Protein mediated glycolipid transfer is inhibited FROM sphingomyelin membranes but enhanced TO sphingomyelin containing raft like membranes

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Abstract

The mammalian glycolipid transfer protein, GLTP, catalyzes the transfer in vitro of glycolipids between membranes. In this study we have examined on one hand the effect of the variations in the donor vesicle composition and on the other hand the effects of variations in the acceptor vesicle composition on the GLTP-catalyzed transfer kinetics of galactosylceramide between bilayer vesicles. For this purpose a resonance energy transfer assay was used, the energy donor being anthrylvinyl-galactosylceramide and the energy acceptor DiO-C$_{16}$. First, we show that the transfer of anthrylvinyl-galactosylceramide from palmitoyl-oleoyl-phosphatidylcholine donor vesicles was faster than from dipalmitoyl-phosphatidylcholine vesicles, and that there is no transfer from palmitoyl-sphingomyelin vesicles regardless of the cholesterol amount. In this setup the acceptor vesicles were always 100% palmitoyl-oleoyl-phosphatidylcholine. We also showed that the transfer in general is faster from small highly curved vesicles compared to that from larger vesicles. Secondly, by varying the acceptor vesicle composition we showed that the transfer is faster to mixtures of sphingomyelin and cholesterol compared to mixtures of phosphatidylcholines and cholesterol. Based on these experiments we conclude that the GLTP mediated transfer of anthrylvinyl-galactosylceramide is sensitive to the matrix lipid composition and membrane bending. We postulate that a tightly packed membrane environment is most effective in preventing GLTP from accessing its substrates, and cholesterol is not required to protect the glycosphingolipid in the membrane from being transferred by GLTP. On the other hand GLTP can more easily transfer glycolipids to 'lipid raft' like membranes, suggesting that the protein could be involved in raft assembly.
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1. Introduction

The suggested presence of sphingolipid-cholesterol microdomains or rafts in biomembranes [1–3] has been stimulating new developments in the area of lipid and membrane science. The strong and preferential lateral interaction that has been proposed to occur between sphingolipids and cholesterol lead to formation of lateral clusters of an ordered nature [4]. The tendency of glycosphingolipids (GSLs) to organize into lateral membrane domains is thought to be a key feature, not only in their own intracellular sorting and trafficking, but also in the sorting and trafficking of proteins, such as glycosylphosphatidylinositol (GPI) anchored proteins [4,5]. The largest contribution to cholesterol-lipid interactions appears to be from van der Waals forces and hydrophobic forces. It has been suggested that the interactions of cholesterol with sphingomyelin can further be strengthened by both intra-

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and intermolecular hydrogen bonding of the cholesterol hydroxyl group to the polar head group and interfacial regions in sphingomyelin [6–8].

Fairly little is known about the factors governing the interfacial interaction of cholesterol with GSLs. Langmuir monolayer studies have concluded that cholesterol shows a clear condensing effect on liquid-expanded but not as strongly on liquid-condensed monoglycosylceramides such as galactosylceramide (GalCer) [9]. This indicates that the acyl chain composition of the GSLs is one major factor controlling the way different species interact with cholesterol [9–11]. The thermal behavior of palmitoyl-GalCer in dipalmitoyl-phosphatidylcholine (DPPC) have been studied and indicate that palmitoyl-GalCer has a limited solubility above 23 mol% in DPPC gel and liquid-crystal bilayers at temperatures just above the DPPC gel to liquid crystal transition (42 °C) [12]. If the temperature is increased, the solubility of palmitoyl-GalCer also increases and is completely miscible at all molar ratios above its T_m [12–14]. These extensive studies show clearly that the thermal properties of the matrix lipids have a major impact on how the GSLs are solubilized.

Glycolipid transfer proteins (GLTPs, 24 kDa, pI 9.0) have been identified in a wide variety of cell and tissue types, including mammalian brain, liver, kidney, spleen, as well as in spinach chloroplasts [15,16]. GLTP is specific for various glycolipids including neutral glycosphingolipids and gangliosides [17,18]. Several characteristics of bovine, porcine and human GLTPs suggest that these proteins are different from other known lipid transfer proteins. The bovine GLTP have been crystallized [19] and the crystal structure of human GLTP has been solved [20].

Previously bovine brain GLTP has been used as a tool to study the mixing properties of GalCer in different lipid matrixes, as well as the properties of the protein itself [21,22]. GLTP has proven to be a sensitive tool that can be used to probe membrane interfaces regarding GSL mixing and their lateral organization. In this study we show that the transfer of anthrylvinyl-galactosylceramide is faster from palmitoyl-oleoyl-phosphatidylcholine (POPC) vesicles than from DPPC vesicles and that there is no transfer from palmitoyl-sphingomyelin (P-SPM) vesicles. The presence of cholesterol even up to 25 mol% in these phospholipid membranes has a marginal effect on how well GLTP can transfer galactosylceramide. We also show that the transfer in general is faster from small highly curved vesicles compared to larger vesicles. On the other hand transfer from POPC donor vesicles to acceptor vesicles containing a lipid raft like mixture, with phosphatidylcholine, sphingomyelin and cholesterol is much more suitable as an acceptor membrane for GSLs than pure POPC. Our data would suggest that GLTP could potentially be involved in the assembly of lipid rafts rather than function as a transporter of GSLs from lipid rafts.

2. Materials and methods

2.1. Materials

Cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dipalmitoyl-phosphatidylcholine (DPPC), 1,2-dipalmitoyl-phosphatidylethanolamine (DPPE) and Egg Sphingomyelin were all from Avanti Polar Lipids (Alabaster, AL). N-palmitoyl-D-*erythro*-sphingosylphosphorylcholine (P-SPM) was purified from Egg SPM using reverse-phase HPLC (with the column from SUPELCO, Discovery C18, 5 μm particle size, 250 mm×21.2 mm column dimensions at 9 ml/min). The fluorescent probe, N-[(11E)-12-(9-anthryl)-11-dodecenoyl]-1-O-β-galactosylsphingosine (AV-GalCer) were prepared as described earlier [23,24], DiO-C_{16} (3,3′-dihexadecyloxacarbocyanine perchlorate) was from Molecular Probes (Eugene, OR) and Triton X-100 was from ICN Biomedicals (Aurora, OH). The concentration of the different phospholipids was determined by the Bartlett method [25] and the probes, gravimetrically (MT5, Mettler-Toledo, Columbus, OH), which we found to agree well with determinations based on molar extinction coefficients. Recombinant bovine GLTP was expressed and purified as described earlier [26].

2.2. Preparation of phospholipid vesicles

Small unilamellar vesicles (SUVs) or large unilamellar vesicles (LUVs) consisting of P-SPM, DPPC or POPC with various amounts of cholesterol and the fluorescent probes AV-GalCer (1 mol%) and DiO-C_{16} (3 mol%) were used as donors. The SUVs were prepared by probe sonication [27] and the LUVs by extrusion [28]. Phospholipids and various amounts of cholesterol were mixed with 1% AV-GalCer and 3% DiO-C_{16} from stock solutions in hexane/isopropanol (3:2 v/v) and dried under nitrogen. The mixtures were stored at −20 °C and re-dissolved immediately before sonication/extrusion in sodium phosphate buffer with pH 7.4 containing 10 mM sodium dihydrogen phosphate, 1 mM dithiothreitol and 1 mM EDTA. The suspension with a total concentration of 0.4 mM was sonicated for 10 min on ice with a Branson 250 sonifier, and then centrifuged for 15 min at 15,000 rcf to remove titanium probe particles, multilamellar vesicles, and undispersed lipid (negligible amount). The final concentration of the donor vesicles per assay was 13 μM, the final AV-GalCer concentration in each assay was 0.13 μM. Light scattering measurements (Malvern 4700, Malvern Instruments, Worcestershire, UK) on the sonicated vesicles showed an average diameter of 36 nm (data not shown). The extruded vesicles were prepared by extruding the suspension through two 100 nm polycarbonate filters (Whatman, Kent, UK). The vesicle size was determined to 110 nm with light scattering (data not shown). The POPC acceptor vesicles were prepared by sonication as described above for the sonicated donor vesicles.

2.3. Resonance energy transfer assay

The resonance energy transfer (RET) assay method used for measuring the transfer of glycolipids between two bilayer vesicle populations has been thoroughly described previously [21,22,29]. In this work we have used DiO-C$_{16}$ instead of perylenoyl triolein used previously. This is to avoid the possibility of phase separation of the RET pair that could be caused by cholesterol if triolein is used [30]. The excitation and emission spectra of DiO-C$_{16}$ and AV-GalCer and their molecular structures are shown in Fig. 1A.

2.4. Calculations of the transfer rates and availability

By adding Triton X-100 (final concentration 1%) to the transfer reaction mixture after the initial AV-GalCer transfer, the total fluorescence intensity can be measured (Fig. 1B). Addition of Triton X-100 caused the two fluorescently labeled lipids to become dispersed into lipid–Triton X-100 micelles considerably beyond their Förster distances. The intensity after Triton addition (subtracted with a Triton blank) corresponds to the total concentration of AV-GalCer used in each transfer reaction, which was 1 mol% or 133 nM. The availability was calculated by comparing the steady state plateau with the total fluorescence intensity, galactosylceramide flip-flop has been shown to have half-times of days [31], and therefore it does not affect the availability. By comparing the levels of the fluorescence intensity without GLTP, and 1 min after GLTP was injected, and after the addition of detergent, we can calculate the amount AV-GalCer transferred by GLTP (pmol transferred/s, Fig. 1B). To calculate the transfer rates we need to correct for the potential quenching by Triton X-100 of the anthrylvinyl fluorescence. For this we measured the fluorescence in 13.3 μM POPC-vesicles with 1% AV-GalCer and adding Triton X-100 to a final concentration of 1%. No change in fluorescence (after subtraction of a triton blank) could be showed and therefore we conclude that no quenching occurs (data not shown). The fluorescent intensity of AV-GalCer shows a linear increase in intensity between 0.25 and 4%, as shown in Fig. 1C. Calculating the initial transfer rates at 1 min allows for a much faster collection of data, and a better reproducibility than the previously used transfer rates at the halftime of the transfer reaction [21,22,29,32]. The AV-GalCer availability was calculated by comparing the starting level of intensity with the levels at transfer equilibrium and the total intensity after Triton addition, subtracted by a Triton blank (Fig. 1B).

3. Results

3.1. Glycosphingolipid availability

We can determine the amount of available AV-GalCer in POPC vesicles for GLTP by adding Triton X-100 (final concentration of 1%) to the transfer reaction mixture after the transfer has reached a steady plateau, as described in the Materials and methods section. The excess detergent solubilized the vesicles and allowed the total fluorescence intensity of all of the AV-GalCer in the system to be obtained. The results in Fig. 2 indicate clearly that there is no change in the available AV-GalCer amount as a function of increasing the cholesterol in the mixed POPC vesicles, and that there is about 60% AV-GalCer available for the highly curved probe sonicated POPC SUVs at 37 °C. The less curved extruded vesicles showed an average availability of about 50%.

3.2. Transfer of AV-GalCer from phospholipid/cholesterol donors at 37 °C

Classically the role of cholesterol in phospholipid bilayer gel to liquid crystal transition is to diminish the apparent enthalpy change by increasing the gel-state hydrocarbon chain packing disorder, while increasing the chain packing order in the liquid crystal state. We therefore studied the possible effects that cholesterol could have on the GLTP mediated AV-GalCer transfer from cholesterol/phospholipid bilayers, as well as the effect that a vesicle curvature or bending could have on the transfer.

We have analyzed the GLTP-mediated AV-GalCer transfer from probe sonicated (36 nm in diameter) donor vesicles consisting of POPC, DPPC, P-SPM and cholesterol up to 25 mol% at 37 °C. Fig. 3A shows the AV-GalCer transfer in pmol/s for 1 μg of GLTP as a function of a successive increase in the cholesterol content. The AV-GalCer transfer rate from the single chain mono-unsaturated POPC and increasing cholesterol amounts was faster compared to the fully saturated DPPC (Fig. 3A). POPC and DPPC both displayed a successive decrease in the transfer rate, whereas no transfer was detected from P-SPM vesicles regardless of the cholesterol amount. For the larger extruded vesicles (110 nm in diameter) the transfer rate was for the POPC system almost identical compared to the probe sonicated vesicles, except for a slightly lower AV-GalCer transfer for the fraction containing no cholesterol (Fig. 3B). For the DPPC vesicle system the transfer rate successively increased with increasing cholesterol amount, whereas AV-GalCer transfer from P-SPM and P-SPM/cholesterol mixed bilayers was not detectable (Fig. 3B).

3.3. Transfer of AV-GalCer from phospholipid/cholesterol donors at 50 °C

The POPC/cholesterol system is at 37 °C with low cholesterol concentrations in a fluid phase, and at molar fractions of cholesterol between 0.15 and 0.5 in a phase co-existence of fluid and liquid-ordered phases, and at 50 °C in a fluid phase at all cholesterol amounts [33]. At 37 °C the DPPC and P-SPM system is in a gel phase and addition of cholesterol generate gel and liquid-ordered coexistence
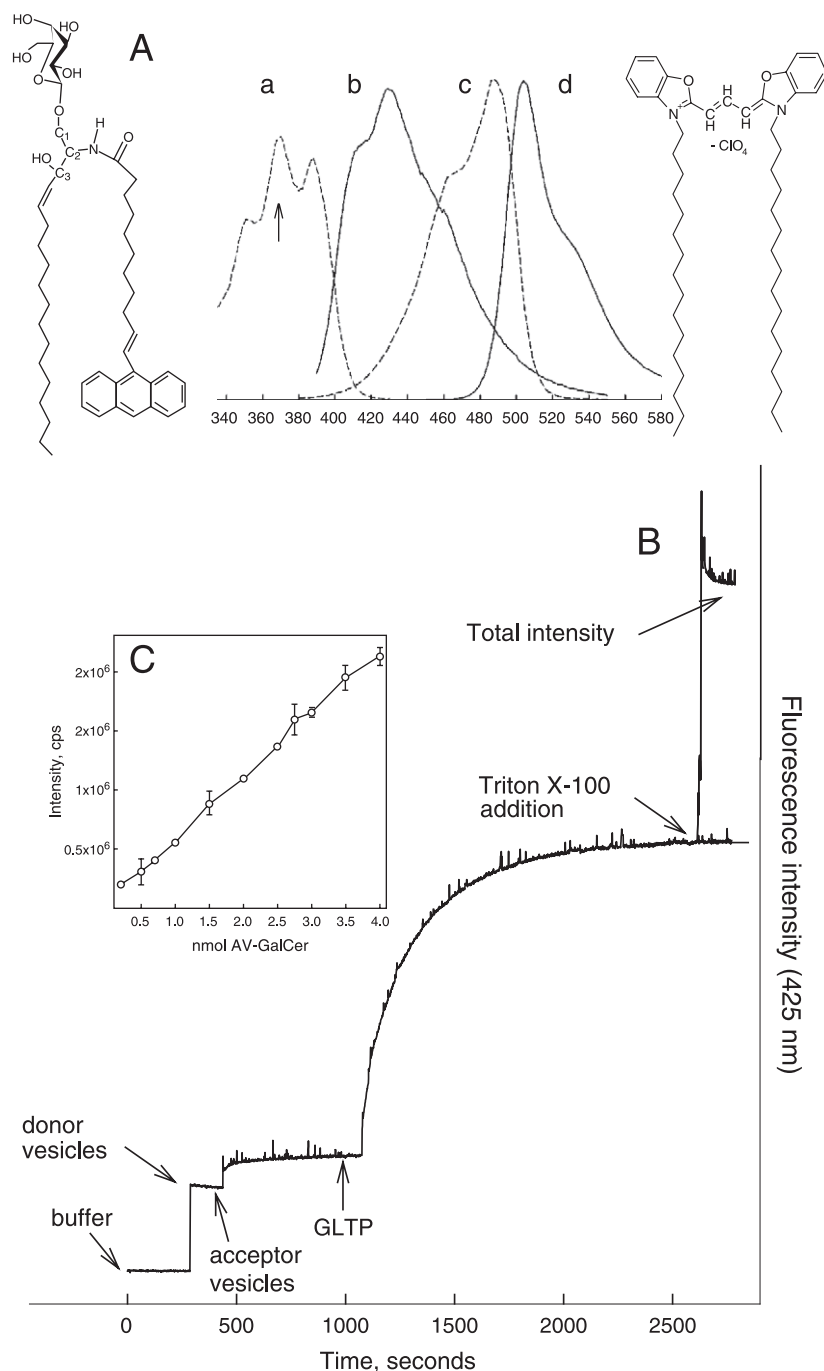
Fig. 1. (A) The fluorescent probe anthrylvinyl-galactosylceramide, AV-GalCer (left) and 3,3′-dihexadecyloxacarbocyanine perchlorate, DiO-C16 (right) were used to monitor the GLTP mediated glycosphingolipid transfer. The graph shows the normalized spectra of the fluorescence resonance energy transfer pairs AV-GalCer (energy donor) and DiO-C16 (energy acceptor) in ethanol. (a) Excitation of AV-GalCer (λem=440 nm). (b) Emission of AV-GalCer at λex=370 nm (arrow). (c) Excitation of DiO-C16 (λem=580 nm). (d) Emission of DiO-C16 at λex=440 nm. (B) POPC donor vesicles (13.3 μM) were added to a sodium phosphate buffer (pH 7.4) and allowed to equilibrate for 5 min at 37 °C. Acceptor vesicles were added (133 μM), and the system was allowed to equilibrate for another 5 min. Next, 1 μg GLTP was added and the change in fluorescence intensity at 425 nm was recorded. At the 1 min time point the initial transfer rate for the transfer reaction was calculated using the value for total fluorescence intensity after Triton X-100 addition. (C) Linear increase in AV-GalCer fluorescence as a function of increasing fluorophore concentration in POPC SUVs.

phases. At 50 °C the DPPC [34] have phase coexistence of fluid and liquid ordered phase states, the P-SPM system is in an fluid phase at low cholesterol concentrations, but have phase co-existence of fluid and liquid ordered phase states with molar fractions of cholesterol between 0.1 and 0.35 [33].

At 50 °C the activity loss due to the higher temperature was minimal due to recording only in the first minute after GLTP addition. At 50 °C the transfer of AV-GalCer from sonicated POPC, DPPC or P-SPM donor vesicles with increasing cholesterol displayed no significant change in the
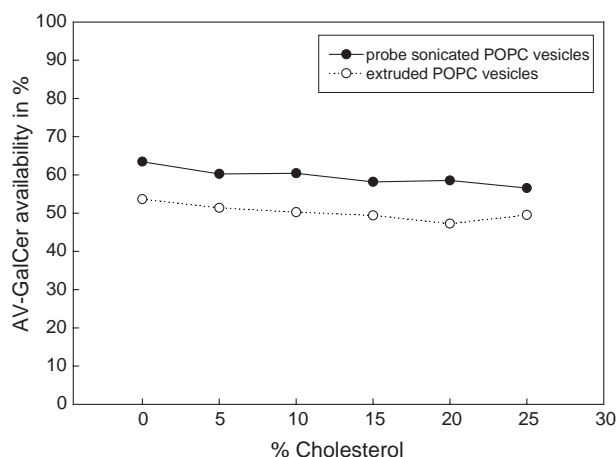
Fig. 2. Amount of AV-GalCer accessible to GLTP in POPC/cholesterol SUVs at 37 °C. The levels are expressed as percents of available AV-GalCer. The average size of the probe sonicated vesicles were 36 nm, and the 100 nm filter extruded vesicles 110 nm in diameter determined with light scattering. Values are averages of at least three measurements.

transfer rate. The POPC system followed an almost steady rate of about 1.7 pmol/s, and a barely detectable transfer rate for DPPC and P-SPM was observed (Fig. 4A). At 37 °C the
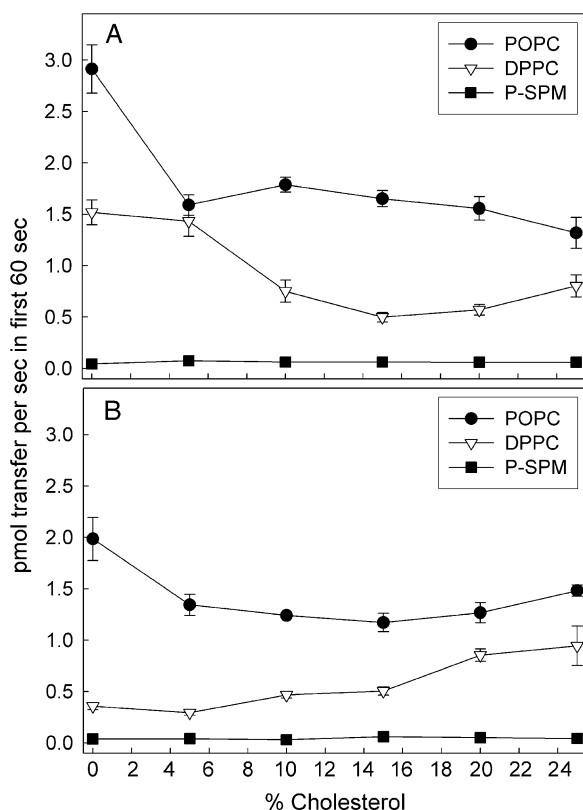

Fig. 3. GLTP mediated (14 nM) AV-GalCer (1 mol%) transfer rates from unilamellar phospholipid vesicles composed of either POPC, DPPC or P-SPM without or with increasing cholesterol amount at 37 °C. (A) pmol AV-GalCer initial transfer rates (1 min) versus increasing mol% cholesterol in probes sonicated small (on average 36 nm in diameter) phospholipid unilamellar vesicles. (B) Initial transfer rates for extruded phospholipid unilamellar vesicles on average 110 nm in diameter. Values are averages of at least three measurements.
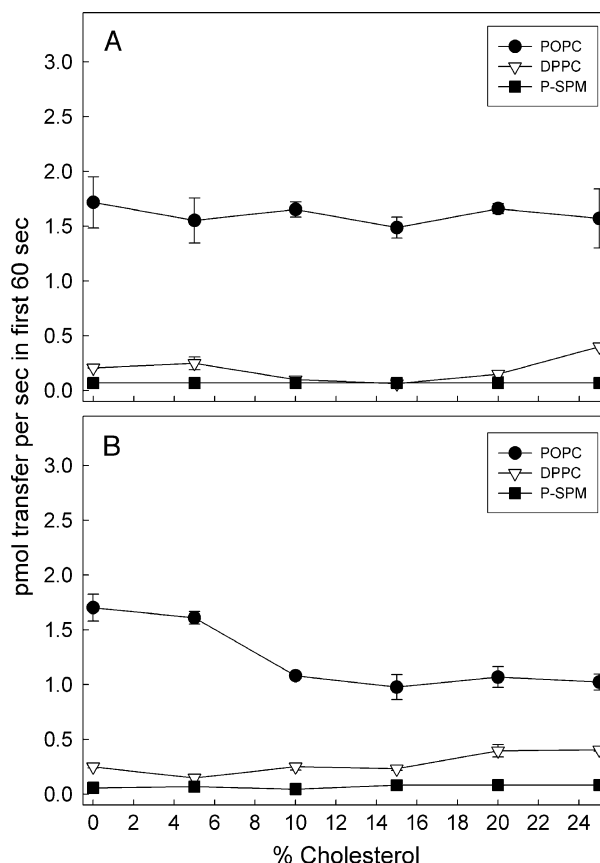

Fig. 4. GLTP mediated (14 nM) AV-GalCer (1 mol%) from unilamellar phospholipid vesicles composed of either POPC, DPPC or P-SPM without or with increasing cholesterol amount at 50 °C. (A) pmol AV-GalCer initial transfer rates (1 min) versus increasing mol% cholesterol in probes sonicated small (on average 36 nm in diameter) phospholipid unilamellar vesicles. (B) Initial transfer rates for extruded phospholipid unilamellar vesicles on average 110 nm in diameter. Values are averages of at least three measurements.

DPPC mixed donors displayed an AV-GalCer transfer but not at 50 °C (compare Figs. 3A and 4A open triangles). When the bilayer curvature and bending in the mixed phospholipid/cholesterol donor vesicles decreases the GLTP mediated AV-GalCer transfer rate also decreases, Fig. 4B. Both the POPC and DPPC system show an average drop of 0.5 pmol/s. AV-GalCer transfer decreases slightly with increasing cholesterol amount in POPC, whereas in the DPPC system a slight increase was detected.

3.4. GLTP heat stability measurements

The slightly lower transfer rates after a raise in the temperature prompted us to examine the stability of the GLTP to heat. If the protein was heat treated at 50 °C for 20 min we observed a reduction in the activity (40% reduction), Fig. 5. The protein was first kept at the indicated temperature for 20 min, and then assayed for transfer activity. Above 55 °C a complete loss of activity was observed.
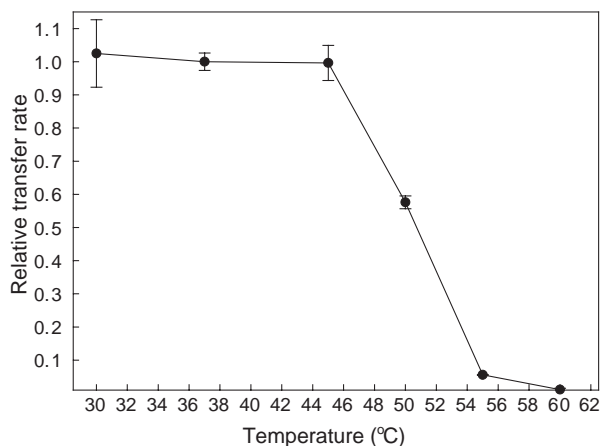
Fig. 5. AV-GalCer transfer rates from POPC vesicles versus increasing temperature. GLTP was heat treated at the indicated temperature for 20 min prior to the transfer activity measurements. 100% indicates the transfer at 37 °C. Values are averages of four measurements.

3.5. Transfer of AV-GalCer to different phospholipid/cholesterol acceptors

In Fig. 6, the transfer rates for AV-GalCer from POPC vesicles to different phospholipid/cholesterol acceptor vesicles are examined. The probe sonicated vesicles (both donors and acceptors) are prepared as described in the Materials and methods section. The results in Fig. 6 show that the transfer rate to acceptor vesicles composed of pure phospholipids decreases in the order POPC, DPPC, P-SPM. As a control we substituted 10% of POPC for the positively charged DPPE, which did not significantly change the transfer rate. We previously showed that negatively charged donor vesicles have a negative effect on the GLTP mediated transfer, but only a marginal effect with negatively charged acceptor membranes [21]. The transfer is increased when POPC vesicles are mixed with different molar fractions of DPPC, cholesterol does slightly decrease the transfer rate (Fig. 6). The AV-GalCer transfer to POPC and P-SPM mixed acceptors is in a nonlinear fashion dependent on the molar fraction, being fastest to vesicles where POPC and P-SPM are mixed 1 to 1. The transfer is decreased by adding 33 mol% cholesterol to these vesicles.

4. Discussion

In previous studies where the GLTP mediated AV-GalCer transfer is measured and the effects of different charged lipids in POPC donors, or the effects of increasing amounts of different sphingomyelins in POPC donors are examined, the transfer reactions reached different equilibrium levels [21,22]. In the charged system the levels were always 60% [21]. This is consistent with the theoretical value of about 60–65% accessible AV-GalCer in the SUV outer leaflet in highly curved vesicles. The theoretical value for the amount of AV-GalCer in the outer leaflet for highly curved vesicles

with a diameter of 35 nm can be calculated assuming that AV-GalCer is mass distributed between the outer and inner bilayer leaflets, similar to the POPC matrix. For vesicles composed of sphingomyelin, only between 20% and 25% of the total AV-GalCer was accessible to GLTP, but substituting DMPC or DPPC for SPM did not lower the amount of accessible AV-GalCer as much as SPM [22].

In this study we found that, in general, cholesterol had little effect on the AV-GalCer availability, but we could observe a clear difference in the availability as a function of vesicle size and matrix lipid composition. Probe sonicated
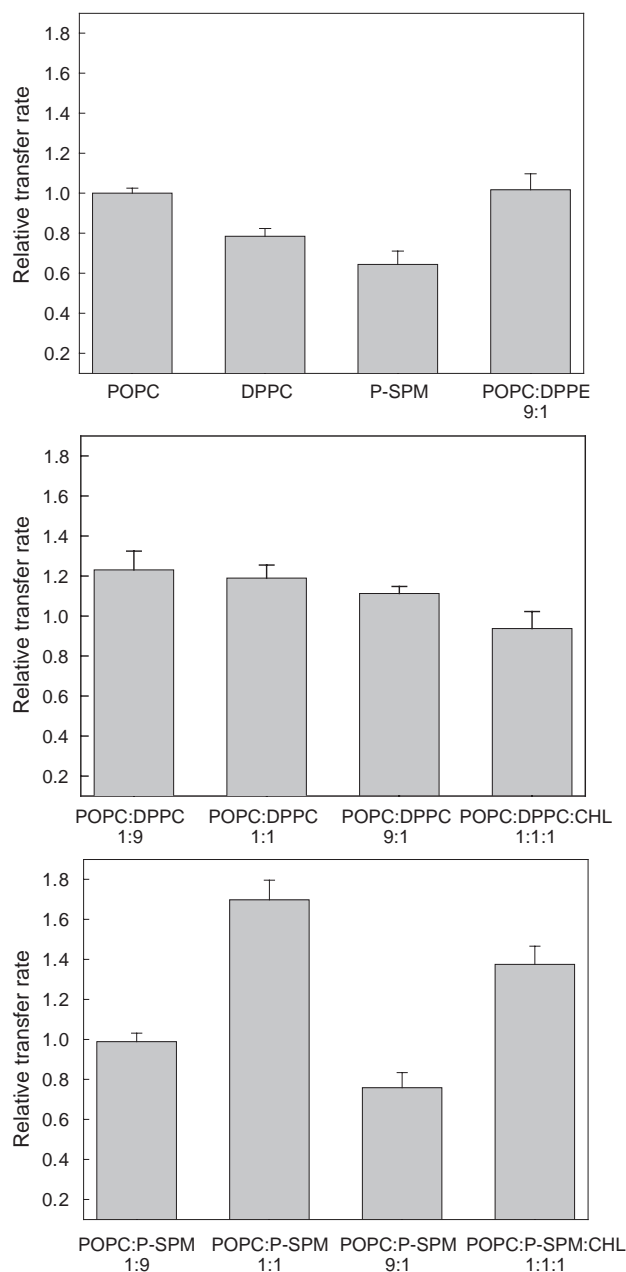

Fig. 6. GLTP mediated (14 nM) AV-GalCer (1 mol%) transfer rates from unilamellar POPC vesicles to different phospholipid/cholesterol acceptor vesicles at 37 °C. The transfer is normalized to 1.0 for POPC vesicles. Values are averages of at least three measurements.

POPC/cholesterol SUVs had a availability value at about 60% which agrees well with the theoretical value for a vesicle with such a diameter and with a mass distribution of 3:2 outer to inner leaflet ratio [35,36]. The larger extruded POPC vesicles had an availability value of about 50%, which is also in agreement with a mass distribution of close to 1.2:1 outer to inner leaflet for vesicles with a diameter of around 110 nm. To be able to compare all lipid mixtures in both a fluid and liquid-ordered phase state we analyzed the transfer at 37 and at 50 °C, well above the transition temperature between gel and liquid phase for both DPPC and P-SPM (41 °C). The slightly lower transfer rates observed at the higher temperature were probably only in part due to a lower specific activity of GLTP. The other differences in the transfer rates should reflect a different packing nature of the matrix lipids. Since the transfer rate measurements were done only 1 min after GLTP addition the protein activity loss was minimal.

Previously it has been shown that increasing the SPM content of POPC donor vesicles decreases the transfer rate in a nonlinear fashion. Decreased transfer rates were clearly evident at SPM mol fractions of 20 mol% or higher. The pool of AV-GalCer available for GLTP mediated transfer was also smaller in donor vesicles containing high SPM content. In contrast, AV-GalCer was more readily transferred from donor vesicles composed of POPC and different disaturated phosphatidylcholines. GLTP mediated GalCer intermembrane transfer is substantially diminished as the donor vesicle matrix content of SPM increases in SPM/PC mixtures. This suggested stronger lipid–lipid interactions between the glycolipid and SPM [22]. Here we show that the addition of cholesterol to PC or SPM donor vesicles had a minimal effect on how well the GSL was transferred from these membranes by GLTP. The saturated more ordered acyl chains of DPPC would allow for a stronger association of AV-GalCer in the bilayer than in the more disordered chains of POPC. Calorimetric data confirms that palmitoyl-GalCer below 23 mol% is fully miscible in DPPC bilayers [13], whereas the miscibility of bovine brain GalCer over a range of 0–70 mol% is limited in POPC bilayers [37].

The addition of 5 mol% cholesterol to POPC donor vesicles resulted in a decrease in the AV-GalCer transfer rates. This could be explained by the generation of a more tightly packed liquid-ordered POPC/cholesterol phase that would more strongly protect AV-GalCer from GLTP mediated transfer. The liquid-ordered phase, however, would not be as favorable an environment for the glycolipid as would a gel phase like P-SPM or DPPC. Since sphingolipids can further form both inter- and intramolecular hydrogen bonding networks [38], these environments protect the glycolipid even more from being transferred by GLTP.

The faster transfer rates of AV-GalCer from the more highly curved probe sonicated donor vesicles (36 nm in diameter) compared to the more planar extruded vesicles (110 nm in diameter) could be explained by the higher bending and curvature stress in the leaflets of the smaller vesicles. The higher stress would create a less optimal packing environment for the glycosphingolipid, and hence allow for a faster GLTP transfer activity. In the larger vesicles the packing of the GLTP substrate is stronger and more optimal and reflects in a slower transfer rate.

We also measured if a variation in the acceptor vesicle composition had any effect on the transfer rate. Here the composition of the donor vesicles were always POPC with 1% AV-GalCer and 3% DiO-C$_{16}$ and the acceptor vesicle composition was varied. As the results in Fig. 6 clearly report, it did indeed have effects, the transfer being faster to raft like acceptor vesicles. We show that transfer of AV-GalCer from POPC donor vesicles is faster to mixtures of POPC and P-SPM acceptor vesicles than to vesicles composed of a pure phospholipid. The transfer of AV-GalCer to vesicles of equimolar P-SPM and POPC content was faster than to vesicles where either of the phospholipids was in majority. Hypothetically this would suggest that GLTP might be involved in formation of lipid rafts rather than in using them as platforms for extracting glycolipids. Here we show that the transfer of AV-GalCer from raft like donors is extremely slow both at physiological relevant temperatures and even at 50 °C where the membrane phase state has a more fluid nature.

Taken together, we have demonstrated that the effect of cholesterol is marginal on the GLTP mediated transfer of AV-GalCer from donor vesicles composed of PC and SPM sterol mixtures. It appears that the miscibility of the glycolipid in different membrane matrixes is a key factor governing the rate of not only the GLTP transfer ability from donors, but also the transfer to different membrane matrixes. The effect of the curvature and bending stress is also acting as a parameter that can significantly increase lateral diffusion and organization of lipids [39,40] as well as transbilayer distribution [41,42], and consequently have impact on GLTP mediated glycolipid transfer.

Acknowledgements

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