

Short communication

**THE CERAMIDE STRUCTURE OF GM1 GANGLIOSIDE  
 DIFFERENTLY AFFECTS ITS RECOVERY IN LOW-DENSITY  
 MEMBRANE FRACTIONS PREPARED FROM HL-60 CELLS WITH  
 OR WITHOUT TRITON-X100**

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**Abstract:** Gangliosides are characteristically enriched in various membrane domains that can be isolated as low density membrane fraction insoluble in detergents (detergent-resistant membranes, DRMs) or obtained after homogenization and sonication in 0.5 M sodium carbonate (low-density membranes, LDMs). We assessed the effect of the ceramide structure of four [<sup>3</sup>H]-labeled GM1 ganglioside molecular species (GM1s) taken up by HL-60 cells on their occurrence in LDMs, and compared it with our previous observations for DRMs. All GM1s contained C18 sphingosine, which was acetylated in GM1(18:1/2) or acylated with C<sub>14</sub>, C<sub>18</sub> or C<sub>18:1</sub> fatty acids (Fas),

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Abbreviations used: cPMs – crude plasma membranes; CT – cholera toxin; DRMs – detergent-resistant membranes; Fa – fatty acid; GM1 ganglioside – Galβ3GalNAcβ4 (Neu5Acα3)Galβ4GlcCer (GM1s are abbreviated according to Palestini *et al.* [39] as follows: GM1(18:1/2), GM1 with N-acetylated C18 sphingosine; GM1(18:1/14), GM1 with myristic acid-acylated C18 sphingosine; GM1(18:1/18), GM1 with stearic acid-acylated C18 sphingosine; and GM1(18:1/18:1), GM1 with oleic acid-acylated C18 sphingosine); LDMs – low-density membrane fraction; *ld* – liquid disordered; *lo* – liquid ordered; medium H – RPMI 1640 medium containing 10 mM Hepes buffer, pH 7.3, and 5 μg/ml insulin and transferrin; PBS-G – PBS containing 0.1% gelatin; sodium carbonate buffer – a solution consisting of 2.5 mM Tris, 500 mM Na<sub>2</sub>CO<sub>3</sub>, 5 mM NaCl, 2.5 mM EDTA, pH 11.0, 2 mM Pefabloc SC and chymostatin, leupeptin, antipain, and pepstatin, each at 5 μg/ml; TX – Triton X-100

respectively in GM1(18:1/14), GM1(18:1/18) or GM1(18:1/18:1). The recovery of the GM1s in the LDMs was unrelated to their preference for the liquid-ordered phase, and differed from that reported for DRMs in terms of dependence on the length and saturation of the Fas and cholesterol content in the cell membranes. Sonication resulted in the redistribution of GM1s from the LDMs to membranes of higher buoyant density. This process depended on the ceramide structure of the GM1s, with GM1(18:1/14) recovered in the highest proportion; on the intensity of sonication; and on the character of the sample. The greater recovery of GM1(18:1/14) in LDMs may be due to its enrichment in membrane domains different from those containing the other GM1s. Cross-linking all the GM1s with cholera toxin increased their resistance to sonication-induced redistribution to membranes of higher density. Extraction of sonicated samples containing GM1(18:1/18) with Triton X-100 resulted in the recovery of at least 75% of this ganglioside in the low density DRMs. Moreover, sonication strongly reduced the LDM CD59 content, but did not significantly affect that of CD14.

**Key words:** Ceramide, Gangliosides, GM1, Membrane domains, Myristic acid, Sonication

## INTRODUCTION

Gangliosides, sialic acid-containing glycosphingolipids, are the most complex cell lipids [1-3] with a propensity to occur in membranes as microdomains [4-6]. In cell membranes, gangliosides are not only directly involved in signal transduction [7-9], but they also affect the association of proteins with various domains, and consequently the activity of those proteins [10-13]. Apart from its physiological effects [14, 15], the ganglioside GM1, due to its specific binding by the cholera toxin, is frequently used as a marker in studies on membrane domains. Therefore, the objective of our previous [16] and current studies was to find out if the structure of the ceramide moiety of tritium-labeled GM1 ganglioside taken up by HL-60 cells from their culture medium affects its non-random distribution in the membranes. We previously demonstrated that the length and unsaturation of the fatty acid residue of GM1 affects its occurrence in the detergent-resistant membrane fraction (DRM) [16]. However, detergent extraction has both merits and limitations as a method to study membrane domains [17-20].

The objective of this study was to find out if the ceramide structure of GM1, previously found to affect its occurrence in DRMs, also influences its abundance in the low-density membrane fraction (LDMs) prepared with a detergent-free method [21]. We determined the abundance of the following [<sup>3</sup>H]GM1s in the LDMs: GM1(18:1/2), which is not synthesized by cells and is susceptible to detergent extraction; GM1(18:1/18:1), which contains unsaturated oleic (*cis*-9-octadecenoic) acid, and is significantly less resistant to detergent extraction than GM1s with saturated fatty acids not shorter than C<sub>12</sub>; GM1(18:1/14), which is resistant to detergent extraction to the same extent as GM1s with long-chain

saturated fatty acids; and GM1(18:1/18), the major GM1 of the bovine brain, which is resistant to detergent extraction, and is used in most experiments on the uptake of exogenous gangliosides [22, 23]. We detected that sonication decreases the recovery of GM1s in LDMs, so we determined the effect of this treatment on CD59 and CD14, GPI-anchored proteins, enriched in the DRM fraction of HL-60 cells [24] and supposed to occur in the same membrane domains as gangliosides [10].

## MATERIALS AND METHODS

Antibodies were purchased from the following suppliers: goat anti-human CD59 polyclonal antibody (AF 1987), R&D Systems; rabbit anti-CD14 polyclonal antibody (M-3-5), Santa Cruz Biotechnology; HRP-conjugated goat anti-rabbit IgG antibody, Jackson ImmunoResearch; HRP-conjugated rabbit anti-goat immunoglobulins, DacoCytomation.

Protease inhibitors were obtained from Merck; the cholesterol assay kit from Boehringer; and cholera toxin, Sigma-Fluor liquid scintillation mixture, and the remaining chemicals from Sigma-Aldrich. The GM1s with different fatty acids were the same preparations as described in [16]. After labeling with tritium [25], they were purified and characterized as detailed in [16]. The specific radioactivity of the [<sup>3</sup>H]GM1s used in this study ranged from 2.0 to 3.6 Ci/mmmole, while the purity exceeded 99% as determined after high performance thin layer chromatography by Phosphor imager analysis.

### Cell culture and uptake of [<sup>3</sup>H]GM1 by cells

Human promyelocytic leukemia HL-60 cells obtained from ATCC were cultured at 37°C under 5% CO<sub>2</sub> in RPMI 1640 medium containing 20% fetal calf serum (both from GIBCO/BRL Life Technologies), supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin, to a density of 1-1.5 ×10<sup>6</sup>/ml, and further treated as described in [16]. [<sup>3</sup>H]GM1s were added to the cells in RPMI 1640 medium without serum but containing 10 mM Hepes buffer, pH 7.3, and 5 µg/ml of insulin and transferrin, to final concentrations of 0.5 µM or less, so that their uptake after 1 h did not exceed 1 fmole/10<sup>4</sup> cells [16]. Following incubation with [<sup>3</sup>H]GM1s, the cells were washed three times with PBS-G by centrifugation.

### Preparation of LDMs

To prepare the LDMs, the procedure of Song *et al.* [21] was used with a minor modification to increase the reproducibility of the results. Briefly, after incubation with [<sup>3</sup>H]GM1s and washing with PBS-G, 8×10<sup>7</sup> cells were suspended in 1.3 ml of a solution consisting of 5 mM Tris, 10 mM NaCl and 5 mM EDTA, pH 7.5, also containing 4 mM Pefabloc SC and chymostatin, leupeptin, antipain, and pepstatin, each at 10 µg/ml. The sample was left on ice for 15 min and after the addition of 1.3 ml of 1 M Na<sub>2</sub>CO<sub>3</sub>, pH 11.1, the suspension was homogenized by pushing through syringe needles [16]. For sonication, an

ultrasonic disintegrator model Mk2, 150 W (MSE, England) with a 9.5-mm diameter titanium probe was used. The sample, in a polypropylene conical tube, was placed in ice and subjected to one or three bursts of sonication at 20 kHz, with a peak-to-peak amplitude at the tip of the probe of about 66  $\mu\text{m}$  (MSE manual), and the tip of the probe inserted about 5 mm into the sample. Sonication bursts lasting 20 s each were interrupted by short intervals when the probe was cooled in an ice slurry. After homogenization, followed where indicated by sonication, a 1.5 ml aliquot was withdrawn, mixed in an ultracentrifuge tube with 1.5 ml of 86% sucrose in 150 mM NaCl buffered with 25 mM MES, pH 6.5, overlaid with 6 ml of 35% sucrose and topped with about 3 ml of 5% sucrose, both solutions in 25 mM MES, pH 6.5, 150 mM NaCl and 250 mM  $\text{Na}_2\text{CO}_3$ . After centrifugation (Beckman SW41 rotor, 36,000 rpm, 16-18 h), twelve gradient fractions, about 1 ml each, were collected from the top of the tube, and their radioactivity, and cholesterol and protein contents were determined.

#### **Preparation of the crude plasma membrane fraction, cPMs**

After incubation with [ $^3\text{H}$ ]GM1s,  $1 \times 10^8$  cells were suspended in 1.1 ml of a solution consisting of 5 mM Tris, 10 mM NaCl and 5 mM EDTA, pH 7.5, also containing 4 mM Pefabloc SC and chymostatin, leupeptin, antipain, and pepstatin, each at 10  $\mu\text{g}/\text{ml}$ . After 15 min on ice, the sample was mixed with 1.1 ml of a solution containing 20 mM Tris, 100 mM NaCl, 5 mM EDTA and 200 mM  $\text{Na}_2\text{CO}_3$ , adjusted to pH 11.0 with NaOH. The cells were homogenized as described for the preparation of LDMs. 2 ml of the homogenate was mixed in an ultracentrifuge tube with 2 ml of 86% sucrose, and overlaid with 5 ml of 40% sucrose followed by about 3 ml of 5% sucrose. The sucrose solutions were prepared in a 25 mM Tris, 150 mM NaCl, 5 mM EDTA, pH 7.5 buffer. After centrifugation as above, twelve gradient fractions were collected from the top of the tube and their radioactivity, and cholesterol and protein contents were determined. The cPM fraction, collected at the interface between the 5% and 40% sucrose solutions, was diluted with PBS containing 1 mM PMSF and recovered after centrifugation (SW41 rotor, 36,000 rpm, 45 min). The pellet, containing cPMs corresponding to about  $8 \times 10^7$  cells, was suspended in 0.5 ml of 0.5 M  $\text{Na}_2\text{CO}_3$ , pH 11.0. For experiments on the redistribution of [ $^3\text{H}$ ]GM1s from the cPMs to the membranes of the whole-cell homogenate, these aliquots were used directly, while for sonication of the cPMs alone, they were diluted with 2.1 ml of 0.5 M  $\text{Na}_2\text{CO}_3$ .

#### **SDS/PAGE and immunoblot analysis**

LDM fractions collected after density gradient centrifugation were pooled and diluted with water containing 1 mM PMSF. After centrifugation (SW41 rotor, 36,000 rpm, 1 h), membranes were recovered in the pellets while the supernatants were discarded after it was checked that they contained none of the analyzed proteins. The samples were dissolved in 10% SDS, and the aliquots were mixed with equal volumes of Laemmli electrophoresis buffer with (for

CD14) or without (for CD59) 2-mercaptoethanol. The proteins were resolved by SDS/PAGE (10% gel) and transferred onto nitrocellulose (Schleicher & Schuell). The membranes were blocked overnight in a cold room with 5% BSA in PBS containing 0.05% Tween 20. Anti-CD59 or anti-CD14 antibodies, respectively diluted 1:1000 or 1:500, were added and, after incubation for 2 h at room temperature, the nitrocellulose membranes were washed extensively with PBS containing 0.05% Tween 20. After the addition of HRP-conjugated secondary antibodies, incubation for 1 h at room temperature, and washing with PBS containing 0.05% Tween 20, the signal from HRP was detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce).

#### **Other procedures**

To verify if the radioactivity determined in these studies is associated with the [<sup>3</sup>H]GM1s taken up by the cells, homogenized and sonicated samples were dialyzed, lyophilized and extracted with chloroform and methanol [16]. Aliquots of the extracts, 50,000 dpm each, were analyzed on HPTLC in a chloroform/methanol/0.25% aqueous CaCl<sub>2</sub> (60:35:8) solvent system. The radioactive material was detected on the plates by fluorography.

The relative abundance of CD59 and CD14 proteins in the LDMs was determined densitometrically on immunoblots with Kodak 1D image analysis software. For the cross-linking of the [<sup>3</sup>H]GM1s taken up by the cells, CT at a final concentration of 20 nM was used. The remaining procedures were as described [16]. Statistical analyses were performed using Student's *t* test with the significance level at *p* < 0.05.

## **RESULTS AND DISCUSSION**

### **The abundance of [<sup>3</sup>H]GM1s in LDMs is affected by their ceramide structure but follows a different pattern than that detected for DRMs**

After density gradient centrifugation, LDMs were collected at the boundary between the 5% and 35% sucrose solutions (fractions 2-5, Fig. 1). The abundance of [<sup>3</sup>H]GM1s in the LDMs depended on their ceramide structures, but this relationship was not the same as that reported earlier for DRMs, where the formation of the *lo* phase seems important [16]. LDMs prepared without sonication contained similar amounts of GM1(18:1/14) and GM1(18:1/2), but less of GM1(18:1/18) and GM1(18:1/18:1), the latter two occurring in a similar proportion (Tab.1). After one, and even more evidently after three bursts of sonication, the recovery from the LDMs of all [<sup>3</sup>H]GM1s as well as of cholesterol and protein were significantly reduced. However, the extent of this decrease was not the same for all [<sup>3</sup>H]GM1s. It was the most pronounced for [<sup>3</sup>H]GM1(18:1/2), and the least pronounced for [<sup>3</sup>H]GM1(18:1/14). Again [<sup>3</sup>H]GM1(18:1/14) was significantly more abundant in the LDMs than GM1(18:1/2) or GM1(18:1/18), but not than GM1(18:1/18:1). We previously reported that GM1(18:1/2) and GM1(18:1/18:1) occur in DRMs in a much lower proportion than GM1(18:1/18) [16].

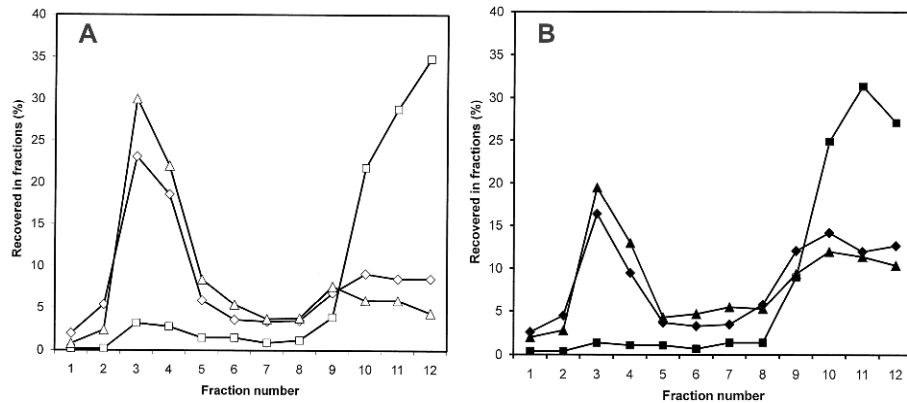


Fig. 1. The effect of sonication on the distribution of [ $^3\text{H}$ ]GM1(18:1/18), cholesterol and protein in sucrose density gradient fractions prepared from HL-60 cells. About  $8 \times 10^7$  cells were incubated with [ $^3\text{H}$ ]GM1(18:1/18) for 1 h at  $37^\circ\text{C}$ , washed with PBS-G, and homogenized in sodium carbonate buffer. A – A 1.5-ml aliquot of the homogenate was analyzed by sucrose density gradient centrifugation as described in the Materials and Methods. Radioactivity ( $-\circ-$ ), and cholesterol ( $-\Delta-$ ) and protein contents ( $-\square-$ ) were determined in the fractions. B Before centrifugation, the cell homogenate was subjected to three bursts of sonication. The radioactivity ( $-\bullet-$ ), and cholesterol ( $-\blacktriangle-$ ) and protein contents ( $-\blacksquare-$ ) were determined. The values are expressed as a percentage of the sum of all the fractions except the sediment. The figure represents a single, typical experiment. Assays were run in triplicate with values differing by less than 10% from the means. The data from three such experiments is presented in Tab. 1.

Tab. 1. The effect of sonication on the recovery of different [ $^3\text{H}$ ]GM1 molecular species, cholesterol, and protein from LDMs. Cells were incubated with [ $^3\text{H}$ ]GM1s for 1 h at  $37^\circ\text{C}$  and further treated as specified in the Materials and Methods. The cell homogenate prepared from  $8 \times 10^7$  cells was analyzed by density gradient centrifugation directly or after one ( $1\times$ ) or three ( $3\times$ ) bursts of sonication as specified in the Materials and Methods. Where indicated, the cells were incubated with 20 nM CT before homogenization, followed by three bursts of sonication. The values represent the percentage of a given compound recovered in LDMs in relation to all the gradient fractions except the sediment, and are shown as means ( $\pm\text{SD}$ ) from three independent experiments. ND, not determined.

	Recovery, (% $\pm\text{SD}$ ) in LDMs			
	No sonication	Sonication, $1\times$	Sonication, $3\times$	CT preceding sonication, $3\times$
GM1(18:1/2)	64.4 $\pm$ 3.9	45.0 $\pm$ 2.6	25.8 $\pm$ 2.9	68.8 $\pm$ 2.4
GM1(18:/14)	65.9 $\pm$ 1.9	58.1 $\pm$ 1.4	41.5 $\pm$ 1.8	68.5 $\pm$ 1.5
GM1(18:1/18)	58.6 $\pm$ 1.2	50.1 $\pm$ 2.6	31.3 $\pm$ 4.0	66.7 $\pm$ 2.2
GM1(18:1/18:1)	60.6 $\pm$ 1.7	52.9 $\pm$ 2.6	35.6 $\pm$ 2.6	68.2 $\pm$ 2.8
Cholesterol	65.7 $\pm$ 3.0	59.6 $\pm$ 3.2	40.5 $\pm$ 2.2	ND
Protein	8.5 $\pm$ 0.8	6.1 $\pm$ 0.8	4.2 $\pm$ 0.4	ND

As shown in Tab. 1, this is not the case for the LDMs. Irrespective of the intensity of sonication, the differences in the abundance in LDMs between these three GM1s were not significant. Another property differentiating LDMs from DRMs is the effect of cholesterol depletion. Previously, it was detected that depleting about 70% of the cell cholesterol did not significantly reduce the occurrence in DRMs of GM1s acylated with long-chain saturated fatty acids, whereas it diminished the recovery of GM1(18:18:1) and even more strongly that of GM1(18:1/2) [16]. As determined for LDMs in this study, the effect of cholesterol depletion was not significant not only for GM1(18:1/18) but also for GM1(18:1/18:1) and GM1(18:1/2) (not shown).

Interestingly, cross-linking with CT of all the GM1s taken up by cells significantly increased their recovery in LDMs (Tab. 1), just as it enhanced their resistance to solubilization with TX [16]. It seems possible that cross-linking results in the redistribution of all the GM1 molecular species into domains more resistant not only to detergent extraction but also to sonication.

#### **Sonication reduces the recovery of CD59 from LDMs**

Since gangliosides are supposed to occur in membrane domains also occupied by GPI-anchored proteins [10], we determined the effect of sonication on the recovery of CD59 and CD14 in LDMs. With proteomic analysis, these proteins were recently found to be enriched in the DRMs of HL-60 cells [24].

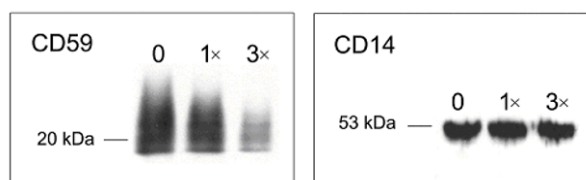


Fig. 2. The effect of sonication on the recovery of CD14 and CD59 in LDMs. Aliquots of LDMs obtained from a cell homogenate without sonication (lane 0); after a single burst of sonication (lane 1×); and after three bursts of sonication (lane 3×) were analyzed with SDS/PAGE. After immunoblotting, CD14 and CD59 were detected as described in the Materials and Methods. Similar effects were found with LDMs prepared in three independent experiments. The multiple bands for CD59 are due to differences in the glycosylation.

As shown in Fig. 2, sonication strongly decreased the LDM content of CD59 but not of CD14. After one and three bursts of sonication, the recoveries of CD59 were respectively reduced to 64.7% ( $\pm 13.9$ ,  $n = 3$ ) and 18.9% ( $\pm 8.3$ ,  $n = 3$ ) as compared with unsonicated samples. For CD14, the effects of sonication were not significant. This is an interesting observation as it reveals that sonication affects the recovery in LDMs not only of exogenous GM1s but also of CD59, a DRM marker protein. On the other hand, the not significant effects on CD14 indicate that these proteins may differ in their distribution between various membrane domains. Indeed, it has been reported that DRM markers can be

detected in separate domains in intact cells [26]. Moreover, various glycoforms of CD52, also a GPI-anchored protein, have been found to associate with different DRM fractions [27]. It is also important to note that sonication reduced the whole protein content of LDMs (Tab. 1) while not significantly affecting CD14. Thus, a roughly two-fold enrichment of this protein in LDMs could be achieved.

### **Sonication results in the redistribution of [<sup>3</sup>H]GM1s from the LDMs to membranes of higher buoyant density**

From our earlier experiments, it could be inferred that GM1s taken up by HL-60 cells reside mostly in the plasma membrane, thus mimicking endogenous gangliosides [16]. To learn more about the relationship between the ceramide structure of [<sup>3</sup>H]GM1s and their redistribution from the LDMs to fractions of higher buoyant density, for some experiments, we used not the whole-cell homogenate, but the less heterogeneous cPMs. LDMs obtained from cPMs without sonication had at least 87% ( $\pm 5.8$ ) of the radioactivity detected in the gradient fractions, in a similar proportion for all the [<sup>3</sup>H]GM1s (Tab. 2). A single burst of sonication significantly reduced these values but, surprisingly, an additional two bursts had little effect. After three bursts, the relationship between the occurrence of different [<sup>3</sup>H]GM1s in LDMs and their ceramide structure followed a complex pattern. GM1(18:1/14) was significantly more abundant than GM1(18:1/2) and GM1(18:1/18), while the difference between GM1(18:1/14) and GM1(18:1/18:1) was not significant. Again, just as for the whole-cell homogenate, GM1(18:1/18), GM1(18:1/2), and GM1(18:1/18:1) were found in LDMs in a similar proportion.

The decreased recovery of [<sup>3</sup>H]GM1s in LDMs after sonication could have resulted from the release of plasma membrane fragments containing the labeled gangliosides and their coalescence with cellular membranes of higher buoyant density. To verify this assumption, we prepared cPMs containing [<sup>3</sup>H]GM1s and mixed them with a whole-cell homogenate containing no radioactive material. The data from Tab. 3 indicates that this treatment did not affect the recovery of [<sup>3</sup>H]GM1s in LDMs prepared without sonication. However, when this mixture was sonicated, significantly less [<sup>3</sup>H]GM1s were detected in LDMs than in experiments where cPMs were sonicated separately (Tab. 2 versus Tab. 3).

The effect of sonication depended on the ceramide structure of the GM1s: after three bursts GM1(18:1/14) remained significantly more abundant in the LDMs than GM1(18:1/2) and GM1(18:1/18), but not than GM1(18:1/18:1) (Tabs 1 and 3). Also, the data presented in Tab. 2 indicates that sonication of cPMs results in the release and redistribution of [<sup>3</sup>H]GM1s from lighter to heavier membrane fractions. A compositional analysis of the LDMs prepared from cPMs without and after sonication revealed that about 49% of the protein, 12% of the cholesterol, and 11-13% of the [<sup>3</sup>H]GM1s in cPMs occur in membrane fractions



Tab. 2. The recovery of different [ $^3\text{H}$ ]GM1 molecular species, cholesterol, and protein in cPMs isolated from HL-60 cells, and in LDMs prepared from cPMs under diverse sonication conditions. Cells were incubated with [ $^3\text{H}$ ]GM1s and used for the preparation of cPMs as described in the Materials and Methods. cPMs corresponding to about  $8 \times 10^7$  cells were used for the isolation of LDMs directly, or after one (1 $\times$ ) or three (3 $\times$ ) bursts of sonication. The radioactivity, and the cholesterol and protein contents were determined in the gradient fractions. The values represent the percentage of a given compound recovered in cPMs or LDMs in relation to all the gradient fractions except the sediment, and are shown as means ( $\pm$ SD) from three independent experiments. Recoveries in cPMs apply to the preparation of this fraction.

	Recovery (% $\pm$ SD) in cPMs	Recovery, (% $\pm$ SD) in LDMs prepared from cPMs following		
		No sonication	Sonication, 1 $\times$	Sonication, 3 $\times$
GM1(18:1/2)	69.6 $\pm$ 3.4	87.0 $\pm$ 5.8	65.0 $\pm$ 5.6	58.5 $\pm$ 2.6
GM1(18:1/14)	76.3 $\pm$ 4.0	89.3 $\pm$ 4.2	71.0 $\pm$ 5.2	70.8 $\pm$ 1.1
GM1(18:1/18)	72.0 $\pm$ 5.6	88.7 $\pm$ 3.2	68.7 $\pm$ 4.6	63.4 $\pm$ 3.2
GM1(18:1/18:1)	70.7 $\pm$ 3.3	87.0 $\pm$ 6.7	69.4 $\pm$ 5.1	63.6 $\pm$ 7.2
Cholesterol	73.6 $\pm$ 4.3	88.3 $\pm$ 3.7	72.9 $\pm$ 2.7	68.7 $\pm$ 2.7
Protein	13.2 $\pm$ 1.1	50.3 $\pm$ 4.9	37.9 $\pm$ 1.2	33.6 $\pm$ 2.9

Tab. 3. The effect of sonication on the redistribution of different [ $^3\text{H}$ ]GM1 molecular species and protein from cPMs to membranes of higher buoyant density. Cells were incubated with [ $^3\text{H}$ ]GM1s and used for preparation of cPMs. cPMs isolated from  $8 \times 10^7$  cells were suspended in 0.5 ml of 0.5 M  $\text{Na}_2\text{CO}_3$  and mixed with 2.1 ml of a whole-cell homogenate in sodium carbonate buffer also prepared from  $8 \times 10^7$  cells containing no [ $^3\text{H}$ ]GM1s. LDMs were isolated directly, or after three bursts of sonication as described in the Materials and Methods. The values represent the percentage of a given compound recovered in the LDMs in relation to all the gradient fractions except the sediment, and are shown as means ( $\pm$ SD) from three independent experiments.

	Recovery, (% $\pm$ SD) in LDMs	
	No sonication	Sonication, 3 $\times$
GM1(18:1/2)	80.4 $\pm$ 6.3	27.9 $\pm$ 2.3
GM1(18:1/14)	86.6 $\pm$ 6.2	43.8 $\pm$ 2.9
GM1(18:1/18)	83.0 $\pm$ 7.5	35.1 $\pm$ 4.5
GM1(18:1/18:1)	83.8 $\pm$ 5.6	36.9 $\pm$ 3.2
Protein	15.1 $\pm$ 0.8	6.1 $\pm$ 0.8

of high buoyant density (Tab. 2). We assume that during the sonication of the cPMs, this fraction represents the “acceptor membranes” for [ $^3\text{H}$ ]GM1s containing microdomains. As can be calculated from the data in Tab. 2, a single burst of sonication removes about 12% of protein, 15% of cholesterol, and about 20% of radioactivity from the LDMs, redistributing it into the heavier “acceptor

membranes". To explain the much smaller effects of additional sonications, several possibilities should be considered, including redistribution of the released material back to the LDMs. The latter would be in keeping with the observation that LDMs prepared from HeLa cells contained mitochondrial proteins [28]. Obviously, the potential "acceptor membranes" in the whole-cell homogenate represent a more bulky and heterogeneous fraction than those in the cPMs. Therefore, sonicating samples containing whole-cell homogenate should result in greater losses of [<sup>3</sup>H]GM1s to the heavier fractions than those observed after the sonication of cPMs. A comparison of the data from Tabs 1 and 3 with that in Tab. 2 supports this possibility.

We do not expect the "acceptor membranes" to be of physiological importance. For cPMs, they could represent a heavier membrane fraction enriched in cytoskeletal elements, while for the whole-cell homogenate, they could also contain fragments of cellular organelles released during harsh sonication. It could be suspected that the difficulties in the preparation of LDMs reported by us result from poorly selected sonication conditions. This does not seem to be the case. Since we did not know the details of the original procedure [21] in the preliminary experiments, we prepared LDMs from [<sup>3</sup>H]GM1(18:1/18)-containing cells changing the sonication amplitude, duration, and the sonifier probe. Under all the applied conditions, the decrease in LDM protein content was accompanied by a similar loss of [<sup>3</sup>H]GM1(18:1/18).

#### **GM1s in LDMs versus DRMs**

It was previously reported that in HL-60 cell membranes, a high proportion of [<sup>3</sup>H]GM1(18:1/18) occurs in DRMs [16]. Assuming that DRMs are not artifacts [29-31], it can be expected that within LDMs, this ganglioside is also enriched in microdomains resistant to detergent extraction. To determine if these domains are not destroyed during sonication, we subjected sonicated, [<sup>3</sup>H]GM1(18:1/18)-containing samples to extraction with TX. As shown in Tab. 4, the detergent extraction of sonicated whole-cell homogenate, cPMs, and a mixture of cPMs with the whole cell homogenate, yielded similar recoveries of [<sup>3</sup>H]GM1(18:1/18) in DRMs, ranging from 75% ( $\pm$  5.4) to 85% ( $\pm$  0.9), not different from the previously reported data on the occurrence of [<sup>3</sup>H]GM1s in this membrane fraction prepared without sonication [16]. As expected, when compared with LDMs, the protein content of DRMs prepared by this procedure was considerably reduced, especially where 1% TX was used (Tab. 4).

Some of the data presented here concurs with previously reported observations on the characteristics of DRMs and LDMs. A typical feature of DRMs is an enrichment of their lipid constituents in saturated fatty acids [32-34] known to promote formation of the *lo* phase [35]. This property is not shared by LDMs, with their high content of arachidonic acid [36]. Thus, the similar abundance of GM1(18:1/2), GM1(18:1/18:1) and GM1(18:1/18) in LDMs, in contrast to their different proportions in DRMs, is not surprising.

Tab. 4. The effect of Triton X-100 on the recovery of [ $^3\text{H}$ ]GM1(18:1/18) and protein in DRMs prepared from sonicated whole cell homogenate, cPMs sonicated separately, and cPMs sonicated with whole cell homogenate. Cells were incubated with [ $^3\text{H}$ ]GM1(18:1/18) and used for the preparation of whole-cell homogenate or cPMs as described in the Materials and Methods. A whole-cell homogenate in 2.6 ml of sodium carbonate buffer or cPMs corresponding to  $8 \times 10^7$  cells suspended in 2.6 ml of 0.5 M  $\text{Na}_2\text{CO}_3$  were subjected to three bursts of sonication. A 1350  $\mu\text{l}$  aliquot was withdrawn, mixed with 150  $\mu\text{l}$  of either 1% or 10% TX, left for 30 min on ice with occasional vortexing, and subjected to density gradient centrifugation. Alternatively, 2.1 ml of whole-cell homogenate prepared from  $8 \times 10^7$  cells containing no radioactive material was mixed with 0.5 ml of the cPM fraction in 0.5 M  $\text{Na}_2\text{CO}_3$  prepared from  $8 \times 10^7$  cells containing [ $^3\text{H}$ ]GM1(18:1/18). The sample was sonicated and treated with TX as above. After centrifugation, the radioactivity and protein levels were determined in gradient fractions. The values represent the percentage of a given compound recovered in DRMs in relation to all the gradient fractions except the sediment, and are shown as means ( $\pm$ SD) from three independent experiments.

Sonicated material	Recovery, (% $\pm$ SD) in DRMs after extraction with			
	0.1% TX		1.0% TX	
	GM1	Protein	GM1	Protein
Cell homogenate	76.5 $\pm$ 5.9	4.1 $\pm$ 0.7	75.0 $\pm$ 5.4	1.9 $\pm$ 0.4
cPMs	84.2 $\pm$ 4.5	19.0 $\pm$ 1.8	78.6 $\pm$ 4.0	15.4 $\pm$ 1.6
cPMs mixed with cell homogenate	83.0 $\pm$ 4.4	6.0 $\pm$ 0.6	85.6 $\pm$ 0.9	4.2 $\pm$ 0.4

Comparing our previous results [16] with these observations, we assume that these GM1s reside within LDMs in microdomains with different resistance to detergent extraction but similar susceptibility to sonication. Also not surprising is the lack of an effect of cholesterol depletion. It was reported by others that this treatment only slightly decreased the association of many proteins with LDMs [28, 37]. The lower losses of GM1(18:1/14) compared to some of the other GM1s from LDMs upon sonication are interesting. This GM1 forms the same number of hydrogen bonds stabilizing it within the membrane as the remaining GM1s. Moreover, containing a shorter fatty acid than GM1(18:1/18), it should be less resistant to release as a monomer. It can be hypothesized that GM1(18:1/14) is enriched in membrane microdomains more resistant to sonication-dependent release, thus different from those in which GM1(18:1/18) is abundant. It remains to be established if these differences, found for cells sonicated on ice in 0.5 M  $\text{Na}_2\text{CO}_3$ , can also be detected under physiological conditions.

It should be mentioned that neither incubation with cells nor the relatively harsh sonication conditions used in this study affected the HPTLC mobility of [ $^3\text{H}$ ]GM1s. In a single experiment, we determined that a lipid extract prepared from sonicated, dialyzed and lyophilized samples contained 87-92% of the radioactivity taken up by cells. Radioactive material thus obtained migrated on HPLC as homogenous bands with the same mobility as the [ $^3\text{H}$ ]GM1s used for incubations.

## CONCLUSIONS

Unlike the case with DRMs [16], the recovery of different GM1s in LDMs does not depend on their preference for *lo* membrane domains. Thus, we assume that LDMs contain membrane microdomains in both the *lo* and *ld* phases. Sonication, successfully employed by Song *et al.* [21] for the purification of caveolae, should be used with caution for the isolation of microdomains enriched with GM1. In spite of its limitations, this procedure allowed us to demonstrate that GM1(18:1/18)-enriched microdomains can withstand relatively harsh sonication and can be recovered in DRMs after detergent extraction. Moreover, sonication made it possible to differentiate between microdomains enriched with GM1(18:1/14) and those where GM1(18:1/18) was more abundant. We assume that GM1s differing in their ceramide structures are unevenly distributed between the various membrane domains, in agreement with the multiple domain model of plasma membrane presented by Babychuk and Draeger [38].

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