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Review

Phosphatidylglucoside: Its structure, thermal behavior, and domain formation in plasma membranes

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ABSTRACT

Phosphatidylglucoside (PtdGlc) is a unique glyco-glycerophospholipid that is found in both bacterial and mammalian cells. The discovery of PtdGlc in mammalian cells is relatively recent (Nagatsuka et al., 2001. FEBS Lett. 497, 141–147). Chemical structural analysis of the PtdGlc found in mammalian organs and cultured cells showed that PtdGlc is composed exclusively of a single pair of saturated fatty acid chains; the *sn*-1 chain is stearic acid (C18:0) and the *sn*-2 chain is arachidic acid (C20:0). PtdGlc forms distinct domains, which are different from cholesterol-based sphingolipid domains, on the outer leaflet of the plasma membrane. In this review, we summarize recent studies of PtdGlc. Special attention is paid to the thermal behavior of PtdGlc in a pure system and in mixtures with other lipid components that may relate to the formation of PtdGlc domains in biomembranes. Finally, we discuss proposed biological functions of PtdGlc based on recent experimental results.

Contents

1. Introduction

The total number of lipid species in mammalian cell membranes is roughly estimated to be in the thousands (van Meer, 2005). According to Yetukuri et al. (2008), the theoretically estimated number of different molecular lipids is about 180,000, taking into account various types of lipids including non-membrane forming lipids such as fatty acids and cholesterol esters. The existence of so many naturally occurring lipids suggests that the functions of lipids are not just limited to the formation of a lipid bilayer that acts as a cell membrane permeability barrier, or to acting as an energy storage molecule, such as is written in general text books. If lipids were only needed for these functions, then a few kinds of lipids would be enough. It can therefore be speculated that each lipid species that exists has a distinct and specific function in biological systems, in a similar manner to the way each protein

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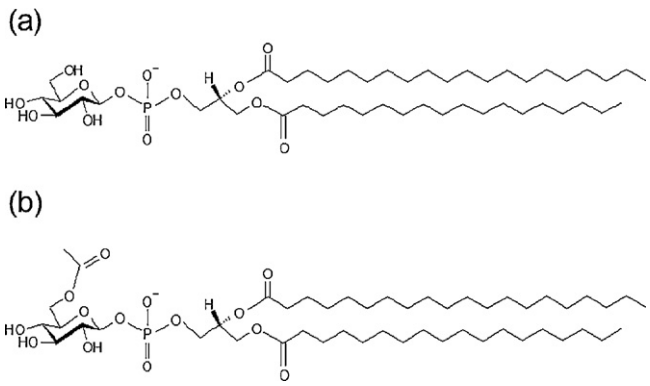

Fig. 1. Structure of (a) 1-stearoyl (C18:0)-2-arachidoyl (C20:0)-*sn*-glycero-3-phospho-β-D-glucoside (phosphatidyl-β-D-glucoside, PtdGlc) and (b) 1-stearyl-(C18:0)-2-arachidoyl (C20:0)-*sn*-glycerol-3-phosphoryl β-D-(6-*O*-acetyl)glucopyranoside (acetylated PtdGlc).

within a cell has a specific function. This mini-review deals with a unique glyco-glycerophospholipid, phosphatidyl-β-D-glucoside (PtdGlc) in which a glucose group is linked glycosidically to the phosphate group of a phosphatidic acid in a β-anomeric configuration (Fig. 1). The first report of the discovery of PtdGlc from a microorganism was published in 1970 (Short and White, 1970). However, until recently, relatively little attention has been paid to PtdGlc. Indeed, PtdGlc was not even mentioned in a recent handbook of lipids that has over seven hundred and fifty pages (Gunstone et al., 2007). Unique and interesting features of PtdGlc isolated from mammalian or cultured cells have recently been shown. One such unexpected feature is that PtdGlc is a single molecular species. It contains a specific saturated fatty acid chain, wherein the *sn*-1 chain is stearic acid (C18:0) and the *sn*-2 chain is arachidic acid (C20:0). As can be expected from this fatty acid composition, PtdGlc forms a distinct PtdGlc-enriched domain on the outer leaflet of plasma membranes. Interestingly, a recent model membrane study (Murate et al., 2010) showed that PtdGlc is phase-separated from sphingomyelin (SM), the main lipid component of so-called lipid rafts (Simons and Ikonen, 1997; Lingwood and Simons, 2010), independently of the presence of cholesterol. This finding suggests that the PtdGlc domain in cell membranes differs from that of sphingolipids/cholesterol complex domains.

Although the biological functions of PtdGlc and the PtdGlc-enriched domains are not currently well understood, in this mini-review we will summarize current knowledge regarding PtdGlc. Since a recent review of PtdGlc by Nagatsuka and Hirabayashi (2008) focused on the biological activities of PtdGlc, we will focus mainly on the discovery of PtdGlc, the determination of its chemical structure, its thermal behavior and its domain formation. In addition, we discuss reports describing those biological activities of PtdGlc that have been published since the previous review, and compare the thermal behavior of PtdGlc with the behavior of major membrane lipids. Finally, we discuss the biological functions of PtdGlc that can be predicted based on our current knowledge.

2. Discovery of phosphatidyl-β-D-glucoside (PtdGlc)

2.1. Discovery of PtdGlc in bacteria

Smith and Henrikson (1965) reported the isolation of a glucose-containing phospholipid from the bacterium *Mycoplasma laidlawii B*. They named this lipid "phosphatidylglucose" and considered that its chemical structure was as follows; the fatty acid chains are attached to glycerol and the glucose is attached to glycerol through a phosphodiester linkage between the C_6 of glucose and the C_3 of glycerol. However, in a paper written together with Shaw and Verheij (Shaw et al., 1970), Smith revised this structure based on the fact that the glucose-containing phospholipid was completely resistant to hydrolysis by phospholipases A, C and D. This finding suggested that the glycerophosphate residue is not esterified with fatty acids. Finally, Shaw et al. (1970, 1972) identified the lipid isolated from *Mycoplasma laidlawii B* as glycerlyphoshoryldiglucosyl diglyceride (Fig. 2). In the same year, Short and White (1970) reported the isolation of phosphatidylglucose from the gram-positive bacterium, *Staphylococcus aureus*. Using chemical analysis, Short and White (1970) showed that phosphatidylglucose contains 2 mol of fatty acid, 1 mol of glucose, and 1 mol of glycerol per mole of phosphate. Treatment with phospholipase C produced diglyceride and glucose-1-phosphate from the lipid. On the basis of these results, Short and White (1970) proposed that the structure of phosphatidylglucose from *Staphylococcus aureus* is 3-*sn*-phosphatidyl-1′-glucose.

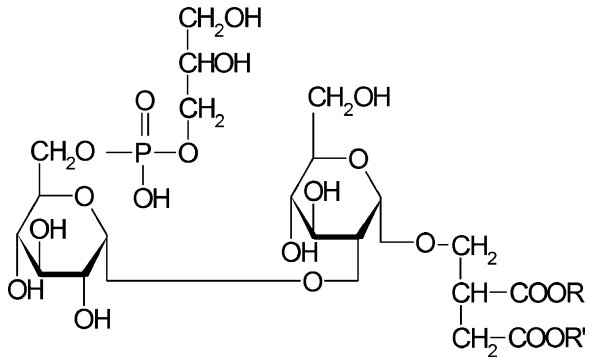

Fig. 2. Structure of glycerlyphosphoryldiglucosyl diglyceride.

2.2. Discovery of PtdGlc in mammalian cells and tissues

PtdGlc was identified in mammalian cell tissues in 2001, approximately 30 years after Short and White's paper, by Nagatsuka et al. (2001) who also isolated PtdGlc from human cord red cells. Four years later, Nagatsuka et al. (2003) found PtdGlc in detergent-insoluble membrane (DIM) fractions of human promyelocytic leukemia cells (HL-60), suggesting that there is a PtdGlc-based lipid microdomain similar to the lipid raft domain that is thought to consist of glycosphingolipids, sphingomyelin and cholesterol (Simons and Ikonen, 1997; Lingwood and Simons, 2010). Yamazaki et al. (2006) generated a PtdGlc-specific monoclonal antibody, DIM21, by immunizing mice with DIMs isolated from HL60. This work was motivated by the finding that the DIM fraction acts as an effective immunogen (Katagiri et al., 2001, 2002).

By using this DIM21 antibody, Nagatsuka et al. (2006) immunochemically detected PtdGlc in the central nervous system of fetal rat brains. A much higher level of expression of PtdGlc was found in astroglial cells of rat brains than in other types of cells. This reported "higher level" was based on the relatively strong fluorescence intensity that was observed in immunofluorescence microscopy. Unfortunately, the exact amount of PtdGlc in cells has not yet been successfully quantitatively analyzed because PtdGlc is much more easily broken down by phospholipases than glycosphingolipids. The final yield of PtdGlc purified from rat brains was about 700 nmol/g of dried brains (Nagatsuka et al., 2006).

PtdGlc has also been found in epithelial cells from various human organs (Kitamura et al., 2007). In addition, Kaneko et al. (2011) recently reported the expression of PtdGlc in neural stem cells in the subventricular zone of adult mouse brains. Oka et al. (2009) and Kina et al. (2011) have found that PtdGlc is expressed in human neutrophils.

3. Determination of the chemical structure of PtdGlc

The chemical structure of the lipid moiety of PtdGlc was characterized using a combination of various methods, including enzymatic methods, mass spectroscopy, NMR spectroscopy, thin layer chromatography (TLC) and gas chromatography. The enzymatic study (Horibata et al., 2007) showed that this lipid is not phosphatidylinositol. This study also showed that the lipid moiety of PtdGlc is sensitive to bee venom and to porcine pancreas phospholipase A2s, *Bacillus cereus* phospholipase C, and *Streptomyces chromofusus* phospholipase D, but it is insensitive to phosphatidylinositol-specific *Bacillus cereus* phospholipase C. The behavior of the lipid moiety of PtdGlc on TLC also differs from that of phosphatidylinositol (Nagatsuka et al., 2001, 2006). An NMR spectroscopic study (Nagatsuka et al., 2001) demonstrated that the chemical shift and coupling constant of proton binding to the C1 atom of the sugar part of the lipid (PtdGlc) are distinctly different from the reported values for phosphatidylinositol (Shibata et al., 1984). Based on the NMR data combined with the results of mass spectroscopy and gas chromatography, it was concluded that the chemical structure of PtdGlc found in fetal rat brain is 1-stearyl-2-arachidoyl-*sn*-glycerol-3-phosphoryl β-D-glucopyranoside (Fig. 1a) (Nagatsuka et al., 2006). It was further found that acetylated PtdGlc, 1-stearyl-2-arachidoyl-*sn*-glycerol-3-phosphoryl β-D-(6-*O*-acetyl)glucopyranoside (Fig. 1b) exists in isolated DIM21 antigen from fetal rat brain. Greimel et al. (2008) reconfirmed that this acetylation occurs at position 6 of the glucose of PtdGlc based on a comparison between NMR data of the acetylated PtdGlc from a natural source and that of an organic chemically synthesized 6-*O*-acetyl PtdGlc.

One of the most unexpected findings from these studies was that there is only one fatty acid combination for PtdGlc isolated from the central nervous system of fetal rat brain, i.e., the *sn*-1 and *sn*-2 chains are exclusively stearic acid (C18:0) and arachidic acid (C20:0), respectively. A single molecular species rarely occurs in natural phospholipids. Furthermore, very few natural lipids are known that have a combination of C18:0 and C20:0.

The chain composition of PtdGlc suggests that the phase transition temperature is higher than physiological temperature and that a gel phase domain containing PtdGlc will form. In the following section, we will describe the thermal properties of this unique lipid, discuss the experimental data regarding the thermal behavior of this lipid, and we will compare these data with those obtained for other membrane lipids.

4. Thermal behavior of PtdGlc

4.1. Pure PtdGlc

A differential scanning calorimetry (DSC) study of the thermal behavior of pure PtdGlc (Murate et al., 2010) showed that pure PtdGlc exhibits two endothermic phase transitions, at 23.7 °C and at 76.4 °C, in a heating scan. Fig. 3 shows a representative DSC thermogram of a pure PtdGlc vesicle sample in Hepes buffer (pH 7.0) containing 100 mM NaCl and 10 mM ETDA. The scan rate was 0.5 °C/min. This thermogram was recorded after more than ten cycles of temperature between 5 °C and 85 °C by the DSC apparatus to ensure that the sample was in an equilibrium state. In Section 4.3, we will describe the mysterious thermal behavior of PtdGlc that was observed during the initial temperature cycling stage. The higher temperature endothermic phase transition peak with an enthalpy value ΔH of 14.2 kcal/mol is due to chain melting, which was confirmed by wide angle X-ray diffraction (unpublished data). There was no diffraction peak reflected from any ordered crystal lattices in the X-ray diffraction pattern. Hence, the lower temperature transition peak at 23.7 °C with a small enthalpy value

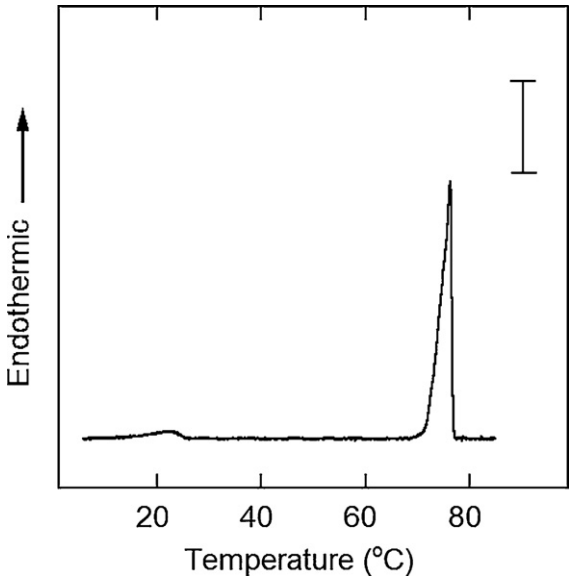

Fig. 3. DSC thermogram of a pure PtdGlc vesicle sample obtained using a heating scan rate of 0.5 °C/min. The vertical scale bars correspond to 0.2 kcal/mol/°C.

(ΔH = 0.97 kcal/mol) is due to a gel-to-gel phase transition. Thus, pure PtdGlc bilayers are in a gel state at physiological temperatures. We discuss below the chain melting transition temperature and enthalpy of PtdGlc in comparison with those of other membrane lipids.

The lipid that shows the highest chemical similarity to PtdGlc is phosphatidylinositol (PI). However, very few experimental data have been reported on the phase transition of PIs with chemically defined hydrocarbon chains. In fact, as far as we know, there are only two such papers. One of these papers (Hansbro et al. 1992), reported that the gel to liquid crystalline phase transition of hydrated dimyristoyl (diC14:0)-PI occurs at 19–21 °C (ΔH = 5.8 kcal/mol). This temperature is fairly similar to that of phosphatidylcholine (PC) with the same fatty acid chains, i.e., dimyristoyl-PC (DMPC, diC14:0-PC, 23.4 °C) (Koynova and Caffrey, 1998). In contrast, the other paper by Mansour et al. (2001) reported that the transition of hydrated dipalmitoyl (diC16:0)-PI occurs at −24.9 °C, which is a much lower temperature than that of dipalmitoyl-PC (DPPC, diC16:0-PC, 41.4 °C) (Koynova and Caffrey, 1998) and is fairly similar to that of unsaturated dioleoyl-PC (DOPC, diC18:1-PC) (Koynova and Caffrey, 1998). These authors did not mention the transition enthalpy value. Based on the difference in melting temperature between myristic acid (54.4 °C) and palmitic acid (62.9 °C), the enormous difference between the melting temperature of DMPI and DPPI was unexpected and the basis for this difference is currently unknown. Since there are insufficient experimental data regarding PI, we have instead compared the data regarding PtdGlc with data of other glycerol-phospholipids and glycerol-glycolipids instead of with PI data.

Before comparing PtdGlc with other membrane lipids, we will discuss the chain length dependence of the melting transition of membrane lipids. Because both *sn*-1 and *sn*-2 chains of PtdGlc are saturated we will only compare PtdGlc with other saturated membrane lipids. Caffrey and co-workers published several review papers regarding the phase transitions of membrane lipids, using the lipid thermodynamic database termed LIPIDAT (http://www.lipidat.tcd.ie) that they established (Caffrey and Hogan, 1992; Koynova and Caffrey, 1994a,b, 1995, 1998). Using the data in these reviews, we constructed the graphs shown in Figs. 4 and 5, which show the chain length dependence of melting transition temperatures of neutral synthetic

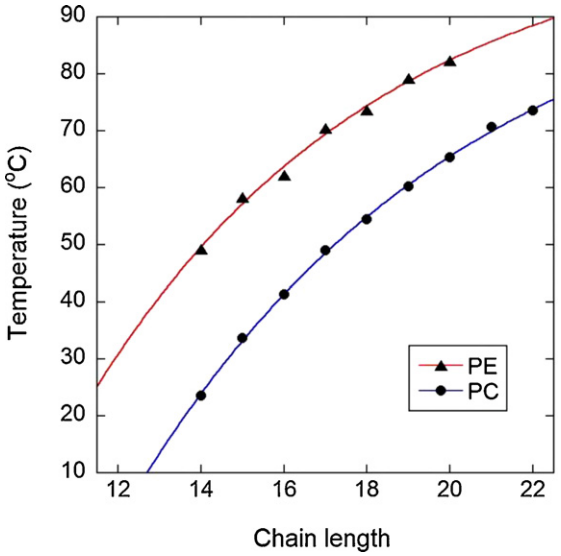

Fig. 4. Dependence of the melting transition (gel-to-liquid crystalline phase transition) temperature on the chain length of saturated symmetric diacylphosphatidylcholines (PCs) (●: closed circles) and phosphatidylethanolamines (PEs) (▲: closed triangles). The lines are best-fitting exponential curves.
Data are from the reviews by Koynova and Caffrey (1994a, 1998).

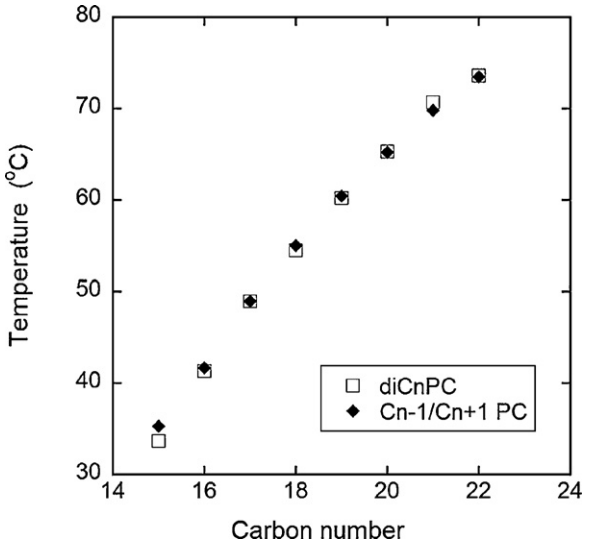

Fig. 6. Comparison of the measured melting temperatures of diC*n*:0-PCs (□: open squares) and the estimated melting temperatures of C($n-1$):0/C($n+1$):0 PCs (⧫: closed lozenges). The horizontal axis is the carbon number, *n*, in a single chain. The data of diC17:0-PC is an estimated value.
Data are from the review by Koynova and Caffrey (1998).

glycerolphospholipids (PCs and phosphatidylethanolamines (PEs)) and glycoglycerolipids (diacyl-β-glucosides (diacyl-β-Glcs) and diacyl-β-galactosides (diacyl-β-Glas)), respectively. These figures demonstrate that the curves for all four of these lipids have almost the same curvature. In other words, the different headgroups only contribute to the absolute value of the melting temperature. Among those four lipids in which the same fatty acid molecular species are compared, the melting temperatures of three of the lipids (excluding PC) are almost identical.

It is well established that there is alternation between even and odd numbers in the carbon number-dependent curves of the melting temperatures of long saturated fatty acids (Scrimgeour and Harwood, 2007). The melting curve of the β-form of triacylglycerols is also zigzag rather than smooth when it is plotted as a function of carbon number (Hagemann and Rothfus, 1983; Foubert et al., 2007). This effect is called the "odd–even effect" (Small, 1986). The β-form is the most thermodynamically stable form in solid states of triacylglycerols (Foubert et al., 2007). Triacylglycerols have some metastable solid forms and one of these metastable forms is called the α-form (Foubert et al., 2007). In the α-form, the fatty acid chains of triacylglycerols are assumed to oscillate with a high degree of molecular freedom. The melting transition from the α-form does not exhibit the odd–even effect (Hagemann and Rothfus, 1983; Foubert et al., 2007). All of the curves seen in Figs. 4 and 5 are smooth, indicating that the odd–even effect is not dominant in the gel-to-liquid crystalline phase transitions of these phospholipids and glycolipids. The most thermodynamically stable phase for many phospholipids and glycolipids is not a gel phase but is a metastable phase. The gel-to-liquid crystalline phase transitions of the lipids shown in Figs. 4 and 5 are similar to the melting transition from the metastable α-form of triacylglycerol rather than that from the most stable β-form. The odd–even effect is generally assumed to be associated with differences in hydrocarbon chain arrangements in a crystalline lattice (Small, 1986).

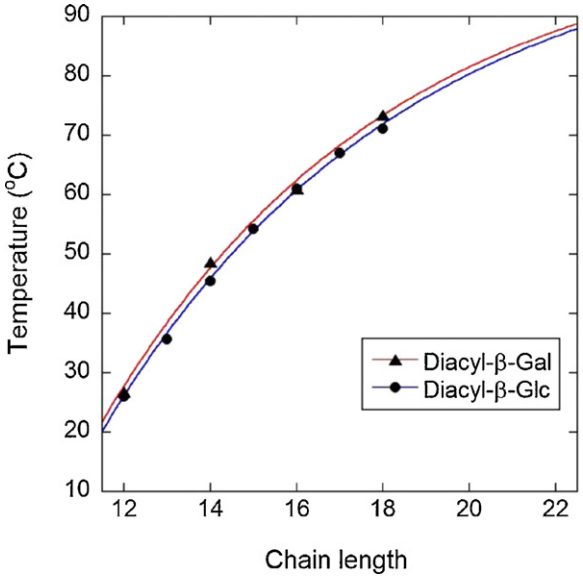

Fig. 5. Dependence of the melting transition (gel-to-liquid crystalline phase transition) temperature on the chain length of saturated symmetric diacyl-β-Glcs (●: closed circles) and diacyl-β-Glas (▲: closed triangles). The lines are best-fitting linear regression lines.
Data are from the reviews by Koynova and Caffrey (1994a, 1994b, 1998).

The data shown in Figs. 4 and 5 are data of symmetric membrane lipids, where the *sn*-1 chain is identical with the *sn*-2 chain. In contrast, PtdGlc is an asymmetric lipid, i.e., its two chains are different (C18:0 and C20:0). Let us therefore next consider the melting temperatures of asymmetric lipids. Based on experimental data regarding gel-to-liquid crystalline phase transitions in asymmetric PCs, an empirical equation has been proposed in order to predict the transition temperatures (Huang, 1991; Cevc, 1991; Marsh, 1992). Using data estimated by using this empirical equation in cases where experimental data were not available, Koynova and Caffrey (1998) created a table in which they summarized the transition temperatures of saturated symmetric and saturated asymmetric PCs. When we examined this table, we noticed that the melting temperatures of C($n-1$):0/C($n+1$):0 PCs, in which the carbon numbers of *sn*-1 and *sn*-2 chain are $n-1$ and $n+1$, respectively, are almost identical with the measured melting temperatures of diC*n*:0-PCs. This similarity in melting temperature is displayed in Fig. 6. Based on the data shown in Figs. 4–6, we assumed that

the melting temperatures of other membrane lipids would show the same relationship to carbon number and chain length as that observed for C($n-1$):0/C($n+1$):0 PCs and diCn:0-PCs (Fig. 6). The temperature of the melting transition, i.e., the gel-to-liquid crystalline phase transition, of diC19:0-PE was reported to be 79.2 °C (Lewis and McElhaney, 1993), which is similar to that of PtdGlc. The melting temperatures of diC19:0-β-Glc and diC19:0-β-Gla can be estimated as 76 °C and 78 °C, respectively from the graph shown in Fig. 4. However, in reality, diC19:0-β-Glc exhibits a direct transition from a gel to an inverted hexagonal (H_{II}) phase at 76.5 °C (Mannock et al., 1988). In any case, the melting temperature of PtdGlc is not unusual and is within the range expected based on its saturated chain length.

The reason for the difference between the melting temperatures of PEs and PCs is thought to be due not only to the fact that the headgroups of PEs are smaller than those of PCs, but also to the fact that there is an additional hydrogen bond between the amine hydrogens and the phosphate groups of neighboring lipids in PEs in the gel phase. This additional hydrogen bond is thought to cause the observed elevation of transition temperatures because additional energy is required to break the hydrogen bonds between the headgroups in order for a melting transition to occur, i.e., a phase transition from the gel phase to the liquid crystalline phase (Koynova and Caffrey, 1994a; Tobochnik et al., 1995).

These data would suggest the existence of a hydrogen bonding network around the PtdGlc headgroup region. A proton NMR study of PtdGlc in DMSO-d_6 suggested the presence of an intramolecular hydrogen bond (Greimel et al., 2008). A further computer simulation study of PtdGlc estimated that the distance between the proton of OH-2 and one of the non-bridging oxygen molecules of the phosphate moiety was only 0.17 nm, indicating the existence of an intramolecular hydrogen bond between these two molecules (Greimel et al., 2008). An intermolecular hydrogen bond might form between neighboring PtdGlcs in water. However, the formation of an intramolecular hydrogen bond has also been reported for PI in a DMSO-d_6 solution (Hansbro et al., 1992).

Next, we consider the melting transition enthalpy of PtdGlc. Similar to our above strategy, by using the data in reviews by Koynova and Caffrey (1994a,b, 1998) we constructed the graph in Fig. 7 which shows the chain length dependence of melting transition enthalpy values of several synthetic symmetric glycerolipids. In contrast to the contribution of the headgroups to the absolute value of the melting temperature, the absolute enthalpy values are almost independent of the structure of the headgroups, and the relationship between chain length and enthalpy is fairly linear, at least for the four glycerolipids analyzed. A simple statistical mechanical theory has been proposed to explain this linear dependence, which considers the equilibrium between 3 states (a trans and two gauche conformations) of the rotation around carbon–carbon bonds of hydrocarbon chains (Heimburg, 2007). One question is, can this chain length linear dependence be generalized to a linear dependence on the total number of carbon atoms in the chains? In other words, can the same statistical mechanical theory be applied to asymmetric lipids? The answer is that it is dependent on the difference in the length of the two acyl chains. If the difference in length is large, the dependence on the total number of carbon atoms would not be valid, but, if the difference is small, this dependence would be valid. Indeed, the melting enthalpy values of C18:0/C16:0 PE (9.2 kcal/mol) (Mason and Stephenson, 1990) and C14:0/C16:0-PC (7.9 kcal/mol) (Koynova and Caffrey, 1998) are almost the same as those of diC17:0-PE (9.4 kcal/mol) (Lewis and McElhaney, 1993) and diC15:0-PC (7.4 kcal/mol) (Koynova and Caffrey, 1998), respectively. Hence, we assume that it would be valid to compare the enthalpy value of PtdGlc (C18:0/C20:0) with the enthalpy values of diC19-glycerolipids. The average value of the melting transition

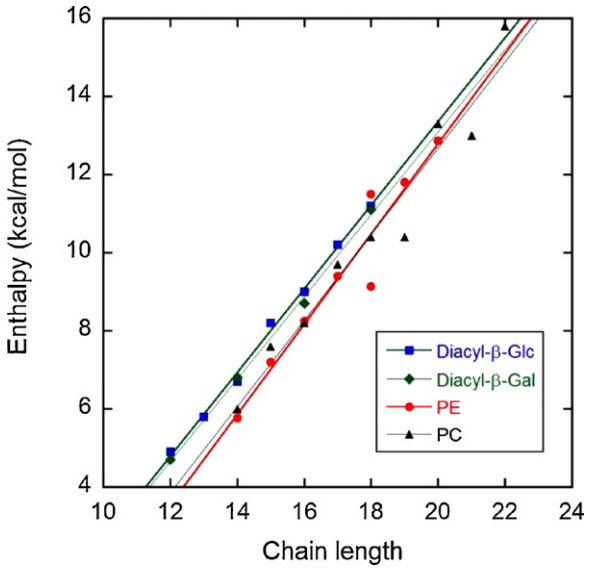

Fig. 7. Dependence of the melting transition enthalpy values on the chain length of saturated symmetric PCs (▲: closed triangles), PEs (●: closed circles). diacyl-β-Glcs (■: closed squares) and diacyl-β-Glas (◆: closed lozenges).

enthalpy of diC19-glycerolipids is estimated to be about 12 kcal/mol, based on Fig. 7. The difference between this value and that of PtdGlc (14.2 kcal/mol) is approximately 15%. Compared with transition temperature measurements, the error of transition enthalpy measurements is fairly large. In fact, the reported values vary considerably even for the same lipid measured under the same condition (see the standard deviations for the transition temperature and enthalpy in Table 1 of Koynova and Caffrey, 1998). Taking this fact into account, we can conclude that, similar to the transition temperature, the transition enthalpy of PtdGlc when measured under a physiological condition, i.e., neutral pH and about 0.1 M ionic strength, is not significantly different from the expected value. However, PtdGlc is a negatively charged lipid and the ionic strength and pH of the solution affect the phase transition behavior of charged lipid bilayers (Träuble and Eibl, 1974; Jacobson and Papahadjopoulos, 1975; Blume and Eibl, 1979; Salonen et al., 1989; Cevc, 1990; Takahashi et al., 1995; Garidel et al., 1997; Kodama et al., 1999; Kinoshita et al., 2009b; Garidel et al., 2011). Therefore, further detailed investigations, such as analysis of the effect of sodium ions or pH on the phase transition of PtdGlc, are required.

4.2. Phase separation of PtdGlc

The behavior of PtdGlc when mixed with other lipids has been studied by means of DSC (Murate et al., 2010). In the next section, we will discuss the strange scan-repeat-number-dependent thermal behavior of PtdGlc, for which experimental conditions are an important issue. The detailed conditions will be described in the next section. To avoid confusion, we will first describe the conclusion of the study.

PtdGlc exhibits low miscibility with all of the lipids investigated in the DSC study. The main components of a lipid raft are sphingomyelin (SM), cholesterol, and glycosphingolipids such as gangliosides (Simons and Ikonen, 1997; Lingwood and Simons, 2010). In the DSC thermogram of a PtdGlc/brain SM mixture (1:1 mol ratio; vesicle samples), two distinct peaks were observed. The peak temperatures were almost identical to those of pure SM and pure PtdGlc, respectively, indicating that PtdGlc exhibits phase separation from brain SM. Interestingly, the phase separation

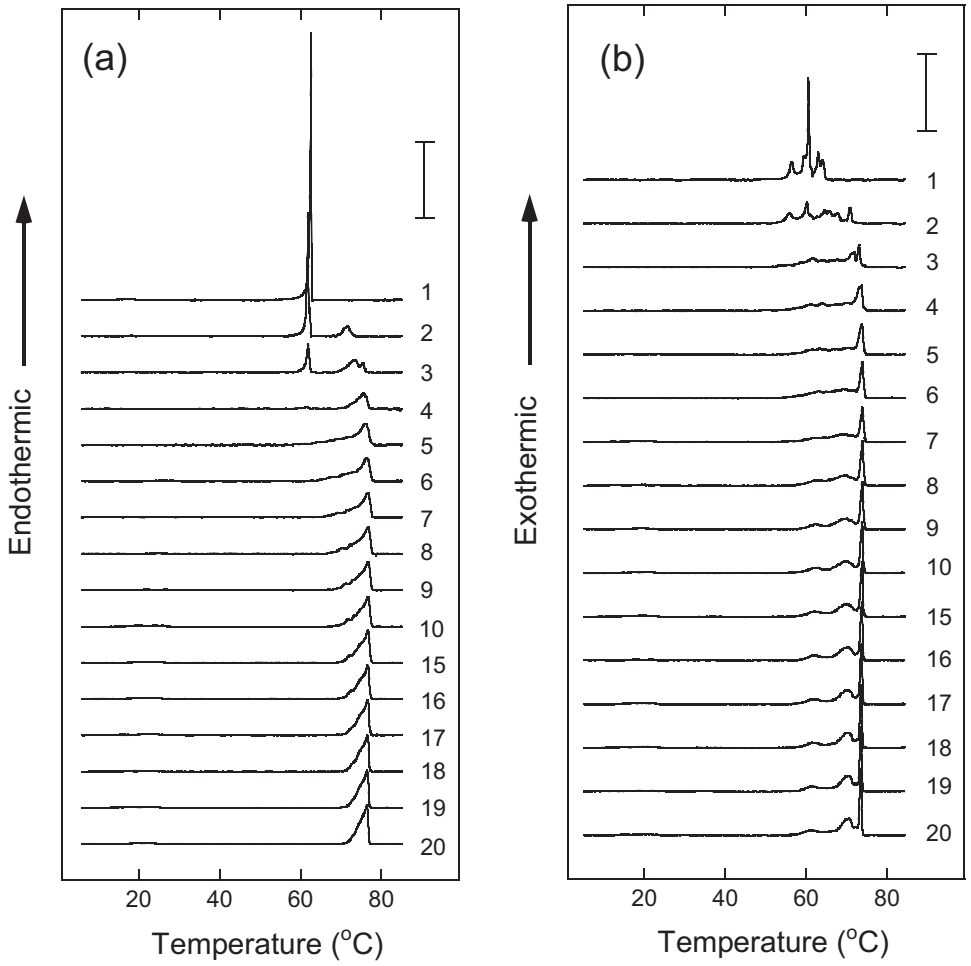

Fig. 8. Successive DSC thermograms of PtdGlc vesicle samples in a heating scan (a) and a cooling scan (b). The vertical scale bars correspond to 1.0 kcal/mol/°C. The numeral on the right side of each curve indicates the repeat number of the scan.

of PtdGlc from brain SM was also observed in the presence of cholesterol (1:1:1 mol ratio). Two reasons can be assumed for this phase separation. The first reason is that there are differences in the backbone structures of SM (sphingolipid) and PtdGlc (glycerolipid). The second reason is that cholesterol interacts more strongly with sphingolipids than with glycerolipids (Masserini and Ravasi, 2001; Barenholz, 2002; Ohvo-Rekilä et al., 2002). The lipid bilayer fluid region of the outer leaflet of mammalian plasma membranes is mainly composed of unsaturated PCs. Immiscibility of PtdGlc with palmitly-oleoyl-PC (POPC, C18:0/C18:1-PC) was also indicated from DSC analysis and small-angle X-ray scattering measurements of these lipids in a 1:1 mol ratio mixture. Thus, the reason for the immiscibility of PtdGlc and POPC is due to the large difference in the fatty acid composition of POPC and PtdGlc.

These results suggest that PtdGlc is not a component of sphingolipid-based lipid raft domains and, further, that it forms a unique lipid domain containing mainly PtdGlc. We will describe the existence of PtdGlc-enriched domains in living biomembranes in Section 5.

4.3. Scan-repeat-number-dependent thermal behavior of PtdGlc

The paper by Murate et al. (2010) dealt only with results regarding equilibrium states of PtdGlc caused by repeated thermal cycling. PtdGlc exhibits extremely strange scan-repeat-number-dependent thermal behavior both in pure vesicle samples (Fig. 8) and in vesicle samples of a PtdGlc-containing mixture (Fig. 9). In the DSC study by Murate et al. (2010), the samples were prepared as follows. Stock solutions of PtdlGlc and D-erythro-N-streroyl glucosyl-C18-siphigoine (C18-GlcCer) were dissolved in a chloroform/methanol (2:1, v/v) mixture. Brain SM, POPC and cholesterol were dissolved in pure chloroform. Lipid mixtures were obtained by mixing these stock solutions. After removal of the solvent by evaporation under a stream of nitrogen gas and storage in a vacuum, the lipid films were hydrated and vortexed in a buffer solution (100 mM Hepes, 100 mM NaCl, 10 mM ETDA, pH 7.0) at room temperature. The hydrated samples were then transferred into the DSC cell. In the DSC instrument, these samples were subjected to successive heating and cooling scans between 5 °C and 85 °C, i.e., at temperatures above and below the chain-melting transitions of the lipids, to ensure that the samples were fully hydrated and homogeneous. Prior to the DSC scans, the samples had not been heated above room temperature.

In the first heating scan of a pure PtdGlc sample, a sharp transition peak was observed at 61.8 °C and the transition enthalpy was 18.6 kcal/mol. In the second heating scan, the peak at 61.8 °C decreased and a new, higher temperature peak appeared at 72.7 °C. After the fourth heating scan, the peak at 61.8 °C had completely disappeared and only the high temperature peak was observed at 76.4 °C. The enthalpy value of this peak at 76.4 °C was 14.2 kcal/mol. The thermograms observed in cooling scans are complex but display the same tendency. This behavior of PtdGlc is not due to chemical degradation. The absence of chemical degradation was indicated by the fact that only a single sample spot was observed on thin-layer chromatography after

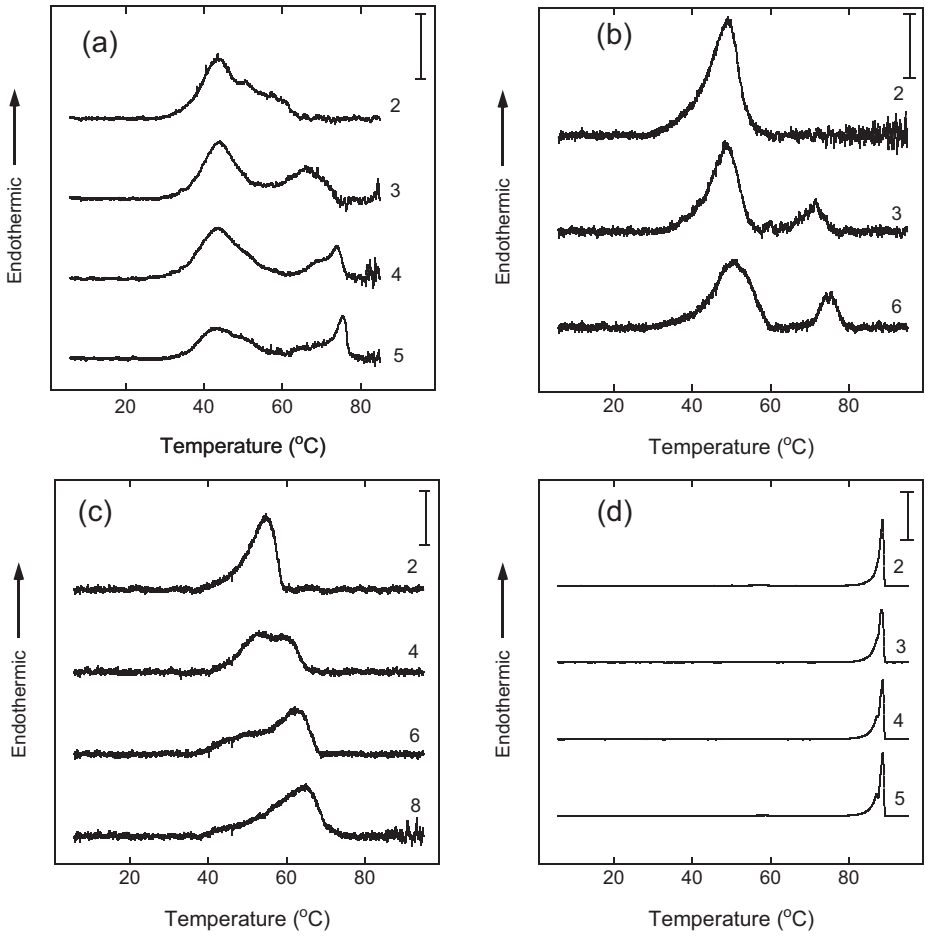

Fig. 9. DSC thermograms of PtdGlc/brain SM (1:1 mol/mol) (a), PtdGlc/brain SM/cholesterol (b), PtdGlc/POPC (1:1 mol/mol) (c), and C18-GlcCer (d). All data were obtained using vesicle samples. Data were normalized with respect to the total mass of lipids (in (b), cholesterol was not included to calculate the lipid mass). The vertical scale bars correspond to 0.5 kcal/mol/°C. The numeral on the right side of each curve indicates the repeat number of the scan.

the final DSC scan. If the heating and cooling cycle does not affect the liquid crystalline state, based on the enthalpy values, the first phase below the phase transition at 61.8 °C should be assumed to be more stable than the low-temperature phase formed after several heating and cooling cycles. At present, the origin of this strange scan-repeat-number-dependent thermal behavior is unclear.

Interestingly, a similar behavior is observed for phase-separated PtdGlc in lipid mixtures (Fig. 9). In mixtures of PtdGlc/brain SM or PtdGlc/brain SM/cholesterol, the transition peak at 76 °C corresponding to pure PtdGlc is not clearly observed in the 1st or 2nd heating scan, but is clearly observed after the 3rd heating scan. In a mixture of PtdGlc/POPC, a clear separation of the peak at 76 °C is not observed, but a similar tendency is evident. The scan shown in Fig. 6d shows successive DSC heating thermograms of C18-GlcCer and displays no scan number-dependent behavior. Thus the scan-dependent behavior of PtdGlc that was observed even in a lipid mixture indicates that the phase-separated PtdGlc region contains few other lipids, i.e., this region closely resembles pure PtdGlc bilayer membranes. Taking the change in shape of the DSC peak of the lipid that separates from PtdGlc into consideration, it is possible that these data reflect the following process. Initially, PtdGlc mixes well with the other lipids in the lipid films that were formed after removal of the solvents. However, subsequently PtdGlc gradually separates from the other lipids during repeated heating and cooling scans. Although the detailed process remains unsolved, evidently PtdGlc that is in the gel phase tends to separate from the main lipid components of the so-called lipid raft domain, which includes lipids such as SM.

5. A PtdGlc domain in plasma membranes

Since pure PtdGlc is in a gel phase at physiological temperatures and tends to separate from both POPC and SM/cholesterol in a mixture, it is predicted that PtdGlc will form a unique domain in biomembranes. In order to confirm the existence of a PtdGlc domain in membranes, the distribution of PtdGlc in cell membranes has been studied using microscopic methods. Immunofluorescence confocal microscopic measurements using fluorescence-labeled antibodies showed that the localization of PtdGlc in cells of the human promyelocytic leukemia cell line, HL60, or in the human alveolar epithelial cell line, A549, is distinct from that of SM and mono-sialoganglioside (GM1) (Nagatsuka et al., 2003; Murate et al., 2010). GM1 is also a main lipid component of the lipid raft domain (Simons and Ikonen, 1997; Lingwood and Simons, 2010). The distribution of the PtdGlc antibody in the immunofluorescence confocal microscopic image displays a dot like pattern. This pattern differs from the pattern of labeled SM or labeled GM1.

Since the resolution of confocal microscopy is only about 0.5 μm, an SDS-digested freeze fracture immunoelectron microscopy technique has been developed to investigate the distribution of specific lipid molecule of biomembranes with a higher resolution. By using two different sizes of colloidal gold particles, Murate et al. (2010)

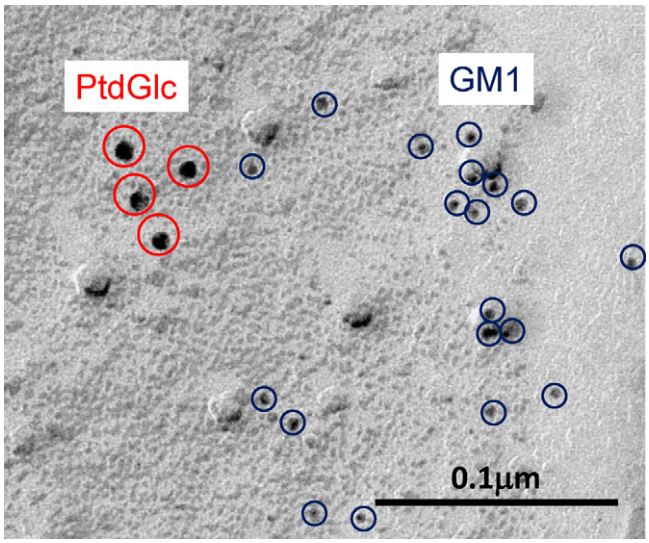

Fig. 10. Distribution of PtdGlc (10 nm gold; indicated by red circles) and GM1 (5 nm gold; indicated by blue circles) on the outer leaflet of the plasma membrane of A549 cells.
Taken from Murate et al. (2010).

studied the distributions of PtdGlc and GM1 using SDS-digested freeze fracture immunoelectron microscopy. In that study (Murate et al., 2010), the distribution of PtdGlc was studied using the DIM21 antibody, and the distribution of GM1 was studied using the cholera toxin B subunit (CTxB). The binding of CTxB to GM1 is well established (De Haan and Hirst, 2004). Anti-biotin antibody conjugated to 5 nm colloidal gold particles and anti-mouse IgM antibody conjugated to 10 nm colloidal gold particles were then used to detect biotinylated CTxB and DIM21, respectively for immunoelectron microscopic observation. Electron microscopy can easily distinguish a 10 nm-particle from a 5 nm-particle (Fig. 10) and one of the great advantages of freeze fracture electron microscopy is that it is possible to distinguish the outer and the inner leaflets of the cell membrane. This immunoelectron microscopic observation showed that both PtdGlc and GM1 are almost exclusively located in the outer leaflet of the plasma membrane of HL60 and A549 cells. Thus, a larger number of the labeled particles were detected on the outer leaflet, whereas a smaller number of the particles were detected on the inner leaflet.

As shown in the freeze fracture immunoelectron microscopic image shown in Fig. 10, PtdGlc domains do not overlap with GM1 domains. In addition, statistical pattern analysis using Ripley's K-function indicated that the radius of the PtdGlc-rich domain is 48 nm in HL60 cells and 69 nm in A549 cells. These results demonstrated that PtdGlc forms specific PtdGlc-enriched lipid domains on the outer leaflet of the plasma membrane and that, furthermore, these PtdGlc domains are distinct from so-called lipid rafts that are composed of sphingolipids/cholesterol complexes.

6. Suggested biological functions of PtdGlc and future directions

Among the many unanswered questions regarding PtdGlc, one of the most important questions is the biological function of the PtdGlc domains in plasma membranes. Based on recent experimental results and observations, several biological functions have been proposed for PtdGlc as follows.

PtdGlc domains are found in astroglial lineage cells of the developing mouse cortex (Kinoshita et al., 2009a) suggesting that these PtdGlc domains act as signaling platforms for astroglial differentiation. The PtdGlc-expressing cells generate neurospheres in cell culture, which differentiate into neurons, astrocytes, and oligodendrocytes (Kaneko et al., 2011), suggesting that PtdGlc-expressing cells have multipotency. It is as yet unclear whether the PtdGlc-domain is directly involved in the regulation of self-renewal or cellular differentiation of neural stem cells. Further detailed studies are needed to clarify the function of PtdGlc in nervous systems.

Since PtdGlc is hydrolyzed by phospholipases A, C and D, it should be investigated whether products of PtdGlc hydrolysis such as lyso-PtdGlc act as a second messenger. As described above, PtdGlc is composed exclusively of 18:0/20:0 fatty acid chains. Since arachidic acid is rarely found in mammals, arachidic acid produced from PtdGlc might act as a unique specific signal messenger. A preliminary result of Hirabayashi and co-workers suggested that Lyso-PtdGlc is involved in axonal migration in the developing chick spinal cord (Kamiguchi et al., unpublished observation).

A recent study of PtdGlc expression on the surface of human neutrophils (Kina et al., 2011) suggested that PtdGlc mediates apoptosis of neutrophil cells. Kina et al. (2011) found that anti-PtdGlc (DIM21) induces neutrophil apoptosis. It has already been reported that there are so-called lipid raft domains (lipid domains consisting mainly of glycosphingolipids) on the plasma membranes of neutrophils and that these domains are involved in apoptosis (Remijsen et al., 2009). Lactosylceramide (LacCer) is the most abundant glycosphingolipid in the cell membranes of human neutrophils. A LacCer-enriched domain mediates neutrophil phagocytosis (Nakayama et al., 2008) and the generation of superoxide (Iwabuchi and Nagaoka, 2002) but not apoptosis. Interestingly, confocal microscopic observation showed that PtdGlc is co-localized with Fas but not with LacCer on the plasma membrane of neutrophils (Kina et al., 2011). Fas is a death receptor on the plasma membranes of cells that leads to apoptosis (Yonehara et al., 1989; Nagata, 1996). Based on these data, it can be inferred that PtdGlc, but not LacCer, is involved in neutrophil apoptosis and that the Fas-mediated pathway is responsible for PtdGlc-mediated neutrophil apoptosis.

Most of the biological functions of PtdGlc have been evaluated by using the DIM21 antibody as an artificial ligand. It is therefore very important to identify natural ligands for PtdGlc or PtdGlc-domains. Identification and cloning of the gene that encodes the enzyme that synthesizes PtdGlc will also contribute to our understanding of the in vivo roles of PtdGlc.

The unique fatty acid composition of PtdGlc is the origin of the driving force that results in the formation of a specific PtdGlc domain on the outer leaflet of plasma membranes. Identification of other lipids and proteins that are associated with PtdGlc-enriched domains is also an important task. In addition, an understanding of the function of PtdGlc-enriched domains is essential for clarification of the differences in the physical properties (such as size, fluidity and stability) between a sphingolipids/cholesterol mixture domain and a PtdGlc-enriched domain. The origin of the unique scan-repeat-number-dependent thermal behavior of PtdGlc, is to our best knowledge, an unique behavior that has never been reported for any other membrane lipid, and should be further investigated from the viewpoint of lipid physical chemistry.

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