Reduction of Glycosphingolipid Levels in Lipid Rafts Affects the Expression State and Function of Glycosylphosphatidylinositol-anchored Proteins but Does Not Impair Signal Transduction via the T Cell Receptor*

Received for publication, July 16, 2003, and in revised form, September 2, 2003 Published, JBC Papers in Press, September 23, 2003, DOI 10.1074/jbc.M307674200

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Lipid rafts are highly enriched in cholesterol and sphingolipids. In contrast to many reports that verify the importance of cholesterol among raft lipid components, studies that address the role of sphingolipids in raft organization and function are scarce. Here, we investigate the role of glycosphingolipids (GSLs) in raft structure and raft-mediated signal transduction in T lymphocytes by the usage of a specific GSL synthesis inhibitor, threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP). Surface GM1 expression and the expression of GSLs in rafts were profoundly reduced by PDMP treatment, whereas the expression of other lipid and protein constituents, such as cholesterol, sphingomyelin, Lck, and linker for activation of T cells, was not affected. T cell receptor-mediated signal transduction induced by antigen stimulation or by antibody cross-linking was normal in PDMP-treated T cells. In contrast, the signal through glycosphosphatidylinositol (GPI)-anchored proteins was clearly augmented by PDMP treatment. Moreover, GPI-anchored proteins became more susceptible to phosphatidylinositol-specific phospholipase C cleavage in PDMP-treated cells, demonstrating that GSL depletion from rafts primarily influences the expression state and function of GPI-anchored proteins. Finally, by comparing the effect of PDMP with that of methyl-β-cyclodextrin, we identified that compared with cholesterol depletion, GSL depletion has the opposite effect on the phosphatidylinositol-specific phospholipase C sensitivity and signaling ability of GPI-anchored proteins. These results indicate a specific role of GSLs in T cell membrane rafts that is dispensable for T cell receptor signaling but is important for the signal via GPI-anchored proteins.

Recently, it was discovered that within the plasma membrane exist microdomains known as lipid rafts, which play important roles in various cellular functions including membrane trafficking, signal transduction, and endocytosis (1–3). In the immune system, the signals mediated through T-cell receptor (TCR), B-cell receptor, Fc receptor, and some cytokine receptors are known to depend on raft integrity (4–6). Moreover, many raft-associated proteins like Src-family kinases and linker for activation of T cells (LAT) are indispensable for lymphocyte signal transduction, indicating the importance of rafts as signaling platforms on the plasma membrane. In addition to physiological functions of rafts in many cellular processes, it has become apparent that several groups of pathogens preferentially utilize rafts when interacting with their target cells (7, 8).

Lipid rafts are highly enriched in cholesterol and sphingolipids (glycosphingolipids (GSLs) and sphingomyelin (SM)) (1, 2). Among these lipid components, cholesterol has a critical function for maintaining raft structure. Cholesterol is thought to fill the space between the hydrocarbon chains of the sphingolipids and to function as glue that keeps the raft assembly together. In fact, cholesterol depletion from cell membranes using drugs, such as methyl-β-cyclodextrin (MβCD), generally results in disruption of raft-mediated cellular functions (1). Thus, despite some concerns regarding side effects of the drug on signaling events (9), this experimental approach has been widely used to verify the importance of cholesterol in raft organization and function.

In contrast to many studies manipulating the cholesterol level in lipid rafts, reports that address a role for sphingolipids in raft function are scarce. A previous study (10) demonstrates...
that reduction of SM levels in Chinese hamster ovary mutant cells induced decreased distribution of cholesterol in lipid rafts. Moreover, it was reported that the addition of exogenous gangliosides to the culture of Madin-Darby canine kidney cells or Chinese hamster ovary.K1 cells displaced glycosylphosphatidylinositol (GPI)-anchored proteins from lipid microdomains (11, 12). All of these studies suggested that changes in sphingolipid contents alter raft lipid and protein constituents. However, none of these studies contributed to our understanding of any functional change in membrane rafts by the manipulation of sphingolipid levels. Although an effect of GSL deficiency on functional raft formation has been suggested previously (13), whether sphingolipids are essential for raft-mediated signal transduction remains to be addressed.

In this study, we investigated a role for sphingolipids in raft organization and function in lymphocytes, focusing particularly on a role for GSLs by the usage of a specific GSL synthesis inhibitor, n-PDMP. We demonstrated that the expression state of GPI-anchored proteins is affected by the reduction of GSL levels. Furthermore, our results showed that a reduced level of GSLs in membrane rafts modulates signaling through GPI-anchored proteins but not through the TCR. By comparing the effect of n-PDMP with that of MβCD, we also identified that GSL and cholesterol depletion have distinctly different influences on GPI-anchored proteins in lipid rafts.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Hybridomas—**Jurkat is a human leukemic T cell line. J.ECoR cells are stable transfectants of the ecotropic retrovirus receptor into Jurkat cells (14). 2B4 is a murine T cell hybridoma that is specific for pigeon cytochrome c plus I-ε (major histocompatibility class II) (15). LK35.2 is a B cell hybridoma and is used as antigen-presenting cells (APC) (16). All of the cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 μg/ml a-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified atmosphere of 5% CO₂.

**DNA Construction and Retroviral-mediated Gene Transfer—**A DNA fragment coding for the full-length mouse Thy-1 was obtained by reverse transcriptase-PCR using the 2B4 T cell hybridoma cDNA as a template. The sense and the antisense primers contained restriction sites for XhoI and for NotI, respectively. The fragment was subcloned into pPCR-Script (Stratagene) and sequenced. The XhoI/NotI fragment was then cloned into the pMX-puromycin retroviral vector (17). The fragment coding for the full-length mouse Thy-1 was obtained by reverse transcriptase-PCR using the 2B4 T cell hybridoma cDNA as a template. The sense and the antisense primers contained restriction sites for XhoI and for NotI, respectively. The fragment was subcloned into pPCR-Script (Stratagene) and sequenced. The XhoI/NotI fragment was then cloned into the pMX-puromycin retroviral vector (17). The resulting construct (pMX-Thy-1) was expressed in J.ECoR cells via retrovirus infection as described previously (18, 19). For stable transfectants, the infected cells were selected in medium supplemented with puromycin at 0.4 μg/ml from 48 h after infection. Growing colonies were expanded in selecting medium, and positive colonies expressing surface Thy-1 were screened by flow cytometry.

**Antibodies and Reagents—**The following mAbs were used: 145–2C11 (20), anti-mouse CD3; OKT3 (American Type Culture Collection), anti-human CD6; G7 (BD Biosciences), anti-mouse Thy-1; MOL171 (21), anti-Leuk mAb; anti-LAT polyclonal Ab (Upstate Biotechnology); anti-PLCγ1 mAb (UBI); PY20 (Transduction Laboratories), anti-phosphotyrosine; anti-β-actin mAb (Sigma); biotin-conjugated anti-CD59 mAb (Exalpha Biologicals); and FITC-conjugated anti-mouse Thy-1.2 mAb (BD Biosciences). FITC-conjugated avidin and FITC-conjugated cholera toxin B (CTx-B) were purchased from BD Biosciences and Sigma, respectively. Antigen pigecytochrome c (amino acids 88–104) was synthesized according to the amino acid sequence as described previously (22).

**PDMP or MβCD Treatment—**Cells were cultured with 10 μM or 1 μM PDMP in 10% fetal calf serum RPMI 1640 medium for 3–4 days. For MβCD pretreatment, cells were incubated with 4 mM MβCD (Sigma) in serum-free RPMI 1640 medium for 40 min at 37 °C. For PDMP or MβCD-treated cells, were washed with RPMI 1640 medium and then used for all of the experiments.

**Isolation of Raft Fraction and Immunoblotting Analysis—**Rat fraction were prepared as described previously (22). 1×10⁶ cells were washed in PBS containing 5 mM sodium orthovanadate and 5 mM EDTA and then were lysed with 1× PBS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 mM sodium orthovanadate, and 5 mM EDTA. The lysate was homogenized with 20 strokes of a Dounce homogenizer, gently mixed with an equal volume of 80% sucrose (w/v) in MES-buffered saline, and placed at the bottom of a 14 × 55-mm clear centrifuge tube (catalog number 344060, Beckman). The sample was then overlaid with 6.5 ml of 50% sucrose and 3.5 ml of 5% sucrose in MES-buffered saline and centrifuged at 200,000 × g for 16 h. Following centrifugation, 12 1-ml fractions (excluding the pellet) were collected from the top of the gradient. Aliquots corresponding to fractions 4 and 5 were combined as raft fractions, and those corresponding to fractions 11 and 12 were combined as non-raft fractions. Immunoblotting analysis was performed as described previously (23).

**Intracellular Calcium Analysis—**PDMP- or MβCD-treated cells were incubated with 3 μM Indo-1 AM (Molecular Probes) for 30 min at 37 °C in Hank’s-buffered saline solution containing 1% fetal calf serum, 1 mM CaCl₂, and 1 mM MgCl₂. Intracellular calcium analysis was performed on a BD LSR (BD Biosciences). Indo-1 was excited by a He-CD laser (325 nm, 8 milliwatts). Indo-1 emission was detected using a 380-nm (violet) low pass filter and a 510/20-nm (blue) bandpass filter. To determine the relative intracellular calcium concentration, Indo-1-loaded cells were warmed to 37 °C, analyzed for 15–30 s to establish baseline calcium levels, and stimulated by the addition of OKT3 (10 μg/ml) or G7 (10 μg/ml). Acquisition was continued in real time up to 200 or 512 s.

**IL-2 Production—**PDMP-treated 2B4 cells (1×10⁶) were stimulated by incubation with immobilized 2C11 mAb (1 μg/ml), soluble G7 mAb

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**Figure 1.** Effect of PDMP on GM1 expression in Jurkat cells. A, the biosynthetic pathway of sphingolipids and the step inhibited by n-PDMP. B, n-PDMP inhibits GlcCer synthase, resulting in depletion of GlcCer and downstream GSLs. C, kinetic changes in surface GM1 expression by n-PDMP treatment. Jurkat cells were incubated with n-PDMP for 1–3 days. The cells were stained with FITC-conjugated cholera toxin B (CTx-B) and analyzed by flow cytometry. The shaded histogram represents cells stained with FITC-conjugated avidin as a negative control. C, surface GM1 expression of n-PDMP-treated cells. Jurkat cells were incubated with L-PDMP for 3 days. GM1 expression was detected by flow cytometry as described in B.
or graded concentrations of antigen pigeon cytochrome c for 24 h. LK35.2 cells (5 × 10⁴) were added as a source of APC. Supernatants of the culture were assayed for IL-2 production by enzyme-linked immunosorbent assay (BD Biosciences).

Cholesterol Staining—To analyze cholesterol expression on the cell surface, biotinylated C₉ derived from perfringolysin O (β-toxin) was used as described previously (24). PDMP- or MβCD-treated Jurkat cells were stained with biotinylated β-toxin (BC) followed by FITC-conjugated avidin and analyzed by flow cytometry. The shaded histogram represents cells stained with FITC-conjugated avidin alone. C, localization of LAT, Lck, and GM1 in rafts from PDMP-treated Jurkat cells. Aliquots corresponding to fractions 4 and 5 were combined as the raft fractions (R), and aliquots corresponding to fractions 11 and 12 were combined as the non-raft fractions (N). These were then electrophoresed and tested for levels of LAT and Lck and GM1 by immunoblotting analysis. D, recruitment of PLC-1 to rafts after TCR stimulation. PDMP-treated Jurkat cells (D−) were either left untreated or stimulated with OKT3 for 10 min before the isolation of the raft and the non-raft fractions. The amount of PLC-1 and LAT was measured by immunoblotting analysis using specific Abs.

**Fig. 2.** Expression of lipid and protein constituents in rafts from n-PDMP-treated Jurkat cells. A, lipid analysis using raft fractions. PDMP-treated Jurkat cells were lysed with MES-buffered saline containing 1% Triton X-100, and the lysates were subjected to equilibrium gradient centrifugation. Aliquots corresponding to fractions 4 and 5 (the raft fractions) were combined. Acidic and neutral lipids from the raft fractions were separated by HPTLC and visualized with orcinol-sulfuric acid (upper) and 3% cupric acetate, 8% phosphoric acid (lower), respectively. The positions of lipid standards are indicated on the left. B, surface cholesterol expression. PDMP- or MβCD-treated Jurkat cells were stained with biotinylated β-toxin (BC) followed by FITC-conjugated avidin and analyzed by flow cytometry. The shaded histogram represents cells stained with FITC-conjugated avidin alone. C, localization of LAT, Lck, and GM1 in rafts from PDMP-treated Jurkat cells. Aliquots corresponding to fractions 4 and 5 were combined as the raft fractions (R), and aliquots corresponding to fractions 11 and 12 were combined as the non-raft fractions (N). These were then electrophoresed and tested for levels of LAT and Lck and GM1 by immunoblotting analysis. D, recruitment of PLC-1 to rafts after TCR stimulation. PDMP-treated Jurkat cells (D−) were either left untreated or stimulated with OKT3 for 10 min before the isolation of the raft and the non-raft fractions. The amount of PLC-1 and LAT was measured by immunoblotting analysis using specific Abs.

**Fig. 3.** The distribution of cholesterol and LAT-GFP in n-PDMP-treated cells. Jurkat-derived transfectants expressing LAT-GFP were incubated with n-PDMP for 3 days. Untreated or n-PDMP-treated cells were stained with biotinylated β-toxin followed by streptavidin-Texas Red. The cells were observed by confocal microscopy. In the left lower panels are differential interference contrast (DIC) images. Colocalization of cholesterol and LAT-GFP is indicated by arrows.

(10 μg/ml), or graded concentrations of antigen pigeon cytochrome c for 24 h. LK35.2 cells (5 × 10⁴) were added as a source of APC. Supernatants of the culture were assayed for IL-2 production by enzyme-linked immunosorbent assay (BD Biosciences).

Cholesterol Staining—To analyze cholesterol expression on the cell surface, biotinylated C₉ derived from perfringolysin O (β-toxin) was used as described previously (24). PDMP- or MβCD-treated cells were incubated with biotinylated C₉ (50 μg/ml) in PBS containing 1 mg/ml bovine serum albumin at room temperature for 20 min. After washing twice with PBS, the cells were stained with FITC-conjugated avidin and analyzed by flow cytometry.

**Confocal Microscopy**—Confocal microscopy was performed with a 63/1.4 oil-objective lens on a Zeiss LSM 510 confocal microscope using laser excitation at 488 and 543 nm. The widths of GFP and Texas Red emission channels were set such that bleed-through across channels was negligible.

Lipid Analysis—Lipid analysis of the raft fractions was performed as described previously (13). The chloroform-rich phase from Folch partition was separated into neutral and acidic lipid fractions by using a DEAE-Sephadex A-25 column (acetate form, 2.4-ml bed volume; Amersham Pharmacia Biotech). The fractions were dried and subjected to methanolic 0.1 M NaOH for ester cleavage. After neutralization, solu-
tyrosine phosphorylation of protein likely to be LAT is indicated. 

**RESULTS**

**Reduction in Surface Expression of GSL by an Inhibitor of Glucosylceramide Synthase, D-PDMP—**To elucidate a role of GSLs in membrane raft organization and function in T cells, we depleted cellular GSLs by using an inhibitor of glucosylceramide (GlcCer) synthase, D-PDMP (25). It has been reported that an analog of ceramide, D-PDMP, could inhibit UDP-glucose:N-acylsphingosine glucosyltransferase, which forms GlcCer from ceramide, the first step of synthesis of a series of GSLs (Fig. 1A). D-PDMP has been used to study biological functions in living cells (26). When we added D-PDMP to the culture of Jurkat cells at a concentration of 10 μM, surface GM1 expression was reduced in a time-dependent manner (Fig. 1B). Surface GM1 expression in Jurkat cells was considerably reduced even 1 day after the addition of D-PDMP, and 3 days after the addition the level of surface GM1 was almost one-tenth of that observed in cells without treatment. Because D-PDMP treatment more than 3 days did not induce a further inhibition of GM1 expression, we treated lymphoid cell lines with D-PDMP for 3 days in this study. With regard to the concentration of D-PDMP, we were able to inhibit GM1 expression in Jurkat cells at a concentration of 10 μM without detriment to cell viability and cell growth (data not shown). As a control for D-PDMP, we used L-PDMP, the stereoisomer of D-PDMP, which has been shown to be inactive in inhibiting GlcCer synthase (27). As previously reported, we did not observe any reduction in GM1 expression by L-PDMP treatment (Fig. 1C).

**Lipid Raft Organization in D-PDMP-treated Jurkat Cells—**We examined whether membrane raft structure was affected by the reduction in cellular GSL levels by D-PDMP. Lipid and protein components in membrane rafts are known to be fractionated into a Triton X-100-insoluble floating, so-called raft or detergent-resistant membrane fraction by sucrose density gradient centrifugation. We prepared raft fractions from untreated, D-PDMP-treated, and L-PDMP-treated Jurkat cells and first analyzed raft-associated lipids by HPTLC using acidic and neutral lipid fractions. We observed that major GSLs expressed in Jurkat cells were GM3 and GM2 and that these GSLs were mostly lost upon D-PDMP but not by L-PDMP treatment (Fig. 2A, top). When we analyzed the neutral lipid fractions, we were able to detect the expression of GlcCer, ceramide, SM, and cholesterol from untreated and L-PDMP-treated cells. However, the expression of GlcCer was almost completely inhibited in D-PDMP-treated cells (Fig. 2A, bottom). It is noteworthy that levels of SM and cholesterol were not changed by the inhibition of GSL synthesis. Normal expression of cholesterol in membrane rafts from D-PDMP-treated cells was also observed by the binding of δ-toxin on the cell surface in flow cytometry and confocal microscopy analysis (Figs. 2B and 3). Perfringolysin O, a cholesterol-binding toxin produced by Clostridium perfringens, is shown to bind selectively to cholesterol-rich microdomains of intact cells (24). Indeed, the distribution of this toxin showed a patchlike pattern on the cell surface, and LAT-GFP, one of raft markers we have previously reported (19), was clearly colocalized with this site. Thus, cholesterol patches seemed to correspond with lipid rafts, and the formation of cholesterol patches was not inhibited by L-PDMP (Fig. 3).

We next investigated the effect of D-PDMP on raft-localized proteins. An adaptor molecule, LAT, and Src-family kinase, Lck, are well known raft-resident proteins in T cells and play important roles in raft-mediated T cell activation (28, 29). As shown in Fig. 2C, LAT and Lck were highly enriched in the raft fraction and this localization was not impaired by D-PDMP treatment. We further analyzed stimulation-induced accumulation of a signaling protein into rafts in D-PDMP-treated cells. In response to TCR stimulation, PLCγ1 has been shown to accumulate into rafts by way of binding with LAT (30) and...
D-PDMP did not inhibit this response (Fig. 2D). In contrast to PLCγ1 accumulation, the localization of LAT was not changed before and after activation. Two forms of LAT at molecular masses of 36 and 38 kDa seem to correspond to palmitoylated and non-palmitoylated forms, respectively, as described previously (28). In addition to the localization of LAT, Lck, and PLCγ1, we observed that total protein content and distribution were similar in the raft fractions from untreated and D-PDMP-treated cells (data not shown). Thus, D-PDMP appeared not to alter protein distribution in rafts from T cells with or without stimulation.

Taken together, these results indicate that D-PDMP preferentially reduces the level of GSLs in rafts without affecting SM and cholesterol levels and the alteration of lipid contents did not induce a significant change in protein distribution in lipid rafts.

**TCR-mediated Signal Transduction in D-PDMP-treated T Cells**—It has been well established that the process of TCR-mediated signaling is dependent upon raft structure and function (31). We next examined whether the reduction of GSLs in lipid rafts modulates TCR-mediated T cell activation. Surface TCR in D-PDMP-treated Jurkat cells was cross-linked with anti-CD3 mAb to activate TCR signaling pathways. As shown in Fig. 4, tyrosine phosphorylation of cellular proteins and Ca\(^{2+}\) mobilization, critical early signaling events for T cell activation, were successfully induced by TCR cross-linking and D-PDMP treatment was not able to inhibit these responses. In addition to these early signaling events, the expression of CD69, a marker for late activation events, was also not impaired by D-PDMP (data not shown). Thus, TCR signaling by anti-CD3 mAb stimulation was not inhibited in D-PDMP-treated cells.

To examine whether the lack of effect of D-PDMP effect on the TCR signal is dependent on the strength of TCR stimulation, we stimulated T cells under more physiological conditions. Mouse T cell hybridomas, 2B4 cells, were treated with D-PDMP for 3 days, stimulated with various concentrations of antigenic peptide in the presence of APC, and then analyzed for IL-2 production. Although D-PDMP induced a similar inhibitory effect of GM1 expression on 2B4 cells (data not shown), IL-2 production was not changed even at low concentrations of antigen between untreated and D-PDMP-treated cells (Fig. 5).

These results clearly demonstrated that raft structure with a reduced level of GSLs is still capable of transducing the signal through the TCR stimulated either with antigen or Ab cross-linking.

**GPI-anchored Proteins Become More Sensitive to PI-PLC Cleavage in D-PDMP-treated Cells**—We next evaluated D-PDMP-induced changes in raft structure by analyzing the expression state of GPI-anchored proteins on the plasma membrane. Previous reports demonstrated that the sensitivity of GPI-anchored proteins to bacterial PI-PLC is heavily influenced by lipid components in membrane rafts (32, 33). Hence, we investigated whether a reduction of GSLs in rafts affects the PI-PLC sensitivity of GPI-anchored proteins in T cells. Untreated, D-PDMP-treated, and L-PDMP-treated Jurkat cells were treated with various concentrations of PI-PLC, and surface expression of CD59 was analyzed (Fig. 6A). Although the expression level per se was not changed, the sensitivity of CD59 to PI-PLC was apparently increased by D-PDMP. Decreased expression of CD59 because of PI-PLC cleavage was more obvious in D-PDMP-treated cells at a concentration of 50 milliunits/ml. To establish whether increased sensitivity to PI-PLC is not limited to CD59, we established a Jurkat-derived transfectant-expressing mouse Thy-1 and analyzed for PI-PLC sensitivity. Again, Thy-1 expressed on Jurkat cells became more cleavable at any concentration of PI-PLC by D-PDMP treatment (Fig. 6B), suggesting that GPI-anchored proteins in general become more susceptible to PI-PLC in D-PDMP-treated cells.

![Fig. 6. Effect of D-PDMP on the PI-PLC sensitivity of GPI-anchored proteins. A, Jurkat cells were cultured with or without PDMP for 3 days and treated with indicated concentrations of PI-PLC for 60 min. The cells were stained with biotin-conjugated anti-CD59 mAb followed by FITC-conjugated avidin. The shaded histogram represents cells stained with FITC-conjugated avidin alone. B, Jurkat-derived transfectants expressing mouse Thy-1 were cultured with or without PDMP and treated with indicated concentrations of PI-PLC. The cells were stained with FITC-conjugated anti-mouse Thy-1 mAb.](image-url)
The Signal through GPI-anchored Proteins Is Augmented in \( \nu \)-PDMP-treated Cells—We next investigated the signaling ability of GPI-anchored proteins in \( \nu \)-PDMP-treated T cells. It has been proposed that cross-linking of GPI-anchored proteins induces clustering of lipid rafts and activation of raft-associated signaling molecules, leading to T cell activation (34, 35). When we cross-linked mouse Thy-1 on stable transfectants by anti-Thy-1 mAb G7, we successfully observed tyrosine phosphorylation of cellular proteins and \( \nu \)-PDMP treatment clearly augmented this response (Fig. 7A). Among phosphorylated proteins, the phosphorylation of proteins likely to be LAT (\( \sim 36 \text{kDa} \)) was enhanced. Although \( \nu \)-PDMP-induced increase in phosphorylation of the 36-kDa protein was not so dramatic, we confirmed that the increase was reproducible and that the 36-kDa protein was indeed LAT by immunoprecipitation analysis (data not shown). To further analyze whether Thy-I-mediated signaling was influenced by \( \nu \)-PDMP in late activation events, IL-2 production from mouse 2B4 T cell hybridomas was determined after cross-linking with anti-Thy-1 mAb (Fig. 7B). We observed that IL-2 production from \( \nu \)-PDMP-treated cells was \( \sim 2 \times \) higher than that of untreated or \( \nu \)-PDMP-treated cells. As described previously, tyrosine phosphorylation of cellular proteins and IL-2 production were almost comparable among untreated, \( \nu \)-PDMP-treated, and \( \nu \)-PDMP-treated cells when these cells were stimulated with anti-CD3 mAb. These results indicated that a reduced level of GSLs in lipid rafts modulates the signal through GPI-anchored proteins but not the signal through the TCR.

Cholesterol Depletion Induces an Effect Opposite to GSL Depletion in Terms of PI-PLC Sensitivity and the Signaling Ability of GPI-anchored Proteins—Cholesterol is an essential component of membrane rafts, and extraction of cholesterol from cell membrane using MβCD has been widely used as a method that disrupts raft function (1, 36). We finally investigated the effect of cholesterol depletion compared with that of GSL depletion in terms of PI-PLC sensitivity and the signaling ability of GPI-anchored proteins. We were able to treat Jurkat cells with \( \sim 4 \text{mM} \) MβCD for 40 min without affecting cell viability and cell growth, and this treatment resulted in \( 50\% \) reduction in total cellular cholesterol by lipid analysis (19) and the loss of perfringolysin O binding by flow cytometry (Fig. 2B). When we analyzed PI-PLC sensitivity in MβCD-treated Jurkat cells, we found that CD59 became more resistant to PI-PLC cleavage (Fig. 8A, left). A similar effect was observed in mouse Thy-1 expressing stable transfectants (Fig. 8A, right). Thus, in clear contrast to the effect of GSL depletion by \( \nu \)-PDMP, cholesterol depletion from membrane rafts rendered GPI-anchored proteins more resistant to PI-PLC. To determine whether the change in expression state of GPI-anchored proteins by MβCD influences the signaling ability, Thy-1 transfectants were treated with MβCD, stimulated with anti-Thy-1 mAb, and then examined for \( \text{Ca}^{2+} \) mobilization (Fig. 8B). We observed a prominent inhibition in \( \text{Ca}^{2+} \) response by MβCD treatment. Collectively, these results demonstrated that alteration of raft structure induced by cholesterol depletion is quite different from that by GSL depletion.

**DISCUSSION**

This study was undertaken to define a physiological role of GSLs in T lymphocytes using GSL synthesis inhibitor, \( \nu \)-PDMP. Although lymphocytes are known to be rich in sphingolipids on the cell surface (37), how these lipids are involved in various lymphocyte functions is still largely unknown. GSLs with saturated fatty acids are fundamental constituents of lipid rafts, and therefore may play an important function in raft-mediated T cell activation. Our study clearly demonstrates that lipid rafts with a reduced level of GSLs contain normal amounts of raft-localized signaling proteins (Fig. 2C) and can mediate the signal through the TCR (Figs. 4 and 5). This result may indicate either of the following two possibilities in terms of rafts and TCR signaling. First, although TCR signaling depends on raft integrity, GSLs may not be essential for the formation of rafts in T cells. Second, reduction of GSLs might actually induce raft disorganization but the progression of TCR signaling occurs independently from raft integrity.

The existence and function of lipid microdomains in T cells...
was originally reported by Xavier et al. (38) and has been the subject of intense investigation over the last few years. Some of the key proteins involved in TCR signaling are constitutively localized in rafts or recruited into rafts upon TCR stimulation. Moreover, raft lipids per se are thought to accumulate into the immunological synapse, the specialized contact surface that is formed between T cells and APCs, during T cell activation (39, 40). The experimental approach widely used to verify the functional importance of rafts in TCR signaling is the manipulation of membrane cholesterol using drugs such as MβCD. Treatment of T cells with MβCD has been shown to result in dissociation of proteins from rafts and inhibit early and late TCR signaling events (36, 38). However, Viola and colleagues (9) recently reported that the inhibition of TCR-induced Ca2+ mobilization by MβCD is possibly because of nonspecific depletion of intracellular Ca2+ stores and plasma membrane depolarization. This result along with the known cytotoxicity of MβCD raises a serious concern regarding the current consensus of a critical role of rafts in lymphocyte signaling. TCR signaling may not depend on raft microdomains, and the present study might be in line with this possibility.

Nonetheless, we still favor an importance of rafts in TCR signaling because of the following reason. Besides the experiments using raft-disrupting drugs, there exist several studies that utilize lipid-processing mutations of signaling proteins (23, 29, 41). We previously demonstrated that, whereas raft targeting of activated phosphatase using the N-terminal portion of LAT inhibits TCR signaling, the targeting of this molecule into the plasma membrane outside rafts using LAT mutants cannot induce this inhibition (23). It was also reported that TCR signaling in LAT-negative T cell lines is severely impaired and cannot be reconstituted by transfection with LAT mutants at residues required for raft localization (41). All of these studies clearly proved that raft localization of signaling proteins is indispensable to initiate TCR signal transduction. Thus, reduction of GSLs cannot alter raft organization that preserves protein components within the microdomain. Because SM levels were not changed in detergent-resistant membrane fractions from d-PDMP-treated cells (Fig. 2A), SM does not seem to replace reduction in raft-associated GSLs. However, it is possible that phospholipids with saturated fatty acids can be incorporated more into raft microdomains when GSL synthesis is blocked. Alternatively, because d-PDMP treatment could not totally abrogate surface GM1 expression (Fig. 1B),
residual GSLs may be enough to maintain raft integrity for TCR signaling. Further studies are necessary to determine the precise lipid composition of rafts when cellular GSLs are decreased.

GPI-anchored proteins are a heterogeneous class of proteins anchored to the membrane via a post-translational lipid modification. The signaling capacity of GPI-anchored proteins has been studied for >10 years especially in lymphocytes (42), but the mechanism by which signal transduction can occur is not yet elucidated. GPI-anchored proteins are mainly localized in raft microdomains, and it has been proposed that cross-linking of GPI-anchored proteins induces clustering of rafts and activation of the raft-associated proteins, leading to lymphocyte activation (35, 43, 44). Thus, elucidation of factors that influence signal transduction through GPI-anchored proteins may promote a better understanding for the mechanism of raft-mediated lymphocyte activation. We here made several novel findings in terms of the effects of GSL deficiency on GPI-anchored proteins in lymphocytes. First, although the expression level per se was not changed, GPI-anchored proteins became more susceptible to PI-PLC in δ-PDMP-treated T cells (Fig. 6). A previous report by Lehto and Sharom (33) using reconstituted lipid bilayer systems demonstrates that lipid fluidity and packing are the most important modulators of PI-PLC activity on GPI-anchored proteins. They found that inclusion of ganglioside GM1 or GT1b in the lipid bilayer composed of dimyristoylphosphatidylcholine reduces the overall PI-PLC activity. Based on these findings, it is possible that GSL depletion increases the fluidity and decreases the packing state of lipids in the plasma membrane of lymphocytes. Alternatively, GPI-anchored proteins are shown to be physically associated with GSLs (12) and reduced association with GSLs in rafts may somehow render GPI-anchored proteins more susceptible to PI-PLC. Consistent with our observation, Hanada and colleagues (10, 32) have demonstrated the increased sensitivity of GPI-anchored proteins to PI-PLC in sphingolipid-deficient Chinese hamster ovary cell lines.

A second and more important observation regarding GSLs and GPI-anchored proteins in this study is the GSL depletion-induced change in the signaling ability of GPI-anchored proteins. Tyrosine phosphorylation of LAT and IL-2 production were clearly induced in response to anti-Thy-1 cross-linking, and these responses were increased in δ-PDMP-treated cells (Fig. 7). Most GPI-anchored proteins have been shown to co-isolate with Src-family kinases in immunoprecipitation assays, and signaling via GPI-anchored proteins is considered to proceed through these kinases (45). Given that the localization of Src-family kinases in rafts is not affected by δ-PDMP treatment (Fig. 2C), the efficiency of interaction between Src-family kinases and GPI-anchored proteins might be elevated by GSL depletion. GSLs may have a negative regulatory role in functional association between GPI-anchored proteins and Src-family kinases in membrane rafts. Studies are in progress to elucidate a molecular mechanism for this negative regulation.

Finally, the present study indicates that, although both GSLs and cholesterol are major lipid constituents of rafts, they differentially contribute signaling cascades initiated in lymphocyte membrane rafts. Whereas cholesterol appears to be essential for signaling both via the TCR and GPI-anchored proteins, GSLs are only capable of modulating the latter pathway. The mechanism for this difference is presently unknown, but further investigation of the functions of individual raft lipids will provide important insights into the process of lymphocyte activation.