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Differences in the domain forming properties of *N*-palmitoylated neutral glycosphingolipids in bilayer membranes

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Abstract

We have compared the domain forming properties of three neutral acyl chain defined glycosphingolipids differing in their head group structures. The aim of the study was to explore if glycosphingolipids and sterols exist in the same lateral domains in bilayer membranes and how the structure of the head group influences the capacity of the glycosphingolipids to colocalize with cholesterol. The glycosphingolipids used in the study were galactosyl-, glucosyl- and lactosylceramides with a palmitic acid in the *N*-linked position. Domain formation in mixed bilayer vesicles was examined using fluorescent reporter molecules associating with ordered domains, together with a fluorescence quencher lipid in the disordered membrane phase. Our results show that the glycosphingolipids studied were poor in forming sterol-enriched domains compared to palmitoyl-sphingomyelin as detected by cholestatrienol quenching. However, the tendency to associate with cholesterol was clearly dependent on the carbohydrate structure of the glycosphingolipids, also when two glycosphingolipids with different head groups were mixed in the bilayer. All palmitoylated glycosphingolipids associated with palmitoyl-sphingomyelin/cholesterol domains. Our results show that the head group structures of neutral glycosphingolipids markedly affect their domain forming properties in bilayers both with and without cholesterol. The most striking observation being that large differences in domain forming properties were seen even between glucosylceramide and galactosylceramide, which differ only in the stereochemistry of one hydroxyl group in the carbohydrate head group.

Keywords: Cholesterol; Cholestatrienol quenching; Galactosylceramide; Glucosylceramide; Lactosylceramide; Membrane domain

1. Introduction

Glycosphingolipids at the cell surface participate in cell–cell interaction and recognition possibly through what has been called glycosignaling domains [1–3]. Glycosphingolipids in the plasma membrane also function as receptors for toxins [4,5] and viruses [6]. The structural features in common for the glycosphingolipids are the sphingoid base (mainly 18–20 carbons in length) and the long, mostly saturated amide-linked acyl chain. The structure of the polar head group may vary significantly, ranging from one neutral monosaccharide residue to big assemblies of carbohydrates and sialic acid, which gives the gangliosides their charged nature. The wide variety in head group structures among natural glycosphingolipids appears likely to reflect their biological function as specific recognition molecules in plasma membranes.

Most animal glycosphingolipids are structurally derived from glucosylceramide (GlcCer), and many also share the lactosylceramide moiety, which contains an additional galactose residue, as a common unit [7]. Another series of glycosphingolipids are derived from galactosylceramide (GalCer), which differ from GlcCer only by an altered stereochemical configuration of one hydroxyl group in the sugar head group. GalCers are highly enriched in the multilamellar layers of the myelin sheet which isolates the axons of neuronal cells [7], while the GlcCer derived glycosphingolipids often are found in the so called glycosynapses of plasma membranes [2,8].

GalCers and GlcCers also follow different sorting pathways in polarized cells [9,10]. Newly synthesized GlcCers are preferentially sorted to the apical plasma membrane domain as shown in studies on HepG2 and MDCK cells, whereas GalCers, like sphingomyelins, are transported to the basolateral membrane [9,10].

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In the late 1990s the raft hypothesis suggested that ordered domains enriched in glycosphingolipids, sphingomyelin and cholesterol are present in biological membranes [11,12]. Since then researchers in the field have taken great interest in the lateral distribution of these lipids in both cell and model membranes. Glycosphingolipids have high T_m values and a tight lateral packing density in bilayer membranes, due to their hydrophobic parts being highly saturated and the opportunity to form extensive hydrogen-bonding networks in the head group region [13–15]. It has been shown that glycosphingolipids have the ability to form gel-phase microdomains or partition into liquid-ordered domains in liquid-disordered membranes [16,17].

Because of their structure glycosphingolipids could also be regarded as good candidates for interacting with cholesterol. However, cholesterol has only moderate effects on domain formation by mixed brain galactosylceramides [18]. It has also been shown that there is solid phase immiscibility of *N*-palmitoyl-galactosylceramide (PGalCer) with cholesterol at temperatures below the T_m of the glycolipid [19]. The conclusion was drawn that hydrogen bonding between PGalCer molecules hindered the interaction with cholesterol. GlcCers and their interaction with sterols in membranes have not been studied as much as GalCers. Yet, studies on pure glycosphingolipid membranes have shown that the thermotropic behavior of the epimers PGalCer and *N*-palmitoyl-glucosylceramide (PGlcCer) are quite similar, although not identical [20,21].

The rafts co-existing with the fluid lipid matrix of the plasma membrane have been proposed to be in the liquid-ordered phase [15,22,23]. Cholesterol seems to be essential for formation of the liquid-ordered phase. However, ordered domains can be formed by saturated sphingolipids in bilayers even without cholesterol. Cholesterol can also be displaced from liquid-ordered domains by other molecules such as ceramides or long-chain alcohols or amides [24–27].

Although glycosphingolipids, sphingomyelin and cholesterol are all found in the detergent resistant fraction of biological membranes, it does not necessarily mean that these lipids are located in the same lateral domains. Some studies have suggested that populations of ordered domains with differing sphingolipid content may be present in biological membranes [8,28]. In a recent study on raft-associated GPI-anchored proteins it was shown that there were significant differences in cerebroside content in distinct detergent resistant domains in the same natural membrane [29].

The aim of this study was to explore whether some specific glycosphingolipids and sterols co-exist in the same ordered domains in mixed bilayer membranes or if they perhaps segregate into different domains, as was recently implicated for ceramides in a study by Chiantia and coworkers [30]. The glycosphingolipids used in this study were galactosyl-, glucosyl- and lactosylceramides with a palmitic acid in the *N*-linked position (PGalCer, PGlcCer and PLacCer, respectively). We also wanted to explore how the structure of the polar head group affected the membrane properties of the glycosphingolipids, bearing in mind that they have different specialized functions in biological systems. Interestingly, we noticed that even such a small structural difference as the stereochemical orientation of one hydroxyl group in the sugar moiety, affected the domain forming properties of the GalCer and the GlcCer.

2. Experimental procedures

2.1. Materials

D-*erythro*-*N*-palmitoyl-sphingomyelin (PSM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL) by reverse-phase HPLC (Supelco Discovery C18-column, dimensions 250×21.2 mm, 5 μm particle size) using 100% methanol as eluent. The purity and identity of the product was verified on a Micromass Quattro II mass spectrometer (Manchester, UK). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids. (7-Doxyl)-stearic acid was obtained from TCI (TCI Europe N.V., Belgium) and was used for the synthesis of 1-palmitoyl-2-stearoyl-(7-doxyl)-*sn*-glycero-3-phosphocholine (7SLPC) according to Szolderits and coworkers [31]. Cholesterol was from Sigma Chemicals (St. Louis, MO). Stock solutions of lipids were prepared in hexane/2-propanol (3:2 vol:vol), stored in the dark at −20 °C, and warmed to ambient temperature before use.

Palmitoyl-galactosylceramide (N-palmitoyl-psychosine) was obtained from Larodan Fine Chemicals (Malmö, Sweden) and used without further purification. (The results of the fluorescence quenching experiments were identical when PGalCer was synthesized similarly as the other glycosphingolipids.) All other acyl chain defined glycosphingolipids were synthesized from the corresponding lyso-derivatives (Avanti Polar Lipids; head group identity verified by NMR by the supplier) and fatty acid anhydrides (Sigma Chemicals). 7 μmol lyso-galactosylceramide (D-galactosyl-β1-1′-D-*erythro*-sphingosine; psychosine), lyso-glucosylceramide (D-glucosyl-β1-1′-D-*erythro*-sphingosine) or lyso-lactosylceramide (D-lactosyl-β1-1′-D-*erythro*-sphingosine), 70 μmol of palmitic acid (or oleic acid) anhydride and 5 μmol of triethylamine were dissolved in 500 μl dry dichloromethane/methanol (4:1, vol:vol). The reactions were carried out at room temperature (or at 35 °C if the components were difficult to dissolve) for 5 h. The glycolipids were purified by reverse-phase HPLC on a preparative Supelco RP-18 column with 100% methanol as the eluent and UV-detection at 203 nm. The purity and identity of the products were verified on a Micromass Quattro II mass spectrometer.

Cholestatrienol (cholesta-5,7,9(11)-trien-3-beta-ol; CTL) was synthesized and purified using the method published by Fisher and coworkers [32]. *trans*-parinaric acid (tPA) was obtained from Molecular Probes (Eugene, OR). D-*erythro*-*N*-*trans*-parinoyl-sphingomyelin (tParSM) was synthesized from tPA and D-*erythro*-sphingosylphosphorylcholine (lyso-SM; Avanti Polar Lipids) according to Cohen and co-workers [33]. The synthesized fluorescent probes were purified by reverse-phase HPLC on a RP-18 column with methanol/acetonitrile (7:3, vol:vol) as eluent for CTL and 100% methanol for tParSM. All compounds were positively identified by mass spectrometry. CTL, tPA and tParSM were stored dry under argon in the dark at −87 °C until solubilized in argon-purged ethanol (CTL) or methanol (tPA and tParSM). Stock solutions of fluorescent lipids were stored in the dark at −20 °C and used within a week.

2.2. Preparation of vesicles

Vesicles used in steady-state fluorescence measurements were prepared at a lipid concentration of 50 μM. The lipid mixtures were dried under nitrogen, redissolved in benzene to assure the lipids were properly mixed, redried with nitrogen and finally kept in vacuum for 30–60 min. The dry lipids were dispersed in argon-purged water and heated to about 60 °C. The warm samples were vortexed and then sonicated for 2 min (25% duty cycle, power output 10 W) with a Branson probe sonifier W-450 (Branson Ultrasonics, Danbury, CT). Sonication at ~60 °C was shown to give similar results in fluorescence quenching studies as sonication at a temperature above the T_m of the pure glycosphingolipids (data not shown). This procedure gives liposomes with a rather large size distribution with a mean diameter of about 200 nm. The lipid

composition did not markedly affect the size distribution in the samples according to light-scattering (Malvern 4700 multiangle laser spectrometer at an angle of 90°). The water used in the experiments was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system, to yield a product with a resistivity of 18.2 MΩcm.

In fluorescence quenching studies, F samples consisted of POPC: 7SLPC: sphingolipids: sterol (30:30:30:10, mol%) and in F_0 samples POPC replaced 7SLPC. These membrane compositions (at least with PSM as the sphingolipid) should experience a phase co-existence region during the temperature scan (10–80 °C) according to previous studies [34,35]. POPC and the nitroxide labeled quencher (7SLPC) mix well at the concentrations used, and together they make up the disordered phase in the vesicles at all temperatures studied (Halling, K.K., Ramstedt, B. and Slotte, J.P., unpublished calorimetric data). The sphingolipids and sterol constitute the ordered domain building lipids of the mixture. The formation of ordered domains was studies with CTL as a selective marker for sterol-enriched domains and tParSM as a marker for sphingomyelin-rich domains. CTL is a fluorescent cholesterol analog that has been shown to mimic the membrane behavior of cholesterol quite well [32,36–39]. In complex lipid bilayers, in which lateral domain formation is expected, CTL can be used as a fluorescent reporter molecule that associates with sterol-enriched domains [40]. tParSM has been used by us in previous studies where we have shown that the probe reports on melting temperatures of sphingomyelin-rich domains in fairly good agreement with DSC results on similar lipid mixtures [26]. The free fatty acid tPA was observed to partition into ordered domains in general and was used to detect ordered domains not enriched in sterols. The probes were included in the bilayers to represent 1 mol% of the total lipid so that CTL and tParSM replaced a fraction of cholesterol and PSM, respectively, and tPA replaced either glycosphingolipid or POPC. The fluorescent probes were protected from light during all steps and solvents were saturated with argon before use in order to minimize the risk of oxidation.

For DSC measurements, vesicles composed of POPC: glycosphingolipid: cholesterol (60:30:10, mol%) were prepared similarly as above. The vacuum dried lipids were hydrated in warm water (about 70 °C) to yield a final lipid concentration of 4 mM. The samples were vortexed briefly followed by 4 min of bath sonication at ~70 °C with a Branson bath sonifier 2510 (Branson Ultrasonics).

2.3. Steady-state fluorescence measurements and quenching of steady-state fluorescence

Fluorescence measurements were performed on a PTI QuantaMaster-2 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ) operating in the L-format. The temperature was controlled by a Peltier element, with a temperature probe immersed in the sample solution. The samples were heated and cooled between 10 and 80 °C at a rate of 5 °C/min during the measurements. The measurements were performed in quartz cuvettes and the sample solutions were kept at constant stirring throughout the measurements. The excitation and emission slits were set to 5 nm and the wavelengths were adjusted with selectively transmitting filters. The wavelengths of the excitation and emission filters used for CTL were 321 nm (range ±5 nm) and >385 nm, respectively. Fluorescence emission of tPA and tParSM was detected at >420 nm, while excitation occurred through a filter centered around 310 nm (range ±5 nm).

The fluorescence quenching method has been discussed in detail in previous publications [26,40]. Briefly, fluorescence intensity of quencher containing F samples is compared to the fluorescence intensity of quencher-free F_0 samples giving the fraction of unquenched fluorescence (F/F_0). In lipid vesicles in which ordered and disordered domains co-exist, the amount of CTL, tParSM or tPA (predominantly associated with ordered domains) exposed to quenching by 7SLPC gives a measure of the extent of ordered domains formed in the system. The stability of the sterol-enriched domains, for example, can be measured by looking at the quenching susceptibility of CTL as a function of temperature. Quenching susceptibility of tParSM similarly reports on stability of sphingomyelin-rich domains. The changes in quenching are reversible in bilayers containing PSM as the domain building lipid after temperature scans to at least 80 °C (results not shown) indicating probe and quencher-stability throughout the experiments.

2.4. Differential scanning calorimetric measurements

DSC measurements were performed in a Calorimetry Sciences Corporation Nano II DSC (Provo, UT). The samples, prepared as described above, were subjected to three sequential heating and cooling scans between 0 and 100 °C at a rate of 0.5 °C/min.

3. Results

3.1. Palmitoyl-glucosylceramide and -lactosylceramide were more prone to associate with cholesterol than palmitoyl-galactosylceramide

Complex bilayers containing POPC (±7SLPC), glycosphingolipid and sterol (60:30:10, mol%) were prepared, where CTL replaced cholesterol to give a final probe concentration of 1 mol % in the bilayer. Fluorescence intensity was measured during heating and cooling scans and the results are shown in Fig. 1A and B, respectively. No melting of sterol-enriched domains as detected by CTL quenching was observed in the heating scan of bilayers containing PGalCer (curve 1) in the temperature interval studied (10–80 °C). In the immediately following cooling scan, an increase in CTL fluorescence intensity between 35 °C and 25 °C was observed, suggesting that some of the sterol population was transiently protected from quenching at these temperatures. The reason for this was possibly that a

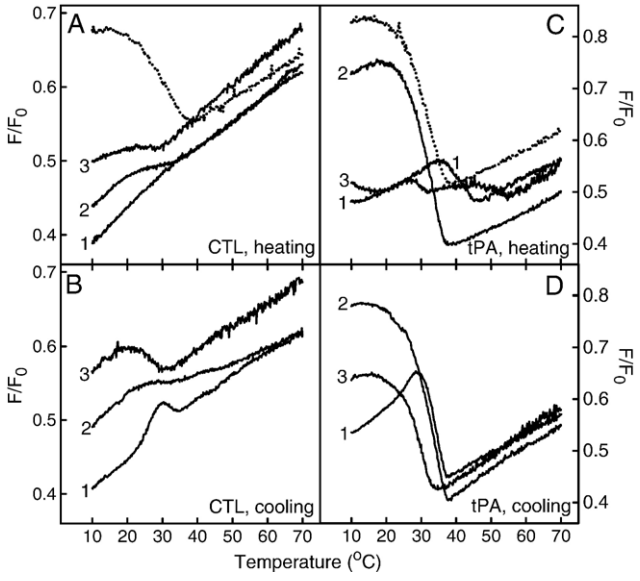

Fig. 1. Formation of ordered domains in complex glycosphingolipid containing lipid bilayers. The quenching of CTL fluorescence (A, B) was used as a measure of the amount of sterol-enriched domains formed in the membranes and the quenching of tPA (C, D) was used as a measure of the amount of ordered (sterol-enriched or sterol-poor) domains formed. The fraction of unquenched fluorescence (F/F_0) is plotted as a function of increasing (A, C) and decreasing (B, D) temperature. The bilayers were prepared to contain POPC (±7SLPC): glycosphingolipid: cholesterol (60:30:10, mol%) where CTL or tPA replaced 1 mol% of cholesterol or glycosphingolipid, respectively. The used glycosphingolipids were PGalCer (1), PGlcCer (2) and PLacCer (3). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min. The dotted lines show domain formation in POPC (±7SLPC):PSM:cholesterol (60:30:10, mol%) with CTL (A) and tPA (C) for reference.

metastable phase in which CTL was included existed around 30 °C. Further cooling seemed to squeeze out the sterol from the protecting environment to again become exposed to the quencher. PGlcCer (curve 2) and PLacCer (curve 3) were able to shield some sterol from the quencher at the starting temperature and up to about 30 °C, as indicated by the slope change in the heating scans of these samples. A larger increase in the F/F_0 value was observed at the same temperature in the cooling scan of the bilayer containing PLacCer, suggesting that more sterols moved into an ordered environment after the sample had been heated to 80 °C. CTL remained protected from quenching in the ordered PLacCer domains at temperatures below 30 °C, opposite to what was observed for PGalCer. Also PGlcCer seemed to end up protecting somewhat more of the probe from quenching in the cooling scan. In such cases, where the F/F_0 value at the end of the cooling scan is slightly higher than at the beginning of the heating scan, it might reflect a temporary probe distribution that will equilibrate with time. It is likely that the probe will be squeezed out more slowly from ordered domains in such samples and that the F/F_0 value at low temperature would gradually decrease with time.

We have shown in previous studies [26,40] that heating of bilayer mixtures with 30 mol% PSM instead of the glycosphingolipid, gives rise to a clear domain melting (dotted line in Fig. 1). Thus, compared to PSM, very little of the sterol was shielded from quenching by the glycosphingolipids as indicated by the small quenching amplitudes and the low F/F_0 values at the initial experimental temperature.

The possible explanations for the continuous increase in F/F_0 with temperature, which is also seen for other lipid compositions in Figs. 3 and 4, are thoroughly discussed in a previous paper by Alanko and co-workers [26]. In brief, the F/F_0 value reflects the fluorescence behavior of CTL as well as reports on fluorescence quenching. CTL fluorescence decreases as a function of temperature, which indirectly gives a smaller change in a highly quenched F value than in the F_0 value. The difference between F and F_0 is therefore larger at low temperatures but becomes smaller at higher temperatures. The gradual increase in F/F_0 that this leads to does not affect our conclusions. CTL fluorescence will also differ somewhat depending on the lipid environment [40], probably resulting in the observed differences in the F/F_0 increase with different lipid mixtures.

3.2. Cholesterol was predominantly excluded from the ordered glycosphingolipid domains

Domain formation by the glycosphingolipids was also studied with tPA as the fluorescent probe. First, we examined if tPA was suitable for detection of ordered domains not enriched in sterols, i.e. domains that CTL fail to report. This was done by comparing the absolute fluorescence intensities of tPA and CTL (at 1 mol%) in various quencher-containing samples (F samples) composed of POPC: 7SLPC: variable lipid: sterol (30:30:30:10, mol%, data not shown). The results showed that the intensity of both probes was low in bilayers where no ordered domains were expected to form, containing an unsaturated lipid, oleoyl-GalCer or POPC, as the variable lipid component. tPA was protected from quenching to a high degree in bilayers containing 30 mol% PGalCer (at 20 °C), whereas CTL was quenched in these samples almost as much as in the POPC sample. Both probes were effectively protected against quenching in samples containing 30 mol% PSM. This indicates that ordered domains were formed both in the PSM and the PGalCer system, but whereas both CTL and tPA partitioned into the domains in the bilayers containing PSM, only tPA was able to partition into the ordered domains in the PGalCer containing bilayers.

Hence, tPA was considered useful for detection of ordered domains not necessarily enriched in sterols. The quenching of tPA was measured as a function of temperature in bilayer vesicles composed of POPC (±7SLPC), glycosphingolipid: sterol (60:30:10, mol%) where tPA replaced 1 mol% of the glycosphingolipid. As seen in the heating scan in Fig. 1C, melting of ordered domains was clearly detected by tPA in PGalCer and PGlcCer containing bilayers, the PGalCer domains being more thermostable. The observed domain melting temperature in the PGlcCer system as detected by tPA (~30 °C) agreed with the temperature detected by CTL, but the F/F_0 curve for tPA had a markedly larger amplitude. Probably, very little of the sterol population was located in the ordered PGlcCer domains, and CTL was therefore less affected by the domain melting than was tPA.

In the PLacCer sample, tPA experienced changes in the bilayer structure at several temperatures as indicated by the variations in the F/F_0 curve. These variations were possibly due to transitions between different phases in which tPA was included. In contrast, tPA detected only one phase change in the PGalCer and PGlcCer samples in the temperature interval studied. We observed that the shape of the PLacCer up scan detected by tPA was changing slightly between different measurements and the discontinuities of the curve shifted in temperature.

Ordered domains formed upon cooling in all the samples (Fig. 1D). In the case of PGalCer and PLacCer (but not PGlcCer), the heating and cooling scans were very different. These differences were reproduced if the samples were subjected to a second heating scan after cooling (results not shown). Domain formation occurred at a lower temperature than the domain melting in the PGalCer sample, but the observed F/F_0 amplitude was larger in the cooling scan. It also seemed like tPA was squeezed out of the ordered PGalCer environment upon cooling below ~30 °C, as was CTL. PLacCer protected tPA from quenching at temperatures below 30 °C after heating, which correlates with the results obtained with CTL. It should again be noted that the absolute F/F_0 value after cooling might change slightly with time when a lipid distribution closer to equilibrium is reached. In bilayers containing 30 mol% oleoyl-glycosphingolipid (OGalCer or OGlcCer), no ordered domains were formed since the F/F_0 curves for both tPA and CTL were continuously linear (results not shown).

The results of the quenching experiments in Fig. 1 agree fairly well with the DSC data for corresponding membrane systems (POPC: glycosphingolipid: cholesterol, 60:30:10, mol%) shown

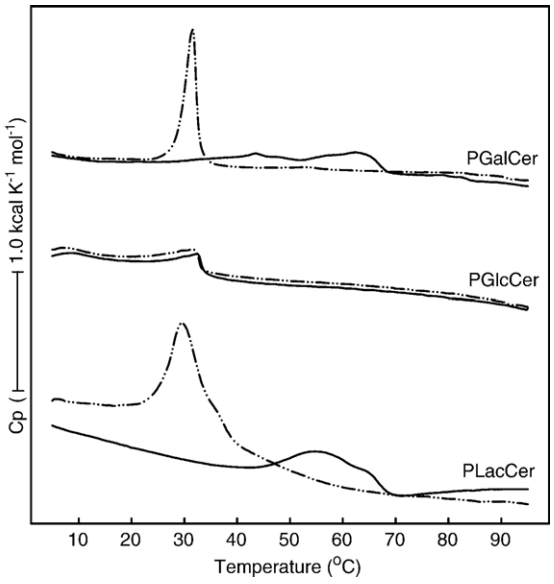

Fig. 2. Representative thermograms of complex glycosphingolipid containing lipid bilayers. The bilayers were prepared to contain POPC:glycosphingolipid: cholesterol (60:30:10, mol%) where the used glycosphingolipids are indicated in the figure. The second heating (solid line) and cooling (dotted line) scans are shown. The total lipid concentration was 4 mM and the temperature gradient 0.5 °C/min.

in Fig. 2. Again, the heating scans differed clearly from the cooling scans of the PGalCer and PLacCer containing bilayers. A distinct transition peak was only seen in the DSC cooling scan of these samples, which correlates with the quenching results where ordered domain formation was more pronounced in the cooling scan after a preceding heating. The transition peak in the PGalCer down scan agreed with the domain formation temperatures observed with CTL and tPA upon cooling. PLacCer domain formation occurred at a slightly lower temperature according to the quenching results compared to the DSC data. The heating thermograms of the PGalCer and PLacCer samples showed a few smaller transitions which appeared or disappeared gradually with increasing number of heating/cooling cycles. Similar variations were also observed with tPA upon heating of the PLacCer containing bilayer and can probably be explained by the complex thermal behavior of the glycosphingolipids [20,41–44]. In the thermogram of the bilayer containing PGlcCer, a single transition peak was observed in both the heating and cooling scans, and the transition temperature was in good agreement with the domain melting and formation temperatures detected with the fluorescence quenching method.

3.3. Effects of neutral glycosphingolipids on sterol- and sphingomyelin-rich domains

Next, we studied how PSM/sterol domains were influenced by the presence of glycosphingolipids. Bilayers consisting of POPC (±7SLPC): glycosphingolipid: PSM: sterol (60:15:15:10, mol%) where studied with CTL, which again replaced cholesterol to give a final probe concentration of 1 mol % in the bilayer. The melting of PSM/sterol domains in bilayers containing 15 mol% PSM, without glycosphingolipids, was measured for reference (curve 4 in Fig. 3). All the palmitoylated glycosphingolipids associated with the PSM/sterol domains and stabilized them as indicated by the increase in F/F_0 amplitude and domain melting temperature compared to the pure PSM/sterol domains. The glycosphingolipid/PSM/sterol domains formed were approximately equally thermostable as detected by CTL quenching (Fig. 3A). They were also of comparable stability as the PSM/sterol domains formed in bilayers composed of POPC (±7SLPC): PSM: sterol (60:30:10, mol %), which we have studied in previous publications [26,40] (dotted line in Fig. 1A). Heating of the glycosphingolipid- and PSM-containing samples did not affect the bilayer structures, since the up and down scans were practically similar (Fig. 3A and B). Inclusion of 15 mol% of unsaturated glycosphingolipid (OGalCer or OLacCer) in the bilayer resulted in destabilization of the PSM/sterol domains as detected by CTL (results not shown), showing that the saturated acyl chain rather than the carbohydrate head group was responsible for the observed stabilizing effect of the palmitoylated glycosphingolipids.

The effect of the glycosphingolipids on PSM/sterol domains was also studied with tParSM as the fluorescent probe. tParSM was included as 1 mol% of the total lipid and was replacing a fraction of the PSM. The quenching of tParSM in bilayers consisting of POPC (±7SLPC): glycosphingolipid: PSM: sterol (60:15:15:10, mol%) is presented in Fig. 3C and D. The melting of PSM/sterol domains as reported by tParSM in bilayers containing 15 mol% PSM is shown for reference. The results

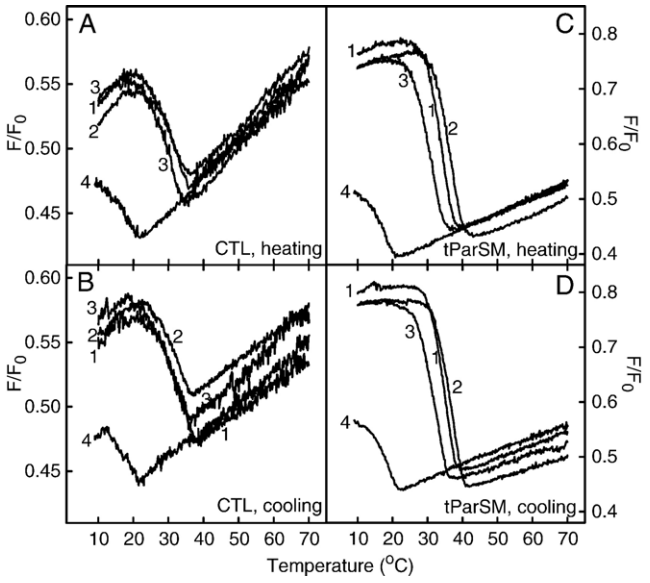

Fig. 3. Formation of sterol- and sphingomyelin-enriched domains in the presence of glycosphingolipids. The quenching of CTL (A, B) and tParSM (C, D) was measured to detect stability of ordered domains enriched in sterols and sphingomyelins, respectively. F/F_0 is plotted as a function of increasing (A, C) and decreasing (B, D) temperature for bilayers prepared to contain POPC (±7SLPC): PSM: glycosphingolipid: cholesterol (60:15:15:10, mol%) where CTL or tParSM replaced 1 mol% of cholesterol or PSM, respectively. The used glycosphingolipids were PGalCer (1), PGlcCer (2) and PLacCer (3). Bilayers containing POPC (±7SLPC): PSM: cholesterol (75:15:10, mol%) were used for reference (4). The total lipid concentration was 50 μM and the temperature gradient 5 °C/min.

clearly show that PGalCer, PGlcCer and PLacCer did associate with the sphingomyelin-rich domains since the amplitude of F/F_0 curve increased remarkably in the presence of the glycosphingolipids. The observed melting temperatures correspond fairly well with the ones detected by CTL, although minor differences in domain stability between the different glycosphingolipid samples were observed only with tParSM. The quenching of tParSM was also reversible and heating and cooling scans were similar in the presence of any of the glycosphingolipids.

3.4. Sterol enrichment into ordered domains in bilayers containing two different glycosphingolipids was clearly dependent on the head group structures

The glycosphingolipids used in this study were not very prone to form sterol-enriched domains on their own. However, the saturated glycosphingolipids were able to participate in sterol-enriched domain formation when PSM was present in the bilayer. Hence, we also wanted to examine if a mixture of two palmitoylated glycosphingolipids with different head groups (15 mol% of each) would be able to accommodate sterols when present in the same membrane. The quenching of CTL was studied in bilayers composed of POPC (±7SLPC): glycosphingolipids: sterol (60:15+15:10, mol%). As seen in Fig. 4A and B, PGlcCer and PLacCer (curve 2+3) were together able to form sterol-enriched domains, which were stable up to around 32 °C. This was expected since these two were the most prone

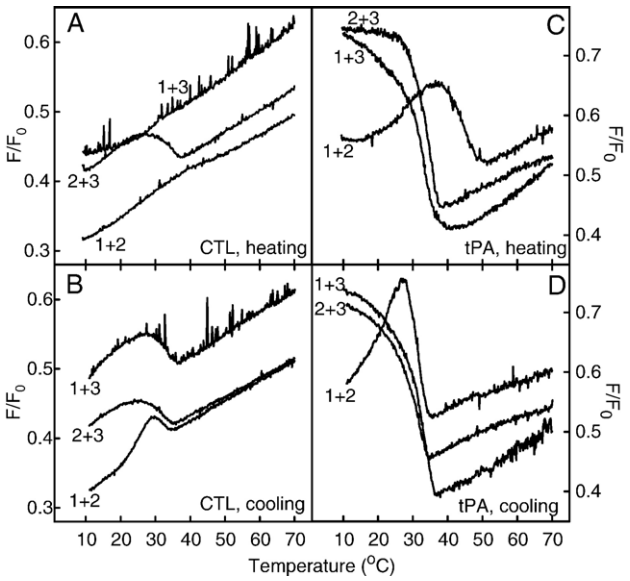

Fig. 4. Formation of ordered (sterol-enriched) domains in bilayers containing two glycosphingolipids with different head groups. Quenching of CTL (A, B) was measured to detect stability of sterol-enriched domains in the membranes and quenching of tPA (C, D) was measured to detect stability of ordered (sterol-enriched and sterol-poor) domains. F/F_0 is plotted as a function of increasing (A, C) and decreasing (B, D) temperature for bilayers prepared to contain POPC (±7SLPC): glycosphingolipids: cholesterol (60:15+15:10, mol%) where CTL or tPA replaced 1 mol% of cholesterol or POPC, respectively. The mixed glycosphingolipids, PGalCer (1), PGlcCer (2) and PLacCer (3), are indicated in the figure. The total lipid concentration was 50 μM and the temperature gradient 5 °C/min.

to associate with cholesterol also when only one glycosphingolipid was present in the bilayer (Fig. 1A and B).

It seemed like PGalCer in mixture with PGlcCer or PLacCer was able to suppress their ability to associate with sterols. When PGlcCer was mixed with PGalCer (curve 1+2) the latter seemed to dominate in the mixture and no sterol-enriched domains were formed. Both the heating and cooling scans for this mixture were very similar to the corresponding scans for the bilayer containing 30 mol% PGalCer (Fig. 1A and B). This glycosphingolipid mixture also seemed to protect some sterol from the quencher transiently at ~30 °C upon cooling, probably because the sterols were included in a metastable phase which existed around 30 °C. The mixture of PGalCer and PLacCer (curve 1+3) was not able to protect CTL from quenching at the starting temperature either, but sterol-rich domains seemed to form at about 32 °C when the sample was cooled after heating to 80 °C. Again, the higher F/F_0 value after the cooling of this sample as compared to before the heating suggests a possible redistribution of the probe at a rate too slow to be reflected in the cooling scan.

Bilayers containing two mixed glycosphingolipids (POPC (±7SLPC): glycosphingolipids: sterol (60:15+15:10, mol%)) were also studied with tPA as the fluorescent probe replacing 1 mol% of POPC in the bilayer. The results showed that all binary glycosphingolipid mixtures were able to form ordered domain as reported by tPA (Fig. 4C and D). Here again, the mixture of PGalCer and PGlcCer (curve 1+2) was dominated by PGalCer. The domain melting temperature detected for this mixture was significantly higher than the domain formation temperature observed when cooling the sample, indicating that the glycolipids (or only PGalCer) were sensitive to the direction of temperature change. PGalCer/PLacCer (curve 1+3) and PGlcCer/PLacCer (curve 2+3) formed equally stable ordered domains, and the heating and cooling scans for these mixtures were practically similar. The domain melting and formation temperatures observed with tPA for these mixtures were in good agreement with the ones observed with CTL. However, the results obtained with tPA showed that ordered domains were present in all mixtures although the sterol was not affected by the domain melting in all of them. Concerning PGlcCer and PLacCer, the enrichment of sterols into the ordered domains was more pronounced when the bilayer contained both glycosphingolipids instead of only one of these two (comparing Figs. 1 and 4). However, sterol enrichment was more extensive into ordered domains formed by a glycosphingolipid and PSM (comparing Figs. 3 and 4).

4. Discussion

The aim of this study was to explore whether some specific acyl chain defined neutral glycosphingolipids are able to associate with sterols in mixed bilayer membranes. We also wanted to find out how the head group structure influences the domain forming properties of the glycosphingolipids. The semisynthetic glycosphingolipids used in this study were galactosyl-, glucosyl- and lactosylceramides with a palmitic acid (or an oleic acid) in the *N*-linked position. The thermotropic

behavior of all these glycosphingolipids has been shown to be very complex [20,41–43]. The phase behavior of galactosyl- and glucosylceramides has been summarized by Koynova and Caffrey in 1995 [21] and that of lactosylceramides was recently discussed by Li and co-workers [42]. All glycosphingolipids used in this study had higher T_m values than PSM (41 °C; [45,46]), which was used as a domain forming reference sphingolipid in this study. The chain-melting phase transition temperatures of the glycosphingolipids are 85 °C for PGalCer [47], 87 °C for PGlcCer [20] and 78 °C for PLacCer [42,43].

The formation of sterol-enriched domains in a fluid bilayer was examined using cholestatrienol as a fluorescent reporter molecule, selective for sterol-enriched domains, together with a quencher in the disordered phase. Our results showed that as reported by the CTL quenching method the glycosphingolipids studied were poor in forming sterol-enriched domains compared to PSM when present as the only sphingolipid in the bilayer. However, the tendency to associate with cholesterol was clearly dependent on the polar head group of the glycosphingolipid. Differences in domain forming properties were even seen between the GlcCer and the GalCer, which differ only in the stereochemistry of one hydroxyl group in the carbohydrate head group. PGlcCer was able to accommodate some sterol at low temperatures, while PGalCer did not shield the sterol from the quencher noticeably, apart from the transient protection at around 30 °C after a preceding heating. The quenching data for tPA showed that ordered domains were formed in both the PGalCer and the PGlcCer systems, although the sterol was not (or only partially) included in the domains. PLacCer, which has a structure identical to PGlcCer but with an additional galactosyl residue linked to the glucosyl residue, also interacted more favorably with cholesterol than did PGalCer. The domain forming behavior of PLacCer showed that the size of the head group was not the major determinant for the capacity of the glycosphingolipids to form sterol-enriched domains or ordered domains in general.

The different domain forming properties observed for the GlcCer and GalCer might be implicated in their sorting between subcellular membranes, knowing that they follow different biosynthetic and sorting pathways in biological systems [7,9]. The tiny structural difference also gives GalCers and GlcCers different specialized functions [7].

Thermotropic studies have in general indicated that the stereochemical difference between PGalCer and PGlcCer has only minor effects on the phase transitions of the pure glycosphingolipids [20,21]. However, the structural difference seems to make them interact differently with other components in mixed bilayer membranes, as we observed in this study. A stereochemical alteration of one hydroxyl group has also been shown to result in dramatic differences in the structural transitions of galacto- and gluco-glycerolipids [48,49] and of long alkyl chain β-galactosides and β-glucosides [50] with identical hydrophobic moieties.

None of the glycosphingolipids studied here were very prone to associate with cholesterol in the mixed membranes. The saturated glycosphingolipids, which have very high T_m-values, were probably forming gel-phase domains in the fluid POPC matrix [16,19]. Packing constraints seem to hinder the partitioning of sterol into these domains. Heating to 80°C loosened up the bilayer structure in the PGalCer and PLacCer systems, and this enabled the sterols to partition into the glycosphingolipid-rich domains upon cooling. According to previous studies on GalCer/cholesterol bilayers, cholesterol is hindered to insert into the cerebroside matrix until the hydrogen bonding interactions between adjacent GalCer molecules are disrupted at high temperatures [19]. Our results still suggested that the sterol (and also tPA) was squeezed out from the PGalCer domains when the bilayer again was cooled below 30 °C, probably because the tightly packed gel-phase domains were regenerated. In PLacCer containing bilayers, on the other hand, both CTL and tPA remained protected from the quencher at low temperatures after a heating/cooling cycle. There might however be a time dependent redistribution of the probes that was not detected with the cooling rate used in this study. Comparative studies on galacto- and glucoglycerolipids [48,49], and on alkyl β-galactosides and -β-glucosides [50], suggest that the galacto compounds consistently confer higher stability to membrane phases and that this results from a more efficient hydrogen bonding network with water in the polar parts of the membrane. This could explain why the sterols were more easily inserted into the ordered PGlcCer domains compared to the PGalCer domains.

The quenching results were in fairly good agreement with the DSC data for similar bilayer systems. The PGalCer and PLacCer containing bilayers seemed to be structurally complex. This is indicative of a polymorphic phase behavior which has been described elsewhere for pure GalCer, GlcCer and LacCer bilayers [20,42–44]. Glycosphingolipid bilayers typically interconvert between different metastable and stable bilayer phases, and the transitions have been shown to depend both on the thermal history of the sample and on the heating/cooling rate [20,43,44]. When domain melting and formation was detected with CTL and/or tPA quenching, these events were in most cases comparable with the transition peaks in the corresponding thermogram. Yet, there were transitions observed in the DSC data that had no counterparts in the fluorescence quenching results. These were possibly due to thermal events taking place in the carbohydrate parts of the molecules or to transitions between different gel states at which the acyl chain arrangement was changed although the packing density of the bilayer was not. Thus, the quenching susceptibility of the probes was necessarily not affected.

The thermogram of the PGlcCer containing bilayer was reversible while the ones for PGalCer and PLacCer containing bilayers were not. Differences between heating and cooling scans were also observed for galactoglycerolipids but not for glucoglycerolipids in a study where the change in specific volume of the glycolipids at the phase transitions was measured [48]. The glucoglycerolipid containing two palmitoyl chains was shown to undergo identical volume changes at the same transition temperatures upon heating and cooling, whereas for the corresponding galacto compound the volume change as well as the transition temperatures were always different in the heating scans compared to the cooling scans [48].

When a sphingomyelin was included in the glycosphingolipid containing membranes the melting of sterol-enriched domains could clearly be detected by the CTL quenching method. Domain formation by PSM and sterol has been studied by the quenching method also in our previous studies [26,40]. Phase co-existence has also been documented for similar membrane compositions in giant unilamellar vesicles as well as multi-lamellar liposomes [34,35]. Probably, the formation of sterol-enriched domains in glycosphingolipid containing bilayers was affected by the lateral packing density in the glycosphingolipid-rich domains. The packing-defects that arose from mixing a glycosphingolipid and PSM gave sterols the opportunity to enter into sterol-enriched domains with saturated glycosphingolipids which were not able to form this kind of domains on their own.

We have shown earlier that the melting temperatures of the pure PSM/sterol domains reported by the quenching susceptibility of CTL and tParSM are in good agreement with each other, indicating that the probes are sensing the same environment [26]. When neutral glycosphingolipids were incorporated into the mixed bilayers the results became slightly more complex. In POPC (±7SLPC): glycosphingolipid: PSM: sterol (60:15:15:10, mol%) bilayers, the results obtained both with CTL and tParSM as the fluorescent probe showed that all palmitoylated glycosphingolipids associated with and stabilized the PSM/sterol domains. The difference between the individual glycosphingolipids was insignificant as reported by CTL. However, when comparing the quenching curves for CTL and tParSM (Fig. 3A, B and C, D) it seems like the sterol was released at the onset of the gradual domain melting process, whereas the sphingomyelins due to differences in affinity for the ordered domain were released later. It is also possible that there were two kinds of ordered domains in the system and that the tParSM probe was shielded from quenching in sterol-poor glycosphingolipid/PSM domains that melted at temperatures higher than those reported by CTL quenching. This could explain the minor differences in domain stability between the different glycosphingolipid containing samples, only seen when detected with tParSM. Similar conclusions have previously been drawn about the behavior of ganglioside GM1 in a sphingomyelin based bilayer, where GM1 ganglioside and cholesterol partitioned into different domains, one sterol-sphingomyelin domain and one GM1-sphingomyelin domain [51].

The melting temperature for PSM/PGalCer/sterol domains observed with CTL was a few degrees lower than what we have reported earlier [40]. However, in the same study, the stability of PSM/PGalCer/sterol domains was also determined by measuring quenching susceptibility of *trans*-parinoyl-GalCer, which reported on a melting temperature in good agreement with the one observed here with CTL [40]. We have observed minor shifts in domain stability between individual experiments and we will therefore point out that the detected domain melting temperatures in the figures are not to be taken as absolute values. Nevertheless, the results shown are representatives of several experiments and the differences between curves reported in this study were highly reproducible.

The different capacities of the glycosphingolipids to associate with cholesterol were also noticeable when two glycosphingolipids with different head groups were mixed in the bilayer. PLacCer and PGlcCer (15 mol% of each) clearly formed sterol-enriched domains together, and these two were also the most prone to colocalize with sterols when only one glycosphingolipid was present in the bilayer. PGalCer, on the other hand, suppressed the ability of both PGlcCer and PLacCer to form sterol-enriched domains. No domain melting was observed with CTL in the heating scans of the PGalCer/PGlcCer or PGalCer/PLacCer containing bilayers, but PGalCer/PLacCer accommodated more sterol at low temperatures after the bilayer had been heated and again cooled. Quenching of tPA revealed that in all systems containing two mixed glycosphingolipids, the extent of ordered (sterol-poor) domains was larger than the extent of sterol-enriched domains.

It was interesting to notice that the mixture of GlcCer and LacCer most favorably interacted and associated with cholesterol, since these three lipids are thought to be components of signaling domains in plasma membranes [2,8]. GalCers, on the other hand, which are typically found in the cholesterol-rich multilamellar layers of myelin, did not show any tendencies to interact with cholesterol in this study. The situation is most likely different in more complex membranes, where also the protein content has a large influence on the formation of functional domains. It is also likely that different kinds of ordered domains are present in complex bilayer membranes [8,28,29,34].

We can conclude, based on what is known from the literature and what we observed in this study, that the distribution of glycosphingolipids between lateral domains in mixed lipid bilayers is a complex process. The head group structure clearly influences the domain forming properties of the glycosphingolipids, although none of the mono- and diglycosylceramides used in this study was very prone to associate with cholesterol when present as the only sphingolipid in the bilayer. We observed that even such a small structural difference as a stereochemical alteration of a single hydroxyl group in the pyranose ring can have dramatic effects on the membrane properties of the glycosphingolipids, which in turn may be reflected in their biological functions.

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