Sphingolipid-enriched Membrane Domains from Rat Cerebellar Granule Cells Differentiated in Culture

A COMPOSITIONAL STUDY*

(Received for publication, November 8, 1999, and in revised form, February 3, 2000)

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Sphingolipid-enriched membrane domains, characterized by a particular protein and lipid composition, have been detected in a variety of cells. However, limited data are available concerning these domains in neuronal cells. We analyzed the lipid and protein composition of a sphingolipid-enriched membrane fraction prepared from primary rat cerebellar granule cells differentiated in culture. Although the protein content of this fraction was only 1.4% of total cellular protein, 60% of the gangliosides, 67% of the sphingomyelin, 50% of the ceramide, and 40% of the cholesterol were located in this fraction. The protein pattern of the sphingolipid-enriched domain fraction was dramatically different from that associated with the cell homogenate. This fraction contained 25% of the tyrosine-phosphorylated proteins and was enriched in two proteins with apparent molecular masses of 135 and 15 kDa. 12% of cellular glycerophospholipids were located in the fraction, with phosphatidylcholine having the highest enrichment. The molar ratio between proteins, glycerophospholipids, cholesterol, sphingomyelin, ceramide and gangliosides in cerebellar granule cells was 1.6:41.6:6.1:1.3:0.3:1 in the cell homogenate and 0.04:8.3:4.0:1.4:0.2:1 in the sphingolipid-enriched membrane fraction. These data indicate that selected proteins segregate with sphingolipids in specialized domains in the membrane of cultured neurons.

The increasing body of evidence, obtained by several different experimental approaches from both artificial and cellular models (1–23), suggests that lipid and protein components in the cell membrane are not randomly or homogeneously distributed but rather organized in domains with peculiar physicochemical and functional properties, different from those of the surrounding membrane environment, confirming the original prediction of Singer and Nicholson (24).

Sphingolipid-enriched domains that are reported to be enriched in gangliosides, sphingomyelin, and cholesterol (17–23) are emerging as membrane compartments with relevant biological functions. They are rich in proteins involved in the mechanisms of signal transduction (1, 18–23, 25–31) and cell adhesion molecules (34). Thus, sphingolipid-enriched microdomains could represent a site within the plasma membrane where different molecules (both lipids and proteins) involved in signal transduction and/or cell adhesion and cell-cell interactions are specifically sorted and concentrated, allowing reciprocal interactions of functional significance. Recent studies have revealed that gangliosides in membrane sphingolipid-enriched domains associate closely and specifically with single or multiple signal transducer molecules. Ganglioside GM3 is closely associated with c-Src, Rho, FAK, and Ras in B16 melanoma cells (1, 21), with c-Src and Csk in neuroblastoma Neuro2a cells (22), and GD3 is associated with Src-family kinase Lyn and the neural cell adhesion molecule TAG-1 in rat brain (33, 35). Such structural units seem to be involved in signal transduction in response to glycosphingolipid-mediated stimulation; GM3-mediated cell adhesion of melanoma B16 cells induces c-Src and FAK phosphorylation and Rho and Ras activation (1); treatment of neuroblastoma Neuro2a cells with exogenous gangliosides induces c-Src and mitogen-activated protein kinase activation, leading to neuronal differentiation (22); and treatment of primary cultured rat cerebellar neurons with anti-GD3 antibody induces Lyn activation with consequent phosphorylation of mitogen-activated protein kinases (33). Neurotrophin-induced p75NTR-dependent sphingomyelin hydrolysis is also localized in a caveolar domain (27).

Procedures for the isolation of sphingolipid-enriched domains have been developed (1–3, 36). They are generally based on the characteristic low density of the domains, which allows their separation by density gradient centrifugation after cell disruption in the presence of detergent (Triton X-100), in hypotonic conditions or by mechanical procedures. Despite the variety of density gradient steps and of cell lysis procedures, procedures for the isolation of sphingolipid-enriched domains have been developed (1–3, 36). They are generally based on the characteristic low density of the domains, which allows their separation by density gradient centrifugation after cell disruption in the presence of detergent (Triton X-100), in hypotonic conditions or by mechanical procedures. Despite the variety of density gradient steps and of cell lysis procedures, procedures for the isolation of sphingolipid-enriched domains have been developed (1–3, 36). 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results obtained with different methods are similar and lead to similar conclusions (1, 3).

Most data concerning the structure and functional role of sphingolipid-enriched domains have been obtained from studies performed on non-neuronal cells such as Madin-Darby canine kidney cells (17, 22, 34). The lack of information on sphingolipid-enriched domains in the nervous system is mainly due to the complexity of this tissue at the cellular and compositional level. Moreover, the need to utilize primary cultures for neurons implies difficult work to collect enough cells for the analytical procedures.

With this investigation we studied the composition of sphingolipid-enriched microdomains obtained from rat cerebellar granule cells differentiated in culture. To overcome the analytical problems related to the relatively limited number of cells, we radiolabeled the cellular lipids and proteins using appropriate radioactive precursors, namely [3H]sphingosine, [32P]orthophosphate, and [35S]methionine.

EXPERIMENTAL PROCEDURES

Materials

Commercial chemicals were the purest available, common solvents were distilled before use, and water was doubly distilled in a glass apparatus. Trypsin, crystalline bovine serum albumin, and all the reagents for cell culture were from basal modified Eagle’s medium and fetal calf serum, which were purchased from Flow Laboratories. Anti-phosphotyrosine mouse monoclonal IgG2b antibody and protein A/G PLUS-agarose were from Santa Cruz Biotechnology. Sphingosine was prepared from cerebroside (37). Sphingolipids and glycolipids to be used as standards were extracted from rat brain, purified, and characterized (38).

Radioactive Compounds

[1-3H]Sphingosine was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium borohydride (39) (radiochemical purity, >98%; specific radioactivity, 2 Ci/mmole). [32P]Orthophosphate (carrier-free) and [35S]methionine corresponded to fraction 5 and was regarded as the sphingolipid-enriched fraction. The bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer and centrifuged (17 h at 200,000 × g) at 4 °C.

Hypertonic Sodium Carbonate Method—Cells were harvested as described above, suspended (8–10 × 10⁶ cells/ml) in 500 mM sodium carbonate, pH 11.0, and homogenized using a loose fitting Dounce homogenizer (20 strokes) and a probe sonicator (three 10-s bursts). 1.5 ml of the cell homogenate thus obtained was mixed with an equal volume of 90% sucrose in MEB, pH 6.5, 150 mM NaCl and overlaid with a discontinuous sucrose gradient (30–5%) in the same buffer containing 250 mM sodium carbonate. Samples were subjected to ultracentrifugation as described above.

Using brain tissue, a discrete light-scattering band under light illumination located at the interface between 5 and 30% sucrose was detectable. After ultracentrifugation, eleven 1-ml fractions were collected starting from the bottom of the tube. The light-scattering band corresponded to fraction 5 and was regarded as the sphingolipid-enriched fraction. The bottom fraction (fraction 11) contained a pellet that was carefully homogenized before analysis. The entire procedure was performed at 0–4 °C in ice immersion.

Analysis of Radiolabeled Protein Patterns

Cell lysates and sucrose gradient fractions obtained after labeling cerebellar granule cells with [32P]orthophosphate or [35S]methionine were analyzed to determine protein content and pattern. In the case of [32P]orthophosphate labeling, samples were extensively dialyzed to remove free [32P]phosphate, and phospholipids were removed by chloroform/methanol extraction (45) before analysis. Similar amounts of proteins, each sample (0.2–1.0 μg) were subjected to SDS-PAGE on 10% acrylamide gels followed by radioactivity detection.

Phosphotyrosine-containing proteins were identified by immunoprecipitation. Aliquots of each fraction obtained from cells labeled with [32P]orthophosphate or [35S]methionine (containing ~15–30 μg of protein) were diluted 10-fold in immunoprecipitation buffer (90 mM Tris-Cl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 5 mM phenylmethylsulfonyl fluoride, 75 milliunits/ml aprotinin, 1% Triton X-100), mixed with protein A/G-Sepharose beads (40 μl packed), and stirred on a rotary stirrer overnight at 4 °C to preclude nonspecific binding. After centrifugation (270 × g for 2 min), 2 μg/ml mouse anti-phosphotyrosine monoclonal IgG2a, or 2 μg/ml normal mouse IgG (as negative control) was added to the supernatants. The mixtures were placed on a rotary stirrer overnight at 4 °C. Immunoprecipitates were recovered by adding protein A/G-Sepharose beads (40 μl packed) and mixing for 2 h. Beads were washed three times with immunoprecipitation buffer, recovered by brief weak centrifugation (270 × g for 2 min), suspended in 50 μl of SDS-sample buffer, heated to 95 °C for 3 min, and centrifuged (1000 × g for 2 min). Supernatants were subjected to SDS-PAGE, and dried gels were analyzed to determine the radioactive protein patterns. The radioactivity associated with immunoprecipitates was quantitatively determined by liquid scintillation counting. The total amount of immunoprecipitable radioactivity was calculated for each fraction and for the cell lysate. Data were expressed for each fraction as the percentages of total immunoprecipitable radioactivity present in the amount of cell

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lysateloaded ongradient. Radioactivity associated with negative controls never exceeded 5% of radioactivity found in immunoprecipitates.

**Analysis of Radioactive Lipids**

The cell lysate, postnuclear supernatant, and sucrose gradient fractions obtained after cell metabolic radiolabeling were analyzed to determine the content of radiolabeled lipids. Samples were dialyzed for 4 days against distilled and decarbonated water (changed two times a day). We found that a long dialysis of samples before lipid extraction is necessary for effective removal of sucrose and that the removal of sucrose is critical for the quality of lipid extraction and analysis. After dialysis, samples were lyophilized, and lipids were extracted twice with 0.5 ml of chloroform/methanol, 2:1 (v/v) (45). The total lipid extract was not subjected to partitioning to avoid any possible loss of material. Aliquots of the lipid extract were analyzed by HPTLC as described below, followed by radioactivity imaging for quantification of radioactivity.

Identity of lipids separated by HPTLC was assessed by co-migration with standard lipids and confirmed by susceptibility of compounds to the following enzymatic and chemical treatments. A sample of the lipid extract was treated at 37 °C for 2 h in 50 μl of water in the presence of 1 milliunit of *Vibrio cholera* sialidase to yield GM1. Sphingomyelin and phospha tidylethanolamine were purified according to the HPTLC blotting technique previously reported (46); they were separated by HPTLC, identified by spraying with primulin, blotted to polyvinylidene difluoride membrane where the corresponding bands were cut, and HPTLC, identified by spraying with primulin, blotted to polyvinylidene.

**Thin Layer Chromatography**

- **3H-Labeled lipids** were separated by monodimensional HPTLC carried out with the solvent systems chloroform/methanol/0.2% aqueous CaCl₂, 50:42:11 or 55:45:10 (v/v/v). **32P-Labeled lipids** were separated by mono- or bidimensional HPTLC using the following solvent systems and conditions. For monodimensional HPTLC, chloroform/methanol/ acetic acid/water, 30:20:2:1 (v/v/v/v) and chloroform/acetic acid/ water, 40:15:13:12.8 (v/v/v/v) were used to specifically analyze the content of phosphatidylinositol phosphates. The separation was carried out on HPTLC plates that were previously impregnated with a 1.3% solution of potassium oxalate in methanol/water, 2:3 (v/v), dried at room temperature, and activated at 110 °C for 15 min. For bidimensional HPTLC, the first run, chloroform/methanol/acetic acid/water, 30:20: 2:1 (v/v/v/v) was used; after the run plates were dried and exposed to HCl fumes for 15 min, they were dried again. For the second run, chloroform/methanol/acetic acid/water, 10:2.4:2:1 (v/v/v/v) was used; this bidimensional HPTLC system allowed the analysis of plasmalogens in the glycerophospholipid mixture. Also for bidimensional HPTLC, for a first run, chloroform/methanol/acetic acid/water, 30:20: 2:1 (v/v/v/v) was used. For the second run chloroform/methanol/water/32% NH₄OH, 48:40:7:5 (v/v/v/v) was used. Cholesterol was separated by monodimensional HPTLC using the solvent system hexane/diethyl ether/acetic acid, 80:20:1 (v/v/v).

**Other Analytical Methods**

Cholesterol was quantitated after separation on HPTLC followed by visualization with 15% concentrated sulfuric acid in 1-butanol. The quantity of cholesterol was determined by densitometry and comparison with known amounts of standard cholesterol using the Molecular Analyst program (Bio-Rad Laboratories). The protein content was determined according to Lowry (47), with the micro BCA assays (Pierce) and as dot spot revealed by Coomassie Blue staining; bovine serum albumin in the presence of sucrose was the reference standard.

The radioactivity associated with cells, with cell fractions, with lipids, and with delipidized pellets was determined by liquid scintillation counting. Digital autoradiography of the HPTLC plates and of the SDS-PAGE gels was performed with a Beta-Imager 2000 instrument (Biospace, Paris, France) using an acquisition time of about 24 h. The radioactivity associated with individual lipids and proteins was determined with the specific β-Vision software provided by Biospace. Autoradiography of 32P- and 35S-labeled proteins was carried out using Kodak Biomax MR and MS films.

**RESULTS**

**Proteins—Feeding 32P-orthophosphate and 35S-methionine to cells** yielded an incorporation of radioactivity into proteins of 5.20 ± 0.51 × 10⁶ cpm/mg cell protein and 44.9 ± 3.60 × 10⁶ cpm/mg cell protein in the cell lysate obtained in the presence of Triton X-100, respectively. After discontinuous sucrose gradient centrifugation, fractions were collected from the top of the gradient (gradient fraction 1) to the bottom (fraction 11). The distribution of protein-associated radioactivity into the eleven fractions is reported in Fig. 1. The distribution pattern of 32P- and 35S-labeled proteins was very similar. Fraction 5 contained only 1.4 ± 0.4% of the total protein-associated radioactivity, which is predominantly (>70%) recovered in fractions 10 and 11. The experimental conditions we used for protein labeling were reported to yield a similar specific radioactivity for most proteins (43). The total protein content of the postnuclear fraction subjected to fractionation in a typical experiment was 4.7 mg. Thus, on the basis of the distribution of radioactivity, we calculated that fraction 5 contained 65 μg of protein. In preliminary experiments performed with standard albumin, we found that sucrose and other components of the gradient buffer interfered with the Lowry, Coomassie Blue, and BCA protein assays. For this reason, the use of a radiolabeling procedure seems to be highly preferable for the precise determination of protein content in fractions prepared by sucrose gradient centrifugation. Fig. 2 shows the patterns of 35S-methionine-labeled proteins in gradient fractions and cell homogenate after separation by SDS-PAGE and autoradiography. The protein pattern of fraction 5 was much different from that of heavier fractions. The main protein present in fraction 5 had an apparent molecular mass of about 135 kDa; a well isolated protein band at about 15 kDa could be also observed. Fractions 6, 7, and 8 contained only trace amounts of proteins; they contained as the main component the protein of about 135 kDa found in...
fraction 5 and were also enriched in a protein of about 46 kDa. Fig. 3 shows the distribution of phosphotyrosine-containing proteins in the eleven fractions as determined by immunoprecipitation with anti-phosphotyrosine monoclonal antibody. Similar amounts of proteins from fractions 5, 9, 10, and 11 and homogenate were subjected to immunoprecipitation with anti-phosphotyrosine mAb as described under "Experimental Procedures." Proteins associated with the immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography. Fractions were collected as described in the legend of Fig. 1. Patterns are representative of those obtained in two different experiments.

**Fig. 3.** Distribution of phosphotyrosine-containing proteins in sucrose gradient fractions from cerebellar granule cells differentiated in culture. Relative amounts of radioactivity associated with each gradient fraction after labeling with [35S]methionine (lower panel) or with [32P]orthophosphate (upper panel) were calculated for each fraction, and data were expressed as percentages of total immunoprecipitable radioactivity present in the homogenate. Fractions were collected as described in the legend of Fig. 1. Data are the means of three different experiments, with the S.D. never exceeding 15% of the mean values.

The radioactivity associated to gangliosides GM1, GD3, GD1a, GD1b, O-Ac-GT1b, GT1b, and GQ1b was 9, 5, 26, 12, 13, 35, and 2%, respectively, of the total radioactivity associated to all gangliosides. This distribution is in very good agreement with the ganglioside pattern of granule cells previously reported (49, 50). Nevertheless, the chosen pulse-chase feeding conditions did not allow introduction of a similar specific radioactivity in ceramide, sphingomyelin, and gangliosides, because of different turnover of each sphingolipid class (49, 50, 53, 54). The radioactivity ratio between gangliosides and ceramide was about 4-fold higher than the mass ratio, and the radioactivity ratio between ganglioside and sphingomyelin was about 3-fold higher than the mass ratio (Table I).

**Fig. 4.** Patterns of phosphotyrosine-containing proteins in sucrose gradient fractions from cerebellar granule cells differentiated in culture after labeling with [35S]methionine. Equal amounts of proteins from fractions 5, 9, 10, and 11 and homogenate were subjected to immunoprecipitation with anti-phosphotyrosine mAb (right panel) or with non-immune mouse IgG as negative control (left panel) as described under "Experimental Procedures." Proteins associated with the immunoprecipitates were separated by SDS-PAGE and visualized by autoradiography. Fractions were collected as described in the legend of Fig. 1. Patterns are representative of those obtained in two different experiments.

**Fig. 2.** Protein patterns in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture after labeling with [35S]methionine. Similar amounts of protein from gradient fractions 4–11 and from the homogenate (lane Hom) were analyzed by SDS-PAGE followed by autoradiography (1000 cpm/lane; time of exposure, 15 days). Fractions were collected as described in the legend of Fig. 1. Patterns are representative of those obtained in two different experiments.

**Fig. 5.** Distribution of radioactive lipids extracted from the cell homogenate after HPTLC separation. Radioactive ceramide, GlcCer, LacCer, sphingomyelin, GM1, GD3, GD1a, GD1b, O-Ac-GT1b, and GQ1b were identified. Radioactive PE was also identified in the lipid extract. The presence of radioactive PE is due to the recycling of the radioactive ethanolamine formed in the catabolism of [1-3H]sphingosine (42). Fig. 6 shows the distribution of radioactivity incorporated into ceramide, sphingomyelin, glycosphingolipids (including neutral sphingolipids and gangliosides), and PE in each sucrose gradient fraction prepared by the Triton X-100 method. All radioactive sphingolipids were largely associated with fraction 5 (50–65% of the radioactivity associated with sphingolipids in the cell homogenate), minor quantities being distributed into fractions 4 and 6–11. In particular, less than 25% of radioactive ceramides and less than 10% of radioactive complex sphingolipids was present in fraction 11, which contained about 60% of cell proteins. Radioactive PE, on the other hand, was predominantly recovered in fractions 9–11, and only a low amount was detectable in fraction 5. Very similar results were obtained analyzing sucrose gradient fractions prepared from [1-3H] sphingosine-fed cells after lysis in hypertonic conditions (500 mM sodium carbonate), i.e. the majority of radioactive sphingo-
Sphingolipid-enriched Domains

Data on the mass sphingolipid and glycerophospholipid contents are from Refs. 49–51, 53, and 54. By these data and by the radioactivity contents in fraction 5 (F 5) and in homogenate (Hom), we calculated the mass content of each class in fraction 5 and molar ratios of proteins and different lipids in fraction 5 and in homogenate. Data on cholesterol have been determined in this study. Molar ratios were normalized to total ganglioside content (nmol of component/nmole of gangliosides in that fraction). Protein radioactivity is from [35S]labeling (as cpm). Sphingolipid radioactivity is from [3H] labeling (as dpm). Glycerophospholipid radioactivity is from [32P] labeling (as cpm). Protein molar content was calculated on the average of the protein molecular mass and must considered an approximate value.

![Fig. 5. Pattern of radioactive lipids from rat cerebellar granule cells differentiated in culture after feeding [1-3H]sphingosine](Image)

**Table I**

Lipid and protein contents in rat cerebellar granule cells differentiated in culture

|                | Hom | F5   | Molar ratios normalized to gangliosides |
|----------------|-----|------|----------------------------------------|
|                | nmol/10⁶ cells | dcpm/10⁶ cells | molar content (nmol/10⁶ cells) | % on Hom, as dcpm | nmol/10⁶ cells |
| Proteins       | 1.25 | 3,420,000 | 58,333 | 1.7 | 0.02 | 1.58 | 0.04 |
| Ceramide       | 0.22 | 576 | 287 | 49.8 | 0.11 | 0.28 | 0.23 |
| Sphingomyelin  | 1.00 | 3,593 | 2,399 | 66.8 | 0.67 | 1.27 | 1.41 |
| Gangliosides   | 0.79 | 8,846 | 5,297 | 59.9 | 0.47 | 1.00 | 1.00 |
| GM1            | 0.06 | 806 | 475 | 58.9 | 0.04 | 0.08 | 0.08 |
| GD3            | 0.04 | 506 | 299 | 59.1 | 0.02 | 0.06 | 0.06 |
| GD1a           | 0.19 | 2,340 | 1,354 | 59.1 | 0.11 | 0.24 | 0.24 |
| GD1b           | 0.09 | 1,085 | 637 | 58.7 | 0.05 | 0.13 | 0.13 |
| O-Ac-GT1b      | 0.10 | 1,086 | 611 | 56.3 | 0.06 | 0.13 | 0.12 |
| GT1b           | 0.28 | 2,884 | 1,809 | 62.7 | 0.17 | 0.35 | 0.37 |
| GQ1b           | 0.02 | 140 | 80 | 57.1 | 0.02 | 0.02 | 0.02 |
| Glycerophospholipids | 32.83 | 70,500 | 7,830 | 12.0 | 3.95 | 41.56 | 8.31 |
| PE             | 6.64 | 6,500 | 357 | 5.5 | 0.55 | 8.41 | 7.75 |
| PS             | 2.59 | 2,833 | 195 | 6.9 | 0.18 | 3.28 | 3.77 |
| PC             | 16.41 | 15,833 | 3,512 | 21.9 | 3.51 | 20.77 | 7.36 |
| PPC            | 0.35 | 13,167 | 237 | 1.8 | 0.01 | 0.44 | 0.01 |
| PI             | 1.22 | 20,333 | 840 | 4.2 | 0.05 | 1.53 | 0.11 |
| PIP            | 0.19 | 8,657 | 274 | 3.2 | 0.01 | 0.30 | 0.03 |
| PIP₂           | 0.24 | 8,167 | 181 | 5.7 | 0.01 | 0.30 | 0.03 |
| Cholesterol    | 4.80 | | | | | |

**Fig. 6** shows the distribution of each radioactive glycerophospholipid into fractions prepared by sucrose gradient centrifugation. The majority of radioactivity (70–95%) for all radioactive glycerophospholipid species was largely recovered in fractions 9–11. Fraction 5 contained less than 10% of the radioactive glycerophospholipid species were dramatically different from each other even within the same fraction. In fraction 5, radioactive phospholipid species were dramatically different from each other even within the same fraction. Fraction 5, radioactive PC and PPC showed the highest (13.2) and lowest enrichment (1.1), respectively.

**Quantitative Determination of Endogenous Sphingolipids and Glycerophospholipids**—The experimental conditions used [32P]orthophosphate, was 0.94 ± 0.05 × 10⁶ cpm/mg protein in the cell lysate obtained in the presence of Triton X-100. Each glycerophospholipid has specific turnover because of different and multiple biosynthetic pathways. The experimental conditions used allowed the introduction of radioactivity in the majority of species (Table I), thus making it possible to also follow those species that are minor or very minor cell components, like PI, PIP, and PIP₂ (52). Fig. 7 shows the patterns of [32P]-labeled lipids from the cell homogenate and gradient fractions after HPTLC separation. Radioactive PE, PS, PC, PI, PPC, PIP, and PIP₂ were identified. Phosphatidylethanolamine plasmalogen and the phosphosphingolipid sphingomyelin were present in trace amounts. The radioactive glycerophospholipid patterns in the sucrose gradient fractions were remarkably different (Fig. 7). Fig. 8 shows the distribution of each radioactive glycerophospholipid into fractions prepared by sucrose gradient centrifugation. The majority of radioactivity (70–95%) for all radioactive glycerophospholipid species was largely recovered in fractions 9–11. Fraction 5 contained less than 10% of the radioactivity associated with each glycerophospholipid, with the exception of PC: about 22% of radioactivity associated with PC was detected in fraction 5. The case of PE, the distribution of radioactivity in the fractions was remarkably similar to that determined after labeling with [1-3H]sphingosine (Fig. 6). The enrichment of single radioactive glycerophospholipids in each fraction (calculated as described above for sphingolipids) is reported in Table II. The highest values were associated with fraction 9, whereas enrichments in fraction 5 were modest. However, enrichments for the different radioactive glycerophospholipid species were dramatically different from each other even within the same fraction. In fraction 5, radioactive PC and PPC showed the highest (13.2) and lowest enrichment (1.1), respectively.

**Enrichment** = \(\frac{\text{dpm in fraction}}{\text{dpm in homogenate}} \times \frac{\text{proteins in homogenate}}{\text{proteins in fraction}}\) (Eq. 1)

All radioactive sphingolipids were highly enriched in fraction 5 (36.6), whereas enrichments calculated for the high density fractions were much lower (<8.5 in fraction 9, ≥1 in fraction 10, and <0.5 in fraction 11). Within each fraction, all of them showed a very similar enrichment (ranging from 29.9 for ceramide to 40.2 for radioactive sphingomyelin, in the case of fraction 5).

**Glycerophospholipids**—Incorporation of radioactivity into cellular glycerophospholipids, by metabolic labeling with
for cell lipid metabolic radiolabeling were designed to incorporate a significant amount of radioactivity in each lipid, allowing the detection and analysis of all species, including the minor components. However, because of the different turnover of each species, it is impossible to achieve the same specific radioactivity for each component. Under our experimental conditions, only gangliosides incorporated radioactive sphingosine proportionally to their endogenous content (Table I). Thus, the percentage of distribution of radioactivity between the $^3$H- or $^{32}$P-labeled lipids within each fraction does not reflect the mass lipid composition in the fraction. This has been calculated on the basis of the endogenous lipid content in the homogenate and the distribution of radioactivity in each lipid within the gradient (Table I). Quantitative data on the total ganglioside, sphingomyelin, ceramide, and glycerophospholipid contents in rat cerebellar granule cells differentiated in culture previously reported (49–51, 53, 54) were used for the calculation. The data we calculated are reported (as molar ratios) in Table I. Comparison of the lipid molar ratio between fraction 5 and the homogenate clearly shows the great difference in their relative composition. In fact, the molar ratio between glycerophospholipids and gangliosides is about 42:1 in the homogenate, and it is reduced to about 8:1 in fraction 5.

**Cholesterol**—The content of cholesterol in rat cerebellar granule cells differentiated in culture was $64 \pm 9$ nmol/mg of protein in the cell homogenate. In this range of cholesterol concentration, S.D. for the method was $\pm 15\%$. The distribution of cholesterol in fractions prepared by sucrose gradient centrifugation is shown in Fig. 9. About 40% of cellular cholesterol was associated with fraction 5 after sucrose gradient centrifugation. Tables I and II show the relative cholesterol content and the cholesterol enrichment in fraction 5 and in the homogenate, respectively.

**DISCUSSION**

The sphingolipid-enriched membrane fraction prepared by the Triton X-100 method (1–3) contains 50–65% of cellular sphingolipids and 40% of cholesterol but only a minor part of the glycerophospholipids (about 10%). The distribution of sphingolipids within the gradient was identical using the sodium carbonate method. On the basis of the distribution of each lipid within the gradient and of the cellular content of each lipid, we calculated the molar composition of the sphingolipid-enriched domain, i.e., of fraction 5 of the sucrose gradient fractionation, and compared it to that of the homogenate, as reported in Table I. Among the plasma membrane complex lipids, gangliosides are the most characteristic of neuronal cells. Thus, we normalized these data (Table I) to the total ganglioside

**TABLE II**

Relative enrichment of lipid classes and single lipids in sucrose gradient fractions from cerebellar granule cells

| GlyceroPL, glycerophospholipids. Data are the means of four experiments. ND, not detected. |
|---------------------------------|---------|---------|---------|---------|---------|---------|---------|
| GlyceroPL | Ceramide | Sphingo | Gangliosides | GM1 | GD3 | GD1a | GD1b | O-Ac-GT1b | GT1b | GQ1b | PE | PS | PC | PPC | PI | PIP | PI2 | Cholesterol |
| Fraction 4 | Fraction 5 | Fraction 6 | Fraction 7 | Fraction 8 | Fraction 9 | Fraction 10 | Fraction 11 |
|------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Sphingolipids | 24.0 | 36.6 | 21.6 | 15.3 | 17.1 | 5.7 | 0.9 | 0.2 |
| Ceramide | 22.5 | 29.9 | 18.9 | 10.3 | 16.8 | 4.7 | 0.9 | 0.4 |
| Sphingomyelin | 25.4 | 40.2 | 24.2 | 17.7 | 14.2 | 5.4 | 0.5 | 0.1 |
| Gangliosides | 25.0 | 36.3 | 21.1 | 16.8 | 18.7 | 6.5 | 1.0 | 0.1 |
| GM1 | 24.0 | 35.5 | 18.8 | 15.1 | 16.5 | 5.7 | 1.0 | 0.2 |
| GD3 | 22.8 | 35.7 | 21.6 | 15.2 | 16.5 | 5.6 | 1.0 | 0.2 |
| GD1a | 25.4 | 35.6 | 19.5 | 16.0 | 17.4 | 6.4 | 1.1 | 0.2 |
| GD1b | 23.4 | 35.4 | 24.1 | 16.1 | 19.4 | 6.5 | 1.0 | 0.1 |
| O-Ac-GT1b | 27.8 | 34.0 | 19.2 | 14.1 | 17.1 | 7.9 | 1.3 | 0.2 |
| GT1b | 33.2 | 38.0 | 21.1 | 15.0 | 15.4 | 6.8 | 0.8 | 0.1 |
| GQ1b | 29.1 | 34.3 | 26.3 | ND | ND | 8.2 | ND | ND |
| PE | 6.6 | 6.5 | 0.0 | 2.1 | 2.00 | 31.9 | 3.0 | 0.6 |
| PS | ND | 4.1 | ND | ND | ND | 32.0 | 0.7 | 1.2 |
| PC | 23.0 | 13.2 | ND | 7.0 | 7.3 | 31.3 | 4.1 | 0.4 |
| PPC | ND | 1.1 | ND | ND | ND | 5.0 | 1.0 | 1.5 |
| PI | 3.0 | 2.5 | ND | 1.7 | 2.7 | 38.2 | 5.2 | 0.4 |
| PIP | ND | 1.9 | ND | ND | ND | 45.3 | 0.6 | 1.0 |
| PIP2 | ND | 3.4 | ND | ND | ND | 58.8 | 0.4 | 0.5 |
| Cholesterol | 3.7 | 24.0 | 9.5 | 6.0 | 6.7 | 7.8 | 2.2 | 0.4 |
Spingolipid-enriched Domains

**Fig. 7.** Patterns of radioactive phospholipids in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture after metabolic labeling with $^{32}$P orthophosphate. Lipids from different fractions and from the homogenate (lane Hom) were extracted as described under "Experimental Procedures" and separated by HPTLC in a solvent system containing chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:8 (v/v/v/v/v) using potassium oxalate-impregnated HPTLC plates. Radioactive lipids were detected by autoradiography (2000 cpm applied on 3-mm line; time of exposure, 48 h). Fractions were collected as described in the legend of Fig. 1. Patterns are representative of those obtained in two different experiments, with S.D. never exceeding 20% of the mean values.

**Fig. 8.** Distribution of radioactivity associated with different glycerophospholipids in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture after metabolic labeling with $^{32}$P orthophosphate. Radioactive lipids were extracted, separated by HPTLC in a solvent system containing chloroform/acetone/methanol/acetic acid/water, 40:15:13:12:8 (v/v/v/v/v) using potassium oxalate-impregnated HPTLC plates. Radioactive lipids were detected by autoradiography (2000 cpm applied on 3-mm line; time of exposure, 48 h). Data are expressed as percentages of total radioactivity, or with unknown adapter proteins cannot be excluded. Patterns are representative of those obtained in two different experiments. LPC, lyso-phosphatidylcholine.

**Fig. 9.** Distribution of cholesterol in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture. The amount of cholesterol in the homogenate and in gradient fractions was determined after separation by HPTLC as described under "Experimental Procedures." Data are expressed as percentages of total cholesterol present in the homogenate. Fractions were collected as described in the legend of Fig. 1. Data are the means of three different experiments, with S.D. never exceeding 20% of the mean values.

Leading to the formation of lipid domains, independent of the presence of particular scaffolding proteins. Spontaneous segregation of gangliosides and other sphingolipids has been well described in artificial and cellular models (4–14). Clustering of glycosphingolipids in microdomains could provide a favorable membrane geometry to accommodate their large hydrophilic headgroups (55). The possibility of forming hydrogen bonds because of the amide and hydroxyl group of ceramide (56) should further stabilize their clustering. All of these interactions contribute to the formation of a very rigid microenvironment (57), providing a clear explanation for the resistance of sphingolipid-enriched domains to detergent solubilization, extraction with sodium carbonate, or mechanical disruption. Cholesterol is also predominantly recovered in the sphingolipid-enriched fraction prepared from cerebellar granule cells. However, its molar ratio to gangliosides varies from about 6:1 in homogenate to about 4:1 in fraction 5, indicating that it segregates together with sphingolipids, contributing to the formation of a rigid phase, but without representing a major contributor to this phenomenon, as confirmed by studies on model membranes (6, 10). The segregation process of sphingolipids excludes the majority of cellular glycerophospholipids; their molar ratio to gangliosides dramatically decreases from 42:1 in the homogenate to about 8:1 in fraction 5. Interestingly, not all glycerophospholipids are excluded to the same extent from the sphingolipid-enriched domain. In particular, a higher portion of PC (that is the glycerophospholipid bearing the largest headgroup), with respect to other glycerophospholipids segregates together with sphingolipids.

Even more dramatic is the exclusion of proteins from the sphingolipid-enriched domain (a rough protein to ganglioside molar ratio, calculated on the basis of the average protein molecular mass, ranges from about 1.6:1 in the homogenate to 0.04:1 in fraction 5). This strongly indicates that only selected protein molecules are associated with these domains, as confirmed from their particular protein patterns and enrichment in phosphotyrosine containing proteins. Actually, many proteins reported to be present in sphingolipid-enriched domains bear some structural features, such as lipid modifications, GPI anchors, or transmembrane domains, which would explain their segregation with sphingolipids. However, not all proteins bearing these features associate with sphingolipid-enriched domains, suggesting that other elements are relevant in determining their segregation. Specific interactions with lipid components or with unknown adapter proteins cannot be excluded.

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Sphingolipid-enriched Membrane Domains from Rat Cerebellar Granule Cells Differentiated in Culture: A COMPOSITIONAL STUDY
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J. Biol. Chem. 2000, 275:11658-11665.
doi: 10.1074/jbc.275.16.11658

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