Journal of Neurochemistry, 2003, **85**, 224–233 doi:10.1046/j.1471-4159.2003.01655.x

The adhesion protein TAG-1 has a ganglioside environment in the sphingolipid-enriched membrane domains of neuronal cells in culture

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Abstract

We studied the interactions between gangliosides and proteins at the exoplasmic surface of the sphingolipid-enriched membrane domains by ganglioside photolabeling combined with cell surface biotin labeling. After cell photolabeling with radioactive photoactivable derivatives of GM3, GM1 and GD1b gangliosides, followed by cell surface biotin labeling, sphingolipid-enriched domains were prepared and immunoprecipitated with streptavidin-coupled beads, under experimental conditions preserving the integrity of the lipid domain. About 50% of the total radioactivity linked to proteins was associated with acylated tubulin, about 10% with a 135-kDa protein present as a series of species with pI ranging from 6.5 to 8.0, about 5% with a protein of about 70 kDa and with pI near to 6.5. By immunoprecipitation with streptavidin-coupled beads under conditions disrupting the integrity of the lipid domain, the 135 kDa protein was recovered in the immunoprecipitate, that did not contain tubulin. Thus, the 135 kDa protein has an exoplasmic domain, and it was then identified as the GPI-anchored neural cell adhesion molecule TAG-1. Remarkably, TAG-1 was cross-linked in a similar extent by the photoactivated ganglioside GM3, GM1 and GD1b. The three gangliosides bear different oligosaccharide chains, suggesting that the ganglioside/TAG−1 interaction is not specifically associated with the ganglioside oligosaccharide structure.

Keywords: gangliosides, lipid domains, neuronal cells, photolabeling, TAG-1.

J. Neurochem. (2003) **85,** 224–233.

Several aspects of the functional role of gangliosides at the cell surface can be ascribed to their ability to modulate the activity of plasma membrane proteins, such as cell surface receptors or transporters (reviewed in Ledeen 1989; Hakomori 1990; Yates and Rampersaud 1998). The best examples are probably represented by tyrosine kinase receptors, including epidermal growth factor receptor, platelet-derived growth factor receptor, neurotrophin receptors of the Trk family and insulin receptor. Despite the tremendous number of papers describing

Received November 6, 2002; revised manuscript received December 12, 2002; accepted December 20, 2002.

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Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm and the IUPAC-IUBMB (1997) recommendations.

Abbreviations used: Cer, ceramide, *N*-acyl-sphingosine; GM3, II^{3}Neu5AcLacCer, α-Neu5Ac-(2–3)-β-Gal-(1–4)-β-Glc-(1–1)-Cer; [11-^{3}H-(*Neu5Ac*)]GM3-N_3, α-Neu5[^{3}H]Ac-2–3-β-Gal-(1–4)-β-Glc-(1–1)-{(2*S*,3*R*,4*E*)-2-[12-(2-nitro-4-azidophenyl)amino-dodecanoyl]amino-3-hydroxyoctadec-4-ene}; GM1, II^{3}Neu5AcGg$_4$Cer, β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; [11-^{3}H-(*Neu5Ac*)]GM1-N_3 (position 11 of sialic acid is the acetyl carbon), β-Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5[^{3}H]Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-{(2*S*,3*R*,4*E*)-2-[12-(2-nitro-4-azidophenyl)amino-dodecanoyl]amino-3-hydroxyoctadec-4-ene}; GD1b, II^3(Neu5Ac)$_2$Gg$_4$Cer, β-Gal-1–3-β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-Cer; [6-^{3}H(*IV-Gal*)]GD1b-N_3, β-[6-^{3}H]Gal-(1–3)-β-GalNAc-(1–4)-[α-Neu5Ac-(2–8)-α-Neu5Ac-(2–3)]-β-Gal-(1–4)-β-Glc-(1–1)-{(2S,3R,4E)-2-[12-(2-nitro-4-azidophenyl)amino-dodecanoyl]amino-3-hydroxyoctadec-4-ene}; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SEMF, sphingolipid-enriched membrane fraction; SNIP, supernatant obtained after separation of immunoprecipitate.

a modulatory effect of gangliosides and ganglioside metabolites on cell surface receptors, several basic questions remain to be resolved. First, the majority of experimental pieces of evidence has been obtained by evaluating the receptor or postreceptor functions in cultured cells or cell-free preparations after adding exogenous gangliosides. Thus, the physiological significance of receptor modulation by gangliosides needs sometimes to be critically evaluated. Moreover, an unifying view on the mechanisms underlying this possible role at the molecular level is still lacking.

A possible and widely accepted working hypothesis is that the fine tuning of receptor functions is achieved by dynamic lateral interactions with glycosphingolipid molecules belonging to a specific microenvironment of the protein within the plasma membrane. These interactions would induce conformational changes in the receptor, directly (e.g. changing its intrinsic tyrosine kinase activity) or indirectly (e.g. changing its association with regulator or substrate proteins) affecting its biological functions. In other words, plasma membrane glycosphingolipids would act as allosteric regulators of the receptor.

To test this hypothesis, we need to deepen the knowledge of forces that drive proteins and glycosphingolipids together within the cell membrane, allowing their reciprocal interactions. Unfortunately, only a few experimental tools are available to study this kind of complex lateral interaction of amphiphiles.

The segregation of sphingolipids together with a specific subset of plasma membrane proteins within membrane lipid domains, i.e. areas with a peculiar composition [highly enriched in sphingolipids and cholesterol, and containing phosphatidylcholine, mainly dipalmitoylphosphatidylcholine (Prinetti *et al*. 2000a), as the main glycerophospholipid (Brown and Rose 1992; Iwabuchi *et al*. 1998; Prinetti *et al*. 1999; Prinetti *et al*. 2000a; Prinetti *et al*. 2001a)] seem to represent a novel, important clue to understand the regulation of plasma membrane proteins by gangliosides (Hakomori *et al*. 1998; Hakomori 2000). Among others, the interaction between GD3 ganglioside, the Src-family tyrosine kinase Lyn and the GPI-anchored neural adhesion molecule TAG-1 in the sphingolipid-enriched membrane domain from cerebellar neurons has been described (Kasahara *et al*. 1997; Kasahara *et al*. 2000; Prinetti *et al*. 2001b). Lyn is a myristoylated protein, associated with the membrane inner lipid layer. TAG-1 is a GPI-anchored protein, interacting with the membrane outer lipid layer. Thus, these proteins offer different possible modes of interaction with gangliosides, belonging to the outer layer of the membrane, as both are possible hydrophobic interactions involving the myristoyl chain (in the case of Lyn) or the phosphatidylinositol fatty acid tail (in the case of TAG-1) and the ceramide moiety of the ganglioside. In the case of TAG-1, bearing a domain protruding in the extracellular environment, hydrophilic interactions with the ganglioside sialyloligosaccharide chain could be involved.

We developed procedures to synthesize radioactive and photoactivable gangliosides, and introduced their use to study the ganglioside/protein interactions (Sonnino *et al*. 1989; Chigorno *et al*. 1990; Sonnino *et al*. 1992; Chigorno *et al*. 2000; Prinetti *et al*. 2000b). The photoactivable group, a reactive azide, is at the end of the acyl chain. After being added to cells, the photoactivable ganglioside become part of the plasma membrane with the azide deeply inserted in the membrane lipid core. Thus, as shown in Fig. 1, acylated proteins, transmembrane proteins as well as GPI-anchored proteins can be cross-linked by gangliosides after illumination.

By means of cross-linking experiments with photoactivable, radioactive ganglioside derivatives and of immunosep-

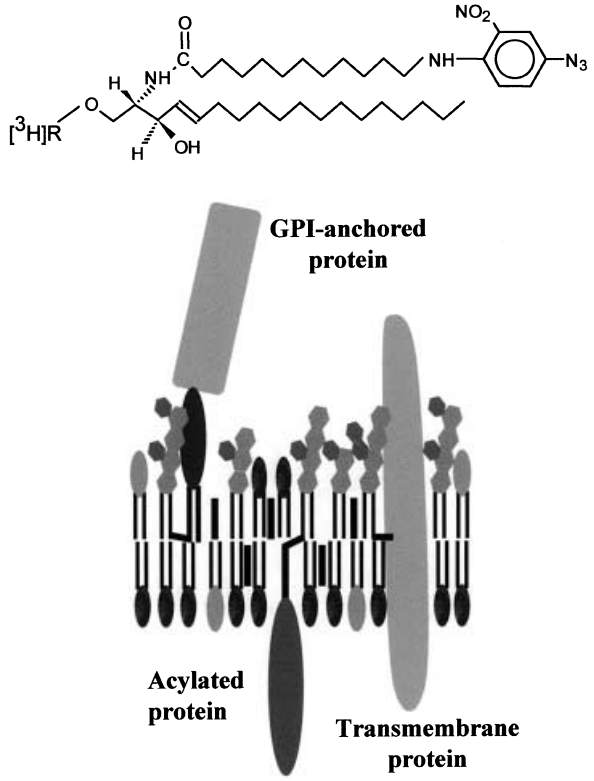

Fig. 1 Schematic representation of the three possible ganglioside–protein linkages available in the plasma membrane by cell photolabeling with radioactive photoactivable gangliosides. Cells are fed the radioactive and photoactivable ganglioside for a time that allows the ganglioside insertion into the plasma membrane extracellular leaflet. By illumination, the ganglioside photoactivable group (the structure is reported in the upper part of the figure where [^{3}H]R is the radioactive oligosaccharide of GM3, GM1 or GD1b) is transformed into nitrene that very rapidly links to proteins. According to position of the azide, deeply inserted into the membrane lipid core, the formed covalent linkage can be with the acyl moiety of an acylated protein inserted into the cytoplasmic leaflet, with the acyl chains of a GPI anchored protein inserted into the extracellular leaflet, and with a transmembrane protein.

aration techniques using antigangliosides antibodies, we showed that (i) Lyn actually interacts with gangliosides in sphingolipid-enriched membrane domains from cerebellar neurons via hydrophobic interactions; and (ii) Lyn and TAG-1 belong with other signaling proteins to a complex membrane lipid environment (Prinetti *et al*. 2000b; Prinetti *et al*. 2001b). In this paper, we describe a direct interaction of TAG-1 with gangliosides in sphingolipid-enriched membrane domains from cerebellar neurons.

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, crystalline bovine serum albumin, and reagents for cell culture were from Sigma Chemical Co. (St Louis, MO, USA), except for basal modified Eagle's medium and fetal calf serum, which were purchased from Flow Laboratories (Irvine, UK). Sulfo-NHS-biotin and horseradish peroxidase-conjugated streptavidin were from Pierce. Rabbit polyclonal antibody to TAG-1 were raised against baculovirus produced protein (Prinetti *et al*. 2001b). Monoclonal antibodies against α- and β-tubulin were from Sigma. Horseradish peroxidase-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Streptavidin-coupled magnetic beads (Dynabeads M-280 streptavidin) were from Dynal (Oslo, Norway). [^{35}S]Methionine (specific radioactivity 1175 Ci/mmol) was purchased from NEN. GM1 and GD1b were extracted from bovine brain (Tettamanti *et al*. 1973) and purified (Acquotti *et al*. 1991). GM3 was prepared from GM1 by chemical reactions (Mauri *et al*. 1999). Radioactive photoactivable gangliosides [11-^{3}H(*Neu5Ac*)]GM3-N_3, [11-^{3}H(*Neu5Ac*)]GM1-N_3 and [6-^{3}H(*IV-Gal*)]GD1b-N_3 were prepared as previously reported (Sonnino *et al*. 1989; Chigorno *et al*. 2000; Prinetti *et al*. 2000b; Mauri *et al*. 2003).

Cell cultures

Granule cells, obtained from the cerebellum of 8-day-old Sprague–Dawley rats, were prepared and cultured as described (Gallo *et al*. 1982). 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 seventh and eighth 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 seventh 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 (Prinetti *et al*. 2000a). After pulse, cells were washed and maintained for 1 h in serum starvation and then incubated with 0.25 mg/mL of sulfo-NHS-biotin in phosphate-buffered saline (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 (Altin and Pagler 1995). 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) (Cole and Ashman 1987).

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

Cells at the eighth day in culture were incubated with a mixture of 10^{-6} M GM3 and 10^{-6} M [11-^{3}H(*Neu5Ac*)]GM3-N_3, of 10^{-6} M GM1 and 10^{-6} M [11-^{3}H(*Neu5Ac*)]GM1-N_3 or of 10^{-6} M GD1b and 10^{-6} M [6-^{3}H(*IV-Gal*)]GD1b-N_3 for 6 h in serum-free basal modified Eagle's medium. After incubation, cells were washed five 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 (v = 360 nm) on ice (Sonnino *et al*. 1989; Chigorno *et al*. 1990; Sonnino *et al*. 1992; Prinetti *et al*. 2000b). All procedures before exposure to UV light were performed under red safelight. In some experiments cells, were treated, before harvesting, with 2 mL of 0.1% trypsin in PBS for 4 min to remove the portion of gangliosides that strongly interacts with proteins protruding from the membrane surface (Chigorno *et al*. 1985; Sonnino *et al*. 1992).

Preparation of sphingolipid-enriched membrane domains by sucrose gradient centrifugation

After metabolic labeling with [^{35}S]methionine or photolabeling with [^{3}H]ganglioside derivatives followed by 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 (Prinetti *et al*. 2000a). 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 × 10^7 cells/mL) and Dounce homogenized (10 strokes, tight). Cell lysate was centrifugated (5 min, 1300 *g*) to remove nuclei and cellular debris. The postnuclear fraction was mixed with an equal volume of 85% sucrose (w/v) in 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, placed at the bottom of a discontinuous sucrose concentration gradient (30–5%) in the same buffer, and centrifugated (17 h, 200 000 *g*) at 4°C. After ultracentrifugation, 11 fractions were collected starting from the top of the tube. The light-scattering band located at the interface between 5 and 30% sucrose and corresponding to fraction 5 was regarded as the sphingolipid-enriched membrane fraction (SEMF). The entire procedure was performed at 0–4°C in ice immersion.

Analysis of protein patterns

Sucrose gradient fractions obtained after cell labeling with [^{35}S] methionine or after cell photolabeling with [^{3}H]ganglioside derivatives and then biotinylated were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) or two-dimensional electrophoresis (isoelectric focusing on a pH gradient from 3.5 to 10 in the presence of 9.5 M urea and 1% NP-40, followed by 10% acrylamide SDS–PAGE for the second dimension) (Bravo 1984; Prinetti *et al*. 2000b). After separation, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. [^{35}S]-labeled proteins were detected by autoradiography and proteins

cross-linked to [^{3}H]ganglioside derivatives were analyzed by digital autoradiography. The PVDF membranes were probed with horseradish peroxidase-conjugated streptavidin to detect biotinylated proteins. The presence of TAG-1 and tubulin was assessed by immunoblotting with specific antibodies, followed by reaction with secondary horseradish peroxidase-conjugated antibodies and enhanced chemiluminescence detection (Pierce Supersignal).

Immunoprecipitation experiments

Aliquots of fraction 5 (200 μL, containing 10–20 μg protein) obtained from cells photolabeled with [^{3}H]ganglioside derivatives, 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. Under these conditions, we preserved the organization of lipid domains (Brown and Rose 1992; Prinetti *et al*. 2001b). In some experiments, the immunoprecipitate (IP) was treated with 1% SDS in lysis buffer at 100°C for 5 min, then diluted 10 times with lysis buffer (0.1% SDS final concentration) and re-immunoprecipitated with streptavidin-coupled magnetic beads. These conditions are known to break up the membrane organization (Kasahara *et al*. 1997) and allow to immunoprecipitate, by streptavidin-coupled magnetic beads, only proteins belonging to the exoplasmic surface of the plasma membrane. Equal relative amounts (usually corresponding to one-fifth of the sample) of the IP, the re-IP and the corresponding supernatant were analyzed by SDS–PAGE (Prinetti *et al*. 1999).

Other experimental procedures

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

Results

Protein composition of SEMF from cultured rat cerebellar neurons

The protein composition of rat cerebellar granule cells at the eighth day in culture was analyzed by labeling of total cell proteins with [^{35}S]methionine followed by biotinylation of cell surface proteins. In agreement with previous observations, the SEMF contained about 1.5% of the total cell protein-associated radioactivity. Figure 2 shows the [^{35}S]-methionine-labeled protein patterns in the cell homogenate (lane A) and in the SEMF (lane B) after separation by SDS–PAGE and autoradiography. The protein pattern of the SEMF was much different of that of cell homogenate. In particular, the SEMF was highly enriched in two radioactive proteins, showing apparent molecular masses of about 135 and 15 kDa. To allow the identification of cell membrane proteins with exoplasmic domain(s), cell proteins were labeled with sulfo-NHS-biotin under experimental conditions

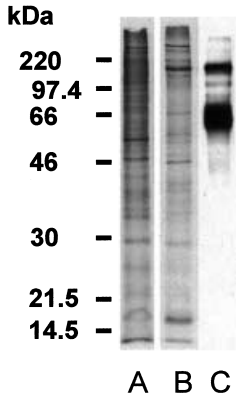

Fig. 2 Protein patterns in the SEMF from cultured rat cerebellar neurons. After metabolic labeling of cell proteins with [^{35}S]methionine and labeling of cell surface proteins with biotin, SEMF were prepared by sucrose gradient centrifugation. About 0.1 μg of proteins from the cell homogenate and from the SEMF were analyzed by SDS–PAGE. Proteins were transferred to PVDF membranes; radioactive proteins were visualized by autoradiography (about 2000 cpm; time of exposure: 15 days) and biotinylated proteins by reaction with horseradish peroxidase–streptavidin and enhanced chemiluminescence detection. Lane A, [^{35}S]proteins from homogenate; lane B, [^{35}S]proteins from SEMF; lane C, biotinylated proteins from SEMF. Patterns are representative of those obtained in three different experiments.

that did not allow the internalization of the biotin derivative (cell surface biotin labeling). The cell surface biotinylated protein pattern of the SEMF after separation by SDS–PAGE is shown in Fig. 2 (lane C). If compared with the total protein pattern of the SEMF (Fig. 2, lane B), only a limited number of SEMF proteins were biotin-labeled under these conditions. The larger part of SEMF biotinylated proteins was concentrated in the molecular mass range between 50 and 70 kDa, but some proteins showing higher molecular mass were also present. An intense biotin-labeling was associated with a band showing an apparent molecular mass of 135 kDa, co-migrating with the main radioactive spot recognized in SEMF after [^{35}S]methionine labeling. SEMF proteins were also separated by two-dimensional PAGE. Figures 3(a) and (b) show the patterns of [^{35}S]methionine- and biotin-labeled proteins in SEMF, respectively. The two-dimensional PAGE analysis confirmed that only a few of the [^{35}S]proteins are also biotinylated, thus belonging to the exoplasmic surface of the plasma membranes, and allowed to resolve the biotinylated proteins with molecular mass ranging from 50 to 75 kDa in several different spots on the basis of their isoelectric points.

Ganglioside photolabeling of proteins in SEMF from cultured rat cerebellar neurons

The interactions between gangliosides and proteins in the SEMF have been studied by incubating cells in the presence of three radioactive and photoactivable ganglioside derivatives, [11-^{3}H(*Neu5Ac*)]GM3-N_3, [11-^{3}H(*Neu5Ac*)]GM1-N_3

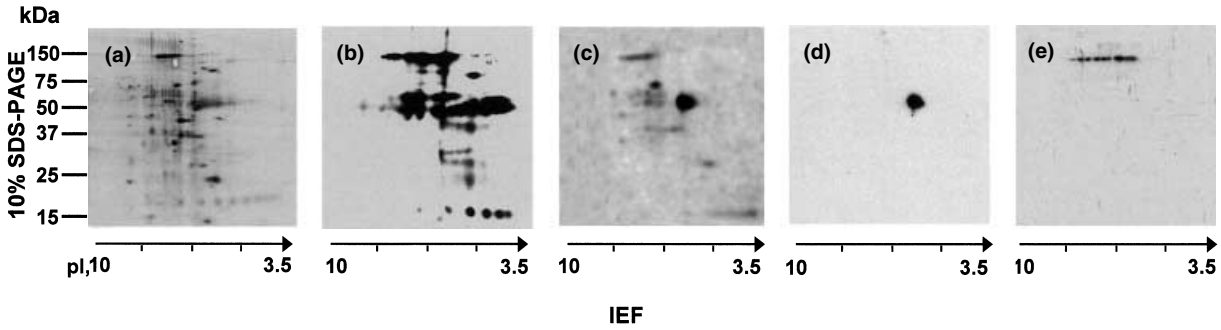

Fig. 3 Two-dimensional protein patterns in the SEMF from cultured rat cerebellar neurons. After metabolic labeling of cell proteins with [^{35}S]methionine or photolabeling with [^{3}H]ganglioside derivatives, the cell surface proteins were labeled with biotin, SEMF were prepared by sucrose gradient centrifugation and SEMF proteins were separated by two-dimensional electrophoresis. (a) [^{35}S]Proteins visualized by autoradiography (about 10 000 cpm; time of exposure: 15 days); (b) biotinylated proteins visualized by reaction with HRP-streptavidin and enhanced chemiluminescence detection; (c) proteins cross-linked to photoactivated [^{3}H]GM_3 derivative, visualized by digital autoradiography (about 30 000 dpm; time of acquisition: 60 h); (d) western blotting using specific antitubulin antibody; (e) western blotting using specific anti-TAG-1 antibody. Patterns are representative of those obtained in three different experiments.

and [6-^{3}H(*IV-Gal*)]GD1b-N_3. After pulse incubation with ganglioside derivatives, the amount of radioactive ganglioside weakly bound to the cells was removed by washing in the presence of fetal calf serum, cells were shortly chased to allow a more physiological distribution of the ganglioside derivative within the cell membrane, cell surface proteins were biotinylated, cells were illuminated under UV light to achieve cross-linking of cell proteins to radioactive gangliosides and a SEMF was prepared.

The distribution of radioactivity within fractions collected after sucrose gradient centrifugation is shown in Fig. 4. Independently from the ganglioside structure, about 15% of total radioactivity associated with cells was recovered in the SEMF, while the majority of it was associated with the heaviest fractions of the gradient. This, in agreement with previous results (Chigorno *et al*. 2000) should reflect the notion that only a minor portion of the photoactivable gangliosides administered to cells in culture become components of the cell membranes. In fact, even after removing the serum-labile portion, a relevant amount of ganglioside derivatives remains that is more stably associated with the cell surface, but not yet inserted in the lipid bilayer and can be removed by a trypsin treatment (Chigorno *et al*. 1985). The radioactivity associated with the heavy fractions of the gradient (containing more than 80% of cell proteins) should correspond to those trypsin-labile ganglioside molecules that after illumination artifactually and randomly bind the extracellular moiety of cell membrane components, particularly proteins. In fact, trypsin treatment of cells before gradient centrifugation did not alter the amount of radioactivity associated with the SEMF but greatly reduced that recovered in the high density fractions (Fig. 5).

The SEMF proteins cross-linked to different ganglioside derivatives were analyzed by SDS–PAGE followed by digital

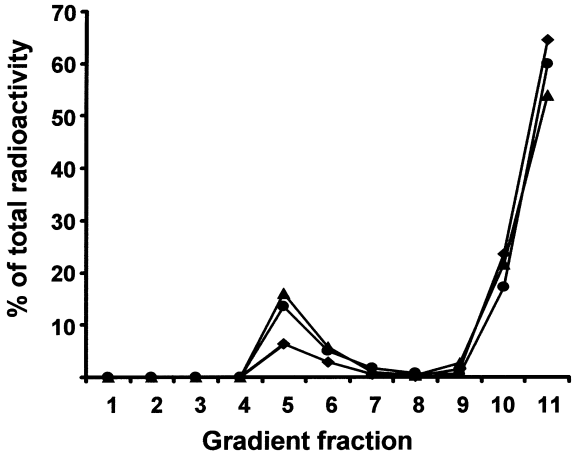

Fig. 4 Radioactivity distribution in sucrose gradient fractions from cultured rat cerebellar neurons in culture after incubation with photoactivable radioactive ganglioside derivatives. Cells were incubated in the presence of [11-^{3}H(*Neu5Ac*)]GM1-N_3 (●), [6-^{3}H(*IV-Gal*)]GD1b-N_3 (▲) and [11-^{3}H(*Neu5Ac*)]GM3-N_3 (◆) photoactivable gangliosides 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 cells were 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 SD never exceeding 10% of the mean values.

radioimaging. The radioactive protein patterns are shown in Fig. 6 in comparison with the corresponding patterns of biotinylated proteins. The patterns of cross-linked proteins in

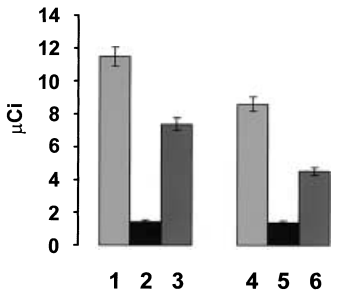

Fig. 5 Effect of trypsin treatment on radioactivity distribution in sucrose gradient fractions from cultured rat cerebellar neurons in culture after incubation [6-^{3}H(*IV-Gal*)]GD1b-N_3. Cells were incubated in the presence of [6-^{3}H(*IV-Gal*)]GD1b-N_3 photoactivable ganglioside and illuminated as described in the legend of Fig. 4, and were treated with 0.1% trypsin in PBS for 4 min as described under 'Materials and Methods' (bars 4–6) or PBS only (bars 1–3). Cells were subjected to sucrose gradient ultracentrifugation for the preparation of SEMF and fractions were collected as described in the legend of Fig. 4. The radioactivity associated with the postnuclear fraction and each gradient fraction was determined by liquid scintillation counting. Data are expressed as absolute radioactivity present in the postnuclear fraction (bars 1 and 4), in the SEMF fraction (fraction 5, bars 2 and 5) and in the high density fraction of the gradient (fractions 9–11, pooled; bars 3 and 6). Data are the means of three different experiments ± S.D.

the SEMF were not sensitive to trypsin treatment (Fig. 6b, lane 3). The patterns obtained with different gangliosides were essentially similar, even if some quantitatively minor differences could be observed. To allow a better resolution, SEMF proteins cross-linked to radioactive gangliosides were also analyzed by two-dimensional electrophoresis followed by digital autoradiography (Fig. 3c). Under these experimental conditions, only a limited number of protein molecules were cross-linked to the ganglioside, if compared with the complex total and cell surface protein patterns in this membrane subfraction (Figs 3a and b, respectively). The main radioactive ganglioside-cross-linked proteins showed molecular mass ranging from 50 to 75 kDa. The major radioactive protein, containing about 50% of the total protein associated radioactivity, had a molecular mass of 55 kDa and an isoelectric point of about 5.5, and was immunologically identified as tubulin (Fig. 3d). This is in agreement with previous data, showing that palmitoylated tubulin is the main protein cross-linked by a photoactivable diazirine GM1 derivative in neuronal cells (Palestini *et al*. 2000). Two other radioactive bands at 60 and 53/57 kDa, respectively, and isoelectric points between 6 and 7 (Prinetti *et al*. 2000b) were previously identified as the non-receptor protein tyrosine kinases c-Src and Lyn (Prinetti *et al*. 2001a). The fact that the major part of radioactivity is associated with proteins belonging to the cytosolic surface of the plasma membrane is in agreement with the minor changes induced by trypsin treatment (Fig. 6b, lane 3). A band with a

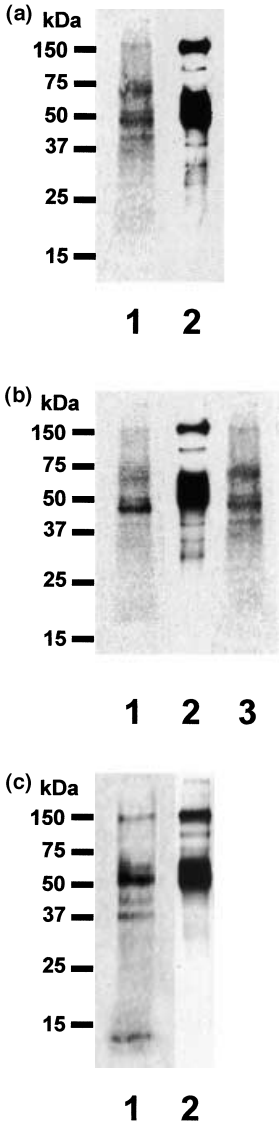

Fig. 6 Patterns of photolabeled proteins in the SEMF from cultured rat cerebellar neurons after incubation with photoactivable radioactive ganglioside derivatives. Cells were incubated in the presence of [11-^{3}H(*Neu5Ac*)]GM1-N_3 (a), [6-^{3}H(*IV-Gal*)]GD1b-N_3 (b) and [11-^{3}H(*Neu5Ac*)]GM3-N_3 (c) photoactivable gangliosides 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. Similar amount of SEMF proteins (2.5–5 μg) were analyzed by SDS–PAGE. Proteins were transferred to PVDF membranes; radioactive proteins were visualized by digital autoradiography and biotinylated proteins by reaction with HRP-streptavidin and enhanced chemiluminescence detection. Lane 1, proteins cross-linked to [^{3}H]gangliosides (about 15 000 dpm; acquisition time: 48 h); lane 2, biotinylated proteins; lane 3, proteins cross-linked to [^{3}H]gangliosides after trypsin treatment as described in the legend of Fig. 5 (about 15 000 dpm; acquisition time: 48 h). Patterns are representative of those obtained in three different experiments.

molecular mass of 135 kDa contained about 10% of the total protein associated radioactivity, being thus intensely cross-linked by photoactivable gangliosides. As revealed by cell surface biotin labeling experiments, this protein belongs to the plasma membrane exoplasmic side; it was immunologically identified as the GPI-anchored neural cell adhesion molecule TAG-1 (Fig. 3e), a GPI-anchored protein that is a typical component of the SEMF obtained from cerebellar neurons (Kasahara *et al*. 2000; Prinetti *et al*. 2001b). The other radioactive proteins, one of which carrying about 5% of total protein associated radioactivity at pI near to 6.5 of about 70 kDa, remain unknown.

SEMF prepared from cells treated with the three different ganglioside derivatives and subjected to cell surface biotinylation were immuno-isolated with streptavidin-coupled magnetic beads under experimental conditions that preserve the organization of lipid domains (Prinetti *et al*. 2001b). Figure 7 shows the patterns of proteins cross-linked to the radioactive ganglioside derivative (lane 1) and the biotinylated protein patterns (lane 2). As expected (Prinetti *et al*. 2001b), the radioactive and biotinylated protein patterns in the immunoprecipitate were very similar to those found in the SEMF (Fig. 6). One of the proteins of the immunoprecipitate was stained by a specific antibody against the neural cell adhesion molecule TAG-1 (Fig. 7, lane 3). The protein stained by the anti-TAG-1 antibody showed a molecular mass of about 135 kDa, was intensely biotinylated and cross-linked by all three radioactive ganglioside derivatives. Moreover, the immunoprecipitates contained a protein band that was stained by an anti-tubulin antibody (Fig. 7, lane 4), confirming that immunoprecipitation under these experimental conditions allowed the separation of the whole membrane domain, comprising both its exo- and endoplasmic membrane layer.

The immunoprecipitates were boiled in the presence of high detergent concentration to break up the membrane organization, and a second immunoprecipitation with streptavidin-coupled beads was performed. Under these experimental conditions, the immunoprecipitate did not contain anymore an intense radioactive band at 60 kDa, that was recovered in the corresponding supernatant (Fig. 8, lane A). This band was not biotinylated (Fig. 8, lane B) and was positive to antitubulin antibody (Fig. 8, lane C) confirming that the structure of the lipid domain was disrupted and that the immunoprecipitate contains only exoplasmic membrane proteins.

On the other hand, as shown in Fig. 8, in the immunoprecipitate a radioactive (Fig. 8, lane A), biotinylated (Fig. 8, lane B) and anti-TAG-1 positive protein (Fig. 8, lane D) with molecular mass of 135 kDa was quantitatively recovered.

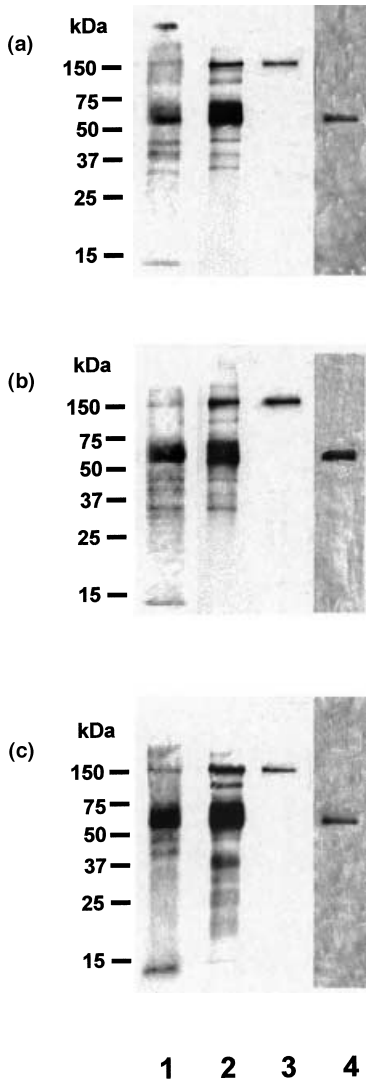

Fig. 7 Lipid domain separation with streptavidin-coupled magnetic beads from SEMF prepared from cultured rat cerebellar neurons in culture after incubation with photoactivable radioactive ganglioside derivatives followed by cell surface biotinylation. Cells were incubated in the presence of [11-^{3}H(*Neu5Ac*)]GM1-N_3 (a), [6-^{3}H(*IV-Gal*)]GD1b-N_3 (b) and [11-^{3}H(*Neu5Ac*)]GM3-N_3 (c) photoactivable gangliosides 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. Similar amount of SEMF proteins (10–20 μg) were subjected to precipitation with streptavidin-coupled magnetic beads under experimental conditions that preserve the integrity of lipid domains. Proteins in the immunoprecipitates (corresponding to one-fifth of the total sample) were separated by SDS–PAGE and transferred on PVDF membranes. Lane 1, pattern of proteins cross-linked to photoactivated [^{3}H]ganglioside derivatives, visualized by digital autoradiography; lane 2, pattern of biotinylated proteins visualized by reaction with HRP-streptavidin and enhanced chemiluminescence detection; lane 3, western blotting using specific anti-TAG-1 antibody; lane 4, western blotting using specific antitubulin antibody. Patterns are representative of those obtained in three different experiments.

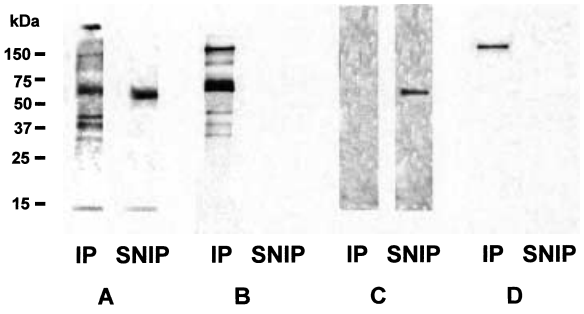

Fig. 8 Separation of biotinylated proteins from lipid domains prepared from cultured rat cerebellar neurons in culture after incubation with photoactivable radioactive ganglioside derivatives followed by cell surface biotinylation. The immunoprecipitates obtained as described in the legend of Fig. 7 from cells subjected to photolabeling with [11-^{3}H(*Neu5Ac*)]GM1-N_3 were boiled in the presence of high detergent concentration to disrupt the supramolecular organization of the lipid domain. Biotinylated proteins were separated using streptavidin-coupled magnetic beads. Proteins in the immunoprecipitates (IP) and the supernatants remaining after the immunoprecipitation (SNIP) were separated by SDS–PAGE and transferred onto PVDF membranes. Lane A, pattern of proteins cross-linked to photoactivated [^{3}H]ganglioside derivatives, 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 antitubulin antibody; lane D, western blotting using specific anti-TAG-1 antibody. Patterns are representative of those obtained in three different experiments.

Discussion

Several experiments on cultured cells have shown that exogenously administered gangliosides bind to cells, become components of the cell membrane, and enter the physiological sphingolipid pools (Radsak *et al*. 1982; Chigorno *et al*. 1985; Chigorno *et al*. 1996). Similar results were obtained by administering radioactive photoactivable ganglioside derivatives to cultured cells (Sonnino *et al*. 1989; Sonnino *et al*. 1992). Based on this observation, we developed techniques for the photolabeling of cells as a tool to study lipid–protein interactions (Sonnino *et al*. 1989; Chigorno *et al*. 1990; Sonnino *et al*. 1992; Chigorno *et al*. 2000; Prinetti *et al*. 2000b). These techniques rely on the administration of photoactivable radioactive gangliosides to cultured cells and allow to obtain membrane proteins cross-linked to ganglioside molecules that are inserted into the membrane and located in the protein microenvironment. However, since a relevant amount of radioactive ganglioside is weakly bound to the cells, as always in the case of exogenous ganglioside administration (Chigorno *et al*. 1985), after cross-linking, a significant amount of ganglioside derivatives will be artifactually linked to the extracellular moiety of cell membrane components. Several preliminary experiments indicated that to avoid the presence of artifactual cross-linking, it is necessary to analyze a sphingolipid-enriched membrane fraction. The amount of ganglioside radioactivity associated with this fraction is relatively small (Fig. 4), but it can be ascribed to ganglioside derivative molecules that are inserted in the plasma membrane in a way that closely resembles that of endogenous gangliosides. In fact, trypsin treatment (that is effective in removing ganglioside molecules associated with the cell surface but not stably inserted in the lipid bilayer) (Radsak *et al*. 1982; Chigorno *et al*. 1985) does not affect the amount of radioactivity associated with the SEMF (Fig. 5) and only marginally affects the pattern of cross-linked proteins in this fraction (Fig. 6b).

Cell surface biotinylation allowed labeling several SEMF proteins

Some of the proteins belonging to the exoplasmic side of the sphingolipid-enriched domain were also cross-linked to radioactive gangliosides. Among these, the main radioactive band was identified as a typical neuronal SEMF component, the GPI-anchored cell adhesion molecule TAG−1. The association of gangliosides with proteins in the sphingolipid-enriched membrane domains has been previously described. In the case of neuronal cells, the strong association between sphingolipids and some protein components of the sphingolipid-enriched domains was strong enough to allow reciprocal co-immunoprecipitation, as demonstrated using anti-c-Src, anti-Lyn, anti-Csk (Prinetti *et al*. 2000b), or anti-GD3 antibodies (Prinetti *et al*. 2001b). In particular, the interaction between GD3 and the Src-family tyrosine kinase Lyn seems to play a role in affecting the distribution of TAG-1 in the lipid membrane domain (Kasahara *et al*. 1997; Kasahara *et al*. 2000). The partners involved in this interaction are interacting with the cell membrane in very different ways. Thus, the possible modes for reciprocal interactions are different, offering an interesting model for the study of the lipid environment of membrane proteins. Lyn is a myristoylated protein, associated with the membrane inner lipid layer and it was demonstrated to interact with a complex lipid domain in the neuronal plasma membrane (Kasahara *et al*. 1997; Prinetti *et al*. 2001b). This interaction is likely to be ruled by hydrophobic interactions involving the myristoyl chain of the protein. TAG-1 is a GPI-anchored protein, belonging to the outer layer of the membrane, as is the case for gangliosides. We can hypothesize the existence of hydrophobic interactions involving the fatty acid tails of the GPI anchor and the ceramide moiety of the ganglioside. However, the strong cross-linking of TAG-1 by gangliosides suggests that hydrophilic interactions between the ganglioside sialyoligosaccharide chain and the extracellular domain of the protein or the oligosaccharide chain (Bovin 1996) of the GPI anchor could be involved. The latter process involves ‘side-by-side’ carbohydrate–carbohydrate interactions between molecules belonging to the same membrane. This kind of interaction was first described between

glycophorin and Forssman glycolipid, or globoside, in a liposomal membrane model (Endo *et al*. 1982), but was never described in cell membranes.

Interestingly, and irrespectively by the fact that amino acids or sugars of TAG-1 are involved in the GPI–protein/ganglioside interaction, we detected a comparable amount of radioactivity associated with TAG-1 after photolabeling with three ganglioside derivatives (GM3, GM1 and GD1b) that deeply differ for their sugar composition. Moreover, the three gangliosides GM3, GM1 and GD1b display different dynamic properties of their oligosaccharide chain more external portion (Brocca and Sonnino 1997). This would indicate that this interaction requires only a limited, conserved region of the oligosaccharide chain (i.e. the disaccharide -β-Gal-(1–4)-β-Glc- at the oligosaccharide reducing end of the three gangliosides). Alternatively, the segregation of molecules bearing a saccharide moiety in a restricted surface area could create a tight network of hydrophilic interactions mediated by water molecules. Water is a natural component of the sugar shell being attracted by the hydrophilic character of sugars and by the necessity to avoid repulsion between the ganglioside negative charged oligosaccharide (Brocca *et al*. 1998) and it has been determined that each ganglioside oligosaccharide chain is surrounded by 20–60 water molecules (Bach *et al*. 1982). This would be a new sight on the forces that are contributing to the stabilization of sphingolipid-enriched membrane domain. Of course, further investigation relying on the analysis of endogenous lipids will be necessary to confirm the results presented in this paper. However, the sensitivity of currently available analytical procedures does not allow this kind of investigation.

Acknowledgements

This research was supported by CNR (grant 99–02 PF49 to SS) and COFIN-PRIN 2000 and 2001.

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