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Biochimica et Biophysica Acta 1778 (2008) 342 – 347
Biochimica et Biophysica Acta
BBA
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Lipocalin-interacting-membrane-receptor (LIMR) mediates cellular internalization of β-lactoglobulin

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Received 17 August 2007; received in revised form 4 October 2007; accepted 9 October 2007
Available online 18 October 2007

Abstract

β-Lactoglobulin (BLG) is a member of the lipocalin protein family and a major food-borne allergen in humans. Numerous *in vitro* studies have suggested a role for BLG in molecular transport processes; however, its physiological role remains enigmatic. A cellular receptor for BLG has been proposed, but has not yet been identified. Here we show that human LIMR, known to act as an endocytic receptor for lipocalin-1, also binds bovine BLG and mediates its cellular uptake. The specificity of this interaction is corroborated by a complete block of cellular uptake of BLG in the presence of LIMR antibodies or LIMR downregulation by antisense RNA. Furthermore, heterologous expression of human LIMR in insect cells mediates cellular internalization of FITC-BLG. Since LIMR is highly expressed in the human intestine, it might also function in the uptake of food-borne BLG.
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Keywords: β-Lactoglobulin; Endocytosis; Receptor; Lipocalin; LIMR

1. Introduction

β-Lactoglobulin is a core member of the lipocalin family. It is abundant in the milk of ruminants, and is present in many other mammals, except primates [1–3]. BLG binds a variety of hydrophobic molecules *in vitro*, such as fatty acids [4–8], retinol and retinol derivatives [6,9], cholesterol [10], porphyrin [9], bilirubin [11], alkaloids like piperine and aromatic compounds like benzo(α)pyrene [12,13]. Furthermore, bovine BLG is one of the three most common allergens in cow's milk [14]. However, no definite physiological function could be assigned to BLG so far. The currently most favoured hypothesis attributes to it a role in molecular transport [1,3]. Several attempts have been made to identify cellular receptors of BLG. Binding of BLG to specific cell surface receptors in the intestine of neonate calves has been described [15], and also to bovine germ cell plasma membrane fractions [16]. Transcytosis of intact BLG across rabbit ileum in tissue chambers and across CaCo-2 cells has been reported [17,18]. However, the identity of the receptor(s) has remained unclear.

To date, four receptors for lipocalins have been identified. Megalin, a member of the low-density lipoprotein receptor family, is known to bind a large variety of mostly hydrophobic ligands [19]. Among them there are several lipocalins, including mouse retinol-binding protein, mouse urinary protein (MUP), mouse odorant-binding protein, apolipoprotein M and human Lcn-2 [20–23]. Novel cell surface receptors for mouse Lcn-2 (24p3R) and retinol-binding protein have been reported recently [24,25]. In a previous study, we described a novel receptor for Lcn-1, the lipocalin-interacting-membrane-receptor (LIMR), which is present in a wide variety of organisms and is highly conserved in mammals [26,27].

BLG has significant amino acid sequence homology and structural similarity to Lcn-1 and binds some ligands, which are known to be *in vivo* ligands of Lcn-1 [28,29]. Therefore, in the

Abbreviations: BLG, β-lactoglobulin; b, bovine; Lcn-1, lipocalin-1 (also called tear lipocalin, or von Ebner's gland protein); Lcn-2, lipocalin-2 (also called Ngal); LIMR, lipocalin-interacting-membrane-receptor; FITC, fluorescein-isothiocyanate

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doi:10.1016/j.bbamem.2007.10.010

present study we investigated whether human LIMR is able to interact with BLG and to function as an endocytic receptor for BLG.

2. Materials and methods

2.1. Purification of bBLG and FITC labeling of bBLG

bBLG was purified from milk of cows, homo-allelic for BLG, by a procedure described previously [30]. FITC labeling of bBLG was done by incubating purified bBLG with a 20-fold molar excess of FITC in 200 mM sodium bicarbonate buffer, pH 9.0 for 2 h at room temperature. The reaction was stopped by addition of NH_4Cl to 50 mM and incubation for 1 h. Unconjugated FITC was removed by gel-filtration on PD-10 columns (Amersham Biosciences).

2.2. In vitro binding assays

For a binding assay in solution, a 6x His-tagged N-terminal fragment (aa 1-59) of LIMR (10–20 μg) bound to Ni-NTA beads (50–100 μl) was incubated with 2 μg of purified bBLG in 1 ml of 50 mM NaH_2PO_4/300 mM NaCl (pH 8.0) at 4 °C for 2 h. The beads were then washed twice with 50 mM NaH_2PO_4/300 mM NaCl (pH 8.0) buffer containing 1% Triton X-100. The His-tagged LIMR fragment and any associated proteins were eluted with 250 mM imidazole; 20 μl of each eluate was subjected to SDS-PAGE with subsequent Coomassie blue staining or immunoblotting using a bBLG-specific antiserum (ICL, Inc).

2.3. Cellular uptake studies

NT2 cells were grown on coverslips for 18 h at 37 °C in complete medium (Minimal Essential Medium) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin). SF9 cells were grown on coverslips for 18 h at 27 °C in Grace's medium (Invitrogen). Cells were washed once with serum-free medium and incubated for 3 h in serum-free medium at 37 °C (NT2 cells) or 27 °C (SF9 cells). FITC-bBLG was added (final concentration, 50 μg/ml each) followed by incubation for 1 h at 37 °C or 27 °C, respectively. To remove cell surface-bound ligands that had not been internalized, cells were washed twice with phosphate buffered saline (PBS) and treated with 50 mM glycine, 150 mM NaCl, pH 3.0, for 2 min. Cells were washed again with PBS and fixed in 100% ice-cold acetone for 5 min.

2.4. Fluorescence microscopy and image analysis

Analysis of FITC-bBLG uptake was performed using a Carl Zeiss Axioplan fluorescence microscope or by laser scanning confocal microscope. When employing the latter, cells were imaged with an inverse IX70 Olympus microscope mounted to the Ultra*VIEW* RS 3-line confocal System (Perkin Elmer) equipped with a three-line Argon-Krypton laser (488/568/647 nm) and a ×100/×40 PlanApo (numerical aperture 1.4 oil) objective (Olympus). Image acquisition and processing was carried out with Ultra*VIEW*™software (Perkin Elmer). Confocal images captured as a stack of images at different focal points (at 0.2 μm steps) were processed with Volocity™ 3D/4D visualisation software (Improvision Inc.).

2.5. Antisense down regulation of LIMR expression in NT2 cells

Vector construction of a LIMR antisense plasmid and transfection of NT2 cells was done essentially as described previously [27].

2.6. Construction of a LIMR expression vector and transfection of SF9 cells

To construct a vector for expression of LIMR in SF9 cells, a PCR fragment was amplified from a plasmid [26] using upstream primer: 5′-TCC CCC GGG ATG GAA GCA CCT GAC TAC-3′ and downstream primer: 5′-GCT CTA GAT CAC TGG TGC TGG GTC TT-3′. The PCR product was gel-purified, digested with *Sma*I and *Xba*I and ligated with the *Sma*I/*Xba*I digested vector pOPRSVI/MCS (Stratagene). Transfection of SF9 cells was done using Cellfectin Reagent (Invitrogen) according to the manufacturer's recommendations.

3. Results

3.1. bBLG binds to the N-terminal domain of LIMR

In a previous study we showed that the N-terminal extracellular domain of LIMR is essential for its interaction with Lcn-1 [26]. To examine if this domain of LIMR is able to interact with BLG, we have incubated purified bBLG with a His-tagged recombinant N-terminal fragment (aa 1–59) of LIMR immobilized to Ni-NTA beads. Potential complexes bound to Ni-NTA-agarose beads were eluted and analyzed by

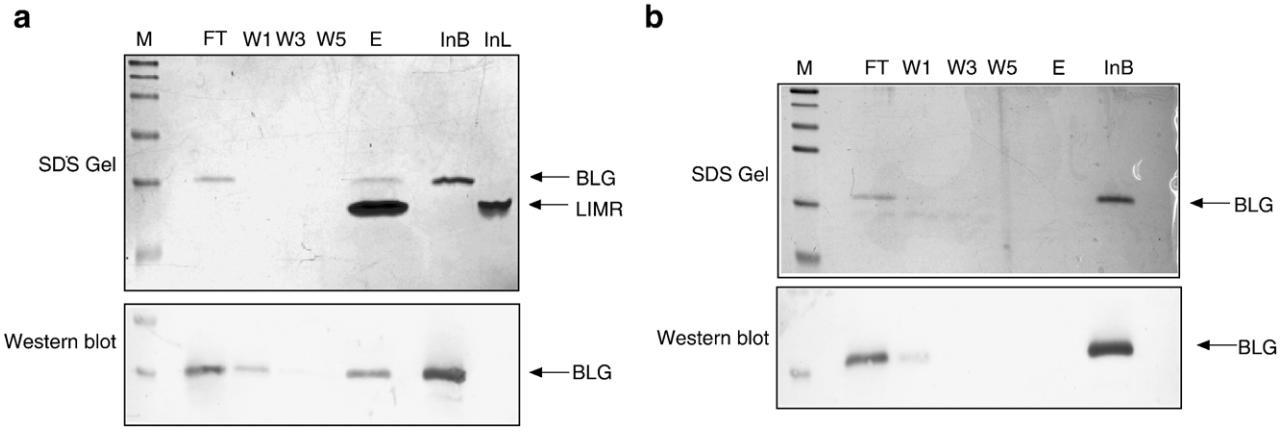

Fig. 1. Interaction of LIMR with bBLG *in vitro*. (a) A 6x His tagged N-terminal LIMR fragment (aa 1–59) bound to Ni-NTA beads was incubated with bBLG in solution. Proteins were analyzed by 18% SDS-PAGE and Coomassie blue staining, and Western blotting using bBLG antibodies. Lane M, marker; lane FT, flow-through of the Ni-NTA column; lanes W1, W3 and W5, first, third and fifth wash with 1% Triton X-100/phospate buffer (pH 8.0); lane E, elution of bound proteins by 250 mM imidazole; lane InB, input of purified bBLG; lane InL, input of recombinant His-tagged N-terminal LIMR fragment. (b) For a control Ni-NTA beads without LIMR were treated as above and analyzed by 18% SDS-PAGE and Coomassie blue staining, and Western blotting (b). Lane M, marker; lane FT, flow-through of the Ni-NTA column; lanes W1, W3 and W5, first, third and fifth wash with 1% Triton X-100/phosphate buffer (pH 8.0); lane E, eluate; lane InB, input of purified bBLG.

SDS-PAGE and immunoblotting using a bBLG-specific antiserum (Fig. 1a). When bBLG was incubated with LIMR-coated beads, it was retained on the beads even after several washings and was eluted by 250 mM imidazole (Fig. 1a, lane E). In contrast, no binding of bBLG was observed with Ni-NTA beads lacking LIMR (Fig. 1b, lane e), indicating that bBLG physically interacts with the N-terminal fragment of LIMR.

3.2. Cellular internalization of BLG by LIMR in human NT2 cells

In a previous study, we found that human NT2 cells express LIMR [26]. Therefore, NT2 cells were incubated with FITC-labeled bBLG and examined by fluorescence microscopy to study the cellular uptake of BLG. Endocytic uptake of FITC-

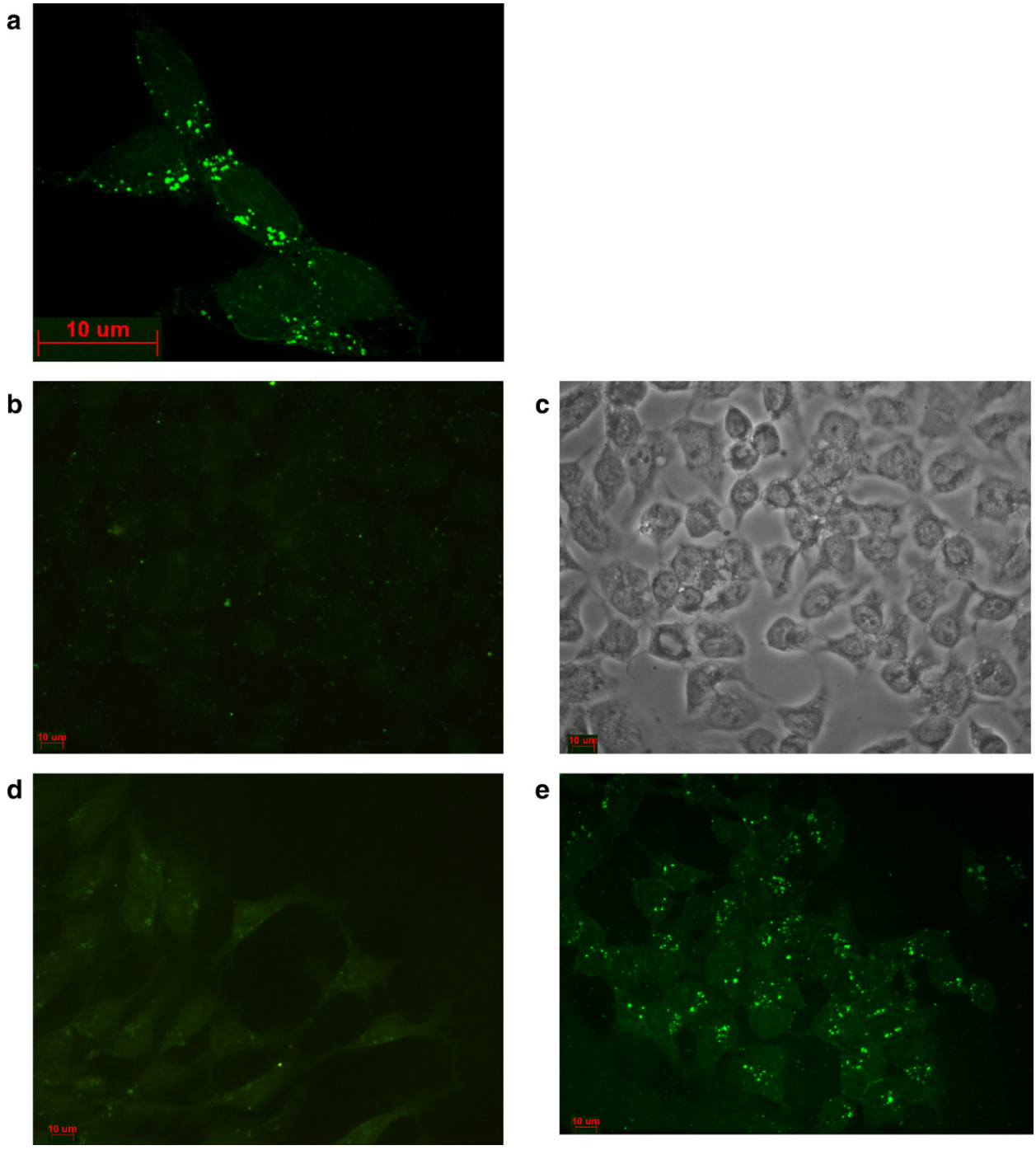

Fig. 2. Analysis of LIMR-mediated cellular internalization of FITC-bBLG in NT2 cells. Confocal fluorescence microscopy of NT2 cells incubated with FITC-bBLG (a). Uptake studies in the presence of anti-LIMR antibodies: fluorescence microscopy (b), and corresponding phase contrast image (c). Uptake studies with NT2 cells expressing LIMR-antisense RNA (d), and mock-transfected cells (e).

bBLG is evident from the appearance of a bright granular intracellular staining detected by confocal fluorescent microscopy (Fig. 2a).

Blocking experiments, using polyclonal antibodies against the N-terminal fragment of LIMR as well as knock-down of LIMR by the expression of antisense RNA clearly demonstrated that LIMR is responsible for the observed internalization of bBLG. Pretreatment of the NT2 cells with anti-LIMR antibodies completely blocked cellular uptake of bBLG (Fig. 2b). Also, a drastic decrease in intracellular fluorescence intensity was observed in the NT2 cells expressing antisense LIMR RNA (Fig 2d). In contrast, the mock-transfected cells showed a similar cytoplasmatic fluorescence staining as the wild type NT2 cells (Fig. 2e).

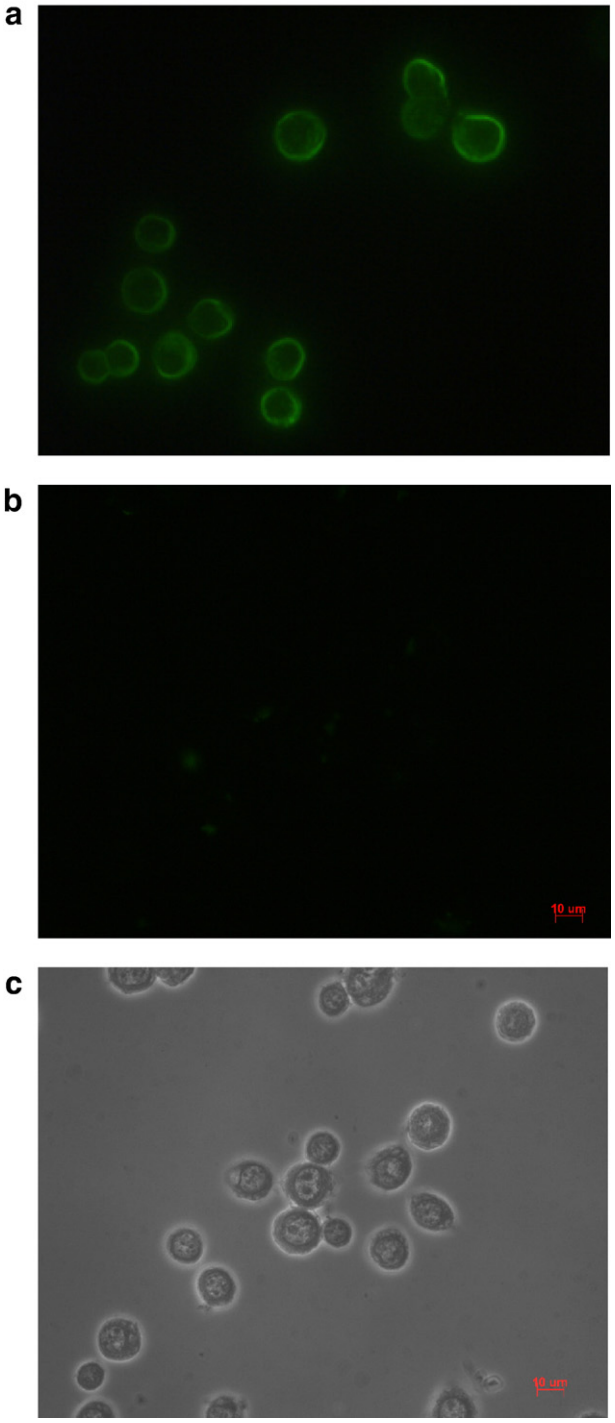

Fig. 3. Expression of LIMR in insect SF9 cells. Fluorescence microscopy of SF9 cells transfected with a LIMR cDNA expressing vector stained with anti-LIMR antibodies (a). No LIMR specific staining was obtained with mock-transfected SF9 cells (b). Phase contrast image of mock-transfected cells (c).

3.3. Cellular internalization of BLG by heterologous expression of LIMR in insect SF9 cells

In order to confirm the results obtained with human NT2 cells, we expressed human LIMR in a heterologous insect cell system (SF9 cells from *Spodoptera frugiperda*). Immunofluorescence analysis of the transfected cells using LIMR-specific antibodies revealed high levels of LIMR expression (Fig. 3), with the more intense staining of the cell membrane indicating correct cellular targeting of the recombinant receptor (Fig. 3a). No significant staining was obtained with mock-transfected SF9 cells (Fig. 3b). Next, we incubated the LIMR-transfected SF9 cells and control cells with FITC-bBLG. The LIMR-expressing SF9 cells produced a bright intracellular fluorescence (Fig. 4a), and confocal fluorescence microscopy revealed in LIMR-expressing SF9 cells a granular cytoplasmic staining very similar to that obtained with the NT2 cells (Fig. 4b). In contrast, no accumulation of fluorescence was detected in the mock-transfected cells (Fig. 4c).

4. Discussion

Delivery of their hydrophobic ligands through binding to specific cell surface receptors seems to be a general feature of most lipocalins [31–33]. Several types of receptors have been suggested to mediate ligand transfer through the cell membrane [32]. These include pore-like receptors, internalization of the entire ligand–lipocalin complex by endocytic receptors, or a mechanism where the ligand is released from the lipocalin outside the cell and is actively transported by the receptor to a cytosolic binding protein. However, with the exception of the transport of retinol by retinol-binding protein, which was shown to be delivered by this latter type of receptor [25,34,35], all lipocalin receptors identified so far act as endocytic receptors [23,24,27]. Our experiments provide clear evidence in support of the concept that BLG also is internalized by an endocytic mechanism mediated by the Lcn-1 receptor LIMR. This result is not entirely unexpected, since Lcn-1 and BLG share high structural similarity and very similar ligand binding activities [36,37]. In addition, LIMR has been found to mediate cellular uptake of uteroglobin [38], which shares several features with lipocalins. Our findings are in agreement with earlier observations on the transport of BLG across the rabbit ileum, indicating that BLG is efficiently absorbed by the intestinal mucosa in its intact antigenic form and demonstrating that this transport is significantly reduced by the microtubule assembly inhibitor colchicine [17].

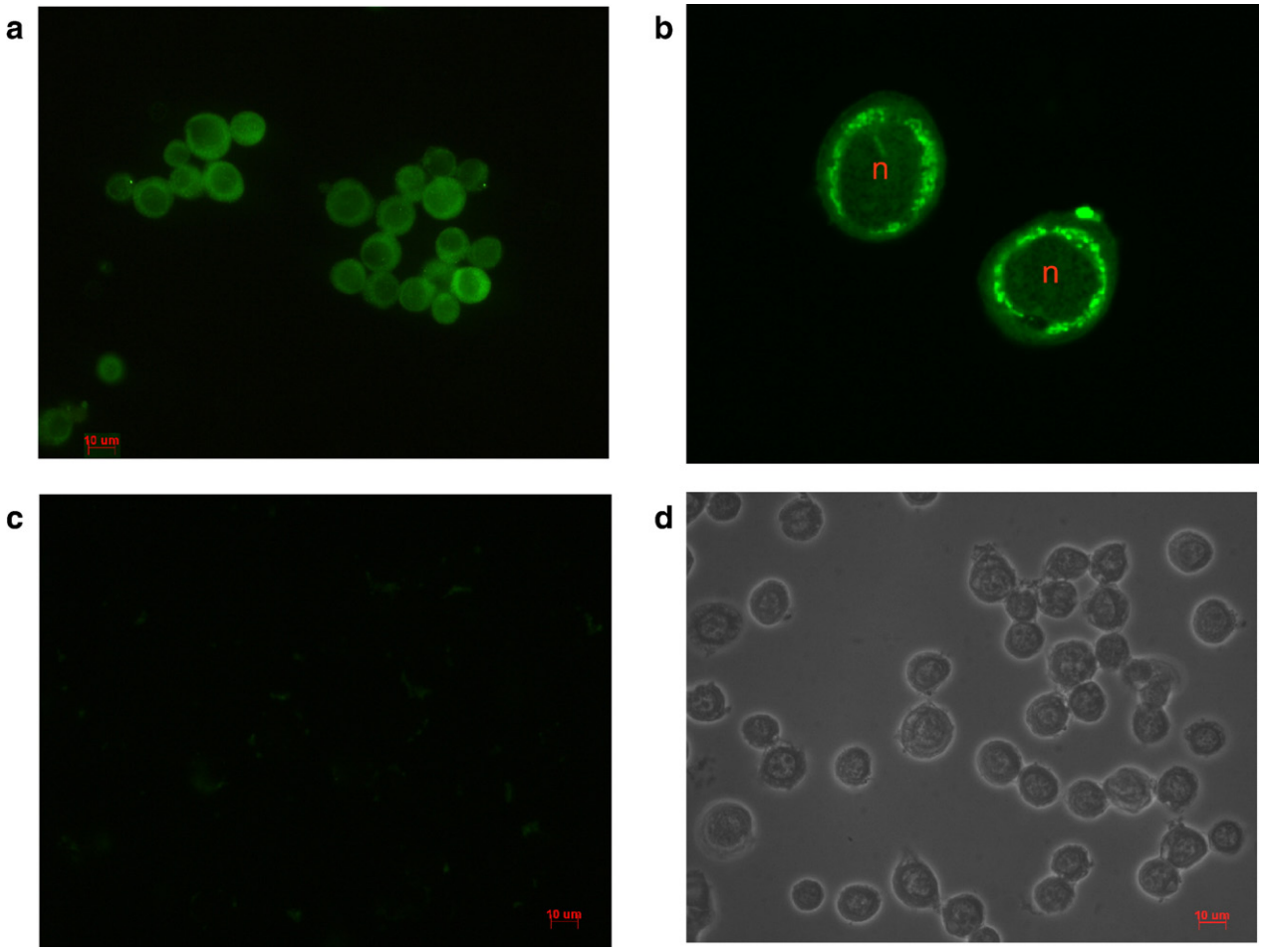

Fig. 4. Uptake of FITC-bBLG in LIMR expressing SF9 cells. Fluorescence microscopy (a) and confocal laser microscopy (b) of LIMR expressing SF9 cells incubated with FITC-bBLG; n, nucleus. Mock-transfected cells incubated with FITC-bBLG (c). Phase-contrast image of mock-transfected cells (d).

We used human LIMR for our studies, since the bovine LIMR has not yet been isolated. Nevertheless, a partial cDNA clone (BC111204.1) was isolated from *Bos taurus*, which contains an open reading frame showing 90% sequence identity with the sequence of human LIMR suggesting that a LIMR-like protein is expressed in the cow.

Since the physiological ligand of BLG is still not known, we cannot draw any conclusion on the biological function of the LIMR mediated BLG uptake in BLG producing animals. Cellular internalization of BLG by LIMR might be a mechanism for scavenging of potentially harmful ligands, leading to their detoxification and degradation, as supposed for Lcn-1 [39]. On the other hand, LIMR-mediated internalization of BLG might lead to more complex reactions, as described with uteroglobin. LIMR dependent cellular uptake of uteroglobin has been found to trigger anti-inflammatory and anti-chemotactic properties [38,40].

Humans do not possess a BLG gene. Nevertheless, we suggest that LIMR might play an important role in the uptake of BLG from food sources, because it is highly expressed in tissues of the human gastrointestinal tract [26]. In this process, LIMR might act in two opposing ways. It might trigger immunoreactions associated with BLG as a food-borne allergen that is responsible for milk protein immunoreactivity and intolerance [41]. Alternatively, it might help to internalize BLG for degradation, thus preventing allergic response.

Acknowledgements

This study was supported by the Austrian Science foundation (FWF) grant P17552-B11. The authors wish to thank Alexandra Lusser for technical comments and Rajam Csordas for critical reading and correction of the manuscript.

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