Desialylation of extracellular GD1a-neoganglioprotein suggests cell surface orientation of the plasma membrane-bound ganglioside sialidase activity in human neuroblastoma cells

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Abstract The orientation of the catalytic site of a ganglioside-specific sialidase in the plasma membrane of SK-N-MC neuroblastoma cells was probed using water-soluble GD1a-neoganglioprotein substrate on intact cells and GM1-product detection by cholera toxin B. Desialylation of substrate was readily observed, whereas specific sialidase inhibitors prevented the reaction, and conditioned medium was inactive. Inhibitors of endocytosis and acidification had no effect on substrate degradation, and lowering temperature to 18°C reduced activity but did not abolish it. We conclude that the ganglioside sialidase activity is cell surface-orientated and displays an in situ specificity that mirrors enzyme preparations in vitro.

Key words: Sialidase; Glycosidase; Ganglioside; Neoganglioprotein; Plasma membrane; Cell surface

1. Introduction

Gangliosides of the plasma membrane are modulators of various cellular functions including cell adhesion, cellular interactions, cell growth, differentiation, and neural repair [1–3]. Control of a cell's ganglioside lining may therefore be of profound impact on cellular behavior. Such control can be based on both biosynthetic and degradative processes. Major sites of ganglioside metabolism are the endoplasmic reticulum and the Golgi apparatus for biosynthetic reactions, and the lysosomal compartment for final degradation [3]. The only known reactions for in situ modelling of the cell surface ganglioside pattern are that of a sialyltransferase presumably located at the outer surface of synaptosomal membranes from calf brain [4], and that of a ganglioside-specific sialidase in neuronal calf brain membranes [5] or in the plasma membrane of human SK-N-MC neuroblastoma cells [6]; the neuroblastoma cell enzyme acted specifically on gangliosides with terminal sialic acids, yielding a shift from higher sialylated species to GM1 and a conversion of GM3 to lactosylceramide [7]. Since inhibition of the enzyme by inclusion of specific inhibitors in the medium of SK-N-MC neuroblastoma cultures led to a release from contact inhibition of growth and a loss of differentiation markers, a role of the ganglioside sialidase on growth control and differentiation in this neuronal cell line was suggested [8].

The plasma membrane sialidase of neuronal tissues was recently purified and characterized [9,10] and has just been cloned [11–14]. In transfected COS-7 cells a protein protection assay suggested that the sialidase might be an atypical type I membrane protein and it was speculated that ganglioside desialylation would occur on the cytoplasmic side of the plasma membrane or in intracellular vesicles formed by plasma membrane budding [12]. In contrast, however, incubation of intact cells in the presence of millimolar concentrations of Cu^{2+} at low temperature completely inactivated the plasma membrane-associated sialidase activity [6], and inclusion of the sialidase inhibitor 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (NeuAc2en) in the culture medium totally blocked ammonia-insensitive desialylation of plasma membrane-inserted radiolabelled ganglioside GM3 [8], both observations pointing to a cell surface-orientated sialidase activity. In order to further proceed in understanding the sialidase's cellular functions, it would therefore be helpful to definitely establish the orientation of the enzyme in the plasma membrane. To this end, we probed the sialidase activity of intact neuroblastoma cells towards a soluble radiolabelled GD1a-neoganglioprotein as an extracellular substrate that does not insert into membranes.

2. Materials and methods

Lyso-ganglioside GD1a was obtained from Calbiochem (Bad Soden, Germany), succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and *N*-succinimidyl-*S*-acetylthioacetate (SATA) were from Pierce (Rockford, IL, USA), BSA (fatty acid- and globulin-free), vinblastine, heparin (sodium salt, high molecular weight, from porcine intestinal mucosa), heparan sulfate (sodium salt, from bovine intestinal mucosa), colominic acid and fetuin (from fetal calf serum) were from Sigma (Munich, Germany). Chondroitin sulfate A (sodium salt, from shark notochord) and chondroitin sulfate B (sodium salt, from pig skin) were purchased from ICN (Eschwege, Germany). *N*-Acetylneuraminic acid (NeuAc) and 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (NeuAc2en) were from Roche (Mannheim, Germany).

For preparation of GD1a-neoganglioprotein, lyso-GD1a was treated with SMCC producing maleimidyl-derivatized ganglioside, which was subsequently coupled to SATA-reacted BSA. The whole procedure was exactly as described by Mahoney and Schnaar [15]. For iodination, 20 μg GD1a-neoganglioprotein was incubated at room temperature for 15 min in 50 mM sodium phosphate, pH 7.4, with 74 MBq carrier-free $Na^{125}I$ (Hartmann, Braunschweig, Ger-

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Abbreviations: NeuAc2en, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; SMCC, succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SATA, *N*-succinimidyl-*S*-acetylthioacetate; BSA, bovine serum albumin; PBS, phosphate-buffered saline

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many). Labelled protein was separated from free iodine by gel filtration on Sephadex G-25 (PD-10 column, Amersham Pharmacia, Freiburg, Germany). Specific radioactivity of the labelled protein was 770 kBq/μg.

Neuroblastoma cells (SK-N-MC) were seeded in 96-well tissue culture plates at an initial density of 10 000 cells per well and cultured for 5 days at 37°C in Eagle's minimal essential medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (PAA, Linz, Austria), penicillin (100 IU/ml), streptomycin (100 μg/ml) and non-essential amino acids in an atmosphere of 95% air and 5% CO_2. Then 5 kBq ^{125}I-labelled GD1a-neoganglioprotein was included in the medium and GM1-neoganglioprotein formed was determined after 4, 8, 12 or 16 h of incubation at 37 and 18°C, respectively. To quantitate the GM1-neoganglioprotein content of the medium, it was transferred to 96-well Maxisorp plates (Nunc, Roskilde, Denmark) that had been coated with 1 μg cholera toxin B in 100 μl 100 mM Na_2CO_3 buffer, pH 9.6 for 12 h at 4°C, blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and washed three times with PBS/0.05% Tween 20. The plates were agitated in the cold room for 8 h on a reciprocating shaker before the supernatants were removed, the plates washed three times with 200 μl PBS/Tween, and cholera toxin B-bound radioactivity solubilized with 200 μl 0.5 N NaOH and measured in a liquid scintillation counter with external standardization (Canberra Packard TRICARB 2900, counting region 0–70 keV).

Conditioned medium was taken from cultures after 5 days and transferred to empty 96-well plates and incubated with labelled GD1a-neoganglioprotein as above.

Cellular protein was measured after removal of the medium and two washes with PBS by solubilization of the cells with 20 μl 0.2 N NaOH and conducting the Lowry protein determination [16] directly in the culture wells using a microtest plate reader (Anthos 2010).

Plasma membrane-bound ganglioside sialidase activity in neuroblastoma cell homogenates was assayed as described previously [6].

3. Results and discussion

Neoganglioproteins, such as lyso-GD1a-derivatized BSA, are excellent tools for the characterization and identification of ganglioside-recognizing proteins [15], because they circumvent experimental problems caused by the amphipathic nature of gangliosides that result in their hydrophobic binding to and spontaneous insertion into cell membranes. Although they retain the lipid structure, the covalently bound gangliosides are prevented from inserting into cell membranes, and so appear useful for detecting plasma membrane-bound ganglioside sialidase activity of whole cells towards extracellularly presented substrate. The result of such an experiment is shown in Fig. 1A, where GD1a-neoganglioprotein was included in the culture medium of SK-N-MC neuroblastoma cells and the product of the sialidase reaction, GM1-neoganglioprotein, determined at various time points using cholera toxin B in a solid phase assay. The amount of GM1-neoganglioprotein increased in a time-dependent manner, indicating sialidase activity towards substrate presented from outside of the enzyme's membrane. Addition of the specific sialidase inhibitor NeuAc2en to the culture medium totally abolished the desialylation reaction. Activity measurements in the conditioned medium from neuroblastoma cultures excluded the possibility that a soluble sialidase either originating from secretion [17] or from the fetal calf serum present in the culture medium might contribute to the observed neoganglioprotein desialyation. The present novel methodology for the measurement of ganglioside sialidase activity using a neoganglioprotein substrate might also prove useful for other applications where the use of detergents for substrate solubilization has to be avoided, e.g., when intact cells or subcellular organelles are to be used.

It was suggested that ganglioside substrates and the plasma

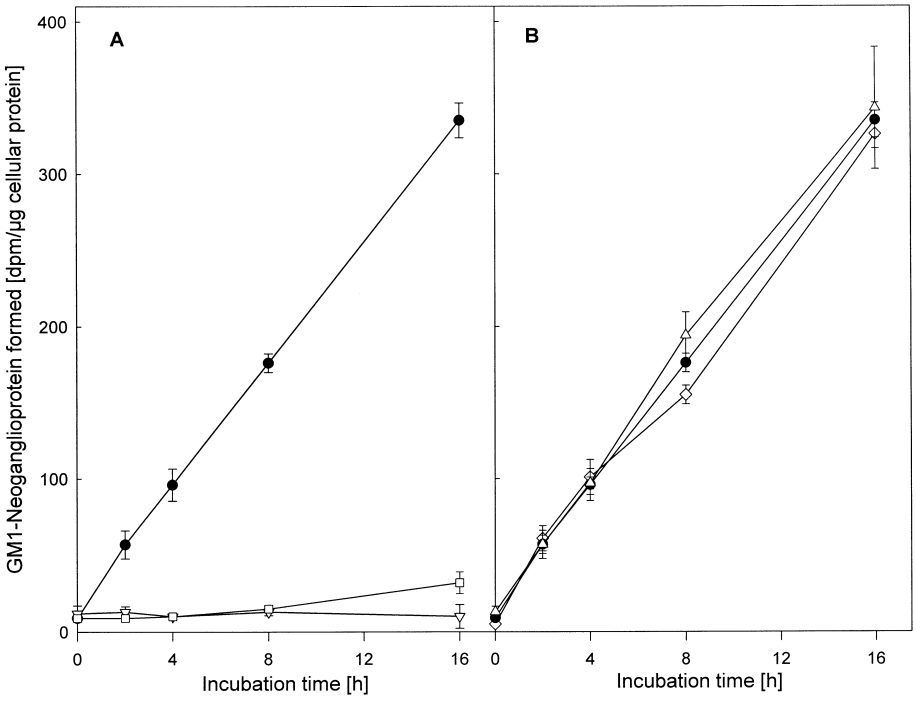

Fig. 1. A: Sialidase activity towards extracellular GD1a-neoganglioprotein: Neuroblastoma cells were grown in 96-well plates for 5 days. Then the cells were incubated in the absence (●) or presence (□) of 250 μM NeuAc2en with 5 kBq of iodinated GD1a-neoganglioprotein for the indicated time periods. Conditioned medium (▽) of the cultures was likewise incubated with labelled GD1a-neoganglioprotein in 96-well plates. B: Effect of vinblastine or ammonium chloride on desialylation of GD1a-neoganglioprotein: Sialidase activity towards extracellular GD1a-neoganglioprotein was assayed in absence (●) or in presence of (◇) 50 μM vinblastine or (△) 20 mM ammonium chloride. GM1-neoganglioprotein formed by sialidase action was determined by its binding to cholera toxin B subunit in solid phase assay as described in Section 2. Results are the means of eight determinations, error bars representing S.E.M.

membrane sialidase become internalized into some intracellular acidic compartment like endosomes for desialylation reaction to occur [12]. This would require intracellular vesicle transport and acidification of the compartment. However, vinblastine, a microtubule inhibitor, and ammonium chloride, a neutralizer of acidic compartments [18] did not affect GD1a-neoganglioprotein desialylation (Fig. 1B). As a further test, incubations were performed at 18°C where no endocytotic uptake of extracellular substrate can occur. Although reaction velocity was lower, considerable GD1a-neoganglioprotein desialylation at this temperature was observed (Fig. 2). The reduction of the sialidase activity in comparison to incubations at 37°C seems to be a direct effect of the lowered temperature on enzyme catalysis, as it was also observed when cell homogenates were assayed in vitro for Triton-activated plasma membrane sialidase activity towards ganglioside GM3 (Fig. 3). Both observations, lack of inhibition by vinblastine or ammonia and sustained activity at 18°C, favor the supposition that the sialidase directly acts on the cell surface rather than in secondarily formed intracellular vesicles. This point of view is also supported by the fact that the neoganglioprotein product is found in the extracellular space and is not degraded by the endosomal/lysosomal pathway as should be expected in the case of intravesicular action of the sialidase.

In previous studies, the influence of various potential inhibitors on the plasma membrane sialidase had been determined in detergent-activated neuroblastoma homogenates [8]. The data shown in Fig. 4 indicate that the results now obtained with the enzyme in its native environment closely correspond to the results previously obtained in an artificial system. In particular, besides the specific inhibitor NeuAc2en, the extracellular matrix and cell surface constituent heparan sulfate, as well as heparin, are potent inhibitors. The chondroitin sulfates A and B, on the other hand, had only small effects, and NeuAc at 1 mM was not inhibitory.

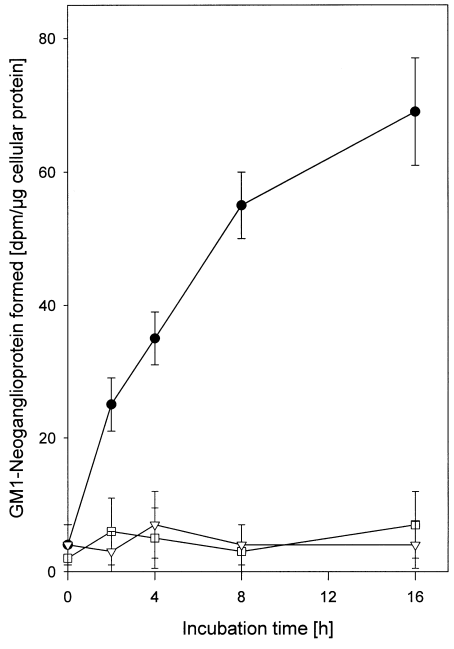

Fig. 2. Sialidase activity towards extracellular GD1a-neoganglioprotein at 18°C. GD1a-neoganglioprotein desialylation was measured in (●) absence or (□) presence of NeuAc2en and in (▽) conditioned medium of neuroblastoma cultures as described for Fig. 1, with the exception that the incubation temperature was lowered from 37 to 18°C. Results are the means of eight determinations, error bars representing S.E.M.

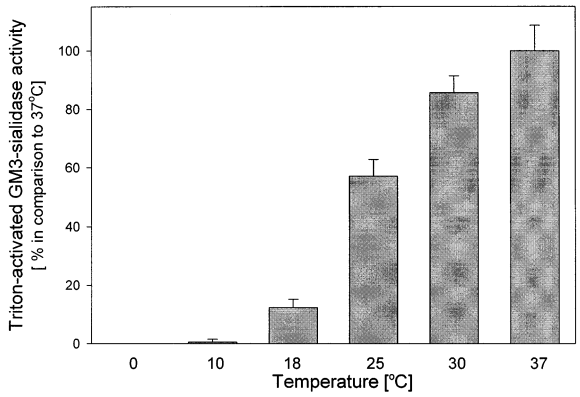

Fig. 3. Temperature dependence of the plasma membrane ganglioside sialidase activity. Plasma membrane sialidase activity towards tritium-labelled ganglioside GM3 was assayed in homogenates of neuroblastoma cells after specific activation with Triton X-100 at the indicated incubation temperatures. Results are the means of four determinations, error bars representing S.E.M.

The membrane-bound ganglioside sialidase purified from human brain [9] or that present in plasma membranes of SK-N-MC cells [7] did not desialylate non-lipid sialoglycoconjugates such as sialoglycoproteins, sialyloligosaccharides, or polysialic acids, and it was anticipated that this also holds true for the enzyme in situ. Indeed, we did not detect any competitive effect of 3′-sialyllactose, fetuin, or colominic acid on the desialylation of GD1a-neoganglioprotein in the cell cultures (Table 1), thus establishing the enzyme's strict ganglioside specificity also in its intact natural environment. Our results confirm and extend previous observations by Schengrund et al. of an ecto-sialidase in transformed hamster cells [19].

The finding that the cell surface sialidase can attack sub-

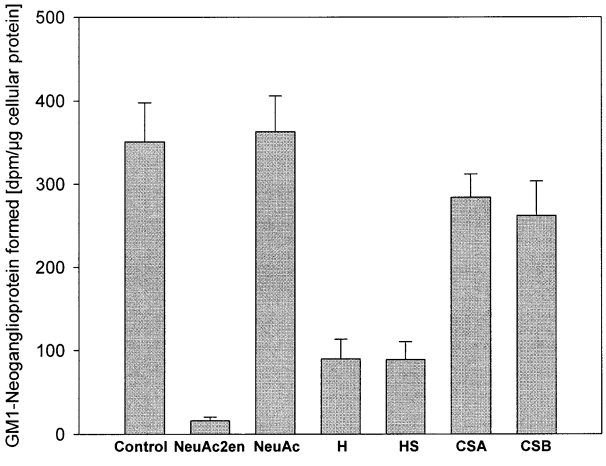

Fig. 4. Effect of sialidase inhibitors on desialylation of GD1a-neoganglioprotein. Neuroblastoma cells were grown in 96-well cell plates for 5 days. Then cells were cultured in absence (control) or presence of potential inhibitors with 5 kBq of iodinated GD1a-neoganglioprotein for 16 h before GM1-neoganglioprotein product was determined: NeuAc2en, 250 µM; NeuAc, 1 mM; H = 100 µg/ml heparin; HS = 100 µg/ml heparan sulfate; CSA = 100 µg/ml chondroitin sulfate A; CSB = 100 µg/ml chondroitin sulfate B. Results are the means of four determinations, error bars representing S.E.M.

Table 1
Effect of potential non-lipid substrates on GD1a-neoganglioprotein sialidase activity of cultured neuroblastoma cells

Compound	GM1-neoganglioprotein formed (dpm/μg cellular protein)
Control	351 ± 46.8
10 mM NeuAc residues bound to fetuin	361 ± 23.8
10 mM 3′-sialyllactose	357 ± 46.9
10 mM NeuAc residues bound in colominic acid	347 ± 31

5 kBq iodinated GD1a-neoganglioprotein and potential competitive substrates were included in the culture medium for 16 h before formed GM1-neoganglioprotein was determined as described under Section 2. Results are the means of four measurements ± S.E.M.

strates beyond its own membrane might be of considerable physiological importance. Sialidase-modulated changes in the behavior of neuroblastoma cells such as growth inhibition or differentiation are observed at cell densities bringing about direct intercellular contact [6,8]. Our present results point to the possibility that the plasma membrane sialidase might be able to desialylate gangliosides on the surface of adjacent cells, thereby acting as some kind of sensor for cell contact. Further investigations are to be conducted to prove this attractive possibility.

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