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Prosaposin and prosaptide, a peptide from prosaposin, induce an increase in ganglioside content on NS20Y neuroblastoma cells

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Prosaposin has been recently identified as a neurotrophic factor eliciting differentiation in neuronal cultured cells (NS20Y). In this paper we investigate whether prosaposin and its active peptide (prosaptide) may modify the ganglioside pattern in neuroblastoma cells. The analysis by high performance thin layer chromatography did not reveal qualitative changes in the ganglioside pattern of NS20Y cells incubated in the presence of prosaposin, compared to control cells, but it did reveal an increase of the content of all three major resorcinol positive bands (GM3, GM2, GD1a). Cytofluorimetric and immunofluorescence microscopic analysis revealed that the increase of the ganglioside content was at the plasma membrane level. These findings suggest that the neurotrophic activity of prosaposin on NS20Y neuroblastoma cells might be mediated in part by the increase of cell surface gangliosides.

Keywords: gangliosides, prosaposin prosaptide, neuroblastoma cells, differentiation

Introduction

Gangliosides are sialic acid containing glycosphingolipids, which consist of a hydrophobic portion, ceramide, and a hydrophilic portion. They are ubiquitous constituents of cell membranes [1] and are implicated in a large variety of biological events occurring at the cell surface [2].

Exogenously added gangliosides are rapidly taken up by the cell [3] where they may function as endogenous gangliosides. There is evidence that gangliosides are involved in cell differentiation and development [4–6]. Although synthesis and expression of nervous tissue gangliosides are strictly developmentally regulated [7, 8], an increased level of specific gangliosides on the cell surface can induce cell differentiation in specific cell lines, including neuronal cells [4, 9].

The change of Ca^{2+} influx, the change in intracellular pH through Na^{+}/K^{+} antiporter [10], or the regulation of protein kinase C (PKC) activity [11] are all mechanisms through which gangliosides may modulate cell growth, but it remains unknown how modifications in the amount and organization of gangliosides on the cell surface can promote a signal transduction. It seems likely that signal transduction might originate from a specific ganglioside-protein interaction process occurring at the membrane level. The observation that gangliosides facilitate the binding of certain viruses [12] and bacterial toxins [13] demonstrated that proteins are capable of binding specific gangliosides. Binding of the ganglioside by the protein would then initiate a chain of events that could lead to a variety of cellular responses.

It has been recently demonstrated that prosaposin, a glycoprotein of 66 kDa exists as a secretory protein in body fluids [14], as well as a neuronal surface membrane component [15]. Prosaposin, the precursor of saposins A, B, C and D [14], binds gangliosides with high affinity and facilitates their transfer from micelles to membranes [16]. Prosaposin has been recently identified as a neurotrophic factor eliciting differentiation in neuronal cultured cells (NS20Y) [17]. The neurotrophic activity of

prosaposin resides in the saposin C domain; the treatment of neuronal cells with saposin C triggers signal transduction cascade by binding to a high-affinity receptor that induces protein phosphorylation [17]. An analysis of the effect of different synthetic peptides from saposin C on neurite outgrowth of NS20Y cells revealed that only the aminoterminal fragment was active; using a set of overlapping peptides the active sequence was located to a region between aminoacid residues 8–30. Nanomolar concentrations of this 22-mer peptide (CEFLVKEVTKLIDNNKTEKEIL), named prosaptide, selectively stimulated neurite outgrowth in neuroblastoma cells [18].

Since prosaposin [16], as well as gangliosides [4, 9], have been shown to promote neurite outgrowth in cultured neuronal cells, we investigated whether prosaptide may modify the ganglioside pattern in neuroblastoma cells.

Materials and methods

Cells

Murine neuroblastoma cells (NS20Y) which extend neurites in response to gangliosides [19] were from Professors K. Uemura and T. Taketomi. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Bio Whittaker, Walkersville, Maryland, USA), containing 10% foetal calf serum plus penicillin and streptomycin. After about 24 h, the medium was replaced with DMEM + 0.5% foetal calf serum plus effectors, 100 ng ml^{-1}. Cells were cultured for an additional 24 h and analysed for ganglioside pattern and neurite outgrowth.

Effectors

Prosaposin was prepared as previously described from human milk [14].

Synthetic peptides were obtained commercially (Peptide Synthesis Facility, UCSD). All peptides were purified by High Performance Liquid Chromatography on a Vydac C-4 column to purer than 95% before use. Prosaptide as described above is a 22-mer which is active as a neurotrophic factor [18]. Peptide 769-M is prosapeptide containing a single aminoacid transfersion of the first asparagine to aspartic acid (14N − D); 769-M is inactive as a neurotrophic factor [18]. Epopep is an 18 aminoacid residue peptide derived from human erythropoetin; it is active as a neurotrophic factor, stimulating neurite outgrowth in the nanomolar range [20]. Peptide 9 is a 20-mer derived from human ciliary neurotrophic factor; it is also active as a neurotrophic factor (unpublished data).

Ganglioside extraction

Gangliosides were extracted from untreated and peptide treated neuroblastoma cells (NS20Y) according to the method of Svennerholm and Fredman [21], with minor modifications. Briefly, cells (plus GT1b, Sigma Chemical Co., as an internal standard), were extracted twice in chloroform:methanol:water (4:8:3) (by vol) and subjected to Folch partition by the addition of water to give a final chloroform:methanol:water ratio of 1:2:1.4. The upper phase, containing polar glycosphingolipids, was purified of salts and low molecular weight contaminants using Supelclean LC-18 tubes, 3 ml (Supelco Inc., Bellefonte, PA, USA) [22].

The eluted glycosphingolipids were dried down and separated by high-performance thin-layer chromatography (HPTLC), using silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), preactivated by heating to 100 °C for 30 min. Chromatography was performed in chloroform:methanol:0.25% aqueous KCl (5:4:1) (by vol).

Gangliosides GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b, (Sigma Chemical Co., St Louis, USA) were used as standards. Plates were air-dried and gangliosides visualized with resorcinol [23].

Quantitative analysis of gangliosides

Quantitative analysis of gangliosides was performed by the following methods: (i) by scanning densitometry (Hoefer Scientific Instr. model GS300, San Francisco, CA); (ii) measuring the lipid-bound sialic acid of either total ganglioside extract or ganglioside-scraped TLC bands, by the periodate-resorcinol method outlined by Jourdian *et al.* [24]. The absorbances were measured at 630 nm with a Perkin-Elmer Lambda 4B spectrophotometer. The quantitative data are the mean ±SD for $n = 6$ experiments.

Immunofluorescence and flow cytometry analysis

Cells (1×10^6 in 1 ml of PBS) were fixed in 4% formaldehyde in PBS for 1 h at room temperature.

After washing three times in phosphate-buffered saline (PBS) pH 7.4, cells were incubated for 1 h at 4 °C with anti-GM3 (GMR6) [25] or anti-GM2 (GMB28) monoclonal antibody [25] (kindly provided by Dr T. Tai, The Tokyo Metropolitan Institute of Medical Science), followed by the addition of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (μ-chain specific) (Sigma Chemical Co., St Louis, USA) diluted 1:128 in PBS and incubated for another 30 min at 4 °C. After washing with PBS at 4 °C, green fluorescence intensity was analysed on an EPICS profile cytometer (Coulter Electronics, Hialeah, FL). Vital cells were gated on the basis of forward angle light scatter and 90° light scatter parameters; 5000 cells were counted for each histogram. The cytometry data is the mean ± SD for $n = 5$ experiments.

Formaldehyde fixed NS20Y cells treated and untreated

with prosaptide, labelled with anti-GM3 or anti-GM2 monoclonal antibody and FITC-conjugated goat anti-mouse IgM as above, were also analysed by immunofluorescence microscopy.

Analysis of cell differentiation

Cell differentiation was evaluated determining neurite outgrowth in NS20Y cells assayed by the method described by Uemura [19].

In particular, cells (2×10^4) were plated onto glass coverslips in 30 mm petri dishes and then treated as above. Neurite outgrowth was scored under a phase contrast microscope. Cells bearing neurites longer than one cell diameter were scored as positive and 100 cells were counted in triplicate from different portions of each dish. Each assay was carried out in duplicate dishes. The average error of duplicates (44 assays) was ±5%.

Results

Ganglioside analysis in NS20Y cells by HPTLC and subsequent staining with resorcinol revealed the presence of three main bands comigrating with GM3, GM2 and GD1a in both untreated and prosaposin treated cells (Fig. 1). One example of densitometric analysis is shown in Fig. 2.

The identity of GM3 and GM2 was confirmed by TLC

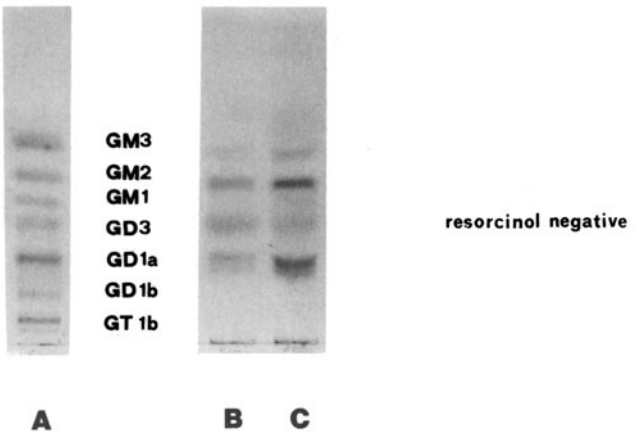

Figure 1. HPTLC analysis of gangliosides extracted in chloroform:methanol:water from 1×10^7 neuroblastoma cells (NS20Y). Lane A, standard gangliosides: GM3 (Fidia Research Laboratory, Abano Terme, Padua, Italy), GM2, GM1, GD3, GD1a, GD1b, GT1b (Sigma Chemical Co., St Louis, USA); lane B, gangliosides obtained from 1×10^7 neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the absence of prosaposin; lane C, gangliosides obtained from neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the presence of prosaposin, 100 ng ml^{-1}; The plate was stained with resorcinol (ganglioside-specific stain). The figure shows the presence of three main bands comigrating with GM3, GM2 and GD1a in both untreated and treated cells.

immunostaining using GMR6 and GMB28 monoclonal antibodies (not shown).

The analysis did not reveal qualitative changes in the ganglioside pattern of NS20Y cells incubated in the presence of prosaposin, compared to control cells, but it did reveal an increase in the content of all three major resorcinol positive bands.

The total content of gangliosides, determined as lipid-bound sialic acid (mean ±SD of six experiments), was 1.1 ± 0.24 μg per mg protein of untreated cells and 1.75 ± 0.36 μg per mg for prosaposin treated cells.

The GM3 content (as bound NeuAc) was 1.08 ± 0.19 μg per 10^7 untreated cells and 1.52 ± 0.25 μg per 10^7 prosaposin treated cells; the content of GM2 (as bound NeuAc) was 2.70 ± 0.29 μg per 10^7 untreated cells and 3.7 ± 0.35 μg per 10^7 prosaposin treated cells; the GD1a content (as bound NeuAc) was 2.99 ± 0.39 μg per 10^7 untreated cells and 5.50 ±0.55 μg per 10^7 prosaposin treated cells.

In order to verify the effect of the synthetic peptides on the ganglioside pattern, NS20Y cells were incubated in the presence of prosaptide, Epopep, peptide 769-M, peptide 9, rabbit polyclonal antibody, anti-prosaptide or rabbit preimmune serum (Fig. 3a).

The total ganglioside content, determined as bound NeuAc (mean + SD of six experiments), was 1.70 + 0.30 μg per mg protein of prosaptide treated cells, compared to 1.1 + 0.24 μg per mg protein of untreated cells, as above. The recovery after ganglioside extraction was virtually the same in treated and untreated cells, as verified using GT1b as an internal standard (Fig. 3b). None of the other synthetic peptides under test, including P769-M, which differs by only one residue from prosaptide, were able to induce a significant modification of the ganglioside content in NS20Y neuroblastoma cells (Fig. 3a). Thus, the effect of prosaptide and prosaposin on the gangliosides content was virtually the same.

With the aim of clarifying whether the increase in ganglioside content was at the cell plasma membrane level, a cytofluorimetric analysis was performed using two different antiganglioside MoAbs (anti-GM3, GMR6 and anti-GM2, GMB28). It revealed significantly higher ($p < 0.001$) staining for GM3 on the prosaptide treated cells (33.9 ± 6.1%) compared to untreated NS20Y cells (2.2 ± 0.9%) (mean of five experiments). No significant difference was shown in the staining with the anti-GM2 MoAb, since in both prosaptide treated and control cells about 87% of the cells were reactive with the antibody. However, the mean fluorescence intensity was higher in the prosaptide treated cells (13.23) as compared to the untreated control cells (11.71) (Fig. 4).

To analyse the pattern of ganglioside distribution on the cell surfaces we observed, by immunofluorescence microscopy, NS20Y cells immunostained with anti-GM3 or anti-GM2 MoAbs (Fig. 5). The analysis showed that

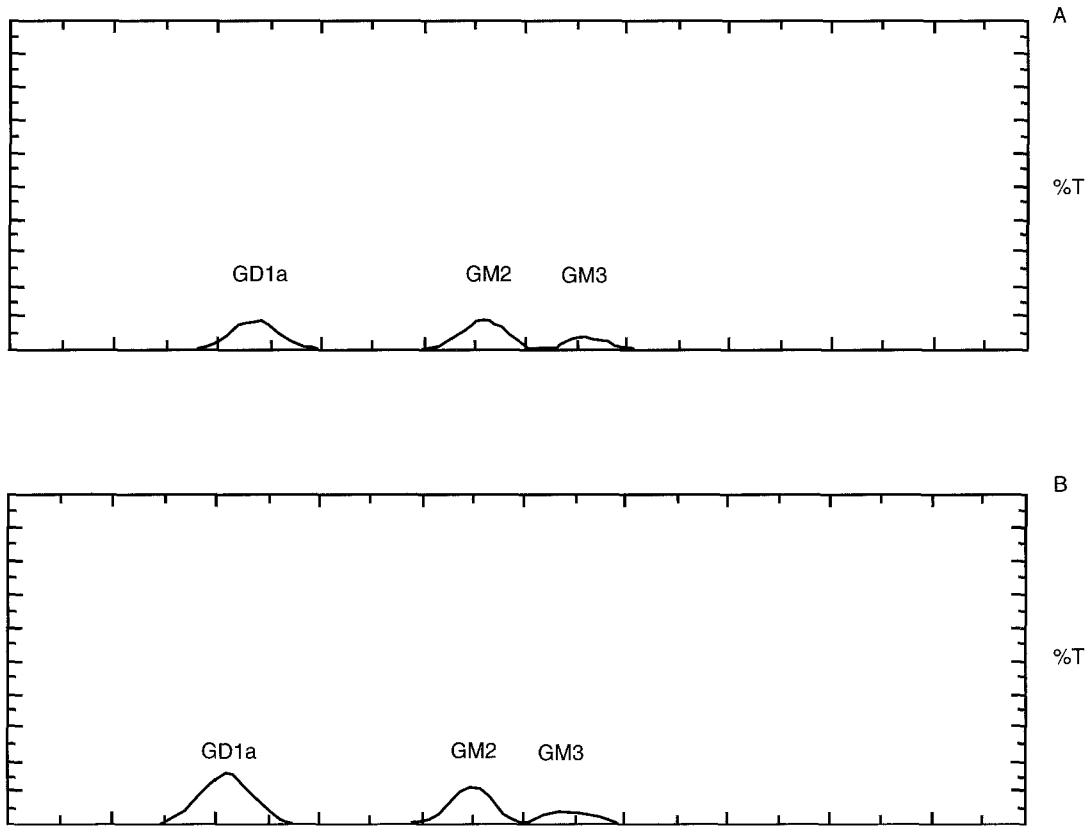

Figure 2. Densitometric analysis of GM3, GM2 and GD1a content. The analysis program excludes every resorcinol-negative band. (A) gangliosides obtained from 1×10^7 neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the absence of prosaposin; (B) gangliosides obtained from neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the presence of prosaposin. The peaks areas were calculated by a Gaussian integration analysis. The figure shows the increase of ganglioside content in cells incubated in the presence of prosaposin compared to control cells.

most of the cells (either untreated or treated with prosaptide) were positive for anti-GM2 MoAb; the number of anti-GM3 immunostained cells was significantly higher ($p < 0.001$) in prosaptide treated NS20Y cells as compared to untreated control cells, in accordance with the cytofluorimetric results. In all positive cells both anti-GM2 and anti-GM3 signals appeared uneven and punctate on the plasma membranes. The prosaptide was active in stimulating neurite outgrowth in about 15% of NS20Y cells. Virtually all neurite extending cells were immunostained with anti-GM3 and anti-GM2 MoAbs.

Discussion

The present study demonstrates that prosaposin induces a significant increase of the three major ganglioside constituents of NS20Y neuroblastoma cells, which comigrate with GM3, GM2 and GD1a. Prosaposin and its derived proteolytic products, named saposins (sphingolipid activator proteins) are involved in lysosomal hydrolysis of several sphingolipids, including gangliosides. Different enzymatic pathways can be activated by prosaposin; in particular it possesses stimulative activity for cerebroside β glucosidase, GM1 ganglioside β galactosidase and sphingomyelinase [26]. Moreover, it has been reported that saposins accumulate in tissues of patients with lysosomal storage diseases, especially GM1 or GM2 gangliosidosis [27]. In these patients saposins accumulate in the same tissues that glycosphingolipids accumulate. However, prosaposin was also recently revealed as a cell differentiation factor capable of stimulating neurite outgrowth and increasing ChAT activity in neuroblastoma cells [17]. Since it was shown that the neurotrophic activity of prosaposin resides in the saposin C domain and in particular in a prosaptide derived from this region [18], we decided to analyse whether the same portion of the molecule is responsible for the observed modifications of the ganglioside pattern. The analysis of the effect of different synthetic peptides on the cell ganglioside pattern clearly showed that the prosaptide selectively induced the increase in the ganglioside content, suggesting that only

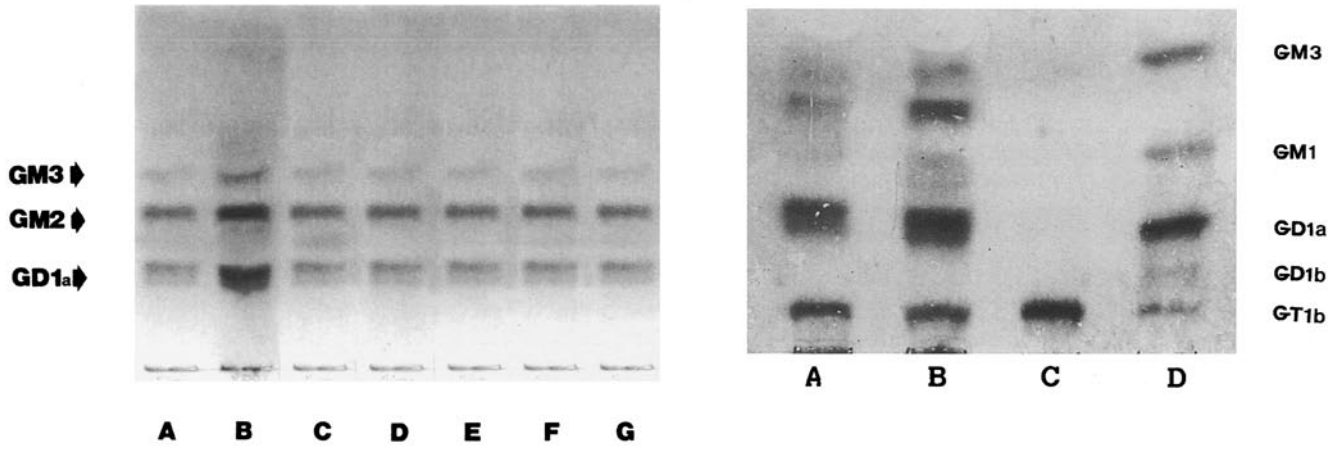

Figure 3. HPTLC analysis of gangliosides extracted in chloroform:methanol:water from 1×10^7 neuroblastoma cells (NS20Y).

(a) Lane A, gangliosides obtained from 1×10^7 neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the absence of effectors: Lanes B, C, D, E, F, G, gangliosides obtained from neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the presence of prosaptide (B), Epopep (C), P769-M (D), peptide 9 (E), 100 ng ml^{-1} each, rabbit polyclonal antibody anti-prosaptide (F) or rabbit preimmune serum (G). The plate was stained with resorcinol (ganglioside-specific stain). The figure shows that the increase in ganglioside content was only observed in prosaptide treated cells. On the contrary, none of the other synthetic peptides under test, including P769-M, and the rabbit polyclonal antibody anti-prosaptide, were able to induce a significant modification in ganglioside content in NS20Y neuroblastoma cells.

(b) Lane A, gangliosides obtained from 1×10^7 neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the absence of prosaptide, plus GT1b (Sigma Chemical Co., St Louis, USA), 5 μg as an internal standard; Lane B, gangliosides obtained from neuroblastoma cells (NS20Y) incubated for 24 h at 37 °C in the presence of prosaptide 100 ng ml^{-1}, plus GT1b (Sigma), 5 μg as an internal standard; Lane C, standard GT1b (Sigma), 5 μg; Lane D, standard gangliosides: GM3 (Fidia Research Laboratory, Abano Terme, Padua, Italy), GM1, GD1a, GD1b, GT1b (Sigma); The plate was stained with resorcinol (ganglioside-specific stain). The figure shows that GT1b recovery was virtually the same in lanes A and B compared to lane C (about 80%).

the aminoterminal fragment of the molecule was active. This result is consistent with the demonstration that prosaptide binds to a cell surface receptor with high affinity ($K_d = 70$ pM) and increases tyrosine phosphorylation in NS20Y cells [18].

In order to assess whether the increase in ganglioside content was at the plasma membrane level, a cytofluorimetric analysis was performed, using an anti-GM3 and an anti-GM2 MoAbs, which showed highly restricted binding specificity [25]. This revealed that the previous treatment of NS20Y cells with the prosaptide had induced a significant increase in the number of anti-GM3 reactive cells. It was reported [28] that the reactivity of gangliosides with specific monoclonal antibodies is strictly dependent on the concentration of the glycolipids at the cell surface. Possibly, in this case, there is a threshold value for immunological expression of cellular glycolipids and the reactivity with antibody could be controlled by an 'all or none' phenomenon. Our findings are in good agreement with these observations, revealing that the increase in GM3 content (from 0.99 to 1.45 μg per 10^7 cells) is accompanied by an increase in the number of anti-GM3 reactive cells, whereas GM2, which is present at a higher concentration in the NS20Y untreated cell (2.76 μg per 10^7 cells), is immunostained by the MoAb (about 87% reactive cells). However, the mean fluorescence intensity was higher in the prosaptide treated cells as compared to control NS20Y cells.

Immunofluorescence microscopy with the anti-GM3 MoAb confirmed the cytofluorimetric data which showed a higher reactivity of prosaptide treated NS20Y cells compared to untreated cells. In both treated and untreated cells the pattern of staining appeared uneven and punctate, suggesting a clustered distribution of GM3 and GM2 molecules on the cell surface, similar to that observed in other cell types, such as human skin fibroblasts [29], peripheral blood lymphocytes and Molt-4 lymphoid cells (M. Sorice *et al.*, unpublished observations). Furthermore, the immunofluorescence microscopy findings, together with the selective action of the prosaptide on both neurite outgrowth and modification of the ganglioside pattern, suggest that the increase in ganglioside content could be related to cell differentiation induced by prosaposin. There is increasing

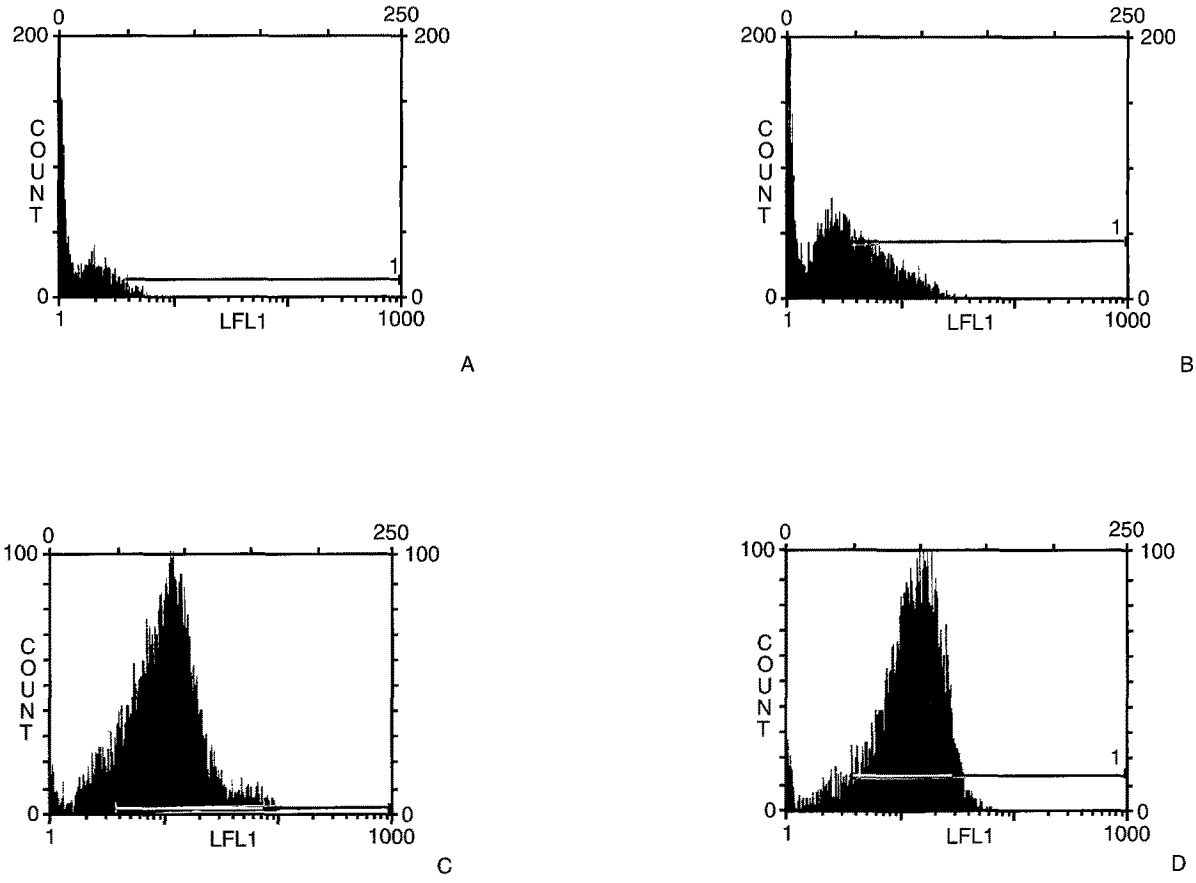

Figure 4. Cytofluorimetric analysis of anti-GM3 and anti-GM2 reactivity. A shows the reactivity of untreated neuroblastoma cells (NS20Y) with anti-GM3 mAb GMR6, followed by incubation with FITC-conjugated goat anti-mouse IgM, compared to cells treated for 24 h with prosaptide, 100 ng ml^{-1} (B). C shows the reactivity of untreated NS20Y cells with anti-GM2 mAb GMB28, compared to prosaptide treated cells (D). Histograms represent log fluorescence vs cell number. Cell number is indicated on the y-axis and fluorescence intensity is represented in three logarithmic units at the x-axis. The cursor shows the specific reactivity. Incubation with prosaptide induced a significant increase in the number of anti-GM3 immunostained cells. No significant difference was shown in the staining with the anti-GM2 MoAb; however, the mean fluoresence intensity was higher in the prosaptide treated cells (13.23) as compared to the untreated control cells (11.71).

evidence that glycolipids, especially gangliosides, are, in addition to or in conjunction with membrane proteins, involved in neuronal differentiation and development. In particular it has been reported that NS20Y-treatment with exogenously added gangliosides results in increased projections (neurites) on the cell surface and fine structures projecting from the cell processes (branches). Taken collectively, these findings prompt us to suggest that the neurotrophic activity of prosaposin on NS20Y neuroblastoma cells might be mediated in part by the increase in cell surface gangliosides. This hypothesis is supported by the preliminary observation that both effects of the prosaptide and prosaposin, stimulation of neurite outgrowth and increase in the ganglioside content on NS20Y plasma membrane, were inhibited by previous treatment of the cells with monensin (data not shown), which is known to be an inhibitor of ganglioside anabolism [30].

In conclusion, our findings demonstrate that prosaposin and prosaptide treated NS20Y cells show a significant increase in plasma membrane ganglioside content. Further studies are in progress to clarify whether the prosaposin-induced increase of ganglioside content may be involved in neural cell differentiation mechanism(s).

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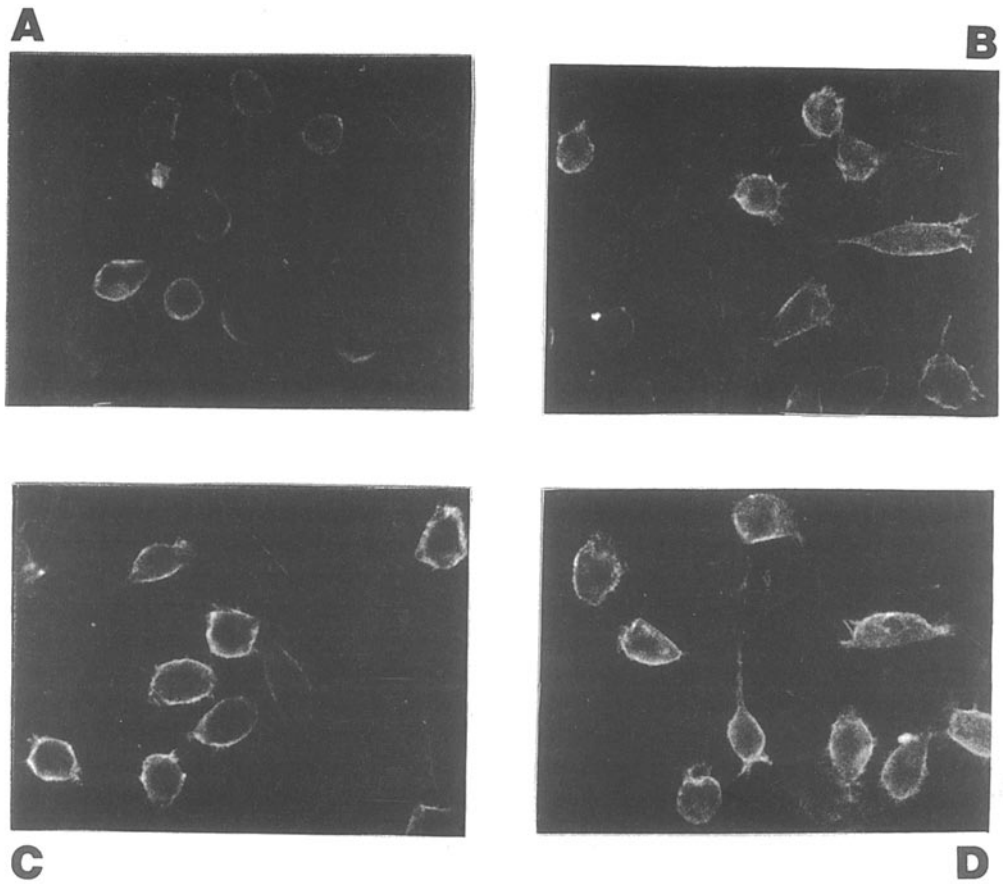

Figure 5. Immunofluorescence analysis of the distribution of GM3 and GM2 on the cell plasma membranes. In both prosaptide untreated (A) and treated (B) cells the anti-GM3 immunolabelling appears uneven and punctate over the cell surfaces. Both anti-GM2 immunostained prosaptide untreated (C) and treated (D) cells show an intense and uneven distribution over the cell plasma membranes.

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