Recent data suggest that membrane microdomains or rafts that are rich in sphingolipids and cholesterol are essential in signal transduction and membrane trafficking. Two models of raft structure have been proposed. One proposes a unique role for glycosphingolipids (GSL), suggesting that GSL-head-group interactions are essential in raft formation. The other model suggests that close packing of the long saturated acyl chains found on both GSL and sphingomyelin plays a key role and helps these lipids form liquid-ordered phase domains in the presence of cholesterol. To distinguish between these models, we compared rafts in the MEB-4 melanoma cell line and its GSL-deficient derivative, GM-95. Rafts were isolated from cell lysates as detergent-resistant membranes (DRMs). The two cell lines had very similar DRM protein profiles. The yield of DRM protein was 2-fold higher in the parental than the mutant line, possibly reflecting cytoskeletal differences. The same amount of DRM lipid was isolated from both lines, and the lipid composition was similar except for up-regulation of sphingomyelin in the mutant that compensated for the lack of GSL. DRMs from the two lines had similar fluidity as measured by fluorescence polarization of diphenylhexatriene. Methyl-β-cyclodextrin removed cholesterol from both cell lines with the same kinetics and to the same extent, and both a raft-associated glycosyl phosphatidylinositol-anchored protein and residual cholesterol showed the same distribution between DRMs and the detergent-soluble fraction after cholesterol removal in both cell lines. Finally, a glycosyl phosphatidylinositol-anchored protein was delivered to the cell surface at similar rates in the two lines, even after cholesterol depletion with methyl-β-cyclodextrin. We conclude that GSL are not essential for the formation of rafts and do not play a major role in determining their properties.

Recent studies suggest that plasma membrane lipids do not always mix homogeneously but that membranes may contain microdomains or rafts that are rich in sphingolipids and cholesterol (1–3). Rafts may be concentrated or stabilized in caveolae. Rafts have been observed in caveolae and also exist in cells that lack caveolae. Rafts may be concentrated or stabilized in caveolae (1–3). Rafts may be concentrated or stabilized in caveolae. Rafts have been always mix homogeneously but that membranes may contain microdomains or rafts that are rich in sphingolipids and cholesterol (1–3). Rafts may be concentrated or stabilized in caveolae. Rafts have been observed in caveolae and also exist in cells that lack caveolae.

Detergent-resistant Membrane Rafts in Melanoma Cells

Glycosphingolipids Are Not Essential for Formation of Detergent-resistant Membrane Rafts in Melanoma Cells

METHYL-β-CYCLODEXTRIN DOES NOT AFFECT CELL SURFACE TRANSPORT OF A GPI-ANCHORED PROTEIN*

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Recent data suggest that membrane microdomains or rafts that are rich in sphingolipids and cholesterol are essential in signal transduction and membrane trafficking. Two models of raft structure have been proposed. One proposes a unique role for glycosphingolipids (GSL), suggesting that GSL-head-group interactions are essential in raft formation. The other model suggests that close packing of the long saturated acyl chains found on both GSL and sphingomyelin plays a key role and helps these lipids form liquid-ordered phase domains in the presence of cholesterol. To distinguish between these models, we compared rafts in the MEB-4 melanoma cell line and its GSL-deficient derivative, GM-95. Rafts were isolated from cell lysates as detergent-resistant membranes (DRMs). The two cell lines had very similar DRM protein profiles. The yield of DRM protein was 2-fold higher in the parental than the mutant line, possibly reflecting cytoskeletal differences. The same amount of DRM lipid was isolated from both lines, and the lipid composition was similar except for up-regulation of sphingomyelin in the mutant that compensated for the lack of GSL. DRMs from the two lines had similar fluidity as measured by fluorescence polarization of diphenylhexatriene. Methyl-β-cyclodextrin removed cholesterol from both cell lines with the same kinetics and to the same extent, and both a raft-associated glycosyl phosphatidylinositol-anchored protein and residual cholesterol showed the same distribution between DRMs and the detergent-soluble fraction after cholesterol removal in both cell lines. Finally, a glycosyl phosphatidylinositol-anchored protein was delivered to the cell surface at similar rates in the two lines, even after cholesterol depletion with methyl-β-cyclodextrin. We conclude that GSL are not essential for the formation of rafts and do not play a major role in determining their properties.

Recent studies suggest that plasma membrane lipids do not always mix homogeneously but that membranes may contain microdomains or rafts that are rich in sphingolipids and cholesterol (1–3). Rafts may be concentrated or stabilized in caveolae but also exist in cells that lack caveolae. Rafts have been proposed play important roles in signal transduction; for instance, recruitment of signaling proteins to rafts in T cells (4–8) and basophils (9–11) appears to be required for signaling. Rafts may also play a role in intracellular sorting. For instance, depletion of cholesterol, sphingolipids, or certain raft-associated proteins affects sorting of apical proteins in epithelial cells (12–17) and axonal proteins in neurons (18, 19). Recent studies also suggest that rafts play a role in sorting in the endocytotic pathway (20, 21).

Two models for the organization of lipids in rafts have been proposed. The first was developed by Simons and colleagues (1, 22, 23) as part of a model for sorting of apical and basolateral proteins in the trans-Golgi network of polarized epithelial cells. In this model, clusters of GSL form spontaneously in the trans-Golgi network. Apical proteins partition into these clusters, and the resulting rafts are packaged into apical transport vesicles. In early versions of the model, it was suggested that GSL might self-associate through hydrogen bonds between GSL headgroups and between the hydroxyl groups of the sphingosine base and the hydroxy fatty acid present on many sphingolipids (22). More recently, it was suggested that sphingolipids might interact through weak interactions between the GSL carbohydrate headgroups (1). Because cholesterol is now known to be an important component of rafts, it was proposed that cholesterol, with its small headgroup, is recruited to rafts because of its ability to fill gaps in the bilayer created by the discrepancy in size between the large GSL headgroups and their acyl chains (1).

The second model for the structure of rafts was developed from studies of rafts isolated by their insolubility in nonionic detergents such as Triton X-100 (3, 24, 25). This model postulates that interactions between lipid acyl chains play a key role in raft formation and that rafts exist in membranes as domains in the liquid-ordered (l_0) phase (3). In particular, the high acyl chain melting temperature (T_m) of sphingolipids was proposed to promote phase separation and formation of l_0 phase domains in the presence of high amounts of cholesterol.

Several pieces of evidence support the importance of acyl chain interactions in raft formation. First, sphingomyelin (which has the same phosphocholine headgroup as phosphatidylcholine) and GSL are equally enriched in DRMs isolated

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1 The abbreviations used are: GSL, glycosphingolipids(s); DRM, detergent-resistant membrane; GPI, glycosyl phosphatidylinositol; l_0, liquid-ordered; PLAP, placental alkaline phosphatase; DMEM, Dulbecco’s modified Eagle’s medium; MBCD, methyl-β-cyclodextrin; DPPC, dipalmitoyl phosphatidylcholine; DOPC, dioleoyl phosphatidylcholine; DPH, diphenylhexatriene; PBS, phosphate-buffered saline; TNE, Tris-NaCl-EDTA; PAGE, polyacrylamide gel electrophoresis; T_m, melting temperature.
Glycosphingolipids Are Not Essential for Raft Formation

from cells (26). Further support came from studies of sphingoli-

lipid- and cholesterol-rich model membranes that contain 1,2

phase domains and can form DRMs upon detergent extraction.

When two phosphatidylcholine species, one with saturated acyl

chains and a high $T_m$, and the other with unsaturated acyl

chains and a low $T_m$, were incorporated into these membranes

and subjected to detergent extraction, the high $T_m$ lipid but not

the low $T_m$ lipid associated with DRMs (25). Finally, a GPI-

anchored protein associated with detergent-insoluble gel phase

dipalmitoyl phosphatidylcholine (DPPC) domains in model

membranes (27), showing that sphingolipid-specific hydrogen

bonding is not required for organization of these domains.

The use of detergent insolubility in studying rafts has raised

concern about potential detergent-induced artifacts. Indeed, we

found that detergent insolubility can underestimate raft asso-

ciation of proteins and lipids (27, 28) and is therefore not a

quantitative measure of raft association. However, several po-

tentially serious detergent effects (including artificial cre-

ation of domains from previously homogeneous bilayers and

recruiting of nonraft associated proteins and lipids into rafts

during lysis) do not occur (3, 27, 29). Thus, enrichment of

proteins or lipids in DRMs appears to be a good indication that

they associate with rafts in vivo, and DRMs have proven to be

very useful tools for studying rafts.

Glycosphingolipids play important roles in cell recognition,

signaling, and other processes (30, 31) and are essential for

mammalian development (32). However, some mammalian

cells that lack glycosphingolipids can be grown in culture (33).

In this study, we tested the role of GSL in raft formation in vivo

using the GM-95 melanoma cell line, which is deficient in GSL

because it lacks ceramide glucosyltransferase, the first enzyme

in GSL synthesis (34). Sphingomyelin is up-regulated in GM-

95, and the total amount of sphingolipid is similar in GM-95

and the parental MEB-4 line (35). Up-regulation of sphingomy-

elin in GM-95 is a direct effect of the lack of glycosphingolipids,

as shown by the fact that expression of recombinant ceramide

glycosyltransferase restores both GSL and sphingomyelin to

wild-type levels (35). We compared DRMs and several other

parameters that might reflect differences in raft structure be-

tween MEB-4 and GM-95 cells.

We also examined the transport kinetics of placental alka-

line phosphatase (PLAP), a GPI-anchored protein, through the

secretory pathway to the cell surface. GPI-anchored proteins

have a high affinity for rafts and are apically targeted in epithe-

lial cells (36). Although melanoma cells are not polarized,

two separate transport pathways, cognates of apical and baso-

lateral pathways, have been shown to exist in nonpolarized

cells (37–39). Thus, if the presence of GSL in rafts were crucial

for the incorporation of GPI-anchored proteins into transport

vesicles, transport would be slowed in GSL-negative cells. How-

ever, PLAP was delivered to the cell surface at the same rate in

the two cell lines. Depletion of cholesterol with MBCD did not

affect PLAP transport in either line.

MATERIALS AND METHODS

Cells—The MEB-4 subline of the B16 murine melanoma cell line (40)

and the glycosphingolipid-negative GM-95 cell line derived from MEB-4

(33, 34) were from the RIKEN Cell Bank (Tokyo, Japan). B16-F1 and

B16-F10 cells were from Dr. I. J. Fidler (University of Texas, M. D.

Anderson Cancer Center, Houston, TX). Cells were grown in Dulbecco’s

modified Eagle’s medium (DMEM) with 5% iron-supplemented calf

serum (growth medium). Cells were stably transfected (41) with PLAP

(42), cotransfecting with a G418 resistance marker.

Antibodies—The following antibodies were used: rabbit polyclonal

antibodies; anti-PLAP, Dako (Carpinteria, CA); anti-Ga, and Ga1,2,3-

Oncogene Research Products (Cambridge, MA); anti-pβ23 (Yes), as

described (43); anti-β-galactosidase, Cappel (Durham, NC); anti-caveo-

lin, Transduction Laboratories (Lexington, KY); horseradish pero-

diase-conjugated goat anti-mouse IgG (M,Jackson Labs (West Grove,

PA); horseradish peroxidase goat anti-rabbit IgG, Sigma.

Lipids—Dioleoyl phosphatidylcholine, DPPC, and TLC standards

(brain phosphatidylcholine, bovine liver phosphatidylethanolamine,

brain phosphatidyserine, bovine liver phosphatidylinositol, brain cere-

brosides, and cholesterol) were from Avanti Polar Lipids. Diphenyl-

hexadecylphosphorylcholine (DPPH) was from Avanti Polar Lipids.

Other Materials—MBCD was from Sigma. The Renaissance chemi-

luminescence reagent, [1,2,6,7-3H]-cholesterol (75.1 Ci/mmol, 1 mCi/

ml) referred to as [3H]cholesterol, and EXPRE3SS protein labeling mix

(>1000 Ci/mmol) referred to as [3H]sphingomyeline were from NEN

Life Science Products. (Labeling medium lacked methionine but not

[3H]cysteine. The labeling mix probably accounted for only a small fraction of total protein labeling.) [3H]Acetic acid (sodium

salt, 5 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis,

MO). Prestained protein molecular weight standards were from Bio-

Rad or Life Technologies, Inc. The detergent-compatible protein assay

kit (used for all protein determinations) was from Bio-Rad. Sulfo-NHS-

biotin, sulfo-LC-NHS-biotin, and streptavidin immobilized on aga

roase beads were from Pierce. Parsbin heat-kill Staphylococcus aureus

cells were from Calbiochem (San Diego, CA). All other materials were

from Sigma or Fisher Scientific.

DRM Preparation—DRMs were prepared by ultracentrifugation of

cold Triton X-100 cell lysates in sucrose step gradients, either harvest-

ing the DRM band or fractionating the gradient, as described (43) with

the DRM band. The lysis buffer but not gradient fractions contained

0.1 mM sodium carbonate, pH 11. Lysates were passed through a 22

gauge needle to shear DNA before mixing with Tris-NaCl-EDTA

(TNE) buffer (43) containing 80% sucrose and placing at the bottom of

the centrifuge tube. These conditions allowed more reproducible recov-

ery of DRMs, which otherwise stuck variably to cytoskeleton and pe-

leted in the sucrose gradients. Under these conditions, sucrose gradient

pellets were small and contained very little protein. Except where

noted, cells in each 10-cm dish were lysed in 1 ml of TNE/carbonate (43)

containing enough Triton X-100 to give the indicated w/v ratio of
t
detergent to total cell protein, determined by first measuring cell

protein in a parallel dish. In a pilot experiment, equal amounts of DRM

protein were recovered if 10-cm dishes of MEB-4 cells were extracted

with 1 ml of TNE/carbonate containing 2% Triton X-100/dish or with 2

ml of TNE containing 1% Triton X-100/dish, showing that the detergent:
protein ratio and not the concentration of Triton X-100 deter-

DRM yield. For lipid analysis, ultracentrifugation was overnight

at 24K RPM in an SW28 rotor (Beckman, Palo Alto, CA); for other

experiments, it was for at least 3 h, and sometimes overnight at 28K

RPM in an SW41 rotor. To measure [3H]cholesterol, 0.1-ml aliquots of

each 1-ml fraction were analyzed by scintillation counting. The fraction

of [3H]cholesterol in DRMs was determined by dividing the cpm in

gradient fractions 9–12 by the total cpm in the gradient.

Metabolic Labeling, Gels, and Blots—Metabolic labeling with

[3H]sphingomyelin (24) and SDS-polyacrylamide gel electrophoresis

(PAGE), Western blotting, and fluorography (44) were as described.

Lipid Analysis—For extraction of whole cell lipids, cells were lysed

with 1 ml of TNE after rinsing with phosphate-buffered saline (PBS)

(43).

Lysates were transferred to glass tubes with Teflon-lined caps with

several methanol rinses, brought to 1:1 chloroform:methanol, sonicated,

and incubated overnight at room temperature with rocking. In harvest-

ing DRM bands from the interface between 5 and 38% sucrose gradient

steps for lipid analysis, care was taken to avoid a triglyceride-rich fat

ease that floated on the 5% layer. DRMs were extracted with 1:1

chloroform:methanol as for whole cell lipids. Neutral and acidic lipids

were separated (45, 46) for quantitation of DRM lipids but not for

quantitation of cholesterol from whole cell extracts. In all cases, lipids

were desalted and (except for GM3) analyzed by quantitative TLC by

charring in the presence of cupric acetate and densitometry in compar-

ison with standards on the same plate (45, 46) with minor modifi-

cations as described (24). Quantitation was performed using a Bio-Rad

GS-670 imaging densitometer.

Because GM3 is the only major ganglioside in MEB-4 cells, it was

quantitated (assuming a molecular weight of 1182) by measuring or-

ganic sialic acid by a standard resorcinol assay as follows. Lipids were

dried under N₂ and dissolved in 500 μl of H₂O. 500 μl of resorcinol

reagent (made as follows: 50 mg of resorcinol were dissolved in 2.2

ml of concentrated HCl) containing 0.0625 ml of 0.1 M CuCl₂

was added; volume was brought to 25 ml with H₂O, and samples

were boiled for 15 min, chilled on ice 2 min, and extracted with

0.8 ml of butyl acetate/butanol 85:15. The absorbance of the upper

layer was measured at 580 nm and compared with a standard curve

(2-30 μg/ml sialic acid).

Fluiddity Measurements—Fluorescence polarization of DPH in large
unilamellar liposomes prepared by suspension of dried lipids in PBS, sonication, and three freeze-thaw cycles was measured as described (25). DRMs isolated from four confluent 10-cm dishes of each cell type were suspended in 2 ml of PBS with sonication and divided in half. DPH was added to one tube to a final concentration of 100 nM from a 100 μM stock solution in tetrahydrofurin; the other was used as a background control. Fluorescence polarization was measured as described (25). Control experiments showed that DPH fluorescence polarization was not affected by the ratio of DPH to DRM.

Labeling with [3H]Cholesterol—Cells in 10-cm dishes were incubated with 3 μCi of [3H]cholesterol/10 ml normal growth media for 2 days, trypan blue stained, and transferred to a fresh dish to avoid contamination of lysates with [3H]cholesterol that adhered to the dish (47). After growth for about 6 h to allow adhesion to the dish, medium was replaced with serum-free DMEM plus 1% bovine serum albumin, to minimize esterification of [3H]cholesterol (47), for growth overnight before the experiment.

Cholesterol Depletion—Except for surface delivery experiments, (described separately below), cells were grown to confluence in 10-cm dishes. Medium was removed, and monolayers were rinsed once with PBS. 2.5 ml of DMEM without serum was added to each dish. At various times, this medium was replaced with 2.5 ml of the same medium containing 10 mM MBCD. Times of addition of MBCD were staggered so that all cells were exposed to serum-free medium for the same length of time. Cells remained adherent to the dish for up to 60 min. (Most cells detached after 150 min.) After MBCD treatment, cells were processed as described above (for cholesterol determination) or placed on ice and extracted with TNE/Triton X-100 for DRM preparation.

Surface Delivery of PLAP—Determination of surface delivery rate was as essentially as described (48, 49). Briefly, MEB-4 or GM-95 cells stably expressing PLAP in 35-mm dishes were incubated 45 min with or without 10 mM MBCD in 1 ml of DMEM lacking methionine and serum; pulse labeled 20 min with 100 μl of DMEM containing 2 mM/ml [3H]methionine without methionine or serum; chased for 0, 15, 30, 45, 60, 120, or 180 min in DMEM without serum containing 5 mM methionine and 0.1% bovine serum albumin; subjected to four rounds of cell surface biotinylation with 0.5 mg/ml sulfo-NHS-biotin or sulfo-NHS-LC-biotin (with similar results) in PBS; quenched; and subjected to sequential immunoprecipitation and collection of biotinylated proteins on streptavidin-coated agarose beads and fluorography (hexane/isopropyl ether/acetic acid 65:35:2). The cholesterol was visualized with iodine vapor and scraped from the plate, and quantitated in a scintillation counter.

Determination of [3H]cholesterol (47), for growth overnight before the experiment. The values of [3H]cholesterol-bound PLAP were normalized for the total amount of cell protein (Fig. 1, circles). As expected, more DRM protein was recovered at lower detergent:protein ratios. Recovery appeared to plateau at detergent:protein ratios greater than 5 or 6. Slightly less DRM protein was recovered from GM-95 than MEB-4 cells at each detergent:protein ratio. We repeated the experiment, lysing MEB-4 and GM-95 cells at a detergent:protein ratio of 10, after determining protein concentration from a dish grown in parallel. As shown in Fig. 1 (squares), we recovered 0.18 ± 0.04% of total cellular protein in DRMs from MEB-4 cells and 0.09 ± 0.02% from GM-95 (n = 4, ± S.D.). In further experiments, we prepared DRMs from cells lysed at known detergent:protein ratios.

Similar MEB-4 and GM-95 DRM Protein Profiles—Although similar amounts of DRM protein were isolated from the two cell types, it was possible that some proteins might require GSL for DRM association. To test this idea, we examined the protein profile of DRMs from the two cell types. Cells were labeled to steady state with [35S]methionine and extracted at detergent:protein ratios of 2.5:1, 5:1, and 10:1. DRMs prepared from the lysates were subjected to SDS-PAGE and proteins detected by fluorography (Fig. 2). DRMs from the two cell types had very similar protein profiles, showing that most proteins do not require glycosphingolipids to associate with DRMs. Consistent with differences in overall DRM protein recovery (Fig. 1), more protein was recovered from each cell type at a detergent:protein ratio of 2.5:1, whereas recovery was similar at ratios of 5:1 and 10:1. However, the differences were more quantitative.
than qualitative; similar protein profiles were seen under all conditions, but more of each protein was recovered when less Triton X-100 was used.

**DRM Marker Proteins Are Equally Abundant in MEB-4 and GM-95 DRMs**—Because cytoskeletal contaminants can be prominent DRM proteins (43), it was important to examine individual proteins known to associate specifically with DRMs (51, 52). We first chose three endogenous proteins, the Src family kinase Yes and the heterotrimeric G proteins Go and Ga. 5 µg of DRM protein from cells extracted at detergent: protein ratios of 3, 5, or 9 were subjected to SDS-PAGE and transferred to nitrocellulose, and Yes, Go, and Ga were detected by Western blotting (Fig. 3A). All three proteins were present at similar levels in DRMs from the two cell types. We next examined DRM association of a GPI-anchored protein in the two cell lines, using cells stably expressing PLAP. PLAP was detected by Western blotting and ECL. For each protein, all panels were from the same exposure of the blot. B, MEB-4 (top) or GM-95 (bottom) cells expressing PLAP in one 10-cm dish were extracted at a Triton X-100: protein ratio of 10:1 and subjected to sucrose gradient ultracentrifugation. The gradient was fractionated from the bottom (left side of figure), and aliquots of each fraction were subjected to SDS-PAGE, transfer to nitrocellulose, and Western blotting for PLAP. Fractions containing the lysate and DRMs are indicated. C, Triton X-100 whole cell lysates of MEB-4 (lane 1), GM-95 (lane 2), B16-F1 (lane 3), B16-F10 (lane 4), or NIH 3T3 (lane 5) cells containing 43 g of protein were subjected to SDS-PAGE and Western blotting, probing for caveolin (arrow).
averages of two experiments that diverged by a maximum of 0.01.

(b) Values are the somes of the indicated lipid composition or in MEB-4 or GM-95 DRMs after extraction at a Triton X-100:protein ratio of 10. Values are the averages of two experiments that diverged by a maximum of 0.01.

| Lipid          | MEB-4 | GM-95 |
|----------------|-------|-------|
| Cholesterol    | 6.2 ± 0.9 | 6.1 ± 1.1 |
| PC             | 1.6 ± 0.1 | 1.3 ± 0.2 |
| PE             | 0.5 ± 0.2 | 0.4 ± 0.2 |
| Sphingomyelin   | 0.9 ± 0.2 | 2.8 ± 0.9 |
| Cerebrosides    | 0.7 ± 0.2 | ND      |
| GM3            | 0.8 ± 0.1 | ND      |
| Total SPL      | 2.4 ± 0.4 | 2.8 ± 0.9 |
| Total DRM lipid| 10.8 ± 1.6 | 10.6 ± 2.3 |

MG-95 values for GM-95 are shown after MBCD. No reproducible loss of any other lipid species was observed after MBCD treatment. Averages and range of obtained values are shown (error bars; MEB-4 on the left, and GM-95 on the right); each point is the average of 2–6 trials.
examined the effect of cholesterol depletion with MBCD on transport of PLAP in MEB-4 and GM-95 cells. Treatment with 10 mM MBCD for 45 min, to remove about 60% of the total cholesterol (Fig. 4), did not affect transport in either cell line (Fig. 6, A, panels 2 and 4, and B, open circles and squares).

We did not incubate cells with an inhibitor of cholesterol synthesis during the assay, and we cannot exclude the possibility that newly synthesized cholesterol rescued a transport defect in GM-95. However, cholesterol synthesis is almost completely repressed in cells grown in serum-containing medium (65). We found that cholesterol synthesis was induced less than 2-fold from this low repressed level following MBCD removal under the conditions of our delivery assay (Fig. 6C), suggesting that newly synthesized cholesterol did not contribute significantly to cellular cholesterol pools. Similarly, others have shown that cellular cholesterol levels do not increase appreciably in a 2-h incubation following MBCD removal (11).

**DISCUSSION**

DRMs from MEB-4 and GM-95 cells were very similar in protein and lipid yield, protein composition, membrane fluidity, and response to cholesterol removal with MBCD. DRM lipid composition was also very similar in the two cell lines, except for the loss of GSL and a corresponding increase in sphingomyelin in GM-95 DRMs. We conclude that GSL headgroup interactions are not required for raft formation.

**Cholesterol and Membrane Phase Behavior**—Before starting this study, one reason to suspect different behavior of MEB-4 and GM-95 DRMs was the very high \( T_m \) of many GSL. Our data have shown that \( T_m \) is an important determinant of whether lipids will associate with rafts (25, 27, 29), so the high \( T_m \) of GSL might be expected to affect the properties of rafts and DRMs. If so, this should have been especially evident in the DPH fluorescence polarization at 50 °C, above the \( T_m \) of sphingomyelin. However, this parameter was very similar in the two cell types (Table II). This similarity can be explained by the profound effect of cholesterol on membrane phase behavior. Cholesterol can markedly enhance both detergent insolubility (27) and phase separation (29, 56) of order-preferring lipids, in a crude sense effectively raising their \( T_m \). Thus, the effect of cholesterol on the temperature dependence of DRM acyl chain order was much greater than that of any difference between the \( T_m \) of pure GSL and sphingomyelin. This phenomenon was previously observed in phospholipid-sphingolipid-cholesterol model membranes (29). The phase behavior of these membranes was the same whether the sphingolipid consisted of a mixture of sphingomyelin and cerebrosides or was entirely sphingomyelin.

**Glycosphingolipids Are Not Essential for Raft Formation**

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**Sphingolipid Levels and Membrane Phase Behavior**—Sphingomyelin expression is up-regulated in GM-95 cells, so that the total sphingolipid levels in GM-95 and MEB-4 cells (35) and DRMs prepared from them (our data) are very similar. This similarity supports the idea that maintaining the correct plasma membrane phase behavior is important to cells and is an important function of sphingolipids and sterols. Studies in yeast also support this idea; the lethality of sphingolipid deficiency can be suppressed by novel phospholipids that contain unusually long, saturated acyl chains (66).

**DRM Protein Yield**—The major difference between MEB-4 and GM-95 DRMs (aside from the sphingolipid composition) was the 2-fold higher yield of protein in MEB-4 DRMs. We do not know the basis of this difference. Some proteins may have a slightly higher affinity for DRMs that contain GSL, although we did not see significant differences in overall DRM protein composition or enrichment of specific DRM marker proteins in MEB-4 DRMs. Alternatively, it is known that cytoskeletal proteins can associate with DRMs, possibly as contaminants (43).

![Fig. 5](image_url) **Effect of cholesterol removal on DRM association of \([3H]cholesterol in MEB-4 and GM-95 cells.** MEB-4 or GM-95 cells stably expressing PLAP (one 10-cm dish for each time point) were treated with MBCD for 10, 30, or 60 min or left untreated as indicated. After extraction of cells at a Triton X-100:protein ratio of 10:1, lysates were subjected to sucrose gradient ultracentrifugation. 1-ml fractions were collected from the bottom (left side of figure). Aliquots of each fraction were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by Western blotting for PLAP.
MEB-4 and GM-95 cells have markedly different morphologies (33), presumably reflecting differences in cytoskeletal organization. At least part of the difference in DRM protein recovery might simply reflect variability in nonspecific adherence of cytoskeleton to DRMs.

**Effect of Detergent:Protein Ratio on DRM Protein Yield**—We recovered more protein in DRMs as the detergent:protein ratio was lowered (Fig. 1). This may simply mean the low detergent concentrations were insufficient to solubilize bulk nonraft-associated membrane. However, the selectivity of the increase in DRM protein at low detergent concentrations argues against this interpretation. Instead of seeing a more complex DRM protein profile at low detergent concentrations (reflecting increasing amounts of contaminants), we saw better recovery of proteins that were also prominent in high detergent DRMs (Fig. 2). We observed similar behavior of the DRM-associated neuronal protein GAP-43 (28). These results suggest that detergent may partially solubilize rafts, and this effect may be more pronounced with more detergent. In agreement with this idea, detergent can sometimes partially solubilize liquid phase lipids in two phase model membranes containing liquid disordered domains (27). We conclude that detergent insolubility can demonstrate qualitatively that a protein or lipid has a high affinity for rafts. However, detergent insolubility may not be a reliable quantitative measure of the in vivo raft association of proteins or lipids.

**Rafts and Intracellular Sorting**—The raft model for sorting postulates that organization of proteins and lipids into rafts in the trans-Golgi network facilitates their sorting into a special class of transport vesicles in polarized and nonpolarized cells (1, 22). Because PLAP was transported to the cell surface as rapidly in GM-95 as in MEB-4 cells, efficient packaging of this protein into the correct transport vesicles does not seem to require GSL.

In contrast with results of Keller and Simons on influenza hemagglutinin transport (13), we found that cholesterol depletion with MBCD did not slow cell surface transport of PLAP. This discrepancy could be explained by a differential effect of MBCD on raft association of GPI-anchored and transmembrane proteins. Sheets et al. (11) showed that two molecules that are anchored in the outer leaflet of the bilayer (Thy1, a GPI-anchored protein, and the ganglioside GD1b) associated with DRMs even after MBCD treatment. (Similarly, PLAP still associated with DRMs after depletion of cholesterol by inhibition of synthesis (67) and by MBCD treatment as shown in this study.) In contrast, the Src family kinase Lyn (which associates with the inner leaflet) and the high affinity immunoglobulin E receptor, a transmembrane protein that is normally recruited to rafts when clustered, no longer associated with DRMs after depletion of cholesterol by inhibition of synthesis (77) and by MBCD treatment as shown in this study.) In contrast, the Src family kinase Lyn (which associates with the inner leaflet) and the high affinity immunoglobulin E receptor, a transmembrane protein that is normally recruited to rafts when clustered, no longer associated with DRMs after depletion of cholesterol by inhibition of synthesis (77) and by MBCD treatment as shown in this study.) 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