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Interactions between gangliosides and proteins in the exoplasmic leaflet of neuronal plasma membranes: A study performed with a tritium-labeled GM1 derivative containing a photoactivable group linked to the oligosaccharide chain

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Interactions between gangliosides and proteins at the exoplasmic surface of the sphingolipid-enriched membrane domains can be studied by ganglioside photolabeling combined with cell surface biotin labeling. In the present paper, we report on the results obtained using a novel radioactive photoactivable derivative of GM1 ganglioside, carrying the photoactivable nitrophenylazide group at the external galactose.

After cell photolabeling with the radioactive photoactivable derivative of GM1 and cell surface biotin labeling, sphingolipid-enriched domains were prepared from rat cerebellar neurons differentiated in culture and further purified by immunoprecipitation with streptavidin-coupled beads. Among proteins belonging to the sphingolipid-enriched domains that were biotin labeled, thus bearing an exoplasmic domain, a few were also cross-linked by the radioactive photoactivable ganglioside. In particular, two protein bands showing apparent molecular mass of 135 and 35 kDa were intensely photolabeled. The 135 kDa protein was immunologically identified as the GPI-anchored neural cell adhesion molecule TAG-1. These data suggest that hydrophilic interaction between the exoplasmic domains of the protein and the ganglioside sialooligosaccharide chain could exist.
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Keywords: **gangliosides, ganglioside-protein interactions, TAG-1, neuronal cells, lipid domains, photolabeling**

Abbreviations: **GM1, II^{3}Neu5AcGg$_4$Cer, ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-Cer; [^{3}H]GM1, [3-^{3}H*(sphingosine)*]GM1, ß-Gal-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-[^{3}H]Cer; [^{3}H]GM1-N$_3$; ß-[6-(2-nitro-4-azidophenyl)]GalN-(1-3)-ß-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-ß-Gal-(1-4)-ß-Glc-(1-1)-[^{3}H]Cer; GalN, 6-aminogalactose; Cer, ceramide, *N*-acyl-sphingosine; [^{3}H]Cer, *N*-acyl-[3-^{3}H]sphingosine Sph, sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-octadecene; DMF, N,N-dimethylformamide; GPI, glucosyl phosphatidyl inositol; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.**

Introduction

It is widely accepted that glycosphingolipids (and in particular gangliosides) at the level of the plasma membrane can affect the biological functions of protein molecules, such as cell surface receptors (*e.g.* tyrosine kinase receptors, including epidermal growth factor receptor, platelet-derived growth factor receptor, Trk family neurotrophin receptors and insulin receptor) or transporters [reviewed in 1–3]. Moreover, gangliosides are known to segregate together with other sphingolipids, cholesterol, saturated-chain phosphatidylcholine,

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and a specific subset of plasma membrane proteins (including receptor and non-receptor tyrosine kinases, G protein-coupled receptors, protein kinase C isoenzymes) within membrane lipid domains ("sphingolipid-enriched membrane domains") [4–8]. Thus, dynamic lateral interactions between gangliosides and proteins belonging to this specific plasma membrane microenvironment could be responsible for the modulation of the functional properties of membrane proteins participating in signal transduction [9,10]. To provide a unifying view on these events at the molecular level, it is necessary to better understand the forces that drive together proteins and glycosphingolipids within the cell membrane and that are able to induce conformational changes in the protein molecule, affecting its biological functions either directly or indirectly, *e.g.* changing its association with regulatory proteins or substrates.

An interesting example of such interactions is represented by the GPI-anchored protein TAG-1, an axonal 135 kDa glycoprotein transiently expressed in neurons and glial cells in the developing mammalian nervous system. TAG-1 is a neural cell adhesion molecule that plays a role in axonal pathfinding and in axoglial interactions [11,12]. In primary cerebellar cultures, TAG-1 interacts with GD3 ganglioside and the Src-family tyrosine kinase Lyn within the sphingolipid-enriched membrane domains [13–16], and antibody-mediated cross-linking of TAG-1 or GD3 induced Lyn activation and the tyrosine phosphorylation of a putative Lyn substrate [13,14,16]. The study of this supermolecular lipid-protein assembly is particularly intriguing from the topological point of view. In fact, TAG-1, a GPI-anchored protein, belongs to the membrane outer lipid layer, while Lyn is a myristoylated protein associated with the membrane inner layer. For both these proteins, it is possible to speculate the existence of hydrophobic interactions with gangliosides, belonging to the outer layer of the membrane, involving TAG-1 phosphatidylinositol fatty acid tail or Lyn myristoyl chain and the ceramide moiety of the ganglioside. By means of cross-linking experiments with photoactivable, radioactive ganglioside derivatives carrying the reactive azide group at the end of the acyl chain, we were able to show that these hydrophobic interactions do actually occur for both TAG-1 and Lyn in the sphingolipid-enriched membrane domains from rat cerebellar neurons differentiated in culture [17–19]. In the case of TAG-1, bearing a domain protruding in the extracellular environment, the question remains to be answered: can hydrophilic interactions with the ganglioside sialyloligosaccharide chain also be invoked? Results from photolabeling experiments showed that TAG-1 was similarly cross-linked by three ganglioside derivatives with different oligosaccharide chains (GM3, GM1, and GD1b) having the photoactivable group at the hydrophobic moiety [18]. In this paper, we describe the use of a novel radioactive photoactivable derivative of GM1 ganglioside, carrying the photoctivable nitrophenylazide group at an external galactosamine (Figures 1 and 2), and we show that TAG-1 in sphingolipid-enriched domains from rat cerebellar neurons is efficiently photolabeled by this derivative.

Materials and methods

Materials

Commercial chemicals were of the highest purity available, common solvents were distilled before use, and water was doubly distilled in a glass apparatus. Trypsin and crystalline bovine serum albumin were from Sigma Chemical Co., reagents for cell culture were from Hy-clone. Sulfo-NHS-biotin and horseradish peroxidase-conjugated streptavidin were from Pierce. Rabbit polyclonal antibody to TAG-1 was raised against baculovirus-produced protein [15]. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology. Streptavidin-coupled magnetic beads (Dynabeads M-280 streptavidin) were from Dynal. [^{35}S]methionine (specific radioactivity 1175 Ci/mmol) was purchased from NEN. GM1 was extracted from bovine brain [20] and purified [21]. Isotopic [3-^{3}H*(sphingosine)*]GM1, [^{3}H]GM1, was prepared by the dichloro-dicyanobenzoquinone/[^{3}H]sodiumborohydride method folowed by HPLC purification [22]. [1-^{3}H]sphingosine (2 Ci/mmol) was prepared as described [7].

Synthesis of photoactivable and radioactive GM1 derivative, [^{3}H]GM1-N_3

The synthesis of [^{3}H]GM1-N_3 having the photoactivable group linked to the oligosaccharide moiety was carried out from [^{3}H]GM1 by combining and adapting experimental procedures previously developed for the synthesis of ganglioside analogues [23, 24]. The general scheme for the preparation of [^{3}H]GM1-N_3 is reported in Figure 2. Characterization of [^{3}H]GM1-N_3 showed a chemical and radiochemical homogeneity over 99% and specific radioactivity of 0.5 Ci/mmol.

Reaction 1: Oxidation of [^{3}H]GM1

[^{3}H]GM1 (0.41 mCi) was dissolved in 500 μl Triton X-100/1-propanol (18 mg/ml) and the mixture was slowly dried under N_2 flux. The residue was dissolved in 500 μl of 25 mM phosphate buffer, pH 7.0, 5 mM EDTA, and 450 mU of galactose oxidase were added. The mixture was stirred at 37°C for 5 h. 450 mU of galactose oxidase were further added and reaction allowed to proceed overnight in the same conditions. The reaction products were characterized by HPTLC (solvent system chloroform/methanol/water, 55/45/10 by vol).

Reaction 2: Reductamination of radioactive GM1 derivative containing the oxidised galactose

The reaction mixture was dried under vacuum and dissolved in 1 ml of 2.5 mM $NaCNBH_3$, 1 M CH_3COONH_4 in methanol, and the reaction was allowed to proceed overnight at room temperature. The reaction mixture was dried under vacuum and applied onto a silica gel 100 column chromatography (60 cm × 1.2 cm), equilibrated and eluted with 1 l chloroform/methanol/water 60/35/5 by volume and then with 1 l chloroform/methanol/water

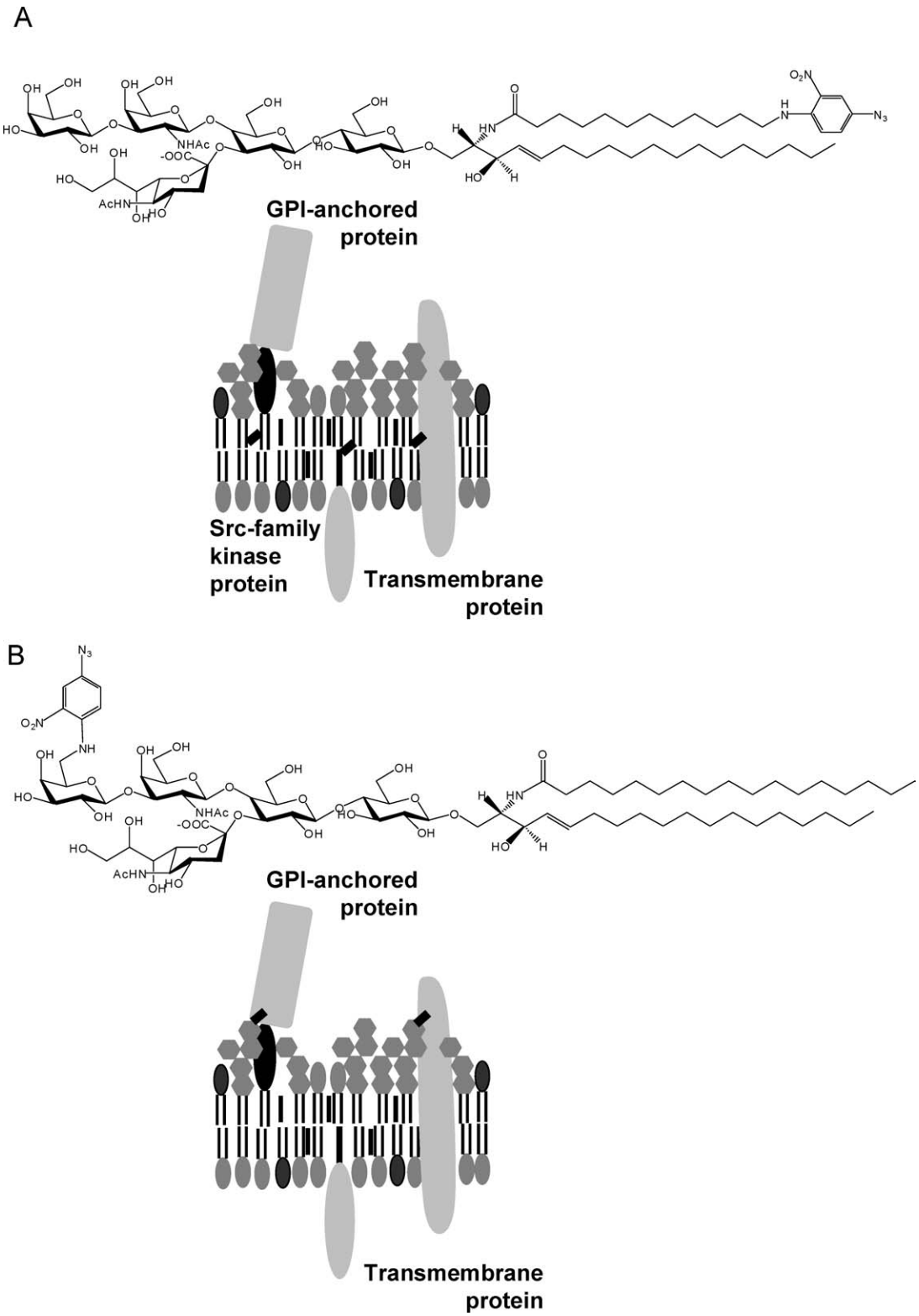

Figure 1. Schematic representation of the possible ganglioside-protein cross-linking in the plasma membrane by cell photolabeling using radioactive photoactivable gangliosides carrying the reactive group ad different moieties of the molecule. Panel A: ganglioside derivatives with the photoactivable group at the end of the ceramide fatty acid; Panel B, ganglioside derivatives with the photoactivable group at the external galactose of the oligosaccharide chain. After incubating cells in the presence of radioactive photoactivable gangliosides, gangliosides become inserted in the plasma membrane. By UV illumination, the photoactivable nitrophenylazide group is transformed into a nitrene that rapidly reacts with adiacent molecules forming a covalent bond.

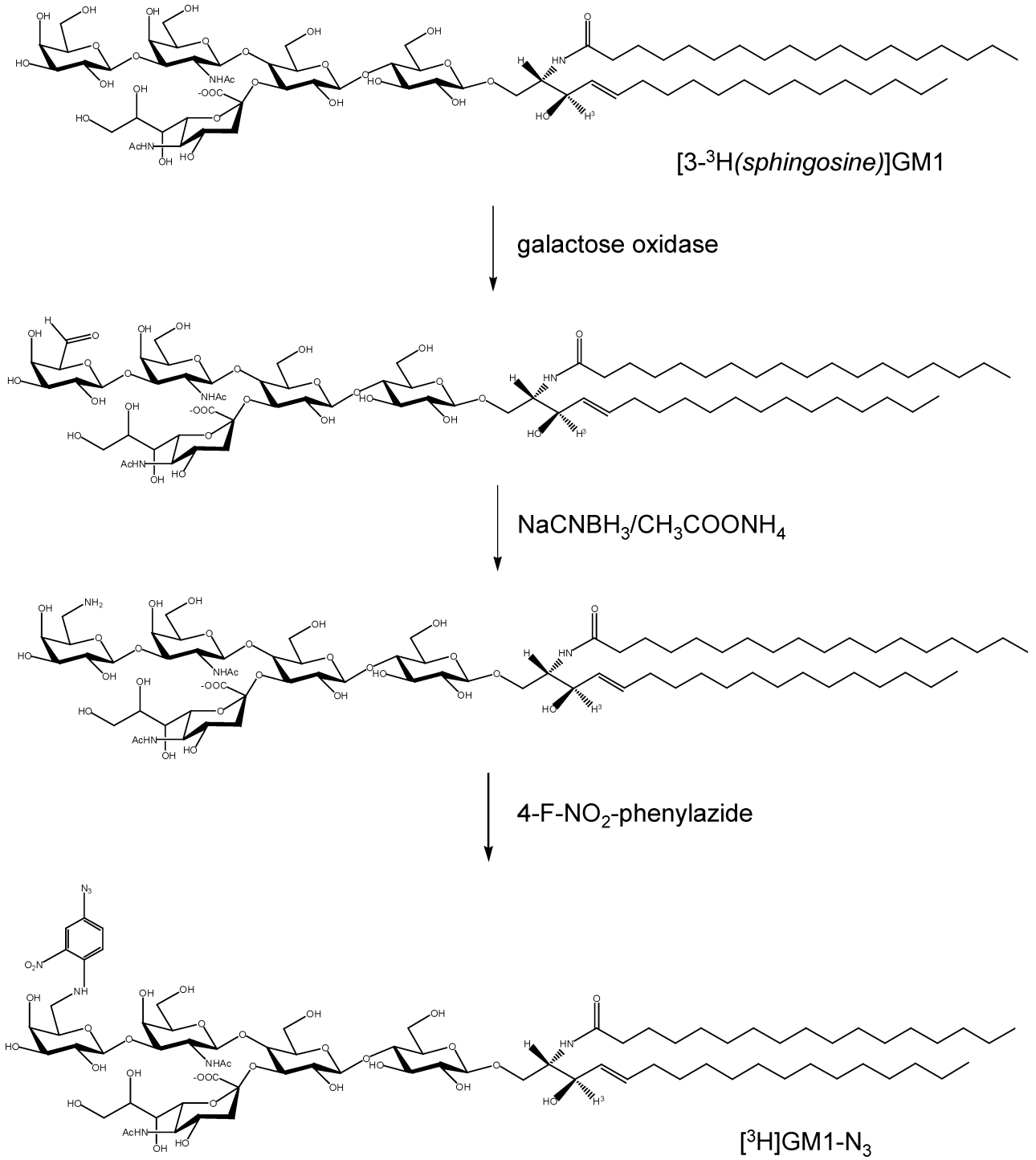

Figure 2. Scheme of the synthetic process for the preparation of [^{3}H]GM1-N_3.

50/40/10 by vol. The reaction products were characterized by HPTLC (solvent system chloroform/methanol/$CaCl_2$ 0.2%, 50/42/11, by volume). Fractions with homogeneous galactosamine containing a radioactive GM1 derivative were dried and the residue immediately solubilized in 100 μl anhydrous DMF.

Reaction 3: Preparation of photoactivable ganglioside from galactosamine containing radioactive GM1 derivative

To the solution of galactosamine containing radioactive GM1 derivative, 1 μl triethylamine and 1.5 μmole 4-F-3-NO_2-phenylazide, dissolved in 27 μl ethanol, were added. The

mixture was vigorously stirred overnight at 80°C. The reaction mixture was dried, and [^{3}H]GM1-N_3 was purified from 4-F-3-NO_2-phenylazide in excess on a reverse phase RP18 column (20 cm × 1 cm) equilibrated and eluted with methanol/water 15/4 by volume. Fractions containing partially purified [^{3}H]GM1-N_3 were dried and applied on a silica gel 100 column (80 cm × 1.2 cm) equilibrated and eluted with chloroform/methanol/water 70/26/4 by volume. Fractions containing homogeneous [^{3}H]GM1-N_3 were dried and the residue immediately solubilized in methanol (1.45 μCi/ml) and stored at +4°C.

Cell cultures

Granule cells, obtained from the cerebellum of 8-day-old Sprague-Dawley rats, were prepared and cultured as described [25]. Cells were 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. Experiments were performed at the 7th and 8th day in culture. Average protein content at this time was 700 μg protein/dish.

[^{35}S]methionine metabolic labeling and cell surface biotinylation

Cells at the 7th day in culture were preincubated in methionine-free medium for 2 h and subsequently incubated in the presence of 25 μCi/ml L-[^{35}S]-methionine (5 ml/dish) for 20 h, to radiolabel proteins [7]. After treatment with radioactive methionine, cells were washed and maintained for 1 h in serum starvation and then incubated with 0.25 mg/mL of sulfo-NHS-biotin in PBS pH 7.4 (5 mL/dish) (a biotin ester not permeable to the plasma membrane due to the presence of the sulfonate group) for 30 min at 4°C [26]. Under these experimental conditions, the internalization of the biotin derivative does not occur, and biotinylation is restricted to the cell surface, allowing the identification of cell membrane proteins with exoplasmic domain(s) (cell surface biotin labeling) [27].

Treatment of cell cultures with radioactive photoactivable gangliosides and cell surface biotinylation

Cells at the 8th day in culture were incubated with a mixture of 1.25×10^{-6} M [^{3}H]GM1-N_3 and 1.25×10^{-6} M GM1 for 6 h in serum-free basal modified Eagle's medium. After incubation, cells were washed 5 times with culture medium containing 10% fetal calf serum and then serum-starved for 1 h.

Cell surface biotinylation was accomplished as described above. After biotin labeling, cells were rinsed twice with ice-cold PBS, 4 ml ice-cold PBS were added and cells were illuminated for 45 min under UV light ($\lambda = 360$ nm) on ice [17,23,28–29]. All procedures before exposure to UV light were performed under red safelight.

Preparation of sphingolipid-enriched membrane domains by sucrose gradient centrifugation

After metabolic labeling with [^{35}S]methionine or photolabeling with [^{3}H]GM1-N_3 and biotinylation, cells were subjected to ultracentrifugation on discontinuous sucrose gradients, after lysis and homogenization in the presence of 1% Triton X-100, as previously described [7]. 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 Na_3VO_4, 1 mM PMSF, and 75 mU/ml aprotinin, $5–8 \times 10^7$ cells/ml) and Dounce homogenized (10 strokes, tight). Cell lysate was centrifugated (5 min, 1,300g) to remove nuclei and cellular debris. The post-nuclear 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 Na_3VO_4, placed at the bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer, and centrifugated (17 h, 200,000g) at 4°C. After ultracentrifugation, eleven fractions were collected starting from the top of the tube. The light-scattering band that was located at the interface between 5% and 30% sucrose, corresponding to fraction 5, was regarded as the sphingolipid-enriched membrane fraction (SEMF). The entire procedure was performed at 0 to 4°C in ice immersion.

Sphingolipid analysis

Cells at the 8th day in culture were incubated in the presence of 3×10^{-8} M [1-^{3}H]sphingosine (5 ml/dish) in a cell-conditioned medium for a 2 h pulse followed by a 48 h chase to achieve steady-state metabolic labeling of cell sphingolipids [7]. Sucrose gradient fractions from [1-^{3}H]sphingosine-labeled cells were dialyzed, lyophilized, and lipids were extracted with chloroform/methanol 2:1 by volume [7]. The total lipid extract was analyzed by HPTLC with the solvent systems chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 by volume, followed by radioactivity imaging (Beta-Imager 2000 Z instrument, Biospace, Paris).

Analysis of protein patterns

Sucrose gradient fractions obtained after cell labeling with [^{35}S]methionine or after cell photolabeling with [^{3}H]GM1-N_3 and biotinylation were analyzed by SDS-PAGE. After separation, proteins were transferred to PVDF membranes. [^{35}S]proteins were detected by autoradiography and proteins cross-linked to [^{3}H]GM1-N_3 were analyzed by digital autoradiography. The PVDF was probed with horseradish peroxidase-conjugated streptavidin to detect biotinylated proteins. The presence of TAG-1 was assessed by immunoblotting with a specific antibody, followed by reaction with secondary horseradish peroxidase-conjugated antibody and enhanced chemiluminescence detection (Pierce Supersignal).

Immunoprecipitation experiments

For a further purification of the sphingolipid-enriched membrane domain, aliquots of fraction 5 (200 μl, containing 10–20 μg protein) obtained from cells photolabeled with [^{3}H]GM1-N_3 and biotinylated at the cell surface, were immunoprecipitated with 50 μl of streptavidin-coupled magnetic beads previously washed twice with PBS buffer. The mixtures were stirred overnight at 4°C, then recovered by centrifugation [15]. Equal relative amounts (usually corresponding to 1/5 of the sample) of the IP and the corresponding supernatant were analyzed by SDS-PAGE [6].

Other experimental procedures

The radioactivity associated with cells and with cell fractions, was determined by liquid scintillation counting. Digital autoradiography of the PVDF membranes and HPTLC plates was performed with a Beta-Imager 2000 instrument (Biospace, Paris). The radioactivity associated with individual proteins or lipids was determined with the specific Beta-Vision software provided by Biospace. Autoradiography of [^{35}S]proteins was carried out using Kodak Biomax MR and MS films.

Results

Exogenously administered gangliosides are able to become components of the cell membrane, virtually indistinguishable from cell membrane gangliosides [29–31]. Thus, the administration of photoactivable gangliosides to cultured cells followed by illumination allows to obtain membrane proteins cross-linked to ganglioside molecules located in the protein microenvironment, reflecting the interactions occurring between ganglioside and protein molecules within the membrane. Previously, we studied the interactions between gangliosides and proteins in the SEMF by incubating cells in the presence of radioactive and photoactivable ganglioside derivatives, carrying a reactive nitrophenylazide group at the end of the ceramide acyl chain [17–19]. These derivatives were well suited for the study of ganglioside-protein interactions involving the proximity of hydrophobic moieties of the lipid and protein molecules, deeply inserted into the core of the membrane lipid bilayer (Figure 1).

To study possible ganglioside-protein interactions involving hydrophilic parts of these molecules, we synthesized a radioactive and photoactivable derivative of GM1 ganglioside carrying the reactive nitrophenylazide group at the neutral non-reducing end of the ganglioside oligosaccharide chain (Figures 1 and 2), and optimized its use in cultured neurons. After a 6 h pulse with [^{3}H]GM1-N_3 the loosely bound radioactive ganglioside was removed by washing cells in the presence of fetal calf serum, and cells were shortly chased to allow a more physiological distribution of the ganglioside derivative within the cell membrane, according to experimental protocols previously established for the administration of exogenous gangliosides and ganglioside derivatives to cultured cells [17–19,24,28–32]. Subsequently, cells were illuminated under UV light to achieve cross-linking of cell proteins to radioactive gangliosides and a SEMF was prepared by lysis in the presence of 1% Triton X-100 and sucrose gradient ultracentrifugation. The distribution of radioactivity within sucrose gradient fractions is shown in Figure 3. In the same figure, the distribution of natural [^{3}H]GM1 added to cells and the distribution of cellular GM1 ganglioside, as determined after sphingolipid metabolic labeling with radioactive sphingosine as a tracer, are reported for comparison. In differentiated rat cerebellar granule cells, GM1 represents about 10% of cell gangliosides [7,33]. As previously reported for all sphingolipids in these cells [7], GM1 was largely associated with the SEMF (fraction 5 of the sucrose gradient), about 60% of

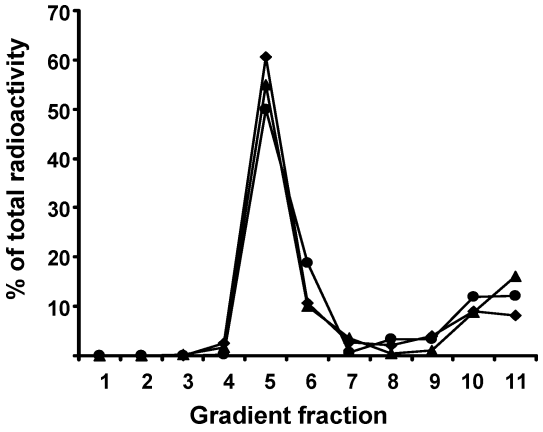

Figure 3. Radioactivity distribution in sucrose gradient fractions from cultured rat cerebellar neurons in culture after incubation with photoactivable radioactive ganglioside derivatives. Cells were incubated in the presence of a mixture of 1.25×10^{-6} M [^{3}H]GM1-N_3 and 1.25×10^{-6} M GM1 (circle) for 6 h as described under "Materials and Methods", the cell surface proteins were labeled with biotin, cells were illuminated for 45 min under UV light to allow cross-linking of cellular components with photoactivated gangliosides, and finally subjected to sucrose gradient ultracentrifugation for the preparation of SEMF. Eleven fractions were collected from the top of the tube, fraction 5 corresponding to the SEMF. The radioactivity associated with each fraction was determined by liquid scintillation counting. Data are expressed as percentages of total radioactivity loaded in the gradient and are the means of three different experiments, with the S.D. never exceeding 10% of the mean values. For comparison, SEMF were prepared from cells after sphingolipid labeling with [1-^{3}H]sphingosine, and radioactive lipids were extracted from each fraction, separated by HPTLC and detected by digital autoradiography (about 250 dpm/lane, applied on a 3 mm line. Time of acquisition: 24 h). The distribution of radioactivity associated with GM1 in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture is shown (diamond). Data are expressed as percentage of total radioactivity associated with GM1 present in homogenate. Figure shows also the distribution of radioactive GM1 (triangle) in membrane fractions prepared from cells fed [^{3}H]GM1.

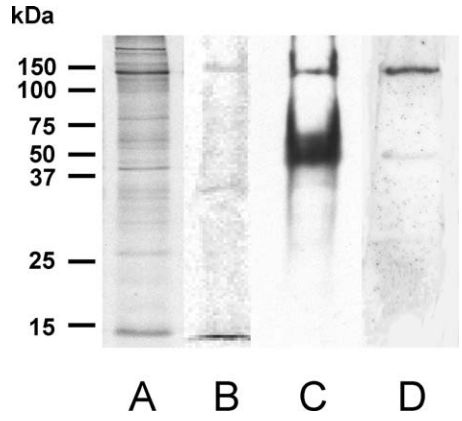

Figure 4. Protein patterns in the SEMF from cultured rat cerebellar neurons. Cells were incubated in the presence of [^{3}H]GM1-N_3 for 6 h as described under "Materials and Methods", the cell surface proteins were labeled with biotin, cells were illuminated for 45 min under UV light to allow cross-linking of cellular components with photoactivated gangliosides, and SEMF were prepared by sucrose gradient ultracentrifugation. After SDS-PAGE separation the SEMF proteins were transferred to PVDF membranes and radioactive proteins were visualized by digital autoradiography and biotinylated proteins by reaction with HRP-streptavidin and enhanced chemiluminescence detection. To allow the detection of total SEMF protein pattern, cell proteins were labeled with [^{35}S]methionine, SEMF were prepared by sucrose gradient centrifugation and about 0.1 μg of proteins from the SEMF were analyzed by SDS-PAGE, transferred to PVDF membranes, and visualized by autoradiography (about 2000 cpm; time of exposure: 15 days). Lane A, [^{35}S]proteins from SEMF; lane B, proteins cross-linked to [^{3}H]gangliosides (about 15,000 dpm; acquisition time: 48 h); lane C, biotinylated proteins visualized by reaction with HRP-streptavidin and enhanced chemiluminescence detection; lane D, Western blotting using specific anti-TAG-1 antibody. Patterns are representative of those obtained in three different experiments.

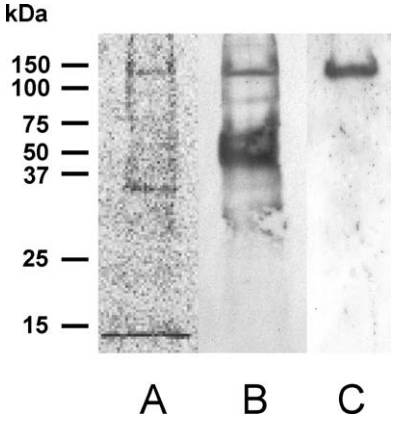

Figure 5. Lipid domain separation with streptavidin-coupled magnetic beads from SEMF prepared from cultured rat cerebellar neurons in culture after incubation with photoactivable radioactive ganglioside derivatives followed by cell surface biotinylation. Cells were incubated in the presence of [^{3}H]GM1-N_3 for 6 h as described under "Materials and Methods", the cell surface proteins were labeled with biotin, cells were illuminated for 45 min under UV light, and SEMF were prepared by sucrose gradient ultracentrifugation. A similar amount of SEMF proteins (10–20 μg) were subjected to precipitation with streptavidin-coupled magnetic beads. Proteins in the immunoprecipitates (corresponding to 1/5 of the total sample) were separated by SDS-PAGE and transferred on PVDF membranes. Lane A, pattern of proteins cross linked to [^{3}H]GM1-N_3, visualized by digital autoradiography; lane B, pattern of biotinylated proteins visualized by reaction with HRP-streptavidin and enhanced chemiluminescence detection; lane C, Western blotting using specific anti-TAG-1 antibody. Patterns are representative of those obtained in three different experiments.

cell GM1 being recovered in this fraction (Figure 3). Cells incorporated about 1.5% of administered [^{3}H]GM1-N_3 and the majority of [^{3}H]GM1-N_3 associated with cells was recovered in the SEMF, the radioactivity distribution within the gradient closely resembling that of endogenous GM1, suggesting that under these experimental conditions the ganglioside derivative is inserted in the membrane lipid bilayer in a "physiological" way.

The SEMF proteins cross-linked to [^{3}H]GM1-N_3 were analyzed by SDS-PAGE followed by digital radioimaging. The radioactive protein patterns are shown in Figure 4, in comparison with the total SEMF [^{35}S]protein pattern and with the SEMF cell surface biotinylated protein pattern.

In agreement with our previous findings [15,18], the SEMF from rat cerebellar granule cells differentiated in culture was peculiarly enriched in a radioactive protein with an apparent molecular mass of about 135 kDa (Figure 4, lane A). When cell proteins were labeled with sulfo-NHS-biotin under experimental conditions that did not allow the internalization of the biotin derivative, only a limited number of protein molecules were linked to biotin. An intense biotin-labeled band was detected (Figure 4, lane C), co-migrating with the 135 kDa main radioactive spot recognized in SEMF [^{35}S]protein pattern. However, most biotin-labeled SEMF proteins were detected in the molecular mass range between 50 and 70 kDa. The 135 KDa, biotin-labeled protein was immunoreactive with a monoclonal antibody directed against the neural cell adhesion molecule TAG-1 (Figure 4, lane D), a GPI-anchored protein that is a typical component of the SEMF obtained from cerebellar granule cells [15]. Remarkably, under these experimental conditions only two protein molecules with apparent molecular masses of 135 and 35 kDa were intensely cross-linked to the [^{3}H]GM1-N_3 (Figure 4, panel B).

SEMF prepared from cells treated with the [^{3}H]GM1-N_3 and subjected to cell surface biotinylation were further immunoisolated with streptavidin-coupled magnetic beads [15]. Figure 5 shows the patterns of proteins cross-linked to the [^{3}H]GM1-N_3 radioactive ganglioside derivative (Lane A) and the biotinylated protein patterns (Lane B) and the Western blot with anti-TAG1 antibody (Lane C). Also in the case of immunoisolated SEMF, the protein band at 135 kDa was intensely biotinylated,

cross-linked by the [^{3}H]GM1-N_3 and immunoreactive with the anti-TAG-1 antibody.

Discussion

The experimental tools currently available for the study of complex lateral interactions of amphiphiles within cell membranes are very limited. However, these interactions are probably underlying several important biological events at the cell surface, such as the modulation of receptor functions by glycosphingolipids. A possible approach to this problem relies on the administration of exogenous ganglioside derivatives to cultured cells. Exogenously administered gangliosides become components of the cell membrane, virtually indistinguishable from physiological cell sphingolipid pools [30–33]. A similar behavior was observed by administering radioactive photoactivable ganglioside derivatives to cultured cells [23,29]. These derivatives carry a reactive nitrophenylazide group at the end of the ceramide fatty acid [19]. After the ganglioside becomes inserted in the membrane, the reactive group is deeply embedded in the hydrophobic core of the membrane [19], and a cross-linkage might occur with the lipid moiety of a GPI-anchored protein [18], with a hydrophobic amino acidic stretch of an integral membrane protein [24], or with the fatty acid anchoring a protein to the cytoplasmic side of the membrane [17], as exemplified in Figure 1. Previously, we used techniques based on the administration of photoactivable radioactive gangliosides to rat cerebellar granule cells differentiated in culture to study ganglioside-protein interactions within a particular membrane microenvironment, the sphingolipid-enriched membrane domains. The main protein of sphingolipid-enriched membrane domains from these neuronal cells is the GPI-anchored neural cell adhesion molecule TAG-1. This protein associated with sphingolipids and other protein components (*i.e.* the protein tyrosine kinases c-Src, Lyn and Csk) of the sphingolipid-enriched domains, forming a supermolecular assembly that was stable enough to be immunologically purified [15,17,18]. The studies of Kasahara *et al.* [13,14,16] underscored that the association between GD3 ganglioside, the Src-family tyrosine kinase Lyn and TAG-1 in the sphingolipid-enriched membrane domain from cerebellar neurons has a functional relevance, representing the module where a TAG-1 and/or ganglioside-mediated signal is transduced *via* Lyn activation and the trigger of an intracellular phosphorylation cascade. Using photoactivable derivatives, such as those described above, we were able to show that gangliosides, typical components of the outer membrane layer, interact with both Lyn, a myristoylated protein associated with the membrane inner lipid layer, and TAG-1, a GPI-anchored protein associated with the membrane outer lipid layer. Remarkably, in photolabeling experiments with ganglioside derivatives that deeply differ for their sialooligosaccharide chain, we detected very similar patterns of cross-linked, radioactive proteins. This might not be surprising for c-Src and Lyn. These proteins are associated with the membrane inner lipid layer due to their fatty acid residues (palmitoyl and/or myristoyl). Thus, it can be easily hypothesized that their interactions with gangliosides are mediated by hydrophobic interactions involving the fatty acyl chain of the protein and the ceramide moiety of the ganglioside. In the case of TAG-1, hydrophobic interactions involving the phosphatidylinositol fatty acid tail are still possible, but hydrophilic interactions between the ganglioside sialyloligosaccharide chain and the extracellular domain of the protein or the GPI anchor oligosaccharide chain (through "side-by-side" carbohydrate-carbohydrate interactions [34,35]) cannot be excluded.

To further investigate this point, we prepared a novel radioactive photoactivable derivative of GM1 ganglioside, carrying the photoctivable nitrophenylazide group linked to the external galactose modified to galactosamine (Figure 1), and thus well suited for the study of interactions mediated by the hydrophilic moieties of membrane molecules. Rat cerebellar granule cells were incubated in the presence of this radioactive and photoactivable ganglioside derivative under experimental conditions similar to those previously used to allow the uptake of exogenous gangliosides or ganglioside derivatives [17–19,30,31,33], cell surface proteins were biotinylated and cells were subsequently illuminated to allow cross-linking of cell proteins to radioactive gangliosides. When cells were fractionated on sucrose gradient after lysis in the presence of 1% Triton X-100 to prepare a sphingolipid-enriched membrane fraction, about 60% of ganglioside radioactivity was recovered in the SEMF. This distribution closely resembles the distribution of exogenous natural GM1 and of endogenous GM1 (Figure 3) within the gradient. Remarkably, the percentage of radioactivity associated with the SEMF in the case of [^{3}H]GM1-N_3 having the azide at the oligosaccharide moiety is much higher than that measured for photoactivable derivatives with the azide group at the ceramide moiety [18], indicating that the former derivative is able to interact with the cell membrane in a more physiological way.

The patterns of proteins cross-linked to radioactive gangliosides is the SEMF, purified by sucrose density centrifugation or by immunoprecipitation of surface biotin-labeled proteins, were very simple (Figures 4 and 5). One of the two main proteins labeled by [^{3}H]GM1-N_3 had a molecular mass of 135 kDa; it was biotynilated, as expected for a protein bearing an exoplasmic domain, and was immunoreactive with a monoclonal anti-TAG-1 antibody. Thus, we concluded that [^{3}H]GM1-$N_3$is able to efficiently label TAG-1 within sphingolipid-enriched membrane domains from differentiated cerebellar neurons, indicating that the ganglioside oligosaccharide chain is close enough to the exoplasmic domain or to the GPI sugar chain of TAG-1 to allow a direct interaction.

According to the above results we confirm that the interactions between gangliosides and Tag-1 involve the ganglioside oligosaccharide chain, but the question remains to be answered if the ganglioside oligosaccharide chain interacts with the aminoacyl chain of TAG-1 or with the oligosaccharide moiety of the TAG-1 GPI.

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