- and holo-Lf uptake was significantly reduced by hypertonic sucrose treatment and clathrin gene suppression and co-localization of LfR and clathrin was indicated by confocal microscopy, verifying that both apo- and holo-Lf are internalized through a clathrin-mediated endocytosis pathway in Caco-2 cells, consistent with reports in mammary gland carcinoma cells (Legrand, Vigie et al. 2004). Significantly,

our co-immunoprecipitation results clearly indicated a direct interaction between LfR and AP2, a critical component of the clathrin adaptor protein complex, demonstrating that LfR is responsible for mediating Lf endocytosis via binding to Lf followed by recruitment of AP2 and clathrin assembly. In general, there are four adaptor protein complexes, AP-1, AP-2, AP-3 and AP-4. Only AP2 complexes work with clathrin to mediate rapid endocytosis from the plasma membrane (Kirchhausen 2000; Boehm and Bonifacino 2001). AP2 complexation plays an essential role in endocytosis; they not only recruit receptors but also facilitate clathrin scaffold assembly. AP2 complexes, composed of α, β2, μ2, and σ2 subunits, interact with internalization signals present in the cytosolic tails of endocytosed receptors, and initiate rapid internalization (Roth 2006). Consistent with our direct empirical evidence, AP2 binding domains have been predicted in LfR (66–69, 120–123, 139–142, http://www.elm.eu.org/). Thus, these studies provide direct evidence that LfR is associated with AP2 and facilitates clathrin-mediated endocytosis of both apo-and holo-Lf in Caco 2 cells. Our results indicate that apo- and holo-Lf have a similar binding affinity and can be internalized to a similar extent in Caco-2 cells. Consistent with these results, a similar number of binding sites and binding affinity for apo- and holo-Lf have been observed in another human enterocyte model (HT-29 cells) (Mikogami, Heyman et al. 1994; El Yazidi-Belkoura, Legrand et al. 2001) and mouse intestinal cells (Hu, Mazurier et al. 1988). Studies in HT-29-18-Cl intestinal cells showed that fully iron-saturated human Lf binds to cells with similar parameters as human Lf with minimal iron saturation (3%) (Mikogami, Heyman et al. 1994), which indicates that, similar to our observations, iron saturation had no effect on the binding of human Lf to intestinal cells. In addition, LfR in mouse enterocytes binds mouse apo- and holo-Lf with similar affinity (Hu, Mazurier et al. 1988). The association constants (K_{ass}) obtained for Lf binding to Caco-2 cells in the present study were very similar, and of similar magnitude to those we have reported earlier for human infant brush border membrane vesicles (BBMV) (Kawakami and Lonnerdal 1991), piglet BBMV (Gislason, Iyer et al. 1994; Suzuki, Shin et al. 2001) and recombinant human LfR (Suzuki, Shin et al. 2001).

It is curious that apo- and holo-Lf are both taken up to the same extent by LfR in Caco-2 cells, while apo- but not holo-Lf is taken up by LfR in BeWo cells. It has been reported that different cells types (e.g., HeLa, a cervical cancer cell and MDA-MB-468, an invasive breast cancer cell) respond differently to the same concentration of EGF as a result of differences in EGFR expression at the plasma membrane (Yu, Hale et al. 2009). In addition, Hansen et al. (Hansen, Hansen et al. 2009) reported that the heterodimerization pattern of angiotensin II type I receptor (AT1R) and the bradykinin B2 receptor (B2R) in Cos-7 and HIH3T3 cells is different from the one reported in HEK 293 cells (AbdAlla, Lother et al. 2000), suggesting that even though the same receptor is expressed, the receptor may function differently in different cells. Thus, we speculate that the difference in the LfR-mediated uptake of apo- and holo-Lf between placenta (BeWo cells) (Lopez, Kelleher et al. 2008) and enterocytes may reflect differences in cell-specific functions.

Despite the fact that both apo- and holo-Lf display similar binding and uptake kinetics, our data illustrate that apo-Lf but not holo-Lf stimulates proliferation of Caco-2 cells. It has been suggested that Lf is a modulator of intestinal development through direct effects on proliferation and differentiation of intestinal epithelial cells during infancy. Previous studies indicate that the degree of iron saturation of Lf can elicit different biological effects in various cell types (Oguchi, Walker et al. 1995; Norrby 2004). The physiological relevance of this query reflects the fact that in human milk, the majority of Lf is not iron-saturated and the predominance of apo-Lf in milk may play an ontogenic role in intestinal development. To investigate the role of Lf iron saturation on enterocyte proliferation, we utilized Caco-2 cells and determined that apo-Lf stimulated cellular proliferation, whereas holo-Lf did not have the same effect. In agreement with our results, Buccigrossi et al. (Buccigrossi, de

Marco et al. 2007) found that Lf (with a low degree of iron-saturation) more strongly stimulates proliferation of Caco-2 cells than does holo-Lf. In contrast, Oguchi et al. reported that the iron-saturated form of human Lf enhanced cell proliferation, while the iron-unsaturated form suppressed it (Oguchi, Walker et al. 1995). However, Oguchi et al. used apo- or holo-Lf to treat Caco-2 cells in serum free medium for 6 days and assessed cellular proliferation in differentiated Caco-2 cells. As proliferative mechanisms are likely diminished in differentiated Caco-2 cells, it is reasonable to assume that effects of Lf on differentiated cells would be different from effects on highly proliferative, non-differentiated cells. Our data support a role for the predominant form of Lf in milk (apo-Lf) (Fransson and Lonnerdal 1980) in facilitating intestinal proliferation in the developing intestine.

An important question is, how does apo- but not holo-Lf stimulate proliferation of intestinal epithelial cells? We previously demonstrated that LfR mediates apo- but not holo-Lf uptake and only apo-Lf may be involved in cellular invasion in placental trophoblasts (BeWo cells) (Lopez, Kelleher et al. 2008), suggesting that apo- and holo-Lf enter cells through different mechanisms. Our data in Caco-2 cells clearly refutes this possibility in this cell-type. Both apo- and holo-Lf activated ERK signaling, but apo-Lf had a more potent effect on this pathway than did holo-Lf. Previous studies have shown that Lf exerts its multiple functions through the interaction of specific LfRs with cellular signaling pathways (Grey, Banovic et al. 2004; Lin, Chiou et al. 2005) or directly enters the nucleus and serves as a transcription factor to activate specific gene expression (Garre, Bianchi-Scarra et al. 1992; Mariller, Benaissa et al. 2007). The Ras-dependent extracellular signal-regulated mitogen-activated protein kinase cascade (ERK) is an important intracellular signaling pathway that transmits a variety of extracellular signals that promote cellular proliferation (Troppmair, Bruder et al. 1994). It has been reported that proliferation in various cell types was significantly inhibited after the Ras-ERK pathway was blocked by specific inhibitors (Meloche and Pouyssegur 2007). Thus, it is inferred that Lf might stimulate the proliferation of intestinal epithelial cells by activating the Ras-ERK cascade. The current study indicates that both apo- and holo-Lf are capable of initiating ERK signaling transduction, but apo-Lf stimulates ERK significantly more than holo-Lf, which might lead to the different effects of apo- and holo-Lf on cellular proliferation. Moreover, Apo-Lf-induced proliferation was significantly inhibited by a specific inhibitor of the ERK1/2 signaling cascade, suggesting that apo-Lf stimulates proliferation via activating the ERK1/2 signaling cascade. Biophysical studies illustrate that the tertiary structure of iron-saturated Lf is much more compact than the iron-free form (Grossmann, Neu et al. 1992). Moreover, the affinity of peptide phage libraries for apo-Lf was much stronger than holo-Lf (Noppe, Vanhoorelbeke et al. 2004). A binding motif, implicated to be involved in binding to the receptor, has been recently documented in the Lf molecule (Sakamoto, Ito et al. 2006). It is possible that the binding domain of Lf that activates LfR and thus initiates signaling transduction is partly within the teritiary structure of holo-Lf, but is exposed in apo-Lf. The mechanisms behind this observation are not understood. Therefore, studies are underway to investigate whether intestinal LfR is also a signaling receptor, and whether apo- and holo-Lf can initiate different signaling pathways via activated LfR.

More importantly, our data is the first to demonstrate that apo-Lf-induced proliferation significantly decreased when clathrin was suppressed ~95% by clathrin siRNA, indicating that clathrin-dependent endocytosis is involved in controlling apo-Lf-induced cellular signaling cascades and thus influences its physiological functions. Endocytosis regulates many cellular signaling pathways by decreasing the number of functional receptors by transferring receptors from the plasma membrane to intracellular compartments (von Zastrow and Sorkin 2007).

In conclusion, our data clearly indicate that LfR located at the plasma membrane of Caco-2 cell is responsible for the uptake of both apo- and holo-Lf through clathrin-mediated endocytosis. However, this is not an exclusive mechanism and clarification of these processes may help us understand how Lf plays a pivotal role in iron absorption, mucosal immunity, and proliferation of intestinal epithelial cells during infancy.

Acknowledgments

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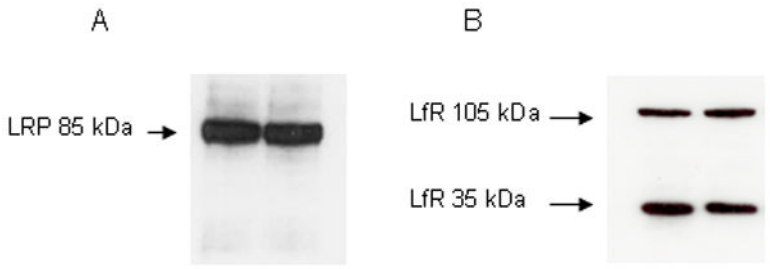

Fig. 1.
LRP and LfR are both expressed in Caco-2 cells. Total protein of d16 Caco-2 cells was extracted and subjected to immunoblotting (50 μg/lane) with anti-LRP (A) or LfR (B).

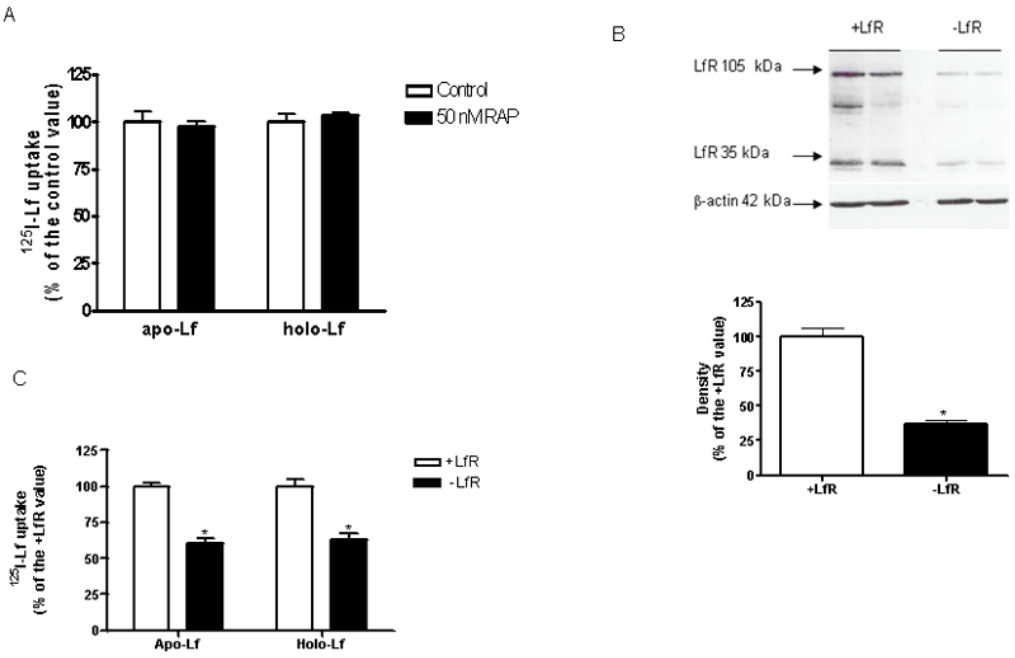

Fig. 2.

LfR but not LRP mediates Lf endocytosis in Caco-2 cells. (A) Effects of receptor associated protein (RAP) on apo- or holo-Lf uptake by Caco-2 cells. D16 Caco-2 cells were pre-treated with RAP (50nM) for 1 h at 37°C and then were incubated with RAP solution in SFM containing ^{125}I-apo- or holo-Lf (100,000 cpm/well) for 10 min at 37°C. Lf uptake was measured as described in Material and Methods. Data are mean ± S.D.; n=6. (B) Subconfluent cells (40~50% confluence) were transiently transfected with LfR siRNA (2.0 μg/well) for 48 h. Total protein was extracted and subjected to immunoblotting. Control and siRNA treatment are shown as +LfR and −LfR respectively. The density of total LfR, 105 kDa and 35 kDa bands, was quantified by the Chemi-doc Gel Quantification System (Bio-Rad Laboratories, Hercules, CA). * P<0.001, compared with the +LfR group. (C) Control or LfR suppressed cells were incubated with SFM containing ^{125}I-apo- or holo-Lf (100,000 cpm/well) for 10 min and then Lf uptake was measured as described in the Material and Methods.

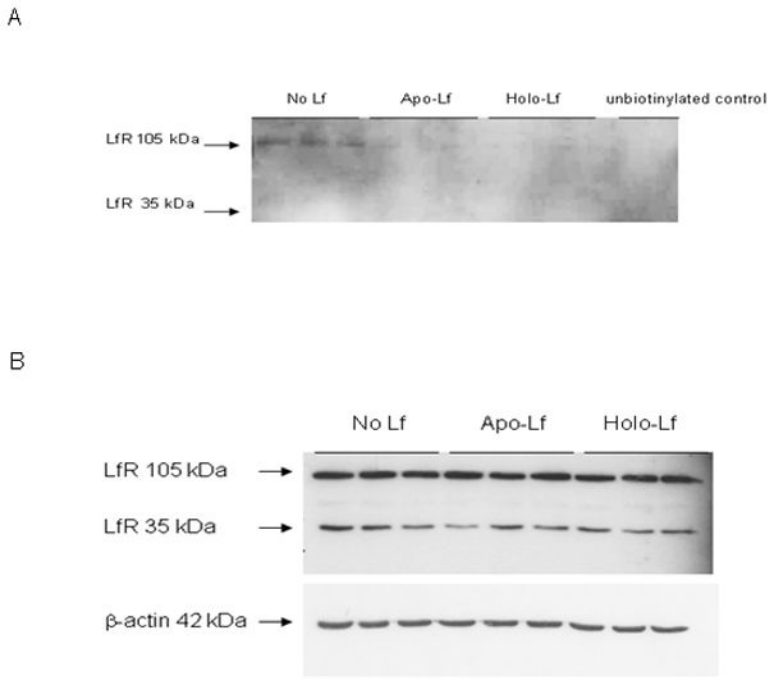

Fig. 3.

Effects of Lf on the localization of LfR at the plasma membrane and total abundance of LfR in Caco-2 cells. (A) After D16 Caco-2 cells were treated with or without apo- or holo-Lf (3 μM) for 60 min, the cells were rinsed twice with ice-cold PBS. Subsequently, the plasma membrane proteins were biotinylated with Sulfo-NHSS biotin (0.5 mg/mL, Pierce) at 4°C for 1 h. Biotinylated proteins were then collected by Ultralink-neutravidin beads and subjected to immunoblotting. Cells without biotin treatment were used as negative control to verify the lack of non-specific binding of proteins to the Ultralink-neutravidin beads, which is indicated as unbiotinylated control in the figure. (B) To determine if total LfR abundance was affected after apo-/holo-Lf internalization by Caco-2 cells, d16 cells were incubated with or without apo- or holo-Lf (3 μM) for 60 min at 37°C, and total protein was extracted and subjected to immunoblotting (50 μg/lane).β-actin was used to verify equal protein loading.

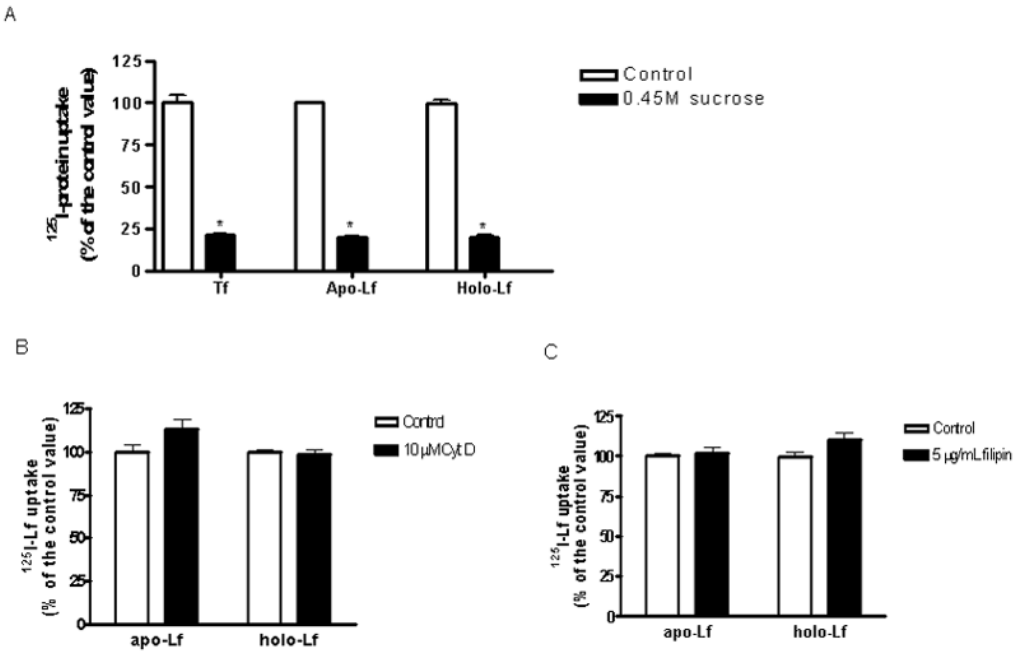

Fig. 4.

Lactoferrin internalization is mediated by clathrin but not macropinocytosis, micropinocytosis and caveolae endocytosis. D16 Caco-2 cells were pre-treated with hypertonic sucrose solution (0.45 M sucrose in SFM) or cytochalasin D (Cyt D, 10 μM in SFM) or filipin (5 μg/mL in SFM) for 2 h at 37°C and then incubated with hypertonic (A) or Cyt D (B) or filipin (C) solution containing ^{125}I-labeled Tf or apo- or holo-Lf (100,000 cpm per well) for 10 min at 37°C. Lf uptake was measured as described in Material and Methods. * P<0.001 compared with the control group.

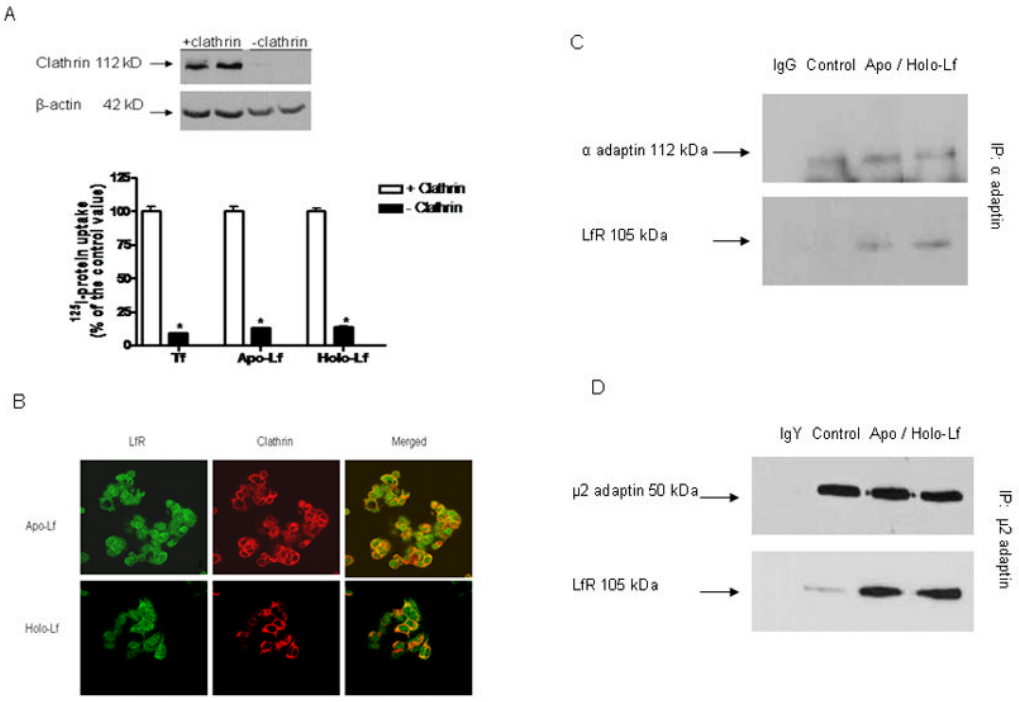

Fig. 5.

Lactoferrin endocytosis is clathrin dependent. (A) Subconfluent cells (40~50% confluence) were transiently transfected with clathrin siRNA (1.6 μg/well) for 24 h. Total protein was extracted and subjected to immunoblotting. The control is indicated as +clathrin and the siRNA treatment is indicated as −clathrin. (B) Control and clathrin-suppressed cells were incubated with SFM containing ^{125}I-apo- or holo-Lf (100,000 cpm/well) for 10 min at 37°C and Lf uptake was then measured. Data are mean ± S.D.; n=6. * P<0.001, compared with the control group. (C) Confocal microscopy was performed as described in Material and Methods to show the co-localization of LfR with clathrin after Caco-2 cells were treated with apo- or holo-Lf (3 μM) for 10 min. (D) and (E) Lactoferrin receptor associates with AP2. After D16 Caco-2 cells were incubated with or with apo-/holo-Lf (3μM) for 15 min, the total cell lysates were immunoprecipitated with adaptin α (D) and μ2 (E) antibodies conjugated to Protein A/G agarose (25 μL) and subsequently subjected to immunoblotting with anti-adaptin α or μ2 to verify if the corresponding antibodies pulled down adaptin α or μ2. Blots were then incubated in Restore Western Blot Stripping buffer for 15 min at room temperature, blocked for 1h and then reprobed with anti-LfR antibody. In addition, total cell protein (500 μg) was immunoprecipitated with normal mouse IgG (2μg) or chicken IgY (2μg) conjugated to Protein A/G agarose to evaluate the specificity of immunoprecipitation.

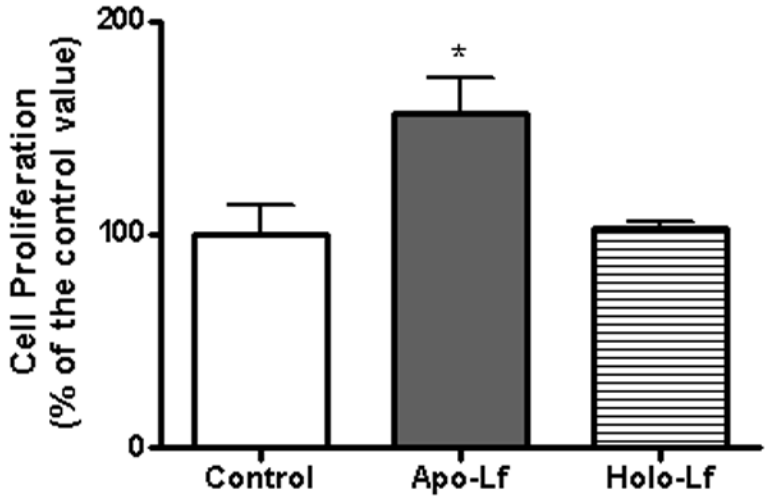

Fig. 6.

Effects of apo- and holo-Lf on proliferation of Caco-2 cells. Subconfluent cells (~50%) were treated with apo- or holo-Lf (400 μg/mL) for 12 h. Effects of apo- and holo-Lf on proliferation of cells were evaluated by a BrdU proliferation kit. Data are mean ± S.D. of two independent experiments; n=6 in each group. * $P<0.001$ compared with the control group.

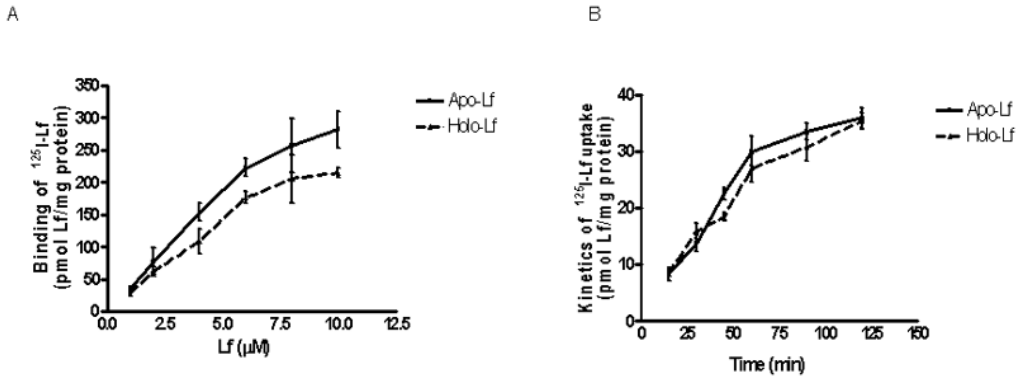

Fig. 7.

Binding and kinetics of internalization of ^{125}I-labeled apo- and holo-Lf by Caco-2 cells. (A) Varying concentrations of ^{125}I-labeled apo- or holo-Lf (1–10 μM) were added to D16 cells and then incubated at 4°C for 90 min. Cellular ^{125}I radioactivity was determined by a gamma-counter. (B) D16 Caco-2 cells in 24-well cell culture plates were incubated with ^{125}I-labeled apo- or holo-Lf (100,000 cpm/well) at 37°C for different times (15–90 min). Intracellular ^{125}I radioactivity was determined by a gamma-counter. Data are mean ± S.D. of two independent experiments; n=6/concentration or time point.

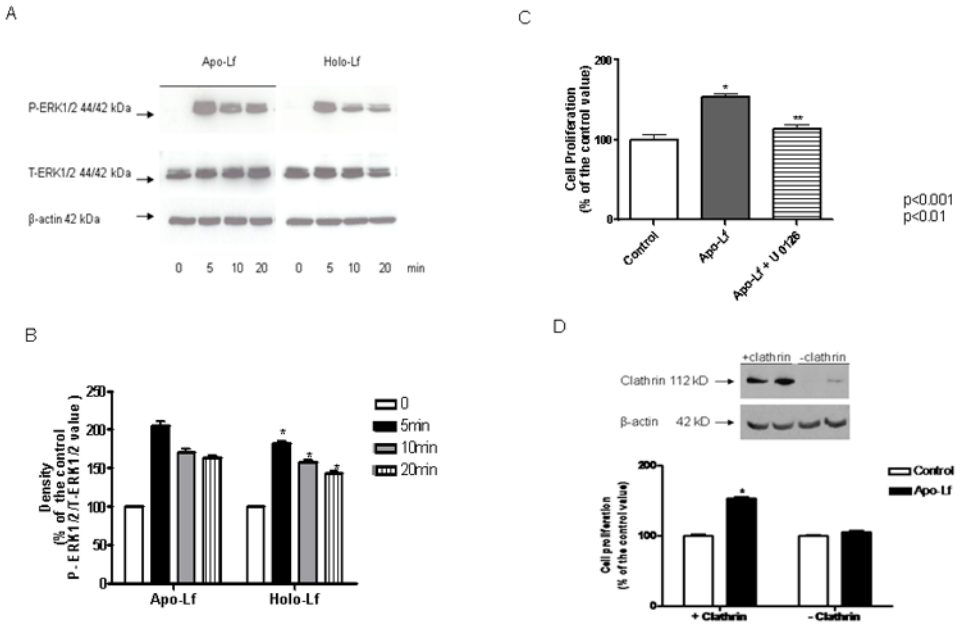

Fig. 8.

Apo- and holo-Lf activate ERK signaling transduction. (A) Subconfluent Caco-2 cells (~50% confluence) were treated with apo- or holo-Lf (400 μg/mL) for 5, 10 and 20 min. Whole cell lysates were subjected to SDS-PAGE and immunoblotted with antibodies to phospho-ERK1/2 (P-ERK1/2) or total ERK1/2 (T-ERK1/2).β-actin was used to verify equal protein loading. (B) Data show the ratio of phosphorylated ERK1/2 to total ERK1/2 of apo-Lf or holo-Lf treatment (mean ± S.D.) for three independent experiments. The ratio at 0 min is indicated as an arbitrary number of 100%. * $p<0.05$ compared with apo-Lf treatment. (C) Effects of U0126 on apo-Lf-induced proliferation was evaluated. Subconfluent Caco-2 cells were pre-incubated with U0126 (50 μM) for 30 min at 37°C and then incubated with apo- Lf (400 μg/mL, in cell culture medium containing 2% FBS) for 12 h at 37°C. The effect of U0126 on apo-Lf induced proliferation was evaluated by a BrdU proliferation kit. Data are mean ± S.D. of two independent experiments; n=6 in each group. * $P<0.001$ and ** $P<0.01$ compared with the control group. (D) Effects of clathrin-suppression on apo-Lf-induced proliferation. Clathrin was attenuated as described above and the effect of clathrin suppression on apo-Lf induced proliferation was determined by a BrdU proliferation kit. Data are mean ± S.D. of two independent experiments; n=6 in each group. * $P<0.001$ compared with the control group.