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Glycolipid transfer protein: Clear structure and activity, but enigmatic function

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Introduction

Glycosphingolipids comprize a small (typically 5–10% by weight) but vital fraction of membrane lipids in eukaryotes (Holthuis et al., 2001). They provide the plasma membrane with chemical and mechanical stabilities and take part in fundamental biological processes including differentiation, cell–cell interaction, and transmembrane signaling. For these lipids, as for most lipids in general, local metabolism and selective transport are important determinants (Sprong et al., 2001). Little is known about these processes, but it is clear that several key players in the organization and control of sphingolipid composition remain to be identified. A protein purified from bovine spleen cytosol specifically transferring glycolipids was already described more than 20 years ago (Metz and Radin, 1980; Radin and Metz, 1982). An absolute specificity was found for glycolipids containing a β-linked sugar to the hydrophobic backbone (Yamada et al., 1986). Glycolipid transfer proteins (GLTPs, EC number not assigned) are water-soluble proteins of average size of 24 kDa that have been studied extensively *in vitro* (Abe and Sasaki, 1985; Abe et al., 1982; Brown et al., 1990; Gammon et al., 1987; Metz and Radin, 1982). Very recently, their structural and *in vitro* functional properties were

Abbreviations: CD, circular dichroism; DTT, dithiothreitol; Egg PC, L-α-phosphatidylcholine; FMN, flavin mononucleotide; GlcCer, glucosylceramide; GlcCer*pyr*, pyrene-labeled glucosylceramide; GLTP, glycolipid transfer protein; HSQC, heteronuclear single quantum correlation; Photo-CIDNP, photochemically induced dynamic nuclear polarization; PMSF, phenyl methyl sulfonylfluoride.

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reviewed in detail (Brown and Mattjus, 2007). These proteins have been isolated from different sources ranging from spinach chloroplasts to mammalian brain, liver and kidney (Brown et al., 1990; Sasaki, 1985, 1990). GLTP orthologs in fungi and plants were found to be involved in programmed cell death (Brodersen et al., 2002; Mattjus et al., 2003; Saupe et al., 1994) but their function in mammals is largely unknown. The activity of GLTP is preserved after expression in *E. coli* (Godi et al., 2004; Lin et al., 2000; Malinina et al., 2004; Rao et al., 2004) allowing for detailed structure–function studies *in vitro*.

Crystal structures of GLTP in the absence and presence of bound glycolipid were solved revealing a new folding motif among lipid-binding proteins (Malinina et al., 2004). In combination with mutational studies the structures provided new insights into glycolipid binding (Malakhova et al., 2005). The sugar head group of the glycolipid is specifically recognized by a network of hydrogen bonds involving Trp96 while the acyl chains are embedded in a hydrophobic tunnel flanked by α-helices. It remains to be investigated, however, in what way GLTP binds to membranes and how glycolipids are being transferred from their membrane environment into the binding pocket of GLTP and subsequently donated to acceptor membranes. In this study we investigate the mechanism of lipid uptake by GLTP. With the help of fluorescent techniques (Li et al., 2004) in combination with photochemically induced dynamic nuclear polarization (photo-CIDNP) (Kaptein et al., 1978; Mok and Hore, 2004) and chemical shift perturbation methods (Bax et al., 1990; Otting, 1993; Zuiderweg, 2002) we have studied interaction of GLTP with vesicular membranes and conditions of lipid uptake.

Materials and methods

Chemicals were from Sigma (St. Louis, MO) and used in the highest purity available, unless indicated otherwise. Silica TLC plates were from Merck (Darmstadt, Germany), organic solvents were from Riedel de Haën (Darmstadt, Germany), [*N*-methyl-^{3}H] choline, 1,2-dipalmitoyl, and [9,10-^{3}H]-palmitic acid were from Amersham (Buckinghamshire, UK). Pyrene-hexanoic acid was from Molecular Probes (Eugene, OR). Porcine glucosylceramide (GlcCer), L-α-phosphatidylcholine (egg PC) and cholesterol were purchased from Sigma–Aldrich. Pyrene-labeled glucosylceramide (GlcCer*pyr*) was synthesized from pyrene-hexanoic acid and 1-β-D-glucosylsphingosine (Sigma–Aldrich) as described (Kishimoto, 1975), purified by two-dimensional thin layer chromatography (TLC) as described previously (Sprong et al., 2000), and quantified spectrophotometrically at $\lambda_{ex} = 342$ nm and $\lambda_{em} = 378$ nm. Deuterium oxide (^{2}H, 99.9%) was obtained from Cambridge Isotope Laboratories, Inc.

Vesicle preparations

In titrations of GLTP

Vesicles used in the titration experiments contained a fixed amount of cholesterol (40%), while the total molar fraction of GlcCer and egg PC was kept at 60%. The molar fraction of GlcCer was varied from 0 mol% to 30 mol%. Lipids were mixed in chloroform/methanol, or ethanol solution, dried under a stream of nitrogen and hydrated in Tris buffer (20 mM Tris, 5 mM EDTA, 200 mM NaCl, pH 7.4). The dispersion was subjected to 6–7 freeze–thaw cycles to get a uniform distribution of buffer solutes between lipid bilayers. After each thawing cycle at 60 °C the lipid dispersion was thoroughly mixed. The lipid suspension was then extruded by 11 passes through 400 nm polycarbonate membrane, and 11 passes through 200 nm polycarbonate membrane using

a hand held extruder (Avanti). For preparation of ^{3}H-labeled vesicles trace amounts of ^{3}H-labeled PC or GlcCer were added to the lipid mixture before lipid drying under nitrogen.

In titrations of vesicles with GLTP

Vesicles were prepared with fixed amounts of egg PC (30 mol%), cholesterol (40 mol%) and GlcCer (25 mol%). In addition these vesicles contained 5 mol% GlcCer*pyr*. These vesicles were made essentially as described above for unlabeled vesicles.

Expression and purification of GLTP

The N-terminal 6 × His-tagged plasmid pQE9-gltp expressing bovine GLTP was a kind gift of Dr Peter Mattjus (West et al., 2004). The pQE9-gltp vector was transformed into *E. coli* BL21 cells and grown in LB medium at 37 °C until the cell density A_{600} reached 0.6. Expression of the His-tagged GLTP was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. The cells were incubated for 2 h at 37 °C, harvested (3300*g*, 30 min, J6-HC Beckman centrifuge, rotor JS-4.2) and lysed by incubation with (1 mg/ml) lysozyme and (1 mg/ml) PMSF in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole and 10% glycerol pH 8.0) followed by sonication. The clarified lysate was purified by affinity chromatography as described before (West et al., 2004). The protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole and 10% glycerol pH 7.5), and transferred into phosphate buffer (25 mM phosphate, 1 mM DTT, 150 mM NaCl, pH 6.8) to a final concentration of imidazole of 0.1 mM. The purity was confirmed by analysis on an SDS-PAGE gel (15%) and estimated to be at least 98% pure. GLTP was concentrated to 7–9 mg/ml with a protein concentrator (Millipore). Concentration of protein was calculated from optical density measurements at 280 nm using $\varepsilon_{280nm} = 13\ M^{-1}\,cm^{-1}$ for 1% (by weight) protein solutions. Transfer activity measurements under standard assay conditions (Mattjus et al., 1999) yielded a second order rate constant of $8 \times 10^{4}\ M^{-1}\,s^{-1}$ at 20 °C.

For expression and purification of ^{15}N-labeled GLTP the pQE9-gltp vector was transformed into *E. coli* BL21 cells and grown in minimal M9 medium (containing 0.5 g/L $^{15}NH_4Cl$) at 37 °C until the cell density A_{600} reached 0.5. Purification of the ^{15}N-labeled GLTP was carried out essentially as described for the unlabeled protein.

Intrinsic fluorescence emission of GLTP

Intrinsic fluorescence emission measurements of GLTP were described before (Li et al., 2004). Fluorescence measurements were performed using a PTI – Photon Technology International fluorometer. Excitation and emission slid widths were set both to 2 nm. Experiments were performed using an excitation wavelength of $\lambda_{ex} = 285$ nm at 37 ± 0.5 °C with constant stirring. Emission was monitored from 300 nm to 500 nm. The concentration of GLTP in all the measurements was 0.5 μM. Vesicles were added in varying amounts (ranging from 5 μM to 160 μM total lipid) to fresh protein solutions. Difference spectra of all fluorescence measurements were obtained by subtracting the spectra of GLTP loaded with vesicles from spectra of GLTP itself recorded each time prior to the addition of vesicles and corrected for dilution.

Quantification of lipid content

GLTP (0.25 mM) was incubated with ^{3}H-labeled vesicles (composition and total lipid concentration are varied) followed by ultracentrifugation at 4 °C for 1 h at 100,000*g* (Optima™ MAX

Ultracentrifuge, Beckman Coulter, rotor TLA 55). Radioactivity was measured for supernatant and pellet separately using a Liquid Scintillation Analyzer (Packard, TRI-CARB 2300TR).

Photo-CIDNP NMR

Surface exposed aromatic residues in proteins can be highlighted using photo-CIDNP NMR (Kaptein et al., 1978; Mok and Hore, 2004). These NMR measurements were performed on a Bruker Avance 500 MHz spectrometer. An Argon laser was used as light source (Spectra Physics, Stabilite 2017). The output power was 5 W. The light beam was directed to the sample in the NMR tube by using an optical fiber. The photo-CIDNP radical reaction was initiated by FMN as a laser-reactive dye. The irradiation leads to the generation of peptide–dye radical pairs involving dye-accessible (and therefore surface exposed) Tyr, Trp and His residues. The laser light was gated into pulses of 400 ms using a computer controlled mechanical shutter. Spectra were recorded after a short delay of 5 ms. The re-cycle delay of the experiments was 6 s. Light and dark spectra were recorded in the same experiment in interleaved scans in order to maintain as much of the same conditions for the two experiments as possible. For each spectrum a total number of 64 scans was accumulated. The final photo-CIDNP data were obtained by subtraction of the dark from the light spectra. Apo-GLTP (0.25 mM) was monitored with 0.26 mM FMN. The effect of different types of vesicles (0% GlcCer, 40% cholesterol, 60% egg PC and 30% GlcCer, 40% cholesterol, and 30% egg PC) was studied as well. Before monitoring by NMR GLTP was harvested after incubation with each type of vesicles (2.5 mM total lipid) by centrifugation for 1 h at 100,000*g* as described before. As a control 1D NMR and photo-CIDNP spectra of FMN (0.26 mM in 25 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 6.8) were recorded. Experiments were carried out at 20 °C and 40 °C.

Chemical shift perturbation experiments

The chemical shifts of amide protons are very sensitive to their chemical and structural environments (Otting, 1993). Therefore, $^{1}H-^{15}N$ correlation NMR experiments (HSQC, Bax et al., 1990) can be used to monitor chemical shift changes in the course of a titration or addition of a ligand and thereby reveal an interaction or binding surface on a protein (Zuiderweg, 2002). Uniformly ^{15}N-labeled GLTP was dissolved in phosphate buffer (25 mM phosphate, 150 mM NaCl, 1 mM DTT, pH 6.8, containing 9% D_2O) at a concentration of 0.3 mM. GLTP was studied either as such or after loading with GlcCer as described above. HSQC spectra were recorded at 20 °C before and after addition of GlcCer using on a Bruker Avance 600 MHz spectrometer.

CD measurements

CD spectra of GLTP itself and GLTP pre-incubated with vesicles containing PC/cholesterol as such or with GlcCer (30 mol%; for details see Vesicle preparations) were monitored at temperatures ranging from 20 °C to 60 °C with 1 °C/min increments at a constant wavelength of 220 nm. Protein samples were pre-scanned at 20 °C between 190 nm and 280 nm. All measurements were performed using a Jasco J-810 spectropolarimeter equipped with a CDF-426S temperature controller.

Results

GLTP has a high affinity for glucosylceramide

The glycolipid transfer activity of GLTP from donor to acceptor vesicles *in vitro* is routinely monitored using a fluorescence based resonance energy transfer assay (Abe et al., 1984; Mattjus et al., 1999; Sahoo et al., 2000). Self-quenching due to excimer formation of pyrene-labeled lipids is also used to study lipid transport (Sahoo et al., 2000; Somerharju, 2002). We applied this technique to study single turnover transfer of partially pyrene-labeled glycolipid (GlcCer*pyr* and GlcCer mixed in 1:5 molar ratio) from donor vesicles to GLTP only. As is shown in Fig. 1 addition of GLTP to a solution of vesicles (9.8 μM total lipid) containing fixed amounts of partially pyrene-labeled GlcCer (30 mol%), egg PC (30 mol%) and cholesterol (40 mol%) leads to an increase in fluorescence intensity at 378 nm. This increase takes place within seconds after mixing protein and vesicles and remains stable thereafter. It is assumed that the fluorescence increase results from complex formation of GLTP–GlcCer complex (X) is then related to the measured fluorescence emission intensity, Fem, using Eq. (1):

$$\mathrm{Fem} = \mathrm{Fem}(0) + C[X] \tag{1}$$

where Fem(0) is the fluorescence emission intensity before addition of GLTP and C is a constant. An apparent binding constant (K_d) for GlcCer taken up by GLTP is obtained by solving Eq. (2):

$$K_d = [([E_{tot}] - [X])([\mathrm{GlcCer}] - [X])]/[X] \tag{2}$$

allowing only one physically possible root as solution:

$$[X] = 0.5a\left(1 - \sqrt{1 - (4[E_{tot}][\mathrm{GlcCer}])/a^2}\right) \tag{3}$$

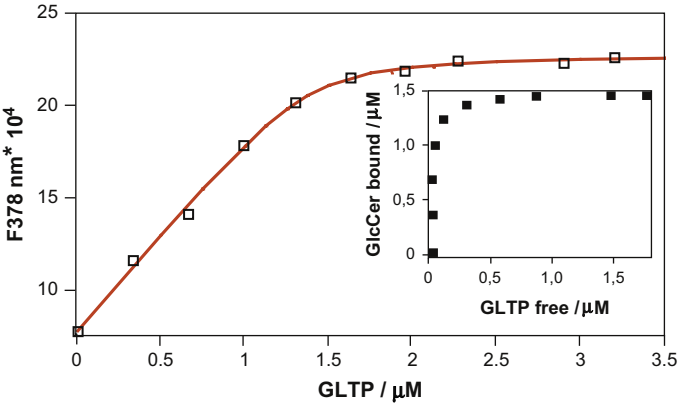

Fig. 1. Titration of unilamellar vesicles (9.8 μM total lipid) containing 25 mol% GlcCer, 5 mol% GlcCer*pyr*, 30 mol% egg PC and 40 mol% cholesterol with GLTP. Fluorescence intensities are measured at 378 nm after rapid mixing of vesicles with GLTP. Inset: replot of the calculated GLTP–GlcCer concentrations as a function of free GLTP concentrations.

where $a = K_d + [E_{tot}] + [\text{GlcCer}]$ and E_{tot} is the total GLTP concentration in μM. While the total concentration of GlcCer is known, the concentration of glycolipid available for GLTP, [GlcCer] in Eqs. (2) and (3) is considered as an unknown.

Substitution of Eq. (3) into (1) gives a value for the function Fem_{calc}. The solid line in Fig. 1 represents the best fit to the data by nonlinear regression where $\text{Fem} - \text{Fem}_{calc}$ is minimized while varying K_d, C and [GlcCer]. Based on this analysis, a replot of the concentration of GLTP–GlcCer complex as a function of free GLTP (inset Fig. 1) demonstrates the high affinity of GLTP for GlcCer, estimated at $K_d = 2.5 \times 10^{-2}$ μM for various concentrations of vesicles containing 30 mol% GlcCer. This K_d value represents the concentration of free GLTP when half of the available GlcCer is bound to the protein. Interestingly, another result of this analysis is the finding that only 50% of total GlcCer present in the vesicles is available for uptake by GLTP.

Intrinsic fluorescence of GLTP changes in response to the molar fraction of GlcCer in vesicles

As is shown in Fig. 2 the emission maxima of apo-GLTP and GLTP treated with vesicles containing egg PC/cholesterol were both found at 340 nm, while for GLTP treated with vesicles containing 30 mol% of GlcCer this maximum was shifted to 328 nm. The inset in Fig. 2 highlights the spectral changes observed by taking difference spectra before and after treatment with vesicles. Such difference spectra were also recorded in the presence of vesicles and are shown in Fig. 3, both for vesicles containing no GlcCer (panel A), or a fixed amount of 30 mol% GlcCer (panel B). The results of titrations of GLTP with vesicles containing various

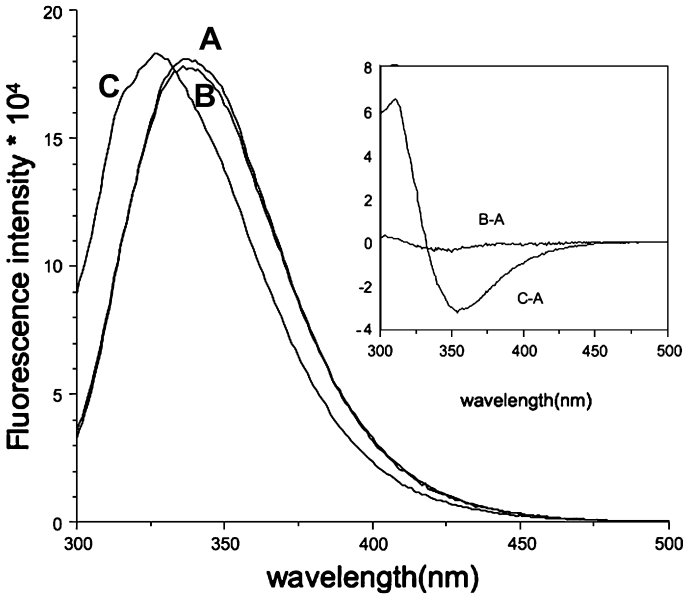

Fig. 2. Fluorescence emission spectra of GLTP (0.5 μM) in the absence of vesicles. A: apo-GLTP, B: GLTP after treatment with vesicles containing egg PC (60 mol%) and cholesterol (40 mol%), and C: GLTP after treatment with vesicles containing 30 mol% GlcCer, egg PC (30 mol%) and cholesterol (40 mol%). Inset: difference spectra for B and C relative to A.

levels of GlcCer are summarized in Fig. 4. Fluorescence signals monitored at 340 nm increase linearly with higher vesicle concentrations. However, this increase was found to arise from light scattering off the vesicles, as similar linear changes were noted in the absence of GLTP (data not shown). While also these titrations do not provide evidence for GLTP binding to the vesicles, an affinity constant for GlcCer can be obtained by plotting the intercepts of the straight lines fitted to the data as a function of mol% GlcCer (inset Fig. 4). From the hyperbolic function fitted to the data an apparent affinity constant of 9 ± 3 mol% was calculated for GlcCer. Taken together with the observation that only 50% of total GlcCer can be accessed by GLTP for the vesicles used, the true affinity constant should be 4.5 mol%.

Vesicular integrity depends on GLTP concentration

In order to check the integrity of the vesicles before and after addition of GLTP trace amounts of either ^{3}H-labeled GlcCer or egg PC were added to the vesicles. The results are summarized in Table 1. The studies confirm that vesicles at all compositions are collected almost exclusively in the pellet (91 ± 3%) after centrifugation in the absence of GLTP. Strikingly, in the presence of a high concentration of GLTP (100 μM) and total lipid (1 mM), 76% of the lipid remains in solution. At low concentrations of GLTP (0.5 μM) and total lipid (50 μM) such as used in the fluorescence experiments we find that vesicles only containing egg PC are not affected by the protein.

A solvent exposed tryptophan residue is involved in binding membrane lipids

Further studies were needed to identify amino acid residues in GLTP that are likely involved in the interaction of the protein with vesicular membrane lipids. In principle, solvent exposed aromatic residues (Trp, Tyr or His) can be identified using photo-CIDNP NMR. Resonance

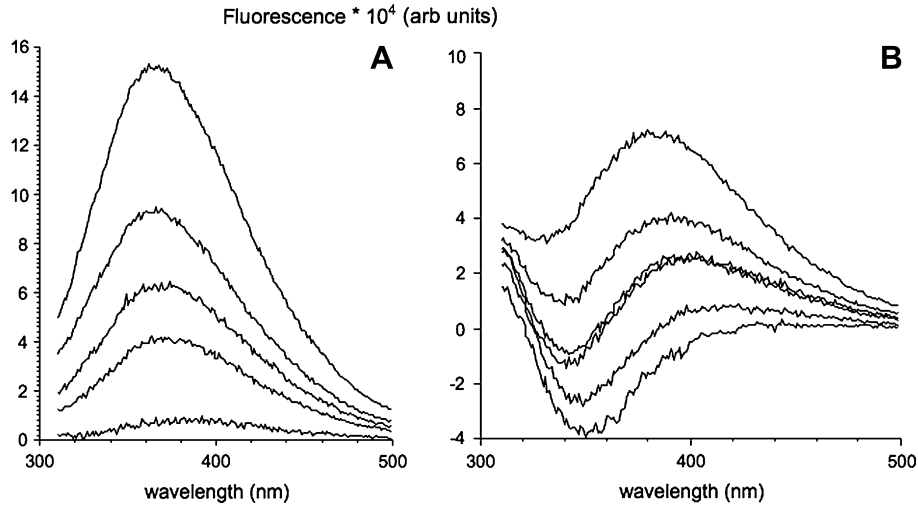

Fig. 3. Fluorescence difference spectra obtained by titration of GLTP (0.5 μM) with increasing amounts of vesicles. Panel A: titration with vesicles (total lipid 10 μM, 45 μM, 65 μM, 100 μM, and 155 μM) containing 60 mol% egg PC and 40 mol% cholesterol and panel B: titration with vesicles (total lipid 10 μM, 24 μM, 45 μM, 65 μM, 100 μM, and 155 μM) containing 30 mol% GlcCer, 30 mol% egg PC and 40 mol% cholesterol.

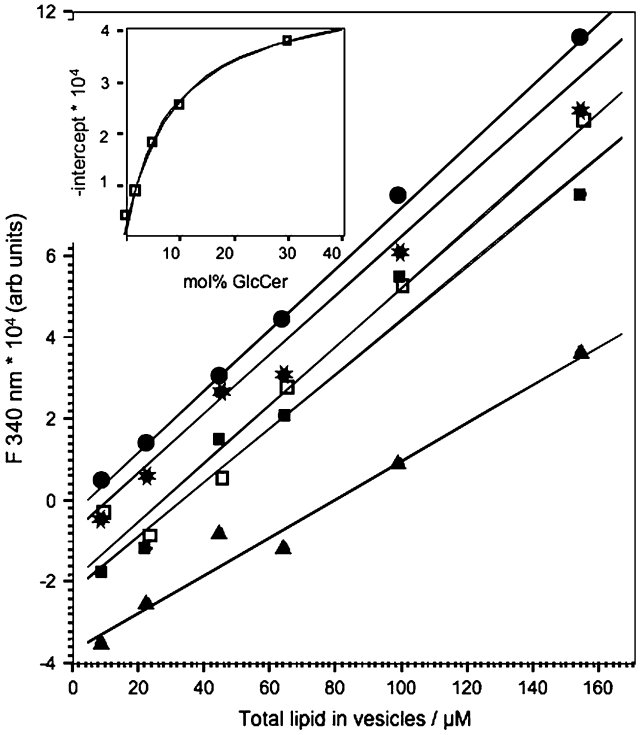

Fig. 4. Titration studies of GLTP (0.5 μM) with vesicles containing several mol fractions of GlcCer (0 mol%, ●; 2 mol%, ✸; 5 mol%, □; 10 mol%, ■; and 30 mol%, ▲). Total lipid concentrations were varied from 5 μM to 160 μM. Straight lines were fitted to data points taken from fluorescence difference spectra at 340 nm. Inset: replot of intercepts derived from the straight lines as a function of GlcCer content in vesicles added. Data are fitted to a hyperbolic function.

signals of these residues could become enhanced after interaction with a laser excited dye, but are attenuated when lipid molecules compete with the dye for interaction with the aromatic residues. Fig. 5 shows the photo-CIDNP NMR spectra of wild type GLTP (trace a), and of GLTP species incubated with vesicles (2.5 mM total lipid), either composed of GlcCer/egg PC/cholesterol (30/30/40 mol%, respectively; trace b) or egg PC/cholesterol (60/40 mol%, respectively; trace c). After centrifugation (1 h, 100,000*g*) spectra were recorded for the GLTP species at

Table 1
Radioactivity (% ^{3}H-labeled lipid in solution and pellet, respectively) measured after centrifugation of mixtures containing GLTP and vesicles

Vesicle composition + 40% cholesterol	Total lipid (mM)	GLTP (μM)	% ^{3}H in solution	% ^{3}H in pellet
60% Egg PC (^{3}H)	0.05	0	6 (±1)	94 (±1)
60% Egg PC (^{3}H)	0.05	1	7 (±2)	93 (±2)
60% Egg PC (^{3}H)	1	100	76 (±8)	24 (±8)
30% GlcCer/30% egg PC (^{3}H)	0.05	0	12 (±3)	88 (±3)
30% GlcCer (^{3}H)/30% egg PC	1	0	10	90
30% GlcCer (^{3}H)/30% egg PC	1	100	87	13

0.25 mM. For both kinds of vesicles the resonance positions of all peaks in the spectra remain the same as for apo-GLTP.

The resonances observed at 6.8 ppm, 6.9 ppm, 7.75 ppm, and 7.8 ppm are assigned to the single highly exposed Trp142 in GLTP (Mok and Hore, 2004) both in apo-GLTP and in GLTP containing glycolipid. Solvent exposure of Trp residues in GLTP was derived using the program DSSP (Kabsch and Sander, 1983). A small resonance at 10.2 ppm is assigned to the indole N1H of Trp142. This resonance is no longer visible in the spectrum for GLTP incubated with vesicles containing egg PC/cholesterol only (trace c). The narrow line widths of the Trp142 resonances in this 24 kDa protein point to a rather high flexibility of this exposed residue. Interestingly, two highly solvent exposed Tyr residues (Tyr153 and Tyr157) are not observed by photo-CIDNP.

In an attempt to observe exposure of aromatic residues other than Trp142 photo-CIDNP NMR studies of GLTP species were carried out at elevated temperatures. However, already at 40 °C the protein was found to denature and precipitate due to aggregation. This denaturation was rather modest for GLTP treated with vesicles containing 30 mol% GlcCer.

Structural consequences for GLTP upon uptake of glycolipid

Direct evidence for structural changes in GLTP resulting from incubations with vesicles containing 30 mol% GlcCer to GLTP was obtained from 2D NMR measurements. Fig. 6a and b shows HSQC spectra of apo-GLTP and an overlay of apo-GLTP with protein incubated with vesicles containing 30 mol% GlcCer, respectively. Careful inspection of the data reveals shifted NH resonances after incubation of GLTP with these vesicles. Due to the limited solubility of the protein and protein denaturation no detailed information about the residues involved in binding of the glycolipid could be obtained, however.

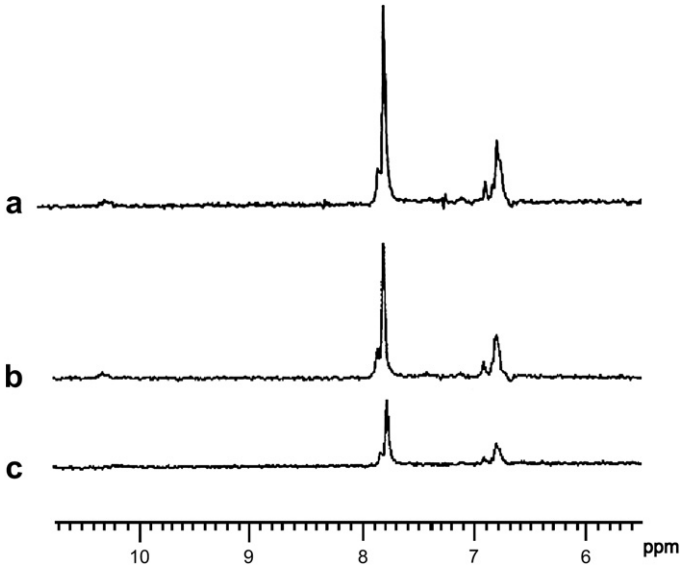

Fig. 5. Photo-CIDNP spectra of apo-GLTP (a), GLTP incubated with vesicles (2.5 mM total lipid) containing GlcCer (30 mol%), egg PC (30 mol%) and cholesterol (40 mol%) (b) or with vesicles (2.5 mM total lipid) containing egg PC (60 mol%) and cholesterol (40 mol%) (c). Protein concentrations are 0.25 mM for all.

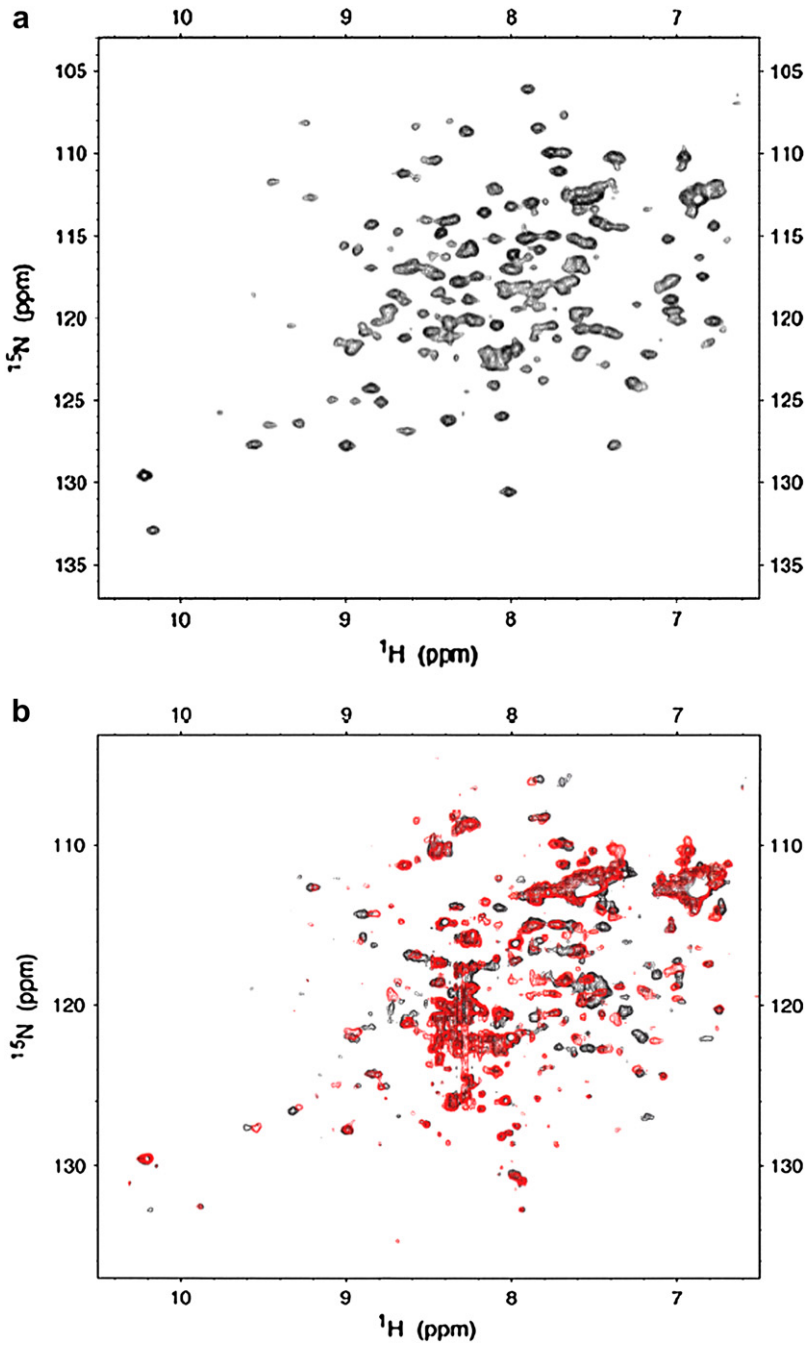

Fig. 6. (a) HSQC (heteronuclear single quantum correlation) spectrum of apo-GLTP and (b) Overlay of HSQC spectra of apo-GLTP (Warnock et al., 1994) and GLTP treated with vesicles containing 30 mol% GlcCer (red). Protein concentrations are 0.3 mM.

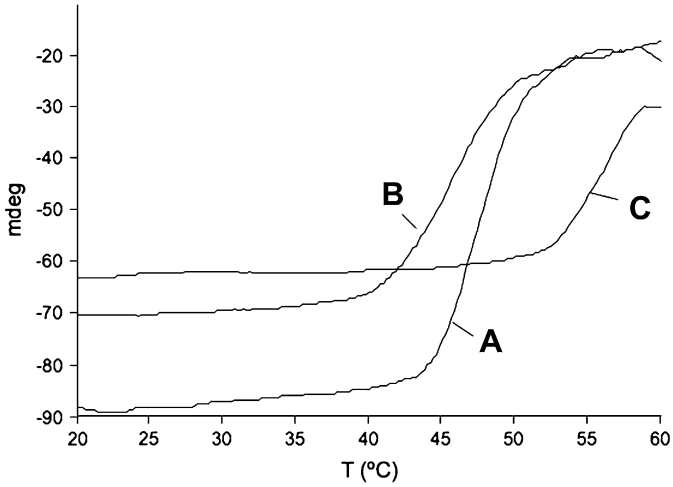

Fig. 7. Changes in α-helical content monitored at 220 nm for several GLTP species as a function of temperature. A: apo-GLTP, B: GLTP treated with vesicles consisting of 60 mol% egg PC and 40 mol% cholesterol, and C: GLTP treated with vesicles containing 30 mol% GlcCer, 30 mol% egg PC and 40 mol% cholesterol. Protein concentrations were 10 μM for A, and 7 μM for B and C, respectively.

Protein denaturation and the influence of bound GlcCer were studied further by CD experiments. The results of these experiments are summarized in Fig. 7. For all GLTP species the loss of α-helical content with increasing temperatures was found to be independent of protein concentration. As is shown in this figure the lipid-free GLTP starts to denature at 43 °C, while GLTP incubated with vesicles only containing PC and cholesterol even appears to be slightly less stable (curve B). In contrast, GLTP treated with vesicles containing 30 mol% GlcCer is much more stable than the other two species, as it starts to unfold at 53 °C.

Discussion

Proteins interacting with poorly water-soluble lipids are generally faced with the problem of how to react specifically and rapidly with lipid molecules when these are present in aggregated form such as micelles or membrane vesicles. The kinetics of molecular processes at lipid–water interfaces require special treatment (Berg et al., 2001). For a protein at least two separate binding steps – and binding domains – are needed for proper interaction with aggregated lipids, one interfacial binding step and another step involving a single lipid molecule. Using unilamellar vesicles containing 30 mol% GlcCer we find single turnover uptake of this lipid by GLTP to be a fast process. Furthermore, we obtained direct evidence that only half of the total amount of glycolipid in the vesicles is accessible to GLTP (Rao et al., 2004; Wong et al., 1984). This indicates that under these experimental conditions the vesicles stay intact even when all glycolipid has been extracted from the outer leaflet of the vesicular membrane. Titrations of fixed amounts of vesicles with GLTP yielded an affinity constant of GLTP for GlcCer uptake from these vesicles of approximately 25 nM. This value is close to the concentration of GLTP (10–30 nM for small or large unilamellar vesicles) at which half maximal transfer activity was observed between donor and acceptor vesicles in previous work (Rao et al., 2004). In our studies we observed signal changes due to glycolipid uptake from donor

vesicles in the absence of acceptor vesicles when GLTP is present in μM amounts. Thus, for obtaining proper kinetic data in fluorescence based resonance energy transfer assays GLTP concentrations should be far below the total concentration of transferred glycolipid.

Attempts were made to study the vesicular binding in more detail by monitoring the intrinsic fluorescence of GLTP when titrated with vesicles containing cholesterol (40 mol%), egg PC and GlcCer. The latter two lipids were varied such that the molar fraction of GlcCer varied between 0 mol% and 30 mol%, while their total remained at 60 mol%. As described before (Li et al., 2004) binding of glycolipid into the binding pocket of GLTP leads to a blue shift in the intrinsic fluorescence. In a recent study using tryptophan variants (West et al., 2006) the importance of Trp96 for glycolipid transfer by GLTP was demonstrated. Therefore, it is very likely that the blue shift in intrinsic fluorescence upon glycolipid uptake can be attributed to perturbation of Trp96 in GLTP. Using this intrinsic fluorescence we conclude from our titrations that GlcCer binding into the binding pocket is sensitive to the mole fraction of GlcCer present in the vesicles rather than its total concentration. In contrast to the high preference for GlcCer uptake no binding constant could be obtained for interaction of GLTP (at 0.5 μM) with the vesicles used up to 160 μM total lipid. Recently, direct evidence of the interaction of GLTP with vesicular membranes was obtained using labeled phospholipids (Rao et al., 2005). From this work it was concluded that GLTP binds to vesicles also in the absence of glycolipid, but binding constants are poor in the absence as well as the presence of glycolipid (around 200 μM total lipid). Given these binding constants it can be questioned whether vesicles stay intact when incubated with GLTP at protein concentrations around 200 μM. From our incubations of GLTP with radiolabeled lipids (Table 1) we concluded that the protein in the low μM ranges does not perturb the vesicular membranes. However, at high protein levels (*e.g.* 0.25 mM, as needed for NMR experiments), vesicular membranes were destroyed indeed leaving most of the lipid bound to the protein. This allowed us to identify amino acids at the surface of GLTP that are likely involved in membrane binding.

Using photo-CIDNP NMR an exposed and flexible Trp residue was clearly seen and identified as Trp142 based on its spectral properties (Mok and Hore, 2004), available structural data (Malinina et al., 2004) and solvent accessibility calculations (Kabsch and Sander, 1983). The photo-CIDNP NMR signals were considerably reduced when GLTP was incubated with vesicles of various compositions. The strongest reduction was noted for 0.25 mM GLTP incubated with 2.5 mM vesicles containing 60 mol% of egg PC. We propose egg PC to cluster near Trp142 thereby suppressing the photo-CIDNP signals. This would indicate that Trp142 together with nearby solvent exposed hydrophobic residues (among which Ile143 and Ile147) form part of the interfacial binding domain of GLTP. In Fig. 8 a model is shown indicating a possible involvement of Trp142 in membrane binding and uptake of GlcCer. The protein does not need to penetrate deeply into the membrane for allowing transport of a glycolipid molecule to its binding pocket. When GLTP is incubated with vesicles containing 30 mol% GlcCer and 30 mol% egg PC (either lipid in three-fold molar excess over GLTP) the photo-CIDNP signals show higher intensities as found for vesicles not containing glycolipid (Fig. 2). This can be explained when a substantial fraction of GlcCer is bound into the glycolipid binding pocket near Trp96 (West et al., 2006; Yamada et al., 1985), which is remote from the interfacial binding site around Trp142. Thus, in contrast with previous suggestions (Rao et al., 2005; West et al., 2006) we do not find evidence for a direct involvement of Trp96 in membrane binding.

The chemical shift perturbation data obtained upon treatment of GLTP with vesicles containing 30 mol% GlcCer clearly indicate that considerable conformational changes take place in the protein due to interaction with glycolipid. Close inspection of the crystal structure data

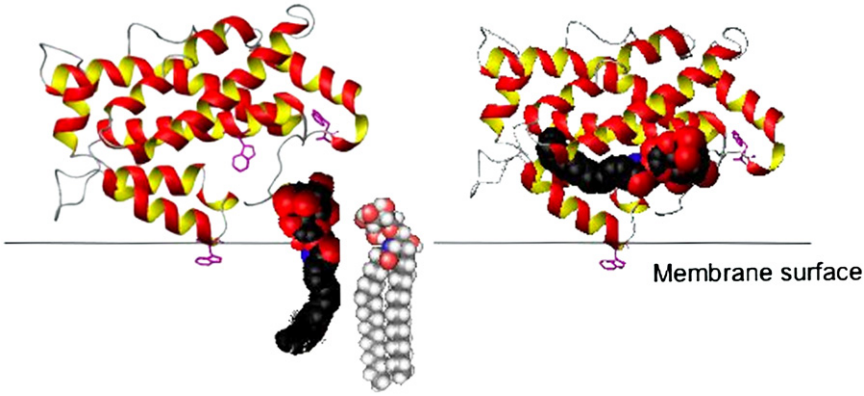

Fig. 8. GLTP structures showing apo-GLTP (from 1swx.pdb, *e.g.* at http://www.ebi.ac.uk/msd) with the location of the three Trp residues present and GLTP containing bound LacCer (from 1sx6.pdb). The suggested role for Trp142 in interfacial binding is indicated. Trp96 becomes hidden by bound glycolipid after transfer to the binding pocket.

for apo-GLTP and protein containing glycolipid (Malinina et al., 2004) leads to the conclusion that about 20 backbone atoms of amino acid residues in close proximity to the glycolipid change their position in the structure. Since protein flexibility is likely needed for proper accommodation of GlcCer, the influence of GlcCer on protein stability was considered to be of interest. The effect of heat treatment on GLTP was investigated by CD measurements. While the α-helical content was found to drop rapidly at temperatures above 43 °C, the presence of bound glycolipid improved the stability of GLTP by 12 °C. In contrast, lipid vesicles without glycolipid slightly destabilized the protein. Heat stability of GLTP has been studied before using glycolipid transfer rates as a measure of protein denaturation (Nylund and Mattjus, 2005); however, the stabilizing effect of bound glycolipid remained unnoticed.

Consequences of high affinity for GlcCer for in vivo action of GLTP

Considering the high affinity of GLTP for GlcCer but the very low affinity for membranes one could assume that GLTP is required to keep GlcCer in a soluble form within the cytosol. As such GLTP could act as a buffer for GlcCer and deliver it when and where it is needed. Compared to other lipid transfer proteins like FAPP2 (EC number not assigned), which has a PH domain that targets the protein to the Golgi apparatus (Godi et al., 2004) or CERT (EC number not assigned), which contains a PH domain for binding to the Golgi and a FFAT domain for binding to VAP proteins in the ER (Kawano et al., 2006) it is difficult to identify membranes that GLTP would bind to. We and others (Rao et al., 2005) find that the efficiency of uptake of glycolipid by GLTP depends on the mole fraction of the glycolipid. This characteristic of GLTP is likely important in defining donor or acceptor membranes *in vivo*. However, up to date there are no techniques available to measure lipid transfer *in vivo*, which would help to answer the questions concerning the identity of donor and acceptor membranes *in vivo*.

Summary

In conclusion, we have found that the efficiency of uptake of glycolipid by GLTP depends on the mole fraction of the glycolipid in the outer leaflet of vesicular membranes. Binding of GlcCer to the protein already occurs at nM levels of GLTP. It is proposed that glycolipid

transfer efficiency will be determined mainly by the rate of release of glycolipid from GLTP. For efficient transfer *in vivo* most likely additional factors are needed for proper GLTP action.

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