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Monitoring glycolipid transfer protein activity and membrane interaction with the surface plasmon resonance technique

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ABSTRACT

The glycolipid transfer protein (GLTP) is a protein capable of binding and transferring glycolipids. GLTP is cytosolic and it can interact through its FFAT-like (two phenylalanines in an acidic tract) motif with proteins localized on the surface of the endoplasmic reticulum. Previous *in vitro* work with GLTP has focused mainly on the complete transfer reaction of the protein, that is, binding and subsequent removal of the glycolipid from the donor membrane, transfer through the aqueous environment, and the final release of the glycolipid to an acceptor membrane. Using bilayer vesicles and surface plasmon resonance spectroscopy, we have now, for the first time, analyzed the binding and lipid removal capacity of GLTP with a completely label-free technique. This technique is focused on the initial steps in GLTP-mediated transfer and the parameters affecting these steps can be more precisely determined. We used the new approach for detailed structure–function studies of GLTP by examining the glycolipid transfer capacity of specific GLTP tryptophan mutants. Tryptophan 96 is crucial for the transfer activity of the protein and tryptophan 142 is an important part of the proteins membrane interacting domain. Further, we varied the composition of the used lipid vesicles and gained information on the effect of membrane properties on GLTP activity. GLTP prefers to interact with more tightly packed membranes, although GLTP-mediated transfer is faster from more fluid membranes. This technique is very useful for the study of membrane–protein interactions and lipid-transfer rates and it can easily be adapted to other membrane-interacting proteins.

1. Introduction

The glycolipid transfer protein (GLTP) is a protein that is able to bind glycosphingolipids and mediate their transfer between two membranes [1,2]. It is a cytosolic protein, but beside its intracellular location, the actual biological role of the protein still remains an enigma [3]. GLTP has been suggested to have a role in glucosylceramide metabolism, since GLTP overexpression leads to a significant increase in cellular glucosylceramide synthesis and transfer [3,4]. Due to GLTPs high specificity for glycosphingolipids, it could also act as an intracellular sensor for glucosylceramide levels instead of as a direct transfer protein *in vivo* [3,5]. We have recently found a FFAT-like (two phenylalanines in an acidic tract) motif in GLTP and we showed that this motif can interact with VAP-A (vesicle-associated membrane protein-associated protein), an integral protein found in the endoplasmic reticulum and ER/Golgi intermediate compartment [6].

The initial interaction of the glycolipid transfer protein with membrane interfaces, the first step in the complete transfer process, is quite nonspecific and GLTP has been shown to interact also with membranes without glycolipids [7–9]. The binding of GLTP to the membrane is weak and nonperturbing [8]. Based on structural studies α-helix 6 has been implicated to be the membrane interacting part of the protein [1,10]. After the initial binding to the membrane interface, GLTP is searching for the glycolipid substrate in the membrane; however, it is not yet clear if the protein itself moves laterally to do this or if this happens by lateral movement of lipids in the membrane [9] or, most likely, a combination of both. The last steps in the process are the formation and the release of the GLTP–glycolipid complex from the membrane interface [2]. GLTP is sensitive to the membrane composition and the miscibility of the glycolipid in the membrane donating the glycolipid for transfer, favoring more fluid membranes for glycolipid removal [11,12].

The crystal structure of GLTP has been solved, both in its apo-form and with bound glycolipid [13,14]. The structure consists of purely α-helices and has a hydrophobic cavity that works with a cleft-like gating mechanism upon substrate binding [13,14]. The α-helical topology of GLTP is unique for transfer proteins, since other transfer proteins generally contain β-structure or extensive disulfide cross-linking, and therefore, GLTP is the founding member of a new family of transfer proteins [2,14]. GLTP contains three tryptophan residues

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; GLTP, glycolipid transfer protein; O-SM, *N*-oleoyl-sphingomyelin; PGalCer, *N*-palmitoyl-galactosylceramide; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; P-SM, *N*-palmitoyl-sphingomyelin; SPR, surface plasmon resonance; RU, response units

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and these have been mutated in earlier studies and the effects of these mutations studied. Tryptophan 96 resides in one of the most widely conserved regions in GLTP [1]. A point mutation of tryptophan 96 to phenylalanine maintained 63% of the transfer activity, while mutation of the same tryptophan to alanine resulted in an inactive transfer protein [7,14]. The four-phosphate adaptor protein 2 (FAPP2) has a GLTP homology domain, and if the tryptophan corresponding to the GLTP W96 in this domain (W407) is mutated to alanine, the glycolipid transfer activity of FAPP2 is also completely lost [15]. Tryptophan 96 (W407 in FAPP2) is located in the sugar recognition center of the protein. The membrane interaction site of the protein is suggested to consist of tryptophan 142, isoleucines 143 and 147 in α-helix 6 [7,10]. Tryptophan 85 seems to be the least important of the three and its mutation to phenylalanine does not inactivate the protein [7,16].

Surface plasmon resonance (SPR) has become one of the most important techniques for studying molecular interactions. The advantages of SPR are that no labeled compounds are needed, very small amount of sample is required, and it is direct, rapid, and easy to use. In the SPR technique, one substance, the ligand, is attached to the surface of the used sensor chip. If the analyte, which is then flowed over the immobilized ligand, binds to the ligand, the mass on the chip surface increases and this leads to an increase in the refractive index of the chip surface. The SPR instrument measures the change in refractive index by an optical method and displays the response signal online as resonance units (RU) versus time [17]. Most of the biochemical studies using SPR have concentrated on protein–protein interactions, while fewer studies have been done on membrane/lipid–protein interactions [18]. Some of the lipid–protein interaction studies have been done with the protein bound to the chip and the lipids floated as monomers over the chip. We find this experimental setup problematic since lipids are not soluble in water/buffer and different lipids have varying critical micelle concentrations. As a result, this approach can only be used with fairly polar lipids with high critical micelle concentrations [19]. Further, lipids as monomers have a much smaller molecular mass than proteins, and since the change in refractive index measured by the SPR instrument is dependent on the mass of the analyte, the response with protein binding to a membrane is much larger [17]. Finally, lipid vesicles can be attached to the chip noncovalently as such, while proteins are attached to the chip by covalent modification or they need some kind of tag [17].

In this study, we utilized the binding of various lipid vesicles to the chip and then measured how well the protein that was floated over the chip surface interacted with the different lipid membrane surfaces containing natural substrates. All the earlier methods used to study the GLTP-mediated transfer process have included fluorescent or radiolabeled lipids [1]. We present here the use of the SPR method to study how variations in the lipid membrane composition and point mutations in a glycolipid transfer protein affects the membrane interaction and the transfer process. We aim at obtaining more detailed information on the first steps in the transfer protein-mediated removal of lipids from specific membranes.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), *N*-oleoyl-sphingomyelin (O-SM), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-palmitoyl-sphingomyelin (P-SM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids) by reverse-phase HPLC (Supelco Discovery C18-column; dimensions 250×21.2 mm, 5 μm particle size) using 100% methanol as eluent. *N*-palmitoyl-galactosylceramide (PGalCer) was synthesized from 1-β-galactosylsphingosine (Avanti Polar Lipids) and palmitic acid anhydride (Sigma Chemicals, St. Louis, MO) as described earlier [20,21]. PGalcer was purified on the same HPLC column as PSM and with the same solvent. The purity and identity of PSM and PGalCer were verified on a Micromass Quattro II mass spectrometer (Manchester, UK). *N*-[(11*E*)-12-(9-anthryl)-11-dodecenoyl]-1-*O*-β-galactosylsphingosine (AV-GalCer) was prepared as described earlier [22]. Stock solutions of glycosphingolipids were prepared in chloroform/methanol (2:1, by volume) and of other lipids in hexane/2-propanol (3:2, by volume). Lipid solutions stored in the dark at −20 °C and warmed to ambient temperature before use. The concentration of phospholipids was determined by the method of Rouser et al. [23], the concentration of PGalCer was determined by careful weighing and of AV-GalCer by fluorescent intensity measurements.

2.2. Expression and purification of wild-type GLTP and tryptophan mutants

The pGEX-6P-1-GLTP(h) vector was used as a template when introducing point mutations in GLTP using PCR according to the manual of Stratagene. Site-directed mutagenesis was performed to obtain the following mutant proteins: W96A, W142A, and W142F. All the made constructs were checked by DNA sequencing.

The vectors of wild-type and mutants were transformed into *Escherichia coli* BL21 cells. The bacteria were grown in yeast–tryptone medium at 29 °C until cell density OD_{600} reached 1.0. Expression of the glutathione-*S*-transferase fusion protein construct was induced with isopropyl-1-thio-β-D-galactopyranoside at a final concentration of 0.5 mM, and the bacteria were grown additionally for 2 h. The cells were harvested and frozen and later lysed by lysozyme and sonication. The cleared lysate was purified on a column of Glutathione Sepharose 4B beads (GE Healthcare). Elution of the protein and cleavage of its GST-tag was done with Prescission Protease (GE Healthcare) in Tris buffer at pH 7.0 (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT). The purity of the protein was checked by SDS–PAGE gel (16%) analysis with Coomassie staining. The purified protein was stored at 4 °C and used within 10 days. The protein concentration was determined according to the method of Lowry using bovine serum albumin as standard [24]. The mutant proteins were always purified and analyzed side by side with wild-type GLTP.

2.3. Preparation of vesicles

Desired lipid mixtures were made from the stock solutions, mixed rigorously and dried under nitrogen. If the mixture contained glycosphingolipids, the dried lipids were redissolved in chloroform, mixed thoroughly, and dried again under nitrogen. All samples were then additionally kept for approximately one hour under vacuum to remove all traces of solvent. Warm buffer (the same Tris buffer as used for protein purification) was then added to the dried lipids, and the samples were vortexed. The lipids were allowed to swell at 60 °C for at least 30 min with vortexing in between. Then the mixtures were sonicated on a water bath for 10 min at 60 °C. Finally, the lipid suspensions were extruded 10 times through two 100 nm polycarbonate filters at 60 °C. The total lipid concentration in the samples was 0.5 mM and the volume extruded was 2 ml. The vesicles were allowed to adjust to RT over night and were used within 2 days.

2.4. Surface plasmon resonance spectroscopy

Membrane–protein interactions and transfer of PGalCer from vesicles to GLTP were studied at 25 °C with a BiacoreX instrument (GE Healthcare). Vesicles with a desired lipid composition were immobilized on a Biacore L1 sensor chip. The sensor chip surface consists of a carboxymethylated dextran matrix with preimmobilized lipophilic groups and vesicles can be captured at the surface noncovalently. The flow rate was set at 5 μl/min throughout the experiments, and the experimental setup was designed according to the Biacore L1 chip manual. All solutions were stored at RT overnight before experiments

were performed (except for the proteins, which were kept on ice or at 4 °C). The same batch of buffer was used for all solutions throughout the experiment, and all solutions were filtered through 0.2-μm membrane filters and then degassed by sonication in a water bath (except for the protein).

At first, the surface of the sensor chip was conditioned with two 2-min injections of 20 mM CHAPS. Then vesicles were immobilized on the surface (10-min injection), and unbound vesicles were removed by washing with 50 mM NaOH in Tris buffer for 2 min. After a stable baseline was reached, the protein of interest was injected for 5–10 min (at varying concentrations, most frequently 0.1 mg/ml). Finally the surface was washed with running buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.0), and then the chip was regenerated with CHAPS, as in the beginning of the experiment. The measurements were always repeated at least two times for each set of samples. Each set of experiments was done with at least two separately purified batches of the protein. An example of a typical experiment is shown in Fig. 1. For the later figures, the sensorgrams were analyzed by Biacore BIAevaluation software, and the signal was adjusted to zero before the addition of the analyte.

3. Results

3.1. Control experiments

Locatelli-Hoops et al. [25] have recently successfully measured the interaction of saposin A with desired lipid vesicles, and they were also able to examine with SPR how saposin A mobilized lipids from the membrane. We started by using similar experimental conditions as described in Locatelli-Hoops et al. [25], and the obtained SPR response was in accordance with theirs (Fig. 1). Immobilizing the vesicles (POPC/PGalCer 9:1) on the chip surface led to a signal increase of 9000–10,000 response units (RUs), and the immobilization usually leveled off in a few minutes. Washing the surface with NaOH was done to remove unbound vesicles and did not have any marked effect on the response. The detergent CHAPS completely removed the immobilized vesicles from the chip surface, since the final response after the regeneration step corresponded exactly to the signal that was obtained in the beginning of the experiment (Fig. 1). The immobilized vesicles that consisted of more saturated lipid species (DPPC and especially P-SM) were harder to wash off and this decreased the lifetime of the used sensor chip.

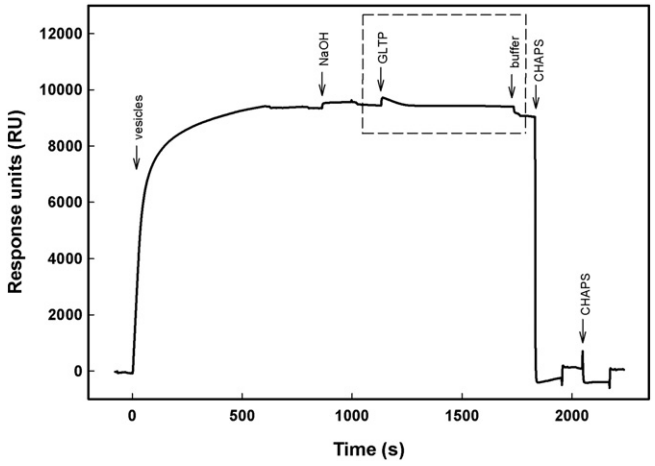

Fig. 1. Description of a typical surface plasmon resonance experiment with GLTP. At first, vesicles (9:1 POPC/PGalCer) were adsorbed to the L1 Biacore chip for 10 minutes. Then, 50 mM NaOH was added to stabilize the baseline and then GLTP (0,1 mg/ml) was injected to start the extraction of glycolipids from the vesicles on the chip surface. After 10 minutes, the eluent was changed to buffer and GLTP easily detached from the vesicle surface. Finally, the chip was regenerated with two injections of 20 mM CHAPS, and the experiment could be started all over again. The box in the figure corresponds to the part shown in the other figures of this publication.

Initially, when testing our experimental setups, we used a batch of GLTP that had been stored in the freezer with 10% glycerol. GLTP has been shown to maintain its activity even after freezing. In the first set of experiments, it was obvious that glycerol gave a marked increase in response units generating a false signal, and therefore, glycerol could not be added to the protein. Fortunately, GLTP is also stable without cryoprotectants, and we found out that it could be stored active at 4 °C without glycerol up to 10 days. Alternatively, the change in refractive index caused by glycerol could be corrected for by injecting the same concentration of glycerol in a reference flow cell.

The effects of the buffer pH and salt strength on the activity of GLTP have been tested before and pH 7.0 and 150 mM NaCl have been shown to be optimal for our protein [7,26]. EDTA and DTT were added because they are present in the last protein purification steps, and we wanted the protein buffer and the experimental buffer to match completely to avoid changes in the response after protein addition.

The optimal concentration of GLTP for the experiments was analyzed in POPC/PGalCer 9:1 vesicles. We were able to detect glycolipid removal with 0.01 mg/ml GLTP, but the process was quite slow (data not shown). With the highest concentrations of GLTP (up to 1.5 mg/ml tested), the response upon protein binding to the membrane increased as compared to the signal gained from the removal of lipids from the vesicles. The total amount of glycolipid removed from the vesicles seemed to be the same regardless of protein concentration (with protein concentrations over 0.1 mg/ml). We concluded that 0.1 mg/ml protein was best for our experiments and raising the protein concentration from this did not lead to an increased amount of glycolipid removed from the membrane, only at a faster rate. This concentration (4.2 μM) is in line with that used by the Sandhoff lab (2.5 μM) [25].

Because of the limitations with the used Biacore instrument, all our experiments were done at 25 °C instead of the more biologically relevant 37 °C. In our earlier studies with the fluorescent assay on GLTP activity, we have observed that the GLTP-mediated glycolipid transfer rate is the same at 30 and 37 °C [28]. For this study, we also determined the transfer rate at 25 °C with the fluorescent assay (data not shown). The transfer rate slows down considerably at temperatures below 30 °C, but there is still detectable transfer at 25 °C.

Finally, we wanted to compare the effects of glycolipid removal that we saw with GLTP to that of other proteins to verify the method. Bovine serum albumin (0.1 mg/ml) did not bind to the POPC/PGalCer 9:1 membranes (data not shown). In its lipid-free form, bovine serum albumin was able to remove a small amount of lipids from the membrane. Bovine serum albumin also serves as a control of the full coverage of the chip surface with vesicles, since it has been shown to bind strongly to an empty L1 chip and not at all to a chip fully covered with lipid vesicles [27]. As a last control experiment, we wanted to have a protein that would bind to the membrane but would not remove any lipids from it. Two of the GLTP mutants used in this study (W96A and W142A) were good controls for proteins that bound to the membrane but did not extract any lipids from it (results shown later in Fig. 5).

3.2. The influence of increasing membrane PGalCer content on lipid removal by GLTP

After the conditions for the experiments were worked out, we started measuring how the glycolipid content in the membrane influenced the amount of PGalCer extraction from the lipid vesicles. This type of experiment with very high concentrations of glycolipids in the donating membrane has been hard to do with the fluorescent probes due to self-quenching. There is only one study that measured GLTP-mediated removal of high concentrations of pyrene-labeled lipids [10]. Although they had some problems in interpreting their

data, they concluded that the efficiency of glycolipid uptake by GLTP depends on the mole fraction of the glycolipid [10]. Fig. 2 shows that the amount of glycolipid extracted from the membrane is dependent upon the concentration of PGalCer in the membrane. The more glycolipid there is in the membrane, the larger the signal decrease after protein binding to the membrane. We were able to detect glycolipid transfer already from a lipid membrane containing 1 mol% of PGalCer. In our experiments, we also included vesicles containing 1 mol% anthrylvinyl-GalCer (AV-GalCer) in a POPC matrix for control purposes. AV-GalCer has been used extensively in our FRET-based method as the fluorescent probe for glycolipids, and therefore, we wanted to compare the transfer of native glycolipids to that of the fluorescent probe. The removal of AV-GalCer was somewhat faster than the removal of PGalCer. The transfer of glycolipids from 1 mol% AV-GalCer containing POPC membranes corresponded to that from 2.5 mol% PGalCer containing POPC membranes judged by the signal decrease after protein addition. The amount of GLTP binding to the membranes was similar and did not seem dependent on the PGalCer concentration. This observation is in line with an earlier study on GLTP partitioning into different vesicle populations with varying glycolipid content [8].

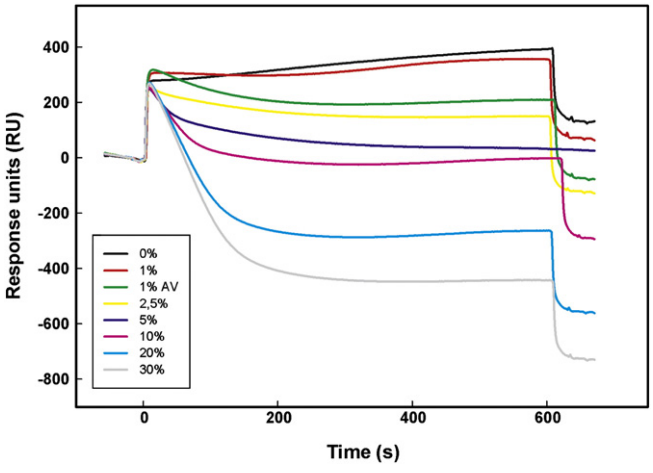

Fig. 2. Increasing PGalCer content leads to an increase in the amount of glycolipid extracted by GLTP from the lipid vesicles. POPC lipid vesicles with varying percentage of PGalCer (from 0% to 30%) were adsorbed to the chip surface (in one set of experiments, 1 mol% AV-GalCer). GLTP-mediated transfer of glycolipids (0.1 mg/ml GLTP) from the vesicle surface was then recorded as a function of time. Washing the membrane surface with running buffer after 10 minutes interrupted the transfer. The figure is representative of several sets of experiments with independently purified protein and new sets of vesicles.

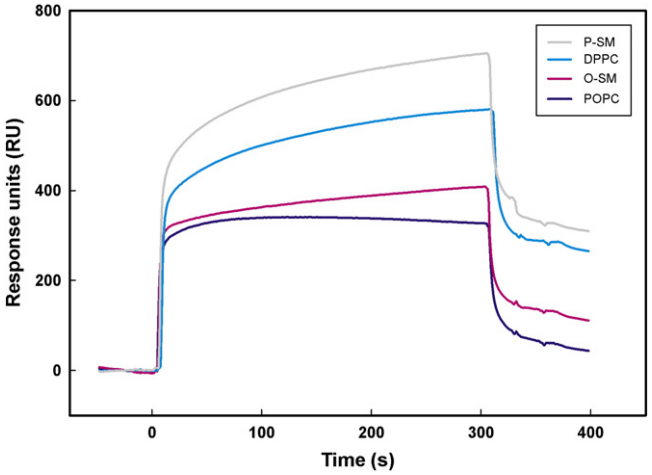

Fig. 3. Binding of GLTP to different lipid vesicle membranes. Different sets of lipid vesicles containing solely one phospholipid class were attached to the chip surface. Binding of 0.1 mg/ml GLTP to the membrane surface was then followed as a function of time. GLTP was washed away from the membrane with running buffer. The figure is representative of several similar sets of experiments.

3.3. The amount of GLTP binding is affected by the phospholipid composition of the membrane

GLTP has also been shown to bind to lipid membranes without glycolipids [7–9]. All of the earlier work has looked at the interaction of GLTP with POPC membranes. We wanted to determine if the extent of GLTP binding was dependent on the phospholipid composition of the membrane. For this purpose, we used two different pairs of phospholipids, one lipid pair containing an unsaturated fatty acyl chain, O-SM and POPC, and the other lipid pair containing only saturated fatty acyl chains, P-SM and DPPC. The response curves in Fig. 3 show that GLTP binds to a higher extent to the more saturated lipid membranes than to the unsaturated membranes. The onset of binding is immediate upon the addition of GLTP to the chip membrane, and this immediate binding is observed with all used lipid species. Likewise, the dissociation of GLTP from the used membranes is fast and immediate, when the GLTP injection is stopped and the surface is washed with buffer. If the extent of binding to the membranes consisting of different lipid classes within the pairs is compared, GLTP binds more to sphingomyelin containing membranes than to membranes containing solely phosphatidylcholine. This can be seen with both pairs of lipid classes. Note that there is no loss of lipids from the vesicles after GLTP binding, since none of the lipid species are substrates for GLTP.

3.4. The immediate glycolipid membrane environment affects GLTP-mediated transfer of glycolipids from lipid vesicles

The lipid environment close to the glycolipid and the physical state of the membrane donating the glycolipid has been shown to affect the amount and rate of GLTP-mediated transfer [12,28]. In the following, we wanted to examine the removal of PGalCer from the different kinds of membranes used in Fig. 3. We chose to use vesicles with 10 mol% PGalCer, since this glycolipid content gave a good response signal with POPC (see Fig. 2). As we expected, the removal of PGalCer was slower and less pronounced from the membranes with the other used lipid species compared to POPC containing membranes (Fig. 4). The GLTP-mediated transfer of PGalCer was quite fast from O-SM/PGalCer and P-SM/PGalCer membranes. We detected a small amount of PGalCer transfer from DPPC/PGalCer membranes. The glycolipid transfer kinetics from P-SM/PGalCer membranes by GLTP differs from the kinetics seen from the other lipid species used. The difference in the magnitude of GLTP binding to the varying membranes can also be seen in this figure in the height of the response signal upon protein binding, just like in Fig. 3.

3.5. The effects of specific tryptophan point mutations in GLTP on the capacity of the protein to transfer glycolipids

Both our and Dr. Brown's research groups have studied the effects of specific tryptophan point mutations on the activity and the membrane interaction of the glycolipid transfer protein [7,14]. In this study, we produced and purified three different tryptophan mutants of GLTP and compared their membrane binding and transfer capacity to that of wild-type GLTP. In contrast to our earlier studies, only one tryptophan out of the three in the protein was mutated at a time in this study, and the other two remained intact. The results are shown in Fig. 5. Panel A compares the binding of the proteins to a POPC membrane. The membrane–protein interaction of the W96A

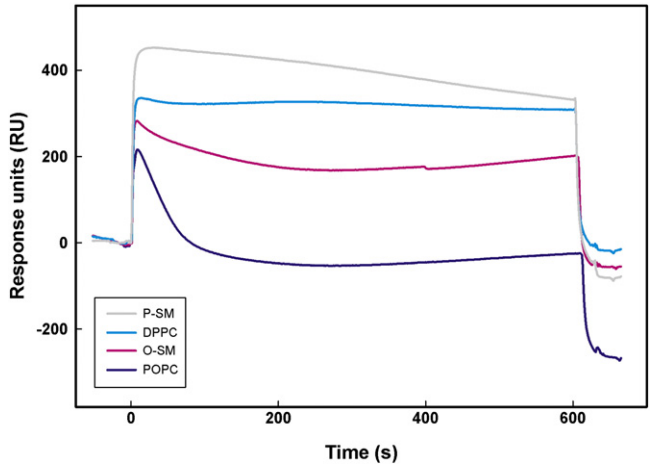

Fig. 4. The immediate glycolipid membrane environment affects GLTP-mediated transfer of glycolipids from lipid vesicles. Mixed vesicles containing a specific phospholipid (90 mol%) with 10 mol% PGalCer were immobilized on the chip surface. GLTP-mediated glycolipid extraction from the vesicle surface was then followed as a function of time. Washing the protein from the membrane surface with running buffer stopped the extraction. The figure is representative of similar sets of experiments.

mutant protein (light blue curve in Fig. 5A) gives a similar response signal to that of wild-type GLTP (blue curve). If tryptophan 142 in the protein is mutated to alanine (pink curve), the amount of membrane binding of the mutant to POPC membranes is lowered by approximately 50% as compared to WT. On the other hand, if the same tryptophan 142 is mutated to the more hydrophobic phenylalanine (grey curve), the protein binding to the membrane clearly increases as compared to W142A and it is even somewhat higher than with wild-type GLTP.

Fig. 5B compares the glycolipid transfer capacity (from POPC/PGalCer 9:1 membranes) of the same mutants to that of WT-GLTP. As expected, the W96A mutant protein (light blue curve) is not capable to mediate transfer at all. This has already been shown in earlier studies using fluorescent assays on this specific tryptophan mutant [14], and this also confirms that it is possible to obtain reliable data on glycolipid transfer by the SPR technique. The obtained data on the transfer capacities of the tryptophan 142 protein mutants is more novel and interesting, since they have not been tested for transfer before. If tryptophan 142 is mutated to alanine, this leads to a protein that, in addition to a lower amount of binding to membranes, is not capable to mediate transfer (Fig. 5B, pink curve). The mutation of the same tryptophan 142 to phenylalanine does not lead to a completely inactive transfer protein (grey curve). A clear protein-mediated transfer of PGalCer is observed, although the glycolipid transfer capacity of the mutant is clearly lower than that of wild-type GLTP (blue curve).

4. Discussion

This study was conducted to investigate the use of the surface plasmon resonance technique for examination of glycolipid transfer proteins. We have looked at GLTP-mediated glycolipid transfer online with natural substrates and outlined the experimental conditions for the use of the method in further studies. The binding of GLTP to varying membranes and how the membrane environment affects the transfer process is especially suitable to address with the SPR approach. We have also looked at how single point mutations in the protein can affect its activity and membrane interaction. Some of the used tryptophan mutants have been used in earlier studies based on fluorescence, and with this approach, we have now been able to reproduce previous data (validating the method) and most importantly to generate totally new information on novel mutants.

It has been shown by several techniques that the vesicles stay intact on the surface of the sensor chip [18,27,29]. Our experimental conditions were very similar to those used by Anderluh et al. [27] when they showed that calcein-loaded vesicles stay intact on the sensor chip until permeabilized by a pore-forming toxin. The binding of zwitterionic vesicles corresponding to a signal increase of 9000–10,000 RUs to the sensor chip in our experiments corresponds to that calculated by their research group for a surface of intact vesicles and is too high for the binding of a single bilayer [27]. Our results on the removal of glycolipids to GLTP also argue for intact vesicles, since GLTP-mediated transfer from planar membranes is very slow [30]. An increase in membrane curvature has been shown to lead to an increased transfer rate [8,30].

From our data on glycolipid-containing membranes, it is not possible to acquire the association and dissociation constants with the BIAevaluation software, since the glycolipid is extracted from the membrane and the kinetic curve therefore becomes complex. However, from the sensorgrams on the pure phospholipid membranes without glycolipids (Figs. 3 and 5A), the binding constants could easily be calculated if a series of experiments were done with

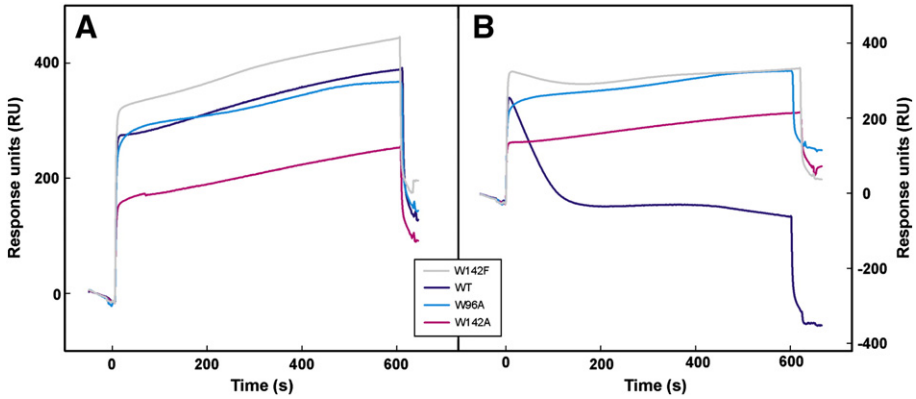

Fig. 5. The effects of specific tryptophan mutations in the glycolipid transfer protein on the capacity of the protein to transfer glycolipids. In all experiments, the protein concentration was 0.1 mg/ml. (A) The binding of wild-type GLTP to a POPC lipid membrane was compared to the binding of glycolipid transfer proteins with mutations in the indicated tryptophan residues within the proteins. (B) POPC/PGalCer 9:1 vesicles were first attached to the chip membrane. The transfer efficacy of wild-type GLTP was then compared to the transfer induced by the same mutant proteins as in panel A. Both panel figures are representative of at least two similar sets of experiments with separately purified protein batches and freshly made lipid vesicles. The mutant proteins used within one figure were always purified and analyzed side by side with WT.

varying protein concentrations. We tried to determine the binding constants but were unable to reach saturation in the binding of GLTP to the membrane for protein concentrations up to 60 μM. It is unlikely that the adsorption of GLTP to the membrane will ever become saturated because the protein will start to pile up on the membrane surface, forming multilayers due to its hydrophobic nature. In this study, we decided not to pursue with the constants since the difference in the magnitude of binding of GLTP and its mutants to the varying membranes can easily be visualized from our experiments. The group of Sandhoff has taken a similar approach in presenting their data on saposins and GM2 activator protein [25,31,32]. Their response signals with saposin-mediated lipid removal from membranes are larger than ours, since up to 86% of the lipids included in their experiments are substrates for their proteins, while we typically had 10% glycolipid as substrate in our experiments. The amount of lipid available for transfer is adjustable, and in our experiments, 10% is enough for a sensitive experimental system, since GLTP has a high specificity for its substrate.

The amount of glycosphingolipids in the membrane donating the lipids for transfer affects the transfer rate, as faster transfer is observed from membranes with a higher concentration of PGalCer (Fig. 2). There seems to be a limit in the amount of glycolipid available for transfer to GLTP, since from all of the vesicle compositions used only 23–30% of the total PGalCer was removed. In an earlier study, Mattjus and coworkers showed that galactosylceramide (total concentration up to 5 mol%) does not distribute evenly in sonicated POPC vesicles but rather seems to prefer the inner leaflet so that only 25% of total GalCer is found in the outer leaflet and is therefore available for transfer [33,34]. This is consistent with our data and suggests that we are extracting out all of the GalCer in the outer leaflet. This also explains why we cannot remove more than 30% of PGalCer from 9:1 POPC/PGalCer membranes by increasing the GLTP concentration.

In the fluorescent AV-GalCer analog used in our previous work, the AV moiety of the probe is positioned in the middle of the bilayer, at the end of the acyl chain. Since the AV part is quite bulky, this certainly weakens the interaction of AV-GalCer with neighboring molecules in the membrane as compared to PGalCer, and this weaker interaction causes AV-GalCer to be more readily removed than PGalCer by GLTP from the membrane (Fig. 2). The acyl chain in AV-GalCer is 12 carbons long and this shorter chain length also decreases its interaction strength with neighboring phospholipids and increases its transfer as compared to PGalCer.

GLTP is known to interact with membranes in the absence of glycolipids [7–9]. All of these studies have been done on vesicles consisting of solely POPC, and therefore, the effect of the membrane lipid composition on the initial membrane interaction of GLTP has not been studied in detail. We addressed this issue by comparing the binding of GLTP to two different sphingomyelin–phosphatidylcholine pairs. Within the pairs used for the membrane interaction studies in Fig. 3, the unsaturated pair is in liquid-crystalline phase at 25 °C and the saturated pair in gel phase [35]. The binding of GLTP to the pure phospholipid membranes seemed to favor gel phase membranes. and the binding was more pronounced to membranes consisting of sphingomyelins than phosphatidylcholines, in both pairs. The same difference in the extent of binding of GLTP to the used membranes was also seen if 10 mol% of the phospholipid was exchanged for PGalCer (Fig. 4). Contrary to our results, Rao et al. found that increasing the sphingomyelin content in mainly POPC-containing vesicles diminished the partitioning of GLTP to the vesicles [8]. In their studies, they used bovine brain sphingomyelin, POPC, 10 mol% porcine brain galactosylceramide and included 10 mol% dansyl-DHPE as quencher. The biological sphingolipids used contain a wide variety of different species varying in acyl chain length and also in unsaturation and hydroxylation [8]. At 37 °C, they have a quite complicated system since some of the sphingolipids are below their T_m and some over; this probably enhances the formation of membrane domains and makes the results on the partitioning of the used dansyl-DHPE probe hard to interpret compared to our simple system. Further, the amount of the quencher is really high in their study and can affect the results and the phase behavior of the lipids.

The transfer rate of PGalCer from vesicles containing 10 mol% glycolipid in a phospholipid matrix was studied for the two pairs of sphingomyelin and phosphatidylcholine (Fig. 4). In earlier studies we have seen a comparable rate of glycolipid transfer from POPC, O-SM, and DPPC vesicles, but we have seldom been able to induce glycolipid transfer from vesicles containing only P-SM and GalCer with our fluorescent assay [11,12,30]. Since there is a difference in the temperature, in the mol% of glycolipids and also in the amount of GLTP that was exposed to the lipids to our recent experiments, the high rate of glycolipid removal in this study from P-SM containing vesicles can probably be explained by these differences. We also analyzed the composition of the flow through after GLTP-mediated transfer from P-SM/PGalCer 9:1 vesicles (data not shown). We found an equal amount of P-SM and PGalCer in the flow through, suggesting that GLTP extracted PGalCer from the membrane, but that a small amount of intact vesicles was also lost during the experiment. If the drop in RU upon GLTP addition would arise from lost vesicles only, the composition of the flow through would need to be 9:1 as in the original vesicles. Besenicar et al. [36] have also noticed that vesicles with a high sphingomyelin content are slightly unstable on the chip surface upon cholesterol extraction by cyclodextrin from the vesicles.

Most of the glycolipid in POPC/PGalCer membranes can be removed by the transfer protein, according to our calculations earlier in the discussion. The total glycolipid removal is smaller from O-SM/PGalCer, DPPC/PGalCer, and P-SM/PGalCer membranes as compared to membranes consisting of POPC/PGalCer (Fig. 4), although the total concentration of GalCer in the outer leaflet should be higher in the sphingomyelin vesicles. While GalCer in POPC vesicles prefers the inner leaflet, it has been shown to favor the outer leaflet in P-SM vesicles [33,34]. Therefore, a considerable amount of glycosphingolipids is left inaccessible to GLTP in the outer leaflet in these lipid membranes, probably due to a better miscibility of the lipids within the membrane. Glycosphingolipids can interact to a higher extent with sphingomyelins due to formation of hydrogen bonds, whereas phosphatidylcholines are unable to form similar bonds with glycosphingolipids, giving a better miscibility of GalCer in SM membranes [37]. The unsaturated membrane lipids and PGalcer are not very miscible due to the difference in their T_m, and this leads to the formation of large lateral PGalCer domains and gives rise to sharp phase boundaries [37,38]. The difference in T_m between the saturated lipid pair and PGalCer is smaller giving a better miscibility and formation of less segregated domains, and consequently less phase boundaries [38]. Since we speculate the phase boundaries to be the best place for GLTP access to the glycosphingolipids poorer miscibility and larger difference in T_m should enhance GLTP-mediated transfer of glycolipids [2]. Phospholipases have been shown to be activated by lipid packing defects, or phase boundaries, in vitro [39–41]. The ability of GLTP to act at phase boundaries, in addition to the higher amount of binding to more saturated membranes, further strengthen the suspicion of GLTP having a role in sensing abnormal glycolipid accumulations within cells. GLTP might be one of the proteins whose activity is regulated by the formation and disappearance of membrane packing defects according to the superlattice model [42].

We found the SPR technique to be very useful in analyzing the extent of membrane binding and lipid transport capacity of lipid transfer proteins. In accordance with previous work, we were able to detect inactive mutants in an efficient way [25,31,32,43]. Our earlier studies on tryptophan mutants have relied on fluorescent methods, and two simultaneous mutations were studied at a time to be able to study the membrane interaction based on tryptophan fluorescence [7]. With SPR we were able to study single point mutations, just as Sugiki et al. in their recent paper on a truncated form of ceramide

transfer protein CERT (the CERT START domain) [43]. Our new data correspond well with the earlier data on tryptophan mutants of GLTP, since the W96A protein is not capable to mediate transfer in our SPR experiments [14]. What has not been shown before is that the membrane interaction of the W96A mutant is unaffected as compared to WT, and therefore W96 does not appear to be involved at all in the membrane-interacting part of GLTP.

Our results on the W142 mutant proteins are novel, since these single mutants have not been studied before. Exchanging this tryptophan to alanine lowers the membrane interaction of the protein to half of WT, and this mutation also confers a protein inactive in mediating transfer (Fig. 5). Mutation of the same tryptophan 142 to phenylalanine does not lead to an inactive transfer protein, although the transfer is markedly lower than that by WT. Further W142F seems to interact with to a higher extent with the vesicle membrane than WT. This can perhaps be explained by the higher hydropathy index of phenylalanine as compared to tryptophan. In the study of West et al. one of the mutants (W96 intact, W85 and W142 mutated to F) retained 72% of its activity. Based on our results, we conclude that this loss in activity is probably solely due to the W142F mutation and the W85F mutation does not play an important role [7]. The tryptophan 142 is according to our studies important for the membrane binding of the protein and its importance has also been confirmed by a recent photo-CIDNP NMR study [10]. In another recent study, Zhai et al. [16] looked at the intrinsic tryptophan fluorescence of GLTP and how the fluorescence shifts upon glycolipid binding from glycolipid/POPC vesicles. They claimed that the entire shift in tryptophan fluorescence upon GLTP-substrate binding came from W96 and none from W142. This does not mean that W142 could not be involved in the initial membrane binding of GLTP; this simply means that it is not possible to detect the importance of W142 by steady-state tryptophan fluorescence, since the immediate hydrophobic environment close to W142 is quite similar in the protein's empty and substrate filled form. We conclude that W142 has an important role for the functionality of the protein.

In conclusion, surface plasmon resonance is a very useful tool in membrane–protein interaction studies. We find it even more valuable for use with membrane-interacting transfer proteins since the transfer process in this way can be measured online and with natural substrates. Further, when binding the lipids to the chip, there is no need to modify the protein; as long as it is pure enough, it can directly be added as the analyte. During the writing process of this publication, Sugiki et al. [43] published a paper on the use of surface plasmon resonance to study a truncated form of the ceramide transfer protein CERT, its START domain. They conclude that, with their new real-time assay method, elementary processes in the transfer process, such as lipid binding and lipid uptake, can now be measured in more detail as compared to conventional assay methods for transfer processes [43]. In our study, in contrast to Sugiki et al., we have analyzed the complete glycolipid transfer protein, enabling also a careful analysis of the initial interaction of the protein with membranes. Like with transfer proteins, SPR is also beginning to be of great importance in studies on membrane–drug interactions and in preclinical drug discovery [18].

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