Ceramide and sphingomyelin species of fibroblasts and neurons in culture

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Abstract The ceramide (Cer) and sphingomyelin (SM) species of cultured differentiated rat cerebellar granule cells and human fibroblasts were characterized by electrospray ionization-mass spectrometry. We identified 35 different species of Cer and 18 species of SM in human fibroblasts, and 35 different species of Cer and 9 species of SM were characterized in rat neurons. The main Cer species of rat cerebellar granule cells contained d18:1 sphingosine linked with palmitic, stearic, or nervonic fatty acid, and the two main SM species were d18:1,16:0 and d18:1,18:0. Both sphingolipids were enriched in detergent-resistant membranes (DRMs; or lipid rafts), and significant differences were found in the sphingolipid patterns of DRMs and of detergent-soluble fractions (DSF) from these cells. In human fibroblasts, the main Cer species were d18:1,16:0, d18:2,16:0, d18:1,24:0, d18:2,24:0, d18:1,24:1, and d18:2,24:1; the most represented species of SM were d18:1,16:0, d18:1,24:0, and d18:1,24:1. In these cells, SM was highly enriched in DRMs and Cer was mainly associated with DSF, and the species found in DRMs were markedly different from those found in DSF.—Valsecchi, M., L. Mauri, R. Casellato, S. Prioni, N. Loberto, A. Prinetti, V. Chigorno, and S. Sonnino. **Ceramide and sphingomyelin species of fibroblasts and neurons in culture. *J. Lipid Res.* 2007.** 48: **417–424.**

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Ceramide (Cer) is the lipid moiety of sphingolipids; it is constituted by a long-chain amino alcohol, 2-amino-1,3-dihydroxy-octadec-4-ene, whose trivial name is sphingosine, connected to a fatty acid by an amide linkage. Like all of the other sphingolipids, Cer is a component of the plasma membranes.

Multiple lines of research have suggested important roles of Cer in several signaling pathways, such as inhibition of cell growth and induction of apoptosis (1, 2). Cer represents the final product of sphingolipid catabolism, which mainly takes place within lysosomes by the involvement of specific glycohydrolases.

Recently, the availability of experimental models able to activate nonlysosomal sphingolipid hydrolases, causing an increase of membrane Cer content, demonstrated that bioactive Cer derives mainly from the in situ degradation of the plasma membrane sphingolipids (3–6). However, this increase of Cer content constitutes a transient phenomenon, because this molecule can be further catabolized or recycled for sphingolipid synthesis (3, 7). Moreover, the Cer increase can be the result of intracellular de novo synthesis (8).

Recent findings indicated that a particular set of lipids and proteins are segregated together within the plasma membrane, forming functional domains that are involved in signal transduction processes (9, 10). These domains, highly enriched in sphingolipids and cholesterol, have been found within the plasma membrane as well as in intracellular membranes, and for their physicochemical characteristics they are resistant to detergent solubilization [from this property derives the term detergent-resistant membranes (DRMs)] (9, 11–13). Cer is a component of DRMs, and it has been suggested that its production and its functional properties are strictly connected to domain organization (14).

In this work, we analyzed by mass spectrometry the content of different species of Cer and sphingomyelin (SM) in cell homogenates and, in particular, their distribution between detergent-soluble fraction (DSF) and DRMs prepared from cultures of differentiated rat cerebellar neurons and human skin fibroblasts. The results suggested that this approach can be applied to the study of changes in Cer and SM content ensuing from cell signaling pathway activation.

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Abbreviations: Cer, ceramide (*N*-acyl-sphingosine); DRM, detergent-resistant membrane; DSF, detergent-soluble fraction; ESI, electrospray ionization; SM, sphingomyelin (ceramide phosphocholine).

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MATERIALS AND METHODS

Materials

Commercial chemicals were the purest available, common solvents were distilled before use, and water was doubly distilled in a glass apparatus. Trypsin and all reagents for cell culture were from Sigma Chemical Co., except for basal modified Eagle's medium and fetal calf serum, which were purchased from Flow Laboratories. Sphingosine was prepared from cerebroside (15); [1-^{3}H]sphingosine was prepared from sphingosine (16). Radioactive sphingolipids were extracted from cells fed with [1-^{3}H] sphingosine, purified, characterized as described previously, and used as chromatographic standards (17). Lyso-SM was from Avanti Polar Lipids. C17-Cer was prepared by acylation of sphingosine and C17-SM of lyso-SM using heptadecanoic anhydride (18).

Cell cultures

Granule cells were obtained from the cerebellum of 8 day old Harlan Sprague-Dawley rats, plated in 100 mm dishes at a density of 9×10^6 cells/dish, and cultured with basal modified Eagle's medium containing 10% fetal calf serum for 8 days, the time necessary for their differentiation (19–21). Normal human skin fibroblasts were obtained by the punch technique, cultured, and propagated as described (22) in 100 mm dishes (~0.35 mg of protein/dish), using Eagle Minimum Essential Medium supplemented with 10% fetal calf serum. When confluent, fibroblasts were used for the experiments.

Metabolic radiolabeling of cell sphingolipids

Cells were incubated with 3×10^{-8} M [1-^{3}H]sphingosine (5 ml/dish) for a 2 h pulse followed by a 48 h chase. The pulse of rat cerebellar granule cells was performed on the 6th day in culture, and the radioactive sphingosine was dissolved in cell-conditioned medium. Human skin fibroblasts were radiolabeled in a preconfluent stage with radioactive sphingosine dissolved in cell culture medium. After the pulse period, the medium was removed and replaced with cell-conditioned medium or with fresh medium (for neurons and fibroblasts, respectively) without radioactive sphingosine for the chase period. This method allowed us to obtain the metabolic radiolabeling of all sphingolipids, including Cer, SM, neutral glycolipids, and gangliosides (23, 24). Tritium-labeled phosphatidylethanolamine was also obtained by recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine.

Sucrose gradient centrifugation

Both rat neurons and human skin fibroblasts were harvested, lysed in a lysis buffer containing 1% Triton X-100, and Dounce homogenized. Cell lysates were centrifuged (5 min, 1,300 *g*) to remove nuclei and cellular debris, thus giving a postnuclear subcellular fraction that was subjected to ultracentrifugation on discontinuous sucrose gradients (17 h, 200,000 *g*) (24). After ultracentrifugation, 11 fractions were collected from the top of the tube. In some experiments, fractions 4–6 were pooled to obtain the DRMs. The other fractions were pooled and regarded as DSF. The entire procedure was performed at 0–4°C in ice immersion.

Lipid extraction

All fractions obtained after ultracentrifugation, as well as postnuclear subcellular fractions, DRMs, and DSF, were dialyzed for 4 days against distilled and decarbonated water (changed two times per day). After dialysis, samples were lyophilized and lipids were extracted with chloroform-methanol (2:1, v/v).

The total lipid extracts of DRMs and DSF were further subjected to two-phase partitioning (25), resulting in the separation of an aqueous phase containing gangliosides and an organic phase containing all other lipids. The organic phases were submitted to alkaline treatment followed by partitioning to remove glycerophospholipids, and the new organic phases were used for mass spectrometry analysis.

HPLC-MS/MS analyses

Mass spectrometry analysis was carried out using a ThermoQuest Finnigan LCQDeca ion-trap mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionization (ESI) ion source, an Xcalibur data system, and a TSP P4000 quaternary pump HPLC apparatus. Separations were obtained on a 5 μm, 250 × 4 mm LiChrospher 100 RP8 column (Merck). Elution was carried out at a flow rate elution of 0.5 ml/min, with a solvent system composed of 5 mM ammonium acetate in methanol-water (9:1, v/v) (A) and 5 mM ammonium acetate in methanol (B). The gradient elution program was as follows: 5 min with solvent A; 5 min with a linear gradient from 100% solvent A to 100% solvent B; 15 min with 100% solvent B; and 5 min with a linear gradient from 100% solvent B to 100% methanol. Methanol was also used to wash the column for 10 min, followed by an equilibration procedure with solvent A for 15 min. Optimum conditions for MS analyses included sheath gas flow of 50 arbitrary units, spray voltage of 4 kV, capillary voltage of −47 V, and capillary temperature of 260°C. The fragmentation voltage used for collision-induced dissociation to obtain MS2 and MS3 was 40–60%. For all experiments, source ion optics were adjusted to accomplish the desolvation of ions while minimizing fragmentation. Mass spectra were acquired over an m/z range of 200–1,000.

As internal standards, synthetic d18:1,17:0 Cer and SM containing d18:1,17:0 Cer were used (for the trivial nomenclature of ceramide/dihydroceramide: first sphingosine/sphinganine, then fatty acid). A 50 μM stock solution of each internal standard in 5 mM ammonium acetate in methanol was prepared and stored at −20°C. Serial dilutions were prepared from these stock solutions and used for calibration curves.

Other analytical methods

Total radioactivity associated with lipids was determined by liquid scintillation counting. Radioactive lipids were separated by high performance TLC with the solvent system chloroform-methanol-0.2% aqueous $CaCl_2$ (55:45:10, v/v) for human fibroblast samples and with the solvent system chloroform-methanol-0.2% aqueous $CaCl_2$ (50:42:11, v/v) for samples from rat cerebellar granule cells. To quantify Cer, high performance TLC was developed with the solvent system ethyl acetate-methanol-chloroform-50 mM aqueous KCl (50:40:10:1.4, v/v). Quantitative radioimaging of TLC plates was carried out with a β-vision instrument (Biospace, Paris, France). The protein content was determined on cell lysates according to Lowry et al. (26) using BSA as a reference standard.

RESULTS

In this study, we report on the Cer and SM species patterns of human fibroblasts and differentiated rat cerebellum granule cells in culture. In particular, we compare data obtained from DRMs and DSF prepared by ultracentrifugation on sucrose gradients, as described in Materials and Methods.

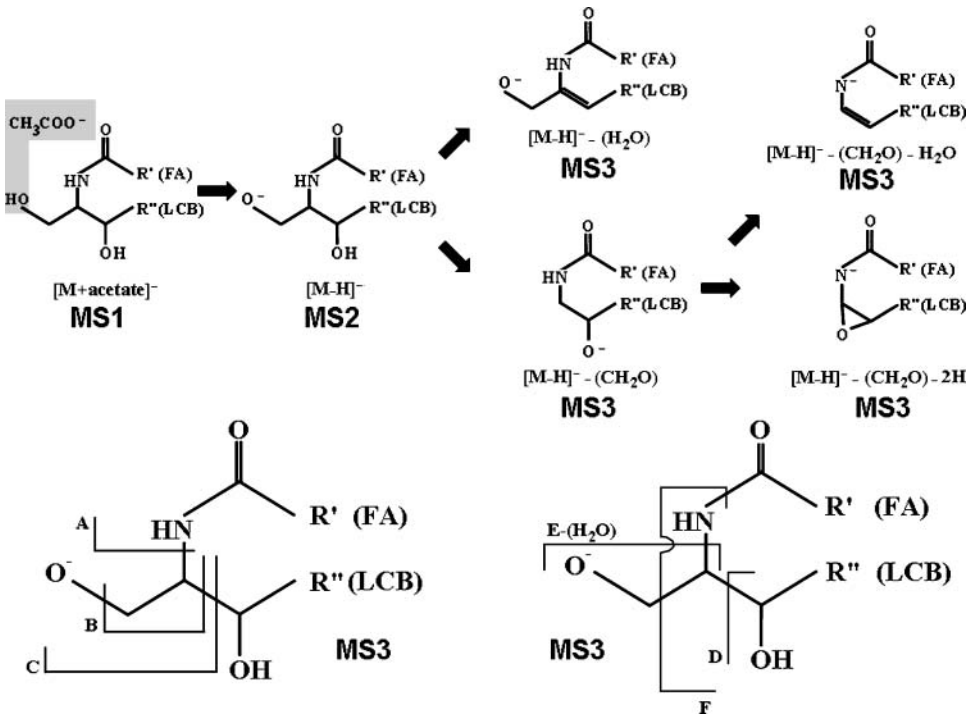

Fig. 1. Proposed electrospray ionization mass spectrometry (ESI-MS) ion fragmentation pattern for ceramide (Cer).

Mass spectrometry of Cer and SM

The molecular species of Cer and SM, differing in the lipid moiety, were characterized by identification of the ions obtained by MS1, MS2, and MS3. The fragmentation patterns for Cer and SM are reported in **Figs. 1**, **2**, respectively. Quantitative data were referred to synthetic internal standards of Cer and SM containing d18:1 sphingosine and 17:0 fatty acid. A linear correlation between a series of solutions of these compounds and the ion peak intensities was obtained by multiple reaction monitoring analysis. We had a correlation coefficient of 0.998 and a slope of 69.64 for Cer in a range from 1 to 100 pmol

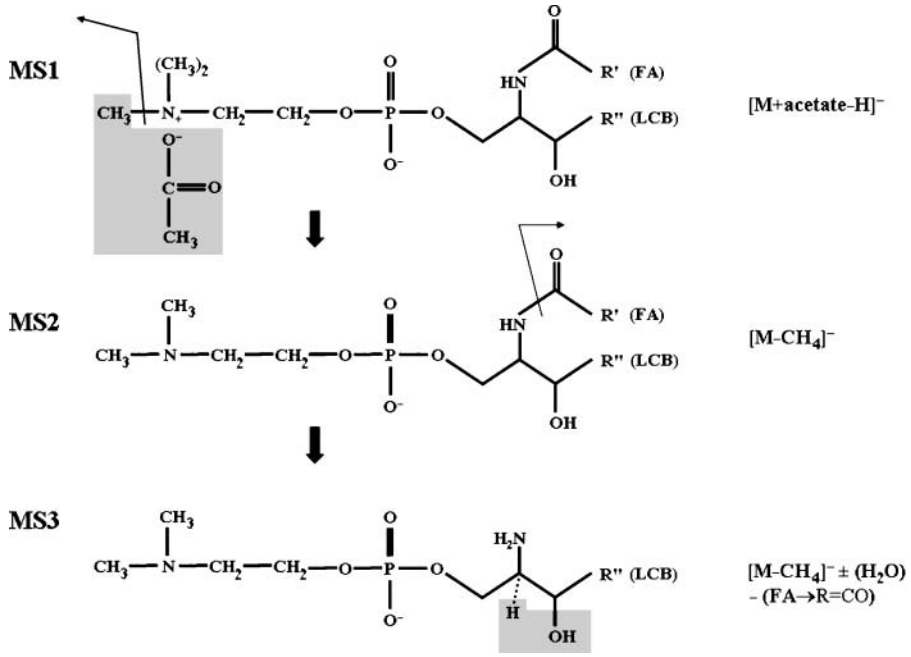

Fig. 2. Proposed ESI-MS ion fragmentation pattern for sphingomyelin (SM).

and a correlation coefficient of 0.999 and a slope of 1.54 for SM in a range from 5 to 1,000 pmol.

The total cell content of Cer in human fibroblasts was 5.502 ± 0.440 nmol/mg cell protein. Previous data on the Cer content in fibroblasts varied from 1 to 9 nmol/mg protein (27–30). By mass spectrometry analysis, we could identify 35 species of Cer containing 7 different long-chain bases and 14 fatty acids (**Table 1**), most of which were minor compounds. The main species were those containing d18:1 with 16:0 or very long fatty acids. d18:1,16:0 represented 19% of the total cell species, and ~42% of it was found in DRMs. Species containing d18:1 linked with very long fatty acids were ~38% of total cell Cer, and ~21% of them were recovered in DRMs. Thus, d18:1,16:0 Cer is the main species in DRMs, representing ~34% of the total Cer species.

High amounts of Cer species containing d18:2 sphingosine (sphingadienine) were found in human fibroblasts, and >90% was in DSF. It has been reported that in mouse brain >10% of complex sphingolipids contain the sphingadienine (31). As described previously, this polyunsaturated sphingosine has a *trans* double bound at C-4 and a *cis* double bound at C-14 (32, 33).

TABLE 1. Pattern of Cer species in cultured human fibroblasts, and distribution between DRMs and DSF

Species	Homogenate	DRMs	DSF
	pmol/mg protein	%	%
d18:1,14:0	49	12	88
d18:1,16:1	20	tr	~100
d18:0,16:1	26	23	77
d16:1,16:0	26	23	77
d18:2,16:0	230	9	91
d18:1,16:0	1,055	42	58
d18:0,16:0	34	41	59
d16:0,18:1	43	tr	~100
d16:1,18:0	20	tr	~100
d18:1,18:0	34	41	59
d18:1,22:1	26	23	77
d18:0,22:1	26	23	77
d18:2,22:0	77	18	82
d18:1,22:0	199	36	64
d18:1,23:1	49	12	88
d18:0,23:1	57	25	75
d18:2,23:0	26	23	77
d18:1,23:0	63	32	68
d18:1,24:2	133	20	80
d18:0,24:2	57	25	75
d16:1,24:1	26	23	77
d18:2,24:1	294	7	93
d18:1,24:1	1,257	18	82
d18:0,24:1	153	30	70
d16:1,24:0	26	23	77
d18:2,24:0	623	9	91
d18:1,24:0	685	27	73
d18:0,24:0	34	41	59
d20:1,24:0	tr	—	tr
d20:0,24:0	34	41	59
d18:1,26:1	20	tr	~100
d18:0,26:1	tr	tr	—
d18:2,26:0	20	tr	~100
d18:1,26:0	34	41	59
d18:0,26:0	34	41	59

Cer, ceramide; DRM, detergent-resistant membrane; DSF, detergent-soluble fraction; tr, trace. Data are means of four experiments with SD in the range of ±15%.

By MS analysis, we calculated that the Cer content in DRMs represents ~30% of total cell Cer. Moreover, as shown in Table 1, the Cer species pattern of DSF is different from the Cer species pattern of DRMs.

In human fibroblasts, we calculated a total SM content of 25.185 ± 2.520 nmol/mg cell protein, in agreement with previous observations (34, 35). Eighteen species of SM, differing in Cer structures obtained combining 5 different long-chain bases and 12 fatty acids, were identified. Their absolute contents and relative enrichments in DRMs and DSF are reported in **Table 2**. DRMs showed a SM pattern very similar to that of whole cell.

In rat cerebellar granule cells, we found 35 species of Cer attributable to the combination of 8 long-chain bases and 19 fatty acids (**Table 3**). The total content of Cer in these cells was 3.643 ± 0.510 nmol/mg cell protein, in agreement with previous observations (36–38). The main species identified were those containing d18:1 sphingosine in association with 16:0, 18:0, and very long fatty acids. Only minor differences between patterns from DRMs and DSF were observed. The Cer content in DRMs determined by MS was 56% of the total.

In the same cells, the total cell content of SM was 12.706 ± 2.280 nmol/mg cell protein, in agreement with previous observations (37–40), and we characterized nine species differing in their Cer structure for the combination of five long-chain bases and two fatty acids (**Table 4**). The SM d18:1,18:0 followed by d18:1,16:0 were the main two species in rat neurons, and they represent 92% of total SM. The species with d18:1,16:0 was more enriched in DRMs than that with d18:1,18:0.

In some cells and tissues, Cer moieties containing hydroxylated fatty acids, mainly belonging to galactoseries sphingolipids, have been described. We did not find any HPLC position and fragmentation pattern associated with hydroxylated Cer or SM in our cells. The sensitivity of our

TABLE 2. Pattern of SM species in cultured human fibroblasts, and distribution between DRMs and DSF

Species	Homogenate	DRMs	DSF
	pmol/mg protein	%	%
d18:1,14:0	126	89	11
d16:1,16:0	112	~100	tr
d17:1,16:0	239	94	6
d18:2,16:0	254	89	11
d18:1,16:0	19,351	90	10
d18:0,16:0	528	84	16
d18:1,17:0	254	89	11
d18:1,18:0	446	75	25
d18:1,22:1	29	tr	~100
d18:2,22:0	239	94	6
d18:1,22:0	280	80	20
d18:1,23:1	29	tr	~100
d18:1,23:0	29	tr	~100
d18:1,24:2	126	89	11
d18:1,24:1	1,593	91	9
d18:2,24:0	294	77	23
d18:1,24:0	1,242	81	19
d18:1,25:1	tr	—	tr

SM, sphingomyelin. Data are means of four experiments with SD in the range of ±15%.

TABLE 3. Pattern of Cer species in rat cerebellar granule cells differentiated in culture, and distribution between DRMs and DSF

Species	Homogenate	DRMs	DSF
	pmol/mg protein	%	%
d18:1,14:0	33	58	42
d18:1,15:0	18	50	50
d18:1,16:1	18	50	50
d18:0,16:1	tr	—	tr
d16:1,16:0	47	70	30
d17:1,16:0	28	67	33
d18:2,16:0	212	49	51
d18:1,16:0	542	58	42
d18:0,18:2	tr	tr	—
d16:0,18:1	33	58	42
d18:1,18:1	14	tr	~100
d18:0,18:1	85	72	28
d16:1,18:0	47	70	30
d18:2,18:0	161	65	35
d18:1,18:0	1,080	58	42
d16:0,20:1	tr	—	tr
d16:1,20:0	tr	—	tr
d18:1,22:1	tr	tr	—
d18:2,22:0	23	39	61
d18:1,22:0	94	55	45
d18:0,23:1	23	39	61
d18:1,23:0	33	58	42
d18:2,24:1	52	37	63
d18:1,24:1	462	49	51
d18:0,24:1	47	70	30
d16:1,24:0	28	68	32
d18:2,24:0	104	50	50
d18:1,24:0	170	61	39
d18:0,24:0	75	56	44
d18:0,25:1	tr	—	tr
d18:1,25:0	33	58	42
d18:0,26:1	23	39	61
d20:0,26:1	tr	—	tr
d18:1,26:0	57	58	42
d20:1,26:0	33	58	42

Data are means of three experiments with SD in the range of ±15%.

methods is on the order of a few picomoles; therefore, if present, these compounds are in traces under this detection limit.

[1-^{3}H]Cer and [1-^{3}H]SM distribution between DRMs and DSF

Cell sphingolipids were metabolically radiolabeled by a 2 h pulse with [1-^{3}H]sphingosine followed by a 48 h chase.

TABLE 4. Pattern of SM species in rat cerebellar granule cells differentiated in culture, and distribution between DRMs and DSF

Species	Homogenate	DRMs	DSF
	pmol/mg protein	%	%
d16:1,16:0	75	tr	~100
d17:1,16:0	99	76	34
d18:2,16:0	99	76	34
d18:1,16:0	3,495	71	29
d18:0,16:0	127	59	41
d16:1,18:0	269	~100	tr
d18:2,18:0	165	68	32
d18:1,18:0	8,151	53	47
d18:0,18:0	226	67	33

Data are means of three experiments with SD in the range of ±15%.

Under these experimental conditions, in agreement with previous studies (24, 25), sphingosine was taken up by the cells and entered into the sphingolipid metabolic pathways, and all of the sphingolipids were radiolabeled. In addition, some radioactive phosphatidylethanolamine was recognized. This is attributable to the recycling of the radioactive ethanolamine produced in the catabolism of [1-^{3}H]sphingosine.

Figure 3 shows the high performance TLC radioactive patterns within fractions collected after cell lysis and centrifugation for DRMs preparation. Fractions 4–6 are the low-density fractions corresponding to DRMs. Phosphatidylethanolamine, in agreement with previous data (24, 25), was largely a component of the high-density fractions. **Figure 4** shows the percentage distribution within fractions of proteins and radioactive lipids. Glycolipids and SM were highly enriched in DRMs from both cell systems. DRM fractions were also enriched in Cer, but to a lesser extent with respect to other sphingolipids. In addition, DRMs from fibroblasts were less enriched in Cer than those prepared from neurons. Cer distributions were confirmed using the solvent system ethyl acetate-chloroform-methanol-50 mM aqueous KCl. Using this solvent system, Cer showed a TLC retention factor of ~0.5, while all other radiolipids remained at the origin (data not shown).

On the basis of the sphingolipid distribution within fractions and collecting together fractions 4, 5, and 6 as DRMs, we determined that DRMs from fibroblasts and neurons contained 77, 81, and 42% and 74, 81, and 61% of total cell glycolipids, SM and Cer, respectively. Radiochemical data for Cer and SM are in good agreement with those obtained by mass spectrometry.

DISCUSSION

Sphingolipids are components of all vertebrate cell membranes. They participate, together with cholesterol, in the organization and stabilization of areas of the membrane with molecular composition and physicochemical properties distinct from the surrounding membrane environment. These portions of the membrane are resistant to detergent solubilization and are thus called DRMs.

In DRMs, several proteins involved in cell signaling are localized. DRMs and their sphingolipid composition are considered responsible for the modulation of these protein activities. In addition to this, we note that several sphingolipid hydrolases, such as sialidase Neu3, sphingomyelinase, β-galactosidase, β-glucosidase, and β-hexosaminidase (6, 41, 42), were recently found associated with plasma membranes and that, among these enzymes, sialidase Neu3 and sphingomyelinase were found associated with the membrane lipid domains (42–44). These enzymes have been demonstrated to be active on natural substrates and, in the case of Neu3, to work through cell-to-cell contacts (45). Thus, changes of enzyme activities are believed to be responsible for changes in the plasma membrane lipid domain composition and for the subsequent modulation of cell signaling processes.

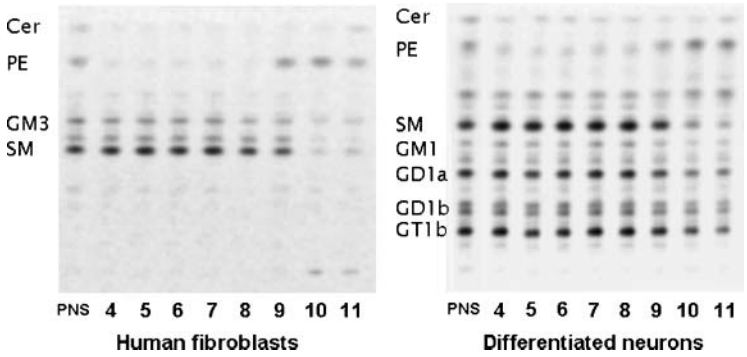

Fig. 3. High performance TLC radioactive lipid patterns of cultured human fibroblasts (left) and differentiated rat cerebellar granule cells (right) fed with [1-^{3}H]sphingosine. The reported patterns are representative of tens of experiments from different cell lines or preparations. Lanes 4–11 show fractions collected from the discontinuous sucrose gradients after ultracentrifugation of detergent cell lysate. Approximately 1,000 dpm/lane were applied. Radioactive lipids were detected by digital autoradiography (48 h acquisition) using a Beta-Imager 2000 instrument (Biospace). PE, phosphatidylethanolamine; PNS, postnuclear subcellular fraction.

For this reason, sensitive methods giving precise information on the lipid structures and their distributions are necessary.

The addition of tritium-labeled sphingosine to cells in culture is a good tool to study all membrane sphingolipids (Fig. 3). Sphingosine is very rapidly taken up by the cells and inserted into the metabolic sphingolipid pathway. Thus, even if this method does not give any information about the amount of each lipid in the cells, it represents a useful and reliable tool for the analysis of sphingolipid distribution among different cell fractions. We used this method to determine the distribution of radioactivity associated with each lipid within each fraction, calculated as percentage value of total radioactivity associated with that specific lipid in the total lysate. Thus, this distribution cannot be affected by the different rates of synthesis or degradation that are typical of each lipid. This method has several advantages: its very high sensitivity, the reduced manipulation of samples before analysis, and the possibility to check the quantitative recovery at each step of the experimental procedure. Nevertheless, no information can be achieved with such a procedure on the structure of the lipid moiety of sphingolipids.

The structure of the sphingolipid molecular species, homogeneous in the head group but heterogeneous in the lipid moiety, was determined by ESI-MS. ESI-MS has been shown to be sensitive enough to detect down to 5 pmol of sphingolipids. Moreover, this technology allows analysis of labile molecular ions that are not easily detectable with MS systems using relatively high energy, and collision-induced dissociation of precursor ions generated from ESI often resulted in novel product ion patterns.

Here, we especially aimed to compare the species patterns of DRMs with those of DSF. Absolute quantification of sphingolipids by MS requires the use of a series of internal standards different in the hydrophobic moiety from natural species. The choice of standards also has to take into account that absolute values of species with a carbon content far from that of the internal standard could be slightly different (27). For our MS analysis, we used synthetic Cer and SM containing d18:1 long-chain base and C17:0 fatty acid.

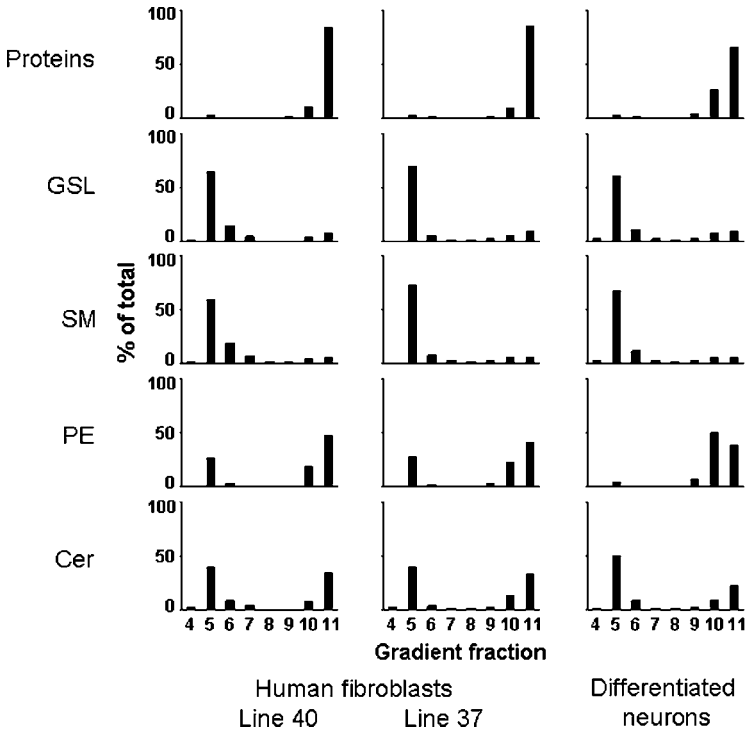

Fig. 4. Protein, sphingolipid, and phosphatidylethanolamine (PE) distribution within fractions collected after discontinuous sucrose gradient ultracentrifugation of detergent cell lysate from human fibroblasts and primary cultured rat neurons (see Materials and Methods). Data from two different fibroblast lines are shown. Protein data are given as mass percentage of the total, and lipid data are given as radioactivity percentage of the total. GLS, glycosphingolipids.

The Cer and SM species were identified according to the MS fragmentation ion schemes reported in Figs. 3, 4, as reported previously (46–48). We determined up to 35 species of Cer and 18 species of SM. Some of these species were present in traces, but their structures could be identified by mean of sequential MS fragmentations.

Together with the confirmation that MS is a very suitable tool for lipidomic analyses (27, 46–52), the most important information from this study is related to the species patterns of Cer and SM in DRMs.

MS analyses gave results on the total enrichment of Cer and SM in DRMs that were comparable to those obtained using radiochemical procedures. Some differences are probably related to the fact that by radiochemical procedure we can follow only, or mainly, species containing d18:1 sphingosine. Interestingly, both procedures confirmed that Cer was much less enriched in DRMs compared with all other sphingolipids. This is in agreement with previous observations (53). In addition to these results, by means of MS analysis, very important information on the species distribution was revealed: *1*) all of the species of SM were highly enriched in DRMs prepared from fibroblasts, and the patterns of SM species in the DSF and insoluble cell fractions were very similar; *2*) in rat neurons, the main SM species (d18:1,18:0) were less enriched in the DRMs with respect to the other species, being equally distributed between DRMs and DSF; *3*) there were significant differences between patterns of DRMs and DSF from neurons; and *4*) there were relevant differences between patterns of DRMs and DSF from fibroblasts. These findings indicate that significant differences exist in the distribution of Cer and SM species between DRMs and other cell membranes and that these differences are influenced by cell type.

SM hydrolysis is regarded as one of the main metabolic mechanisms for the generation of bioactive Cer, and the conversion of SM to Cer is thought to represent a way to alter the lateral organization of specific membrane areas, thus affecting their organization and biological function (e.g., allowing the coalescence of preexisting "resting" lipid rafts into functionally active signaling platforms). Because of the highly hydrophobic nature of Cer, it can be expected that the structure of either the sphingosine or the fatty acid portion of this molecule might play a relevant role in these events. Thus, the generation of peculiar species of Cer from SM, by activation of a membrane-associated sphingomyelinase, yields a new Cer pattern that might be functional for changes in the segregation of this molecule within the membrane with respect to that of SM upstream of Cer production.

In conclusion, our results suggest that MS is a useful tool with which to study Cer and SM species patterns and their changes attributable to metabolic processes.

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