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Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells*

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*Dedicated to Professor Günter Quadbeck on the occasion of his 80th birthday

Gangliosides of the plasma membrane are important modulators of cellular functions. Previous work from our laboratory had suggested that a plasma membrane sialidase was involved in growth control and differentiation in cultured human neuroblastoma cells (SK-N-MC), but its substrates had remained obscure. We now performed sialidase specificity studies in subcellular fractions and found ganglioside GM3 desialylating activity in presence of Triton X-100 to be associated with the plasma membrane, but absent in lysosomes. This Triton-activated plasma membrane enzyme desialylated also gangliosides GD1a, GD1b, and GT1b, thereby forming GM1; cleavage of GM1 and GM2, however, was not observed. Sialidase activity towards the glycoprotein fetuin with modified C-7 sialic acids and towards 4-methylumbelliferyl neuraminate was solely found in lysosomal, but not in plasma membrane fractions.
The role of the plasma membrane sialidase in ganglioside desialylation of living cells was examined by following the fate of [^{3}H]galactose-labelled individual gangliosides in pulse-chase experiments in absence and presence of the extracellular sialidase inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid. When the plasma membrane sialidase was inhibited, radioactivity of all gangliosides chased at the same rate. In the absence of inhibitor, GM3, GD1a, GD1b, GD2, GD3 and GT1b were degraded at a considerably faster rate in confluent cultures, whereas the GM1-pool seemed to be filled by the desialylation of higher gangliosides. The results thus suggest that the plasma membrane sialidase causes selective ganglioside desialylation, and that such surface glycolipid modification triggers growth control and differentiation in human neuroblastoma cells.

Key words: ganglioside/neuroblastoma cells/plasma membrane/sialidase

Introduction

Gangliosides are sialylated glycosphingolipids that are ubiquitous in the plasma membrane of vertebrate cells. In neurons, they comprise approximately two thirds of the glycoconjugate sialic acid content, thereby making an important contribution to the glycocalix surrounding the neural membrane (Yu and Saito, 1989; Ledeen, 1989). Gangliosides are involved in various processes of the nervous system, including cell proliferation, cell differentiation, neural repair, cell–cell recognition and cell adhesion (Ledeen, 1989; Zeller and Marchase, 1992; Hakomori, 1993; Tettamanti and Riboni, 1993).

Specific regulatory effects of gangliosides on cell functions require tight control of their amounts in the plasma membrane. Ganglioside synthesis occurs in the Golgi, and the level of specific glycosyltransferases determines the ganglioside pattern (van Echten and Sandhoff, 1993; Ruan and Lloyd, 1992). On the other hand, ganglioside degradation also appears to influence the ganglioside pattern of the plasma membrane (Schengrund and Repman, 1982; Riboni *et al.*, 1990). Enzymes of ganglioside catabolism are located in the lysosomes with the exception of sialidase which occurs in both lysosomal and nonlysosomal compartments (e.g., Miyagi *et al.*, 1990a and 1990b; Pitto *et al.*, 1992). In cultured fibroblasts and tissues from patients with sialidosis there is a genetic deficiency of a lysosomal sialidase that degrades sialyloligosaccharides, sialoglycoproteins, and gangliosides, whereas a plasma membrane ganglioside sialidase is unaffected (Thomas and Beaudet, 1995).

As already shown in earlier studies, ganglioside sialidase activity was found to be present at the surface of a variety of cells and to be involved in, or modulated by, fundamental processes such as proliferation, cell contact, or phase of the cell cycle. Thus, cell surface ganglioside sialidase activity was clearly detected in transformed, but not in non-transformed, hamster cells (Schengrund *et al.*, 1973). Cell density-dependent changes of surface ganglioside sialidase were studied in mouse fibroblasts, and the activity was found to be greatly enhanced at early stages of contact, but decreased when cultures were confluent (Yogeeswaran and Hakomori, 1975). In cultured murine neuroblastoma or cerebellar granule cells, on the other hand, ganglioside sialidase activity increased with cell density and was therefore considered to be involved in cell differentiation (Schengrund and Repman, 1982; Riboni *et al.*, 1990; Pitto *et al.*, 1992).

In previous work we had shown that cultured human neuroblastoma cells (SK-N-MC) contained two sialidase activities that degrade gangliosides: a plasma membrane-bound activity that was specifically activated by Triton X-100, was readily inactivated by preincubating intact cells in the presence of millimolar concentrations of Cu^{++}, and whose specific activity increased strikingly during cell growth, and a lysosomal activity stimulated by glycodeoxycholate that was protected from inactivation upon exposure of the cells to Cu^{++} and showed no activity increase during cell proliferation (Kopitz *et al.*, 1994). When the specific sialidase inhibitor 2-deoxy-2,3-

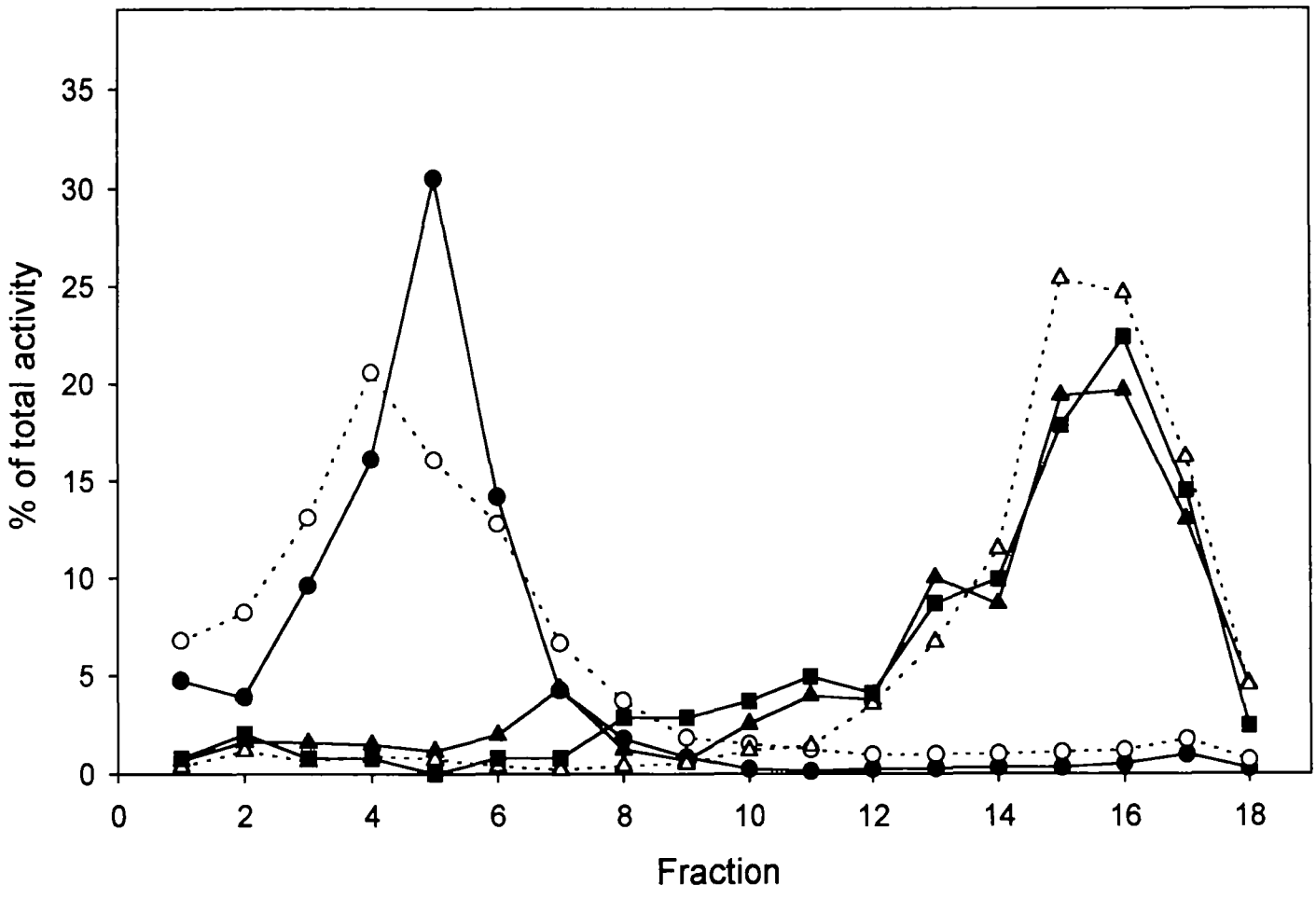

Fig. 1. Subcellular distribution of sialidase activities of human neuroblastoma cells. Isotonic homogenates were fractionated on a Percoll gradient and enzyme activities determined as described in 'Methods' Fractions were collected from top of the gradient and assayed for sialidase activity towards ganglioside GM3 in presence of Triton X-100 (●), for 2'-4-methylumbelliferyl neuraminate (■) or fetuin-desialylating activities (▲), and for the lysosomal marker β-hexosaminidase (△) and the plasma membrane marker alkaline phosphodiesterase (○).

dehydro-N-acetylneuraminic acid (NeuAc2en) was included in the culture medium of the neuroblastoma cells, there was a release from density-dependent control of cell division and loss of the differentiation marker acetylcholinesterase (Kopitz *et al.*, 1994). The results therefore indicated an important role of the sialidase of the plasma membrane in the processes of proliferation control and differentiation. As the sialidase substrates or products transducing these effects had remained undefined, we have now performed specificity studies towards potential ganglioside and other substrates in subcellular fractions of neuroblastoma cells. In addition, the consequences of the inhibition of the plasma membrane sialidase by NeuAc2en on the ganglioside pattern and turnover in living cells were studied in metabolic labelling experiments. The results show that the plasma membrane sialidase activity of neuroblastoma cells specifically desialylates gangliosides with terminal sialic acid residues and demonstrate the direct consequences of this enzymatic action on the ganglioside lining of the cell surface. The sialidase-induced glycolipid modification alone seems sufficient to induce growth control and differentiation in this neuronal cell line.

Results

Topology and specificity of neuroblastoma cell sialidase activities

The subcellular distribution of the sialidase activities of human neuroblastoma cells was examined by fractionation of isotonic homogenates in colloidal silica gradients, and sialidase specificity was tested in incubations with potential ganglioside and other glycan substrates. Highly sensitive sialidase assays were conducted with ganglioside GM3 labelled in the ceramide moiety, and with fetuin which had been tritium-labelled in its sialic acids by the periodate/3H-borohydride reduction method yielding the C-7 analog. As shown in Figure 1, sialidase activity towards ganglioside GM3 in presence of the detergent Triton X-100 was found to co-distribute with the marker for plasma membranes, alkaline phosphodiesterase. On the other hand, sialidase activity towards fetuin was found to fractionate with the lysosomal marker β-hexosaminidase and with activity towards the artificial sialidase substrate 2'-(4-methylumbelliferyl)-α-N-acetylneuraminic acid (4-MU-NeuAc), but was absent in fractions exhibiting the plasma membrane marker alkaline phosphodiesterase. In the lysosomal frac-

Table I. Specific activities of sialidases and marker enzymes in plasma membrane preparation and homogenate of neuroblastoma cells

	Specific activity		
	A Homogenate	B Plasma membranes	B/A ratio
Triton-activated GM3 sialidase	30 μU/mg protein	487 μU/mg protein	16.2
Alkaline phosphodiesterase	3.72 U/mg protein	64.4 U/mg protein	17.3
Na^+/K^+-ATPase	2.21 mU/mg protein	44.1 mU/mg protein	20
Acetylcholinesterase	0.224 mU/mg protein	3.87 mU/mg protein	17.3
Leucine aminopeptidase	29.6 U/mg protein	487 U/mg protein	16.4
5'-Nucleotidase	2.85 mU/mg protein	46.1 mU/mg protein	16.2
GDC-activated GM3 Sialidase	91 μU/mg protein	3.3 μU/mg protein	0.04
4-MU-NeuAc sialidase	295 μU/mg protein	15 μU/mg protein	0.05
β-N-Acetylhexosaminidase	28.22 mU/mg protein	21.54 mU/mg protein	0.76
Galactosyltransferase	49 μU/mg protein	137 μU/mg protein	2.8
Succinate dehydrogenase	285 U/mg protein	357 U/mg protein	1.25
Lactate dehydrogenase	3325 U/mg protein	1069 U/mg protein	0.32
DNA	64.6 μg/mg protein	8.31 μg/mg protein	0.13

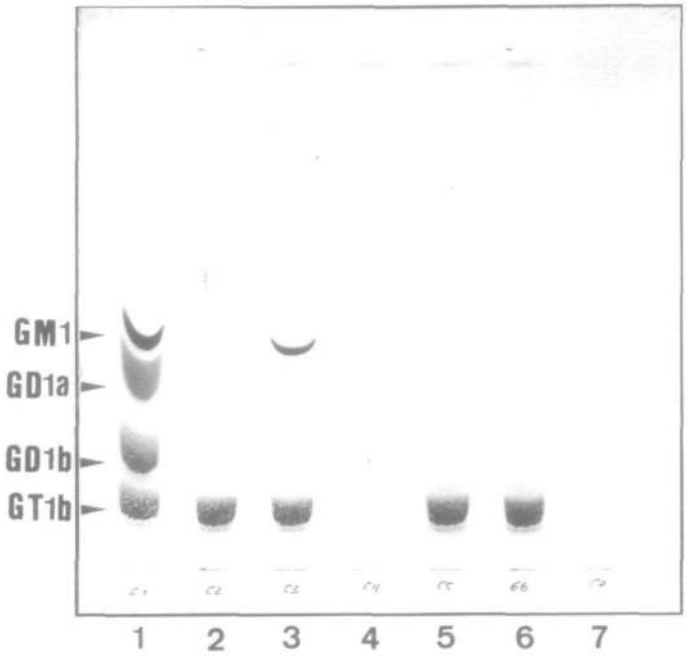

Fig. 2. *Thin-layer chromatography of the products of ganglioside* GT1b degradation by the Triton-activated sialidase. Ganglioside GT1b was incubated in presence of Triton X-100 and neuroblastoma cell homogenate and the reaction products separated by thin-layer chromatography as desribed under 'Methods'. Bands were visualized with orcinol spray. Lane 1: Standards, 50nmol of each ganglioside; lane 2: incubation stopped immediately after mixing by freezing in liquid nitrogen; lane 3: incubation stopped after 8h; lane 4: ganglioside was omitted from the reaction mixture; lane 5: homogenate from Cu^{++}-pretreated cells was used; lane 6: 1mM NeuAc2en was included in the reaction mixture; lane 7: 1mM NeuAc2en was included but homogenate protein and GT1b were omitted from the reaction mixture.

tions, sialidase activity towards ganglioside GM3 could be detected in presence of the detergent glycodeoxycholate, but was rather unstable (results not shown).

The topology of the Triton X-100-activated ganglioside sialidase activity was further tested in a plasma membrane preparation purified by sucessive sucrose and Percoll density gradient centrifugations. As shown in Table I, the specific activity of the Triton-activated ganglioside GM3 sialidase of the plasma membrane preparation was increased over the homogenate by a factor of 16, a similar increase as observed for the plasma membrane markers ouabain-sensitive Na^+/K^+-ATPase, acetylcholinesterase, 5'-nucleotidase, alkaline phosphodiesterase, and leucine aminopeptidase. The markers for microsomes and mitochondria, UDP-galactosyltransferase and succinate dehydrogenase, showed relative increases of 2.8 and 1.3, respectively. The specific activity ratio of the lysosomal marker β-N-acetylhexosaminidase, on the other hand, was decreased to a value of about 0.8, and those for the glycodeoxycholate-activated ganglioside GM3 and the 4-MU-NeuAc sialidases were decreased even further, presumably due to inactivation. The ratios of the cytosolic marker lactate dehydrogenase and the marker for nuclei, DNA, were also markedly diminished.

In order to test the most abundant gangliosides of neural cells as potential substrates of the plasma membrane sialidase, homogenates of neuroblastoma cells were incubated with the gangliosides GM1, GM2, GM3, GD1a, GD1b and GT1b in the presence of Triton X-100. Qualitative analysis of the reaction products was achieved by thin layer chromatography. An example is given in Figure 2, where ganglioside GT1b was tested as substrate. GM1 appeared as the only ganglioside product of the sialidase reaction, and no disialoganglioside was detected (lane 3). A control incubation without addition of the substrate proved that GM1 is indeed the reaction product of GT1b-desialylation and is not derived from the homogenate (lane 4). Cu^{++} pretreatment of intact cells specifically inactivated enzymes on the external surface of the plasma membrane (Kopitz *et al.*, 1994) and consequently no sialidase reaction was observed (lane 5). Likewise, in the presence of the sialidase inhibitor NeuAc2en, no reaction product was detected (lane 6). Applying this procedure to the other gangliosides revealed that also GD1a and GD1b were desialylated by the plasma membrane sialidase, yielding GM1; gangliosides GM1 and GM2, on the other hand, were not degraded (data not shown).

Specific activity of the Triton-activated plasma membrane sialidase activity of neuroblastoma cells towards gangliosides GM1, GM2, GD1a, GD1b and GT1b was quantitated by FPLC-analysis of the reaction products and compared to ganglioside GM3-desialylation (Table II).

Table II. Specificity towards gangliosides of the plasma membrane sialidase in homogenates of neuroblastoma cells

Substrate	Triton-activated sialidase activity (μU/mg protein)
Ganglioside GM3	73.8
Ganglioside GD1a	28.7
Ganglioside GD1b	21.8
Ganglioside GT1b	18.5
Ganglioside GM1	< 0.1
Ganglioside GM2	< 0.1

Homogenates of neuroblastoma cells (0.65 mg protein) were incubated with 25 nmol ganglioside in the presence of 0.04% Triton X-100 for 8 h. Reaction products were separated by FPLC ion exchange chromatography on a Mono Q column (HR 5/5) and quantitated with resorcinol.
For gangliosides GM1 and GM2 radiometric assays with tritium-labelled gangliosides were used additionally, in order to achieve lower detection limits.

When the rate of desialylation of ganglioside GM3 was set to 100 per cent, the rates for gangliosides GD1a, GD1b, and GT1b were 39, 30, and 25 per cent, respectively; gangliosides GM1 and GM2, on the other hand, were not attacked at all. Degradation of gangliosides GM1 and GM2 was not observed either when very sensitive radiometric assays with a detection limit of about 0.1μU/mg protein were applied.

Taken together, the results show that human neuroblastoma cells contain at least two different sialidase activities. One is associated with the plasma membrane and is active on gangliosides GM3, GD1a, GD1b and GT1b, but inactive towards gangliosides GM1, GM2, the artificial substrate 4-MU-NeuAc and the glycoprotein fetuin with modified C-7 sialic acids. The other one is associated with lysosomes, and it desialylates 4-MU-NeuAc, as well as fetuin.

Pattern and catabolism of metabolically labelled gangliosides of neuroblastoma cells

To find out which gangliosides are desialylated by the plasma membrane sialidase in living neuroblastoma cells, cultures were grown in the presence of [^{3}H]galactose, chased with unlabelled medium, and the ganglioside pattern during chase compared to cultures where the plasma membrane sialidase was inhibited by NeuAc2en. For analysis, the gangliosides were extracted from the cells and their labelling pattern determined by HPLC on Lichrosorb-NH_2. Figure 3 shows the ganglioside pattern after seven days of labelling, which consisted of the major species GM3, GM1, GD1a and GM2, whereas GD1b, GT1b, GD2 and GD3 were minor components.

For the determination of ganglioside desialylation during growth of neuroblastoma cells, metabolically labelled cells were trypsinized, seeded at low density in culture flasks and chased for 6 days with unlabelled medium in absence or presence of 250μM NeuAc2en. Because of the negative charge on NeuAc2en, uptake of the inhibitor by the cells should be low (Hirschberg *et al.*, 1976) and its effect directed predominantly towards the plasma membrane sialidase. Determination of the radioactivity of each of the gangliosides at different points of the chase allowed an estimate of their catabolism. In the absence of NeuAc2en the decrease in ganglioside-associated radioactivity should be a function of all cellular ganglioside sialidases, whereas in the presence of the inhibitor, it should mainly depend on lysosomal degradation. The difference between the kinetics in presence or absence of the inhibitor should thus be an approximate measure of the activity of the plasma membrane-bound sialidase. As shown in Figure 4, radioactivity in all gangliosides except GM1 and GM2 decreased faster in the absence than in the presence of NeuAc2en. The difference was greatest with ganglioside GM3, again indicating that it seems the preferred substrate of the plasma membrane sialidase, but was also marked with the other gangliosides having terminal sialic acid residues. The lack of an effect of the inhibitor on the degradation of gangliosides GM1 and GM2 was not unexpected in view of the fact that these were no substrates of the plasma membrane sialidase in the above-mentioned specificity studies. The effects of the plasma membrane sialidase inhibition were not detectable during the initial days of chase but only appeared with increasing cell density. In the presence of the sialidase inhibitor, the degradative profiles of all gangliosides appeared virtually identical with a half-life of about 2.5 days (Figure 4), indicating a common mechanism of membrane internalization followed by lysosomal degradation.

When the relative amounts of radioactively labelled gangliosides at the beginning and at the end of the chase were compared, there was an important change of the labelling pattern in the absence of the sialidase inhibitor. As shown in Table III, the proportion of GM1 more than doubled, whereas that of GM3 decreased to about one fifth and that of GD1a, GD1b and GD2 was also found to be diminished. There was a relative increase of GM2 that is attributed to higher desialylation rates of other gangliosides due to plasma membrane sialidase action. This effect also contributes, besides the filling of the GM1 pool by degradation of higher gangliosides, to the relative increase in GM1 label. In the presence of the inhibitor, on the other hand, the pattern of labelled gangliosides essentially remained unchanged, indicating their common and non-selective endocytosis and catabolism in lysosomes.

Discussion

In a previous investigation, evidence had been presented that in human neuroblastoma cells a plasma membrane-bound sialidase is involved in the processes of proliferation control and differentiation, but the identity of the sialidase substrates had remained unclear (Kopitz *et al.*, 1994). The present study was therefore aimed at elucidating the substrate specificity of the enzyme as a key to an understanding of its function, and to clearly differentiate it from other cellular sialidases.

In subcellular fractionation studies using colloidal silica gradients it could now be shown that human neuroblastoma cells contain two sialidase activities: one that co-distributed with the marker for plasma membrane and desialylated ganglioside GM3 in presence of Triton X-100, and another rather labile one that was associated with lysosomes and degraded the glycoprotein fetuin and the synthetic compound 4-MU-NeuAc. Further evidence for the plasma membrane localization of the Triton-activated ganglioside sialidase was obtained by comparing its specific

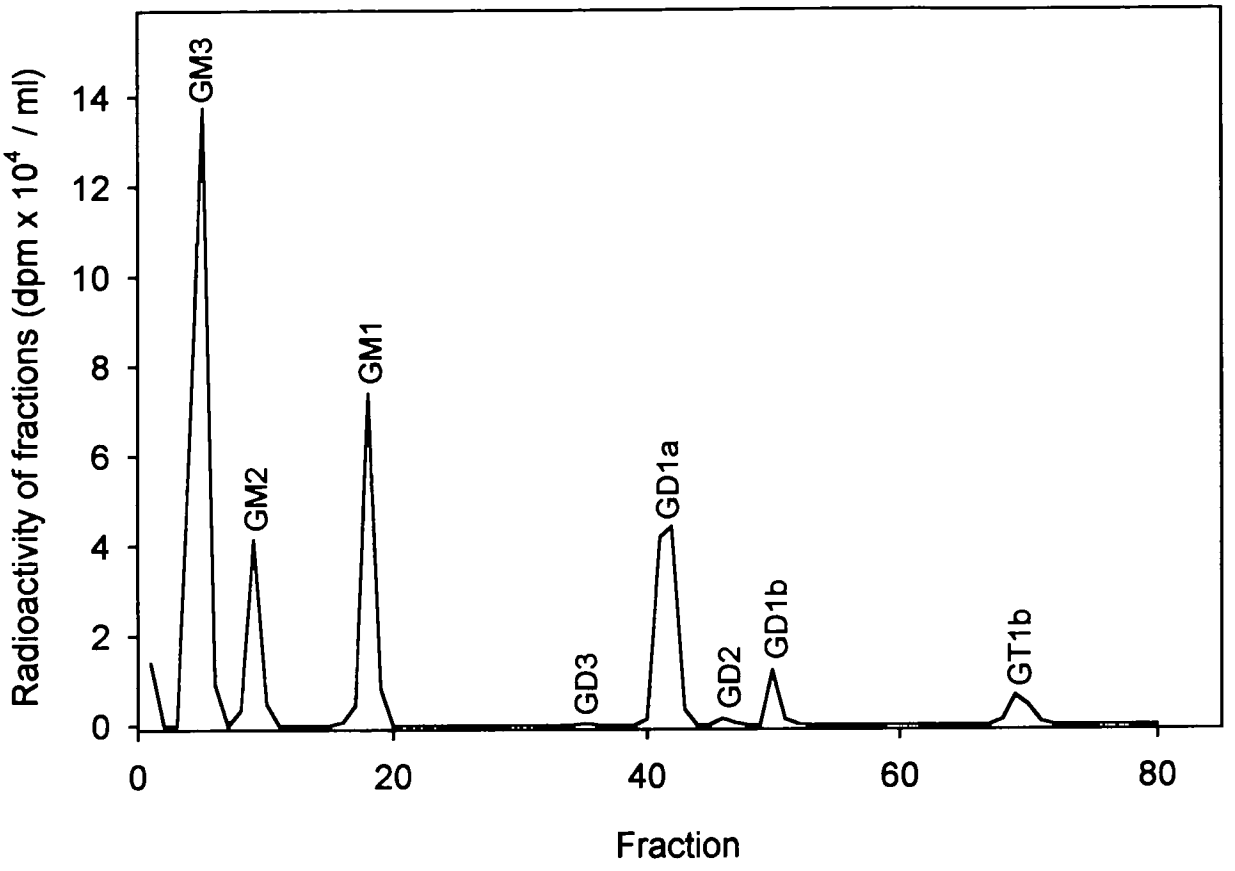

Fig. 3. Pattern of metabollically labelled gangliosides of human neuroblastoma cells. Cells were grown in the presence of [^{3}H]galactose for 7 days and gangliosides extracted quantitatively as described in 'Methods'. One ml of the extract containing 5.5 × 10^5 d.p.m. was separated on a Lichrosorb-NH_2 column, 250 × 4mm I.D., using an acetonitrile-phosphate buffer system (Gazzoti *et al.*, 1985). Fractions of 1ml were collected and counted for radioactivity. The column was calibrated with authentic ganglioside standards that were detected with resorcinol.

activities in the homogenate and a plasma membrane fraction prepared by density gradient centrifugation. The specific activity of the ganglioside sialidase was increased by a factor of 16 in the plasma membrane fraction, similar to the increases observed for five plasma membrane marker enzymes. The specific activities of the lysosomal marker and of the 4-MU-NeuAc and glycodeoxycholate-activated ganglioside GM3 sialidase activities, on the other hand, were found to be decreased in the plasma membrane preparation. The results thus corroborated our earlier findings in cultured human fibroblasts and human neuroblastoma cells of a Triton-activated ganglioside sialidase that was selectively inactivated upon incubation of intact cells in the presence of millimolar concentrations of Cu^{2+} and was therefore considered to reside on the outer leaflet of the plasma membrane, and a lysosomal sialidase that degraded 4-MU-NeuAc and ganglioside substrates and was protected from Cu^{2+} in the above inactivation assay (Schneider-Jakob and Cantz, 1991; Kopitz *et al.*, 1994). The present subcellular fractionation experiment also showed that sialidase activity towards the synthetic substrate 4-MU-NeuAc and the glycoprotein fetuin that co-distributed with the lysosomal marker was absent in the plasma membrane fractions. As the sensitivity of the fetuin sialidase assay was comparable to that using ganglioside GM3 as substrate, the results could either mean that the plasma membrane sialidase is specific only for ganglioside, but not for the sialoglycoprotein, or that the modification of the sialic acid residues produced by the tritium-labelling procedure caused the glycoprotein not to be recognized as a substrate anymore. The latter possibility seems unlikely, however, as the Triton-activated ganglioside sialidase activity that had been purified 1300-fold from human brain tissue failed to desialylate fetuin with unmodified sialic acid residues (unpublished observation). As fetuin contains both α2–3- and α2–6-linked sialic acids (in a 2 to 1 ratio; Baenziger and Fiete, 1979), it also appears unlikely that activity of the plasma membrane sialidase towards the glycoprotein would have been missed due to restricted linkage specificity. When the activity of the plasma membrane sialidase was tested towards potential ganglioside substrates other than GM3, GD1a, GD1b and GT1b were found to be efficiently desialylated to yield GM1. Gangliosides GM1 and GM2, however, were not attacked at all. Taken together, the results suggest that the Triton X-100-activated sialidase activity of the plasma membrane cleaves from gangliosides sialic acid residues that are in a terminal but not in a branching position. On the other hand, the plasma membrane sialidase appears to be inactive towards terminal sialic acid residues in the sialoglycoprotein fetuin and towards 4-MU-NeuAc. A somewhat peculiar finding for an enzyme directed towards the extracellular space is its acidic pH optimum of about 4.5. Although there is at present no direct explanation it must be borne in mind that

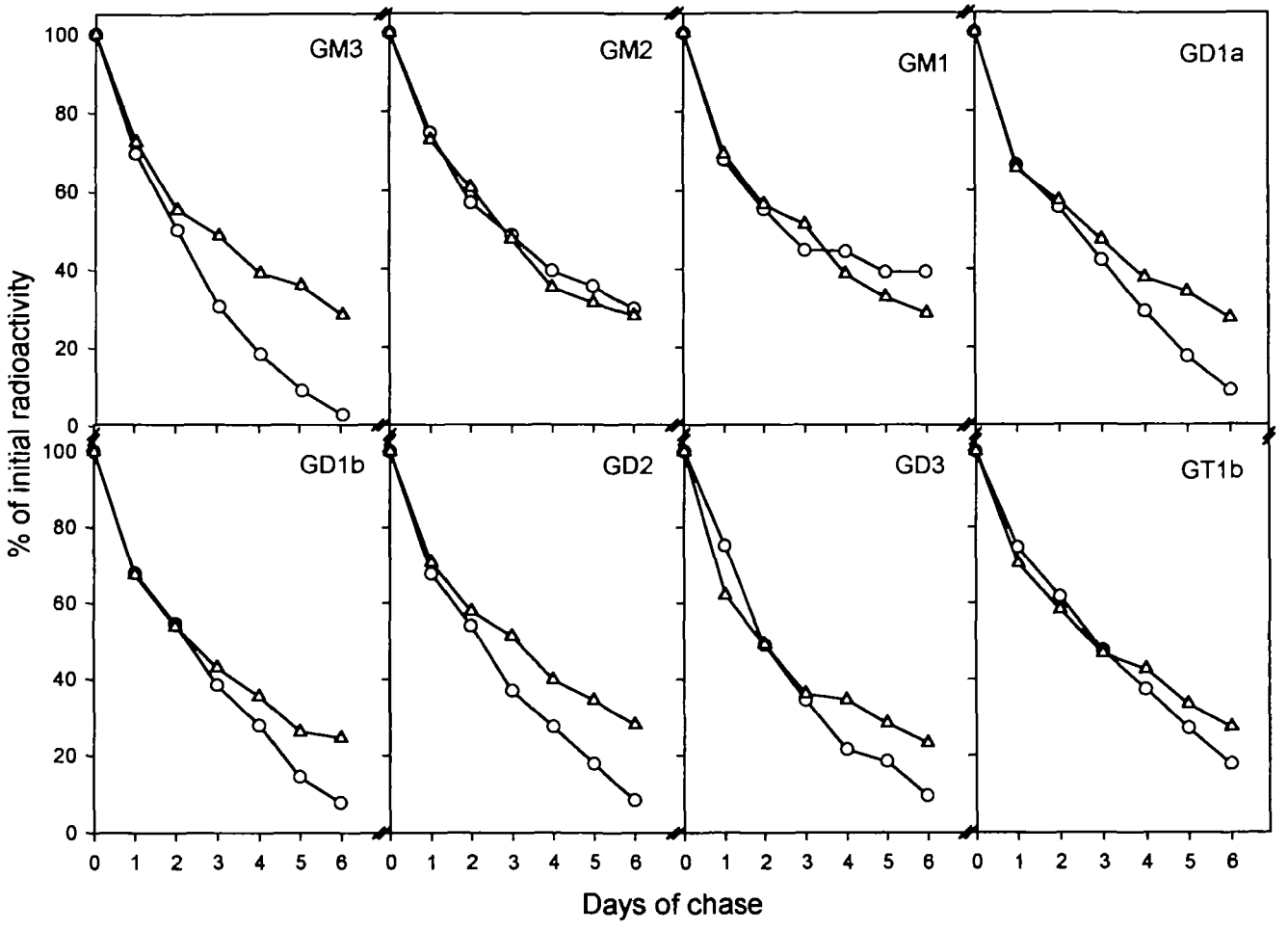

Fig. 4. Chase of metabollically labelled gangliosides of human neuroblastoma cells. Cells cultured in presence of [^{3}H]galactose for 7 days were re-seeded at an initial density of 3.75 × 10^6 cells per 75 cm^2-flask, as described in 'Methods'. They were then chased with non-radioactive medium in absence (○)and presence (△) of NeuAc2en. Radioactivity of the gangliosides was determined after their quantitative isolation and separation by HPLC on Lichrosorb-NH_2 ('Methods'). Curves represent the means of three independent experiments.

Table III. Distribution of radioactivity in metabolically labelled gangliosides of neuroblastoma cells

Ganglioside	% of total[1a] after 7 days of labelling[2]	% of total[1b] after 7 days of labelling and 6 days of chase[2]	% of total[1c] after 7 days of labelling and 6 days of chase in presence of 250 μM NeuAc2en[2]
GM1	26.3 ± 0.4	61.9 ± 1.83	26.8 ± 0.7
GM2	9.6 ± 0.5	16.7 ± 0.6	9.9 ± 0.6
GM3	39.2 ± 1.9	7.1 ± 0.3	39.1 ± 1.7
GD1a	16.8 ± 0.4	8.8 ± 0.5	16.4 ± 0.4
GD1b	5.1 ± 0.2	2.3 ± 0.1	4.5 ± 0.3
GD2	0.4 ± 0 04	0.2 ± 0.03	0.4 ± 0.03
GD3	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01
GT1b	2.9 ± 0.3	2.8 ± 0 1	2.9 ± 0.3

[1]Total radioactivity in ganglioside extract: a, 10797 Bq ± 1620 Bq
b, 1624 Bq ± 340 Bq
c, 2653 Bq ± 443 Bq

[2]Mean values of three independent experiments, ± standard error of the mean

the assay conditions in a homogenate are of course vastly different from the situation of the intact cell where, for instance, the substrate is not presented as a ganglioside-detergent mixed micelle, but is embedded in the membrane of the same or an adjacent cell.

The membrane association and subcellular distribution of ganglioside sialidase has been studied by a number of workers in various cells and tissues. In bovine brain, ganglioside sialidase was found to be highly enriched in isolated synaptosomes (Schengrund and Rosenberg, 1970). In murine neuroblastoma cells (S20Y), ganglioside sialidase activity towards endogenous and exogenous substrates was membrane-bound and increased when the cultures became confluent (Schengrund and Repman, 1982). Cultured human fibroblasts exhibited two ganglioside sialidases, an activity that was plasma membrane-bound and was activated by Triton X-100, and another rather labile activity that was associated with lysosomes, needed activation by deoxycholate and was genetically deficient in cells from patients with sialidosis; the lysosomal sialidase, in contrast to the plasma membrane enzyme, exhibited broad specifity towards sialyloligosaccharides, glycoproteins and gangliosides, and both sialidases could be distiguished by their different sensitivities to inhibitors (Lieser *et al.*, 1989; Zeigler *et al.* 1989; Schneider-Jakob and Cantz, 1991) . Miyagi *et al.* (1990a, 1990b) characterized intralysosomal, cytosolic, and two membrane sialidases present in rat tissues; of the membrane-bound ganglioside-hydrolyzing enzymes, one was associated with brain synaptosomes, whereas the other had multi-substrate specificity (gangliosides, glycoproteins) and was detected in synaptosomal and lysosomal membrane fractions. A subcellular fractionation study of cultured rat cerebellar granule cells found evidence for the existence of possibly two plasma membrane-ganglioside sialidases, differing in pH-optima, in addition to a lysosomal sialidase (Pitto *et al.*, 1992). Our present results in cultured human neuroblastoma cells are in support of a dual subcellular localization of ganglioside-degrading sialidase activities, that is, in the plasma membrane and in lysosomes. Regarding the substrate specificity of the sialidases it has again to be remembered that the assay conditions are highly artificial, and that *in vivo* so-called activator proteins may replace the detergents that were required for the in-vitro activity determinations; indeed, it was shown that degradation of gangliosides by the lysosomal sialidase requires such an activator (Fingerhut *et al.*, 1992). Nevertheless, our present results in human neuroblastoma cells are in general agreement with the published work cited above and indicate that the plasma membrane sialidase activity has a more restricted specificity towards gangliosides, whereas the lysosomal activity is able to attack various classes of substrates such as glycoproteins and gangliosides.

The question as to which gangliosides are actually desialylated by the sialidase in the plasma membrane in growing and differentiating neuroblastoma cells was studied by following the fate of metabolically labelled gangliosides in pulse-chase experiments and in absence and presence of the sialidase inhibitor NeuAc2en. The combination of a procedure for quantitative extraction of gangliosides from cells with an HPLC-method for the analysis of complex ganglioside mixtures allowed exact determination of changes in the labelling pattern. After a 7-day pulse in presence of ^{3}H-galactose, the cells were harvested, reseeded under conditions of logarithmic growth and chased with unlabelled medium for 6 days. Specific activity of the plasma membrane sialidase increased about 15-fold during the logarithmic growth phase under these conditions (Kopitz *et al.*, 1994). Inclusion of the sialidase inhibitor NeuAc2en in the culture medium was used to specifically block the sialidase on the external surface of the plasma membrane. Although an inhibition of the lysosomal sialidase under the conditions employed cannot be rigorously excluded, it should be minimal as the uptake of free sialic acid, and hence NeuAc2en, by cultured cells was reported to be very inefficient (Hirschberg *et al.*, 1976). Any differences in desialylation kinetics of labelled gangliosides in presence and absence of the sialidase inhibitor should therefore be mainly due to the action of the plasma membrane-bound sialidase. Indeed, extracellular NeuAc2en had a marked effect on the decrease of the radioactive label in gangliosides GM3, GD1a, GD1b, GD2, GD3 and GT1b. This effect was hardly detectable during the initial proliferative phase of the cells, but became prominent during logarithmic growth, simultaneously with confluency and with the induction of plasma membrane sialidase activity. Again, ganglioside GM3 was found to be the preferential substrate, but also a considerable share of GD1a, GD1b, GT1b and of the minor gangliosides GD2 and GD3 was desialylated in the plasma membrane. For ganglioside GM2, loss of radioactivity during chase was identical in presence and absence of the inhibitor, confirming that GM2 was not attacked by the plasma membrane enzyme. Radioactive label in ganglioside GM1 decreased faster in presence than in absence of the inhibitor. Obviously, ganglioside GM1 is not a substrate of the plasma membrane sialidase, but the GM1-pool is filled by the desialylation of the higher gangliosides GD1a, GD1b and GT1b, and this 'filling reaction' is prevented by the inhibitor. In presence of NeuAc2en, the rate of disappearance of radioactivity during the chase was the same for all gangliosides and the labelling pattern remained unchanged. This probably means that under these conditions, ganglioside degradation occurs after membrane internalization and transport to lysosomes, the transit time being rate-limiting. The finding of an identical rate of disappearance of radioactivity from all of the gangliosides in presence of NeuAc2en also suggests that lysosomal ganglioside degradation proceeded relatively unimpaired, because an effect of the inhibitor on the lysosomal sialidase, an exohydrolase, would have led to diminished catabolism of gangliosides with terminal sialic acids such as GM3, GD1a, GD1b, and GT1b, but would have spared gangliosides GM2 and GM1 with 'internal' sialic acids. As cultured cells endocytose about half their plasma membrane per h (Steinman *et al.*, 1983), our observation of a half-time of approximately 60 h for intracellular ganglioside degradation may indicate that the membrane is recycled many times before its gangliosides reach the lysosomes for degradation.

The results of the metabolic labelling experiment thus clearly show that certain gangliosides of the plasma membrane are selectively desialylated by the plasma membrane sialidase. Specifically, the action of the enzyme causes a shift from higher gangliosides to GM1, and a strong decrease of GM3 with production of lactosylceramide. This glycolipid change alone seems necessary and sufficient to

cause neuroblastoma cells to engage in cell density-dependent inhibition of growth and neuronal differentiation, as the presence in the medium of the sialidase inhibitor Neu-Ac2en prevented these events (Kopitz *et al.*, 1994). The action of a sialidase that would be secreted into the medium, as described for fibroblasts (Usuki *et al.*, 1988) or Chinese hamster ovary cells (Warner *et al.*, 1993), seems to be excluded here as no activity was detected in the conditioned medium of SK-N-MC cells using highly sensitive sialidase assays (unpublished results). Desialylation of exogenously added gangliosides GD1a and GD1b to GM1 by membrane-bound sialidase had already been demonstrated in primary cultures of cerebellar neurons (Riboni *et al.*, 1991).

Both our results on the substrate specificity of the plasma membrane-bound ganglioside sialidase activity and on the action of the enzyme in living cells thus are in support of each other. We therefore conclude that a selective desialylation of gangliosides with terminal sialic acid residues occurs in the plasma membrane of neuroblastoma cells and that the previously observed effects of the plasma membrane-bound sialidase activity on cellular processes are most likely transduced by such in-situ glycolipid modification. Further knowledge on the complex functions of glycolipids in neural cells is necessary to reveal whether changes of a single ganglioside or global effects on glycolipid composition of the plasma membrane are involved in the plasma membrane sialidase's action on growth and differentiation of neuroblastoma cells.

Materials and methods

Gangliosides were obtained from Dr. Pallmann GmbH (Munich, Germany). Fetuin (from fetal calf serum), V. cholerae neuraminidase and 2-deoxy-2,3-dehydro-N-acetylneuraminic acid were purchased from Boehringer Mannheim (Germany).2'-4-Methylumbelliferyl-α-N-acetylneuraminic acid and orcinol monohydrate were from Sigma (Munich). [^{3}H]-sodium borohydride (2.5TBq / mmol) and D-[4,5-^{3}H(N)]-galactose (2 TBq / mmol) were from DuPont de Nemours (Bad Homburg, Germany). InstaGel from Canberra-Packard (Frankfurt, Germany) was applied for liquid scintillation counting. Percoll was from Pharmacia (Freiburg, Germany). HPTLC$_{60}$ silica gel plates were obtained from Merck (Darmstadt, Germany). Other reagents were from different suppliers and of the highest available purity.

Neuroblastoma cell culture

Neuroblastoma cells (strain SK-N-MC) were obtained from the American Type Culture Collection and cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum (Boehringer Mannheim, Germany), penicillin (100 IU / ml), streptomycin (100 μg / ml) and non-essential aminoacids in an atmosphere of 5% (v/v) CO_2 in air.

For enzyme and other assays, the cells were trypsinized (0.5% (w /v) trypsin (Boehringer Mannheim, Germany) in 0.15M NaCl washed twice with 0.15M NaCl and homogenized in 5mM HEPES, pH 7.4 / 0.5mM EDTA. Pretreatment of intact neuroblastoma cells with 5mM $CuSO_4$ to inactivate the plasma membrane sialidase was exactly as described previously (Kopitz *et al.*, 1994).

Subcellular fractionation

Neuroblastoma cells from 3 flasks (Nunclon Triple Flask™; 500cm^2) were harvested by trypsinization, suspended in 5ml 5mM HEPES, pH 7.6 / 0.25M sucrose / 0.2mM EDTA, and disrupted by nitrogen cavitation (10min, 20bar) in a nitrogen cavitation bomb (Vetter Laborgeräte GmbH, Germany) and the resulting homogenate (100mg protein) was centrifuged (1000g / 10min). The resulting postnuclear supernatant (5ml) was fractionated on 30ml of a self-generating gradient of 30% (v/v) Percoll in 5mM HEPES, pH 7.6 / 0.25M Sucrose / 0.2mM EDTA in a Sorvall rotor T-865 (31000r.p.m., 40min) at 4°C. Fractions (2ml) were collected from top of the gradient.

Purification of plasma membranes

Neuroblastoma cells were homogenized by nitrogen cavitation exactly as described before. The homogenate, 5ml, was layered on 3.5ml 18% (w/v) sucrose / 5mM HEPES pH 7.4 and centrifuged for 17 min at 200g. The resulting supernatant, 7.5ml, was diluted with 1.5ml 5mM HEPES pH 7.4, loaded over 27ml of 1M sucrose / 5mM HEPES pH 7.4 and spun at 100000g for 20min in a Sorvall AH 269 rotor. The first 10ml were collected from top, layered on 27ml 30% Percoll and centrifuged at 100000g for 30min in a Sorvall T-865-rotor. Plasma membranes were collected from a band (3ml / (ρ = 1,05–1,06 g/ml determined with density marker beads) approximately 10 – 13ml from top of the tube. This fraction was diluted 1:11 with 5mM HEPES pH 7.4 and the membranes were pelleted by centrifugation (Sorvall rotor T-865 / 100000g / 60 min). The pellet was resuspended in 1ml 5mM HEPES pH 7.4. The whole procedure was carried out at 4°C.

Radioactive labelling of gangliosides and fetuin

Gangliosides GM1, GM2 and GM3 were [^{3}H]-labelled in their ceramide moiety using 370MBq [^{3}H]-sodium borohydride per μmol of ganglioside and palladium as catalyst according to the method of Schwarzmann (1978). Specific radioactivities were 28,2MBq/mg for GM1, 36MBq/mg for GM2, and 10,9MBq/mg for GM3.

Fetuin was tritium-labelled by a modification of the periodate / [^{3}H]]borohydride reduction method (Van Lenten and Ashwell, 1971). 1 mg of fetuin was dissolved in 300μl 0.1M sodium acetate, 0.15M NaCl, pH 5.6, and 300μl of 5mM sodium periodate were added. After 10min on ice the oxidation was stopped by the addition of 400 μl ethylene glycol. Ultrafiltration in Centricon 10™ (Amicon) was applied for removal of excess ethylene glycol and for changing solvent to 500μl 0.1M sodium phosphate, 0.15M NaCl, pH 7.5. Then 370MBq [^{3}H]-sodium borohydride was added and the reaction mixture was shaken on a Vortex-mixer in an atmosphere of argon at room temperature for 30 min. Reduction was completed by a further incubation for 30 min with 2 mM 'cold' sodium borohydride. The labelled product was purified by gel filtration on a column (5 × 1 cm) of Sephadex G-25 M and concentrated in Centricon 10™. Enzymatic degradation of the product with V. cholerae neuraminidase (Suttajit and Winzler, 1971) released 81% of the radioactivity.

Enzyme assays

Plasma membrane sialidase activity towards tritium-labelled gangliosides was assayed after specific activation with Triton X-100 (Kopitz *et al.*, 1994): 10 – 30μg homogenate protein were incubated with 0.04% Triton X-100, 6μM[^{3}H]ganglioside and 100mM sodium acetate (pH 4.5) at 37°C for 15min in a final volume of 50μl. Reaction was stopped by the addition of 1ml ice-cold methanol. Reaction products were separated from undegraded ganglioside by ion-exchange chromatography on DEAE SepharoseCL-6B minicolumns (Lieser *et al.*, 1989).

For measurement of sialidase activity towards glycoprotein samples, aliquots from Percoll fractions (100μl) were incubated with 3.33KBq of [^{3}H]fetuin in the presence of 100mM sodium acetate (pH 4.5) without detergent in a final volume of 200μl for 2 h at 37°C. The reaction was stopped by the addition of an equal volume of ice-cold 10% trichloracetic acid. After centrifugation (14000g, 4°C, 15min) the supernatant was counted for radioactivity, representing liberated C7-neuraminic acid. Sialidase activity towards 2'-4-methylumbelliferyl-α-N-acetylneuraminic acid was determined without detergent according to Harzer *et al.* (1986).

Alkaline phosphodiesterase was assayed with the substrate thymidine 5'monophosphate p-nitrophenylester (Storrie and Madden, 1990) and β-hexosaminidase was measured fluorimetrically with the substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (Storrie and Madden, 1990).

Recoveries of enzyme activities in Percoll gradient were 62% for Triton-activated sialidase towards ganglioside GM3, 91% for sialidase activity towards fetuin, 85 % for activity towards 2'-4-methylumbelliferyl-α-N-acetylneuraminic acid, 92% for β-hexosaminidase and 95% for alkaline phosphodiesterase.

Ouabain-sensitive Na^+/K^+-ATPase, leucine aminopeptidase, UDP-galactosyltransferase and succinate dehydrogenase were determined according to Graham (1993), lactate dehydrogenase acording to Storrie and Madden (1990), acetylcholinesterase acording to Blume *et al.* (1970), and 5'-nucleotidase according to Chatterjee *et al.* (1979).

In all enzyme assays 1 U corresponds to the turnover of 1 μmol substrate per min.

Specificity of the plasma membrane sialidase activity towards gangliosides

Neuroblastoma homogenate protein, 0.65mg, was incubated at 37°C for 8h with 100μmol ammonium acetate, pH4.5, 0.04% (w/v) Triton X-100 and 25nmol ganglioside (GM1, GM2, GM3, GD1a, GD1b or GT1b) in a final volume of 1ml. Reaction was stopped by freezing the samples in liquid nitrogen. After lyophilization gangliosides were disolved in 100μl methanol and separated on HPTLC plates (silica gel 60; 20 × 20 cm) using chloroform / methanol / 0.02% $CaCl_2$ (55:45:10; v / v/ v) as running solvent. Bands were visualized with orcinol spray (0.2% orcinol monohydrate in 75% sulphuric acid).

Quantitative determination of the plasma membrane sialidase activity towards gangliosides was done by separating the reaction products by FPLC ion exchange chromatography (Pharmacia): the methanolic extracts of the incubations were applied on a Mono Q column (HR 5/5) equilibrated with methanol and eluted with a linear gradient of 0 – 500mM ammonium acetate in methanol (34ml) at a flow rate of 1ml / min and 1ml fractions were collected. Ganglioside-containing fractions were quantified with resorcinol according to Miettinen and Takki-Luukkainen (1959).

Pulse-chase-labelling of neuroblastoma cells

Neuroblastoma cells were seeded in tissue culture flasks (75cm²) at an initial cell number of 3.75 × 10^6 cells and 18.5 MBq [³H]galactose was included in the culture medium. This medium was renewed 4 days after seeding. After 7 days of labelling, cells were trypsinized and their ganglioside pattern analyzed, or the prelabelled cells reseeded at a density of 3.75 × 10^6 cells per flask in nonradioactive medium. After allowing the cells 24h for attachment, they were chased for various time periods in the presence or absence of 250μM NeuAc2en.

Isolation and quantitation of metabolically labelled gangliosides

Cells were trypsinized, washed 3 times with 10ml 0.9% NaCl, and total lipids isolated by Folch extraction (Folch *et al.*, 1957). The methanol extracts (1ml) were dried under a stream of nitrogen and labelled gangliosides were quantitatively isolated from the residue by the procedure of Svennerholm and Fredman (1980). Salts and other nonlipid contaminants were removed using Sep-Pak C18 cartridges (Williams and McCluer, 1980) and the resulting pure ganglioside fraction was lyophilized. The gangliosides were dissolved in 50μl of acetonitrile / 5 mM phosphate buffer, pH 5.6 (83:17; v/v) and analytical high-performance liquid chromatography on a Lichrosorb-NH_2 column (250 × 4 mm I.D.; Merck, Germany) using the solvent system acetonitrile-phosphate buffer at different volume ratios and ionic strengths was applied for separation of the ganglioside mixture (Gazzotti *et al.*, 1985). The resulting 80 fractions (1ml each) were counted for radioactivity. Calibration of the column was achieved by chromatographing pure ganglioside standards and a mixture of all standards, which were detetected with resorcinol (Miettinen and Takki-Luukkainen, 1959). Ganglioside recovery of the whole procedure was between 80 and 90 % and there was no selective loss of any one ganglioside as checked by the addition of an internal standard (mixture of ganglioside standards) during Folch extraction.

Other assays

In samples containing Percoll protein was measured according to Vincent and Nadeau (1983), in all other cases the Lowry procedure was used (Lowry *et al.*, 1951). DNA was determined according to Bashford and Harris (1987).

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Abbreviations

NeuAc2en,2-Deoxy-2,3-dehydro-N-acetylneuraminic acid; 4-MU-NeuAc, 2′-4-Methylumbelliferyl-α-N-acetylneuraminic acid; HEPES, (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; EDTA, Ethylenediaminetetraacetic acid; GDC, Glycodeoxycholate; Gangliosides are abbreviated according to the recommendations of the IUPAC – IUB Commision on Biochemical Nomenclature (1977): GM3, II^3NeuAc-LacCer; GM2, II^3NeuAc-$GgOse_3$-Cer; GM1, II^3NeuAc-$GgOse_4$-Cer; GD1a, IV^3NeuAc,II^3NeuAc-$GgOse_4$-Cer; GD1b, II^3NeuAc_2-$GgOse_4$-Cer; GD2, II^3NeuAc_2-$GgOse_3$-Cer; GD3, II^3NeuAc_2-Lac-Cer; GT1b, IV^3NeuAc,II^3-$NeuAc_2$-$GgOse_4$-Cer;

Enzymes

Sialidase (neuraminidase), EC 3.2.1.18; β-Hexosaminidase, EC 3.2.1.30; Alkaline phosphodiesterase, EC 3.1.4.1; Na^+/K^+-ATPase,EC 3.6.1.3; Acetylcholinesterase, EC 3.1.1.7; UDP-galactosyltransferase, EC 2.4.1.38; 5′-Nucleotidase, EC 3.1.3.5; Succinate dehydrogenase,EC 1.3.99.1; Lactate dehydrogenase, EC 1.1.1.27;Leucine aminopeptidase, EC 3.4.1.1.

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