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Association of Src-family protein tyrosine kinases with sphingolipids in rat cerebellar granule cells differentiated in culture

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Src family kinases play a relevant role in the development and differentiation of neuronal cells. They are abundant in sphingolipid-enriched membrane domains of many cell types, and these domains are hypothesized to function in bringing together molecules important to signal transduction. We studied the association of Src family tyrosine kinases and their negative regulatory kinase, Csk, with sphingolipids in sphingolipid-enriched domains of rat cerebellar granule cells differentiated in culture. We find that c-Src, Lyn and Csk are enriched in the sphingolipid-enriched fraction prepared from these cells. Coimmunoprecipitation experiments show that these and sphingolipids are part of the same domain. Cross-linking experiments with a photoactivable, radioactive GD1b derivative show that c-Src and Lyn, which are anchored to the membrane via a myristoyl chain, associate directly with GD1b. Csk, which is not inserted in the hydrophobic core of the membrane, is not photolabeled by this ganglioside. These results suggest that lipid–lipid, lipid–protein, and protein–protein interactions cooperate to maintain domain structure. We hypothesize that such interactions might play a role in the process of neuronal differentiation.

Keywords: **neuronal cell cultures, gangliosides, sphingolipids, Src family proteins, non-receptor tyrosine kinases, ganglioside enriched domains**

Abbreviations: **Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm, and the IUPAC-IUBMB recommendations [48]. GlcCer, β-Glc-(1-1)-Cer; LacCer, β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM3, II^3-α-Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1, II^3-α-Neu5AcGg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD3, II^3(α-Neu5Ac)$_2$LacCer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1a, IV^3-α-Neu5AcII^3-α-Neu5Ac-GgOse$_4$Cer, α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1b, II^3(-α-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)-(2*S*,3*R*,4*E*)-2-[12-(2-nitro-4-azido-phenyl)amino-dodecanoyl]amino-3-hydroxy-octadec-4-ene}; *O*-Ac-GT1b, IV^3-α-Neu5AcII^3[α-Neu5,9Ac$_2$-(2-8)-α-Neu5Ac-(2-3)] GgOse$_4$Cer; GT1b, IV^3-α-Neu5AcII^3(-α-Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2-3)-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; GQ1b,IV^3(-α-Neu5Ac)$_2$II^3(-α-Neu5Ac)$_2$GgOse$_4$Cer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-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; Cer, ceramide, *N*-acyl-sphingosine; [1-^{3}H]sphingosine, (2*S*,3*R*,4*E*)-2-amino-1,3-dihydroxy-[1-^{3}H]octadecene; SM, sphingomyelin; PE, phosphatidylethanolamine; HPTLC, high-performance thin-layer chromatography; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.**

Introduction

Sphingolipids, in particular gangliosides, are very abundant in the plasma membrane of neural cells, and they have been implicated as modulators of various aspects of neural cell function [1,2]. Sphingolipid patterns in the nervous system undergo marked qualitative and quantitative modifications

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during development, differentiation and neoplastic transformation [3,4]. Their biosynthesis is necessary for the differentiation of neurons in culture [5], and induced expression of GD3 synthetase is able to switch neuroblastoma cells to a differentiated phenotype [6]. Exogenously added gangliosides exert neuritogenic, neurotrophic and neuroprotective effects on a variety of cell systems of neural origin [1,7,8]. The exact mechanism underlying these effects is still poorly understood.

Possible functional interactions between gangliosides and proteins at the level of the plasma membrane have been intensively studied in the past [2,9–11]. More recently, the discovery that lipid and protein components of the cell membrane are not randomly distributed, but rather organized in domains with particular physico-chemical and functional properties, different from those of the surrounding membrane environment [reviewed in 12–14], has given a new interest to this topic. Sphingolipid-enriched domains are rich in gangliosides, sphingomyelin, cholesterol, and proteins involved in the mechanisms of signal transduction (receptor tyrosine kinases—such as receptors for EGF, PDGF, insulin and neurotrophins—non-receptor tyrosine kinases of the Src family, adapter and regulatory molecules of tyrosine kinase signaling, heterotrimeric and small GTP-binding proteins, G protein-coupled receptors, protein kinase C isoenzymes) [15–22]. Thus, sphingolipid-enriched domains could provide a microenvironment within the plasma membrane for reciprocal interactions between lipid and protein molecules involved in the control of signal transduction.

The presence of Src family non-receptor tyrosine kinases in sphingolipid-enriched membrane domains is particularly interesting in the case of neural cells. Many facts indicate that c-Src and other kinases of this family are involved in the process of neuronal differentiation. The expression and the activation of c-Src and Lyn are correlated with the stage of neuronal differentiation in neuroblastomas, neuroblastoma and embryonal carcinoma cell lines [23–27]. The expression of a neuron-specific form of c-Src is increased during the differentiation of cultured neurons from rat embryo striatum [28], and c-Src activation is involved in the control of synaptic transmission mediated by *N*-methyl-D-aspartate receptors [29] and in the induction of long-term potentiation in hippocampal neurons [30]. In PC12 cells, c-Src interacts with synaptosomal and cytoskeletal proteins [31]. Another tyrosine kinase, Csk, is a negative regulator of Src-like kinases, responsible for their C-terminal phosphorylation, keeping them in a repressed form [32]. In neuroblastoma Neuro2a cells, c-Src and Csk are associated with GM3 ganglioside within sphingolipid-enriched membrane domain and neuritogenic concentration of gangliosides are able to induce c-Src activation followed by mitogen-activated protein kinases activation [18]. Moreover, in these cells anti-GM3 antibody is able to induce differentiation [27]. In rat cerebellum and cerebellar neurons, GD3 ganglioside is associated with Lyn and with the neural cell adhesion molecule TAG-1, and antibody-mediated cross-linking of TAG-1 or GD3 induce Lyn activation [33,34].

For these reasons, in the present study we investigated the association of Src-family tyrosine kinases, the regulatory kinase Csk, and sphingolipids within sphingolipid-enriched microdomains obtained from rat cerebellar granule cells differentiated in culture. Non-covalent interactions between different proteins or between lipids and proteins were studied using traditional co-immunoprecipitation approaches, or by photolabeling experiments using radioactive and photoactivable derivatives of gangliosides.

Experimental procedures

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., except for basal modified Eagle's medium and fetal calf serum, which were purchased from Flow Laboratories. Anti-c-Src goat polyclonal IgG (N-16), anti-c-Src (SRC 2), anti-Lyn, anti-Csk rabbit polyclonal IgG antibodies, HRP conjugated secondary antibodies and Protein A/G PLUS-Agarose were from Santa Cruz Biotecnology. Sphingosine was prepared from cerebroside [35]. GD1b was extracted from rat brain [36]. [1-^{3}H]sphingosine (radiochemical purity over 98%; specific radioactivity 2 Ci/mmol) was prepared by specific chemical oxidation of the primary hydroxyl group of sphingosine followed by reduction with sodium boro[^{3}H]hydride [37] (Amersham Pharmacia Biotech). [^{35}S]methionine (specific radioactivity 1175 Ci/mmol) was purchased from NEN. [^{3}H] labeled lipids, ceramide, sphingomyelin, phosphatidylethanolamine, and lactosylceramide were extracted from [1-^{3}H]sphingosine-fed rat cerebellar granule cells, purified, characterized and used as chromatographic standards.

Synthesis of photoactivable and radioactive [6-^{3}H(*IV-Gal)*]GD1b-N_3

The synthesis of [6-^{3}H(*IV-Gal)*]GD1b-N_3 was carried out by combining and adapting experimental procedures previously developed for the synthesis of lysogangliosides and ganglioside analogues [38–40]. A general scheme for the preparation of photoactivable [^{3}H]GD1b derivative is reported in Figure 1. Chemical and radiochemical characterization of [6-^{3}H(*IV-Gal*)]GD1b-N_3 (see below) showed a chemical and radiochemical homogeneity over 99% and a specific radioactivity of 2.57 Ci/mmol.

Preparation of fully deacylated GD1b. 140 μmoles GD1b were solubilized in 82 ml 5 M KOH, 5 M $Na_2S_2O_5$ in anhydrous methanol. The mixture was stirred at 90°C for 60 h. After neutralization with acetic acid and drying, the residue was dissolved in chloroform/methanol/water 2:43:55, by vol., and purified on a RP18 silica gel column, eluted with same solvent, followed by methanol/water 1:1, methanol/

water 4:1, methanol and chloroform/methanol 1:1. The reaction products were characterized by HPTLC (solvent system chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11, by vol.).

N-acetylation of the oligosaccharide chain. 137 μmoles of deacylated GD1b solubilized in 27.4 ml 0.5 M $NaHCO_3$ were mixed with 27.4 ml diethylether and cooled until the aqueous phase was frozen. After the addition of 185 μmoles of 9-fluorenylmethylchloroformate in n-hexane (4.8 ml), the mixture was vigorously stirred at 10°C for 24 h. The reaction mixture was centrifuged at room temperature for 30 min. Phases were treated with acetic anhydride until pH 5.0 was attained. After 3 h, the organic phase was dried under nitrogen and the residue was dissolved in the aqueous phase. The reaction product was dialyzed, lyophilized and purified on a silica gel 100 column (height 70 cm, diameter 12 mm) equilibrated and eluted with isopropanol/water 7:3, by vol. The reaction products were characterized by HPTLC (solvent system isopropanol/water, 7:3, by vol.).

N-Acylation of sphingoid amino group. The reaction product was kept in 32% ammonia in a screw-capped flask for 24 h at room temperature. The mixture was dried and the residue, lyso-GD1b, was dissolved in 5 ml water. The solution was washed three times with 10 ml diethylether and dried. 68 μmoles of lyso-GD1b were dissolved in anhydrous DMF. After adding 75 μmoles of *N*-fluorenylmethylformate-amino-lauric acid pentafluorphenylester, 122 μmoles 1-hydroxybenzotyriazole and 103 μl tributylamine, the mixture was

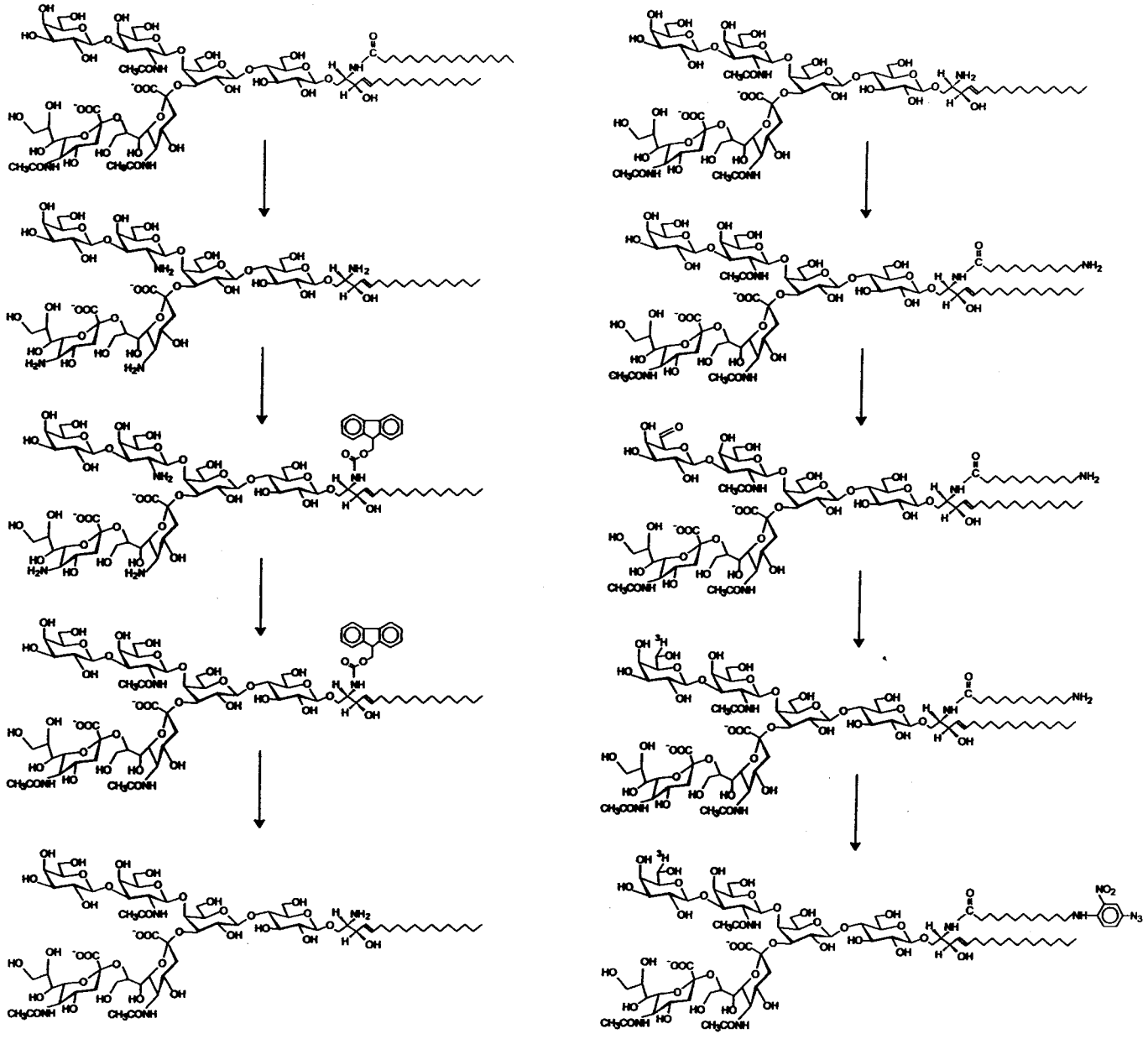

Figure 1. Scheme of the reactions for the preparation of radioactive and photoactivable GD1b, [6-^{3}H(*IV-Gal*)]GD1*b*-N_3, from natural GD1b.

vigorously stirred for 75 min at room temperature. The mixture was dried and the residue was applied to a silica gel 100 column (height 70 cm, diameter 14 mm) equilibrated and eluted with chloroform/methanol/water 30:50:13, by vol., to give homogeneous GD1b-ALAFF. The reaction products were characterized by HPTLC (solvent system isopropanol/water, 7:3, by vol.).

Deblocking of aminoacyl chain. 51 μmoles of GD1b-ALAFF were treated with ammonia as described in the previous paragraph to give GD1b-ALA, GD1b containing the aminododecanoyl acyl chain. The mixture was dried and the residue was purified on a silica gel 100 column (height 70 cm, diameter 14 mm) equilibrated and eluted with chloroform/methanol/water 30:50:13, by vol. Yield for GD1b-ALA was 30.6 μmoles. The reaction products were characterized by HPTLC (solvent system isopropanol/water, 7:3, by vol.).

Labeling of GD1b-ALA. 8 μmoles of GD1b-ALA were dissolved in chloroform/methanol 3:5, by vol. 110 mg of Triton X-100 were added and the mixture was slowly dried. The residue was dissolved in 5 ml of 25 mM phosphate buffer, pH 7.0, 5 mM EDTA, and 450 U of galactose oxidase were added. The mixture was stirred at 37°C for 2.5 hours. 450 U of galactose oxidase were further added and reaction allowed to proceed overnight in the same conditions. The reaction mixture was dried in a reaction tube and subsequently dissolved in 2-propanol and added with 0.1 M NaOH until reaching pH 7.5. 100 mCi NaB^3H_4 (specific radioactivity 12.0 Ci/mmol) were added and reaction was allowed to proceed at room temperature for three days. The reaction mixture was dried and [6-^{3}H(*IV-Gal*)]GD1b-ALA was purified on a silica gel 100 column (height 90 cm, diameter 14 mm), equilibrated and eluted with chloroform/methanol/water 30:50:13. The reaction products were characterized by HPTLC (solvent system chloroform/methanol/water, 30:50:13, by vol.).

Preparation of photoactivable ganglioside. 4 μmoles of radioactive compound were dissolved in 400 μL of anhydrous DMF. 4 mL triethylamine and 9 μmoles 4-F-3-NO_2-phenylazide, dissolved in 150 μL ethanol, were added, and the mixture was vigorously stirred overnight at 80°C. The reaction mixture was dried and [6-^{3}H(*IV-Gal*)]GD1b-N_3 was purified on a silica gel 100 column (height 100 cm, diameter 12 mm) equilibrated and eluted with chloroform/methanol/water 30:50:13, by vol. The reaction products were characterized by HPTLC (solvent system chloroform/methanol/water, 30:50:13, by vol.). Fractions containing [6-^{3}H(*IV-Gal*)]GD1b-N_3 were dried and the residue immediately solubilized in methanol (8.25 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 [41]. 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 between 6th and 8th day in culture. Average protein content at this time was 700 μg protein/dish. Cell viability was assessed by the Trypan blue exclusion method.

Treatment of cell cultures with [1-^{3}H]sphingosine or [^{35}S]methionine

Cells at the 6th day in culture were incubated in the presence of 3×10^{-8} M [1-^{3}H]sphingosine (5 ml/dish) in cell-conditioned medium for 2 hours (pulse) followed by a 48 hours chase as previously described [15]. These experimental conditions allowed metabolic radiolabeling of all sphingolipids (including ceramide, sphingomyelin, neutral glycolipids and gangliosides), and phosphatidylethanolamine (obtained by recycling of radioactive ethanolamine formed in the catabolism of [1-^{3}H]sphingosine). Cells at the 7th day in culture were preincubated in methionine-free medium for 2 hours and subsequently incubated in the presence of 25 μCi/ml L-[^{35}S]-methionine (5 ml/dish) for 20 hours, to radiolabel proteins [42].

Photolabeling experiments

Cells at the 7th or 8th day in culture were incubated with a mixture of 1.0×10^{-6} M GD1b and 1.0×10^{-6} M [6-^{3}H(*IV-Gal*)]GD1b-N_3 for 4 hours in serum-free culture medium, followed or not by different times of chase (2, 4, or 24 hours). After incubation, cells were washed 5 times with basal modified Eagle's medium containing 10% fetal calf serum and once with PBS. 4 ml ice-cold PBS were added and cells were illuminated for 45 min under UV light (wavelength 369 nm) on ice to allow cross-linking. All procedures before exposure to UV light were performed under red safelight. Proteins were analyzed as described below.

Preparation of sphingolipid-enriched membrane domains by sucrose gradient centrifugation

After radiolabeling with [1-^{3}H]sphingosine or [^{35}S] methionine, at the 8th day in culture, cells were subjected to ultracentrifugation on discontinuous sucrose gradients, after lysis and homogenization in the presence of 1% Triton X-100, as previously described [15]. 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 centrifuged (5 min, $1{,}300 \times g$) to remove nuclei and large cellular debris. The postnuclear fraction was mixed with an equal volume of 85% sucrose (wt/vol) in 10 mM Tris buffer (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4. The resulting diluent was placed at the bottom of a discontinuous sucrose concentration gradient (30%–5%) in the same buffer and

centrifuged (17 h, 200,000 × *g*) at 4°C. After ultracentrifugation, eleven fractions were collected starting from the top of the tube. The light-scattering band located at the interface between 5 and 30% sucrose corresponded to fraction 5 and was regarded as the fraction containing the sphingolipid-enriched membrane domains [15]. The entire procedure was performed at 0–4°C in ice immersion. In most experiments, fractions 9 to 11 were pooled together and termed High Density fraction (HD).

Analysis of protein patterns

Cell lysates and sucrose gradient fractions obtained after labeling cerebellar granule cells with [^{35}S]methionine or after photolabeling with [6-^{3}H(*IV-Gal*)]GD1b-N_3 were analyzed by 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) [43]. After separation, proteins were transferred to PVDF membranes. The presence of c-Src, Lyn, Fyn and Csk was assessed by immunoblotting with commercially available specific antibodies, followed by reaction with secondary HRP-conjugated antibody and enhanced chemiluminescence detection (Pierce Supersignal). Proteins cross-linked to [6-^{3}H(*IV-Gal*)]GD1b derivative were analyzed by digital autoradiography.

Immunoprecipitation experiments

Aliquots of fraction 5 and HD fraction obtained from cells labeled with [^{35}S]methionine (containing 5–10 µg protein) were diluted 10-fold in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/ml aprotinin, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate). After preclearing for nonspecific binding with protein A/G-Sepharose, 2 µg/ml rabbit anti-c-Src (SRC 2) or anti-Lyn or anti-Csk polyclonal IgG or 2 µg/ml normal rabbit IgG (as negative control) was added to the samples. In the case of c-Src, some experiments were performed using goat polyclonal IgG (N-16) and normal goat IgG as negative control. The solutions were mixed overnight at 4°C. Immunoprecipitates were recovered by adding protein A/G-Sepharose, and mixing for 2 h. Beads were washed 3 times with RIPA buffer, suspended in SDS-sample buffer, heated to 95°C for 3 min, and recovered by centrifugation. Supernatants were subjected to SDS-PAGE [18]. After separation, proteins were transferred to PVDF membranes and radioactive proteins were visualized by autoradiography. Aliquots of fraction 5 and the HD fraction obtained from cells after labeling with [1-^{3}H]sphingosine containing the same amount of radioactivity were immunoprecipitated with anti-c-Src, anti-Lyn or anti-Csk antibody as described above, except that immunoprecipitation (IP) buffer (50 mM Tris-HCI, pH 7.4, 150 mM NaCl, 2 mM NaF, 1 mM EGTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF, 75 mU/ml aprotinin, 1% Triton X-100) was used instead RIPA buffer. Radioactive lipids associated with the immunoprecipitates were extracted and analyzed as described below.

Analysis of radioactive lipids

The cell lysate, postnuclear supernatant and sucrose gradient fractions, obtained after cell metabolic radiolabeling, were analyzed to determine the content of radiolabeled lipids as previously described [15]. Samples were dialyzed, lyophilized and lipids were extracted with chloroform/methanol 2:1, by vol. Immunoprecipitates were directly extracted. The lipid extracts were analyzed by HPTLC (solvent systems chloroform/methanol/0.2% aqueous $CaCl_2$, 50:42:11 or 55:45:10, by vol.), followed by digital autoradiography for quantification of radioactivity. Identity of lipids separated by HPTLC was assessed by comigration with standard lipids.

Other experimental procedures

The radioactivity associated with cells, with cell fractions, with lipid extracts and with delipidized pellets was determined by liquid scintillation counting. Digital autoradiography of the HPTLC plates and of the PVDF membranes was performed with a Beta-Imager 2000 instrument (Biospace, Paris). The radioactivity associated with individual lipids and proteins 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. Structural characterization of the end product and the intermediates of the synthesis of the radioactive photoactivable GD1b derivative was carried out by ^{1}H-NMR at 500 MHz [44].

Results

Presence of c-Src, Lyn and Csk in the sphingolipid-enriched membrane fraction from rat cerebellar granule cells differentiated in culture. In a previous paper [15], we demonstrated that a low-density membrane fraction prepared from rat cerebellar granule cells differentiated in culture by sucrose gradient centrifugation, after lysis in the presence of 1% Triton X-100, is very rich in sphingolipids and cholesterol, and relatively devoid of proteins and glycerophospholipids. About 60% of sphingolipids were recovered in fraction 5 of the gradient, which contained only about 1.5% of the cell proteins. The high density region of the gradient (fractions 9 to 11), which accounted for the bulk of the cell proteins (>70%), contained less than 25% of cell sphingolipids. For this reason, fraction 5 was termed sphingolipid-enriched membrane fraction.

We analyzed the presence of the Src family tyrosine kinases c-Src and Lyn and of the tyrosine kinase Csk in the sphingolipid-enriched membrane fraction prepared from rat cerebellar granule cells differentiated in culture. As shown in Figure 2, SDS-PAGE followed by immunoblotting with specific polyclonal antibodies revealed that c-Src, Lyn and Csk are present and highly enriched in the sphingolipid-

enriched membrane fraction. On the basis of the relative intensities of the signals and of the protein content of the fractions, we calculated that c-Src, Lyn and Csk are 100-, 75- and 10-fold enriched in fraction 5 respect to the high density fraction, respectively. The presence of c-Src in the sphingolipid-enriched fraction was also confirmed by two-dimensional electrophoresis. Figure 3 shows the pattern of ^{35}S proteins from this fraction, after separation by isoelectrofocusing in the first dimension followed by 10% SDS-PAGE in the second dimension (*left panel*). The same membrane was probed by immunoblotting using a specific anti-c-Src antibody (*right panel*). A band pattern of ^{35}S proteins corresponding to the immunoblotting signal of c-Src was clearly identified. On the basis of the intensity of ^{35}S label, c-Src represents about 0.6% of the total protein content of this fraction.

Co-immunoprecipitation of sphingolipids with c-Src, Lyn and Csk. After labeling of sphingolipids with [1-^{3}H]sphingosine, rat cerebellar granule cells were subjected to sucrose gradient centrifugation. Aliquots of the sphingolipid-enriched membrane fraction and of the high density fraction (fractions 9–11) containing the same amount of lipid-associated radioactivity were immunoprecipitated by specific anti-c-Src, anti-Lyn or anti-Csk antibodies. The immunoprecipitation experiments were carried out using buffers containing 1% Triton X-100 to preserve the integrity of the domain. Figure 4 shows the patterns of radioactive lipids associated with immunoprecipitates after extraction by chloroform/methanol and separation by HPTLC. The radioactivity present in the immunoprecipitates obtained by anti-c-Src, anti-Lyn and anti-Csk antibodies from the sphingolipid-enriched membrane fraction was mainly associated with sphingomyelin and, to a lesser extent, with gangliosides. Negative controls performed using non-immune IgGs did not contain any radioactivity (data not shown). The qualitative distribution patterns of radioactivity among different sphingolipids in the immunoprecipitates obtained by all three antibodies are similar, even if not identical. However, they are quantitatively different from the pattern seen in the sphingolipid-enriched membrane fraction. This could be due to the existence of lipid domains with different composition within this fraction. Immunoprecipitates obtained under the same experimental conditions from the high density gradient fraction did not contain lipid-associated radioactivity. These results indicate that, within the sphingolipid-enriched membrane fraction, c-Src, Lyn and Csk are able to associate with the lipid component of the domain, suggesting that lipid-protein interactions play an important role in the functional assembly of the domain itself.

Reciprocal association of c-Src, Lyn and Csk and their association with other proteins within the sphingolipid-enriched membrane fraction. Aliquots of the sphingolipid-enriched membrane fraction prepared by sucrose gradient centrifugation from rat cerebellar granule cells differentiated in culture, after metabolic labeling of cell proteins with [^{35}S]methionine, containing the same amount of protein were subjected to immunoprecipitation with specific anti-c-Src, anti-Lyn and anti-Csk antibodies. Figure 5 shows the patterns of ^{35}S proteins associated with immunoprecipitates after separation by SDS-PAGE and visualization by autoradiography. Each immunoprecipitate contained a radioactive protein

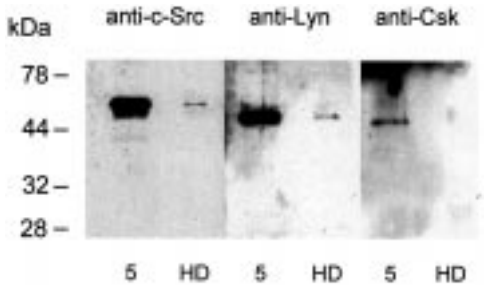

Figure 2. Distribution patterns of c-Src, Lyn and Csk in sucrose gradient fractions from rat cerebellar granule cells differentiated in culture. Cerebellar granule cells differentiated in culture were subjected to discontinuous sucrose gradient centrifugation as described under 'Experimental Procedures'. Fraction 1 was collected at the top of the gradient and the pellet-containing fraction 11 at the bottom. Fraction 5 contained the light-scattering band located at the interface between 5% and 30% sucrose. Similar amounts of protein from gradient fraction 5 and fractions 9 to 11 (pooled together, High Density fraction, HD) were analyzed by SDS-PAGE followed by detection by Western Blotting using respective antibodies, as indicated at the top of each panel. Patterns are representative of those obtained in three different experiments.

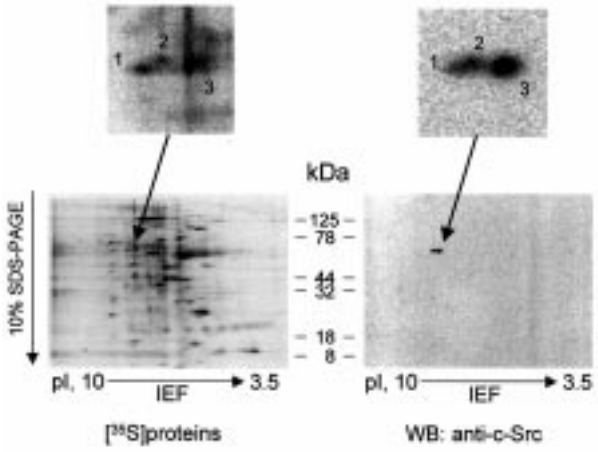

Figure 3. Two-dimensional protein pattern in sphingolipid-enriched membrane fraction from rat cerebellar granule cells differentiated in culture after labeling with [^{35}S]methionine and immunodetection of c-Src. About 1.2 μg (≅3000 cpm) of cell protein from sphingolipid-enriched membrane fraction (Fraction 5) prepared from differentiated granule cells after steady-state metabolic labeling with [^{35}S]methionine was analyzed by two-dimensional electrophoresis followed by autoradiography (time of exposure: 15 days) as described under 'Experimental conditions' (*left panel*). *Right panel*: the same membrane was probed using specific anti-c-Src antibody. *Upper insets*: enlargements of areas indicated by arrows.

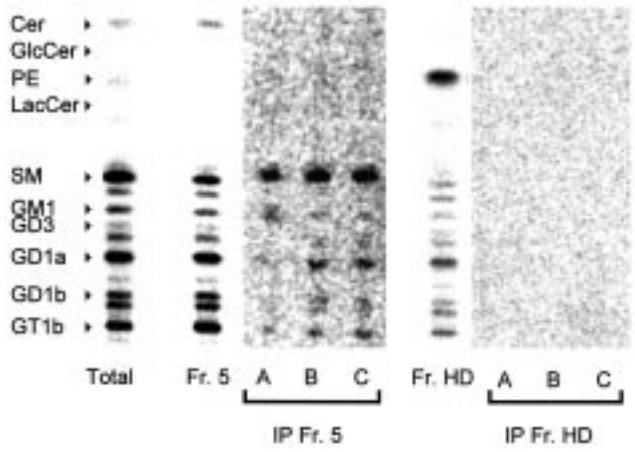

Figure 4. Association of sphingolipids with c-Src, Lyn and Csk in sphingolipid-enriched membrane fraction prepared from rat cerebellar granule cells differentiated in culture after feeding [1-^{3}H]sphingosine. Aliquots of the sphingolipid-enriched membrane fraction (Fraction 5) and the High Density fraction (Fraction HD) prepared from differentiated granule cells after feeding [1-^{3}H]sphingosine, containing the same amount of radioactivity, were immunoprecipitated by polyclonal antibodies to c-Src (*lane A* in both panels), to Lyn (*lane B* in both panels), to Csk (*lane C* in both panels) or normal IgG (as negative control) as described under 'Experimental conditions'. No radioactivity was found in negative controls. Lipids were extracted as described under 'Experimental Procedures' and separated by HPTLC in solvent system chloroform/methanol/0.2% $CaCl_2$ 50:42:11 by vol. Radioactive lipids were detected by digital autoradiography (30–50 dpm applied on a 3 mm line; time of acquisition: 48 hr). Patterns are representative of those obtained in two different experiments.

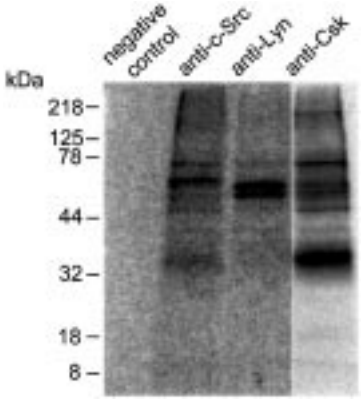

Figure 5. Pattern of radioactive proteins associated to c-Src, Lyn and Csk in the sphingolipid-enriched membrane fraction prepared from rat cerebellar granule cells differentiated in culture after labeling with [^{35}S]methionine. Aliquots of the sphingolipid-enriched membrane fraction (Fraction 5) prepared from differentiated granule cells after steady-state metabolic labeling with [^{35}S]methionine were immunoprecipitated by polyclonal antibodies to c-Src, to Lyn, to Csk, or normal IgG (negative control), as indicated at the top of each panel, as described under 'Experimental conditions'. Radioactivity in negative controls was less than 0.5% of that detectable in innumoprecipitates. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography (time of exposure: 30 days). Patterns are representative of that obtained in two different experiments.

band with an apparent molecular mass corresponding to that expected for the respective antigen (60 kDa for c-Src; a doublet at 53/57 kDa for Lyn; 50 kDa for Csk). The anti-c-Src immunoprecipitate also contained a band comigrating with Csk. The same band at 50 kDA (even if very faint) was detectable in the anti-Lyn immunoprecipitate. On the other hand, the anti-Csk immunoprecipitate contained a clear doublet comigrating with Lyn and a faint band at 60 kDa comigrating with c-Src. Moreover, all three immunoprecipitates showed a band with a molecular mass between 35 and 40 kDa (very strong in the case of the anti-Csk immunoprecipitate), a band between 40 and 44 kDa, and a third band at about 70 kDa. These results indicate that, within the sphingolipid-enriched domain from differentiated rat cerebellar neurons, c-Src, Lyn and Csk are associated with each other and at least three other unknown proteins. The experimental conditions used for these experiments (RIPA buffer containing Triton X-100, SDS and sodium deoxycholate) suggest that these interactions are direct, relatively strong, protein–protein interactions.

Association of Src-family kinases with a photoactivable derivative of GD1b ganglioside. To better understand the interactions between gangliosides and proteins within the sphingolipid-enriched membrane domain from differentiated cerebellar granule cells, photolabeling experiments with a radioactive and photoactivable derivative of GD1b ganglioside, one of the main gangliosides of granule cells, were performed. By incubating cells with [6-^{3}H(*IV-Gal*)]GD1b-N_3, the ganglioside derivative became a component of the plasma membrane with the photoactivable phenylazide group at the fatty acid moiety deeply inserted in the membrane lipid core. By exposure to UV light, a highly reactive nitrene group is formed, that is able to form covalent bonds with a variety of molecules. We set up experimental conditions allowing an efficient photolabeling of proteins in rat cerebellar granule cells differentiated in culture. Figure 6 shows the patterns of cellular proteins cross-linked to radioactive GD1b derivative in granule cells after incubation in the presence of the derivative for 4 hours, followed or not by 2, 4 or 24 hours chase, visualized by digital autoradiography after separation by SDS-PAGE. At all investigated times, a well defined pattern of proteins cross-linked with radioactive ganglioside was detectable. In particular, an intense photolabeling was achieved for protein bands with molecular masses ranging from 35 to 45, from 50 to 60 and from 100 to 130 kDa.

The pattern of photolabeled proteins from differentiated rat cerebellar granule cells, after incubation in the presence of [6-^{3}H(*IV-Gal*)]GD1b-N_3 and illumination, was analyzed by two-dimensional electrophoresis. Figure 7 (*right panels A*, *B and C*) shows typical patterns of proteins cross-linked to ganglio-

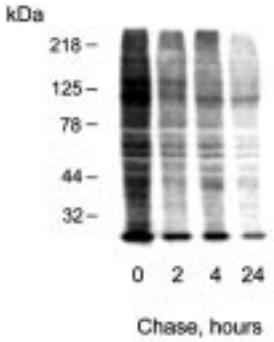

Figure 6. Patterns of photolabeled proteins from rat cerebellar granule cells differentiated in culture after incubation with photoactivable [^{3}H]GD1b derivative. Rat cerebellar granule cells differentiated in culture were incubated in the presence of photoactivable [^{3}H]GD1b derivative as described under 'Experimental conditions' for 4 hours followed or not by different chase times (0-2-4-24 hours). After incubation and exposure to UV light, samples were analyzed by SDS-PAGE (10 μg of cell protein/sample) and proteins cross-linked to radioactive ganglioside were detected by digital autoradiography (18,000–100,000 dpm; time of acquisition: 50 hr). Patterns are representative of that obtained in two different experiments.

sides after detection by digital autoradiography. The pattern of photolabeled proteins was highly reproducible. The main radioactive spots corresponded to a protein doublet with a molecular mass of about 55–60 kDa and an isoelectric point of about 5. Immunoblotting analysis revealed that this band comigrated with tubulin (data not shown). Tubulin already has been reported to be intensely cross-linked by a photoactivated GM1 derivative in these cells [45]. Four well defined protein bands cross-linked to GD1b were detectable with apparent molecular masses ranging from 50 to 70 kDa and isoelectric points from about 6 to about 7. The same membranes (Figure 7, left panels) were probed by immunoblotting using specific polyclonal antibodies to c-Src (*panel A*), Lyn (*panel B*) or Csk (*panel C*). As indicated by the arrows, anti-c-Src and anti-Lyn positive bands comigrated with protein bands cross-linked to GD1b. No photolabeled protein bands were detectable in the area corresponding to the anti-Csk positive bands. Thus, the results of photolabeling experiments indicate that c-Src and Lyn, but not Csk, associate directly with ganglioside molecules.

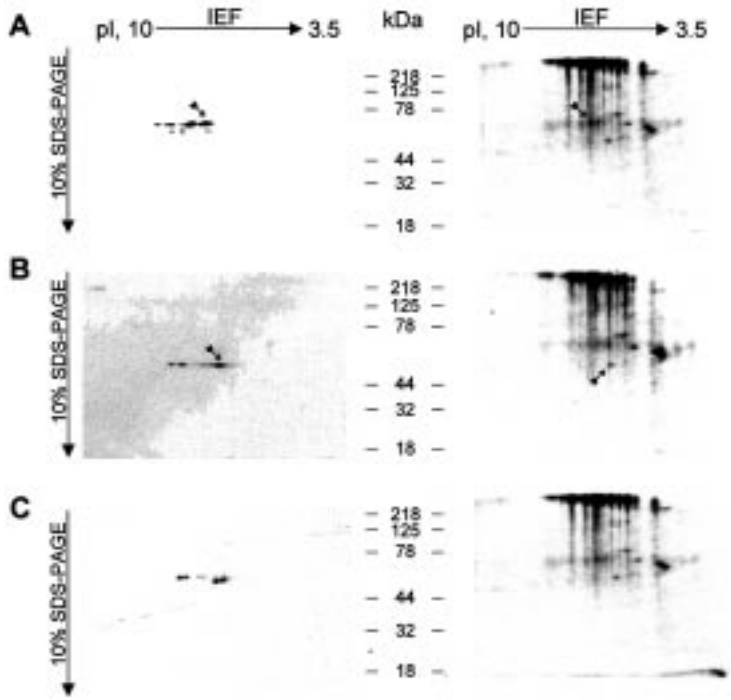

Figure 7. Photolabeling of c-Src, Lyn and Csk in rat cerebellar granule cells differentiated in culture after incubation with photoactivable [^{3}H]GD1b derivative. Rat cerebellar granule cells differentiated in culture were incubated in the presence of photoactivable [^{3}H]GD1b derivative for 4 hours. After incubation and exposure to UV light, aliquots of samples (10 μg of cell protein) were analyzed by two-dimensional electrophoresis as described under 'Experimental conditions', and proteins cross-linked to radioactive ganglioside were detected by digital autoradiography (80,000 dpm; time of acquisition: 24 hr) (*right panels*). *Left panels*: the same membranes were probed using specific antibodies to c-Src (*panel A*) or to Lyn (*panel B*) or to Csk (*panel C*). Patterns are representative of those obtained in two different experiments. Arrows on left panels indicate the positions of proteins detected by Western blotting, corresponding to photolabeled proteins indicated by the arrows on right panels.

Discussion

Sphingolipids, particularly gangliosides, are characteristic components of the plasma membrane of the neurons. Their crucial role in the control of the functional properties of neurons and other cell types of neural origin is indicated by an impressive body of evidence, continuously growing in the last two decades [reviewed in 1,2]. The role of sphingolipids in the maintenance of cellular structure and function can be at least in part explained by their ability to interact with specific proteins at the level of the plasma membrane and to modulate their activity [2,9–11]. Since Src family kinases seem to be so important in neuronal differentiation [23–32], we studied the mode of interaction of Src-family kinases and of the regulatory kinase Csk with sphingolipids in a model of neurons differentiated in culture. Our results indicated that a sphingolipid-enriched membrane fraction prepared from rat cerebellar granule neurons differentiated in culture is rich in c-Src, Lyn and Csk. All these proteins may associate with the sphingolipid components (both sphingomyelin and glycosphingolipids) of the domain, as suggested by the coimmunoprecipitation experiments. However, photolabeling experiments with photoactivable ganglioside indicate that only c-Src and Lyn, but not Csk, associate directly with ganglioside molecules. This is not surprising. c-Src and Lyn are *N*-myristoylated proteins [46]. The myristoyl chain, that is necessary for the binding of these proteins to the membrane, is inserted in the lipid core, and likely represents the site of interaction with the hydrophobic moiety of the ganglioside. The rigid, highly organized lipid environment of the sphingolipid-enriched domain could provide a structure able to trap these proteins, allowing association with other components of the domain itself. Csk, lacking the myristoyl chain, may not directly interact with sphingolipids in the domain. However, Csk could be retained in the domain by specific protein–protein interactions with c-Src, Lyn, and other not yet characterized proteins. Csk is able to interact with other proteins via its SH2 and SH3 domains. Among others, we could detect in all our immunoprecipitates, especially with anti-Csk, a protein with a molecular mass between 35 and 40 kDa. Interestingly, c-Src in the growth cones of differentiating neuroblastoma cells is associated with a 38 kDa protein, of as yet, unknown function [47].

Sphingolipid-protein and protein–protein interactions could cooperate in creating the global structure of the domain. Does this structure have a functional meaning? The association of different cellular components (namely sphingolipids and Src family tyrosine kinases) playing a role in the same cell function is not unexpected. c-Src organized with sphingolipid undergoes activation during ganglioside-induced differentiation in neuroblastoma cells [18]. Activation of c-Src is accompanied by removal of Csk from a complex with c-Src itself within the domain. In addition, anti-ganglioside antibody-induced stimulation of cerebellar neurons leads to activation of Lyn in the domain [33,34]. This suggests that sphingolipid-enriched domains might function as 'signaling domains' during the process of neuronal differentiation. To elucidate this issue, possible changes in the structure of sphingolipid-enriched domain during the development of the neuron need to be investigated.

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