

Immunohistochemical and biochemical analyses of GD3, GT1b, and GQ1b gangliosides during neural differentiation of P19 EC cells¹

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Abstract In an earlier study, we showed that expressions of GD3, GT1b, and GQ1b gangliosides in P19 embryonic carcinoma (EC) cells were enhanced during their neural differentiation induced by retinoic acid. We now further demonstrated that this increase of the b-series gangliosides is due to an increase in their corresponding synthases (sialyltransferase-II, -IV, and -V) in the Golgi. Of the three gangliosides studied, GQ1b appeared to be the best candidate for monitoring such differentiation process. We also used fluorescence-labeled monoclonal antibodies and confocal fluorescence microscopy to obtain direct visual information about the relationship of gangliosides and neural specific proteins in neuron development. Again, GQ1b is the most interesting as it localizes with synaptophysin and neural cell adhesion molecules (NCAMs) on synaptic boutons or dendritic spines in RA-induced neurons (R/N). This suggests that GQ1b could be used as a marker for synapse formation during construction of the neural network.

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Key words: Ganglioside; Sialyltransferase; Retinoic acid; Neural cell differentiation; Confocal laser scanning microscopy

1. Introduction

Gangliosides are sialoglycolipids and are present in high concentrations in the central nervous system (CNS). The levels and types of gangliosides change during neural differentiation and development [1–3]. Retinoic acid (RA) can induce P19 embryonic carcinoma (EC) cells to differentiate into neural cells and this cell system has been shown to be a useful tool for studying the early steps of neural differentiation in vitro [4,5]. Levine and Flynn also showed that it was highly valuable to investigate the localization of the b-series gangliosides in P19 EC cells [6]. The critical ganglioside synthases sialyltransferases (SATs) have been found in the Golgi apparatus derived from rat liver and mouse primary cultured cerebellar neurons [7,8]. SATs changed markedly in hippocampal neurons during axonogenesis and axon elongation, but not during dendritogenesis, dendrite growth or synaptogenesis in polarized neurons [9–11]. We have already shown that the levels of GD3 synthase (SAT-II), GT1b synthase (SAT-IV), and GQ1b synthase (SAT-V) in R/A-induced P19 EC cell aggregates and neurons were significantly higher than those in undifferentiated cells [12]. Neuron specific proteins: growth-associated protein-43 (GAP-43), synaptophysin, and neural cell adhesion molecules (NCAMs) were found located on many growth cones and also uniformly distributed along thin neurites of P19 EC and murine embryonic stem cell cultures using immunofluorescence double-labeling techniques [13–15], while GM1 was shown distributed on the plasma membranes of filopodial and lamellipodia of growth cones during the early stage of neurite outgrowth using electron microscopy [16]. In this present study, we investigate the levels of b-series gangliosides and their synthases in the Golgi and also provide direct visual information on the close relationship of localization, in particular of GQ1b and NCAMs during neural differentiation using double immunostaining of the respective fluorescent monoclonal antibodies (MAbs), and in combination with confocal fluorescence microscopy. Our results indicate that GQ1b is a useful marker for certain neural cell differentiation processes.

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¹ Gangliosides and glycosphingolipids are named according to Svennerholm, L. [23].

Abbreviations: BSA, bovine serum albumin; CNS, central nervous system; EC, embryonic carcinoma; FITC, fluorescein isothiocyanate; Fr(s), fraction(s); Gal, galactose; Gal-T, galactosyltransferase; GAP-43, growth-associated protein-43; HPTLC, high-performance thin-layer chromatography; MAb, monoclonal antibody; NCAMs, neural cell adhesion molecules; NeuAc, N-acetylneuraminic acid; RA, retinoic acid; R/A, RA-induced aggregates; R/N, RA-induced neurons; SAT(s), sialyltransferase(s); TBS-T, Tris-buffered saline-Tween 20; U, undifferentiated cells

2. Materials and methods

2.1. Materials and cells

Retinoic acid (RA, all-*trans* form), bovine brain gangliosides GM3, GD3, GD1b, GT1b, and GQ1b were from Sigma (St. Louis, MO, USA). CMP-[¹⁴C]N-acetylneuraminic acid (NeuAc) (150 mCi/mmol) and UDP-[¹⁴C]galactose (Gal) (250 mCi/mmol) were from NEN Life Science Products (New York, USA). Anti-GD3 MAb R24 was obtained from the American Type Culture Collection (ATCC no. HB8445). Anti-GT1b MAb, clone GMR5 and anti-GQ1b MAb, clone GMR13 were from Seikagaku Co. (Tokyo, Japan). Anti-GAP-43 MAb (clone GAP-7B10) and anti-NCAMs MAb (clone NCAM-OB11) were from Sigma and anti-synaptophysin MAb (clone SY-38) was from Boehringer Mannheim Biochemica (Germany). Horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody was from Amersham Pharmacia Biotech (Buckinghamshire, UK). P19 EC cells (kindly provided by Dr. H. Hamada of Osaka University, Osaka, Japan) were cultured as previously described [12]. Three types of P19 EC cells, undifferentiated cells (U), RA-induced aggregates (R/A), and RA-induced neurons (R/N), were used in this study. R/N were confirmed by morphological changes observed under the microscope and by the expressions of neural marker proteins, GAP-43, synaptophysin, and NCAMs (see Sections 2.6 and 2.7 below).

2.2. Preparation of the subcellular organelle fractions

Four subcellular organelle fractions were isolated at 4°C or on ice from P19 EC cells using previously reported sucrose gradient centrifugation methods and their purity was evaluated by the presence of β 1,4-galactosyltransferase (β 1,4-Gal-T) activity [8,9,17]. Four membrane fractions (Frs.) were collected from the top, Fr. 1, representing 0.3/0.8 M sucrose interface (plasma membrane and synaptic vesicle-enriched fractions [8,17]), Fr. 2, 0.8/1.05 M sucrose interface (*trans*-Golgi-enriched fraction and microsomes), Fr. 3, 1.05/1.3 M sucrose interface (*cis*-Golgi-enriched fraction and mitochondria), and Fr. 4, the pellet (endoplasmic reticulum and nuclei). The membranes were diluted with 50 mM HEPES buffer, centrifuged at 5×10^4 rpm (Beckman TL 100, TLA 100-3 rotor) for 1 h, resuspended in 30 μ l of 0.5 M sucrose in phosphate-buffered saline (PBS) containing 0.3% Triton X-100 detergent, sonicated (0.5 cycle, 90% amplitude, UP 50H, Kubota, Japan), and used as enzyme sources. Protein concentration of the enzyme source was determined by the MicroBCA assay kit (Pierce, Rockford, IL, USA).

2.3. Extraction of lipids and TLC immunostaining

Lipids were extracted from 10^9 cells of U, R/A, and R/N and were separated into neutral and acidic fractions by DEAE Sephadex A-25 column chromatography as previously described [12,18]. Acidic lipids obtained from U, R/A, and R/N were 0.5 mg, 1.4 mg, and 1.3 mg (dry weight) respectively. Lipids were also extracted from the subcellular organelle fractions (Frs. 1–4, see above) of the R/N, but they were not separated into neutral and acidic components because of the limited amount of material available. Lipids were resolved on silica gel 60 high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) using the following solvent systems: solvent (i), CHCl₃/MeOH (1:1, v/v) and solvent (ii), CHCl₃/MeOH/0.2% CaCl₂·2H₂O (40:40:11, v/v/v). Gangliosides were identified by comparing with GD3, GT1b, and GQ1b commercial standards. They were also confirmed by TLC immunostaining with anti-GD3, -GT1b, and -GQ1b MABs as previously described [12]. A study on the neutral glycolipid fraction had already been reported elsewhere [18].

2.4. Assay of β 1,4-Gal-T activity

β 1,4-Gal-T activity was used to evaluate the purity of isolated Golgi-containing membrane fractions and it was measured essentially as described previously [8,19] with slight modification. The assay mixture (50 μ l) contained 20 mg/ml ovalbumin (as acceptor), 10 mM MnCl₂, 0.01% Triton X-100, 20 mM HEPES (pH 7.4) and 10 μ l of the enzyme source. Enzyme assay was started by adding 5 μ l of 10 mM cold and radioactive UDP-[¹⁴C]Gal (4×10^5 CPM; dissolved in H₂O). After 2 h incubation at 37°C, the reaction mixtures were spotted onto a Whatman No. 1 paper (2 × 2 cm). The paper was then washed by dipping into 10% (w/v) trichloroacetic acid, rinsed twice with fresh 10% (w/v) trichloroacetic acid, followed by once with ethanol/diethyl ether (2/1, v/v) and finally with diethyl ether. Radioactivity on the air-dried pa-

per was counted using a liquid scintillation counter. Enzyme activities, averages of two experiments were expressed as CPM-[¹⁴C]Gal/mg protein/h.

2.5. Assay of sialyltransferases

SAT-II, -IV, and -V activities were measured as described previously [10,12]. Briefly, U, R/A, and R/N, and Frs. 1–4 sonicated homogenates were suspended on ice in 15 mM sodium cacodylate buffer, pH 6.5, containing 5% glycerol. SAT activity was measured at 37°C for 2 h in a reaction mixture (20 μ l) comprising membrane homogenate (10 μ l), 1.5 μ M cold and radioactive CMP-[¹⁴C]NeuAc (5×10^4 CPM), 0.4% Triton CF-54, 10 mM MgCl₂, 100 mM sodium cacodylate, pH 6.5, 1 mM 2,3-dehydro-2-deoxy-NeuAc (an inhibitor of endogenous sialidase) and 0.3 mM appropriate acceptor substrate (GM3, GD1b, and GT1b). Enzyme activities of these three SATs were linear up to 4 h under the present conditions. Radiolabeled product and unreacted glycolipids were trapped on a Sep-Pak C18 cartridge (Waters Associates, Milford, MS, USA), washed with water (10 ml), and then eluted with methanol (1 ml) and C/M (1:1, v/v; 1 ml). The total combined eluate was dried, applied onto a silica gel TLC plate, and developed with CHCl₃/MeOH/0.2% CaCl₂·2H₂O (40:40:11, v/v/v). Ganglioside substrates and the newly sialylated products were visualized with orcinol reagent and identified by comparing to cold and radiolabeled authentic standards (NEN Life Science Products, New York, USA). Radioactivity was estimated using a Fujix BAS 2000 Bio-imaging Analyzer (Fuji Photo Film, Tokyo, Japan). Enzyme activities, averages of two experiments were expressed as CPM-[¹⁴C]NeuAc/mg protein/h.

2.6. Western blot analysis

Plasma membrane and synaptic vesicle-enriched fraction (Fr. 1, Section 2.2) was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing condition on 10% or 2–15% gradient gels (Daiichi Pure Chemicals, Tokyo, Japan). Resolved proteins were then transferred to a polyvinylidene difluoride membrane for 2 h in transfer buffer containing 12.5 mM Tris–HCl, 96 mM glycine, and 20% methanol. Blots were blocked for 1 h at room temperature in Tris-buffered saline–Tween 20 (TBS–T) (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% skimmed cow's milk, then washed twice in TBS–T, and incubated with anti-GAP-43, -synaptophysin, and -NCAMs MABs (diluted 1:200) for 1 h at room temperature. After washing with TBS–T, the blots were incubated with goat anti-mouse immunoglobulins conjugated to horseradish peroxidase (diluted 1:5000; Amersham Pharmacia Biotech). Proteins were detected using ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

2.7. Immunohistochemical analysis

Double-label immunostaining was carried out as previously reported [12] with anti-GD3, -GT1b, and -GQ1b MABs, and in combinations with anti-GAP-43, -synaptophysin, and -NCAMs MABs, and appropriate fluorescent-conjugated secondary antibodies (goat anti-mouse IgM-fluorescein isothiocyanate (FITC), goat anti-mouse IgG-rhodamine). Identical batches of MABs were used for both TLC immunostaining and in the Western blotting experiments to eliminate quantitative variations in the antibodies. Briefly, after most of the cells had differentiated into neurons (after 7 days, confirmed morphologically), they were fixed with 4% paraformaldehyde in saline or methanol for 20 min at room temperature [20]. The fixed cells were blocked for 20 min with saline containing 3% bovine serum albumin (BSA) and 10% sheep serum, and then incubated for 16–18 h at 4°C with anti-GD3, -GT1b, and -GQ1b MABs which were diluted to 10 μ g/ml in saline containing 3% BSA and 10% sheep serum. The slides were washed in saline containing 1% BSA and 3% sheep serum, and then incubated with anti-GAP-43, -synaptophysin, and -NCAMs MABs for 16–18 h at 4°C. Then they were washed in saline containing 1% BSA and 3% sheep serum and stained for 1 h with FITC- or rhodamine-conjugated goat anti-mouse IgG or IgM Abs, respectively (1:100 dilution; Biosource International, CA, USA). After washing with saline, the stained cells were observed under a Leica fluorescence confocal microscope (Leica TCS-SP, Leica Microsystems, Germany) using either oil-immersed 10 \times , 40 \times or 63 \times objective lenses. Images were taken and prepared for printing using the Adobe Photoshop software.

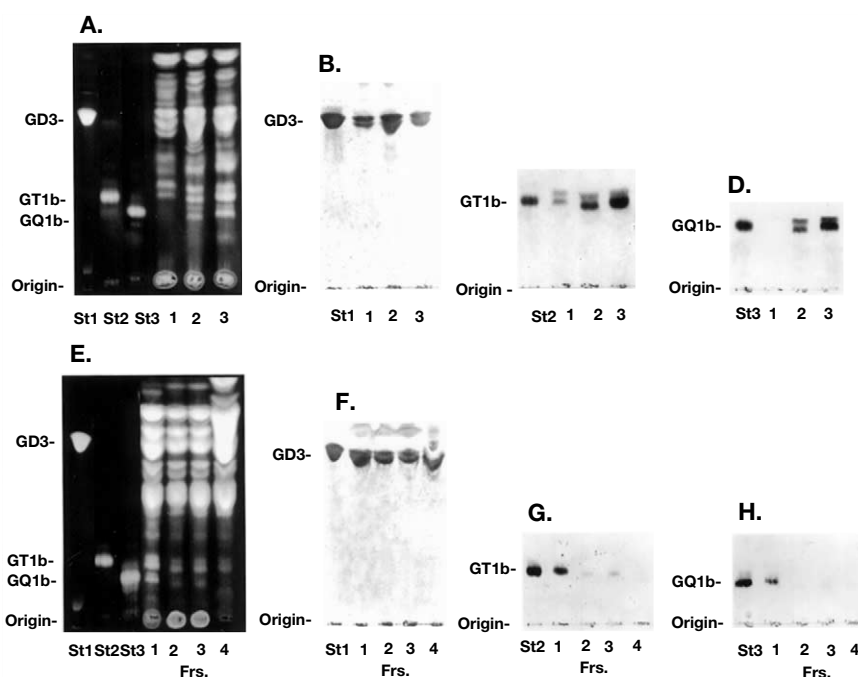


Fig. 1. TLC and immunostaining analyses of lipids in whole cells and in subcellular organelle fractions from RA-induced neural cells. Gangliosides extracted from three types of whole cells (U, R/A, and R/N) and total lipids extracted from subcellular membrane fractions of R/N (Frs. 1–4, see Section 2.2), 2 μ g, were applied and developed on a HPTLC plate with solvent systems (i) $\text{CHCl}_3/\text{MeOH}$ (1/1, v/v), followed by (ii) $\text{CHCl}_3/\text{MeOH}/0.2\% \text{ CaCl}_2 \cdot 2\text{H}_2\text{O}$ (40/40/11, v/v/v). A and E: Primuline staining. B and F, C and G, D and H: Immunostaining analyses of the same samples as in A and E with anti-GD3, -GT1b, and -GQ1b MAbs, respectively. Lanes in A–D: St1, St2, and St3 are GD3, GT1b, and GQ1b standards respectively; lanes 1, 2, and 3 are gangliosides extracts from U, R/A, and R/N respectively. Lanes in E–H: St1, St2, and St3 are standards as in A–E; lanes 1, 2, 3, and 4 are subcellular fractions, Frs. 1–4 respectively. ‘Origin’ points to the position of sample application.

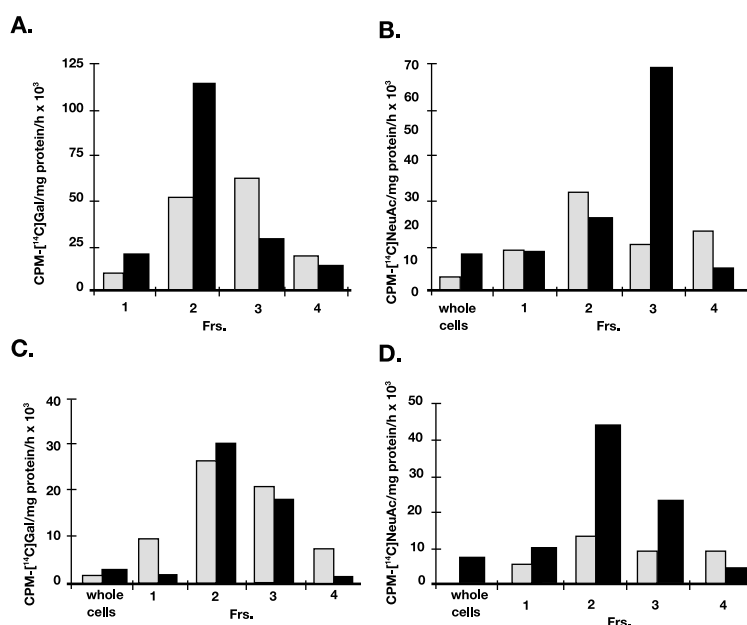


Fig. 2. β 1,4-Gal-T and ganglioside sialyltransferase activities in subcellular organelle fractions from RA-treated cells. A: β 1,4-Gal-T activity. B: SAT-II activity. C: SAT-IV activity. D: SAT-V activity. Lanes 1–4 indicated subcellular organelle fractions Frs. 1–4 (see Section 2.2) obtained from R/A (gray bars) and R/N (solid bars). β 1,4-Gal-T activity was measured using $\text{UDP-[}^{14}\text{C]Gal}$ as donor. SAT-II, -IV, and -V activities were measured using $\text{CMP-[}^{14}\text{C]NeuAc}$ as donor, and GM3, GD1b, and GT1b as acceptor substrates, respectively. All enzyme activities were averages of two experiments.

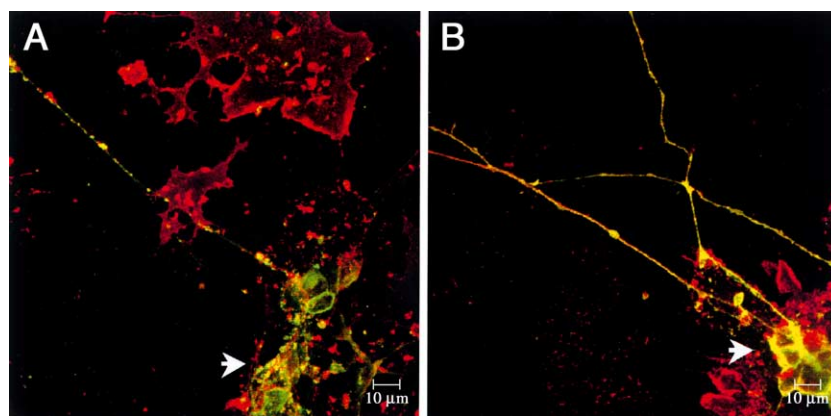


Fig. 3. Localization of GD3, GT1b, and GQ1b in R/N. R/N was double stained with anti-GD3 and -GT1b or -GQ1b MABs. A: Double staining with anti-GT1b (green) and -GD3 (red) MABs. B: Double staining with anti-GQ1b (green) and -GD3 (red) MABs. Scale bars, 10 μ m. Arrowheads point at the cluster of cell bodies.

3. Results

3.1. Expression of GD3, GT1b, and GQ1b in whole cells and in subcellular organelle fractions of R/N

Total acidic glycolipids in whole cells are shown in Fig. 1 and the major gangliosides were confirmed to be GD3, GT1b, and GQ1b both by comparing to standards and by TLC immunostaining using MABs against the respective ganglioside (Fig. 1A–D). GD3 was detected in abundance in all stages of cell differentiation (Fig. 1B). On the contrary, GT1b level showed marked increases from U to R/A and the highest was in R/N (Fig. 1C). GQ1b was observed only in the RA-treated cells (R/A and R/N) (Fig. 1D).

Due to limited amounts of lipid material available in subcellular organelle fractions of R/N, total lipids (Fig. 1E) were analyzed for GD3, GT1b, and GQ1b using TLC immunostaining (Fig. 1F–H). GD3 was the most abundant and was present in almost equal amounts in all fractions (Fig. 1F). GT1b was markedly higher in the plasma membrane fraction, Fr. 1, than in the Golgi-enriched fractions, Frs. 2 and 3 (Fig. 1G). On the other hand, strikingly, GQ1b was only found in the plasma membrane fraction, Fr. 1, which was also enriched with synaptic vesicles (Fig. 1H, [17]).

3.2. β 1,4-Gal-T and ganglioside sialyltransferase activities

As expected, most of the β 1,4-Gal-T activity was localized in the Golgi-enriched fractions (Fig. 2A, Frs. 2 and 3). Highest level was found in the *trans* site (Fr. 2) of the R/N (Fig. 2A, solid bars) and much lower level was displayed in the *cis* site (Fr. 3). There was no significant difference in enzyme activities in the *trans* or *cis* sites of R/A (Fig. 2A, Frs. 2 and 3, gray bars). Similarly, majority of the SAT activities were localized in the Golgi-enriched fractions (Fig. 2B–D, Frs. 2 and 3) and higher activities were found in R/N than in R/A (whole cells). For SAT-II (GD3 synthase), highest activity was found in the *cis* site in R/N (Fig. 2B, Fr. 3, solid bar) but the differences between R/A (Fig. 2B, gray bars) were not significant. In the case of SAT-IV (GT1b synthase), highest activity was displayed in the Golgi-enriched fractions but no difference was observed in the *trans* site (Fr. 2) or *cis* site (Fr. 3) nor when R/A and R/N were compared (Fig. 2C, gray and solid bars, respectively). On the other hand, total SAT-V (GQ1b synthase) activities in R/N whole cells and Golgi mem-

branes (Fig. 2D, Frs. 2 and 3, solid bars) were markedly higher than their R/A counterparts (gray bars). It was significantly higher in the *trans* site (Fr. 2) than in the *cis* site (Fr. 3).

3.3. Localization of GD3, GT1b, and GQ1b in R/N

Fig. 3 shows the results of double immunostaining of b-series gangliosides using anti-GD3 rhodamine-labeled MAB (red), and either anti-GT1b or -GQ1b FITC-labeled MABs (green). GD3 was found to localize with both GT1b and GQ1b, which resulted in appearing as yellow colored areas and were indicated by arrowheads in Fig. 3A and B, respectively. On close examination, staining of GD3 was also localized on non-neuronal cells, which are present underneath neurons. This finding corroborated well with the results observed in Fig. 1B which showed an even distribution of GD3 in all three cell types; U, R/A and R/N. On the other hand, in all cases, GT1b and GQ1b were present only on the cell body of neurons and uniformly distributed along thin neurites, but were absent in any non-neuronal cells (Fig. 3A and B).

3.4. Western blot analyses with anti-GAP-43, -synaptophysin, and -NCAMs MABs

Neural specific proteins GAP-43, synaptophysin, and

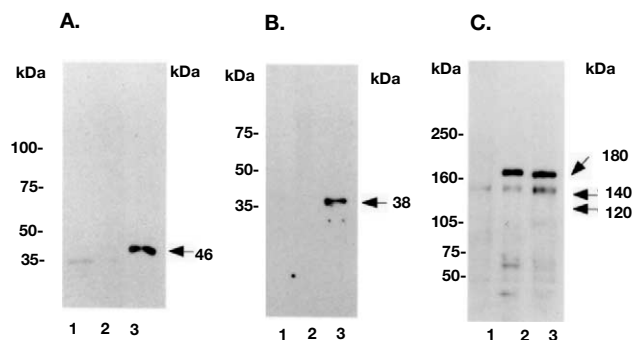


Fig. 4. Western blot analyses of subcellular organelle fraction, Fr. 1 obtained from U, R/A, and R/N with anti-GAP-43, -synaptophysin, and -NCAMs MABs. A: With anti-GAP-43 MAB. B: With anti-synaptophysin MAB. C: With anti-NCAMs MAB. Lanes: 1, U; 2, R/A; 3, R/N. Molecular weight standards (kDa) were shown at the left side of the panels. Arrows indicate the relevant proteins with their molecular weights.

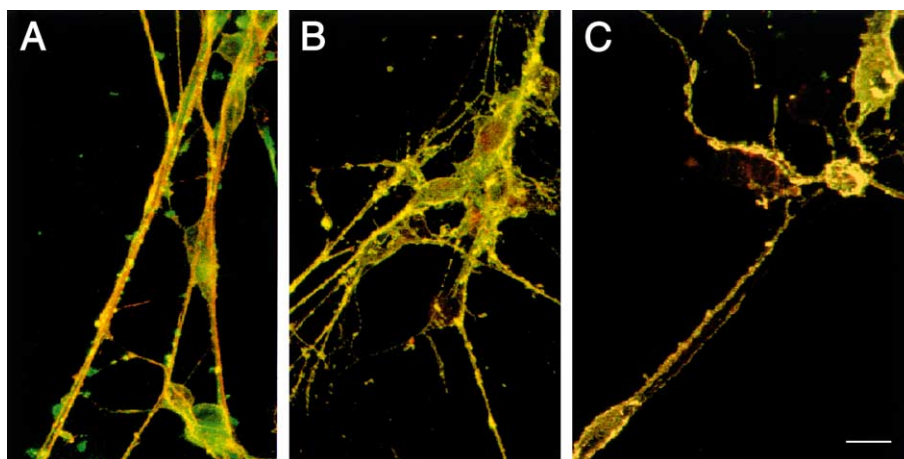


Fig. 5. Double staining of R/N with antibodies against GT1b, GQ1b, GAP-43, synaptophysin, and NCAMs. R/N cells were double stained with relevant antibodies. A: Double staining with anti-GT1b (green) and -GAP-43 (red) MABs. B: Double staining with anti-GQ1b (green) and -synaptophysin (red) MABs. C: Double staining with anti-GQ1b (green) and -NCAMs (red) MABs, and scale bar, 10 μ m.

NCAMs expression were also investigated, in the synaptic vesicle-enriched subcellular organelle fraction, Fr. 1 using immunoblotting with respective MABs (Fig. 4A–C). GAP-43 (46 kDa) and synaptophysin (38 kDa) major proteins were only expressed in R/N (lanes 3, Fig. 4A and B). The anti-NCAMs MAB which recognizes the 140 kDa and 180 kDa isoforms in cells of the CNS and the 120 kDa isoform in glial cells, showed strong NCAMs expression of the 140 kDa and 180 kDa components in both of the RA-treated cells. A very faint band of 140 kDa was also detected in the RA-untreated cells. The amounts of the 140 kDa and 180 kDa NCAMs increased gradually from U, R/A to R/N during differentiation (Fig. 4C).

3.5. Double staining of R/N with MABs against GT1b, GQ1b, GAP-43, synaptophysin, and NCAMs

Fig. 5 illustrates the localization of both b-series gangliosides and neural specific proteins on cell surface of R/N. The MABs used here were the same as those used for Western blots (Fig. 4) so to eliminate batch-to-batch quantitative variations of bindings. GT1b (green) was found to localize with GAP-43 (red), resulting in yellow colored areas in spiny clusters along the edges of the neurites and dendrites, but not on the surface of cell bodies (Fig. 5A). In contrast, GQ1b (green) and synaptophysin (red) were localized with each other and presented as numerous yellow colored spiny clusters on the long neuritic processes and round cell bodies (Fig. 5B). Furthermore, GQ1b clusters were also observed to localize mostly with synaptophysin-labeled synaptic vesicle clusters. GQ1b (green) also localized with NCAMs (red) to form yellow colored regions of numerous synaptic boutons distributing on the surface of cell bodies and spiny clusters along the length of dendrites and neurites (Fig. 5C).

4. Discussion

During RA-induced neural differentiation, the biosynthesis of b-series gangliosides was elevated with increased expressions of GD3, GT1b, and GQ1b of b-series gangliosides [8,9,12,16]. In this study, we confirmed and further showed

b-series ganglioside synthases SAT-II, SAT-IV, and SAT-V were markedly increased in various compartments of the Golgi apparatus. Using fluorescence-tagged MABs and confocal fluorescence microscopy, direct visual localization of both gangliosides and neural specific proteins was obtained. Of the three gangliosides studied, GQ1b is the most interesting as it localizes with synaptophysin and NCAMs on synaptic boutons or dendritic spines in R/N. This suggests that GQ1b could be used as a marker for synapse formation in the study of construction of the neural network. Recent cell biological studies suggested that sphingolipids and cholesterol clustered in biomembranes to form raft-like microdomains [21] and might involve in cell activation and adhesion, signal transduction as well as in the endocytosis of specific molecules [22]. More experiments are therefore required to study in particular the association of the b-series gangliosides and neural specific proteins in microdomains on the neural cell surface.

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