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Glycolipid Transfer Protein Interaction with Bilayer Vesicles: Modulation by Changing Lipid Composition

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ABSTRACT Glycosphingolipids (GSLs) are important constituents of lipid rafts and caveolae, are essential for the normal development of cells, and are adhesion sites for various infectious agents. One strategy for modulating GSL composition in lipid rafts is to selectively transfer GSL to or from these putative membrane microdomains. Glycolipid transfer protein (GLTP) catalyzes selective intermembrane transfer of GSLs. To enable effective use of GLTP as a tool to modify the glycolipid content of membranes, it is imperative to understand how the membrane regulates GLTP action. In this study, GLTP partitioning to membranes was analyzed by monitoring the fluorescence resonance energy transfer from tryptophans and tyrosines of GLTP to *N*-(5-dimethyl-aminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phospho-ethanolamine present in bilayer vesicles. GLTP partitioned to POPC vesicles even when no GSL was present. GLTP interaction with model membranes was nonpenetrating, as assessed by protein-induced changes in lipid monolayer surface pressure, and nonperturbing in that neither membrane fluidity nor order were affected, as monitored by anisotropy of 1,6-diphenyl-1,3,5-hexatriene and 6-dodecanoyl-*N*,*N*-dimethyl-2-naphthylamine, even though the tryptophan anisotropy of GLTP increased in the presence of vesicles. Ionic strength, vesicle packing, and vesicle lipid composition affected GLTP partitioning to the membrane and led to the following conclusion: Conditions that increase the ratio of bound/unbound GLTP do not guarantee increased transfer activity, but conditions that decrease the ratio of bound/unbound GLTP always diminish transfer. A model of GLTP interaction with the membrane, based on the partitioning equilibrium data and consistent with the kinetics of GSL transfer, is presented and solved mathematically.

INTRODUCTION

Glycosphingolipids (GSLs) are essential for proper functioning and development of mammalian cells and are involved in important processes, including cell specific adhesion and cell-cell interactions. In cancer cells, expression of certain GSLs like glucosylceramide has been associated with multidrug resistance (1). In certain viral and bacterial diseases (e.g., HIV, BSE, cholera, *Helicobacter pylori*), GSLs serve as surface attachment sites during infection (2) and may carry out chaperone-like functions to help avoid protein misfolding (e.g., Alzheimer disease) (3). GSL involvement appears to be linked to their enrichment in liquid-ordered membrane microdomains, i.e., rafts and caveolae (4–6), which putatively function as organization sites for many signaling proteins, i.e., epidermal growth factor receptor, insulin growth factor receptor, tyrosine kinase, G-proteins, protein kinase C isozymes, and tumor necrosis factor-α during signal transduction processes (7). Because lipid raft and caveolae constituents play crucial roles in so many cell functions, there is immense therapeutic potential in being able to modulate the composition of lipid rafts (2). For example, cholesterol-depleting substances like β-cyclodextrin are being tested in animal models as a basis for the development of drugs to prevent transmission of HIV-1 (8). Another option for altering lipid raft compositions is to develop protein-based strategies that involve selective transfer of the target lipid constituent to or from the lipid raft domain.

Glycolipid transfer protein (GLTP) is a peripheral protein that promotes selective and net transfer of GSLs between model membranes (9–11). The highly conserved sequence homology among mammalian GLTPs (12–14) and the unique folding conformation used to ligand glycolipid (15) suggest that GLTP defines a newly emerging protein family among lipid transfer/binding proteins. The crystal structures of apo-GLTP (1.65 Å) and of a GLTP/glycolipid complex (1.95 Å) reveal a topology dominated by α-helices, containing a single site for liganding glycolipid and providing a clear picture of how GSL is accommodated by GLTP (15). The liganding site is composed of a sugar headgroup recognition center that uses multiple hydrogen bonds and van der Waals contacts to selectively anchor the sugar-amide moieties to the protein surface, and a hydrophobic tunnel that accommodates the hydrocarbon chains of ceramide. B-factor analyses and comparison of the apo- and GSL-bound forms of GLTP

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Abbreviations used: GSL, glycosphingolipid; GLTP, glycolipid transfer protein; RET, resonance energy transfer; POPC, 1-palmitoyl-2-oleoy-*sn*-glycero-3-phosphocholine; SPM, sphingomyelin; GalCer, galactosylceramide; dansyl DHPE, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine; SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; POPS, 1-palmitoyl-2-oleoy-*sn*-glycero-3-phosphoserine; Trp, tryptophan; Tyr, tyrosine; PI, phosphatidylinositol.

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suggest that glycolipid liganding occurs via an adaptive recognition process. A cleft-like gating mechanism, involving conformational changes to two interhelical loops and one α-helix, appears to facilitate entry of the lipid chains during the liganding process, when the GSL sugar headgroup is tethered to the recognition center. The surface region of GLTP surrounding the glycolipid liganding site appears to serve as a membrane interaction region that is surrounded by many nonpolar amino acids along with four tyrosines, two tryptophans, and six lysines, residues known to interact favorably with membrane interfaces (16,17). This putative membrane interaction region differs from other known membrane interaction motifs, such as the C1 and C2 domains associated with many phospholipases and protein kinases.

From a biotechnological standpoint, GLTP is an attractive candidate to use for probing and remodeling the GSL compositions of lipid rafts because of the protein's selectivity for GSLs. To evaluate the potential of GLTP for remodeling rafts and to continue to gain insights into possible cellular functions, it is imperative to understand the mechanism by which GLTP accomplishes the intermembrane transfer of GSLs and how changes in the lipid composition of membranes affect GLTP action. Recently, significant advances have been made in understanding the mechanism of GLTP action (14) and in showing how changes in membrane lipid composition can affect GLTP activity (18–20). In our kinetic and thermodynamic analyses of GLTP action (14), we proposed that the rate-limiting step of GLTP-mediated GSL intermembrane transfer is formation of the GLTP-GSL complex at the membrane surface rather than the partitioning of GLTP into the lipid-water interface. Our model relied on kinetic analyses of the glycolipid being transferred by GLTP, without the benefit of experimental evidence showing direct interaction of GLTP with vesicles.

The objectives of this study were to develop a means to quantitatively evaluate the interaction of GLTP with membrane vesicles and to assess how membrane characteristics, such as GSL concentration and matrix lipid composition, affect interaction between GLTP and membranes. Because human GLTP has three tryptophans (Trp) and 10 tyrosine (Tyr) residues among its 209 amino acids, the protein is naturally fluorescent (13). The three Trps and several of the Tyr residues are located near the surface of GLTP (13,15), positioning them favorably for possible resonance energy transfer (RET) to membranes containing lipids with headgroup-labeled fluorophores. Here, we report the development of a RET-based approach for directly quantitating the interaction of human GLTP with bilayer vesicles containing phosphatidylethanolamine with a dansyl-labeled headgroup (dansyl-DHPE). The utility of the RET approach was demonstrated by addressing several issues of importance in the regulation of GLTP partitioning to/from membranes. The issues included the effect of: 1), deleting glycolipid versus increasing the glycolipid concentration in the membrane; 2), altering ionic strength; 3), changing the lipid packing using membranes with differing curvature; 4), altering membrane surface charge; and 5), altering membrane sphingomyelin content. We also showed that minimal perturbation of the bilayer lipids occurs when GLTP interacts with the membrane.

MATERIALS AND METHODS

Lipids

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), and porcine brain galactosylceramide (GalCer) and bovine brain sphingomyelin (SPM) were purchased from Avanti Polar Lipids (Alabaster, AL). Avanti indicates the fatty acyl composition of the GalCer to be a 60:40 mixture of nonhydroxylated and 2-hydroxylated acyl chains with the nonhydroxylated fatty acyl distribution being 6% palmitate (16:0), 7% stearate (18:0), 3% arachidate (20:0), 2% arachondate (20:4), 11% behenate (22:0), 22% lignocerate (24:0), and 9% nervonate (24:1). For SPM, the indicated fatty acyl composition is 2% palmitate (16:0), 49% stearate (18:0), 5% arachidate (20:0), 8% behenate (22:0), 6% lignocerate (24:0), and 20% nervonate (24:1). Our previous analyses by capillary gas chromatography have revealed generally similar fatty acyl distributions (48,49). The fluorescent lipids, *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-O-β-galactosylsphingosine [AV-GalCer] and *rac*-1,2-dioleoyl-3-[9-(3-perylenoyl)-nonanoyl]-*sn*-glycero-3-phosphocholine [Per-PC] were prepared as described earlier (21,22). *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (dansyl-DHPE), triethylammonium salt, was purchased from Molecular Probes (Eugene, OR). Stock concentrations of phospholipids were quantitated using the Bartlett method (23) of GalCer, by gravimetric analyses, and of fluorescent lipids, by their extinction coefficients.

GLTP

Recombinant human GLTP was generated by molecular cloning, heterologous expression, and affinity purification as described previously (12,13,15). Protein purity and concentration were determined by SDS-PAGE (13) and bicinchoninic acid (24), respectively.

Preparation of small unilamellar vesicles

The acceptor vesicles were prepared by sonication using a modification of the established procedure by Huang and Thompson (25). Briefly, a lipid film was obtained by slowly evaporating the appropriate mixture in solvents at 37°C on a rotary evaporator, followed by freeze-drying in vacuum for 6 h. The dried lipid film was suspended by vortexing in a sodium phosphate buffer (pH 7.4) to a concentration of 50 mM. The suspension was sonicated with a Heat Systems-Ultrasonics W-225 sonifier on ice, under nitrogen and was then centrifuged for 90 min. at 100,000 $\times$ *g* to remove titanium probe particles and residual multilamellar vesicles. Analysis of the resulting small unilamellar vesicle (SUV) populations by size exclusion chromatography confirmed average diameters of ~25 nm, consistent with previously published values (26,27).

Preparation of large unilamellar vesicles (LUV)

Donor LUV vesicles of appropriate composition were prepared by extrusion through 100 nm size polycarbonate membranes. The appropriate amounts of lipids from stock solutions were thoroughly mixed and dried under nitrogen and then under vacuum for 1 h. The dried lipid mixture was hydrated in the appropriate buffer and subjected to 15 freeze-thaw cycles to ensure uniform distribution of buffer solutes across the bilayers. Rapid freezing was

achieved by immersing the lipid suspension in an isopropanol bath cooled by dry ice. During each thawing cycle, the lipid dispersion was raised above 70°C and vortexed before subsequent freezing. The lipid suspension was then extruded with 21 passes through 100 nm polycarbonate membrane using a hand-held miniextruder (Avanti, Alabaster, AL). The resulting vesicles had a narrow size distribution (28) and mean diameter of 100 nm as measured by size exclusion chromatography using a calibrated Sephacryl S-1000 column (26,27).

Fluorescence measurements

Steady-state fluorescence measurements were performed using a SPEX Fluoromax instrument (Instruments S.A., Edina, NJ). The excitation and emission bandpasses were 5 nm and the cuvette holder was temperature controlled to $T \pm 0.1$°C (Neslab RTE-111, Neslab Instruments, Portsmouth, NH) where T was in the experimental range of 30–44°C. For GLTP partitioning experiments, Trp and Tyr in GLTP were excited at 285 nm while monitoring dansyl-DHPE emission at 513 nm.

Activity of GLTP

To assess activity of GLTP, a fluorescence-based RET assay involving anthrylvinyl labeled glycolipid (AVGalCer) (1 mol%) and a nontransferable perylenoyl-labeled phosphatidylcholine (PerPC) (1 mol%) was used to enable continuous real-time monitoring of GLTP activity. Excitation and emission wavelengths were 370 and 425 nm, respectively. Other details of the assay and measurement of initial velocity of transfer were presented elsewhere (14,29).

Partitioning of GLTP into lipid phase

The interaction of GLTP with lipid vesicles (both SUVs and LUVs) was assessed using fluorescence approaches that measure the RET from the Trp and Tyr residues of GLTP to the dansyl moiety attached to the headgroup of the 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (DHPE) dispersed within the vesicles (Fig. 1). The lipid vesicles were prepared so that the incorporated dansyl-DHPE was always a constant 10 mol% of the total lipid. The remaining 90% of the lipid mixture contained varying amounts of POPC and GalCer. The following equation was used to quantify the amount of bound protein to the vesicles.

$$F(P_0) = \left(\frac{F_b - F_i}{F_i}\right) \times 100. \quad (1)$$

F_b is the emission intensity at 513 nm in the presence of adsorbed protein, and F_i is the emission intensity in the absence of protein. Controls performed using free Trp concentrations comparable to the Trp concentration in GLTP revealed a contribution of <3% of the total signal and enabled corrections by spectral subtraction. Final signals also were corrected for the volume change caused by protein addition. In no instance did the volume corrections exceed 5% of the initial value. The effects of light scattering were minimized by maintaining a constant lipid vesicle concentration and by titrating with increasing amounts of protein. For the experiments involving the addition of dansyl-free SUVs, the vesicle-induced scattering was corrected by parallel measurement of the appropriate scattering contribution. The interaction parameter K_a was measured from $F(P_0)$ by using the following equations;

$$F(P_0) = F_b\left[\frac{P_0 + K_d + L_0/n - \sqrt{(P_0 + K_d + L_0/n)^2 - 4P_0L_0/n}}{2}\right] \quad (2)$$

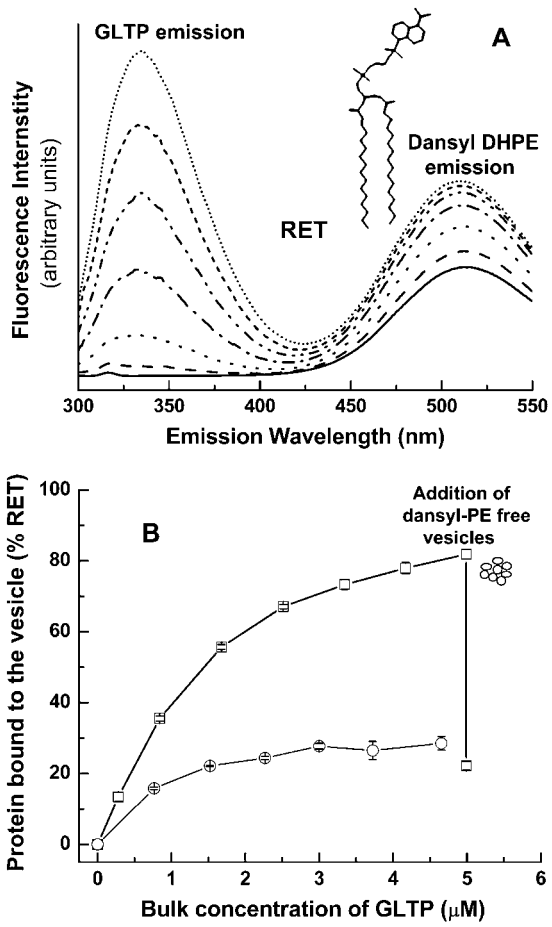

FIGURE 1 (*A*) RET between GLTP and PC vesicles containing dansyl-DHPE and glycolipid. Emission wavelength spectra were acquired while exciting at 285 nm. Samples were stirred continuously before and after adding GLTP to SUVs composed of POPC/GalCer/dansyl-DHPE (70:20:10 mol%). The increasing fluorescence intensity at 513 nm is indicative of dansyl emission via RET from the Trp and Tyr residues of the protein. (*B*) Partitioning isotherm for GLTP to PC vesicles containing GalCer and dansyl-DHPE (70:20:10 mol%). Data in panel *A* representing the emission intensity measured at 513 nm were used to calculate the binding isotherm for GLTP. Protein bound to the vesicles is presented as relative %RET ($F_b - F_i/F_i$) where F_b and F_i are fluorescence emission intensities of vesicles in the presence and absence of protein, respectively. The error bars represent standard deviation of three experiments performed at 37°C in phosphate buffered saline (2.5 ml) at pH 7.4.

$$F(P_0) = F_bP_0\frac{L_0}{nK_d + L_0}. \quad (3)$$

P_0 is the total protein, W is the molar concentration of water (55.3 M at 37°C), L_0 is the total lipid concentration (typically 143 μM), F_b is the emission intensity of the bound protein to the RET, and n is the number of lipid molecules corresponding to the area occupied by the protein at the lipid interface. The derivations of these equations are presented in the Appendix. Briefly, for partition isotherms that exhibited saturation, sets of K_a values were determined by nonlinear regression of Eq. 2 with fitting parameters K_a, n, and F_b. For the isotherms that did not exhibit saturation (linear), K_a was determined using Eq. 3. The surface binding constant for GLTP-GSL complex K_s was determined by nonlinear regression using Eq. 4.

$$K_a = \frac{K_1(1 + x_s K_s)W}{(W - x_s K_s K_1 L_0)}. \quad (4)$$

Equation 4 is derived in the Appendix. K_1 is the interaction parameter when no GSL is present, and x_s is the GSL mol fraction in the lipid phase. All regressions were performed at a confidence interval of 95% using ORIGIN 7.0 software (Origin Lab, Northampton, MA).

Emission anisotropy measurements

DPH and Laurdan anisotropies in the membrane bilayer were measured using excitation and emission wavelengths of 360 and 430 nm for DPH and of 360 and 480 nm for Laurdan. Trp anisotropy of GLTP (2 μM) was measured in the presence and absence of vesicles (140 μM) using excitation and emission wavelengths of 295 and 347 nm (13). The temperature was kept constant at 37 ± 0.1°C. Anisotropy and total fluorescence emission were calculated using the following equations.

$$I_{tot} = I_{vv} + 2GI_{vh}$$
$$r(T) = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}}$$
$$G = \frac{I_{hv}}{I_{hh}}.$$

In these equations, v and h represent the respective orientations (vertical or horizontal) of the excitation and emission polarizers. In all experiments, parallel measurements of intensities were performed for samples and controls using an automated, four-position cuvette holder. Controls were included for buffer and protein alone. The background intensities were subtracted from individual components before calculating the G factor and anisotropy values.

Determination of GLTP penetration into lipid monolayers

A slightly modified experimental design and its application have been described previously in detail (50). Briefly, a cylindrical Teflon trough (surface area) 3.93 cm^2, (volume) 4.7 mL) was filled with HEPES buffered saline. The temperature was held at 24°C. Lipid films were spread from a hexane/isopropanol/water (70:30:2.5) solution (0.6 nM) until the desired surface pressure was reached, as detected using a nichrome Wilhemy wire. After allowing the lipid monolayer to stabilize for 10 min, stirring (90 rpm) was started, and after 20 min, GLTP solution was injected to 115 nM in the buffered aqueous phase. Stirring was continued for 10 min, and the surface pressure was continuously monitored to assess penetration of GLTP into monolayers composed of either POPC or POPC/GalCer (90:10).

RESULTS

To determine whether GLTP interacts directly with lipid bilayers, fluorescence RET was monitored from the Trp and Tyr residues of GLTP to dansyl-DHPE in unilamellar vesicles. Fig. 1, *A* and *B*, show the RET signal responses resulting from the partitioning of GLTP to SUVs comprised of POPC, GalCer, and dansyl-DHPE (70:20:10). Excitation at 285 nm, in the presence of increasing amounts of GLTP, resulted in stepwise increases in the emission intensity of the dansyl moiety of dansyl-DHPE (Fig. 1 *A*; 513-nm peak). To confirm that the observed RET was caused by GLTP association with the vesicles, two kinds of controls were performed. First, measurements of free Trp at concentrations equivalent to GLTP Trp resulted in RET responses that were only ~3% of those observed with GLTP (data not shown). Second, when an excess of dansyl-free vesicles was added to mixtures of GLTP and vesicles containing dansyl PE that had already incubated together for 15 min at 37°C (Fig. 1 *B*), a sudden and dramatic decrease in the emission intensity of the dansyl fluorophore was observed, consistent with rapid dissociation of GLTP from the vesicles containing dansyl-DHPE and redistribution to dansyl-free vesicles.

GSL concentration in PC vesicles and GLTP partitioning

The K_a values associated with the partitioning of GLTP to SUVs containing increasing amounts of glycolipid at 10 mM phosphate are presented in Fig. 2. The partitioning of GLTP depended strongly on the bilayer concentration of GalCer, as shown by the elevations in GLTP associated with the lipid phase when the vesicles contained higher GalCer concentrations. Both the saturation limit and the slope of the partition isotherm were a function of GalCer concentration in the SUVs. The partitioning appeared to reach saturation at lipid/protein ratios near 50:1. However, it is noteworthy that partitioning of GLTP to the lipid phase was clearly observed when POPC vesicles contained no GalCer at all (Figs. 1 *B* and 2), consistent with GLTP having an inherent attraction for the membrane interface. This finding suggested that GLTP interacts with the membrane first and then forms a GLTP/glycolipid complex, a scenario hypothesized as part of a model for GLTP action developed from our previous kinetic studies (14). In this model (Fig. 3), GLTP first partitions into the lipid phase in a nonspecific manner. Once at the lipid-water interface, GLTP interacts with GSL and forms a complex that is released into the bulk. This cycle repeats until a steady state

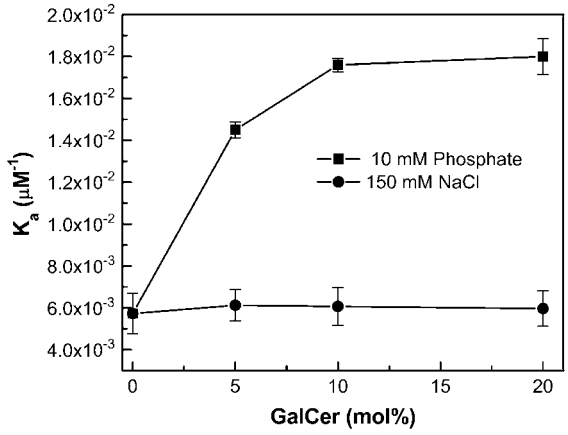

FIGURE 2 Effect of increasing GalCer membrane concentration on GLTP partitioning at low and physiological salt concentrations. The error bars represent standard deviation of three experiments performed at 37°C at pH 7.4.

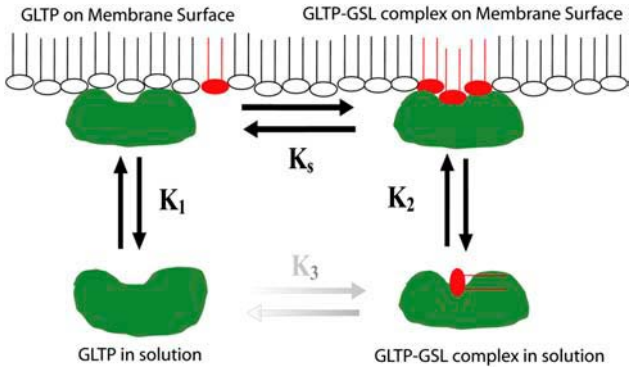

FIGURE 3 Schematic of the proposed mechanism of GLTP binding to vesicles containing GalCer. Free GLTP from the bulk (P_f) partitions nonspecifically into the lipid phase (P_L). P_L then interacts with the GalCer (L_L) at the interface. The GLTP bound to GSL at the interface may be released into the bulk (P_fL_f). The interaction parameter K_a is a function of K_1, K_2, and K_s. $K_a = (K_2(K_1+x_sK_sK_1)/(K_2+x_sK_sK_1))$. In the absence of GSL at the interface, $K_a = K_1$.

is achieved. The rate-limiting step in GSL transfer is either the formation of GLTP-GSL complex or its desorption from the membrane interface. Based on the partitioning isotherms observed between GLTP and PC vesicles and the kinetic model, appropriate equations were formulated for describing the partitioning events (see Materials and Methods; Appendix). Use of Eq. 4 enabled evaluation of the parameters associated with the nonspecific interaction of GLTP with the lipid phase, K_1, and the selective acquisition of GSL by GLTP resulting in GLTP/GSL complex formation at the membrane surface, K_s. The values were $K_1 = 5.83 \pm 0.35 \times 10^{-3}$ μM and $K_s = 4.75 \pm 0.68$, determined by nonlinear regression at a confidence interval of 95% ($R^2 = 0.98$).

Ionic strength affects GLTP partitioning to vesicles

To evaluate the nonspecific interaction of GLTP with PC vesicles and the effect of increasing GalCer membrane concentration on the partitioning of GLTP to the PC vesicle surface, the interaction between GLTP and vesicles was assessed at different increasing salt concentrations (150 and 500 mM NaCl). In general, at higher salt concentrations, the partitioning equilibria shifted toward the bulk phase. With 150 mM NaCl, a threefold decrease in the partitioning of GLTP to the vesicles was observed (Table 1); whereas, no partitioning of GLTP to the vesicles was detectable by RET in the presence of 500 mM NaCl. Fig. 2 also shows that the increase in GLTP partitioning to the lipid phase induced by GalCer at low ionic strength was eliminated at 150 mM NaCl. The K_a values associated with GLTP partitioning to vesicles containing glycolipid (5, 10, or 20 mol%) at 150 mM NaCl were similar to values observed when glycolipid was absent from the vesicles (Fig. 2). Interestingly, however,

TABLE 1 Effect of NaCl on GLTP binding to uncharged SUV with composition 10% dansyl-DHPE, 20% GalCer, 70% POPC

Salt	ΔF_{max}*	v_0 (1/s)*	K_a (μM^{-1})
No salt	2.1 ± 0.1	$1.7 \pm 0.2 \times 10^{-2}$	$5.7 \pm 0.5 \times 10^{-2}$
150 mM NaCl	2.1 ± 0.2	$1.6 \pm 0.2 \times 10^{-2}$	$1.9 \pm 0.3 \times 10^{-2}$
500 mM NaCl	1.8 ± 0.1	$3.0 \pm 0.3 \times 10^{-3}$	†

*All kinetic measurements were performed using SUV donor vesicles containing 1% AV-GalCer and 1% PerPC in POPC matrix, produced by rapid ethanol injection as described previously (14,18,19,29).
†No value detected.

in the presence of 150 mM NaCl, the initial velocity of GLTP-mediated transfer of glycolipid was similar to that observed under low-salt conditions (Table 1). Thus, enhancement of the partitioning of GLTP to membrane vesicles containing glycolipid did not guarantee faster initial rates of glycolipid transfer by GLTP, an observation consistent with the idea that GLTP partitioning to the lipid phase is distinct from the proposed rate-limiting process of GLTP-GSL complexation process within the membrane or GLTP/GSL complex desorption from the membrane. Because nonspecific interaction of GLTP with vesicles is unaffected by salt concentration, our findings also suggested that either GLTP-GSL complex formation or desorption at the membrane surface is sensitive to changing ionic strength.

GLTP interaction with membranes is nonperturbing

To determine whether the partitioning of GLTP to the membrane perturbs the lipid packing of the bilayer, fluorescence anisotropy measurements were performed on the vesicles at 37°C using DPH and Laurdan. These probes provide insights into the order and fluidity of the lipid bilayers (30–33). Fig. 4, *A* and *B*, show the anisotropy values observed for each probe when GLTP amounts sufficient to saturate the RET response were mixed with vesicles containing 0, 10, or 20 mol% GalCer at low ionic strength (10 mM NaCl). GLTP partitioning to the vesicles did not affect the anisotropy values of either membrane localized fluorescent probe over the GalCer range of 0–20 mol%. The observed anisotropy values were consistent with known values for liquid crystalline bilayers and suggested that neither the order nor fluidity of the bilayers is altered by association with GLTP. However, because of inherent uncertainties in quantitating the absolute amount of membrane-bound GLTP by the RET approach, additional experiments were performed to verify the presence of GLTP on the vesicle surface. Fig. 4 *C* shows the steady-state Trp anisotropy of GLTP in the absence and presence of vesicles composed of POPC and of POPC/GalCer (90:10). It is noteworthy that increases in Trp anisotropy were clearly observed when GLTP was incubated with vesicles of either composition, consistent with Trp being involved in the membrane

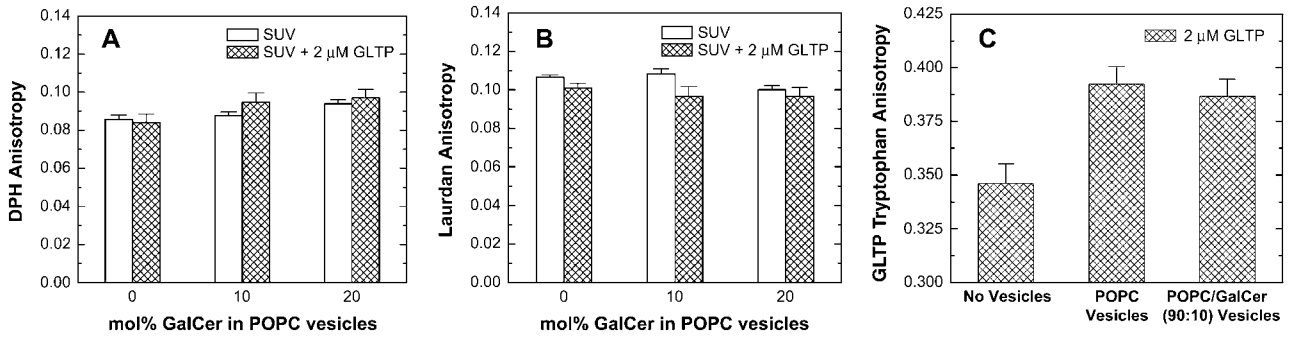

FIGURE 4 Anisotropy of DPH (*A*) and Laurdan (*B*) in bilayer membranes in the presence and in the absence of GLTP. Experimental conditions are described in Materials and Methods. (*C*) The steady-state anisotropy of the tryptophans residues of GLTP in the absence and presence of POPC and POPC/GalCer (90:10) vesicles. Experimental conditions are described in Materials and Methods. In each panel, the error bars represent average of three experiments done at 37°C at pH 7.4.

interaction site of the protein. This involvement is not surprising because our recent structural analyses of GLTP by x-ray diffraction (1.95 Å) show that two of GLTP's three tryptophans residues (Trp-142 and Trp-96) are favorably positioned for involvement in the membrane interaction site of the protein by virtue of close proximity to the glycolipid liganding site (15).

To confirm that GLTP does not perturb the membrane surface during interaction, experiments were performed to determine the surface pressure at which GLTP fails to penetrate a lipid monolayer from the subphase. Fig. 5 shows that GLTP produces no change in surface pressure when the initial surface pressure of the POPC monolayer is 23 mN/m or higher. A similar limiting value for the initial surface pressure ($\pi \approx 22$ mN/m) was observed when GLTP was incubated beneath POPC/GalCer monolayers (90:10). These findings indicate that GLTP does not penetrate monolayers poised at surface pressures of 30–35 mN/m, conditions that mimic the lipid packing environment found in biomembranes (51). Thus, the monolayer data suggest that GLTP interacts in a nonperturbing way with membranes.

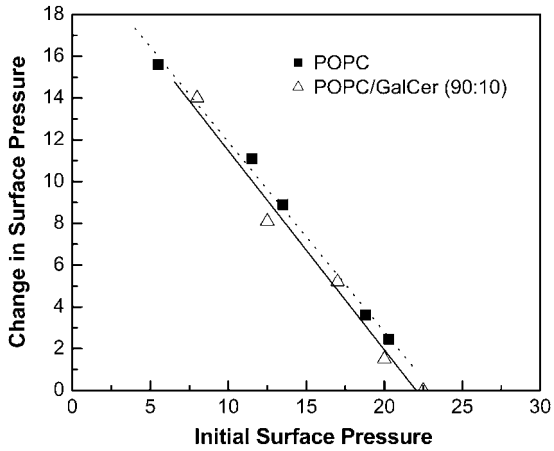

FIGURE 5 Penetration capacity of GLTP for lipid monolayers poised at different initial surface pressures. POPC films are denoted by solid squares. The *x*-intercept denoting $\pi c = 23.1$ mN/m. Linear regression analysis (*dotted line*) resulted in a correlation coefficient of 0.99745. POPC/GalCer films are denoted by open triangles. The *x*-intercept denoting $\pi c = 21.9$ mN/m. Linear regression analysis (*solid line*) resulted in a correlation coefficient of 0.9923.

Membrane curvature affects GLTP partitioning to the bilayer interface

As part of our recent kinetic and thermodynamic analysis of the intervesicular transfer of glycolipid by GLTP (14), we compared the glycolipid transfer rates from SUVs and LUVs and found approximately fivefold faster initial departure rates from donor SUVs (~25 nm diameter) than from donor LUVs (~100 nm diameter). Faster glycolipid transfer from SUVs compared to LUVs also has been observed for bovine GLTP (20). To determine whether SUVs enhance the partitioning of GLTP to the membrane surface compared to LUVs, the RET assay was used to determine the GLTP interaction parameter, K_a, from the respective partitioning isotherms obtained when both the SUVs and LUVs contained 10 mol% GalCer (data not shown). The K_a value for the LUVs was five times smaller ($3.44 \pm 0.3 \times 10^{-3}$ μM) than that for the SUVs ($1.76 \pm 0.5 \times 10^{-2}$ μM). A noteworthy point is that the initial partitioning of GLTP was most affected by vesicle curvature. The elevated partitioning of GLTP to curvature-stressed membranes could have physiological consequences by enabling GLTP targeting to locally curved membrane surfaces involved in processes such as membrane budding and fusion.

GLTP partitioning vesicles containing negatively charged phosphoglyceride

Mattjus et al. (18) found that the presence of charge on the membrane surface impedes the GLTP-mediated intermembrane transfer of GalCer at low ionic strength. Increasing salt levels to physiologic ionic strength restored GLTP transfer rates

such that little difference was observed between charged and zwitterionic membrane donor vesicles. The diminished transfer rate at low ionic strength was attributed to a reduced ''off-rate'' of the GLTP/GSL complex from the membrane. However, this conclusion was based solely on kinetic measurements of glycolipid transfer between SUVs without direct assessment of GLTP partitioning to the membrane and before the realization that GLTP partitioning to the membrane and GLTP/GSL complex formation on the membrane surface are distinct steps in the transfer process. To determine how the presence of negative charge at the membrane surface affects GLTP partitioning, RET measurements were performed to localize GLTP after mixing with vesicles containing various amounts of POPS (0–20 mol%) along with 10 mol% GalCer. Fig. 6 shows the partitioning response observed in the presence of different NaCl concentrations. At low ionic strength, the K_a values increased as POPS content of the membrane increased, resulting in the K_a value at 20 mol% POPS being ~4.5-fold higher than in vesicles containing no POPS. However, no such increases in K_a values were observed in the presence of 150 mM NaCl. Similar responses were observed when other negatively charged phosphoglycerides (e.g, DPPA, POPG) were incorporated into the vesicles (data not shown).

GLTP interaction with vesicles containing sphingomyelin

Changes in membrane lipid composition, other than surface charge, have been found to affect the transfer of glycolipid by GLTP. Increasing SPM content in POPC donor vesicles diminishes the ability of GLTP to transfer GalCer (19,20). The response is nonlinear with respect to SPM membrane content, is particularly evident when SPM mol fractions reach 0.2, and is not duplicated by PCs with saturated acyl chains. What is not known from these earlier studies is whether the presence of SPM significantly alters the partitioning of GLTP to donor vesicles containing GalCer. To address this issue, RET measurements were performed to establish GLTP localization after incubating with vesicles containing 10 mol% GalCer and various amounts of SPM. Fig. 7 illustrates how the K_a value is affected and shows that increasing SPM content diminishes the partitioning of GLTP to the vesicles. Interestingly, the nonlinear response was similar at both low and physiological ionic strength.

DISCUSSION

Understanding the workings of GLTP is important not only for evaluating the potential of this protein for use as a tool to selectively alter the GSL composition of raft microdomains, but also because there is a long history of conflicting data regarding the association of GLTP with membranes. Metz and Radin (9) reported that the diffusion of cerebroside transfer protein from bovine spleen was reduced by mixing with liposomes or red cell ghosts containing glycolipids, and also found that the protein binds small amounts of GalCer (~4%). They speculated that the protein desorbs from the membrane surface as a protein/lipid complex that then rapidly dissociates in solution before reaching an acceptor membrane. Wong et al. (34) detected no glycolipid acquisition by partially purified bovine brain GLTP, but found a substantial fraction of protein coeluting with POPC/GalCer vesicles. Brown et al. (11) used fluorescence approaches to show association of pyrene-labeled glucosylceramide with bovine brain GLTP, but found no evidence of protein association with vesicles containing glycolipid. Sasaki and colleagues showed that porcine brain GLTP acquires pyrene-labeled GalCer from vesicles and forms a complex (35) but found no GLTP/glycolipid complex in the subphase beneath radiolabeled

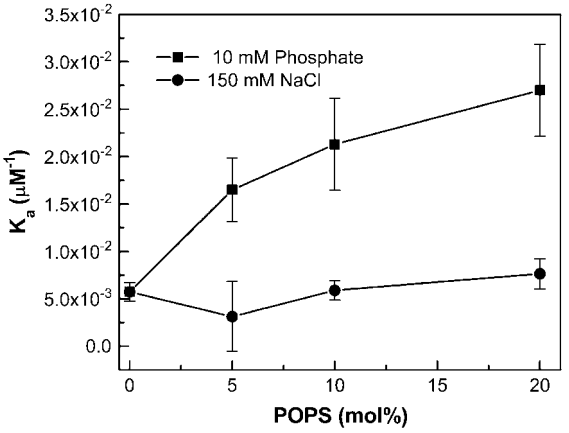

FIGURE 6 Effect of increasing concentration of negatively charged phosphoglyceride in the membrane on GLTP partitioning at low and physiological ionic strength. The error bars represent standard deviation of three experiments performed at 37°C at pH 7.4.

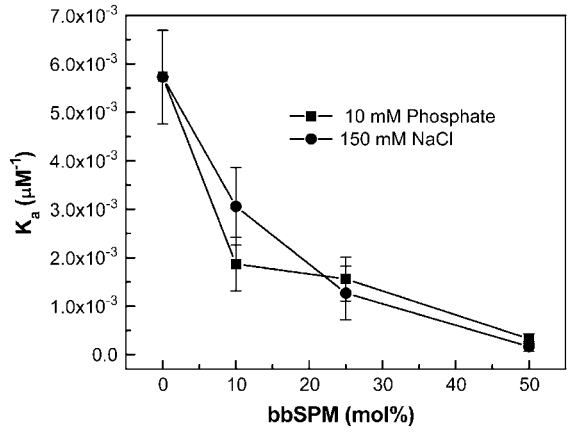

FIGURE 7 Effect of increasing sphingomyelin concentration in the membrane on GLTP partitioning at low and physiological ionic strength. The error bars represent standard deviation of three experiments performed at 37°C at pH 7.4.

GalCer monolayers (36). Some of the difficulties with the earlier studies may have been related to the lengthy purification and instability of certain preparations of GLTP (9,37), which is not highly abundant in animal tissues. Molecular cloning and heterologous expression of GLTP have remedied the situation by enabling abundant amounts of highly purified GLTP to be rapidly obtained in stable form (12,13), making possible recent advances into the structural conformation of human GLTP (13,15) and comprehensive kinetic analyses of different models for the intermembrane transfer of glycolipid by GLTP (14). In the model of GLTP action most consistent with our kinetic studies (Fig. 3), we envision multiple levels of control, represented as separate processes with each defined by its own equilibrium constant. The first process is the interaction of apo-GLTP with the membrane, the second is formation of a GLTP-GSL complex at the interface, and the third is release of the GLTP-GSL complex from the interface. Essential for verification of this model and elimination of alternative models is direct monitoring of the location of GLTP with respect to the membrane.

Although other models have been developed for phospholipid transfer proteins (38–43), these models traditionally rely on ordinary two-substrate enzyme-catalyzed reactions that can be adequately described by ''ping-pong Bi-Bi'' mechanisms. The models are well suited for describing transfer mechanisms involving single component lipid membranes, as is the case for phosphatidylcholine transfer protein (38). However, for cases such as investigated here, a minor lipid component (e.g., GalCer) is being transferred from a two-component lipid membrane in which the major lipid component (POPC) serves as a matrix and itself is not a ''substrate'' for the lipid transfer protein. This situation is more similar, but not identical, to that of the phosphatidylinositol transfer protein (PITP), which displays about a 15-fold preference for PI over PC. In previous modeling studies of PITP action (39,42), it was assumed that the initial transfer velocity can be described solely in terms of donor and acceptor vesicle concentrations to model the functional unit of interaction with PITP. Although this assumption may hold when PI concentrations are relatively high in the membrane vesicles, the situation becomes more complicated in cases where the minor lipid component is present at low concentrations in the membrane (e.g., $<$15 mol%). This means that formation of a protein-lipid complex within the membrane interfacial environment may require lateral diffusion of either lipid or protein or both. This situation appears likely for GLTP and glycolipids that are found at low concentrations in most biomembranes but also have a tendency to locally concentrate in rafts and caveolae.

By relying on the intrinsic fluorescence of GLTP associated with its naturally occurring Trp and Tyr residues and their ability to participate in RET with appropriate energy acceptor fluorophores embedded in the membrane (e.g., dansyl PE), we developed an effective approach for monitoring the partitioning of GLTP to membrane vesicles. Because RET depends critically on the orientation and distance between the donor (Trp and Tyr) and acceptor (dansyl-PE) energy transfer fluorophore pair and does not depend on strong interaction affinity, it is well suited for evaluating the partitioning of proteins to membranes, regardless of whether the interactions are strong or weak. By relying on the intrinsic fluorescence of the naturally occurring Trp and Tyr residues of GLTP, we avoided introduction of extraneous probes at a site(s) that could possibly perturb or alter the conformation of GLTP and/or its membrane interaction region. Because the Trp residues of GLTP have a red-shifted emission wavelength maximum ($\lambda_{max} \approx 347$ nm), are accessible to soluble quenchers, and are localized near the surface of the protein (13,15), a relatively low quantum efficiency was expected. To help circumvent this problem and enhance the sensitivity of the RET approach, we excited at 285 nm to gain a contribution from the 10 Tyr residues of GLTP, and we also used a headgroup-labeled PE containing the dansyl fluorophore as the energy acceptor to minimize the Förster distance upon interaction of GLTP with the membrane. GLTP partitioning to bilayer vesicles was clearly evident in our RET data. Moreover, use of the RET approach, in conjunction with systematic variation of experimental conditions, enabled several conclusions to be drawn about the nature of the GLTP-membrane interaction.

GLTP interacts with membranes containing no glycolipid

In our kinetic model of GSL intervesicular transfer by GLTP (14), we concluded that the rate-limiting step in the transfer process is formation of the GLTP-GSL complex during association of GLTP with the lipid phase and/or the subsequent dissociation of the GLTP-GSL complex from the membrane surface into the bulk phase (denoted by K_s and K_2 in Fig. 6). Because our analyses were based on the initial transfer rates of labeled glycolipid between vesicles, it was impossible to ascertain whether GLTP could directly associate with membranes containing no glycolipid. The RET data in this study provide strong evidence that GLTP does interact with PC vesicles containing no GalCer (Figs. 1 *B* and 2). The RET data are supported by both GLTP Trp anisotropy data (Fig. 4 *C*) and monolayer penetration studies (Fig. 5). The nonspecific interaction of GLTP with the lipid interface may be viewed as the initial step in the transfer process (Fig. 3). Under conditions where no GalCer is present in the membrane matrix, the interaction parameter K_a is equal to the equilibrium constant K_1. The nonspecific interaction between GLTP and the lipid interface is represented by the equilibrium constant K_1 in the proposed model (Fig. 3) and is calculated to be $6.0 \times 10^{-3}\ \mu M^{-1}$ (Fig. 2). It is noteworthy that the K_1 value remains the same at both low and physiologic ionic strengths, suggesting that the initial nonspecific interaction with the lipid-water interface is controlled predominantly by hydrophobic interactions

between GLTP amino acid residues and the lipid bilayer. Consistent with this idea is the observation that the presence of charged phosphoglyceride (e.g., POPS, DPPA, POPG) has no effect on K_1 at physiologic ionic strength, indicating that electrostatic interactions are not primary modulators of the nonspecific partitioning of GLTP. The finding that GLTP partitions to membranes containing no glycolipid also implies that direct interaction of the GLTP/GSL complex with acceptor vesicles is a likely step in the glycolipid transfer process because the presence of POPC vesicles stimulates the release of GSL from the soluble GLTP/GSL complex (44).

GLTP-membrane interactions are transient and weak regardless of glycolipid presence

Several of our findings indicate that GLTP interaction with the membrane occurs in a transient and ''low affinity'' manner. First, the sudden and rapid decrease in the RET signal observed when an excess of dansyl-free vesicles is added to a mixture of GLTP and vesicles containing danysl-PE (Fig. 1 *B*) suggests that GLTP does not associate tightly with the membrane. Second, the increase in K_a observed as a function of increasing GSL concentration in the vesicles at low ionic strength but not at physiologic ionic strength (Fig. 2), is consistent with GLTP acting like a weakly interacting peripheral protein with respect to the membrane. It is noteworthy that the different partitioning responses at low and physiologic ionic strength for GLTP to vesicles containing glycolipid enable separation of the K_a values into their contributing components, K_s, the first-order constant for GLTP-GSL complex formation and the equilibrium constant K_1 for the nonspecific interaction between GLTP and lipid interface, discussed above. The first order constant corresponding to the complex formation K_s was determined to be 4.75 ± 0.68. Thus, formation of GLTP-GSL complex at the interface is marginally favored and can occur spontaneously at the donor vesicle surface. The enhancement in K_a that occurs as a function of increasing GSL concentration at low salt concentrations but not at physiological salt concentrations raise interesting possibilities when viewed within the context of the model for GLTP action shown in Fig. 3. One possibility is that apoGLTP interacts with the membrane at physiological salt concentrations but that GLTP-GSL complex formation, denoted by K_s, does not occur. However, this scenario seems unlikely because GLTP-mediated GSL transfer is observed at salt concentrations as high as 500 mM (18) and a soluble GLTP/GalCer complex can be isolated from the aqueous phase under physiological salt conditions (44). A more likely possibility is that the GLTP-GSL complex equilibrium (K_2 in Fig. 3) shifts toward the bulk phase as salt concentration increases. In any case, it is noteworthy that the presence of negatively charged phosphoglycerides does not interfere with GLTP activity under physiological salt conditions because GLTP appears to reside primarily in the cytoplasm of cells (45) and could play a role in glucosylceramide delivery to various membranes that contain negatively charged phosphoglycerides (e.g., plasma membrane inner surface and/or nuclear membrane). Whether GLTP can also be secreted by cells remains unknown.

GLTP interaction with the membrane is nonperturbing

Consistent with the weak and transient nature of the GLTP-membrane interaction is our finding that the order and fluidity of the bilayer are not altered by the presence of GLTP. DPH and Laurdan anisotropies remain unchanged at membrane saturating concentrations of GLTP regardless of whether GalCer is present or not (Fig. 4, *A* and *B*), suggesting that GLTP interacts peripherally with the membrane and does not penetrate into the bilayer. This finding is further supported by both GLTP Trp anisotropy data (Fig. 4 *C*) and monolayer penetration studies (Fig. 5). The results show that GLTP selectively removes/adds GSLs from/to membranes without directly perturbing them, enhancing the protein's potential usefulness as a membrane modifying agent.

Lipid packing and composition in membranes modulate interaction with GLTP

The increased partitioning of GLTP to PC SUVs compared to PC LUVs, to fluid-phase PC vesicles containing charged phosphoglycerides compared to PC vesicles, and to PC vesicles compared to PC vesicles containing SPM can be summarized in the following way: Conditions that increase the ratio of bound/unbound GLTP do not guarantee increased transfer activity, but conditions that decrease the ratio of bound/unbound GLTP always diminish transfer. This statement is based on the following experimental observations.

From the positive correlation observed between GLTP transfer activity and increased partitioning to SUVs compared to LUVs, one might expect that the increased partitioning of GLTP to negatively charged vesicles would also increase GLTP transfer activity compared to PC vesicles containing no negatively charged phosphoglyceride. In fact, just the opposite response is observed, which emphasizes that placing more GLTP on the donor surface does not guarantee increased formation/desorption of the GLTP/GSL complex and higher GLTP transfer activity. Also equally apparent is the finding that fast and efficient transfer of GalCer by GLTP requires partitioning of GLTP to the membrane. Reduction in the partitioning of GLTP to the membrane, as is the case for vesicles containing SPM, decreases GLTP transfer activity.

CONCLUSIONS

The data presented herein clearly show that GLTP partitioning to the membrane occurs even in the absence of glycolipid being present in the membrane, that the partitioning is weak,

transient, and nonperturbing with respect to the membrane, can be strongly influenced by the membrane lipid composition, and is likely to involve a membrane interaction site that contains Trp-142 and Trp-96. The data support the idea that, once the protein is at the interface, it must find and recognize the carbohydrate moiety on GSL and then form a surface complex that is released into the bulk, consistent with a mechanism involving GLTP acting as a carrier that must desorb from the surface to accomplish GSL transfer. Finally, the studies provide a solid foundation for future site-directed mutagenesis studies aimed at identifying the membrane interaction region of human GLTP and quantitatively assessing the role of select residues in the partitioning of GLTP to the membrane surface.

APPENDIX

Our analyses of GLTP partitioning to the bilayer-water interface and its interaction with GSLs at the interface follows an approach originally developed by White and colleagues (46,47).

Interaction of GLTP with lipid vesicles

When the protein interacts with the lipid (L) surface, it occupies certain areas of the membrane. The number of L molecules it associates with will depend on the area per molecule of the protein and of L. Assuming that the protein interacts with a membrane area corresponding to n L molecules, the dissociation constant for the process can be written as,

$$K_d = \frac{[P_f]/([P_0] + [W])}{(A_{occ})/(A_{free})}. \tag{A1}$$

In this equation, P_f is the free protein in the bulk, P_0 is the total protein, $[W]$ is the concentration of water in the bulk, and A_{occ} and A_{free} are the areas on the vesicle surfaces that are occupied by the protein and that are free, respectively. For the convenience, concentration brackets are dropped.

$$\frac{A_{free}}{A_{occ}} = \frac{A_{tot}}{A_{occ}} - 1 = \frac{L_0/n}{P_L} - 1. \tag{A2}$$

L_0 and P_L are concentrations of the lipid and protein species, respectively, in the lipid phase. In our experiments it is always true that $[W] = 55.3$ M (at 37°C) is $\gg P_0$. Substituting this in the earlier equation,

$$K_d = \frac{P_f}{W}\left(\frac{L_0/n}{P_L} - 1\right). \tag{A3}$$

For the case where the lipid surface is in an excess to the concentration of the bound protein, the condition $L_0 \gg nP_L$ is satisfied. This would be true for conditions where either the saturation limit is very high (large vesicles) or where the bulk concentration of protein is very low. In our experiments, these conditions were reached in the linear portions of partitioning isotherms (Fig. 3). Under these conditions, the partitioning isotherm may be written as,

$$K_d \approx \frac{P_f L_0}{nP_L W}. \tag{A4}$$

Rearranging this equation,

$$P_L = \frac{(L_0/n)}{(1 + WK_d/P_f)}, \tag{A5}$$

or for conditions where saturation was not reached,

$$P_L \approx \frac{P_f L_0}{nK_d W}. \tag{A6}$$

The apparent interaction parameter (K_a) is the reciprocal of the product nK_d. Because total protein P_0 is the sum of bound and free protein, $P_f = P_0 - P_L$. Substituting this in the earlier equations, and solving the resulting quadratic equation,

$$P_L = \frac{P_0 + \frac{W}{nK_a} + \frac{L_0}{2n} - \sqrt{\left(P_0 + \frac{W}{nK_a} + \frac{L_0}{2n}\right)^2 - \frac{4P_0L_0}{2n}}}{2}. \tag{A7}$$

Defining f_b as the fraction of protein associated with the membrane bilayer, and f_f as the fraction of protein free from the lipid bilayer (46),

$$f_b = 1 - f_f = \frac{P_0 + \frac{W}{nK_a} + \frac{L_0}{2n} - \sqrt{\left(P_0 + \frac{W}{nK_a} + \frac{L_0}{2n}\right)^2 - \frac{4P_0L_0}{2n}}}{2P_0}. \tag{A8}$$

For the case where the condition $L_0 \gg nP_b$ is satisfied, the equation is much simpler and is written as,

$$f_b = 1 - f_f \approx \frac{K_a L_0}{W + K_a L_0}. \tag{A9}$$

Analysis of GLTP interaction using FRET

In general the FRET signal F of a solution-containing lipid at concentration L_0 and protein at a concentration P_0 can be written in an equation form as follows;

$$F(P_0) = P_0\{f_b F_b + (1 - f_b)F_f\}, \tag{A10}$$

where f_b and F_b are the fraction of protein bound (see Eq. A8) and the FRET contribution of the bound protein. F_f is the FRET contribution of the free protein and is by definition 0.

The following equation can be written for the proportionality of FRET and protein binding,

$$F(P_0) = F_b\left[\frac{P_0 + \frac{W}{nK_a} + \frac{L_0}{2n} - \sqrt{\left(P_0 + \frac{W}{nK_a} + \frac{L_0}{2n}\right)^2 - \frac{4P_0L_0}{2n}}}{2}\right]. \tag{A11}$$

For the case where the condition $L_0 \gg nP_b$ is satisfied, the equation is much simpler and is written as,

$$F(P_0) = F_b P_0 \frac{K_a L_0}{W + K_a L_0}. \tag{A12}$$

Multistate equilibrium for protein interaction with membranes

Because of specific and nonspecific interaction between protein and membrane surfaces, more than one state at the lipid surface or in the bulk may exist. This situation can be easily incorporated in the model by adding

terms for each state in Eq. A10. For ''i'' states with FRET contribution F_i, the following equation can be written.

$$F(P_0) = P_0 \sum f_i F_i. \quad \text{(A13)}$$

For the special case where GLTP interacts specifically with the GSLs in the membrane, a two-state model is proposed wherein GLTP at the membrane may or may not be bound to GSL. If the state 1 and 2 correspond to the GSL associated and GSL unassociated states, respectively, the following equations can be written.

$$F(P_0) = P_0(f_{b1}F_{b1} + f_{b2}F_{b2}). \quad \text{(A14)}$$

By the definition of K_s, and assuming that the GLTP-GSL association at the surface is of first order,

$$K_s = \frac{f_{b2}}{f_{b1}x_s} \quad \text{(A15)}$$

$$F(P_0) = P_0 f_{b1}(F_{b1} + x_s K_s F_{b2}).$$

Mol balance for the total protein at the interface,

$$f_b = f_{b1} + f_{b2}$$
$$f_b = f_{b1}(1 + x_s K_s). \quad \text{(A16)}$$

If K_1 corresponds to the interaction parameter in the absence of GSL, and using the definition of K_a in rewriting Eq. A16,

$$K_a = \frac{K_1(1 + x_s K_s)W}{(W - x_s K_s K_{a0} L_0)}. \quad \text{(A17)}$$

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