A role of heparan sulphate proteoglycan in the cellular uptake of lipocalins β-lactoglobulin and allergen Fel d 4

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Abstract: Lipocalins, small extracellular hydrophobic molecule carriers, can be internalized by a variety of different cells. However, to date receptors have only been identified for human lipocalins. Here, we specifically investigated uptake mechanisms for lipocalins β-lactoglobulin and Fel d 4 in HeLa and Chinese hamster ovary (CHO) cells. We provide evidence that cell surface heparan sulphate proteoglycan is essential for internalization of these lipocalins. In HeLa cells, lipocalin uptake was inhibited by competition with soluble heparin, enzymatic digestion of cellular heparan sulphate by heparinase and inhibition of its biosynthesis by sodium chlorate. Biochemical studies by heparin affinity chromatography and colocalization studies further supported a role of heparan sulphate proteoglycan in lipocalin uptake. Finally, lipocalin uptake was blocked in CHO mutant cells defective in glycosaminoglycan biosynthesis whereas in wild-type cells it was clearly detectable. Thus, cell surface heparan sulphate proteoglycan represents a novel component absolutely participating in the cellular uptake of some lipocalins.

Keywords: cellular uptake; heparan sulphate; heparinase treatment; lipocalin; proteoglycan.

Introduction

Lipocalins were found to be important carriers of hydrophobic molecules in many species and there is clear evidence that they are involved in a variety of physiological processes such as inflammation, infection, immunity, development, iron metabolism, lipid metabolism and chemosensation (Akerstrom et al. 2006; Brennan and Kendrick 2006; Chakraborty et al. 2012; Conde et al. 2011; Correnti and Strong 2012; Johnson and Wessling-Resnick 2012; Zhao et al. 2012; Kim et al. 2009; Koistinen et al. 2009; Pan et al. 2011; Ruiz et al. 2013; Chamero et al. 2007). They also play a role in reactions of organisms to various stress conditions and in the pathways of signal transduction (Charron et al. 2008; Ganfornina et al. 2008; Lechner et al. 2001; Mulligan et al. 2012; Olsson et al. 2008). However, there is little knowledge regarding the molecular mechanisms by which lipocalins exert their biological effects. Although it is well accepted that lipocalins can bind to specific cellular receptors (Flower 2000), so far only a few human receptors have been identified, which may be functionally grouped into three classes (Burke et al. 2006). Most receptors seem to function in an endocytic manner, mediating uptake of the entire lipocalin-ligand complex. These include megalin, lipocalin-1-interacting membrane receptor (LIMR/LMBR1L), the Lcn-2 receptor NGalR/24p3R/SLC22a17 and basigin (Devireddy et al. 2005; Leheste et al. 1999; Najyb et al. 2015; Wojnar et al. 2001). While megalin and NGalR display promiscuous ligand binding (Christensen and Birn 2002; Langelueddecke et al. 2012), the others possess specific lipocalin binding activity. Another type of lipocalin receptor is STRA6 (Kawaguchi et al. 2007), a receptor for retinol-binding 24p3 protein (RBP). The RBP- STRA6 system represents a small molecule delivery mechanism that involves an extracellular carrier protein but does not depend on endocytosis (Redondo et al. 2008). A third group is typified by CD45, a major protein tyrosine phosphatase receptor, which has been described to bind glycodelin/PP14, an immunoregulatory lipocalin (Rachmilewitz et al. 2003). Thus, the role of these receptors will likely vary according to the physiological function of their lipocalin ligand. In a preliminary study, we found that two animal lipocalins, bovine β-lactoglobulin (BLG) (Kontopidis et al. 2004; Sawyer and Kontopidis 2000) and cat Fel d 4 (Papes et al. 2010; Smith et al. 2004), were internalized by a variety of cells, including several human cell lines. However, to date almost no knowledge on the uptake of exogenous lipocalins exists despite humans being exposed to these proteins daily.
Both lipocalins are relevant human allergens. In addition, BLG was demonstrated to be a potent stimulator of proliferation (Tai et al. 2016) and is used as a nanocarrier for delivering pharmacological agents (Bijari et al. 2019; Shafaei et al. 2017). Therefore, in this work we aimed to identify cell surface components responsible for the cellular uptake of these lipocalins. Here, we present clear evidence that cell-surface heparan sulphate proteoglycan (HSPG) is essential for their internalization and, therefore, represents a novel component involved in lipocalin uptake.

Results

Membrane crosslinking studies indicate close neighbourhood of lipocalins and HSPG binding proteins

Previous studies demonstrated that exogenous animal lipocalins can be internalized by different human cell lines. An example showing uptake and colocalization of bovine BLG and feline Fel d 4 in HeLa cells and CHO cells is depicted in Figure 1. The near perfect colocalization of both proteins in both cell lines, together with the fact that Fel d 4 internalization could be blocked by an excess of BLG (see Supplementary Figure 1), indicated a common uptake mechanism. Therefore, in a first attempt we sought to isolate the corresponding receptor by a crosslinking approach using biotinylated lipocalins which we crosslinked to HeLa cell membranes using SDAD photochemical crosslinker. Then, membranes were solubilized and lipocalin bait-prey complexes were isolated by affinity pull-down and analysed by SDS-PAGE. A silver-stained gel displaying proteins captured by crosslinking lipocalin baits to HeLa membranes is shown in Figure 2. A distinct pattern of protein bands ranging from about 30 to 100 kDa is visible irrespective of lipocalin bait used, although band intensities were weaker for Fel d 4. The most prominent protein bands were excised and identified by mass spectrometry (Table 1). Several heat shock proteins (HSP90, BIP, mtHSP70 and HSP60) and Calnexin, which usually locate to the endoplasmic reticulum, mitochondria and cytosol, were found as well as the cytoskeletal proteins Actin, Vimentin and Emerin. This result was unexpected, but we noticed that a very similar pattern of proteins was identified in an attempt to cross-link lipocalin-type prostaglandin D synthase (PGDS) to NIH3T3 cells (Suk 2012). Later, PGDS was indeed demonstrated to directly interact with cellular and recombinant HSP90 (Binda et al. 2014). Moreover, there is increasing evidence that HSPs can traffic to the cell surface and reinternalize later (Crowe et al. 2017; Okazaki et al. 2000), a mechanism which might be relevant for cellular uptake of PGDS and other lipocalins. To investigate whether BLG and Fel d 4 also directly interact with HSP90, we recombinantly expressed it from E. coli and performed co-affinity precipitation experiments. However, we observed no

Figure 1: Uptake and colocalization of BLG (a, e) and Fel d 4 (b, f) in HeLa and CHO cells, respectively. Cells were simultaneously incubated with 25 µg/mL Carboxyfluoroscein-BLG and 25 µg/mL Hilyte Fluor 594-Fel d 4 in serum-free medium for 2 hours, then fixed in paraformaldehyde and counterstained for nuclei with DAPI (c, g). Merged images (d, h). Scale bar is 10 µm.
direct interaction of HSP90 with BLG or Fel d 4 (see Supplementary Figure 2).

Thus, we speculated that crosslinking of lipocalin baits to cell-surface HSP90 might result from indirect interaction. Heat shock proteins (HSPs) usually locate to the endoplasmic reticulum, mitochondria and cytosol, but have also been found on the cell surface (Bzowska et al. 2017; Gonzalez-Gronow et al. 2009; Soltys and Gupta 1996). These cell-surface exposed HSPs may be bound to heparan sulphate, which has been demonstrated for HSP90 (Snigireva et al. 2015), as they often possess heparin binding activity (Harada et al. 2014; Itoh and Tashima 1993; Ménoret and Bell 2000). This assumption was further supported by the high abundance of Calnexin, an endoplasmic reticulum chaperone and lectin, that has been found to associate with cell surface glycoproteins in HeLa cells (Okazaki et al. 2000). In sum, these indications encouraged us to investigate a potential interaction of lipocalins and heparan sulphate proteoglycan (HSPG).

Lipocalins BLG and Fel d 4 bind to heparin

We first tested whether lipocalins potentially possess heparin or heparan sulphate binding affinity at physiological pH by performing heparin affinity chromatography. Despite net negative charge both BLG and Fel d 4 showed specific retention on a heparin sepharose column (Figure 3) indicating the presence of a heparin-binding motif. In contrast, human Lcn-1, which is similar in molecular weight, structure, isoelectric point and number of basic amino acid residues, did not bind. This observation suggested that HSPG may play a role in binding and internalization of lipocalins.

Lipocalin uptake requires cell surface HSPG in HeLa cells

To verify the above hypothesis, we investigated the effects of different reagents impairing the structure of cellular HSPGs on the internalization of lipocalins. In the first approach, we tested the influence of soluble heparin, a polysaccharide closely related to heparan sulphate, on the endocytosis of BLG (Figure 4). For comparison we used lipocalin-1 (Lcn-1), which is internalized via its specific receptor LIMR (Wojnar et al. 2001). Cellular uptake of BLG...
and Lcn-1 was investigated in untreated HeLa cells or cells pre-treated with heparin prior to the addition of fluorescently labelled lipocalins. Untreated control cells display a circular, diffuse Lcn-1 fluorescence surrounding the nucleus while a more distinct, punctate, non-overlapping BLG fluorescence pattern indicates a different endocytosis route (Figure 4A). Following treatment with heparin, the Lcn-1 signal remains unchanged, which was also evident from image quantification analysis (Figure 4B). However, the vesicular BLG fluorescence is almost completely lost. A similar effect of heparin treatment was also observed on the endocytosis of Fel d 4 (Figure 5). Overall, image quantification revealed a reduction in both BLG and Fel d 4 uptake of about 75% after heparin treatment (Figure 8). 

Next, we treated HeLa cells with different concentrations of heparinase (0.5, 2.0 and 10.0 U/mL) for 30 min prior to the addition of labelled lipocalins. Again, uptake of BLG (Figure 6A) and Fel d 4 (Figure 6B) was markedly reduced. Similar to the results obtained from heparin blocking experiments described above, the amount and intensity of BLG and Fel d 4 fluorescent vesicles was reduced by about 70% in a concentration-dependent manner in comparison to untreated control cells (Figure 8).

Third, we pre-incubated HeLa cells overnight with sodium chlorate, an inhibitor of sulphate transfer onto glycosaminoglycans (GAGs), and then performed uptake experiments. This treatment also resulted in severe reduction of BLG and Fel d 4 uptake (Figures 7A,B). Image quantification demonstrated that the intracellular fluorescence was diminished by about 80% for both proteins (Figure 8).

In sum, these results clearly demonstrated that BLG and Fel d 4 internalization in HeLa cells depends on binding to cell surface heparan sulphate proteoglycan, which is, however, not involved in Lcn-1 uptake.

**Lipocalin uptake is inhibited in glycosaminoglycan-deficient CHO cells**

Furthermore, we investigated the endocytosis of BLG and Fel d 4 in wild-type and glycosaminoglycan-deficient deletion mutant (pgsA-745) CHO cells (Figure 9). These mutant cells have a defect in xylosyltransferase,
the first sugar transfer in GAG synthesis, and do not produce GAG. Wild-type cells display a clear accumulation of fluorescence signal in a perinuclear compartment after 1 hour of incubation with either protein. In contrast, GAG-deletion mutant cells hardly contain any fluorescence signal. This observation again suggests a vital role of GAGs in the endocytosis of BLG and Fel d 4 lipocalins.

HSPG and BLG colocalize on the plasma membrane of HeLa cells

Finally, we were able to detect colocalization of BLG and heparan sulphate on the plasma membrane of HeLa cells by immunostaining (Figure 10). Cell surface HSPG stained in a punctate pattern, which displayed a high degree of colocalization with BLG fluorescence. Additionally,

![Figure 5: Effect of heparin treatment on the internalization of Fel d 4 (red) in Hela cells. (a–c) untreated control cells. (d–f) cells treated with 100 µg/mL heparin. Scale bar represents 10 µm.](image)

![Figure 6: (A) Effect of heparinase treatment on the internalization of BLG in Hela cells. (a–c) untreated control cells. Cells pre-treated with 0.5 (d–f), 2.0 (g–i) or 10.0 (j–l) U/mL heparinase for 30 min. Scale bar is 10 µm. (B) Effect of heparinase treatment on the internalization of Fel d 4 in Hela cells. (a–c) untreated control cells. Cells pre-treated with 0.5 (d–f), 2.0 (g–i) or 10.0 (j–l) U/mL heparinase for 30 min. Scale bar represents 10 µm.](image)
enrichment of BLG is also observed within a perinuclear compartment while the heparan sulphate antibody used in this study was not internalized but was only detectable on the cell surface. The colocalization of heparan sulphate and BLG signals on the plasma membrane further substantiates our assumption of heparan sulphate requirement for lipocalin internalization. This observation is in agreement with our blocking experiments using heparin, heparinase and sodium chlorate as well as the results obtained from the GAG-deletion mutant CHO line pgsA-745.

Discussion

By several different experiments, we have clearly demonstrated that HSPG is essential for the cellular uptake of lipocalins BLG and Fel d 4. Nevertheless, the results of our crosslinking approach require some further discussion. Although the SDAD crosslinker applied in our experimental approach should theoretically be able to link not only protein-protein complexes but any bio-macromolecules (e.g. protein-GAG), we only succeeded in isolating membrane-associated proteins, such as HSPs, Calnexin, Vimentin and Emerin. All of them have been described to locate in cholesterol-rich membrane regions or lipid rafts or to be constitutes of HSPG complexes (Harada et al. 2014; Podyma-Inoue et al. 2016; Snigireva et al. 2015). Mitochondrial HSC70 has even been assigned a crucial role in endocytic HSPG-vesicles (Wittrup et al. 2010). These proteins most likely do not interact directly with our baits but rather localize in close proximity to the actual lipocalin receptor component. Furthermore, the following reason might have hampered the isolation of lipocalin-proteoglycan complexes in our experiments. Proteoglycans consist of a core protein and one to several attached glycosaminoglycan chains resulting in a complex, heterogeneous structure which may run in poorly focused bands in SDS-PAGE. Since we have analysed only prominent protein bands, we might have failed to identify such complexes.

In general, there is increasing evidence for a role of HSPG as a cell surface endocytosis receptor (Christianson and Belting 2014). It was shown that cell penetrating peptides (Poon and Gariépy 2007), polycation-nucleic acid complexes (Mounkes et al. 1998), viruses (Zhu et al. 2011), lipoproteins, growth factors and morphogens among other ligands (Ori et al. 2008) enter cells through HSPG-mediated endocytosis. In addition, HSPG serves as a major receptor for human eosinophil cationic protein, an RNase A superfamily member, highly implicated in asthma pathology, due to its toxicity on bronchial epithelial cells (Fan et al. 2007). Similarly, the transactivator protein Tat of human HIV-1 specifically interacts with HSPG, which is absolutely required for viral particle uptake (Fittipaldi et al. 2003). The mechanism of internalization and trafficking of cell surface proteoglycans and their ligands was also investigated in detail (Payne et al. 2007). In this context, it is not fully surprising to find members of the lipocalin family to be targets of HSPGs. Nevertheless, HSPG-mediated endocytosis represents a novel mechanism for cellular lipocalin uptake, which may not only be restricted to BLG and Fel d 4. Lcn-2 has previously been shown to bind to heparin sepharose (Yang et al. 2002), while lipocalin PP14/glycodelin interacts with cell surface glycoproteins (Rachmilewitz et al. 2003). Since HSPGs are ubiquitously expressed by most mammalian cell types, such as heparin-binding lipocalins may enter numerous cells. This finding is of special interest as BLG has been recently used as a nanocarrier for delivering biological agents such as chemotherapeutics (Bijari et al. 2019; Shafaei et al. 2017), and has also been

Figure 7: (A) Effect of NaClO₃ treatment on the internalization of BLG in HeLa cells. (a–c) untreated control cells. (d–f) Cells pre-treated with 30 mM NaClO₃ overnight. Scale bar is 10 μm. (B) Effect of NaClO₃ treatment on the internalization of Fel d 4 in HeLa cells. (a–c) Untreated control cells. (d–f) Cells pre-treated with 30 mM NaClO₃ overnight. Scale bar is 10 μm.
attributed cytoproliferative effects (Tai et al. 2016). In addition, it was demonstrated that antigens with inherent heparin-binding affinity are supported more efficiently by the immune system (Léonetti et al. 2010), an effect which might shed some light on the enigmatic allergenicity of lipocalins. In addition, it should also be considered that exogenous lipocalins may shuttle potentially harmful hydrophobic ligands via this mechanism.

![Figure 8: Quantification of the effect of HSPG-disturbing treatment on the internalization of lipocalins in HeLa cells. (A) fluorescence intensities of BLG. (B) fluorescence intensities of Fel d 4. Fluorescence signals were quantified by ImageJ, normalized to control levels and depicted as mean signal intensity ± 1 SD (obtained from at least 3 cells per image). Original, uncut images, which the presented quantification is based on, are depicted in Supplementary data (Supplementary Figures 3–8).](image-url)
From our experiments, we cannot rule out the possibility that other classes of the glycosaminoglycan family (which includes heparan sulphate, chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronate) may also participate in lipocalin binding and uptake. Soluble heparin was highly efficient in blocking uptake of both BLG and Fel d 4 in HeLa cells but is probably not an exclusive inhibitor of HSPG-ligand interaction. Heparinase treatment, which is highly specific for HSPGs, was slightly less effective in uptake inhibition (about 70% inhibition compared to about 75% by heparin and about 80% by sodium chlorate). Thus, it is evident that HSPGs are the major component responsible for the lipocalin uptake. Nevertheless, a residual uptake activity, especially in case of BLG, may result from participation of other GAG classes, which are not susceptible to heparinase digestion. Such a mechanism involving both heparan sulphate and chondroitin sulphate is for instance responsible for the internalization of a DNA-binding antibody in HeLa cells (Park et al. 2017).

In addition, we also cannot rule out the possibility that additional components, such as other HSPG binding proteins are involved in lipocalin uptake. However, our results demonstrate that proteoglycans are essential for uptake of BLG and Fel d 4 in the cell lines investigated. Nevertheless, in other cells additional, HSPG-independent uptake mechanisms might exist. In a previous study, we found BLG to be internalized via LIMR, the Lcn-1 receptor (Fluckinger et al. 2008). However, it should be mentioned that this former finding was based on experiments in yeast cells overexpressing recombinant LIMR, which may be a highly artificial system. Concerning Fel d 4 it should be mentioned that the protein used in this study was a recombinant protein produced in E. coli. Fel d 4 has two potential N-glycosylation sites at aa 51 and aa 66. Glycosylation at these sites may enhance the interaction with HSPGs as is known from viral cell entry (Raman et al. 2016). In addition, no specific ligand of Fel d 4 is known at the moment. However, ligands may influence receptor binding which may also lead to use of different or additional receptors.

Materials and methods

Cell culture

HeLa, CHO-K1 and glycosaminoglycan (GAG)-deficient CHO-K1 mutant pgsA-745 cells (Esko et al. 1985) were purchased from ATCC.
All cell lines were grown in DMEM/Ham's F12 medium (Gibco, Thermo Fisher Science, Austria, Vienna) supplemented with 10% FCS, 2 mM glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (further abbreviated “growth medium”) and passaged in 2 to 3-day intervals.

**Biotin and SDAD-labelling of lipocalin baits**

Lipocalins beta-Lactoglobulin (BLG) and Fel d 4 were simultaneously labelled with Sulfo-NHS-SS-Diazirine (SDAD) photoactivatable crosslinker and Sulfo-NHS-LC-Biotin (both from Thermo Scientific, Austria, Vienna). Labelling efficiency was measured as biotin incorporation by a HABA-biotinylation-assay (Thermo Scientific) at 1-2 biotins per protein molecule. SDAD-labelling was estimated to be equally efficient due to similar molecular weight and identical reaction chemistry. Both StrepTactin-binding and crosslinking activity were successfully tested (not shown).

**HeLa membrane preparation**

HeLa cells grown to confluency were washed in PBS and incubated in serum-free growth medium for another 2 hours. Then, cells were washed in ice-cold PBS and harvested using a cell scraper. Cells were collected by centrifugation and the pellet resuspended in cold lysis buffer (250 mM sucrose, 1 mM MgCl2, 10 mM HEPES pH 7.4 supplemented with protease inhibitors). Cells were lysed mechanically by glass beads in a mixer mill (Retsch MM 400, Germany, Haan) set to 25 Hz for 3 min. Membranous compounds were pelleted by ultracentrifugation at 100 000 g at 4 °C for 1 hour. The pellet was resuspended in buffer (250 mM sucrose, 1 mM EDTA, 10 mM HEPES pH 7.4 supplemented with protease inhibitors). Cells were lysed mechanically by glass beads in a mixer mill (Retsch MM 400, Germany, Haan) set to 25 Hz for 3 min. Membranous compounds were pelleted by ultracentrifugation at 100 000 g at 4 °C for 1 hour. The pellet was resuspended in buffer (250 mM sucrose, 1 mM MgCl2, 10 mM HEPES pH 7.4), layered onto sucrose density gradient ranging from 28-43% and centrifuged in a swinging bucket rotor overnight at 100 000 g. Plasma membrane fraction was collected and protein concentration was determined by Bradford assay.

**Isolation and identification of crosslinked proteins from HeLa membranes**

Ten µg of labelled lipocalin bait were combined with 100 µg of HeLa cell membranes in 20 mM sodium phosphate pH 7.4. After 1 hour of incubation at room temperature, crosslinking was induced by UV irradiation at 365 nm for 5 min. Thereafter, the complexes were solubilized by boiling in 1% sodium dodecylsulphate (SDS) and diluted 10-fold in wash buffer (50 mM Tris pH 7.4, 500 mM urea, 500 mM NaCl, 1% Triton X-100). Lipocalin bait-prey complexes were pulled down on StrepTactin sepharose beads (IBA Lifesciences, Germany, Göttingen), extensively washed and finally eluted by reductive cleavage of SDAD’s disulphide bond with 50 mM dithiothreitol (DTT). Supernatants were mixed with 5× Laemmli sample buffer and boiled for 5 min at 95°C. These eluates were then run on a 12% polyacrylamide gel and visualized by mass spectrometry-compatible silver staining. Proteins bands were excised and identified by mass spectrometry (see supplementary information).

**Production and purification of lipocalins and HSP90**

Recombinant Lcn-1 was produced as described previously (Holzfeind and Redl 1994). A codon-optimized Fel d 4 gene was synthesized (GenScript, USA, Piscataway Township), cloned into Qiagen’s pQE70 expression plasmid, produced and purified in M15 E. coli strain according to the manufacturer’s instructions (QIAexpressionist). BLG used in this study originated from bovine milk (L7880, Sigma-Aldrich, Austria, Vienna) and was further purified by gel filtration. The gene encoding HSP90 was amplified from HeLa cDNA (attaching an N-terminal Strep-tag via PCR), cloned into the pET21d+ expression plasmid and expressed in Rosetta(DE3) cells.

**Lipocalin-HSP90 co-affinity precipitation experiments**

Recombinant Strep-tagged HSP90 was directly bound to magnetic StrepTactin beads (MagStrep “type 3” XT beads, IBA Lifesciences) from clarified E. coli lysate. The HSP90-loaded beads were washed extensively in PBS before BLG or Fel d 4 were added at a final concentration of 25 µg/mL in PBS buffer. The reactions were incubated for 1 hour at room temperature to allow binding of lipocalins to HSP90. Afterwards, beads were washed in PBS thrice before bound HSP90 was eluted in 50 mM D-biotin in TBS (100 mM Tris pH 8.0, 150 mM NaCl). Samples collected from supernatant post-incubation, final washing step and eluate were analysed by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-Page). Two control experiments were done applying each protein (HSP90 or lipocalin) alone to StrepTactin beads.

**Heparin affinity chromatography**

Lipocalins (Lcn-1, BLG and Fel d 4) in low salt buffer (buffer A: 20 mM sodium phosphate pH 7.4) were applied to a 2 mL heparin sepharose column (Heparin Sepharose 6 FastFlow, GE Healthcare, Austria, Vienna) connected to an ÄKTA prime chromatography system (Amer sham Pharmacia Biotech, UK, Little Chalfont). Analysis was performed at a flow rate of 1 mL/min. Salt concentration was measured by conductivity and protein concentration by UV absorbance at 280 nm. Samples were loaded and washed under low salt conditions (16 min at 0% buffer B: 20 mM sodium phosphate pH 7.4, 500 mM sodium chloride) and eluted by a linear salt gradient (0 to 100% B over 8 min).

**Fluorescence labelling of lipocalins**

BLG was labelled with an 8-fold molar excess of NHS-Carboxyfluroescin (Sigma) in PBS for 1 hour at room temperature. Lcn-1 and Fel d 4 were labelled with a 5-fold molar excess of Hilyte Fluor 594, SE (Anaspec, Germany, Göttingen) in PBS for 1 hour at room temperature. Then, coupling reactions were quenched and protein-dye conjugates separated from unreacted dye by diafiltration. Degree of labelling was about 1.5 and 1 dye molecules for BLG and Lcn-1/Fel d 4 labelling reactions respectively.

**Fluorescence microscopy**

One day prior to experiments, 2.5×10^7 HeLa or Chinese hamster ovary (CHO) cells were settled in 4-well chamber slides (Nunc Lab-Tek, Thermo Scientific) in 500 µL of growth medium. The next morning, cells were washed in PBS+ (PBS supplemented with 1 mM each of MgCl2 and CaCl2) incubated in serum free medium and treated as described below. Afterwards, samples were washed, fixed in 4% paraformaldehyde,
counterstained with DAPI and imaged on an Axioplan fluorescence microscope (Carl Zeiss, Germany, Jena) equipped with an AcosCam MR3 camera and filter sets 365/445, 470/540 and 565/620. All image processing and analysis was done on Axiovision (Carl Zeiss), Microsoft PowerPoint (Microsoft Corp.) and by ImageJ (image.net).

Heparin, heparinase and sodium chlorate treatment of HeLa cells

HeLa cells were treated with PBS (control), 100 µg/ml heparin (from porcine intestinal mucosa, Sigma), 0.5, 2.0 or 10.0 U/ml heparinase I/III blend (Sigma) in serum-free growth medium for 30 min before 25 µg/ml (equalling 1.25 µM) fluorescently labelled BLG or Fel d 4 were added for 2 hours.

For chlorate experiments, HeLa cells were cultured overnight in growth medium without (control) or with 30 mM NaClO3. The next morning, cells were washed with PBS and incubated in serum-free growth medium for 30 min before labelled lipocalins were added as indicated above.

CHO K1 wild-type and GAG-deficient pgsA-745 mutant cells

CHO K1 wild-type (control) and GAG-deficient pgsA-745 mutant cells were incubated with 25 µg/ml (equalling 1.25 µM) fluorescently labelled BLG or Fel d 4 in serum-free medium for 2 hours and then processed for fluorescence microscopy.

Colocalization of cell surface heparan sulphate and BLG

HeLa cells were simultaneously incubated with Carboxyfluorescein-BSA (25 µg/ml equalling 1.25 µM) and 1 µg of biotinylated heparan sulphate antibody (amsbio FS8-10E4, AMS Biotechnology Ltd, UK, Abingdon) coupled to AlexaFluor594-Streptavidin (Thermo Scientific) for 1 hour in serum-free growth medium. Afterwards, samples were washed, fixed and counterstained with DAPI. Imaging was performed as described above.

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Conflict of interest statement: The authors declare that there are no competing interests associated with the manuscript.

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