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Enterocyte and M-Cell Transport of Native and Heat-Denatured Bovine β-Lactoglobulin: Significance of Heat Denaturation

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The three-dimensional structure, digestibility, and immunological properties of bovine β-lactoglobulin (β-lg) are modified by heat treatments used in processing of liquid milk products. Because it is not known if such treatments also modify the intestinal transport properties of β-lg, the transport of native and heat-denatured bovine β-lg was investigated in experimental cell models using Caco-2 cells and M cells. Transport of β-lg labeled with a fluorescent marker was followed with fluorometric measurements, electrophoretic analyses, and fluorescence microscopy. The data show that both cell types transported native β-lg more efficiently than they did heat-denatured β-lg. In addition, M cells transported native β-lg more than Caco-2 cells. Transport of native and heat-denatured β-lg was transcellular. The electrophoretic data also suggest that heat-denatured β-lg may have degraded more than native β-lg during the transport.

KEYWORDS: β-Lactoglobulin; M cell; Caco-2 cell; heat denaturation; transport

INTRODUCTION

Bovine β-lactoglobulin (β-lg) is the major protein of bovine whey. It belongs to the lipocalin protein family (*1*) and is one of the major milk allergens (*2*). At physiological pH it is a dimer consisting of two amino acid chains (162 amino acids) each having a molecular mass of ∼18 kDa. In addition, β-lg is an exceptionally stable protein in an acidic environment. At pH 2 it only dissociates reversibly to its monomers, but its structure remains native (*3*). Furthermore, native β-lg is practically resistant to pepsin degradation at low pH (*4*, *5*). It has also been shown that bovine β-lg binds small ligands such as fatty acids and vitamins (*1*). Although the physical and chemical properties of bovine β-lg are well characterized (*1*), its biological functions in vivo are not yet known.

Most milk products used for human consumption are heat treated during the commercial milk processing. These heat treatments, such as pasteurization, may cause heat denaturation of β-lg and modify its native structure (*6*). Heat denaturation of β-lg makes it susceptible for proteolytic degradation (*4*, *5*). In addition, heat denaturation of β-lg may expose new antigenic sites (*7*) and change the IgE binding properties of β-lg (*8*). Our recent evidence suggests that heat denaturation also alters the allergenic properties of β-lg (*9*); possible mechanisms for this include changes in its digestibility and/or transport. Effects of

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heat denaturation of β-lg on its intestinal transport properties have not been studied previously.

Mucosal surfaces of the intestinal tract are lined with a single layer of epithelial cells. The most prevalent type of epithelium consists of enterocytes sealed with tight junctions that prevent the paracellular influx of proteins and peptides with a possible antigenic potential. Uptake of macromolecules by enterocytes is an active process, and it is controlled by several mechanisms (*10*). The in vitro transport rate of the unprocessed native β-lg through intestinal enterocytes has been investigated previously in Caco-2 cells that mimic enterocytes (*11*) and in experiments using an intestinal insert from rabbit ileum (*12*). Passage of β-lg across enterocytes has been reported to be transcellular, whereas paracellular leakage is very unlikely (*11*, *13*). In enterocytes transcellular transport of β-lg involves two different pathways. The main pathway is a degradative one (90%) and implies a lysosomal processing of β-lg, whereas the minor route of direct transcytosis allows transport of intact β-lg and is responsible for <10% of the total β-lg transported.

M cells are another important epithelial cell type that occurs over organized mucosal lymphoid follicles (follicle associated epithelium, FAE) in the intestinal tract. M cells deliver foreign material by transepithelial transport from the intestinal lumen to organized mucosa associated lymphoid tissues (O-MALT), and therefore they play an important role in the regulation of immunological reactions to nutritional and other luminal antigens (*14*). Particles and macromolecules that bind to apical plasma membranes of M cells are efficiently endocytozed and transported (*10*). Transport without degradation is the major pathway in M cells, providing nonprocessed antigens to the lymphatic system (*15*), although in rat M cells a lysosomal system capable of processing endocytozed antigens has been detected (*16*). In contrast, most of the antigens transported through enterocytes are processed and degraded in lysosomes.

The uptake of β-lg by M cells has not been investigated previously. However, one study (*17*) has analyzed the absorption of β-lg through the Peyer's patches, which contain M cells, and has shown that absorption of β-lg through the Peyer's patches was less efficient than was observed in the ileum. In vitro absorption studies with M cells were not possible until an M-cell model was introduced in 1997 by Kerneis. The model was used to evaluate the transport of some pathogenic organisms (*18*), and it has also been validated for transport studies with fluorescent microspheres (*19*). Ovalbumin has been shown to be transported through M cells in vitro in a form that it is capable of eliciting cytokine production, unlike when the antigen is transported through Caco-2 cells (*20*). This indicates that the model mimics true M cells in handling of luminal antigens. However, the M-cell model has not been used to evaluate transport of other proteins. Neither have there been any quantitative studies of protein transport by M cells compared to those by enterocytes in vitro.

In the development of food allergies, intestinal degradation and transport properties of allergens are important factors (*33*). The aim of the present study was to compare the in vitro transport of β-lg from bovine milk in enterocytes (Caco-2 cells) and M cells. These epithelial cell types form the two main pathways of intestinal absorption, and transport in M cells in particular may affect the immune response and even allergenicity to dietary antigens in humans. Unlike most other proteins, β-lg stays in vivo largely intact up to the distal small intestinal lumen (*21*). Therefore, in vitro absorption studies of an intact β-lg have physiological relevance. Because structural changes of β-lg induced by heat denaturation might change its transport properties, the effect of heat denaturation of β-lg on the transport was also studied.

MATERIALS AND METHODS

Materials. Tissue culture flasks were purchased from Sarstedt. Polycarbonate membrane transwells (pore size = 3 μm) were from Corning Costar. The human colon adenocarcinoma cell line (Caco-2) was obtained from the European Collection of Animal Cell Cultures, Salisbury, U.K. Dulbecco's PBS, modified Eagle medium with Glutamax I, glucose, and pyridoxal (DMEM), antibiotic antimycotic, trypsin (EC.3.4.21.4)−EDTA, and MEM nonessential amino acids were from Gibco-BRL, Paisley, Scotland. PBS was from Amresco. Fetal calf serum was from CSL. EVOM epithelial voltage meter was purchased from World Precision Instruments (Sarasota, FL). Bovine serum albumin, bovine β-lg, and DAPI were purchased from Sigma (Sigma-Aldrich Pty. Ltd., Sydney, Australia). Electrophoresis apparatus was Miniprotean II from Bio-Rad, Richmond, CA. SDS-PAGE molecular mass proteins were from Fermentas Inc., Hanover, MO, and Bio-Rad. PD-10 gel filtration columns and a Storm 860 laser scanner were from Pharmacia, Uppsala, Sweden. Formalin was obtained from Ajax. Anti-rabbit-IgG antibody conjugated with Alexa 546 fluorescent dye, BODIPY-FL fluorescent dye, and Prolong mounting medium were from Molecular Probes, Eugene, OR. Anti-ZO-1 antibody was from Zymed (San Francisco, CA). Polyclonal antiserum to bovine milk β-lg was produced in rabbits in the biotechnology laboratory/laboratory animal center, University of Oulu, Finland. HRP detection kit for Western blotting and nitrocellulose membrane were from Bio-Rad. Fluorescence intensities were measured with a Fluoroscan FL-plate reader (Labsystems Oy). Microscopy was performed with a Nikon TE300 inverted epifluorescence microscope, equipped with a Lambda 10-2 filter wheel (Sutter Instruments) containing excitation filters for DAPI, fluorescein, and tetramethylrhodamine. Filters and triple-band-pass dichroic mirror were supplied by Chroma Technologies, and the MicroMAx 782 12-bit CCD camera was from Princeton Instruments. Equipment was controlled by MetaMorph software (Universal Imaging).

Methods. *Caco-2 Cell Culture.* Caco-2 cells were seeded at the density of 2×10^5 cells/cm^2 in 20 mL of DMEM supplemented with 10% v/v fetal calf serum, 1% v/v nonessential amino acids, and 1% v/v antibiotics (Caco-2 medium) in 75 cm^2 tissue culture flasks. Cells were incubated at 37 °C, in 5% CO_2 (*19*). Caco-2 cells were seeded (0.1 mL of Caco-2 medium containing 3×10^5 Caco-2 cells) on the inverted transwells. Cells were incubated overnight at 37 °C in 5% CO_2 to attach them to the membranes. Transwells were placed back into their 24-well plates. Caco-2 medium was added to the inner chamber (0.1 mL) and wells (0.6 mL). The cells were incubated at 37 °C in 5% CO_2, and culture medium was changed every 2 days. Cells were grown for 21−28 days to fully differentiate them. Transepithelial electrical resistance (TEER) was measured with EVOM before coculturing to confirm cellular differentiation. Cells were considered to be fully differentiated to enterocyte-like cells when their TEER value was >150 Ωcm^2 (*19*).

Coculture of Caco-2 Cells with Peyer's Patch Cells. Peyer's patch (PP) cells were isolated from the small intestines of BALB/c mice aged between 8 and 12 weeks. Mice were killed by cervical dislocation and the small intestines collected. Intestinal contents were removed by flushing the intestines with PBS containing fetal calf serum and antibiotic−antimycotic solution. PPs were dissected; 5−10 were collected per mouse. Tissues were forced through a nylon mesh with PBS containing fetal calf serum and antibiotic−antimycotic solution, and the cells were collected. The cells were pelleted by centrifugation at 1000 rpm for 10 min and then resuspended in 1 mL of Caco-2 medium, and viable cells were counted (*19*).

Isolated PP cells were diluted in Caco-2 medium at a concentration of 10^7 cells/mL. Medium from the apical chamber of the Caco-2 cells grown for 21−28 days was replaced with the Caco-2 medium. The medium from the basolateral chamber was replaced with 0.1 mL of PP cell suspension (10^6 cells) to grow M cells or with 0.1 mL of Caco-2 medium to grow enterocytes. The cells were incubated for 2 days at 37 °C in 5% CO_2 (*19*).

Labeling of β-Lg. Bovine β-lg (20 mg) was dissolved in 1 mL of 0.1 M sodium carbonate buffer, pH 8.3. BODIPY-FL dye (1 mg) was

dissolved in 100 μL of dimethyl sulfoxide (DMSO). BODIPY-FL is a widely used fluorophore, comparable with fluorescein but with a narrower emission bandwidth and a higher extinction coefficient. Dissolved dye was added slowly to the β-lg solution and then incubated for 1 h at room temperature with continuous stirring and protected from light. The BODIPY-labeled β-lg (β-lg-F) was then separated from nonbound dye with gel filtration using a PD-10 column according to the manufacturer's instructions and PBS as an eluent. β-Lg-F was further diluted in PBS to a concentration of 13 mg/mL and kept at −20 °C until used in transport experiments.

β-Lg-F Transport Experiments. After coculturing of Caco-2 cells with or without PP cells for 2 days, apical culture medium was removed from each transwell and was replaced with 0.6 mL of Caco-2 medium/PBS containing 1 mg/mL native or heat-denatured β-lg-F. For heat denaturation β-lg-F was diluted in PBS at a concentration of 7 mg/mL and was then heat denatured in a water bath at 90 °C for 30 min. Immediately after the denaturation procedure, β-lg-F was further diluted in Caco-2 medium at a concentration of 1 mg/mL and was used in the transport experiments. Thereafter, basolateral medium was replaced with 100 μL of Caco-2 medium, and transwells were incubated at 37 °C for 24 h. Apical and basolateral culture media were collected for further analysis (39 parallel cell cultures).

Trypsination and Fluorometric Measurement. Samples containing β-lg-F from labeling reaction and transport experiments (40μL) were incubated for 24 h at 37 °C with an equal volume (40 μL) of trypsin−EDTA solution (100 μg of trypsin). Before and after trypsination, fluorescence values of the labeled β-lg samples were measured by using a fluorometer with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

Gel Electrophoresis and Western Blot. Reduced and nonreduced SDS-PAGE studies were carried out with 4% stacking gel and 15% separation gel (*22*). Silver staining was performed with a Bio-Rad silver stain kit according to the manufacturer's instructions. Gels were scanned with a Storm scanner for fluorescent label in blue mode with an excitation wavelength of 450 nm and an emission wavelength of >520 nm. For Western blotting proteins were transferred to a nitrocellulose membrane overnight (*22*). Both primary antiserum (anti-bovine milk β-lg) and HRP-labeled secondary antibody were diluted 1:3000 in PBS-Tween. The detection kit for HRP was used according to the manufacturer's instructions.

Immunofluorescence Staining and Microscopy. Transwell membranes were fixed in a solution of neutral-buffered formalin, 10% v/v formalin in Dulbecco's PBS, pH 7.4, for 10 min at room temperature. Cells were permeabilized with 0.1% Triton-X. After three washings in PBS-Tween, the samples were blocked with 2% w/v bovine serum albumin in PBS for 60 min at 37 °C to prevent nonspecific binding of antibodies. Anti ZO-1 antibody (1:1000) was added and incubated 1 h at 37 °C. The samples were then washed three times and probed with anti-rabbit IgG Alexa 546 conjugate (1:100) for 1 h at 37 °C. After three washes, the samples were counterstained with DAPI (1:100) in deionized water for 15 min at room temperature and then washed three times with deionized water before being partially air-dried. The membranes were removed with a scalpel and placed cell side up on glass slides, before mounting with Prolong mounting medium. The medium was allowed to set overnight before the slides were sealed with nail varnish. Cells were examined by epifluorescence microscopy with filters to rhodamine (Alexa 546), DAPI, and fluorescein (BODIPY-FL). Digital photographs were taken, and image analyses were performed by MetaMorph imaging software.

Statistical Evaluation. The data were analyzed with the SPSS 12.0.1 package (SPSS Inc., Chicago, IL). Amounts of transported native and heat-denatured β-lg by Caco-2 cells and M cells were compared using a nonparametric Mann−Whitney test, because the distributions were not normal in all cases. All statistical tests were two-sided, and p values of ≤0.05 were considered to be significant.

RESULTS

Labeling and Heat Denaturation of β-Lg. Labeling of native bovine β-lg was performed using a BODIPY-FL succinimidyl ester conjugate, which reacts with the amine groups of proteins. To get similarly labeled native and heat-denatured β-lg, denaturation was performed after labeling of native β-lg. The fluorescence intensity of the native labeled β-lg (β-lg-F) was 1195 when the β-lg concentration was adjusted to 1 mg/mL in a Caco-2 culture medium. After heat denaturation (90 °C/30 min), the fluorescence intensity of β-lg-F (1 mg/mL) was only 180, indicating a loss of the fluorescence during denaturation.

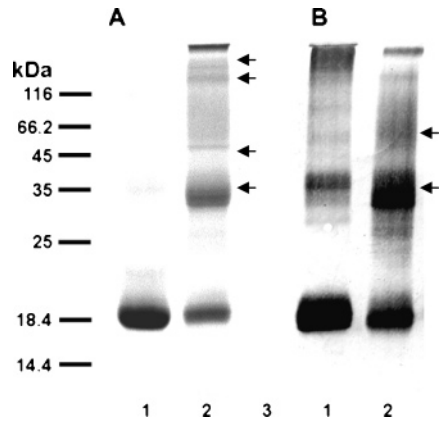

Figure 1. Labeled native and heat-denatured bovine milk β-lg analyzed with nonreduced SDS-PAGE followed by fluorescence scanning or Western blotting: (**A**) fluorescence scanning β-lg [lane 1, native β-lg-F (2 μg, 2 μL); lane 2, heat-denatured β-lg-F (2 μg, 2 μL); lane 3, nonlabeled β-lg (2 μg, 2 μL]; (**B**) Western blotting β-lg [lane 1, native β-lg-F (2 μg, 2 μL); lane 2, heat-denatured β-lg-F (2 μg, 2 μL)]. Arrows in lanes A2 and B2 mark high molecular weight bands representing polymeric β-lg induced by heat denaturation.

Both native and heat-denatured β-lg-F were analyzed by a nonreduced SDS-PAGE. Fluorescent scanning of the native (**Figure 1A**, lane 1) and the heat-denatured β-lg-F (**Figure 1A**, lane 2) shows one main band with a molecular mass around 18 kDa, and in addition the heat-denatured β-lg-F shows some high molecular mass bands (marked with arrows). This indicates clearly that during heat denaturation of the native β-lg (**Figure 1A**, lane 1), the intensity of the main protein band decreases with the simultaneous appearance of new high molecular mass bands (**Figure 1A**, lane 2), in agreement with previously published data (*23*). The nonlabeled β-lg was not visible by fluorescent scanning (**Figure 1A**, lane 3). Furthermore, all protein bands were identified with antisera to bovine milk β-lg (**Figure 1B**). Heat-denatured β-lg-F (**Figure 1B**, lane 2) shows a similar protein profile when either analyzed by fluorescent scanning (**Figure 1A**, lane 2) or identified with antisera to bovine milk β-lg (**Figure 1B**, lane 2), whereas a dimer of native β-lg was detected only with Western blotting (**Figure 1B**, lane 1), probably due to the higher sensitivity of the antisera compared to fluorescent scanning.

Microscopical Assessment of β-Lg Transport. Microscopic examination of immunostained cultured Caco-2 and M cells showed that the human ZO-1 protein, which is a peripheral membrane protein of ∼225 kDa (*24*), was located regularly at the cell junctions (**Figure 2**). This regular staining pattern indicated that the tight junctions between the cells were regularly arranged in both cell types. Additional evidence for an epithelial barrier function before transport studies was obtained by confirming that the TEER value was >150 Ωcm^2. The main reason for studying the ZO-1 immunoreactivity was to identify whether any transport of β-lg could occur across the potential paracellular route.

Figure 2 shows fluorescence micrographs of transported native and heat-denatured β-lg-F in Caco-2 (**Figure 2A**,**C**,**E**)

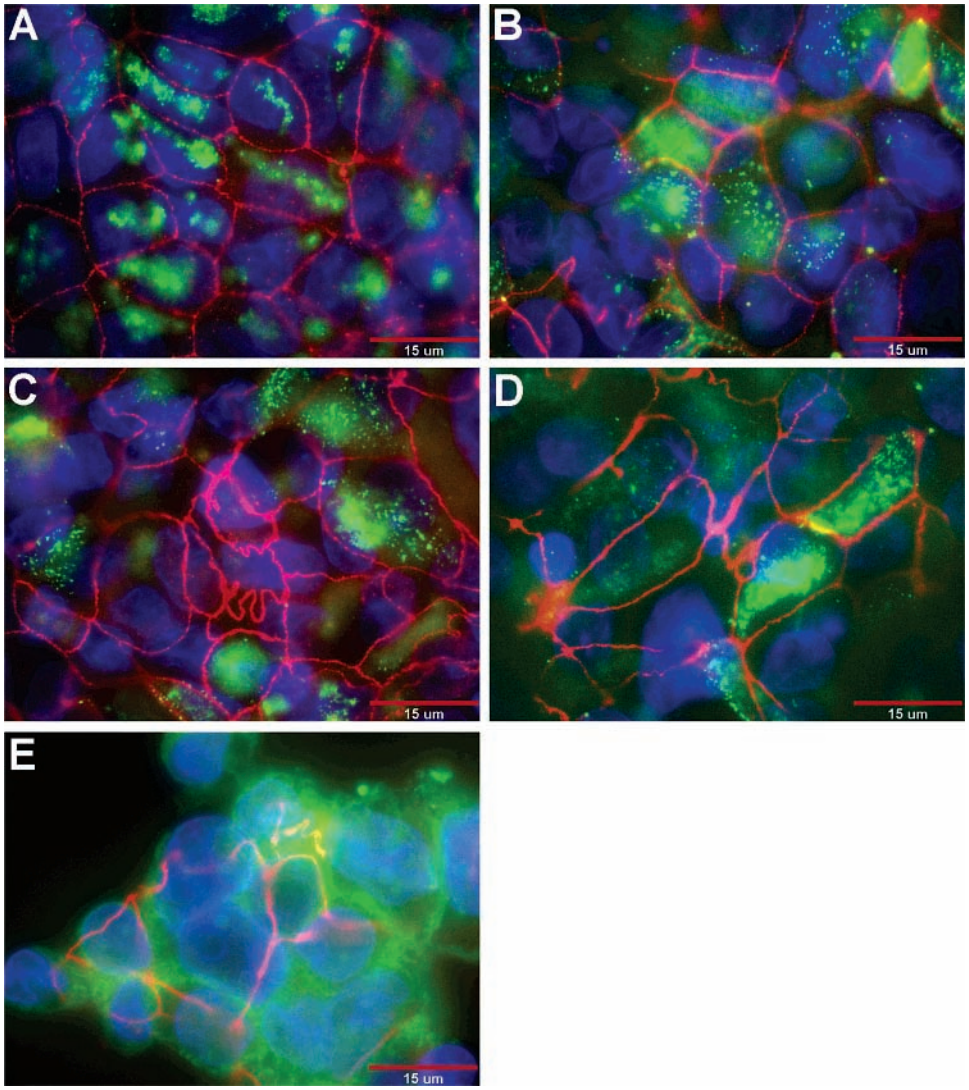

Figure 2. Fluorescence micrographs showing native and heat-denatured labeled β-lg transport in Caco-2 and M cells. In living Caco-2 cells (**A**, **C**) and M cells (**B**, **D**) native (**A**, **B**) and heat-denatured (**C**, **D**) β-lg-F is located in the cytoplasm of the cells, whereas in dead Caco-2 cells (**E**) native β-lg-F is in the intercellular spaces decorated by ZO-1 immunoreactivity. Magnification used was $\times$100; bars represent 15 μm (β-lg, green; ZO-1, red; nuclei, blue).

and M cells (**Figure 2B**,**D**). In living cells (**Figure 2A**−**D**) β-lg-F is located in the cytoplasm of the cells, whereas in dead/injured Caco-2 cells (**Figure 2E**) β-lg-F is located in the intercellular spaces around the tight junctions decorated by ZO-1 immunoreactivity. In healthy cells there was no evidence for fluorescently labeled native or heat-denatured β-lg-F on the tight junction areas, all fluorescence being located in the cytoplasm of the cells. This indicates that the transport of native as well as heat-denatured β-lg-F was transcellular in both Caco-2 and M cells.

Transport of the Labeled β-Lg in Cells. The transported amounts of native and heat-denatured β-lg-F were measured from samples taken from transport experiments in Caco-2 and M cells. Samples from apical and basolateral culture media with native or heat-denatured β-lg-F, incubated for 24 h at 37 °C, were analyzed qualitatively and quantitatively.

Qualitative analyses were completed with a reduced SDS-PAGE followed by fluorescent scanning and Western blotting. Fluorescent scanning (**Figure 3A**,**B**) indicated that native β-lg-F was transported through Caco-2 cells (**Figure 3A**, lane 3) and through M cells (**Figure 3B**, lane 3), because a protein band of a molecular mass of ~18 kDa was detected in the basolateral culture media of Caco-2 and M cells. This protein band was also recognized with antisera to bovine milk β-lg in Western blotting (**Figure 3C**, lanes 1 and 2). Transported heat-denatured β-lg-F could not be visualized with either fluorescent scanning (**Figure 4A**,**B**, lanes 3) or Western blotting using antisera to bovine β-lg (data not shown). This was probably due to degradation of the heat-denatured β-lg-F during the transport, or alternatively it was not transported enough to be detected with these methods.

The quantitative amounts of transported native and heat-denatured β-lg-F in Caco-2 and M cells were estimated by measuring the fluorescence intensities of the transported and nontransported β-lg-F in samples collected from transport experiments. When transported β-lg-F is quantitated, the effects of fluorescence quenching have to be taken into account. Decrease of the fluorescence intensity of β-lg-F caused by heat denaturation may be due to self-quenching of the fluorescence occurring when two identical fluorescent molecules are in close proximity to each other, causing the fluorescence emission to be quenched by intermolecular interactions. It has been shown earlier that protease-catalyzed hydrolysis of a fluorescently labeled protein relieves the quenching (*25*). Accordingly, to be able to compare the amounts of the transported β-lg-F, all of the samples were trypsinated to eliminate the possible self-

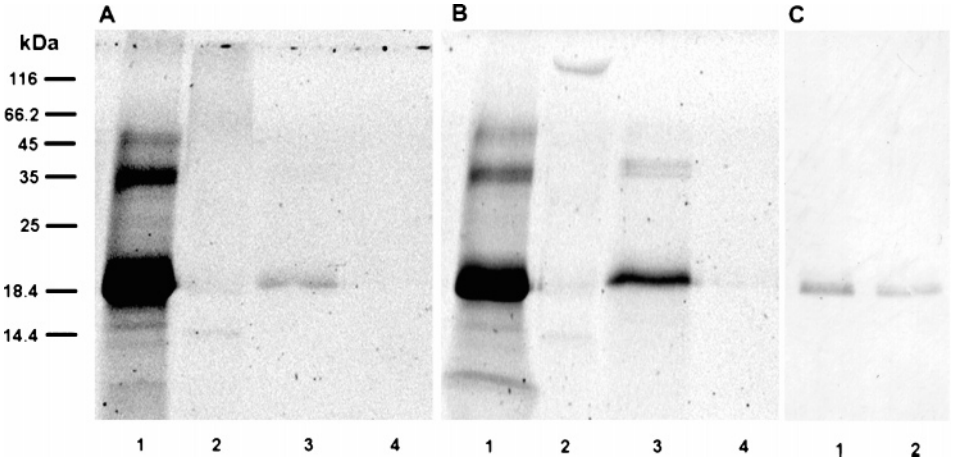

Figure 3. Nontransported and transported native β-lg-F analyzed with SDS-PAGE followed by fluorescent scanning or Western blotting: (**A**) fluorescent scanning of samples from transport experiments in Caco-2 cells after 24 h of incubation [lane 1, nontransported β-lg-F (2.5 μg, 2.5 μL); lane 2, trypsinated nontransported β-lg-F (2.5 μg, 2.5 μL); lane 3, transported β-lg-F (15.6 μL); lane 4, trypsinated transported β-lg-F (7.8 μL); (**B**) fluorescent scanning of samples from transport experiments in M cells [lane 1, nontransported β-lg-F (2.5 μg, 2.5 μL); lane 2, trypsinated nontransported β-lg-F (2.5 μg, 2.5 μL); lane 3, transported β-lg-F (15.6 μL); lane 4, trypsinated transported β-lg-F (7.8 μL); (**C**) Western blotting [lane 1, transported β-lg-F by Caco-2 cells (7.2 μL); lane 2, transported β-lg-F by M cells (7.2 μL).

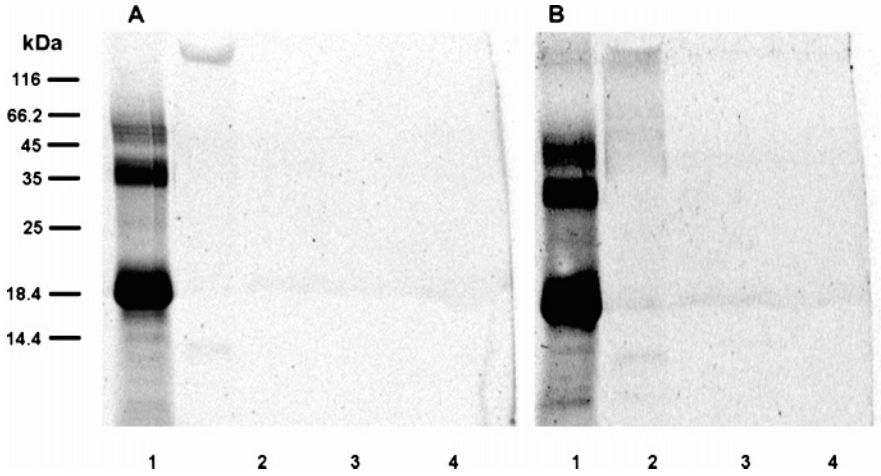

Figure 4. Nontransported and transported heat-denatured β-lg-F analyzed with SDS-PAGE followed by fluorescent scanning: (**A**) fluorescent scanning of samples from transport experiments in Caco-2 cells [lane 1, nontransported β-lg-F (2.5 μL, 2.5 μg); lane 2, trypsinated nontransported β-lg-F (2.5 μL, 2.5 μg); lane 3, transported β-lg-F (15.6 μL); lane 4, trypsinated transported β-lg-F (7.8 μL); (**B**) fluorescent scanning of samples from transport experiments in M cells [lane 1, nontransported β-lg-F (2.5 μL, 2.5 μg); lane 2, trypsinated nontransported β-lg-F (2.5 μL, 2.5 μg); lane 3, transported β-lg-F (15.6 μL); lane 4, trypsinated transported β-lg-F (7.8 μL).

quenching. Trypsinated β-lg-F samples were analyzed by a reduced SDS-PAGE followed by fluorescence scanning or silver staining. According to our results, trypsin degraded both native and heat-denatured β-lg-F, and no intact native (**Figure 3A**,**B** lanes 2 and 4) or heat-denatured (**Figure 4A**,**B**, lanes 2 and 4) β-lg-F could be detected after trypsination.

Fluorescence intensities of the above samples were also measured before and after the trypsin treatment with a fluorescence plate reader. **Table 1** shows that the intensity of the fluorescence of the nontransported heat-denatured β-lg-F elevated drastically (170/2400, mean values) and that of the nontransported native β-lg-F almost doubled (1250/2540, mean values) after trypsin treatment in both cell types. This indicates that by using trypsination, quenching could be greatly eliminated. However, after trypsination, the fluorescence intensity of the heat-denatured β-lg-F was still ~7% less than that of the native β-lg-F (2410/2580, samples before incubation at 37 °C). We reasoned that a permanent loss of the fluorescence or some irreversible quenching might have occurred during the heat denaturation step. To eliminate these effects on the fluorescence

Table 1. Fluorescence Intensities of Transported and Nontransported Native and Heat-Denatured Labeled Bovine Milk β-Lg[a]

	native β-lg		heat-denatured β-lg	
	Caco-2 (n= 10)	M cell (n= 9)	Caco-2 (n= 11)	M cell (n= 9)
Nontransported β-Lg (Apical Chamber)				
before trypsination	1251.50	1248.0	170.3	172.0
after trypsination	2540.0	2530.0	2406.0	2388.0
Transported β-Lg (Basolateral Chamber)				
before trypsination	9.26	12.68	6.44	7.15
after trypsination	9.53	12.62	6.34	7.176
Percentage of Transported β-Lg				
	0.33ab	0.45bc	0.22a	0.25c

[a] Fluorescence intensities of samples (40 μL) from the basolateral and apical (approximate amount of β-lg is 24 μg) culture media, after 24 h of transport were measured before and after treatment with trypsin (40 μL, 100 μg of trypsin; 24 h). Results of 9–11 identical experiments are shown. See Materials and Methods for details. Medians of all values are shown: (a) $p = 0.003$; (b) $p = 0.004$; (c) $p = 0.000$.

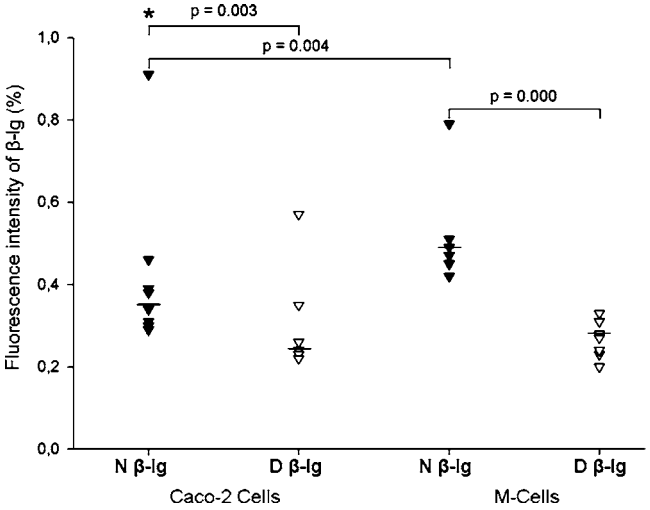

Figure 5. Transport of native and heat-denatured labeled β-lg in Caco-2 and M cells. Fluorescence intensity values are percentages of fluorescence of transported labeled β-lg to respective values of nontransported labeled β-lg (see **Table 1**). Both native (N, ▼) and heat-denatured (D, ▽) β-lg were treated with trypsin before the fluorometric measurements.

intensity in the quantitation of β-lg transport, we calculated the proportion of the transported β-lg-F to that of the nontransported β-lg-F by using the fluorescence intensities measured after trypsin treatment.

The transported proportions of β-lg-F (8−11 parallel transport experiments) are shown in **Table 1** and **Figure 5**. To summarize, native β-lg-F was transported more by both Caco-2 cells ($p = 0.003$) and M cells ($p = 0.000$) than heat-denatured β-lg-F. In addition, native β-lg-F was transported in larger quantities by M cells than by Caco-2 cells ($p = 0.004$), whereas no significant difference between Caco-2 and M cells in the transport of heat-denatured β-lg-F was observed ($p = 0.34$).

Degradation of β-Lg during Transport. Degradation of the β-lg-F during transport in Caco-2 and M cells was assessed qualitatively by a reduced SDS-PAGE followed by fluorescent scanning or Western blotting. Fluorescence scanning (**Figure 4A**,**B**, lanes 3) suggests that heat-denatured β-lg-F is degraded in both cell types during the transport because it was not detected by fluorescence scanning or Western blotting (data not shown). Native β-lg was not totally degraded because it was detected by both fluorescence scanning (**Figure 3A**,**B**, lanes 3) and Western blotting (**Figure 3C**, lanes 1 and 2). In addition, traces of several other proteins not identified by antisera to bovine β-lg were detected in silver staining (data not shown). These high molecular mass proteins might originate from serum added to the culture medium or they might be secreted during the 24 h incubation period, by the Caco-2 and/or M cells.

A second assessment of degradation of transported β-lg was based on the release of the quenched fluorescence, which can be used to monitor degradation of labeled proteins (*25*). **Table 1** shows that trypsin treatment did not increase the fluorescence intensity of transported (basolateral chamber) native or heat-denatured β-lg-F. This might suggest that most of the native and heat-denatured β-lg-F were degraded during transport in Caco-2 and M cells, whereas some native β-lg-F was still intact as seen by fluorescent scanning (**Figure 3A**,**B**, lanes 3; **Figure 3C**, lanes 1 and 2).

DISCUSSION

The significance of bovine milk β-lg as an allergen (*2*) and our recent data suggesting the importance of heat denaturation in the modification its allergenic properties (*9*) prompted us to evaluate the transport properties of this major protein of bovine whey. By using in vitro models, we compared the uptake and transport of native and heat-denatured β-lg labeled with a fluorescent probe in two major types of absorptive intestinal epithelium, enterocytes (Caco-2 cells) and M cells. Our data indicate that β-lg passes through enterocytes and M cells rather than bypassing cells via a paracellular transportation route. Our study demonstrates for the first time that transport of β-lg is quantitatively different in Caco-2 cells compared to M cells, the transport being more effective in M cells. Furthermore, we show that a simple heat denaturation procedure significantly affects the transported amounts of β-lg. Our conclusions are based on several (9−11) identical transport experiments, the results of which showed excellent reproducibility, and the differences in the transport rates between native and heat-denatured β-lg and between cell types are statistically significant.

We show here for the first time that the transport of heat-denatured bovine milk β-lg is transcellular in Caco-2 cells as is the transport of native and heat-denatured β-lg in M-cells. In agreement with previous studies we show also that the transport of native β-lg in Caco-2 cells is transcellular (*13*). Although transcellular transport is active in healthy cells, the paracellular route may be opened after insults leading to cellular dysfunction, degeneration, or death (*26*). This was also noted in the in vitro model used in this study. Native β-lg-F was concentrated in the paracellular area of dead Caco-2 cells, indicating leakage of β-lg through tight junctions (**Figure 2E**). In some pathological situations, intestinal permeability may increase due to defective cell junctions or other mechanisms. The increased permeability is an important factor in the pathogenesis of several conditions such as food allergy (*27*) and Crohn's disease (*28*). Considering the importance of the paracellular route in pathological conditions, it would be interesting to examine further with in vitro studies whether modification of the paracellular route permeability would differently affect the transport of native and heat-denatured β-lg.

Quantitation of cellular transport of proteins marked with a fluorescent label is difficult both due to quenching of fluorescence caused by folding of the protein and due to increase of fluorescence caused by proteolytic degradation during the transport. Heat denaturation induces aggregation of the β-lg molecules in addition to conformational changes (*29*). The observed decrease in the fluorescence intensity related with heat denaturation as shown in this study is likely to be related to self-quenching of the fluorescence occurring when two identical fluorescent molecules are in close proximity to each other (*25*). On the other hand, proteolytic degradation such as trypsination splits the aggregates of β-lg and hydrolyzes it, resulting in 19 peptide fragments containing 1−26 amino acids (*30*). Increasing the distance between the adjacent fluorescent dye molecules, by proteolysis, decreases their interaction and thus increases their fluorescence intensity (*25*). Our finding that the fluorescence intensity of nontransported labeled native bovine milk β-lg increased by trypsination indicates that the labeled β-lg was self-quenched (**Table 1**).

We show here that native β-lg from bovine milk was transported significantly more efficiently than heat-denatured β-lg in both Caco-2 and M cells. There are no previous studies comparing the intestinal transport of native and heat-denatured β-lg, and the mechanisms behind transported amounts remain unknown. A rate-limiting step in the transport might be any part of the process, which starts with adhesion to apical glycocalyx, continues with the uptake across the cell membrane,

a vesicular or lysosomal transport in cytoplasm, and ends up with the exocytosis at the basolateral membrane.

One possible reason for a more efficient transport of native β-lg could be the size difference. Heat denaturation aggregates β-lg molecules, and they can achieve a size of 50 nm in diameter (*29*) compared to 1.75 nm for the monomer (*1*). Time course studies have demonstrated that the transport of polystyrene beads and high molecular weight dextrans at physiological temperature across the M-cell monolayer is size dependent and more rapid that than across the Caco-2 cell monolayer (*20*). This possible size difference between native and heat-denatured β-lg might partly explain the higher transported amounts of native β-lg-F compared to the amounts of heat-denatured β-lg-F; it can also explain the more effective transport in M cells compared to Caco-2 cells.

We wanted to see whether heat denaturation has any impact on the degradation of β-lg during transport in the two cell types. Our data suggest that more intact molecules of native β-lg were transported compared to those of heat-denatured β-lg. This was evident in both Caco-2 and M cells (**Figure 3A**,**B**, lanes 3, versus **Figure 4A**,**B**, lanes 3). Comparison of fluorescence intensities before and after trypsination provides some indirect information on degradation of β-lg, because trypsin would increase fluorescence by releasing the quenched fluorescence in case transported molecules are intact, whereas no increase in fluorescence should occur if the transported β-lg is fully degraded to peptides (*25*). Trypsination did not cause any increase of fluorescence in transported native or heat-denatured β-lg, suggesting that the majority of transported β-lg-F was degraded during the transport. However, our data suggest a qualitative difference in the degradation pattern as seen in the SDS-PAGE gels, where transported native β-lg-F was detected but not heat-denatured β-lg-F (**Figure 3A**,**B**, lanes 3, versus **Figure 4A**,**B**, lanes 3). A significant proportion of the native β-lg-F seems to be transported in intact form, whereas heat-denatured β-lg-F was degraded. This finding suggests that native β-lg is more resistant to degradation during transport than heat-denatured β-lg. To get a more complete picture of degradation, a peptide analysis of the degradation products would be necessary.

Our findings that the transported amount of β-lg is higher in M cells than in Caco-2 cells is largely consistent with the known properties of authentic M cells and the observations made with a M-cell model (*20*). It has been shown earlier that in enterocytes the majority of transported proteins are degraded in lysosomes, whereas transepithelial vesicular transport without degradation is the dominant pathway in M cells (*14*). The presence of the effective lysosomal system in M cells is a matter of controversy (*15*, *16*). M cells surpass Caco-2 cells in the transport of microbes and their fragments (*31*) and also in the transport of inert particles, such as polystyrene beads and high molecular weight dextrans (*20*). M cells in the in vitro model, unlike true M cells, may have some functional differences from authentic M cells (*32*). It should also be noted that in this model there are other cell types including enterocytes that do not transform to M cells as well as mononuclear phagocytes from the Peyer's patches, all capable of contributing to the degradation of antigens (*33*).

Does the difference in transported amounts of native and heat-denatured β-lg have any physiological significance? One starting point of this study was our observation that the immune and mucosal responses in rats are different depending on the type of β-lg used in immunization and exposition (*9*). Our previous results showed that rats treated with native β-lg develop a more intensive rise of total IgE levels than those treated with heat-denatured β-lg, whereas the latter develop a chronic inflammatory type of response in the gastroduodenal mucosa (*9*).

The observed difference in the epithelial transport might be one factor involved in the genesis of different immunological host responses to native and heat-denatured β-lg. It has been established that the dosage of an orally given antigen is important in the genesis of tolerance, although several other factors, such as the presence of additional antigens, adjuvant, and age, are also important. It is assumed that the induction of tolerance requires that the whole antigen molecules are absorbed (*34*), suggesting that the degradation rate during transport through enterocytes and overall transport rates through M cells providing intact antigen molecules are important. In our model, passage of native β-lg molecules was more abundant than that of heat-denatured β-lg, and in M cells native β-lg was more efficiently transported than in Caco-2 cells. In addition, there was some evidence for a less effective degradation of native β-lg compared to that of heat-denatured β-lg-F in both cell types. However, a more detailed analysis of the properties of the transported proteins is necessary before we can conclude if the change in the intestinal transport is an important immunological consequence of the heat denaturation of β-lg.

In conclusion, for the first time this study compares the in vitro transport of an important nutritional protein, bovine β-lg, in enterocytes and in M cells and investigates the significance of heat denaturation of bovine milk β-lg on the transport. The results show that the native β-lg is more abundantly transported in both studied epithelial cell types and is also degraded less during the transport. On this basis our M-cell model is a valuable tool for transport studies of food proteins and allergens, but more studies are needed to elucidate the role of nonepithelial cells in this model in the processing of transported antigens.

ABBREVIATIONS USED

β-Lg, β-lactoglobulin; β-lg-F, β-lactoglobulin labeled with bodipy-FL; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbeccos's modified Eagle medium; EVOM, epithelial voltage meter; O-MALT, organized mucosal associated lymphoid tissue; PBS, phosphate-buffered saline; TEER, transepithelial electrical resistance.

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