Structural Evidence for Adaptive Ligand Binding of Glycolipid Transfer Protein

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Glycolipids participate in many important cellular processes and they are bound and transferred with high specificity by glycolipid transfer protein (GLTP). We have solved three different X-ray structures of bovine GLTP at 1.4 Å, 1.6 Å and 1.8 Å resolution, all with a bound fatty acid or glycolipid. The 1.4 Å structure resembles the recently characterized apo-form of the human GLTP but the other two structures represent an intermediate conformation of the apo-GLTPs and the human lactosylceramide-bound GLTP structure. These novel structures give insight into the mechanism of lipid binding and how GLTP may conformationally adapt to different lipids. Furthermore, based on the structural comparison of the GLTP structures and the three-dimensional models of the related Podospora anserina HET-C2 and Arabidopsis thaliana accelerated cell death protein, ACD11, we give structural explanations for their specific lipid binding properties.

Keywords: crystal structure; homology modeling; conformational change; cavity; fluorescence

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Introduction

Glycolipid transfer protein (GLTP) is a 24 kDa basic cytosolic protein, which has been identified from a variety of organisms and cell types.1,2 GLTPs from mammals share high sequence identity (~90%) and they have in vitro been shown to specifically transfer glycolipids that have glycosyl units attached to the lipid hydrocarbon backbone (either ceramide or diacylglycerol) by β-linkage.3 Glycolipids, and particularly glycosphingolipids, are known to function in many important cellular processes like development, adhesion and cell–cell recognition both in eukaryotes and prokaryotes. The glycosphingolipids can function as cell surface markers and modulators of membrane protein functions, e.g. as binding sites for certain bacteria, toxins and viruses and as stimulators of cell growth, in differentiation and DNA synthesis, and in sorting and trafficking of proteins.4,5 Very little is known about the specific function of GLTP in vivo, but due to the diverse roles of glycolipids in the cell, GLTP could potentially function as a modulator or sensor of glycolipid levels. Most glycosphingolipids are synthesized at the luminal side of Golgi but glucosylceramide, which is the precursor of other glycosphingolipids and the simplest member of the glycosphingolipid family, is synthesized on the cytosolic surface of the Golgi membrane6 and another monohexosylceramide, galactosylceramide, is synthesized at the cytosolic side of the endoplasmic reticulum. It has been suggested that GLTP could participate in the transfer of glucosylceramide from the cytosolic surface of the Golgi membrane to the inner leaflet of the plasma membrane through the cytosol.7,8 GLTP has no sequence homology with other lipid-binding proteins found in the animal kingdom but it shares sequence identity with two lipid-binding proteins found in fungi and plants; the HET-C2 protein from Podospora anserina and the accelerated cell death protein, ACD11, from Arabidopsis thaliana. The inactivation of the het-c gene, which encodes the HET-C2 protein, leads to abnormal ascospore formation in P. anserina,9 while the inactivation of the acd11 gene in A. thaliana causes activation of programmed cell death and expression of defence-related genes.10 HET-C2 has been shown to specifically bind glycosphingolipids...
in vitro in vitro and ACD11 has been proposed to be homologous to GLTP, but it does not bind and transfer glycolipids, instead it has in vitro transfer activity of the single chain lipid sphingosine.10

We have recently reported the preliminary X-ray analysis of GLTP12 and now we present the X-ray structure of bovine GLTP at 1.6 Å resolution (intermediate-GLTP) using the multiple isomorphous replacement (MIR) method. In addition, we have solved two structures with the molecular replacement method at 1.4 Å (apo-GLTP) and 1.8 Å resolution (GM3-GLTP) using the MIR structure as an initial model. The apo-GLTP structure reveals a conformation similar to the apo-conformation of human GLTP13 while the other two structures represent an intermediate conformation of the apo-GLTP and lactosylceramide-bound GLTP (LacCer-GLTP) (Figure 1).13 The apo-GLTP and the intermediate-GLTP structures are bound to a fatty acid while the GM3-GLTP structure is bound to a double-chain glycolipid, ganglioside GM3. Based on the structural analysis of the novel bovine GLTP structures and the known human GLTP structures we suggest a mechanism for lipid recognition and binding by GLTP. In order to provide structural explanations for the specific and distinctive lipid binding of GLTP and the GLTP-related HET-C2 and ACD11 proteins, three-dimensional models of HET-C2 and ACD11 protein were constructed using the GLTP X-ray structures as templates. In addition, the significance of disulfide bonds for the glycolipid transfer activity of bovine GLTP was analyzed.

Results

X-ray structures of bovine GLTP

Glycolipid transfer proteins recognize a wide range of different glycolipids3 and have a high ganglioside transfer activity.14 Mammalian GLTPs share high sequence identity; the amino acid sequences of bovine and porcine GLTP are identical and human and bovine GLTP differ only by five amino acid residues, none of which are involved in ligand binding (Figure 2). In order to investigate the mechanism of lipid binding to GLTP we have solved three different high-resolution X-ray structures of bovine GLTP. All the bovine GLTP structures have a similar fold to the human GLTP,13 a unique two-layer all-α-helical topology with a sugar moiety binding pocket and a channel where hydrophobic ligands can bind (Figures 1 and 4). Furthermore, a large positively charged area formed by four lysine residues (Lys87, Lys137, Lys138 and Lys208) is located on the surface of GLTP in the vicinity of the sugar-binding pocket (Figure 3(a)). The intermediate-GLTP (PDB code 1TFJ) and GM3-GLTP (PDB code 2BV7) structures represent a different conformation than either of the

Figure 1. A stereo view of X-ray structures of bovine GLTP. The superimposed bovine apo (red), intermediate (grey) and GM3-GLTP (blue) structures are shown as cartoons. Secondary structure elements (α-helices) are numbered according to the apo-GLTP structure.
Figure 2. A sequence alignment of 11 GLTP-like sequences. The amino acid numbering follows the bovine GLTP sequence. The a-helices defined by the bovine intermediate-GLTP structure are shown as cylinders. The residues conserved in all sequences are shaded with dark gray, while the residues that are conserved in GLTPs but not in ACD11 are shaded with light gray. Residues of particular interest are indicated in the alignment: lysine residues forming the membrane contact area in bovine GLTP (●), amino acids in the sugar-binding site (○) and amino acids forming the hydrophobic channel (▲). The organisms and sequence accession numbers are referred to as follows: Bovine (P17403); Human (Q9NZD2); Mouse (Q9JL62); Drosophila (Drosophila melanogaster, Q9VXV1); P. anserina (Podospora anserina, Q01494); P. involutus (Paxillus involutus, Q5VK7); FAPP2_Human (Q80W71); FAPP2_Mouse (Q80W71); A. thaliana (Arabidopsis thaliana, Q6NLQ3); C. elegans (Caenorhabditis elegans, Q9BK82); ACD11 (Arabidopsis thaliana, O64587).

Figure 3. Surface properties of GM3-GLTP, HET-C2 and ACD11. The electrostatic potentials of (a) GM3-GLTP, (b) HET-C2 and (c) ACD11 are mapped to the surfaces. The four lysine residues (K87, K137, K138 and K208) forming a locally charged area on the surface of GM3-GLTP are indicated. Only one of these residues is conserved in HET-C2 (K123) and none in ACD11.
recently reported human apo or the LacCer-GLTP structures, whereas the apo-GLTP (PDB code 1WBE) structure resembles the human apo-GLTP. Two of the solved bovine GLTP structures, the apo and intermediate-GLTP, have a bound fatty acid (Figure 4(a) and (b)), which is likely to be a decanolic acid according to mass spectrometry analysis (data not shown) and originate from the bacterial expression of the protein used for crystallization. Moreover, the intermediate-GLTP structure contained uninterpretable electron density near the bound fatty acid within the hydrophobic cavity (Figure 4(b)). The third bovine GLTP structure, GM3-GLTP, contains a bound GM3 ligand (Figure 4(c), the structure of GM3 is shown in Supplementary Figure 1), which was introduced during the crystallization as ganglioside micelles and co-crystallized with the protein. In our GM3-GLTP structure, electron density is observed only for the first sugar unit (glucose), whereas the other sugar units are not visible in the electron density maps, probably due to high thermal motion. The sugar unit and the acyl chain of GM3-GLTP are at the same positions and stabilized by similar hydrogen bonds and hydrophobic interactions as in the human LacCer-GLTP complex structure (Figure 5(a)). The acyl chain fits very well into the hydrophobic channel, but electron density is not observed for all of its carbon atoms (Figure 5(c)). The eight first carbon atoms of the sphingosine chain, which could be traced, are located on the surface of GM3-GLTP structure unlike in the human LacCer-GLTP structure where the sphingosine chain is buried inside the protein (Figure 4(c), (d) and (e)).

**Lipid binding to GLTP**

A hydrophobic channel is located between the two layers of helices that are characteristic for the GLTP fold. The major difference between the known GLTP structures is seen in the shape and size of this channel, particularly at the location where the sugar-binding pocket is linked to the acyl chain binding end of the channel (the acyl chain cavity) upon lipid binding (Figures 4 and 5). In the apo-GLTP structure as well as in the intermediate-GLTP structure the connection between the acyl chain cavity and the sugar-binding pocket is blocked by the side-chain of Ile45 from the α2 helix and Phe148 from the α6 helix. In GM3-GLTP, the side-chain of Ile45 is shifted in such a way that a connecting channel between the acyl chain cavity and the sugar-binding pocket is formed. The channel is clearly narrower in the GM3-GLTP structure than in the human LacCer-GLTP structure, which is mainly caused by the side-chain of Phe148. In GM3-GLTP, the side-chain of Phe148, which is in the same position as in the apo-GLTPs (Figure 5(a)), partially blocks the opening between the sugar-binding pocket and the acyl chain cavity, while in the human LacCer-GLTP structure Phe148 has turned aside to accommodate the sphingosine chain within the hydrophobic cavity. In the bovine GM3-GLTP, the sphingosine chain does not fit into the narrower channel but lies on the surface of the protein.

In all known GLTP structures the acyl chain cavity, which is located between the α1, α7 and α8 helices, is structurally conserved. Small differences in the acyl chain cavities of human and bovine GLTP structures are, however, seen probably due to the crystal packing or different N termini of the crystallized proteins. All crystallized bovine GLTP structures reported here contained an N-terminal 6xHis-tag, whereas in the recently reported human GLTP structures the His-tag was proteolytically cleaved off. In the bovine GLTP structures and the human apo-GLTP structure the acyl chain cavity is open and hence accessible for solvent, while in the human LacCer-GLTP structure the acyl chain cavity is partially blocked by the side-chain of the N-terminal Leu4. Interestingly, the acyl chain cavity of the bovine intermediate-GLTP extends through the GLTP fold forming a tunnel through the structure (Figure 4(b)). A similar extension of the acyl chain cavity is also seen in the bovine GM3-GLTP structure (Figure 4(c)) but in the other currently known GLTP structures the acyl chain extension is blocked (in the bovine apo-GLTP by Phe42, in the human apo-GLTP by Phe42 and Leu152, and in the human LacCer-GLTP by Phe148) (Figures 4(a) and (d) and 5(a)).

Altogether, the differences in the cavity architecture of the GLTP structures are mainly achieved by shifting the α2 and α6 helices and by changing the side-chain conformations of residues Phe42, Ile45, Phe148 and Leu152 that may act as switches critical in controlling the ligand binding process of GLTPs. These structural changes are likely to occur step by step, gradually widening the connecting channel between the acyl chain cavity and the sugar-binding pocket during ligand binding. The intermediate-GLTP and the GM3-GLTP structures seem to represent transitional stages in the lipid binding process of GLTP. They share structural features with both the human apo and LacCer-GLTP structures: (1) the conformation of the loop between helices α1 and α2 (loop α1-α2) in the intermediate-GLTP and GM3-GLTP structures resembles the human LacCer-GLTP conformation. (2) The N terminus of the α2 helix of both the intermediate-GLTP and GM3-GLTP structures is bent compared to the apo-GLTP conformation but it is not in the same orientation as in the human LacCer-GLTP structure. (3) The α6 helix, which in the human LacCer-GLTP structure has shifted outwards with respect to the α2 helix, is in the apo-GLTP conformation in the intermediate-GLTP and GM3-GLTP structures.

**Structural models of HET-C2 and ACD11**

Proteins that are homologous to the mammalian GLTPs can be found in many different species of eukaryotes. Based on the multiple sequence alignment, the known mammalian GLTPs are highly similar to each other, having sequence identities of
Figure 4. (Legend opposite)
over 90% (Figure 2). On the other hand, the GLTPs from <i>Drosophila melanogaster</i>, <i>P. anserina</i>, <i>Paxillus involutus</i>, <i>A. thaliana</i> and <i>Caenorhabditis elegans</i> have low sequence identities (20–30%) to the mammalian GLTPs. In mammalia, there is also a phosphatidylinositol-4-phosphate adaptor protein-2 (FAPP2), which contains a C-terminal domain with 30% sequence identity to GLTP, but its biological function is unknown. These proteins could be grouped as a glycolipid sensor protein family, since they all likely recognize glycolipids. 7,11,15 Another protein, ACD11 from <i>A. thaliana</i>, has been proposed to be homologous to GLTP, 10 even though it has less than 20% sequence identity to the mammalian GLTPs. The glycolipid transfer activity has been tested for three non-mammalian proteins; GLTP from <i>P. anserina</i> (HET-C2), 11 and GLTP from <i>A. thaliana</i> (sequence accession number Q6NLQ3, data not shown) have been shown to transfer glycolipids in vitro, while ACD11 has been reported to transfer sphingolipids instead of glycolipids. 7,11,15

Three-dimensional models of HET-C2 with a bound lactosylceramide (LacCer-HET-C2) and of ACD11 without a ligand were constructed in order to compare their lipid-binding properties to GLTP. The conserved amino acid residues of the GLTPs are mainly located in the sugar-binding pocket, not in the hydrophobic channel. The hydrophobic nature of the channel is, however, conserved. The polar amino acids Asp48, Asn52, Trp96, and His140 are in the sugar-binding pocket of the mammalian GLTPs (Figure 5(a)). Based on our sequence alignment and structural models, the sugar-binding pocket is much more charged in ACD11 than in HET-C2 and bovine GLTP (Figure 3). This is mainly caused by the substitution of residues Asn52 and Trp96 in mammalian GLTP with a lysine and an arginine, respectively, in ACD11 while in HET-C2 the two amino acids are conserved. Even though the overall sequence identity between the human GLTP and HET-C2 is only 28%, as many as 15 of the 22 residues (68%) at 4 Å distance from the lactosylceramide in the human LacCer-GLTP structure are identical between HET-C2 and human GLTP (Supplementary Table 1). However, in ACD11 only seven of these 22 residues (31%) are identical to human GLTP. The amino acids Ile45 and Phe148, controlling the shape and size of the connecting channel between the acyl chain cavity and the sugar-binding pocket of the mammalian GLTPs, correspond to Val42 and Phe134 in HET-C2 and to Ala47 and Val141 in ACD11, respectively (Figure 5). The connecting channel is open in the model of apo-ACD11, while in the apo-HET-C2 model (data not shown) the side-chains of Val42 and Phe134 block the opening between the hydrophobic cavity and the sugar-binding pocket, similarly to apo-GLTP. The positively charged surface area, formed by the lysine residues 87, 137, 138 and 208 in the bovine GLTP, is not totally conserved in the other sequences (Figure 2). Three of the lysine residues are conserved in mammalian GLTPs but only one (Lys137) is conserved in HET-C2 and none in ACD11. Consequently, there is no large positively charged area at the same location on the surface of the LacCer-HET-C2 and the ACD11 models as in the bovine GLTP structures (Figure 3).

**Disulfide bonds affect transfer activity**

There are three conserved cysteine residues in mammalian GLTPs. Two of these are buried inside the protein and can make an intra-subunit disulfide bond, while the third one is located on the surface and can form an inter-subunit disulfide bond, thus forming a GLTP dimer. 16,17 In order to analyze the biological significance of the conserved cysteine residues in GLTPs, the effects of copper sulfate and NEM (sulfhydryl-specific reagent N-ethylmaleimide) on disulfide formation of bovine recombinant GLTP (rGLTP) were analyzed by non-reducing SDS-PAGE and glycolipid transfer activity measurements. Copper sulfate is known to promote both intra- and inter-subunit disulfide bond formation, whereas NEM is believed to block surface cysteine residues only.

Two different forms of bovine rGLTP, which correspond to monomers with and without an internal disulfide bridge, are detected on non-reducing SDS-PAGE (Figure 6(a), lane 1). The treatment of rGLTP with 1 mM copper sulfate for 60 min decreases the amount of the GLTP monomer without the internal disulfide bond and results in the formation of a GLTP dimer too (Figure 6(a), lane 2). After the treatment of rGLTP with 0.45 mM (or higher concentrations) of NEM for 30 min, only the monomeric rGLTP without the disulfide bond is detected on SDS-PAGE, while at concentrations lower than 0.05 mM NEM both monomers are visible (Figure 6(a), lane 3 and 4). The addition of 1 mM copper sulfate to the NEM-treated (0.45 mM)
Figure 5. Glycolipid-binding site of GLTPs. (a) Comparison of the superimposed bovine apo (red), intermediate (white) and GM3-GLTP (blue) as well as human apo (cyan) and LacCer-GLTP (magenta) structures. The side-chains of D48, N52, W96 and Y207 at the sugar-binding site and the side-chains F42, I45, and L152 that are important for regulating the size and shape of GLTP cavities are labeled. Two alternative rotamers are shown for F148 of bovine apo-GLTP. The GM3 ligand in GM3-GLTP is shown in green and the hydrophobic cavity in GM3-GLTP is shown as a transparent surface. (b) Comparison of the superimposed X-ray structures of bovine apo-GLTP (red) and human LacCer-GLTP (magenta) together with the homology models of LacCer-HET-C2 (yellow) and ACD11 (cyan). The same side-chains of GLTP structures as in (b) and the corresponding residues in the HET-C2-LacCer and ACD11 structures are shown. The labels for residues of HET-C2 and ACD11 are indicated by an asterisk (*) and a double asterisk (**), respectively. The acyl chain binding cavity (I), connection channel (II) and extension to the acyl chain cavity (III) are indicated. The LacCer ligand of LacCer-HET-C2 model is shown in green and the ligand-binding cavity of LacCer-GLTP is shown as a transparent surface. (c) The weighted $2F_o - F_c$ electron density map (blue) is drawn with a 2 Å radius around the atoms of the GM3 ligand (green Cα atoms) and the residues D48, N52 H140 and Y207 (white Cα atoms) of bovine GM3-GLTP structure. Contours are shown at 1.0σ.
rGLTP leads to only minor increase in the amount of the GLTP monomer with the internal disulfide bond (Figure 6(a), lane 5).

The glycolipid transfer activity of the bovine rGLTP is also clearly affected by the copper sulfate and the NEM treatment. The treatment with 0.05 mM NEM results in a slight reduction (10%) of the glycolipid transfer activity, but treating with a higher concentration of NEM diminishes more than half of the transfer activity compared to the untreated rGLTP (Figure 6(b)). These results indicate that the internal disulfide bond is important for glycolipid transfer activity. The copper sulfate treatment of rGLTP reduces the transfer activity dramatically (70%), which can be explained by the formation of inactive GLTP dimers. Interestingly, the transfer activity of rGLTP treated with both NEM and copper sulfate is roughly the same as for the 0.45 mM NEM-treated rGLTP or about 50% of that of the control rGLTP. Combined with the gel electrophoresis results, this suggests that the NEM treatment blocks the internal cysteine residues as well as the surface cysteine, thus preventing the copper sulfate-aided formation of the internal disulfide bond.

**Discussion**

The X-ray structures of bovine apo, intermediate and GM3-GLTP were refined to 1.8 Å or better resolution and, thus, provide reliable information for studying the structure-function relationship of GLTPs. The bovine apo-GLTP structure is similar to the human apo-GLTP structure, whereas the intermediate and GM3-GLTP structures represent novel intermediate conformations sharing features of both the apo-GLTPs and the human LacCer-GLTP. The structural differences in the GLTP structures are mainly seen in the α2 and α6 helices and in the loop α1-α2 (Figure 1). The acyl chain cavity of the bovine apo and intermediate-GLTP structures contains a fatty acid, which probably originates from the expression host. Similarly, the human apo-GLTP structure has been reported to have an undefined hydrocarbon in the acyl chain cavity. Since the acyl chain cavity in these structures is solvent accessible the presence of a ligand is probably favorable for the stability of the GLTP structure. The GM3-GLTP structure has a glycolipid ligand, GM3, which was added at the crystallization setup. Only one of the sugar units, the glucose that is β-linked to the ceramide, is seen in the electron density maps of the sugar-binding pocket. The other hydrophilic sugar units most likely extend into the solvent and are highly flexible. The sphingosine chain of GM3 lies on the surface of GLTP and only the acyl chain is inserted into the hydrophobic channel. Therefore, GM3 binds to GLTP in a different way than lactosylceramide in the human LacCer–GLTP complex, where the sphingosine and acyl chains of lactosylceramide are both inside the channel.

The lipid binding to GLTP induces changes in the shape and size of the hydrophobic channel (Figure 4). The conformational changes during lipid binding do not seem to be simultaneous, since the rearrangement of the loop α1-α2 and the bending of the N terminus of α6 helix are independent of the movement of the α6 helix. The helices can move several Ångströms with respect to each other, which makes the hydrophobic channel very flexible and allows adaptive binding of lipids. The flexibility is enhanced by the conformational changes of a few key residues (Phe42, Ile45 and Phe148). Ile45 from the α2 helix functions as a gate; it may open the connecting channel between the acyl chain cavity and the sugar-binding site by rotating its side-chain. Phe148, which is located near Ile45, has a similar role; it can accommodate a second lipid chain in the hydrophobic channel by...
altering its side-chain conformation. In the human LacCer–GLTP complex structure Phe148 blocks the extension of the acyl chain cavity, which is observed in the bovine intermediate and GM3-GLTP structures (Figure 5(a)), whereas it is blocked by Phe42 in the bovine apo-GLTP structure and by Phe42 and Leu152 in the human apo-GLTP structures. Interestingly, the glycolipid transfer activity of GLTP was reduced when the hydrophobic gate residues, Ile45 and Phe148, were mutated to the polar residues asparagine and serine, respectively.

The sugar-binding pocket of GLTP has several conserved residues of which Asp48, Asn52, His140, and, especially Trp96, are known to be important for ligand recognition and binding. A similar aspartate, asparagine and tryptophan environment has been observed in the sugar-binding site of other proteins like in the *Escherichia coli* galactose chemoreceptor protein (PDB code 2GBP). Interestingly, the residues in the sugar-binding pocket and the hydrophobic channel of GLTP are well conserved in HET-C2, which suggests that the lipid binding of GLTP and HET-C2 are similar. However, in ACD11 neither the sugar-binding nor the gate residues are well conserved and, in addition, the surface potentials of the ACD11 model are markedly different from those of GLTP and HET-C2 (Figure 3). Thus, our study provides structural explanation for why ACD11 is unable to transfer glycolipids even though it is homologous to GLTP.

ACD11 has been shown to transfer sphingosine in vitro but the binding mode is still unclear. Sphingosine is a component of both GM3 and lactosylceramide, but the binding mode of sphingosine to ACD11 might be different than it is in the bovine GM3–GLTP or human LacCer–GLTP complex. The sphingosine could bind to the putative acyl chain cavity of ACD11 either in a similar manner to the acyl chain in the GM3-GLTP structure or as the fatty acid in the bovine apo and intermediate-GLTP structures. Based on these results we suggest that ACD11 should not be classified as a GLTP protein, even though it is homologous to GLTP.

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affect the sugar binding by signaling through the α8 helix. Secondly, the loop α1-α2 rearranges, the N terminus of the α2 helix bends and the side-chain of Ile45 rotates to open the connecting channel between the sugar-binding pocket and the acyl chain cavity. This permits the acyl chain of the glycolipid ligand to displace the bound fatty acid or hydrocarbon chain. Thirdly, in order to enable the sphingosine chain to enter the hydrophobic channel, the α6 helix shifts outwards and the side-chain of Phe148 in the connecting channel rotates into the extension of the acyl chain cavity.

Materials and Methods

Crystallization and heavy atom soaking

The bovine GLTP protein was expressed, purified and crystallized as described recently. For the purposes of MIR method, five different GLTP crystals were heavy-atom derivatized with (1) potassium tetra-chloroplatinate (II) (Cl4K2Pt), (2) potassium dicyano-aurate (I) (KAu(CN)2), (3) mercury (II) chloride (HgCl2), (4) 4-(chloromercuri)benzensulfonic acid sodium salt (C6H4ClHgO3SNa) and (5) potassium tetranitroplatinate (II) (K2Pt(NO2)4), which correspond to JBScreen Heavy (Jena Bioscience) tubes 1, 2, 4, 5 and 6, respectively. Heavy-atom soakings were carried out at 25°C for 1–48 h using 2–5 mM heavy-atom concentrations. Three out of five heavy-atom derivatives were found to bind to the crystals (see Supplementary Table 1). In the case of the apo-GLTP data set, the crystal was soaked with 12 nM GM3 for 30 min at 25°C before the diffraction data collection, but the soaked ligand was not seen in the final structure. The GM3-GLTP crystals were produced by co-crystallizing GLTP with GM3 in a molar ratio of 1:1.

Table 1. Data collection and refinement statistics

<table>
<thead>
<tr>
<th>Data collection*</th>
<th>Intermediate-GLTP</th>
<th>Apo-GLTP</th>
<th>GM3-GLTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>55.4, 34.9, 58.2</td>
<td>55.7, 35.4, 58.4</td>
<td>55.5, 34.6, 58.3</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
<td>90, 116, 90</td>
<td>90, 117, 90</td>
<td>90, 116.3, 90</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>20-1.6(1.70-1.61)</td>
<td>25-1.36(1.45-1.36)</td>
<td>25-1.8(1.9-1.8)</td>
</tr>
<tr>
<td>Rmerge (%)</td>
<td>6.7(41.4)</td>
<td>3.9(49.7)</td>
<td>8.1(52.1)</td>
</tr>
<tr>
<td>L/cell</td>
<td>12.2(3.3)</td>
<td>21.6(2.55)</td>
<td>16.6(4.2)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.1(95.1)</td>
<td>94.7(73.2)</td>
<td>99.5(99.7)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>4.1(3.8)</td>
<td>6.1(3.6)</td>
<td>6.1(6.1)</td>
</tr>
<tr>
<td>Refinement</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Resolution (Å)</td>
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<td>25-1.4</td>
<td>25-1.8</td>
</tr>
<tr>
<td>No. reflections</td>
<td>105,133</td>
<td>256,245</td>
<td>115,280</td>
</tr>
<tr>
<td>Rwork/Rfree</td>
<td>18.2/22.1</td>
<td>18.4/21.9</td>
<td>18.9/25.7</td>
</tr>
<tr>
<td>No. atoms</td>
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<tr>
<td>Protein</td>
<td>1698</td>
<td>1774</td>
<td>1639</td>
</tr>
<tr>
<td>Ligand/ion</td>
<td>19</td>
<td>12</td>
<td>51</td>
</tr>
<tr>
<td>SO₄</td>
<td>–</td>
<td>2</td>
<td>–</td>
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<tr>
<td>Fatty acid/glycolipid</td>
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<td>12</td>
<td>43</td>
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<tr>
<td>Glycerol</td>
<td>6</td>
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<td>–</td>
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<tr>
<td>Water</td>
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<td>292</td>
<td>119</td>
</tr>
<tr>
<td>R-factors*</td>
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<tr>
<td>Protein</td>
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<td>21.9</td>
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<td>31.3</td>
<td>39.0</td>
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<td>Water</td>
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<td>28.7</td>
</tr>
<tr>
<td>r.m.s. deviations</td>
<td>Bond lengths (Å)</td>
<td>0.018</td>
<td>0.018</td>
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<tr>
<td>Bond angles (deg.)</td>
<td>1.7</td>
<td>1.7</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* One crystal was used for each dataset. Values for the highest resolution shell are shown in parenthesis.

** Rmerge = Σ(hkl) Σ(i) |Fobs(i,hkl)| - |Fcalc(i,hkl)| / Σ(hkl) Σ(i) |Fobs(i,hkl)|.

*** Rwork/Rfree = Σ(hkl) |Fobs(i,hkl)| - |Fcalc(i,hkl)| / Σ(hkl) |Fobs(i,hkl)|.

*Performed on 5% of the reflections.

Average value for all atoms.
resolution using the program DM\textsuperscript{24} of the CCP4 suite.\textsuperscript{25} The initial model was used as an input for an automatic model building with ARP/wARP.\textsuperscript{26} The model was refined with Refmac5\textsuperscript{27} (CCP4 suite) and modified and rebuilt with O.\textsuperscript{28} Solvent atoms were added to the model with the automatic procedure of ARP/wARP.\textsuperscript{29} The apo-GLTP structure (PDB code 1WBE) was built with ARP/wARP using the intermediate-GLTP structure as an existing model, rebuilt manually with O and refined with Refmac5. The DKA ligand was built with the ARP/wARP LigandBuild program\textsuperscript{30} and solvent atoms were built as above. The GM3-GLTP structure was solved with MOLREP of the CCP4 suite using the intermediate-GLTP structure as the search model, rebuilt manually with O and refined with Refmac5. All structures were analyzed with the programs PROCHECK\textsuperscript{31} and WHATIF.\textsuperscript{32}

Homology modeling

The sequences of 11 GLTP-like proteins from seven organisms were aligned (Figure 2) with the program MALIGN\textsuperscript{33} within the Bodil visualization and modeling package.\textsuperscript{34} Homology models of HET-C2 and ACD11 were made using the program MODELLER\textsuperscript{35} based on the pair-wise alignment derived from the multiple alignment and the crystal structures of human LacCer-GLTP\textsuperscript{13} and bovine apo-GLTP, respectively.

SDS-PAGE analysis and glycolipid transfer activity measurements

Treatment of 0.4 mg/ml GLTP with NEM and CuSO\textsubscript{4} was done under stirring for 30 min and 60 min, respectively. The GLTP samples were incubated in Laemmli buffer without β-mercaptoethanol at 95°C before the non-reducing SDS-PAGE analysis. The SDS-PAGE gel was stained with the silver staining procedure kit (Pierce, Rockford, IL, USA). Commercially raised rabbit polyclonal anti-GLTP antibody (MedPro, Oslo, Norway) was used in Western blot analysis of GLTP. The energy transfer assay for measuring anthrylvinyl-galactosylceramide (AV-GalCer, N-[11E]-12-(9-anthryl)-11-dodecenoyl]-1-O-β-D-galactosylphosphoglycerol) transfer was presented recently\textsuperscript{36} and is based on the method described.\textsuperscript{37} Briefly, AV-GalCer transfer from probe sonicated donor vesicles consisting of 1% AV-GalCer, 3% dihexadecyloxacarbocyanine perchlorate and 96% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) to probe sonicated pure POPC acceptor vesicles was started by GLTP injection. The assay as well as the vesicle preparation was done in a sodium phosphate buffer containing 10 mM sodium dihydrogen phosphate, 1 mM dithiothreitol and 1 mM EDTA at pH 7.4.

Miscellaneous methods

Figures 1 and 3–5 were created with the PyMOL Molecular Graphics System\textsuperscript{†} and/or Corel Draw 11 software. Electrostatic potentials were calculated using the APBS software package\textsuperscript{‡} plugin in PyMOL. Figure 3 was rendered using POV-Ray 3.6.§ The secondary structure elements were assigned with the DSSP program.\textsuperscript{38} Cavities were calculated with the program Surfnet\textsuperscript{39} using 1.4 A and 4.0 A radii for minimum and maximum gap spheres, respectively. Figure 2 was generated with the program ALSCRIPT.\textsuperscript{40} Average B-factors over all atoms were calculated using MOLEMAN.\textsuperscript{41}

Protein Data Bank accession code

The structures have been deposited in the RCSB Protein Data Bank with accession codes 1TFJ, 1WBE and 2BV7.

Acknowledgements

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Supplementary Data

Supplementary data associated with this article can be found in the online version at doi 10.1016/j.jmb.2005.10.031

References


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\text{http://www.pymol.org} \\
\text{http://agave.wustl.edu/apbs} \\
\text{http://www.povray.org/}
\]


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