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Identification and Characterization of the Lipid Binding Property of GrlR, a Locus of Enterocyte Effacement Regulator

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Abstract

Lipocalins are a broad family of proteins identified initially in eukaryotes and more recently in gram-negative bacteria. The functions of lipocalin or lipid binding proteins are often elusive and very diverse. We have recently determined the structure of GrlR which plays a key role in the regulation of locus of enterocyte effacement (LEE) proteins. GrlR adopts a lipocalin-like fold which comprises of eight stranded β-barrel followed by an α-helix at the C-terminus. GrlR has a highly hydrophobic cavity region and could be a potential transporter of lipophilic molecules. To verify this hypothesis, we carried out structure based analysis on GrlR, determined the structure of lipid-GrlR complex and measured the binding of lipid to recombinant GrlR by isothermal titration calorimetry. In addition, we identified that phosphatidylglycerol and phosphatidylethanolamine are the endogenously bound lipid species of GrlR using electrospray ionization mass spectrometry. Further we have shown that the lipid binding property of GrlR is similar to its closest lipocalin structural homolog β-lactoglobulin. Our studies demonstrate the hitherto unknown lipid binding property of GrlR.

Keywords

Lipocalins; Lipids; TTSS; β-barrel proteins

Introduction

Enterohemorrhagic Escherichia coli (EHEC) and *enteropathogenic E. coli (EPEC)* are the two pathogenic *E. coli* that are responsible for diseases like diarrhea and hemorrhagic colitis in humans and belong to the family of attaching and effacing (A/E) pathogens. During infection, these pathogens use the type III secretion systems (T3SS) to inject effector proteins into the host cells and hijack the normal host cell function to benefit the bacteria. The components and related proteins of T3SS are encoded by 41 genes, organized in five

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major operons named locus of enterocyte effacement 1 (LEE1) through LEE5. GrlR is a regulatory protein which plays a major role in the regulation of LEE proteins through its interaction with GrlA, another key regulator. Previously we have reported the structure of GrlR [1].

Structural analysis of GrlR revealed the presence of a hydrophobic cavity in its β-barrel architecture, a feature most commonly observed in lipocalins. Lipocalins are a widespread family of proteins identified in eukaryotes and in gram-negative bacteria [2, 3]. Lipocalins are the carriers of lipophilic molecules but their functions are often elusive and very diverse [4]. The amino acid sequences of lipocalins are poorly conserved except the most general sequence signature GXW motif at the N-terminus [5, 6, 7]. However, GrlR contains a GXY motif at the N-terminus instead of the highly conserved GXW motif. Lipocalins play important role in cryptic coloration, enzymatic synthesis and pheromone transport; they have also been implicated in immune response regulation and modulation of cell homeostasis [8]. The lipocalin fold comprises of an 8 stranded β-barrel followed by an α-helix at the C-terminus [9, 6]. Blc is the first bacterial lipocalin structurally characterized from *E. coli* [10]. The most prominent structural feature of the lipocalin fold is the presence of a large cup-shaped cavity within the β-barrel and a loop scaffold at its entrance. The selectivity of lipocalin is determined by the amino acid composition of the cavity and loop scaffold, as well as its overall size and conformation [11]. GrlR shares a very high structural similarity with other well-characterized lipocalins, which prompted us to investigate the lipid binding property of GrlR.

As the continuation of our efforts to understand the structure and function of GrlR, here we report the identification and characterization of the lipid binding property of GrlR. Based on structural analysis combined with literature search, we hypothesize that lysophosphatidic acid (LPA) is likely to be the candidate substrate that interact with the hydrophobic cavity of GrlR. To verify this hypothesis, we have determined the crystal structure of the LPA-GrlR complex and performed isothermal calorimetry experiments with LPA and GrlR. Further we have studied and compared the lipid binding property of GrlR with its lipocalin structural homologs. Subsequently, we have identified the physiologically relevant lipid species for GrlR using a mass spectrometry approach. Our studies demonstrate the lipid binding property of GrlR and identify the physiologically relevant family of lipid species that are recognized by GrlR for its hitherto unknown second function.

Materials and methods

Protein purification and Crystallization

Plasmid DNA was transformed into *E. coli* BL21 and the cells were grown in LB medium at 37°C to 0.6 AU at OD_{600}. One liter of culture was induced with 100 μM IPTG and continued to grow at 20°C overnight. Cells were then harvested by centrifugation and resuspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 10 mM βmercaptoethanol and one tablet of CompleteTM protease inhibitors (Roche Diagnostics). The protein was purified in three steps, using DEAE-Sepharose (Amersham Pharmacia), NI-NTA (Qiagen) and Gel Filtration (Superdex75) columns, respectively. The His-fusion tag was not cleaved. Drops containing 1 μl GrlR lipid complex (4 mg/ml) and 1 μl reservoir solution were equilibrated by hanging drop vapor diffusion at 21°C. The best crystals were grown from 25% ethylene glycol, 4% tert-butanol and 4% trifluoroethanol (same condition as the apoprotein) with the protein in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl. Crystals measuring ~0.2 mm in length grew over the course of 3 days, belonged to space group $P2_12_12_1$ with a=43.73, b=66.02, c=83.46Å and contained two molecules in the asymmetric unit. The Matthews [12] coefficient is 2.2 $Å^3$/Da, giving a solvent content of 45%. The X-ray data collection and refinement statistics were given in Table 1.

Data collection, structure solution and refinement

Crystals were cryo-protected in the reservoir solution supplemented with 40% ethylene glycol and flash cooled at 100°K. X-ray diffraction data were collected at beam line X29A, Brookhaven National Laboratory using a Quantum-4 CCD detector (ADSC). A total of 180 images were collected at 1.1Å wavelength. All the data sets were processed with HKL2000 [13]. The structure was determined using co-crystallized protein crystals by molecular replacement method using Phaser [14] program in the CCP4 crystallographic suite [15]. Native GrlR model was used to obtain the phasing and the structure was further modeled with CNS [16]. The remaining residues of the molecules were added after several cycles of manual model building using O [17] and followed by refinement using CNS [16]. Finally, 264 well-defined water molecules were added, and refinement was continued until the R-value converged to 0.231 (R_{free} = 0.278) for reflections $I>\sigma(I)$ to 2.5Å resolution. The model had good stereochemistry, with all residues falling within the allowed regions of the Ramachandran plot (Table 1) analyzed by PROCHECK [18].

Isothermal titration calorimetry

ITC was performed using a VP ITC (MicroCal). GrlR protein was purified using affinity chromatography and gel filtration in 20 mM Tris-HCl buffer at pH 7.5. The purified protein sample was analyzed using mass spectrometry to confirm that no lipid species are bound to it. Bovine β-lactoglobulin (purchased from Sigma Aldrich) was used without further purification, and dialyzed into 20mM Tris-HCl buffer. All lipid samples were at pH 7.5 in 20 mM Tris-HCl. Titrations were performed by injecting consecutive 5-10 μl aliquots of lipid solution (0.9–1.0 mM) into the ITC cell containing GrlR or β-lactoglobulin (0.015 mM) at pH 7.5 in 20 mM Tris-HCl. The ITC data were corrected for the heat of dilution of the titrant by subtracting mixing enthalpies for injections of lipid solution into protein-free buffer. Two to four independent titration experiments were performed at 20°C to determine the binding constant of lipid to GrlR. The ITC data analysis was performed using software developed in our laboratory implemented in Origin 7.0 (OriginLab). Because both proteins GrlR and β-lactoglobulin are homodimeric, the protein dimer was employed as the functional unit. A general model based on the overall binding constants for two ligand binding sites, or the model with two identical cooperative ligand binding sites was used in the data analysis.

Lipid Extraction

The PBS washed Ni-NTA precipitates extracted with 600 μl of ice cold chloroform: methanol (1:2). The tubes were vortexed for 1 min and then transferred to a roller shaker in cold room for 1 h. Following incubation, 300 μl of ice cold chloroform was added and the tube was vortexed for 30s. The phase was broken with the addition of 200 μl of ice cold water. The lipids were extracted by vortexing for 2 min. The phases were separated by centrifugation at 9000 rpm for 5 min. The lower organic phase was transferred to a fresh tube. To the remaining aqueous phase, 300 μl of chloroform was added. The phases were vortexed for 2 min and then separated by centrifugation. The lower re- extracted organic phase was pooled with the initial organic phase. The lipids were dried in a speed vacuum. The dried lipids were stored at −80°C till further analysis. Before analysis the lipids were resuspended in 150 μl of chloroform: methanol (1:1).

Mass Spectrometry Analysis

For general profiling, the lipids were initially separated on XTerra C18 reverse phase column (1 mm × 150 mm) (Waters Corporation) before entering into the mass spectrometer. Typically, 5 μl of sample was injected for analysis. The inlet system consisted of a CapLC auto sampler, and a CapLC pump. Chloroform-methanol 1:1 (v/v) with 15 mM piperidine

was used as the mobile phase for isocratic elution at a flow rate of 10 μl/min. The column elutes were measured using electrospray ionization mass spectrometry (ESI-MS) through a Micromass Q-Tof micro mass spectrometer (Waters Corporation) operated in the negative ion mode. The capillary voltage and sample cone voltage were maintained at 3.0 kV and 50 V, respectively. The source temperature was 80°C and the nanoflow gas pressure was maintained at 0.7 bar. The mass spectrum was acquired from m/z 00 to 1200 in the negative ion mode with an acquisition time of 25 min; the scan duration was 1.2 s. Individual molecular species were identified using tandem mass spectrometry and the collision energy used ranged from 25 to 80 eV.

Quantification of individual lipid molecular species was performed using multiple reaction monitoring (MRM) with an Applied Biosystems 4000 Q-Trap mass spectrometer. Samples were directly introduced into the mass spectrometry using an Agilent autosampler. Each individual ion dissociation pathway for the lipid species was optimized with regard to collision energy to minimize variations in relative ion abundance due to differences in rates of dissociation. An optimized 15 μl of samples was injected per run per set with chloroformmethanol 1:1 (v/v) as the mobile phase at the flow rate of 200 μl/min. The run was carried out for 2 min.

Results

GrlR has a lipocalin like fold

A search on the coordinates of GrlR (PDB code 2ovs) for structurally similar proteins was performed using the program DALI [19]. This search identified several lipocalins with significant structural similarity to the β-barrel architecture of GrlR. While having poor or no sequence homology, lipocalins are structurally very similar to each other. The BLAST [20] (NCBI) search on the GrlR sequence revealed no significant sequence similarities with other lipocalins that are structurally similar to GrlR (Fig. 1A). Lipocalins posses a structurally conserved C-terminal helix at one side of the eight-stranded β-barrel. In GrlR the C-terminal last 11 residues are not clearly visible in the electron density map and this region is presumably disordered. However based on the sequence analysis and modeling this region is likely to form a two turn helix. We speculate that the C terminal region of GrlR is a flexible region with a minimum of two turn α-helix. The highest structural similarity is observed between GrlR and the lipid-binding domain of β-lactoglobulin, a core lipocalin, yielding an rmsd of 2.8Å for 92 Cα atoms (PDB code 1beb; 15% sequence identity). This is followed by the retinol binding protein (PDB code 1aqb; rmsd=3.3Å for 98 Cα atoms; 12.6% sequence identity). In addition, 23 lipocalin-like structures or lipid binding proteins revealed significant structural homology with GrlR (Supplementary Table 1). It is worth mentioning that a T3SS secretin pilot protein from *Shigella flexneri* bound with lipids also shows high structural similarity with GrlR [21] (rmsd of 2.8Å for 77 Cα atoms; 16% sequence identity; PDB code 1y9t). All the lipocalins have a hydrophobic cavity through which they bind with lipid molecules. For instance, the folds of lipocalins Blc, the first structurally known bacterial lipocalin [10] and Mxim [21] are identical to each other and very similar to GrlR. These observations suggest that the β-barrel architecture is an evolutionarily conserved structural feature for the lipocalins or lipid binding proteins, while at the same time the amino acid sequence of these proteins has diverged to acquire different lipid specificities for their functional roles (Fig. 1B). Based on the size, hydrophobic nature of the β-barrel cavity and structural similarity with other lipocalins, three lipid molecules were identified to interact with GrlR. The lipid molecules which are likely to interact with GrlR are (1) 1, 2-dioctanoyl-*sn*-glycero-3-phosphate, (2) 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) and (3) 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine. However due to the limited solubility of these lipids only HHGP was suitable to conduct the ITC and structural studies. In order to demonstrate the lipid binding property of GrlR is similar to β-

lactoglobulin (a well-known lipocalin structurally homologous to GrlR) we have studied the binding of HHGP with β-lactoglobulin.

Isothermal Titration Calorimetric studies

Initially ITC experiments were conducted for the three identified lipid molecules to determine the binding affinities. However 1, 2-dioctanoyl-*sn*-glycero-3-phosphate and 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine are not completely soluble in Tris-HCl buffer at 20°C, and we were able to obtain an interpretable data only for 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP). The results indicated that 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) exhibits a micromolar binding affinity for GrlR and β-lactoglobulin, a closest lipocalin structural homologue of GrlR (Fig. 2). Titrations of GrlR and β-lactoglobulin with HHGP showed negative power deflections indicating an apparent exothermic reaction during the binding. GrlR is a dimer in solution as well as in crystal [1], and two ligand molecules were found to bind to one dimer of GrlR. Likewise, β-lactoglobulin is a homodimeric protein. Therefore, the dimer was considered as the functional unit with two binding sites, and the concentration of protein was employed in a dimer basis (0.015 mM). The calorimetric titrations observed with HHGP binding to both proteins do not correspond to a system with identical binding sites, and nonidentical and/or cooperative binding sites must be assumed. This is another evidence for considering the dimer as the functional entity. Because the protein is a homodimer, with two identical binding sites in the absence of ligand, the two binding sites must exhibit (positive or negative) cooperativity.

Experimental data were analyzed employing a general model based on the overall association constants, β_i, and binding enthalpies, ΔH_i, associated with the formation of complex ML_i [22], from which information on the type of binding sites may be directly inferred (see Supplementary Information).

The non-linear regression analysis indicated that two ligands are binding to a GrlR dimer with $\beta_1 = (8.4 \pm 0.9)\cdot 10^5\ M^{-1}$, $\Delta H_1 = -0.1 \pm 0.4$ kcal/mol, $\beta_2 = (8.4 \pm 0.7)\cdot 10^{11}\ M^{-1}$, $\Delta H_2 = -7.1 \pm 0.4$ kcal/mol, $n = 1.08 \pm 0.06$. The parameter n is not the stoichiometry, already accounted for in the model, but the fraction of active protein, as indicated in the Supplementary Information. The cooperativity parameter $4\beta_2/\beta_1^2$ is equal to 4.7, which indicates that the binding sites exhibit positive cooperativity. Further, the experimental data were analyzed employing a positive cooperativity model with two identical binding sites, using explicitly the microscopic association constant, k, and binding enthalpy, ΔH, for each binding site, and the cooperativity constant, **κ**, and the cooperativity enthalpy, Δh (see Supplementary Information). The non-linear regression analysis indicated that two ligands are binding to a GrlR dimer with $k = (4.2 \pm 0.4)\cdot 10^5\ M^{-1}$, $\Delta H = -0.1 \pm 0.4$ kcal/mol, $\kappa = 4.7 \pm 0.3$, $\Delta h = -6.8 \pm 0.4$ kcal/mol, $n = 1.08 \pm 0.06$. These values are identical to the ones calculated directly from the parameters obtained with the general model based on the overall parameters using the appropriate linking equations between the two models (see Supplementary Information).

The non-linear regression analysis also indicated that two ligands are binding to a β-lactoglobulin dimer with $\beta_1 = (1.4 \pm 0.3)\cdot 10^6\ M^{-1}$, $\Delta H_1 = -2.2 \pm 0.4$ kcal/mol, $\beta_2 = (1.8 \pm 0.4)\cdot 10^{10}\ M^{-1}$, $\Delta H_2 = -13.9 \pm 0.4$ kcal/mol, $n = 0.98 \pm 0.08$. The cooperativity parameter $4\beta_2/\beta_1^2$ is equal to 0.038, which indicates that the binding sites are non-identical or they exhibit negative cooperativity. β-lactoglobulin is homodimeric, and hence the experimental data were analyzed employing a negative cooperativity model with two identical binding sites using explicitly the microscopic association constant, k, and binding enthalpy, ΔH, for each binding site, and the cooperativity constant, **κ** and the cooperativity enthalpy, Δh (see Supplementary Information). The non-linear regression analysis indicated that two ligands

are binding to a β-lactoglobulin dimer with k = (7.2 ± 0.9)·10^5 M^{-1}, ΔH = −2.2 ± 0.4 kcal/mol, κ = 0.038 ± 0.05, Δh = −9.5 ± 0.4 kcal/mol, n = 0.98 ± 0.08.

The ITC data suggests that each monomer of GrlR interact with one molecule of 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) molecule. We speculate that lipids with two longer hydrocarbon tails may engage simultaneously with the dimeric GrlR, with one tail for each monomer. The highly hydrophobic nature of longer chain lipids prevented us from performing further ITC experiments.

Structure of GrlR Lipid complex

We attempted to co-crystallize each of the three lipid molecules with GrlR, however, diffraction quality crystals were obtained only for the 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) with GrlR. In our previous study, the lipid binding cavity of GrlR is occupied by a Triton-X100 molecule which was present in the lysis buffer. During the cocrystallization of GrlR with HHGP, we titrated the protein with HHGP using ITC and concentrated the resulting complex for setting up the crystallization screens. Moreover, we did not use any Triton-X100 during the lysis and purification stage. The structure of recombinant GrlR in complex with HHGP was solved by the molecular replacement method using a synchrotron data set and refined to a final R-factor of 0.231 (R_{free}=0.278) at 2.5Å resolution. There are two GrlR-lipid complex molecules in the asymmetric unit (Fig 3). The simulated annealing omit map (Fig 4) of the bound HHGP molecules shows well defined electron density map. The model has been refined with good stereochemical parameters (Table 1). All the residues of GrlR including the N-terminal linker were well defined in the electron density map, except for the last ten residues at the C-terminus.

The GrlR molecule in the GrlR-HHGP complex is highly similar to that of our previously determined GrlR structure [1]. GrlR is a single domain β-barrel protein. The β -barrel consists of eight anti-parallel β-strands running from one side of the molecule to the other. The inner cavity region of the lipocalins is found to be highly hydrophobic and assumed to be a prerequisite for lipid binding. The diameter of the hydrophobic pore of GrlR is comparable to most of the well known lipocalins (Fig 1B). In the GrlR-HHGP complex there are 30 hydrophobic side chains having extensive hydrophobic interactions with the bound lipid molecule. The ends of the β -barrel are closed off by the N-terminus (Met1 to Lys4) and plug residues Tyr59 to Asp70. This indicates that transport through the hydrophobic cavity is restricted.

Structural comparisons of GrlR with lipocalins revealed an extended loop (L1) (the plug region Tyr59 to Asp70 of GrlR) on one side of the β-barrel, which is significantly longer than other loops (Supplementary Fig 1). This loop may act as a plug to close and open the pore. Several highly conserved hydrophobic residues are located at the middle of this loop. The conformation and nature of this extended loop scaffold (plug region) is similar in all lipocalins. In retinol binding proteins, this loop is shown to be partially responsible for the binding of lipid molecules [8]. The loop (L3) in GrlR, located opposite to the extended loop (plug) has two conserved residues (D17, S18) (Supplementary Fig 1). Similar conserved residues are also observed in PagP (D76 and S77) [23] and Mxim (D124 and T125) structures. These two conserved residues have been reported to be important for the catalytic acyltransferase activity in PagP [23]. Moreover, MxiM has been proposed as a soluble acyltransferase that may act to lipidate components of the T3SS [21]. Similar to the structure of PagP, the loops of GrlR are flexible to accommodate the binding of lipid molecules. A small change in phi-psi angle at both end of the extended loop (L1) may facilitate the opening and closing of the pore, and thus regulate the movement of the lipid molecules through the pore. Considering the high structural similarities of GrlR with PagP and Mxim

and the presence of conserved residues, we speculate that GrlR may also carry an acyltransferase activity.

Mass spectrometry analysis to identify the physiological lipid species bound to GrlR

In order to establish a physiological role of the lipid binding property and to identify the relevant lipid species, mass spectrometry experiment was carried out. His tagged GrlR was over expressed in bacterial cells and purified using Ni-NTA resins, and was used for lipid extraction. The extracted lipid was analyzed by Q-TOF mass spectrometry to obtain a complete lipid profile of the lipid species that co-purified with GrlR. A representative lipid profile for the GrlR bound species is shown in Fig. 5B. The total cell lipid extract was taken as controls (Fig 5A). In order to discriminate with non-specific bindings, a reference protein, ribosomal modifying methyltransferase (PrmB), which is not reported to bind to any lipids, was also included in the analysis. His-tagged PrmB was over expressed and purified with Ni-NTA. The lipids were extracted from the protein in a similar fashion as that of the GrlR protein. The lipid profile generated from PrmB is shown in Fig 5C. Comparison between the GrlR and the PrmB lipid profile showed drastic differences. Species with the m/z ratio of 688, 714, 716, 719, 742, 745, 747 and 773 were observed to be significantly higher in the GrlR extracts compared to the PrmB extract. These species were observed to be absent in the Ni-NTA lipid extracts from the un-induced bacterial cultures. Identification of the species was carried out using tandem mass spectrometry. Phosphatidylglycerol and phosphatidylethanolamine species with fatty acid chains of 16:0, 16:1 or 18:1 were observe to be bound to the protein (Fig 6). Interestingly, only specific types of lipid species are observed to be bound to the protein.

A multiple reaction monitoring (MRM) based approach was used to quantify these lipid species in the extracts. In these experiments, the first quadrupole, Q1, is set to pass the precursor lipid ion of interest to the collision cell, Q2, where it underwent collision-induced dissociation. The third quadruple, Q3, was set to pass the structure specific product ion characteristic of the precursor lipid of interest. MRM is very sensitive and is able to quantify the lipid species with less interference from other lipid species which could also be of the same m/z. The MRM method was built for the identified lipids and the extracts were used for quantification. As expected, the levels of the identified lipids were observed to be significantly higher in the GrlR extracts as compared to that in the un-induced control and the PrmB extracts (Fig 7). The data obtained from Q-Tof analysis was confirmed with the MRM data which also showed phosphatidylglycerol and phosphatidylethanolamine species with fatty acid chains of 16:0, 16:1 or 18:1 were bound to the protein GrlR.

Discussion

Structural analysis of GrlR showed that its fold is highly similar to lipocalins or lipid binding proteins. A Triton-X100 molecule was bound in the hydrophobic cavity of the native GrlR. Based on the cavity size and analysis of homologous structures, we predicted that lysophosphosphatidic acid (LPA) can bind to the cavity and subsequently HHGP a representative member of LPAs was chosen for this study. We have co-crystallized the GrlR-HHGP complex and determined its structure. The crystal structure of GrlR with bound lipid ligand showed a dimeric arrangement with a lipid molecule bound to each monomer of the protein. Furthermore, we have performed isothermal calorimetry studies and identified the parameters for the binding of HHGP to GrlR. In addition the binding of HHGP with β-lactoglobulin has been studied and compared with GrlR.

Although GrlR and β-lactoglobulin bind HHGP, according to the data analysis of the ITC experiments the two binding sites in GrlR and β-lactoglobulin dimers show positive and negative cooperativity, respectively. The cooperativity interaction parameter value of 4.7 for

GrlR corresponds to a cooperativity Gibbs energy value Δg of −0.9 kcal/mol, whereas the value of 0.038 corresponds to +1.9 cal/mol. The estimated value of the Hill coefficient for GrlR and β-lactoglobulin are 1.37 and 0.33, respectively, compared to a value of 1 for independent ligand binding.

The intrinsic HHGP binding to GrlR and β-lactoglobulin is characterized by a similar moderate affinity ($k = (4.2 \pm 0.4)\cdot 10^5\ M^{-1}$, kd = 2.4 μM for GrlR; $k = (7.2 \pm 0.4)\cdot 10^5\ M^{-1}$, k_d = 1.4 μM for β-lactoglobulin) with a Gibbs energy of binding of −7.5 kcal/mol and −7.9 kcal/mol, respectively, and it is entropically driven (ΔH = −0.1 ± 0.4 kcal/mol, -TΔS = −7.4 ± 0.4 kcal/mol for GrlR; ΔH = −2.2 ± 0.4 kcal/mol, −TΔS = −5.7 ± 0.4 kcal/mol for β-lactoglobulin). Therefore, the intrinsic parameters for the binding of HHGP to GrlR and β-lactoglobulin are similar: micromolar binding affinity, small binding enthalpy, and binding dominated by entropy. This is what would be expected for the binding of an apolar molecule: hydrophobic desolvation as the predominating phenomenon, resulting in a remarkable entropy gain and a small enthalpic contribution.

The binding of physiological lipid species with GrlR is further confirmed using mass spectrometric studies which compared the profiles from non-induced and induced bacterial culture. We also identified phospatidylglycerol (PG) and phosphatidylethanolamine (PE) as the two major lipid species which bound to GrlR. It is well known that PE and PG are the major lipid components of bacterial membranes. The inner membrane of *E. coli* contains 70– 80% PE and 20–25% PG [24]. The phosphatidylglycerols and phosphatidic acids differ to some extent in terms of their chemical entities. Phosphatidic acid with a glycerols group becomes phosphatidylglycerol. HHGP which showed micromolar binding affinity in ITC experiment has a head group of phosphatidic acid. Previous studies shows that a major urinary protein (MUP) of mouse (PDB code 1MUP) [25, 26] binds with a number of odorant molecules with varying affinity. A similar multiple binding was also reported in odorant binding protein [27]. Using mass spectrometry experiments we have identified the binding of two different lipid species in GrlR.

Further our ITC experiments with HHGP and β-lactoglobulin demonstrate that HHGP binds to β-lactoglobulin. β-lactoglobulin is a well studied lipocalin and its crystal structures have been reported in complex with palmitic acid (PDB code 1b0o [28]), 12-bromododecanoic acid (PDB code 1bso [29]) and retinol (PDB code 1gx8 [30]). The structural comparison of the β-lactoglobulin-lipid complexes compares well with GrlR-HHGP structure. Moreover, the binding of HHGP with other known lipocalins like blc [10] and Mxim [21] were previously reported. Besides, the co-crystal structure of Mxim with HHGP is similar to GrlR: HHGP complex. HHGP showed micro-molar binding affinity with both GrlR and Mxim [21].

Future studies will be directed towards understanding the relationship, if any, between the regulatory and lipid binding function of GrlR. We speculate that GrlR might be having multiple functions as a regulatory protein as well as lipid binding/transport protein and these functions may be changed according to the physiological condition of the bacteria. In this context it is worth mentioning here that the bacterial outer membrane enzyme PagP was found to transfer a palmitate chain from a phospholipid to lipid A [23]. PagP is a close structural homologue of GrlR, suggesting that GrlR has the potential to carry lipid molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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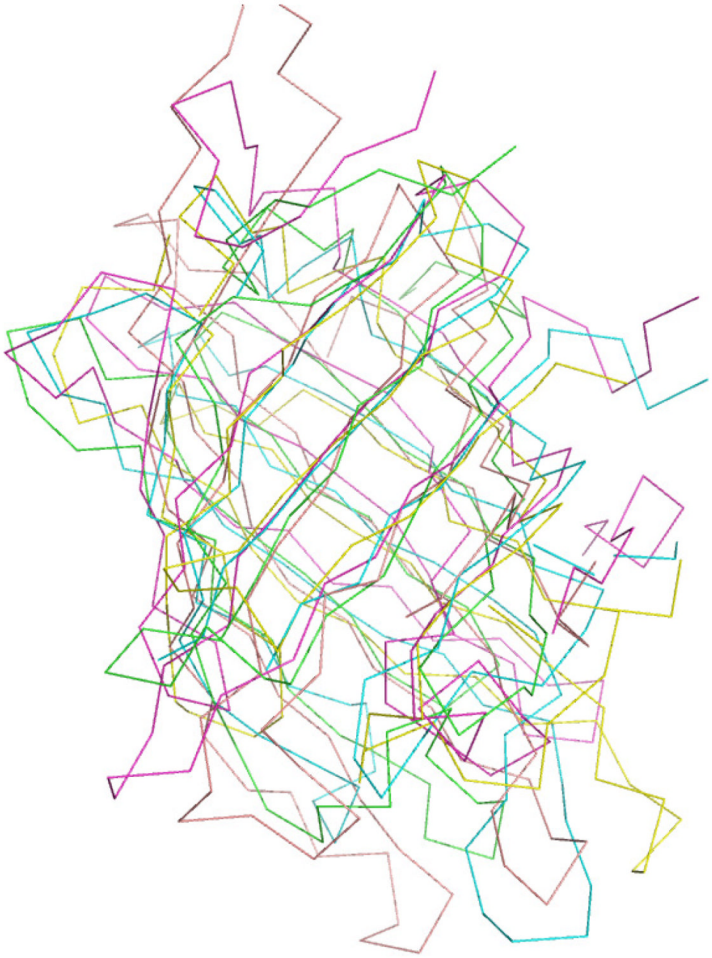


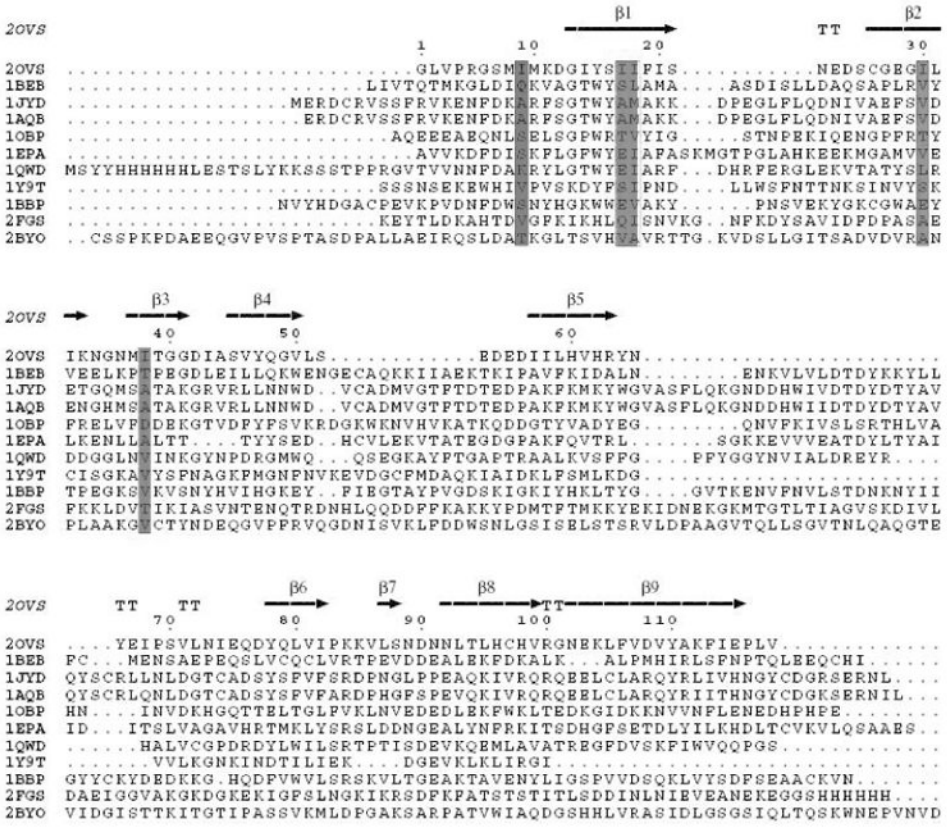


Fig. 1.

A. Superposition of GrlR with major lipocalins. GrlR - green, Retinol binding protein (PDB code 1JYD) - blue, Major Urinary protein (pdb code 1MUP) - violet, Outer membrane lipoprotein blc (PDB code 1QWD) - yellow, Outer membrane enzyme pagp (PDB code 1THQ)-brown. This figure was prepared using Pymol software [31].

B. Sequence alignment of GrlR with major lipocalins. Pdb codes - 2ovs: GrlR, 1beb: bovine β-lactoglobulin, 1jyd: human serum retinol-binding protein, 1aqb: pig plasma retinol-binding protein, 1obp: bovine odorant binding protein, 1epa: epididymal retinoic acid binding protein, 1qwd: blc, 1y9t: Mxim, 1bbp: bilin binding protein, 2fgs: Campylobacter Jejuni YceI Periplasmic Protein, 2byo: LppX (http://www.rcsb.org). The alignment was performed using ClustalW [32] and the figure was prepared using Espript [33].

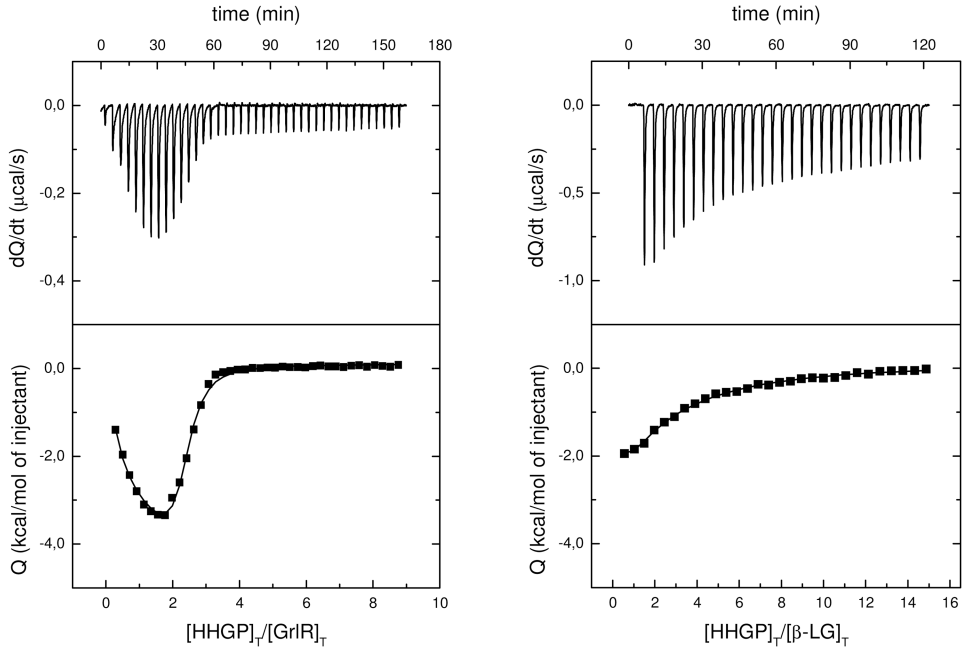


Fig 2.

ITC data for the titration of (Left) 0.9 mM hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) into 0.015 mM GrlR, and (Right) 1.0 mM hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP) into 0.015 mM β-lactoglobulin. The upper panels contain the baseline-corrected raw data, and the lower panels show the peak-integrated, concentration normalized heats of reaction versus the molar ratio. The solid line in the lower panels represents the best fit of the data using the general model using the overall binding constants for two binding sites, or the model with two identical binding sites with positive (GrlR) or negative cooperativity (β-lactoglobulin).

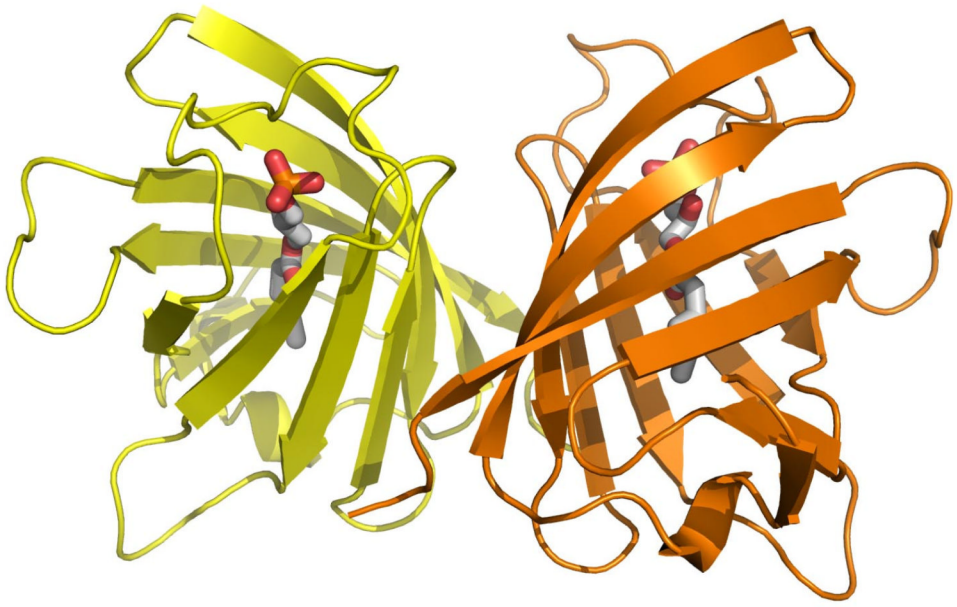


Fig 3.

Overall structure of GrlR with bound 1-hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (HHGP). Ribbon diagram of dimeric GrlR and bound HHGP is shown in stick model. The C-terminal 11 residues are disordered and were not included in this structure. This figure was prepared using Pymol [31].

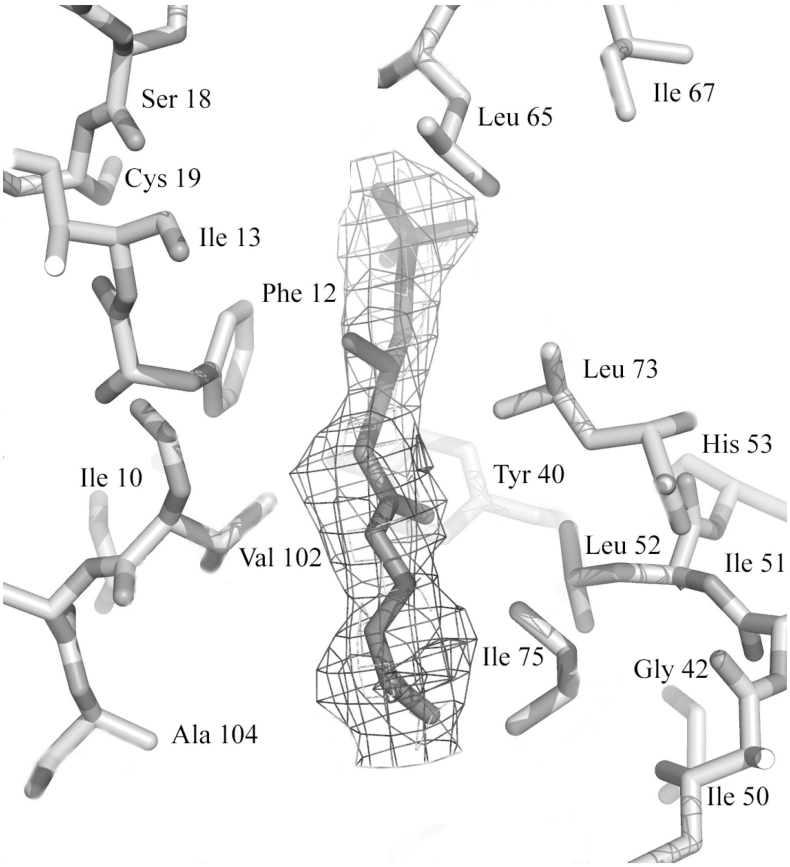


Fig. 4.

Simulated annealing Fo-Fc omit map in the pore region of GrlR. The bound hexanoyl-2-hydroxy-*sn*-glycero-3-phosphate (lipid) molecule and all atoms within 2 A° of the lipid molecule were omitted prior to refinement. The map contoured at a level of 3σ. This figure was prepared using Pymol [31].

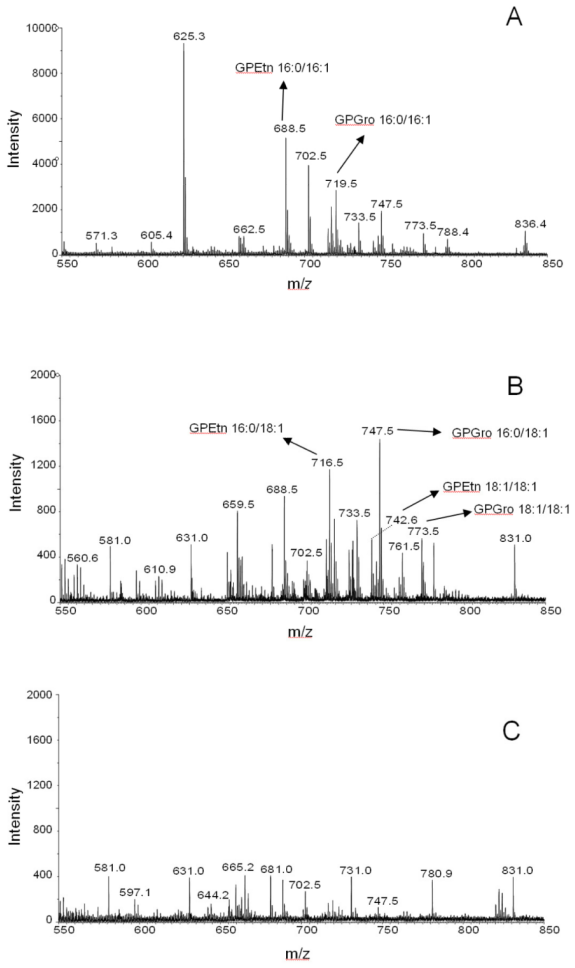
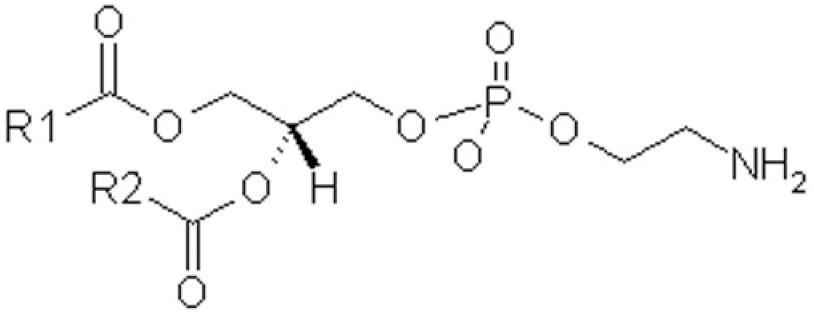
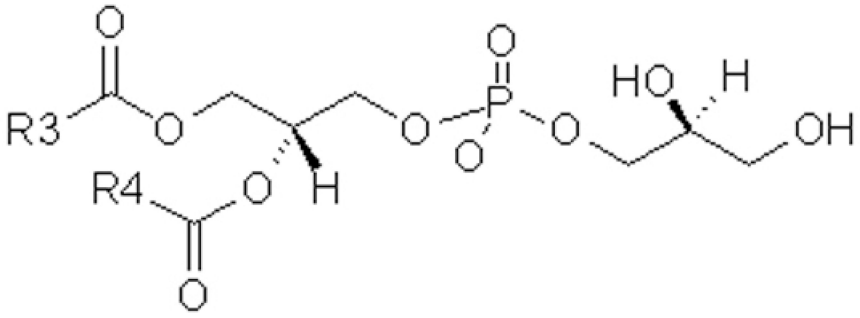


Fig 5. Lipid profile using mass spectrometry

The lipid were extracted from (A) GrlR over-expressed cells (B) Ni-NTA extracts from GrlR over-expressed cells and (C) Ni-NTA extracts from methyl transferase over-expressed cells. The data is the representative profile of 3 different set of experiments.



Phosphatidylethanolamine



Phosphatidylglycerol

Fig 6. Basic structure of phosphatidylglycerol and phosphatidylethanolamine. R1, R2, R3 and R4 indicates the fatty acids

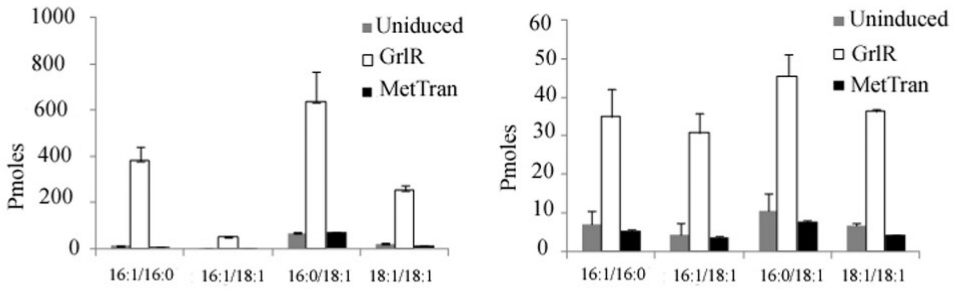


Fig 7. MRM based quantification of lipids

The Ni-NTA lipid extracts from the uninduced (grey box), GrlR over expressed (open box) and the methyl transferase over expressed (black box) cells were passed through a MRM based quantification for phosphatidylglycerol and phosphatidylethanolamine. The number below the bars indicates the fatty acid composition of the respective lipid class. The data is from 3 replicates and is the representative of 3 different sets of experiments.

Table 1
Data collection and refinement statistics

Data collection	**High resolution**
Space group	$P2_12_12_1$
Cell dimensions	
a, *b*, *c* (Å)	43.73, 66.02, 83.46
α, β, γ (°)	90, 90, 90
Resolution (Å)	2.5
$^aR_{sym}$	10.3
$I / \sigma I$	8.2
Completeness (%)	98.9
Redundancy	6.7
Refinement	
Resolution (Å)	20- 2.5
No. reflections	8751
$^bR_{work}/^cR_{free}$	0.231 / 0.278
Number of atoms	
Protein	1836
Ligand/ion	34
Water	264
***B*-factors**	
Protein	34.256
Ligand/ion	36.48
Water	51.869
R.m.s deviations	
Bond lengths (Å)	0.01
Bond angles (°)	1.7
Ramachandran Plot	
Most favorable regions (%)	84.2
Additional allowed regions (%)	13.9
Generously allowed regions (%)	2.0
Disallowed regions (%)	0

$^aR_{sym} = \Sigma|I_i - \langle I \rangle| / \Sigma|I_i|$ where I_i is the intensity of the i^{th} measurement, and $\langle I \rangle$ is the mean intensity for that reflection.

$^bR_{work} = \Sigma|F_{obs} - F_{calc}| / \Sigma|F_{obs}|$ where F_{calc} and F_{obs} are the calculated and observed structure factor amplitudes, respectively.

$^cR_{free}$ = as for R_{work}, but for 5.0% of the total reflections chosen at random and omitted from refinement.