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Transport properties of bovine and reindeer β-lactoglobulin in the Caco-2 cell model

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Abstract

β-Lactoglobulin (βLG) is a protein that binds ligands like fatty acids and retinol into the hydrophobic pocket. Our purpose was to study bovine and reindeer βLG as transporter molecules and compare their transport properties across Caco-2 cell membrane. The reindeer βLG has more valuable binder characteristics than bovine βLG because it has only one genetic phenotype and it seems to exhibit better immunological properties. The permeation of βLG in Caco-2 cells was evaluated by immunoblotting, and the permeation of the model substances retinol, palmitic acid and cholesterol with and without βLG was determined using [^{3}H]-labelled ligands. Both bovine and reindeer βLG were able to pass across a Caco-2 cell monolayer similarly. Unbound and βLG-bound [^{3}H]retinol and [^{3}H]palmitic acid were equally transported across the Caco-2 cell layer, whereas [^{3}H]cholesterol could not pass across Caco-2 cells with or without βLG at any of the studied circumstances. Thus, the bovine and reindeer milk βLG is not a suitable protein to enhance transport of ligands across the Caco-2 cell membrane, used for predicting intestinal absorption.

Keywords: β-Lactoglobulin; Caco-2 monolayer; Ligand; Transport; Reindeer

1. Introduction

β-Lactoglobulin (βLG) is the whey protein that exist in bovine milk (Pérez and Calvo, 1995) and also in the milk of other ruminants such as reindeer (Rytkönen et al., 2002). βLG belongs to the lipocalin protein family, and the other members are retinol-binding protein (RBP) and fatty acid-binding protein (FBP) (Flower et al., 1993). The structures of lipocalins are quite similar (Flower et al., 1993). βLG exists as a dimer in physiological pH and the molecule consists of an anti-parallel β-sheet (Papiz et al., 1986). The core of βLG is an eight-stranded, anti-parallel β-barrel. The molecular weight of the subunit is about 18 kDa (Papiz et al., 1986). The real function of βLG is not known. βLG's ligand binding properties have been widely studied (a.o. Narayan and Berliner, 1997; Wang et al., 1997a,b; Sawyer and Kontopidis, 2000; Riihimäki et al., 2006). These studies have shown that βLG can bind hydrophobic ligands like retinol, retinoic acid, long-chain fatty acids and aromatic compounds. The retinol-binding site is inside the β-barrel, in the central cavity (Narayan and Berliner, 1997), also known as calyx (Flower et al., 1993; Brownlow et al., 1997). Since βLG, for its peculiar biochemical properties, had been proposed as an oral drug carrier (McAlpine and Sawyer, 1990; Eberini et al., 2006), we have an interest in its properties to transport compounds through the intestinal membrane and to elaborate whether βLG could be used as a carrier molecule for drugs or nutrients. As reindeer βLG appears to be less complex compared to bovine milk βLG (Rytkönen et al., 2002; Heikura et al., 2005) we were

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interested to explore if it would have different properties in comparison to bovine βLG in this aspect.

Peptide-conjugated cargo systems have been studied to enhance cellular or brain uptake of a drug (Temsamani and Vidal, 2004). Studies with cell and animal experiments have provided evidence that these cargo systems can successfully transport drugs across membranes of different cell types. This can improve both beneficial effects and reduce the costs. The transport of bovine βLG across the intestinal barrier has been studied using differentiated monolayer cultures of human colon adenocarcinoma cell line Caco-2 cell monolayers, which exhibit enterocyte-like characteristics and are widely accepted and used in *in vitro* modelling for predicting intestinal absorption by epithelial cells (Caillard and Tome, 1995; Puyol et al., 1995; Artursson et al., 1996; Tanabe et al., 2003). The M-cell model, which is epithelial cell phenotype that occurs over organized mucosal lymphoid follicles in the intestinal track, has also been used to study the transport of bovine βLG (Neutra et al., 1999; Rytkönen et al., 2006).

The structure of reindeer βLG is very similar with bovine βLG (Oksanen et al., 2006) but reindeer βLG is more homogeneous encoded only by one genetic variant instead of main variants A and B in case of bovine βLG (Rytkönen et al., 2002; Heikura et al., 2005). The amino acid composition and sequence for reindeer βLG have recently been shown to be related with those of bovine βLG, respectively (Rytkönen et al., 2002). Furthermore, the X-ray structure of reindeer βLG has been determined in pH 6 (Oksanen et al., 2006). The structure was shown to be almost identical with bovine βLG; for example, the molecular mass and the conformation of the binding calyx were almost similar, respectively and the reindeer βLG occurs also as a dimer at physiological pH. In a very recent study the immunological cross-reactivity of bovine specific anti βLG IgE with reindeer's milk βLG in patients with cow's milk allergy was investigated (Suutari et al., 2006). The study showed that reindeer milk βLG inhibits less IgE binding to the capturing antigen than bovine βLG. Thus reindeer βLG could also be less allergenic than bovine βLG (Suutari et al., 2006). Due to these characters the reindeer βLG might have more valuable binder characteristics than bovine βLG and be a safer carrier for divergent aliments and medicines. We studied the transport of bovine and reindeer βLG across the Caco-2 cell monolayers and βLG as a transport molecule for three different model ligands: [^{3}H]retinol, [^{3}H]palmitic acid and [^{3}H]cholesterol. All these model ligands have been shown to bind to bovine βLG (Wang et al., 1997a,b). Our binding studies have also shown that retinol bind to both bovine and reindeer milk βLG (Riihimäki et al., 2006).

2. Materials and methods

2.1. Materials

Sephadex^{TM} G-25 Fine was purchased from Amersham Biosciences (Uppsala, Sweden). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids, fetal bovine serum, Hank's balanced salt solution (HBSS), and Hepes solution were purchased from Gibco Invitrogen (Life Technologies, Paisley, Scotland, UK). Phosphate-buffered saline (PBS) 0.0067 M, pH 7.3–7.5 (PO_4) without calcium and magnesium, and L-glutamine and antibiotic mixture (100 IU/ml penicillin G, 100 μg/ml streptomycin) were supplied by BioWhittaker (Cambrex Bio Science, Verviers, Belgium). Retinol [11,12-^{3}H(N)]-, palmitic acid [9,10-^{3}H]- and cholesterol [1,2-^{3}H(N)]- were purchased from Perkin Elmer (Life Sciences, Boston, USA). Bovine and reindeer β-lactoglobulins were isolated at the Sotkamo Biotechnology Laboratory (Kajaani University Consortium, University of Oulu, Finland). Native bovine βLG was isolated from fresh cow milk (De Jongh et al., 2001), cooled to +4 °C, and isolation of βLG started within 3 h of milking. Reindeer (*Rangifer tarandus tarandus* L.) milk was obtained from Reindeer Research Station (Kaamanen, Finland), was stored at −20 °C and βLG from reindeer milk was isolated modifying the method of De Jongh et al. (2001) as described by Heikura et al. (2005).

2.2. Cell cultures

Caco-2 cells (originating from American Type Culture Collection) were kindly donated by professor Arto Urtti, Drug Discovery and Development Technology Center, Faculty of Pharmacy, University of Helsinki, Finland. The cells were cultured according to Laitinen et al. (2003, 2004). Briefly, the cells were grown in a medium composed of DMEM containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% (v/v) 200 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml). Cultures were maintained at 37 °C in an atmosphere of 5% CO_2, 95% air, and at 95% relative humidity.

Human hepatoma cells (HepG2) were obtained from American Type Culture Collection (Rockville, MD, USA) and maintained at 37 °C in an atmosphere of 4–5% CO_2 in Dulbecco's modified Eagle's medium containing penicillin (100 IU/ml) and (100 μg/ml), along with 10% fetal calf serum (FCS).

2.3. Binding of retinol, palmitic acid and cholesterol to β-lactoglobulin

The binding of ligand to βLG were adapted by the Puyol et al. (1995). 8 μCi [^{3}H]retinol (22.4 μM, 44.5 Ci/mmol) in ethanol, 40 μCi [^{3}H]palmitic acid (0.11 mM, 45.0 Ci/mmol) in ethanol or 8 μCi [^{3}H]cholesterol (19.6 μM, 51.0 Ci/mmol) in ethanol were added to 500 μl of bovine and reindeer βLG (1.88 and 2.25 mg/ml, respectively). The ethanol content was 1.6%. The solution was incubated at 37 °C overnight. A 2–3 molar excess of unlabelled retinol, palmitic acid or cholesterol was then added in protein solution to saturate binding sites followed by incubation for 2 h at 37 °C. The protein solution was passed through a Sephadex G-25 column to remove the unbound ligand. The fractions were collected in Eppendorf tubes. To select the fractions to transport studies the radioactivity of ligands and βLG concentration in the fractions were measured, and the most radioactive and βLG concentrated fractions were chosen.

To determine the radioactivity of fractions 50 μl of each were transferred to white-walled 96-well plates (IsoPlate TC, Perkin Elmer Life and Analytical Sciences/Wallac Oy, Turku, Finland) and 150 μl of liquid scintillator (Optiphase Supermix, Fisher Chemicals, UK) was added. Before the radioactivity measurements the plate was shaken for 10 min on a DELFIA® Plateshake (Perkin Elmer Life and Analytical Science/Wallac Oy, Turku, Finland). The radioactivity of the ligand was measured with a liquid scintillation counter (Microbeta® Trilux, Perkin Elmer Life and Analytical Science/Wallac Oy, Turku, Finland). The βLG concentration of fractions were determined with a Varioskan scanning spectrofluorometer and spectrophotometer (Thermo Electron corporation, Vantaa, Finland) using a UV 96-well plate (UV Flat Bottom Microtiter® Plates, Thermo Labsystems, Franklin, USA).

2.4. Transport studies

For transport studies, the Caco-2 cells were seeded at 6.8×10^4 cells/cm^2 on polycarbonate membranes with a pore size of 0.4 μm in 12-well plates (Transwell, Corning Costar, Cambridge, MA, USA). Cells at passage of 32–43 were used in experiments at the age of 21–27 days and the cultures were fed three times a week. Prior to the transport studies, cell monolayers were washed twice with DMEM medium or HBSS buffer. The apical solution was then changed to 550 μl DMEM medium or HBSS buffer containing the βLG-ligand complex or the bovine or reindeer βLG alone. Samples were collected from the apical compartment at the beginning of the experiment and at the end of experiment after 6 or 24 h, and from the basolateral compartment at the end of experiment after 6 or 24 h. The experiment was performed in an incubator (Stuart, orbital incubator S150, Bibby Sterilin Limited, USA), in humified atmosphere and shaking 75 rpm at 37 °C. The plates for 24 h transport studies were incubated at 37 °C in an atmosphere of 5% CO_2, 95% air, and 95% relative humidity. At the end of every experiment, cells were washed three times with PBS buffer, after that the polycarbonate membranes were cut and dissolved in 100 μl trypsin. The solution was stirred and 100 μl DMEM medium was added. The polycarbonate membranes were then removed and the solution was centrifuged at 10,000 rpm for 2 min. The supernatant was removed and the cells were diluted in 75 μl PBS buffer. Protein inhibitor cocktail (Protease inhibitor cocktail complex tablets, Roche Diagnostics, Mannheim, Germany) was added to all samples at a concentration of 20 μl/ml to prevent degradation of βLG. After the transport experiment, the radioactivity of the collected samples was measured with a liquid scintillation counter. The quantity of βLG was estimated by immunoblotting. The monolayer integrity was assessed by measuring the transepithelial electrical resistance (TEER) both before (after the DMEM medium wash) and after the experiment (EVOMX voltohmmeter, EVOMTM, World Precision Instruments Inc., USA). If the TEER value was below 250 Ω, monolayer was not used. The amount of radioactive ligands retained in polycarbonate membranes and well walls was determined by dissolving polycarbonate membranes in 75 μl methanol and adding 2 ml methanol in the empty wells. The samples were measured with a liquid scintillation counter as described before.

2.5. Western blotting analysis

The quantity of transported βLG was determined by Western blotting. Samples from the apical compartment were diluted 1:3 in DMEM medium. Samples from the basolateral compartment and cell samples were not diluted. Samples from the apical and basolateral compartments were then diluted 1:2 in 2× loading buffer [65.8 mM Tris–HCl (pH 6.8), 2% SDS, 26% glycerol, 0.01% bromophenol blue]. All samples were denaturated at 98 °C for 5 min. Fifty microliters were loaded onto 15% SDS-PAGE gel. Then the βLG was transferred onto a 0.45 μm nitrocellulose membrane (Hybond-^{TM}C Extra, Amersham Biosciences, Bucks, UK) and the membranes were blocked with 1× TBS 1% TWEEN 20 overnight at 8 °C. After blocking, the membranes were incubated with a rabbit polyclonal antiserum against bovine βLG (Biotechnology Laboratory, University of Oulu, Finland) for 1 h at room temperature. After washing the membranes were incubated with goat anti-rabbit IgG (H + L)-HPR conjugate (Bio-Rad, Hercules, CA, USA) diluted to 1:10 000 for 1 h at room temperature. After washing (3× 10 min in washing buffer), the membranes were developed with an enhanced chemiluminescence kit (SuperSignal Western Blotting kit, Pierce, Rockford, USA) according to the manufacturer's instructions. Detection and calculations were made with Syngene's GeneGnome instrument (Cambridge, UK).

2.6. Expression of NPC1L1 protein in Caco-2 cells

The RT-PCR technique was used to study the expression of Niemann-Pick C1-Like 1 (NPC1L1) protein in Caco-2 cells, and HepG2 as a reference cell line. The Caco-2 cells were collected as previously described above. RNA was isolated using an RNeasy mini kit (Qiagen). The cDNA was performed using an M-MLV Reverse transcriptase RNase kit (Promega, Madison, USA). PCR amplification was performed using an Eppendorf® Mastercycler personal device**.** The primers used were 5′-TATCTTCCCTGGTTCCTGAACGAC-3′ and 5′-CCGCAGAGCTTCTGTGTAATCC-3′ according to Davies et al. (2005). The amplification cycles used were 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 20 s, 40 cycles. The sense primer 5′-TCGACCGTCAACTCTCTGAACTG-3′ was also used, in which case the amplification cycles were 95 °C for 10 s, 62 °C for 20 s, 72 °C 30 s, 40 cycles. In the end, the PCR products were visualized with 1.4% agarose gel.

2.7. Statistical analysis

Results were means ± S.D. ($n = 3$) and statistically confirmed using two-sample t-test combined with Bonferroni adjusted probability and Dunn–Sidak adjusted probability tests using SYSTAT® version 11 (SYSTAT Software Inc., Richmond, CA, USA). Values of $p < 0.05$ were considered significant.

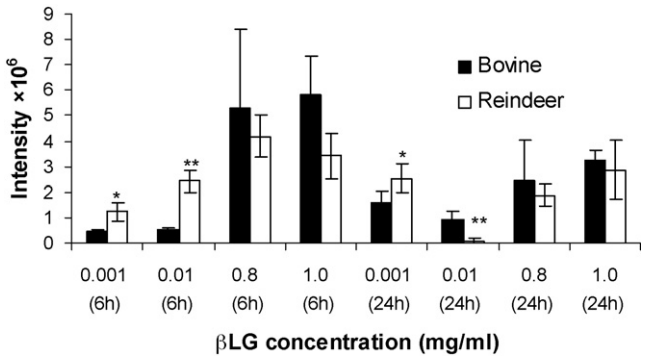

Fig. 1. The permeability of bovine and reindeer βLG across the Caco-2 cell monolayer. Results represent the amount of βLG in the basolateral compartment. The results were means ($n = 3$). p-Values were calculated between bovine and reindeer βLG, $^{*}p < 0.1$, $^{**}p < 0.05$.

3. Results

3.1. Transport of bovine and reindeer βLG across Caco-2 cell monolayers

The uptake and permeation of bovine and reindeer βLG was studied using the Caco-2 cell monolayer. To achieve the best experimental conditions DMEM medium was used in the transport experiments. Normally HBSS buffer is used, but the experiment duration of 6 and 24 h were too long for the cells to survive in the HBSS buffer (TEER values ∼100 Ω). The TEER values, which reflect the tightness and confluence of monolayers, were high in DMEM medium (>250 Ω).

To study the influence of the amount of bovine or reindeer βLG in the experiments, concentrations of 0.001, 0.01, 0.8 and 1.0 mg/ml were studied. The βLG concentration was found to effect on the amount of βLG in the basolateral compartment (Fig. 1). After 6 h more βLG was found in the basolateral compartment when the concentration was increased up to 0.8 mg/ml. After 24 h almost as much βLG were found in the basolateral compartment at all concentrations except 0.01 mg/ml of reindeer βLG. The amount of βLG in the basolateral compartments wase also studied in the βLG-ligand transport studies. Comparison of these results showed that βLG is transported across Caco-2 cell monolayer similarly alone and with ligand (Table 1). To study the permeation of βLG's across the Caco-2 cell monolayer, the concentration 0.001 mg/ml was chosen because the intensity was detectable both in the apical and basolateral compartments by immunoblotting without sample dilutions, giving a better reproducibility, while in the transport of βLG-ligand studies the concentration 0.8–1.0 mg/ml was chosen because these concentrations were best detectable with UV-spectrophotometer (data not shown), as well as to bind higher amounts of the ligands. At the highest concentrations there also seemed to be a saturation of the βLG-transportation in these cells.

Table 1
The permeability of bovine and reindeer βLG alone and with ligand across the Caco-2 cell monolayer studied with Western blotting

	βLG		βLG + ligand
	0.8 mg/ml	1.0 mg/ml	ca. 1.0 mg/ml
Bovine			
6 h	5.3×10^{-6}	5.8×10^{-6}	1.2×10^{-6}
24 h	2.5×10^{-6}	3.2×10^{-6}	1.7×10^{-6}
Reindeer			
6 h	4.2×10^{-6}	3.4×10^{-6}	0.6×10^{-6}
24 h	1.9×10^{-6}	2.9×10^{-6}	2.5×10^{-6}

Results represent the average amount of βLG (intensity) in the basolateral compartment.

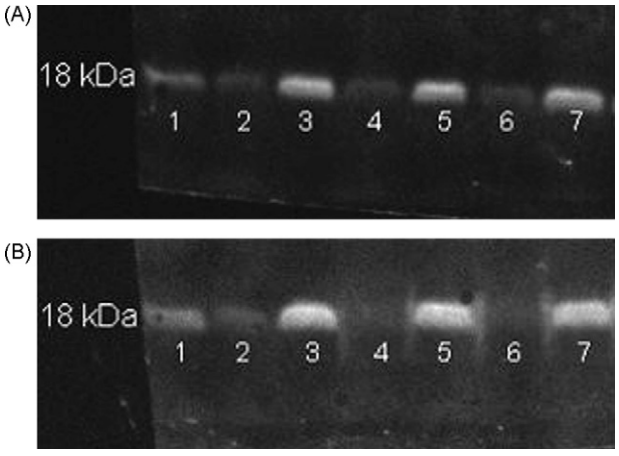

Fig. 2. Transport of bovine (A) and reindeer (B) βLG (0.001 mg/ml) across Caco-2 cells performed using the Western blotting determination ($n = 3$): (1) βLG marker, (2, 4 and 6) basolateral compartment, (3, 5 and 7) apical compartment.

We demonstrated that bovine and reindeer βLG were able to permeate across the Caco-2 cell monolayer (Fig. 2). Results with reindeer βLG were quite similar to those with bovine βLG, the reindeer βLG crossed the Caco-2 cell monolayer somewhat better at the shorter time exposure. With the 0.001 mg/ml concentration, the proportions of bovine and reindeer βLG crossing the membrane after 6 h were 4.6% (S.D. 2.2%) and 17.6% (S.D. 9.5%), respectively, while after 24 h the amounts were 11.2% (S.D. 2.6%) and 16.8% (S.D. 7.4%).

3.2. Transport of βLG-ligand across Caco-2 cell monolayers

The passage of [^{3}H]retinol bound to bovine and reindeer βLG and [^{3}H]retinol alone across Caco-2 cell membrane were studied. The samples were added to the apical compartment of Caco-2 cell culture plates. Fig. 3A shows the percentage of retinol passed across the Caco-2 cell membranes after 6 and 24 h. After 6 h the amount of retinol-bovine βLG was 8.2% and retinol-reindeer βLG 5.9% in basolateral compartment, whereas 15.0% of retinol alone crossed the membrane. The concentrations were the same after 24 h, 8.6 and 5.7%, for βLG samples, but for retinol alone 22.6%. After 6 h the amount of retinol to be retained by the cells was 3.1% with bovine βLG and after 24 h 7.2%. The values with reindeer βLG were 3.0% and 6.0%, respectively and the values without βLG were 17.0% and 15.8%, respectively.

In experiments with [^{3}H]palmitic acid, the percentage of palmitic acid bound to bovine βLG crossing the membrane after

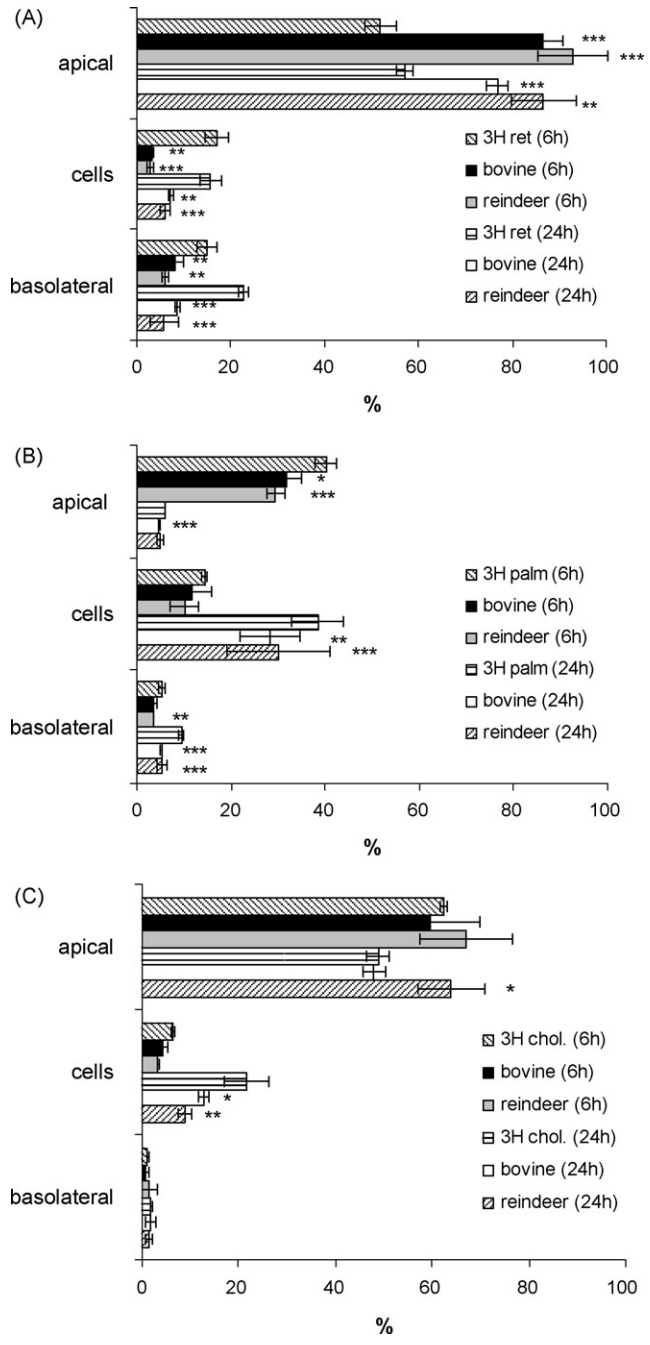

Fig. 3. The percentage of [^{3}H]retinol (A), [^{3}H]palmitic acid (B) and [^{3}H]cholesterol (C) transported across Caco-2 cell membranes after 6 and 24 h. *p*-Values were calculated between [^{3}H]ligand and ligand bound to protein ($n = 3$). $^*p < 0.1$, $^{**}p < 0.05$, $^{***}p < 0.01$.

6 h was 3.5% and of that bound to reindeer βLG was 3.6% (Fig. 3B). The transport of palmitic acid without βLG was 5.2%. After 24 h the amounts were somewhat higher: 5.1% for bovine βLG, 5.3% for reindeer βLG and 9.4% without βLG. The differences between 6 and 24 h were observed in the amount of palmitic acid found in the apical compartment. After 24 h it was much lower than after 6 h. The values with bovine βLG were 31.8% (6 h) and 4.7% (24 h), with reindeer βLG 29.4% (6 h) and 5.0% (24 h) and without βLG 40.2% (6 h) and 6.0% (24 h). The amount of palmitic acid retained by the cells after 6 h were11.7% (6 h) and after 24 h 28.3% (24 h) with bovine βLG. The values with reindeer βLG were 10.1% and 30.2%, respectively. Without βLG the values were 14.4% and 38.4%, respectively. These results demonstrated that most of the βLG-palmitic acid was retained by the cells after 24 h. Because the recovery of palmitic acid was only about 60%, 100 μl methanol was added on the polycarbonate membranes with the cells to enhance the collection of the ligand. This experiment showed that the missing palmitic acid was mainly retained by the cells (data not shown).

Fig. 3C shows the percentage of [^{3}H]cholesterol transported across the Caco-2 cell monolayer after 6 and 24 h. After 6 h only 0.5–1.3% of cholesterol had been transported across the cell membrane, with little change after 24 h (1.4–1.9%). The amount of cholesterol retained in cells was 3–6% after 6 h and 9–21% after 24 h. Most of the cholesterol was found in the apical compartment. The results for bound and unbound cholesterol were quite similar. Also small amounts (3–12%) of the added cholesterol were retained in the polycarbonate membranes and in the well walls. Because it has been reported that the Niemann Pick C1-like 1 protein (NPC1L1) facilitates the transport of cholesterol (Davies et al., 2005), we also studied the expression of NPC1L1 in our Caco-2 cells. Human NPC1L1 was expressed in both the reference cell line HepG2 and the Caco-2 cells, which could be shown with both sense primers used.

4. Discussion

We observed that bovine and for the first time reindeer βLG can be transported across Caco-2 cells. Our results demonstrated that increasing the bovine or reindeer βLG concentration had some influence on the amount of βLG crossing the Caco-2 cell monolayer. The differences between bovine and reindeer βLG, between different concentrations and between 6 and 24 h could be due to degradation of βLG (Caillard and Tome, 1995; Rytkönen et al., 2006), but cannot be explained only by this. Probably other unknown factors have contributed to these variations as well. Previous studies have shown that the passage of bovine βLG across enterocytes is mostly transcellular (Heyman and Desjeux, 1992; Caillard and Tome, 1995; Rytkönen et al., 2006). More than 90% of bovine βLG is transported via a degradative pathway, but almost about 10% transported directly (Heyman and Desjeux, 1992). The transcellular pathway for bovine βLG in Caco-2 cell monolayers has recently been confirmed by following the transcytotic route by tracking the βLG using fluorescence microscopy (Bernasconi et al., 2006). In our results 4.6% of bovine βLG crossed the membrane after 6 h and 11.2% after 24 h. On the other hand, Puyol et al. (1995) have used thrichloroacetic acid-insoluble fraction of [^{125}I]βLG and reported that as much as 75% of bovine βLG were able to cross after 24 h. Comparison of the experimental setups in these investigations shows differences between transport percentages, which can be attributed to differences in βLG concentrations, Caco-2 cell densities or analytical procedures. Some studies have found evidence that the plasma membranes from hybridoma cells bind to bovine βLG (Mansouri et al., 1998). In an earlier study the transport of bovine βLG was compared to the

transport of α-lactalbumin, which also is present in bovine milk (Caillard and Tome, 1995). This study showed that the uptake of bovine βLG was approximately 1.5 times higher than the uptake of α-lactalbumin. Puyol et al. (1995) have studied the transport of bovine βLG and albumin and demonstrated that about 50% of bovine βLG and about 30% albumin were transported across Caco-2 cell monolayer.

The transport of ligands with and without bovine and reindeer βLG across the Caco-2 cell layer was studied using [^{3}H]retinol, [^{3}H]palmitic acid, and [^{3}H]cholesterol. Retinol was chosen to represent the retinoids, which can regulate cell growth, differentiation and apoptosis (Wu et al., 2002). *In vitro* studies have also demonstrated that the retinoids inhibit the growth and invasion of cancer cells (Wu et al., 2002). Palmitic acid was chosen because it binds to bovine βLG very well and could be used as a linker molecule with a drug or nutrient that does not bind to βLG directly, or enhancing uptake of a drug to obtain better therapeutic effects (Riihimäki et al., 2006). The binding of palmitic acid to bovine βLG has been demonstrated by cocrystallisation of βLG and palmitic acid (Wu et al., 1999). Also previous analysis of vitamin A lipid metabolism in the Caco-2 cells has suggested, that this cell line would be a suitable model for studying intracellular events in vitamin A and lipid transports and metabolisms in the small intestine (Quick and Ong, 1990; Levin, 1993; Bellovino et al., 2003). Cholesterol has been proved to bind to βLG (Wang et al., 1997b), but it is known that cholesterol cannot pass through Caco-2 cells alone (Field et al., 1998; Davies et al., 2005). If bovine and/or reindeer βLG could be a proprietary carrier molecule for cholesterol, this could lead to an enhanced uptake, with possible non-beneficial health effects.

Our results showed that bovine and reindeer βLG did not increase the amount of retinol in the basolateral compartment. In fact, more unbound retinol was transported across Caco-2 cells in accordance with Puyol et al. (1995). This was also in the case of reindeer βLG. Majority of bound retinol was in the apical compartment. Previously it has been demonstrated that free retinol is able to diffuse and accumulate in the membranes (Bellovino et al., 2003). Normally cellular retinol-binding protein type I (CRBP-I), present in most cell types, protects retinol from the cellular environment and the cellular membranes from retinol. We did not see membrane accumulation of retinol, as the amount of retinol is constant at 6 and 24 h, but rather an increase in transported retinol between these time points. Binding to CRBP-II, expressed only in the mucosal cells of the small intestine, is required for the retinol intestinal esterification. The Caco-2 cell lines are known to express both CRBP-II and retinol esterification enzymes (Quick and Ong, 1990; Suruga et al.,1999; Bellovino et al., 2003). This could be one reason for unbound retinol to pass in much higher amounts and/or for the bound retinol to be delayed in passing during our experiment.

Bovine and reindeer βLG transported similarly palmitic acid, whereas slightly more palmitic acid was transported across Caco-2 cells alone. The difference between retinol and palmitic acid was that more palmitic acid was retained by the cells after 24 h. Palmitic acid has been reported by Puyol et al. (1995), and in studies with other fatty acids like oleic acid (Levin et al., 1992), to remain in cell layers. Puyol et al. (1995) showed that for [^{3}H]palmitic acid more than 90% of total radioactivity was retained by Caco-2 cells after 24 h. Fatty acid uptake by Caco-2 cells has been studied by using the long-chain fatty acids palmitate and oleate and the short-chain fatty acid octanoate (Trotter et al., 1996). Trotter et al. (1996) demonstrated that the uptake of long-chain fatty acids was saturable, while that of short-chain fatty acids was linear. It is also known that long-chain fatty acids are esterified in enterocytes before secretion (Trotter et al., 1996). Previous studies have demonstrated that palmitic acid is degraded to acetyl-CoA via oxidation and after that it will metabolize through the tricarboxylic acid cycle (Moncla et al., 1983). It has also been reported that Caco-2 cells have high glucose concentration which might increase fatty acids oxidation (Trotter and Storch, 1991). Murthy et al. (1988) have studied the incorporations of palmitic acid into cellular lipids and incubated Caco-2 cells with palmitic acid for 4, 8, and 22 h. In that study 60% of the palmitic acid was remaining in triglycerides after 22 h and smaller amounts were found in phospholipids and fatty acids. Also van Greevenbroek et al. (1995) have showed using Caco-2 cells that when increasing the concentration of palmitic acid more was subsequently accumulated in cell membranes. Burczynski et al. (1990) have studied the uptake of palmitate, which is an ester or salt of palmitic acid, by hepatocyte monolayers and by polyethylene membranes using βLG as a binding protein. In hepatocyte monolayers, when palmitate was free the clearance was weakly, but when palmitate was bound to βLG the clearance was about five times larger. In polyethylene membranes the clearance of bound and unbound palmitate was weakly. Burczynski et al. (1989) have also demonstrated similar results with palmitate bound to albumin in their previous studies. In our study about 30% and 40% of palmitic acid was retained in the Caco-2 cells with or without βLG respectively after 24 h.

We also studied the transport of cholesterol and cholesterol with βLG across Caco-2 cells using both bovine and reindeer milk βLG. Cholesterol has been shown to bind to bovine βLG (Wang et al., 1997b). Our results suggested that cholesterol could not be transported across Caco-2 cells very well (only about 1% was passed). No improvement was obtained by binding the cholesterol to bovine or reindeer βLG. Some earlier studies suggest that cholesterol cannot be transported across Caco-2 cells (Field et al., 1998; Davies et al., 2005). Altmann et al. (2004) have noticed that the Niemann Pick C1-like 1 protein (NPC1L1) is critical for the uptake of cholesterol across plasma membrane of the intestinal enterocyte. Davies et al. (2005) have studied the expression of NPC1L1 in a Caco-2 cell line and compared the results with those for the liver-derived hepatocellular carcinoma cell line, HepG2. The results obtained showed that human NPC1L1 was expressed at much higher levels in HepG2 cells than in Caco-2 cells. Recently Yamanashi et al. (2007) have shown that the uptake of cholesterol into Caco-2 cells from apical side was increased by NPC1L1 overexpression. Our Caco-2 cells did express the NPC1L1 gene, but apparently at too low levels to increase the uptake of cholesterol. Further, the cholesterol homeostatis in Caco-2 cells have been reported by Field et al. (1998), where they concluded that external cholesterol

influx did not alter the percent of newly synthesized cholesterol transported to the plasma membrane. These results showed also that inhibitors of *p*-glycoprotein function interfere with newly synthesized cholesterol reaching to the plasma membrane (Field et al., 1998). In view of this, in our studies the low concentration of cholesterol in basolateral compartment might partly be explained by the efflux transport by *p*-glycoprotein. The functionality of *p*-glycoprotein has been reported by using Caco-2 cells obtained from ATCC, for the passages of 32–43, same passages we used in this study (Siissalo et al., in press). We have also determined that our cells contain *p*-glycoprotein in Caco-2 experiments, but using 96-well plates (unpublished results). After 24 h we found increased levels of cholesterol incorporated to the cells, and more free cholesterol than βLG-bound, thus the accumulation into cells appeared over time, but was partly prevented by βLG.

The bovine and reindeer βLG-ligand results showed that neither bovine nor reindeer milk βLG do raise the amount of ligand in the basolateral compartment in the Caco-2 cell experiments. In fact, more retinol and palmitic acid were transported across Caco-2 cells alone than with βLG. Cholesterol was not transported across Caco-2 cell monolayer alone or with βLG. It might be possible that these ligands are dissociated from βLG before associating with cells or transporting across the monolayer. The results suggest that bovine and reindeer βLG might not carry the ligands across epithelial cells. Further, the Western blotting results showed that as much βLG was transported across Caco-2 cells alone as with ligand. This indicates that ligands do not block the transport of bovine and reindeer βLG across Caco-2 cells. In future studies it would be interesting to study one model drug that bind to βLG strongly and does not affect its transport. This model drug would also show strong pharmacological functions at low concentration and it could not pass through monolayer and would exhibit very low oral bioavailability. For drugs with above properties, βLG might have potential as drug carriers across the intestinal barrier. The finding that the residues lining the hydrophobic calyx of bovine and reindeer βLG have practically identically conformations (Oksanen et al., 2006) and that both βLGs act in the same way paves a possibility implementing homogeneous reindeer βLG as a biological tool.

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