Changes in the Lipid Turnover, Composition, and Organization, as Sphingolipid-enriched Membrane Domains, in Rat Cerebellar Granule Cells Developing in Vitro*

Received for publication, November 27, 2000, and in revised form, March 13, 2001
Published, JBC Papers in Press, March 22, 2001, DOI 10.1074/jbc.M010666200

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In the present paper, we report on the properties of sphingolipid-enriched domains of rat cerebellar granule cells in culture at different stages of neuronal development. The major lipid components of these domains were glycerophospholipids and cholesterol. Glycerophospholipids were 45–75% and cholesterol 15–45% of total lipids of the domains. This corresponded to 5–17% of total cell glycerophospholipids and 15–45% of total cell cholesterol. Phosphatidylcholine, mainly dipalmitoylphosphatidylcholine, was 66–85% of all the glycerophospholipids associated with these domains. Consequently, the palmitoyl residue was significantly enriched in the domains. The surface occupied by these structures increased during development. 40–70% of cell sphingolipids segregated in sphingolipid-enriched membrane domains, with the maximum ganglioside density in fully differentiated neurons. A high content of ceramide was found in the domains of aging neurons. Then, the sphingolipid/glycerophospholipid molar ratio was more than doubled during the initial stage of development, whereas the cholesterol/glycerophospholipid molar ratio gradually decreased during in vitro differentiation. Phosphorylated phosphoinositides, which were scant in the domains of undifferentiated cells, dramatically increased during differentiation and aging in culture. Proteins were minor components of the domains (0.1–2.8% of all domain components). Phototyrosine-containing proteins were selectively recovered in the sphingolipid-enriched domain. Among these, Src family protein-tyrosine kinases, known to participate in the process of neuronal differentiation, were associated with the sphingolipid-enriched domains in a way specific for the type of kinase and for the developmental stage of the cell. Proteins belonging to other signaling pathways, such as phosphoinositide 3-kinase and its downstream target, Akt, were not associated with the domains.

Neuronal development involves many different spatially and temporally regulated events (reviewed in Refs. 1–4), including cell migration (which implies changes in the adhesion properties of the neuronal cell and in its interactions with the changing local environment and other cells), and deep morphological and biochemical changes leading to the processes of neuritogenesis and synaptogenesis, and to the complex spatial organization which is typical of the nervous tissue. In all these developmental events, cell surface properties, determined by membrane components, are of primary importance. Lipids are responsible for the physicochemical properties of the membrane itself, largely determining its asymmetry, fluidity and plasticity features, and its organization in domains. Moreover, many membrane lipids play more specific functional roles, and are in many different ways directly involved in the machinery devoted to the control of information in the neuronal cell. Inositol phospholipids (5), sphingolipids (6, 7), and plasmalogens (8) are precursors of bioactive molecules. Glycosphinolipids, asymmetrically located in the outer leaflet of the membrane bilayer, are of primary importance as receptors and cell surface antigens (9). A particular function of lipids at the cell surface is linked to their ability to interact with membrane proteins, such as receptors or ion channels (6, 10, 11), and to modulate the functional activity of the protein itself. Some membrane lipids undergo segregation, creating a lipid phase distinct from the bulk phase of the membrane. Recent findings indicate that a particular set of lipids and proteins are segregated together within the plasma membrane, forming functional units that are involved in signal transduction processes (12, 13). Spontaneous segregation of membrane sphingolipids seems to be the driving force of these structures, hence termed sphingolipid-enriched membrane domains. A functional role for these domains is particularly well documented in neural cells (reviewed in Ref. 14), where the specific interaction of sphingolipids with protein kinases of the Src family is particularly relevant. The nonreceptor protein-tyrosine kinases belonging to this family seem to be involved in signaling pathways that regulate the function of postmitotic, terminally differentiated cells. They regulate multiple cell functions, including cytoskeletal organization, cell-substrate, and cell-cell adhesion, and cell-cell communication (15, 16). In the nervous system, many lines of evidence indicate that they are involved in the process of neuronal differentiation, suggesting a tight link between Src family kinases and neurite extension and guidance. In the developing cerebellum, the expression of c-Src coincides with the onset of neuronal differentiation (17). The expression and activation of c-Src and Lyn are increased during differentiation (18–22), and postmitotic neurons from the central nervous system express high levels of structurally distinct forms of c-Src and Lyn (15, 23). c-Src and Lyn are required in the

* This research was supported by the Cofinanziamento Ministero dell’Università e delle Ricerca Scientifica e Tecnologica Progetti di Interesse Nazionale (MURST PRIN) 1997 (to S. S. and G. T.), Cofinanziamento MURST PRIN 1998 (to V. C.), and by Consiglio Nazionale delle Ricerche (Target project Biotechnology to G. T. and S. S.), Italy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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response to different neural cell adhesion molecules in cerebellar neurons (24, 25), participating in the control of neurite extension. c-Src, Fyn, and Yes are enriched in growth cones of neurons and neuroblastoma cells as a complex with other proteins (26). c-Src and Fyn also have a role in the mature synapse, related to the induction of long-term potentiation (27, 28). A close association between these proteins and sphingolipid-enriched domains has been demonstrated in many cell types, including neurons (29–32). Moreover, several GPT-anchored proteins, which are usually associated with the sphingolipid-enriched domains, have been implicated in signal transduction mediated by Src family tyrosine kinases. In the present paper, we analyze the changes in lipid composition, turnover, and membrane organization in cerebellar neurons at different stages of development in vitro. The data we obtained clearly indicate a strict correlation between the functional status of the neuronal cell and the level of lipid and protein organization within sphingolipid-enriched membrane domains.

EXPERIMENTAL PROCEDURES

Materials—Commercial chemicals were the purest available, commercial reagents were purchased from Sigma Chemical Co., except for basal modified Eagle’s medium and fetal calf serum, which were purchased from Flow Laboratories. Anti-phosphotyrosine mouse monoclonal IgG antibody, anti-c-Src goat polyclonal IgG (N-16), anti-c-Src (SRC-2), anti-Lyn, anti-Fyn, anti-Akt1/2, anti-PISK (p110) rabbit polyclonal IgG antibodies, horseradish peroxidase-conjugated secondary antibodies, and Protein A/G PLUS-agarose were from Santa Cruz Biotechnology. Sphingosine was prepared from cerebroside (33). Standard sphingolipids and glycosphospholipids were extracted from rat brain, purified, and characterized (34). [1-3H]Sphingosine was prepared by specific chemical oxidation of the principal glycolipid group of sphingomyelin followed by mass-sodium borohydride (35) (radiochemical purity over 98%; specific activity 2 Ci/mmole). [32P]Orthophosphate (carrier-free) and [3H]Methionine (specific radioactivity 1175 Ci/mmmole) were purchased from Amersham Pharmacia Biotech and PerkinElmer Life Sciences, respectively. [3H]-Labeled lipids were extracted from [1-3H]sphingosine-fed cells, purified, characterized, and used as chromatographic standards.

Cell Culture—Granule cells, obtained from the cerebella of 8-day-old Harlan Sprague-Dawley rats, were prepared and cultured as described previously (36–38). The cells were plated in 100-mm dishes at a density of 9 x 10^6 cells/dish and cultured with 10 ml of basal modified Eagle’s medium containing 10% fetal calf serum for up to 17 days. After the 8th day in culture, the medium was supplemented with 1 mg/ml glucose. The cultures also contained about 5% of the cells are immature excitatory granule cells. The 8th day in culture corresponded to differentiation and neuritogenesis. At this stage, the cells already emit short neurite-like processes (38). The 8th day in culture corresponded to the morphology of axo-axonic synapses also observed. From the biochemical point of view, the primary cultures at this stage of in vitro development are characterized by the expression of voltage-dependent sodium channels, neurotransmitter receptors, neuronal surfaceialoglycoproteins. Moreover, they acquire the ability to synthesize and release glutamate under depolarizing conditions (36, 39). The 17th day in culture corresponded to a late stage of neuronal development and is characterized by the onset of age-induced apoptosis of about 25% of cerebellar granule cells (40). Thus, the primary cultures are a valuable model for the study of neuronal development in vitro. Typical protein content at these times were 240, 700, and 770 μg of protein/dish.

Analysis of Endogenous Lipids—Cells (4–7 x 10^5) at the 2nd, 8th, and 17th day in culture were harvested and lyophilized. Lipids were extracted with chloroform/methanol 2:1 (v/v), and the total lipid extract was subjected to a two-phase partitioning as previously described (41), resulting in the separation of an aqueous phase containing gangliosides and in an organic phase containing all other lipids. The ganglioside content was determined in the aqueous phase as lipid-bound sialic acid by the method of Svennerholm (42). The phospholipid content was determined in the organic phases as phosphate after perchloric acid digestion by the method of Beinert and Hanson (43). Gangliosides, free cholesterol, and phospholipids, cholesterol, and ceramide from the organic phases were separated by HPTLC as described below. Identification of lipids after separation was assessed by comigration with standard lipids and confirmed by their susceptibility to enzymatic and chemical treatments as previously described (44). After chromatographic separation, compounds were chemically detected and their amounts were determined by densitometry as described below.

The mass lipid composition of the sphingolipid-enriched membrane fractions was calculated on the basis of the endogenous lipid content in the cell homogenate and the percent distribution of the radioactivity associated with each lipid species in the fraction, as described below. 

Treatments of Cell Cultures with [3H]Sphingosine or [3H]Methionine or [32P]Orthophosphate—Cells at the day of preparation (0 day in culture), at the 8th and at the 15th day in culture, were incubated in the presence of 3 x 10^-8 M [1-3H]sphingosine (5 ml/dish) in cell-conditioned medium for a 2-h pulse followed by a 48-h chase. Under these conditions, free radioactive sphingosine was hardly detectable in the cells, and all sphingolipids and phosphatidyethanolamine (obtained by recycling of radioactive ethanolamine formed in the catalabolism of sphingosine) were metabolically radiolabeled. At the 1st, 7th, and 16th day in culture were preincubated in methionine-free medium for 2 h and subsequently incubated in the presence of 25 μCi/ml 1-[3H]Methionine (5 ml/dish) for 20 h, to achieve steady-state radiolabeling of proteins (44–46). Cells at the 2nd, 5th, and 17th day in culture were incubated in the presence of 50 μCi/ml carrier-free [32P]Orthophosphate (5 ml/dish) in phosphate-free culture medium for 4 h (44, 47). These experimental conditions allowed introduction of radioactivity into phosphoproteins and glycosphospholipids, these latter including the very minor ones, such as the phosphorylated phosphoinositides (47).

Sucrose Gradient Centrifugation—After metabolic radiolabeling with [1-3H]sphingosine, or [3H]Methionine, or [32P]Orthophosphate, at the 2nd, 8th, and 17th days in culture were subjected to ultracentrifugation on discontinuous sucrose gradients as previously described (44). Briefly, cells were harvested, lysed in lysis buffer (1% Triton X-100, 10 mM Tris buffer, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM NaVO_4, 1 mM phenylmethylsulfonyl fluoride, and 75 μmimnes/ml aprotinin, 5–8 x 10^7 cells/ml) and Dounce homogenized (10 strokes, tight). Cell lysate was centrifuged (5 min, 1500 x g) to remove nuclei and cellular debris. The postnuclear fraction was mixed with an equal volume of 85% sucrose (w/v) in 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM NaVO_4, placed at the bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer, and centrifuged (17 h, 200,000 x g) at 4°C. After ultracentrifugation, eleven fractions were collected starting from the top of the tube. The light-scattering band located at the interface between 5 and 30% sucrose and was typically mixed to fraction 5-6, the glycosphospholipid enriched membrane fraction (SEMF) (44). The entire procedure was performed at 0–4°C in ice immersion.

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 and lyophilized, and lipids were extracted

1 The abbreviations used are: GPI, glycosylphosphatidylinositol; SEMF, sphingolipid enriched membrane fraction; Cer, ceramide, N acyl-sphingosine; [1-3H]Sphingosine, (25S,3R,4E)-2-amino-1,3-dihydroxy-1-[1-3H]octadecene; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PPE, phosphatidylethanolamine; PPE, phosphatidylserine; PE, phosphatidylycerine; PS, phosphatidylserine; PIP_2, phosphatidylinositol 4,5-bisphosphate; HITPC, high performance thin-layer chromatography; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; DIC, Day(s) in culture.
with chloroform/methanol 2:1 (v/v) (41). The total lipid extract was analyzed by HPTLC as described below, followed by radioactivity imaging for quantification of radioactivity. Identification of lipids separated by HPTLC was accomplished by comigration with standard lipids and confirmed by susceptibility of compounds to following enzymatic and chemical treatments (44). A sample of the lipid extract was evaporated to dryness at 37 °C for 2 h, in 50 μl of water, in the presence of 1 milliunit of Vibrio cholerae sialidase, to yield GM1. Sphingomyelin, phosphorylcholename, and phosphatidylcholine were purified according to the HPTLC-blotting technique previously reported (48): they were separated by HPTLC, identified by spraying with primulin, blotted to PVDF membrane, and where the corresponding bands were cut and subjected to elution. Sphingomyelin was treated at 37 °C overnight in 30 μl of 100 mM Tris-HCl, pH 7.4, 0.5 mM MgCl₂, 0.05% sodium deoxycholate, in the presence of 11 milliunits of Bacillus cereus sphingomyelinase, to yield ceramide; phosphorylcholename was characterized following its degradation under alkaline conditions. The enzymatic or chemical reaction mixtures were separated by HPTLC, and the reaction products were identified by chromatographic comparison with standard lipids.

Thin Layer Chromatography—H-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). Endogenous gangliosides were carried out by bidimensional HPTLC using the solvent system chloroform/methanol/0.2% aqueous CaCl₂, 50:42:11 for the 1st and hexane/diethylether/acetic acid, 80:20:1, and hexane/chloroform/methanol/acetic acid/water, 40:15:13:28 (v/v), to specifically analyze the content of each ganglioside and phospholipid. The separation was carried out on potassium oxalate-impregnated HPTLC plates. Bidimensional HPTLC: (a) first run, chloroform/methanol/acetone/acidic acid/water, 30:20:2:1 (v/v), for the separation of PE, phosphatidylinositol, PS, PC, and sphingomyelin; (b) chloroform/acetone/methanol/acidic acid/water, 10:2:4:2:1 (v/v), with intermediate exposure to HCl vapoors for 15 min; this bidimensional HPTLC system allowed the analysis of plasmalogens in the glycerophospholipid mixture.

Ganglioside and glycerophospholipid species were quantified after separation on HPTLC followed by specific detection with a d-p-D-methylaminobenzaldehyde reagent (50), or a molbydate reagent (51), respectively. The relative amounts of each ganglioside or of each phospholipid were determined by densitometry, and their mass content was calculated from the percent distribution and total ganglioside and phospholipid content, determined as described above.

Cholesterol and ceramide were separated by monodimensional HPTLC using the solvent systems hexane/diethylether/acidic acid, 80:20:1, and hexane/chloroform/acetone/acidic acid, 10:35:10:1, respectively. Cholesterol and ceramide were quantified after separation on HPTLC followed by visualization with 15% concentrated sulfuric acid in ethanol, placed on a rotary stirrer overnight at 4 °C. Immunoprecipitates were recovered using protein A-Sepharose beads, washed three times with IP buffer, recovered by centrifugation (270 × g, 2 min), suspended in 50 μl of SDS-sample buffer, heated to 95 °C for 3 min, and centrifuged (1000 × g, 2 min). The radioactivity associated with immunoprecipitated radiolabeled lipid was 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 percentage of total immunoprecipitable radioactivity present in the amount of cell lysate loaded on gradient. Radioactivity associated with negative controls never exceeded 5% of radioactivity found in immunoprecipitates.

Other Analytical Methods—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 PVDF membranes was performed with a Betamer 2000 instrument (Biospace, Paris) 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 Bio-Rad Laboratories. Autoradiography of 3P- and 35S-labeled proteins was carried out using Kodak Biomax MR and MS films. Protein content was determined according to Lowry (55) using bovine serum albumin as reference standard.

RESULTS

Protein and Lipid Compositions of Rat Cerebellar Granule Cells Developing in Vitro—The protein and lipid composition of cerebellar granule cells at different stages of neuronal development in vitro are reported, as absolute and relative values, in Tables I and II. An overall increase in cell protein and lipid content was observed during the development in culture. As expected, glycerophospholipids, which represent the bulk membrane lipids, comprised the highest lipid content of these cells at all investigated stages of development, 79–82% of total cell lipids. The glycerophospholipid content significantly increased within the days in culture. We observed a 2.7-fold increase from the 2nd to the 8th day in culture. A further 4-fold increase from the 8th to the 17th day in culture. Within the different glycerophospholipids, PC and PE were the most abundant at all investigated stages of development (Table II), being about 50 and 20% of total glycerophospholipids, respectively. The glycerophospholipid patterns (Table II) were very similar during development in culture, with two major exceptions, the
plasmalogen species of PE and PC. In undifferentiated cells, PPC was relatively abundant, representing 11.7% of total glycerophospholipids; its relative amount dramatically dropped to 1–3% at later stages of development. Relative PPE content increased from 3.7% at the 2nd day in culture to 12.6% at the 8th day in culture (corresponding to a 9-fold increase in the absolute mass content, unique among all phospholipids) and remained relatively high at the latest investigated time (6.9%).

Cholesterol was the second major lipid in granule cells at the 2nd day in culture to 1.00 nmol/10⁶ cells (2.52% of total cell lipids) at the 8th day in culture, respectively. Comparing the different sphingolipids at the 8th and 17th day in culture, the molar ratio of sphingolipid to glycerophospholipid not decreased.

In agreement with previous reports (56, 57), some remarkable differences were observed in the relative amounts of the different gangliosides during the time in culture (Table I).

Palmitic acid was the main fatty acid detected in the total complex lipid mixture, (40%), followed by oleic acid (34%), and stearic acid (22%). These data are in good agreement with the fatty acid composition of PC and PE in differentiated cells, previously reported in the literature (58).

**Lipid Metabolism in Rat Cerebellar Granule Cells Developing in Vitro—**Fig. 1 shows the patterns of radioactive lipids extracted from cerebellar granule cells at different stages of development in culture after HPTLC separation. Data on the incorporation of radioactivity into different lipid classes are reported in Table I. The incorporation of radioactivity into sphingolipids was very similar at all investigated days in cul-

### Table I

| Lipid Class          | 2nd DIC | 8th DIC | 17th DIC |
|----------------------|---------|---------|----------|
|                      | nmol/10⁶ cells | % dcpm/10⁶ cells | nmol/10⁶ cells | % dcpm/10⁶ cells | nmol/10⁶ cells | % dcpm/10⁶ cells |
| Proteins             | 0.44     | 1.25    | 1.43     |
| Ceramide             | 0.040    | 0.22    | 0.34     |
| Sphingomyelin        | 0.260    | 1.00    | 1.05     |
| Gangliosides         | 0.074    | 0.79    | 1.02     |
| Glycerophospholipids | 12.29    | 38.24   | 46.40    |
| Cholesterol          | 2.77     | 4.80    | 5.02     |

### Table II

Data on the mass sphingolipid and glycerophospholipid contents at 8th DIC are from Refs. 53, 56, 58, 60. Sphingolipid radioactivity is from 3H labeling (as dpm). Glycerophospholipid radioactivity is from 32P labeling (as cpm). Protein radioactivity is from 35S labeling (as cpm).

| Lipid Species | nmol/10⁶ cells | % dcpm/10⁶ cells |
|---------------|----------------|-----------------|
| GM3           | Trace          | 1.260           |
| GM1           | 0.003          | 3.5             |
| GM2           | 0.011          | 15.1            |
| GD1a          | 0.020          | 27.5            |
| GD1b          | 0.009          | 11.6            |
| O-Ac-GT1b     | 0.003          | 4.6             |
| GT1b          | 0.025          | 33.8            |
| O-Ac-GQ1b     | 0.001          | 0.7             |
| PE            | 2.47           | 20.1            |
| PPE           | 0.45           | 3.7             |
| PPC           | 6.42           | 52.2            |
| PS            | 0.75           | 6.1             |
| PI            | 0.52           | 4.2             |
| PPI           | ND             | 4.718           |
| PIP₁          | ND             | 2.630           |
| PIP₂          | ND             | 668             |

### Table III

| Lipid Species | nmol/10⁶ cells | % dcpm/10⁶ cells |
|---------------|----------------|-----------------|
| GM3           | Trace          | 1260 ± 240      |
| GM1           | 0.003          | 3.5 ± 0.7       |
| GM2           | 0.011          | 15.1 ± 2.1      |
| GD1a          | 0.020          | 27.5 ± 0.2      |
| GD1b          | 0.009          | 11.6 ± 0.1      |
| O-Ac-GT1b     | 0.003          | 4.6 ± 0.1      |
| GT1b          | 0.025          | 33.8 ± 0.0      |
| O-Ac-GQ1b     | 0.001          | 0.7 ± 0.0       |
| PE            | 2.47           | 20.1 ± 2.1      |
| PPE           | 0.45           | 3.7 ± 0.1      |
| PPC           | 6.42           | 52.2 ± 0.2      |
| PS            | 0.75           | 6.1 ± 0.1      |
| PI            | 0.52           | 4.2 ± 0.1      |
| PPI           | ND             | 4.718 ± 0.1     |
| PIP₁          | ND             | 2.630 ± 0.0     |
| PIP₂          | ND             | 668 ± 0.0       |
Pattern is representative of that obtained in three feeding \([1-3H]sphingosine\).

Granule cells at different stages of development in culture after sphingolipid-enriched membrane fractions from rat cerebellar

cells were extracted as described under "Experimental Procedures" and separated by HPTLC in the solvent system chloroform/methanol/0.2% CaCl2 50:42:11 (v/v). Radioactive lipids were detected by digital autoradiography (250 dpm applied on a 4-mm line. Time of acquisition: 24 h). Pattern is representative of that obtained in three different experiments.

Percentage of total cell gangliosides, indicating that the turnover of ceramide are significantly increased in cells at a late stage of development. This is in good agreement with a role of ceramide (Cer), sphingomyelin (SM), gangliosides (G), neutral glycosphingolipids (NGSL), glycosphospholipids (GPL), and cholesterol (Chol) recovered in the sphingolipid-enriched membrane fractions prepared from rat cerebellar granule cells at the 2nd (light gray), 8th (black), and 17th (dark gray) day in culture. Data relating to sphingolipids and glycosphospholipids were obtained from the distribution of lipids-associated radioactivity after labeling with \([1-3H]sphingosine or \([32P]\)orthophosphate, respectively. Endogenous cholesterol content was directly determined as described under "Experimental Procedures." Data are expressed as percentages of the total amount of each lipid present in the homogenates, and are the means of three different experiments ± S.D.

Fig. 1. Patterns of radioactive lipids in homogenates and sphingolipid-enriched membrane fractions from rat cerebellar granule cells at different stages of development in culture after feeding \([1-3H]sphingosine\). Lipids from the homogenates (Hom) and from the sphingolipid-enriched membrane fractions (SEMF) prepared from rat cerebellar granule cells at the 2nd (A), 8th (B), and 17th (C) day in culture were extracted as described under "Experimental Procedures" and separated by HPTLC in the solvent system chloroform/methanol/0.2% CaCl2 50:42:11 (v/v). Radioactive lipids were detected by digital autoradiography (250 dpm applied on a 4-mm line. Time of acquisition: 24 h). Pattern is representative of that obtained in three different experiments.

Fig. 2. Lipid distribution in the sphingolipid-enriched membrane fractions prepared from rat cerebellar granule cells at different stages of development in culture. Relative amounts of ceramide (Cer), sphingomyelin (SM), gangliosides (G), neutral glycosphingolipids (NGSL), glycosphospholipids (GPL), and cholesterol (Chol) recovered in the sphingolipid-enriched membrane fractions prepared from rat cerebellar granule cells at the 2nd (light gray), 8th (black), and 17th (dark gray) day in culture. Data relating to sphingolipids and glycosphospholipids were obtained from the distribution of lipids-associated radioactivity after labeling with \([1-3H]sphingosine or \([32P]\)orthophosphate, respectively. Endogenous cholesterol content was directly determined as described under "Experimental Procedures." Data are expressed as percentages of the total amount of each lipid present in the homogenates, and are the means of three different experiments ± S.D.
from 32P labeling (as cpm). The mass composition of the sphingolipid-enriched membrane fraction was calculated on the basis of the endogenous lipid and protein content in the homogenate (reported in Table I) and the distribution of radioactivity in each species within the gradient.

SEMF from rat cerebellar granule cells at different stages of culture was always significantly higher in SEMF (an increase in neonatal development). Despite this variability, GD1a relative precision of 12 h, a lapse of time not negligible at this stage of development. This variability associated with each sphingolipid in the cell homogenate). Thus, these structures are more enriched in sphingolipids than in cholesterol. The incorporation of radioactivity into different lipid classes and the lipid composition of SEMF from cerebellar granule cells at the 2nd, 8th, and 17th days in culture are reported in Table III. The data on the cholesterol content were derived from direct chemical analysis. The data on the sphingolipid and glycerophospholipid contents were calculated from the radioactivity distribution in SEMF after labeling cells with [1-3H]sphingosine or [32P]orthophosphate, respectively, and from the total lipid composition of these cells, as reported in Table I. These data clearly indicate that the enrichments (relative to the homogenates) of SEMF in sphingolipids and cholesterol with respect to glycerophospholipids linearly decreased with the number of days in culture. However, the percent lipid composition of SEMF during development in culture varied in a complex way, as indicated by the data in Table III. In particular, the relative ganglioside and sphingomyelin content was higher in fully differentiated cells, ceramide increased after the initial stage and then remained almost unchanged, while cholesterol gradually decreased along the days in culture.

At all the days in culture, the distributions of different gangliosides within SEMF were very similar to the patterns observed in the homogenates (Fig. 1 and Table IV), with a major exception, related to the content of GD1a in SEMF at the 2nd day in culture. The pattern of radioactive gangliosides observed at the 2nd day in culture was relatively changeable, compared with those at other stages of development. This variability between different experiments can be easily explained keeping in mind the very rapid variations of ganglioside content and isoelectric pattern in differentiated cells, respectively. The SEMF always contained a very minor portion of cell proteins, but this portion dramatically increased from the 2nd to the 8th day in culture (0.3 ± 0.1%, 1.6 ± 0.2%, and 2.3 ± 0.3%, of the total cell protein at the 2nd, 8th, and 17th day in culture, respectively) (Fig. 5, left panel). Fig. 6 shows the two-dimensional 35S-protein patterns of SEMF prepared from granule cells at different days in culture. The protein pattern in differentiated cells comprised a wide variety of species, encompassing the whole molecular mass and isoelectric point range. On the other hand, the protein patterns of cells representing 44.2–73.0% of total SEMF lipids. Because glycerophospholipids account for the bulk of membrane lipids, these data suggest that the total surface area occupied by the sphingolipid-enriched domains gradually increases during development in culture. A relevant (25–40%) amount of cellular cholesterol was associated with SEMF; cholesterol was very abundant in SEMF in the first stage of culture, about 45% of total SEMF components, then gradually decreased to 15% at 17th day in culture. As clearly shown, all sphingolipids (namely ceramide, sphingomyelin, gangliosides, and neutral glyco-sphingolipids) are largely associated with SEMF, regardless from the stage of development (40–70% of the radioactivity associated with each sphingolipid in the cell homogenate). Very relevant differences were observed among different glycerophospholipids and different days in culture. At all the stages of development, the fraction of PC recovered in SEMF was always much higher than that of any other glycerophospholipid, thus making PC by far the most abundant lipid in SEMF (93 and 75% of total SEMF glycerophospholipids at the 2nd and 17th day in culture, respectively, Table IV). In the case of other glycerophospholipids, the fraction associated with SEMF greatly varied among the different days in culture, thus resulting in very different patterns (Table IV). In particular, the relative amount of PE in SEMF gradually increased during differentiation from 2.9% to 26.4%; PPE was particularly high in cells at the latest stage of development; the amount of radioactivity associated with phosphorylated phosphoinositides dramatically increased (especially for phosphatidylinositol 4,5-bisphosphate) during differentiation.

Palmitic acid was the main fatty acid of complex lipids from SEMF. Palmitic acid was significantly enriched in SEMF respect to the cell lysate. In fact, its relative content was 1.4-fold higher in SEMF than in the cell lysate. In parallel, we observed a slight reduction in the relative amount of unsaturated fatty acids in SEMF. SEMF enrichment in palmitic acid was mainly due to dipalmitoyl-PC, that represented the main glycerophospholipid specie in this fraction. PC isolated from the total cell homogenate and from SEMF was analyzed by ESI-MS according with previous methods (61). The MS spectrum of PC contained the molecular ions at m/z 756, 782, 810 corresponding to the dipalmitoyl-, the 1-palmitoyl-2-oleoyl-, and 1-stearoyl-2-oleyl- species. Fig. 4 clearly shows the large increase in the relative abundance of the molecular ion corresponding to dipalmitoyl-PC, at m/z 756, in SEMF.

### TABLE III

| Protein Compositions of Sphingolipid-enriched Membrane Fractions from Rat Cerebellar Granule Cells Developing In Vitro—Previous evidence indicated that proteins associated with sphingolipids-enriched membrane domains are selected and are potentially involved in signal transduction events (12, 30, 44). Thus, we analyzed the protein composition of SEMF prepared from cerebellar granule cells at different stages of development in culture. The protein content of granule cells increased during development, as expected, from 26.2 to 85.6 mg/10⁶ cells. To compare these data to those for lipids, we calculated that at the 2nd, 8th, and 17th day in culture the approximate molar protein content was 0.44, 1.25 and 1.43 ± 20 nmol/10⁶ cells, respectively. The SEMF always contained a very minor portion of cell proteins, but this portion dramatically increased from the 2nd to the 8th day in culture (0.3 ± 0.1%, 1.6 ± 0.2%, and 2.3 ± 0.3%, of the total cell protein at the 2nd, 8th, and 17th day in culture, respectively) (Fig. 5, left panel). The protein patterns of cells

| Protein | 2nd DIC | 8th DIC | 17th DIC |
|---------|---------|---------|---------|
| Proteins | 0.001 | 0.016 | 0.131 |
| Ceramide | 0.016 | 0.035 | 0.171 |
| Sphingomyelin | 0.131 | 0.171 | 0.035 |
| Gangliosides | 0.035 | 0.047 | 0.47 |
| Glycophospholipids | 0.70 | 3.95 | 7.030 |
| Cholesterol | 0.70 | 4.42 | 26.8 |
Sphingolipid radioactivity is from $^3$H labeling (as dpm). Glycerophospholipid radioactivity is from $^{32}$P labeling (as cpm). The mass composition of the sphingolipid-enriched membrane fraction was calculated on the basis of the endogenous lipid content in the homogenate (reported in Tables I and II) and the distribution of radioactivity in each species within the gradient.

**Table IV**

| Sphingolipid | 2nd DIC | 8th DIC | 17th DIC |
|--------------|---------|---------|----------|
| GM3 | Trace | % | dcpm/10⁶ cells |
| GM1 | 0.001 | 2.4 | 199 | 0.9 | 5.9 | 4.8 | 0.01 | 0.2 |
| GD3 | 0.004 | 9.5 | 1,073 | 31.9 | 0.02 |
| GD1a | 0.013 | 30.9 | 433 | 12.9 | 0.11 | 23.4 | 1,384 | 26.1 | 0.12 | 33.3 | 1,250 | 33.9 |
| GD1b | 0.005 | 11.9 | 383 | 11.4 | 0.05 | 10.6 | 637 | 12.0 | 0.04 | 11.1 | 343 | 9.3 |
| O-Ac-GT1b | 0.003 | 4.8 | 167 | 5.0 | 0.06 | 12.8 | 611 | 11.5 | 0.03 | 8.3 | 280 | 7.6 |
| GT1b | 0.007 | 16.7 | 585 | 17.4 | 0.17 | 36.2 | 1,809 | 34.2 | 0.12 | 33.3 | 1,192 | 32.3 |
| O-Ac-GQ1b | ND | 2.4 | 50 | 1.5 | 0.02 | 4.2 | 80 | 1.5 | 0.01 | 2.8 | 96 | 2.6 |
| QG1b | 0.001 | 2.4 | 50 | 1.5 | 0.02 | 4.2 | 80 | 1.5 | 0.01 | 2.8 | 96 | 2.6 |
| PE | 0.02 | 2.6 | 23 | 1.3 | 0.35 | 8.6 | 357 | 6.0 | 2.12 | 23.3 | 556 | 11.4 |
| PPE | 0.02 | 2.6 | 70 | 4.0 | 0.05 | 1.2 | 88 | 1.5 | 0.50 | 5.5 | 168 | 3.4 |
| PC | 0.65 | 84.4 | 1,105 | 63.6 | 3.41 | 83.8 | 3,512 | 59.1 | 6.03 | 66.2 | 2,562 | 51.8 |
| PPC | 0.03 | 3.9 | 378 | 21.8 | 0.04 | 0.2 | 237 | 4.0 | 0.12 | 4.4 | 139 | 4.4 |
| PS | 0.04 | 5.2 | 52 | 3.0 | 0.18 | 4.4 | 195 | 3.3 | ND |
| PI | 0.01 | 1.3 | 55 | 3.2 | 0.05 | 1.2 | 840 | 14.1 | 0.14 | 1.5 | 857 | 17.5 |
| PPI | ND | 11 | 0.6 | ND | 260 | 4.4 | 301 | 6.1 |
| PIP | 0.02 | 2.4 | 42 | 4.6 | 0.01 | 0.2 | 274 | 4.6 | 0.226 | 4.6 |
| PIP$_2$ | ND | 0.01 | 0.2 | 181 | 4.0 | 0.01 |

FIG. 3. Phospholipid distribution in the sphingolipid-enriched membrane fractions prepared from rat cerebellar granule cells at different stages of development in culture. Relative amounts of phosphatidylethanolamine (PE), phosphatidylethanolamine plasmalogens (PPE), phosphatidylcholine (PC), phosphatidylcholine plasmalogens (PPC), phosphatidylyserine (PS), phosphatidylserine (PI), phosphatidylserine plasmalogens (PPI), phosphatidylinositol-4-phosphate (PIP), phosphatidylinositol 4,5-diphosphate (PIP$_2$), and phosphatidylethanolamine plasmalogens (PE) were extracted and separated by mono- and bidimensional HPTLC as described under “Experimental Procedures.” Radioactive lipids were detected by autoradiography (typically 2000 cpm applied on a 3-mm line. Time of exposure: 48 h). The radioactivity associated with individual lipids was determined by densitometry. Data are expressed as percentages of the total amount of each lipid present in the homogenate and are the means of three different experiments ± S.D.

FIG. 4. Positive-ion ESI mass spectra of PC from cerebellar granule cells at the 8th day in culture. PC was purified from the total lipid extracts obtained from the homogenate and the sphingolipid-enriched membrane fractions from cells at the 8th day in culture as described under “Experimental Procedures.” A, mass spectrum of PC from cell homogenate; B, mass spectrum of PC from the sphingolipid-enriched membrane fraction. Data are expressed as percent relative abundances. The three main species dipalmitoyl-PC, 1-palmitoyl-2-oleyl-PC, and 1-stearoyl-2-oleyl-PC with molecular ions [M+Na]$^+$ at m/z 756, 782, and 810, respectively, were recognized. Each selected ion was characterized by MS$^2$ and MS$^3$ by loss of quaternary amino and phosphoethyl groups, respectively.

at 2nd and 17th DIC were much simpler and particularly lacking in high molecular mass proteins. A low molecular mass protein (about 20 kDa) was particularly abundant in SEMF from cells at the latest stage of development.

Fig. 5 (right panel) shows the distribution of phosphotyrosine-containing proteins in SEMF, as determined by immunoprecipitation with anti-phosphotyrosine antibody. The portion of phosphotyrosine-containing proteins associated with SEMF was relatively high and markedly increased along the development in culture (7.7%, 22.3%, and 32.1% of total cell phosphotyrosine-containing proteins at 2nd, 8th, and 17th day in culture, respectively).

Among proteins belonging to the SEMF, three members of the Src family were identified by immunoblotting with specific antibodies. Fig. 7 shows the distribution patterns of Src-family tyrosine kinases c-Src, Fyn, and Lyn in SEMF and cell homogenates from cerebellar granule cells at different stages of development in culture. From the relative intensities of the immunoblotting signals, we calculated that the amount of c-Src associated with SEMF was about 20% of total c-Src present in these cells, indicating a very high enrichment of c-Src in this fraction. The amount of c-Src recovered in SEMF was not significantly changed during the development. Fyn was also highly enriched in SEMF, but the amount of Fyn associated with SEMF increased from 15% of total Fyn at the 2nd day in...
protein of these cells to remain vital in culture for long time, we analyzed the distribution patterns of PI3K and of the serine-threonine kinase Akt (a downstream target of PI3K signaling known to be involved in the control of neuronal survival), in SEMF and cell homogenates from cerebellar granule cells at different stages of development in culture. As shown in Fig. 7, both proteins were expressed in our cells but could not be detected in the SEMF under our experimental conditions.

**DISCUSSION**

Membrane lipids have been reported to change to some extent during the development, maturation, or aging of the nervous system, or during the onset of other physiological or pathological changes in neuronal functions. In our study, the changes in the composition and turnover of each membrane lipid class and individual lipid during the development of cultured neurons were related to changes in the organization of sphingolipid-enriched membrane domains. As a model, we used primary cultures of rat cerebellar granule cells, which represent a very homogeneous population of neurons. This highly simplified model obviously lacks the ability to provide any information about the interactions between neurons and glia. However, these cells spontaneously undergo morphological and biochemical changes similar to those occurring during the different stages of in vivo neuronal development, thus representing a valuable model for the study of neuronal differentiation.

We performed our experiments at three different stages of in vitro development: the 2nd day in culture, corresponding to the initial stage of neuronal differentiation and neuritogenesis, the 8th day in culture, corresponding to morphologically and biochemically fully differentiated neurons, and the 17th day in culture, corresponding to a late stage of neuronal development, followed by the eventual age-induced apoptotic cell death. The content of all membrane lipid classes (glycerophospholipids, cholesterol, and sphingolipids) greatly increased during the progression from undifferentiated to fully differentiated granule cells, indicating an overall increase in the surface and complexity of neuronal plasma membrane, corresponding to the extension of a very elaborate neurite network. This event is also characterized by a great increase in the cell protein content. This increase is higher in the case of sphingolipids, particularly gangliosides, than of other lipids, in agreement with previously reported data (56, 57, 60). Thus, the surface density of gangliosides in the membrane of fully differentiated neurons is much higher than in undifferentiated cells. The lipid turn-
over also varied during this phase of development, being higher in undifferentiated cells both for phospholipids and sphingolipids. The latter is in agreement with the previous finding that, in these cells, the activity of 3-ketosphinganine synthase markedly decreases during differentiation (62). In particular, ganglioside turnover at the 2nd day in culture was about 11-fold faster than at the 8th day in culture, whereas in the case of phospholipids this difference was 1.5-fold. The turnover of gangliosides was always more rapid than sphingomyelin and ceramide turnover, and this difference was the highest in undifferentiated cells (12- and 4-fold, for sphingomyelin and ceramide, respectively). In undifferentiated cells, the turnover of some ganglioside species, namely GM3 and GD3, was surprisingly high, as indicated by the high amount of radioactivity associated with these species compared with their mass content. Moving toward a later stage of development, resembling that of aging neurons, the density of gangliosides in the cell membrane showed little or no change, as indicated by the constant mass ratios between gangliosides and glycerophospholipids or gangliosides and cholesterol.

The turnover of glycerophospholipids was significantly lower in aging cerebellar neurons (this fact probably reflects a slower overall renewal of the plasma membrane), whereas the turnover of gangliosides was similar to that in fully differentiated cells.

We focused our attention on the organization of sphingolipid-enriched domains, which are emerging as a compartment within the plasma membrane, where most cell gangliosides are concentrated and segregated together with selected proteins and other lipids. The results we obtained are summarized in Tables III and IV. Our data indicate that a membrane compartment, characterized by a very high enrichment in sphingolipids and cholesterol and for this reason named sphingolipid-enriched domain, always exists in cerebellar granule neurons, regardless of the stage of development. The bulk structure of these domains is largely determined by PC, and, within the different PC species, dipalmitoyl-PC was much more abundant than in the total cell membrane. Thus, the lipid core of the SEMF results highly enriched in palmitic acid respect to the cell membrane.

The surface occupied by these structures seems to increase during development in culture, in a way that is not simply proportional to the overall cell membrane surface, as indicated by their relative glycerophospholipid content. Moreover, a particularly high ganglioside density (as indicated by the ganglioside/glycerophospholipid mass ratio) within the domains seems to be always required, independent of the developmental stage. In fact, in undifferentiated neurons, where the ganglioside content is lower and the neurons are rapidly synthesizing these lipids, we observed in the sphingolipid-enriched domain the highest enrichment in gangliosides with respect to the cell homogenate, an 8-fold increase in the ganglioside/glycerophospholipid molar ratio. A similar behavior was also observed for sphingomyelin. The SEMF from undifferentiated neurons is concentrated and segregated together with selected proteins within the plasma membrane, where most cell gangliosides are enriched domains, which are emerging as a compartment for sphingolipids and cholesterol and for this reason named sphingolipid-enriched domain, always exists in cerebellar granule neurons, regardless of the stage of development. The bulk structure of these domains is largely determined by PC, and, within the different PC species, dipalmitoyl-PC was much more abundant than in the total cell membrane. Thus, the lipid core of the SEMF results highly enriched in palmitic acid respect to the cell membrane.

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be a mechanism of regulation. In Neuro2a neuroblastoma cells, the activation of c-Src in the sphingolipid-enriched domain, that mediates ganglioside-induced neuritogenesis, is accompanied by the removal of Csk from the domain (29). Because Csk is an inhibitory regulatory kinase of c-Src, this is a good example of regulation mediated by an alteration of sphingolipid-enriched domain structure. Thus, it will be very interesting to investigate whether the activity of these proteins within sphingolipid-enriched domains actually changes during in vitro development and whether the transient association with other protein molecules could be responsible for this change.

More specific differences in the levels, turnover, and organization of single lipid species were observed. Some of them are intriguing, even if still difficult to interpret. For example, a single ganglioside, GD1a, is significantly more enriched than others in sphingolipid-enriched domains from undifferentiated cells. This could indicate a specific, yet unknown role for GD1a. PPE is particularly enriched in sphingolipid-enriched domains from aging neurons, and several lines of evidence indicate that the receptor-mediated breakdown of plasmalogens is a relevant event in neural membranes during neurodegeneration (8). The turnover of phosphorylated phosphoinositides is particularly intense in sphingolipid-enriched domains from fully differentiated neurons. Thus, lipid-mediated signaling pathways other than sphingolipid signaling could play a role within these domains. This will not be surprising, because our data clearly indicate that non-sphingoid lipids also undergo a selection process leading to the segregation within domains. Nevertheless, neither the phosphoinositide 3-kinase nor the serine-threonine kinase Akt, which is known to be one of the phosphorylated phosphoinositide signaling downstream targets, were detected in the sphingolipid-enriched domains.

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J. Biol. Chem. 2001, 276:21136-21145.
doi: 10.1074/jbc.M010666200 originally published online March 22, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M010666200

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