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Characterization of cytosolic sialidase from Chinese hamster ovary cells|qlPart II. Substrate specificity for gangliosides

Johannes Müthing,* Monika Burg

Institute of Cell Culture Technology, Faculty of Technology, University of Bielefeld, PO Box 100131, D-33501 Bielefeld, Germany

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Abstract

Cytosolic Chinese hamster ovary (CHO) cell sialidase has been cloned as a soluble glutathione S-transferase (GST)-sialidase fusion protein with an apparent molecular weight of 69 kD in *Escherichia coli*. The enzyme has then been produced in mg quantities at 25-L bioreactor scale and purified by one-step affinity chromatography on glutathione sepharose (Burg, M.; Müthing, J. *Carbohydr. Res.* **2001**, *330*, 335–346). The cloned sialidase was probed for desialylation of a wide spectrum of different types of gangliosides using a thin-layer chromatography (TLC) overlay kinetic assay. Different gangliosides were separated on silica gel precoated TLC plates, incubated with increasing concentrations of sialidase (50 μU/mL up to 1.6 mU/mL) without detergents, and desialylated gangliosides were detected with specific anti-asialoganglioside antibodies. The enzyme exhibited almost identical hydrolysis activity in degradation of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc). A slightly enhanced activity, compared with reference *Vibrio cholerae* sialidase, was detected towards terminally α(2-3)-sialylated neolacto-series gangliosides IV^3-α-Neu5Ac-nLc_4Cer and VI^3-α-Neu5Ac-nLc_6Cer. The ganglio-series gangliosides G_{D1a}, G_{D1b}, and G_{T1b}, the preferential substrates of *V. cholerae* sialidase for generating cleavage-resistant G_{M1}, were less suitable targets for the CHO cell sialidase. The increasing evidence on colocalization of gangliosides and sialidase in the cytosol strongly suggests the involvement of the cytosolic sialidase in ganglioside metabolism on intracellular level by yet unknown mechanisms.

Keywords: Desialylation; Ganglioside specificity; GST-sialidase; Neuraminidase; Thin-layer chromatography (TLC) overlay kinetic assay

1. Introduction

Four types of mammalian sialidases have been identified so far, differing in their subcellular location, catalytic activity, chromatographic, and immunological properties. These are intralysosomal, cytosolic, and two membrane-associated sialidases I and II. Plasma membrane sialidase I has been, at least partly, purified from various mammalian tissues, e.g., rabbit[1] and human erythrocytes,[2] rat liver,[3] brain,[4] and cerebellar granule cells,[5] human fibroblasts,[6] leukocytes,[7] brain,[8] and neuroblastoma cells.[9] Membranous sialidases preferentially hydrolyze sialylated glycosphingolipids (GSLs), and certain detergents such as Triton X-100, octylglucoside or deoxycholate

* Corresponding author. Tel.: +49-521-1066320; fax: +49-521-1066328.

E-mail address: jm@zellkult.techfak.uni-bielefeld.de (J. Müthing).

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are required for maximum enzyme activity. Lysosomal membrane sialidase II has been isolated, e.g., from rat liver,[10] mouse brain,[11] human fibroblasts[12] and placenta.[13] This type degrades both, water-soluble and membrane-bound sialyl glycoconjugates, i.e., glycoproteins and gangliosides, respectively, whereas the intra-lysosomal (located in the lysosomal matrix) sialidase hydrolyses only low-molecular-weight substrates such as oligosaccharides and glycopeptides.[14] Sialylated glycoconjugates available to cytosolic sialidase could not be expected to be present in the cytosol. However, soluble extracts from rat brain, liver, and skeletal muscle displayed sialidase activity.[15–17] Both, rat liver and Chinese hamster ovary (CHO) cell derived soluble sialidases, were active not only toward sialooligosaccharides and sialoglycoproteins (discussed in detail in the preceding paper[18]), but also toward gangliosides. Some gangliosides were degraded by the sialidases and for both enzymes, the requirement of cholic acid as a solubilizing agent in the assay for optimal activity for these substrates has been reported.[16,19] To obtain further insights into the significance of physiological desialylation, the cytosolic sialidase from rat skeletal muscle[20] and CHO cells[21] have been cloned in *Escherichia coli* and insect cells, respectively. To our knowledge, there are no data of the ganglioside hydrolyzing specificity of recombinant mammalian cytosolic sialidases cloned so far.[20,21]

The GST-sialidase fusion protein used in this study was produced on a mg scale and purified from *E. coli* homogenates by one-step affinity chromatography in a soluble and active form.[18] The detailed substrate specificity was elucidated with gangliosides G_{M3}-(Neu5Ac), G_{M3}(Neu5Gc), and gangliosides of the ganglio- and neolacto-series using *Vibrio cholerae* sialidase as reference enzyme. For that purpose, a thin-layer chromatography (TLC) overlay kinetic assay without the requirement of any detergents was employed.

2. Results

TLC overlay kinetic assay.—Terminally sialylated gangliosides can be easily cleaved by sialidases generating respective neutral GSLs, also termed 'asialogangliosides'. In situ desialylation directly on HPTLC plates after chromatographic separation followed by immunochemical detection of asialogangliosides enables qualitative and quantitative determination of unknown sialidase activity and specificity.[22,23] The procedure does not require purified single gangliosides, but only mixtures of gangliosides with known structures (reference gangliosides). Steps to be performed are (i) separation of gangliosides on HPTLC plates; (ii) plastic fixation of the silica gel; (iii) treatment with sialidase; and (iiii) incubation of the plates with antibodies specific for respective asialogangliosides. Alkaline-phosphatase-conjugated second antibodies are used to visualize bound first antibodies by generating a blue dye from 5-bromo-4-chloro-3-indolylphosphate. In order to establish the specificity of the GST-sialidase, purified gangliosides G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), and ganglioside mixtures from human brain (HBG, comprising G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}) and human granulocytes (HGG, comprising IV^3-α-Neu5Ac-nLc_4Cer, IV^6-α-Neu5Ac-nLc_4Cer, and VI^3-α-Neu5Ac-nLc_6Cer) were separated on HPTLC plates (for ganglioside structures see Table 1). Plasticized TLC plates were then incubated with increasing concentrations of sialidase (50 μU/mL up to 1.6 mU/mL), and desialylated gangliosides were detected with respective anti-asialoganglioside antibodies. *V. cholerae* sialidase was chosen as reference enzyme for direct comparison with GST-sialidase. This procedure was named 'TLC overlay kinetic assay' and enables the elucidation of unknown enzyme specificities requiring only small quantities of ganglioside mixtures.

Desialylation of G_{M3}.—The desialylation of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) with reference *V. cholerae* sialidase on HPTLC plates without detergents is shown in Fig. 1(A and B), respectively. Both, G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), chromatograph as double bands due to substitution with C24- (upper band) and C16-fatty acid (lower band, Fig. 1, resorcinol stained R lanes of the inserts). Asialogangliosides were detected with an anti-LacCer antibody, which specifically binds to

Table 1
Structures of G_{M3}, ganglio- and neolacto-series gangliosides employed for TLC overlay kinetic assays

Abbreviation [a]	Structure [b]	Symbol
G_{M3}-specimens		
II^3-α-Neu5Ac-LacCer	Neu5Acα(2-3)Galβ(1-4)Glcβ(1-1)Cer	G_{M3}(Neu5Ac)
II^3-α-Neu5Gc-LacCer	Neu5Gcα(2-3)Galβ(1-4)Glcβ(1-1)Cer	G_{M3}(Neu5Gc)
Ganglio-series		
II^3-α-Neu5Ac-Gg_4Cer	Galβ(1-3)GalNAcβ(1-4)(Neu5Acα(2-3))Galβ(1-4)Glcβ(1-1)Cer	G_{M1}
IV^3-α-Neu5Ac, II^3-α-Neu5Ac-Gg_4Cer	Neu5Acα(2-3)Galβ(1-3)GalNAcβ(1-4)(Neu5Acα(1-3))Galβ(1-4)-Glcβ(1-1)Cer	G_{D1a}
II^3-α-$(Neu5Ac)_2$-Gg_4Cer	Galβ(1-3)GalNAcβ(1-4)(Neu5Acα(2-8)Neu5Acα(2-3))Galβ(1-4)Glcβ-(1-)Cer	G_{D1b}
IV^3-α-Neu5Ac, II^3-α-$(Neu5Ac)_2$-Gg_4Cer	Neu5Acα(2-3)Galβ(1-3)GalNAcβ(1-4)(Neu5Acα(2-8)Neu5Acα(2-3))-Galβ(1-4)Glcβ(1-1)Cer	G_{T1b}
Neolacto-series		
IV^3-α-Neu5Ac-nLc_4Cer	Neu5Acα(2-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glcβ(1-1)Cer	IV^3nLc4
IV^6-α-Neu5Ac-nLc_4Cer	Neu5Acα(2-6)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glcβ(1-1)Cer	IV^6nLc4
VI^3-α-Neu5Ac-nLc_6Cer	Neu5Acα(2-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)Galβ-(1-4)Glcβ(1-1)Cer	VI^3nLc6

[a] According to the IUPAC-IUB recommendations.[57]
[b] Symbols of G_{M3} and ganglio-series gangliosides follow the original designation of Svennerholm.[58]

asialo-G_{M3} (examples are shown in lanes 2 and 6 of the inserts of Fig. 1 after incubation with 100 μU/mL and 1.6 mU/mL of *V. cholerae* sialidase, respectively) and which does not react with G_{M3} (Fig. 1, lanes 7 of the inserts). Using G_{M3}-amounts of 1 μg per lane and increasing sialidase activities (50, 100, 200, 400, 800, and 1600 μU/mL corresponding to columns 1–6, respectively), the TLC overlay kinetic reveals a concentration dependent degradation of both, G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), with preferential desialylation of G_{M3}(Neu5Ac), particularly at low enzyme concentrations. The desialylation of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) with the GST-sialidase was performed exactly under the same conditions as with *V. cholerae* sialidase (see Fig. 1) and is shown in Fig. 2(A and B), respectively. Both gangliosides were degraded to asialo-G_{M3} (lactosylceramide) by the CHO cell derived cytosolic sialidase fusion protein. The TLC kinetic pattern of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) desialylation were similar to those of *V. cholerae* sialidase hydrolytic cleavage (see Fig. 1). Minor differences, i.e., some lowered enzyme activity towards G_{M3}(Neu5Ac) at concentrations from

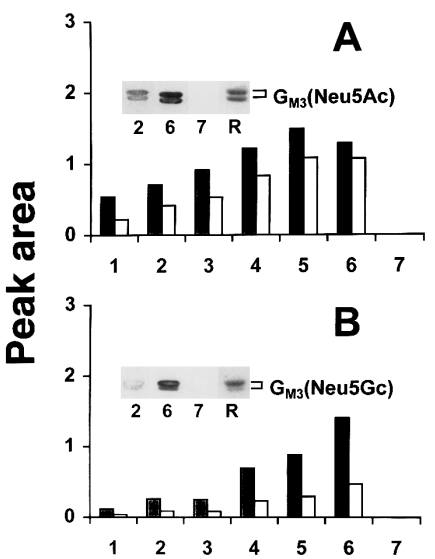

Fig. 1. TLC overlay kinetic: *V. cholerae* sialidase treatment of G_{M3}(Neu5Ac) (A) and G_{M3}(Neu5Gc) (B) and subsequent immunostaining with anti-LacCer antibody. In lanes 1–7, 1 μg of G_{M3} and for resorcinol stain (R) 5 μg of G_{M3} were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/ml, lanes 1; 100 μU/mL, lanes 2; 200 μU/mL, lanes 3; 400 μU/mL, lanes 4; 800 μU/mL, lanes 5; 1.6 mU/mL, lanes 6; *V. cholerae* sialidase buffer as control, lanes 7. Immunostained bands were quantified by quintuple TLC scanning and are presented as peak area columns. The inserts show the TLC immunostains using 100 μU/mL (lanes 2), 1.6 mU/mL (lanes 6), and buffer as control (lanes 7) in comparison to the resorcinol stained G_{M3} (R).

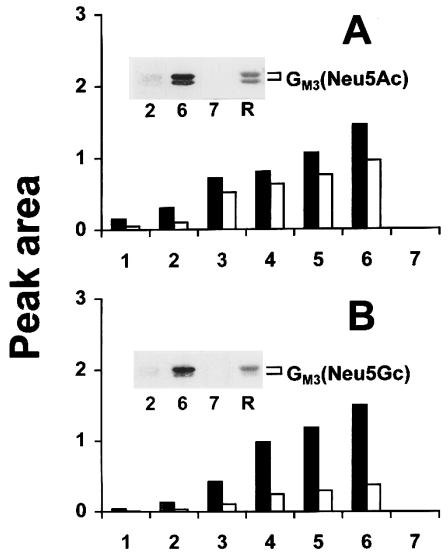

Fig. 2. TLC overlay kinetic: GST-sialidase treatment of G_{M3}(Neu5Ac) (A) and G_{M3}(Neu5Gc) (B) and subsequent immunostaining with anti-LacCer antibody. In lanes 1–7, 1 μg of G_{M3} and for resorcinol stain (R) 5 μg of G_{M3} were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/mL, lanes 1; 100 μU/mL, lanes 2; 200 μU/mL, lanes 3; 400 μU/mL, lanes 4; 800 μU/mL, lanes 5; 1.6 mU/mL, lanes 6; GST-sialidase buffer as control, lanes 7. Immunostained bands were quantified by quintuple TLC scanning and are presented as peak area columns. The inserts show the TLC immunostains using 100 μU/mL (lanes 2), 1.6 mU/mL (lanes 6), and buffer as control (lanes 7) in comparison to the resorcinol stained G_{M3} (R).

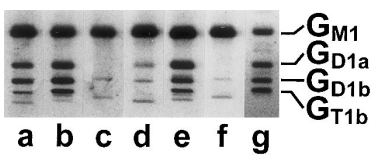

Fig. 3. Sialidase treatment of human brain gangliosides (HBG) and subsequent immunodetection of G_{M1} (hydrolysis product of G_{D1a}, G_{D1b}, and G_{T1b}) with choleragenoid and anti-choleragenoid antibody. In lanes a–f, 0.2 μg of HBG were chromatographed; lane g shows the resorcinol stain of 20 μg HBG. The HPTLC plates were fixed and incubated for 2 h with increasing activities of *V. cholerae* sialidase (100 μU/mL, lane a; 1.6 mU/mL, lane b; *V. cholerae* sialidase buffer as control, lane c) and GST-sialidase (100 μU/mL, lane d; 1.6 mU/mL, lane e; GST-sialidase buffer as control, lane f). The entire TLC kinetics of *V. cholerae* and GST-sialidase are shown in Figs. 4 and 5, respectively.

50 to 800 μU/mL and some enhanced activity towards G_{M3}(Neu5Gc) at concentrations from 200 to 800 μU/mL, were observed for GST-sialidase (Fig. 2(A and B), respectively) compared with reference *V. cholerae* sialidase (see Fig. 1(A and B), respectively). According to these data, it is obvious that the CHO cell cytosolic sialidase is capable of hydrolyzing the ganglioside G_{M3} without any detergents and without discriminating between Neu5Ac- and Neu5Gc-substitution of lactosylceramide.

Desialylation of ganglio-series gangliosides.—G_{M1} can be visualized on HPTLC plates with choleragenoid, and G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b} by conversion with sialidase to G_{M1} prior to treatment with choleragenoid.[24] *V. cholerae* sialidase converts major gangliosides of human brain to G_{M1}, whereas G_{M1} itself remains intact due to its sialylation at position II of the Gg_4Cer core. The suitability of this assay is demonstrated in Fig. 3 by means of 0.2 μg reference HBG per TLC run. G_{M1} was detected as a single band by choleragenoid without (Fig. 3, lanes c and f) and the higher sialylated gangliosides with G_{M1}-backbone after enzyme treatment of, e.g., 100 μU/mL and 1.6 mU/mL *V. cholerae* sialidase (Fig. 3, lanes a and b, respectively). At low enzyme concentrations the GST-sialidase showed only poor hydrolysis activity towards G_{D1a}, G_{D1b}, and G_{T1b} (Fig. 3, lane d), but enhancement of enzyme concentrations increased desialylation of higher ganglio-series gangliosides to G_{M1} (Fig. 3, lane e). The entire TLC overlay kinetics of HBG desialylation by *V. cholerae* and CHO cell sialidase are shown in Figs. 4 and 5, respectively. The direct comparison of both kinetics demonstrates the preference of G_{D1a}, G_{D1b}, and G_{T1b} as substrates for *V. cholerae* sialidase particularly at low enzyme concentrations, whereas considerable higher concentrations of GST-sialidase were required for HBG desialylation. Degradation of α(2-3)-linked Neu5Ac (exemplified with G_{D1a}) and of α(2-8)-linked Neu5Ac (exemplified with G_{D1b}) was much more efficient with *V. cholerae* sialidase at enzyme concentrations in the range from 50 up to 400 μU/mL (Fig. 4) while at least 400 and 800 μU/mL of GST sialidase are required for reasonable hydrolysis of G_{D1a} and G_{D1b}, respectively (Fig. 5). Because both α(2-3) and α(2-8) sialic acid cleaving activities are required for the hydrolysis of G_{T1b}, the degradation of this ganglioside to G_{M1} showed most clearly the substrate preference of ganglio-series gangliosides of *V. cholerae* sialidase.

The attempt to desialylate G_{M1} and higher G_{M1}-core gangliosides (G_{D1a}, G_{D1b}, and G_{T1b}) to Gg_4Cer failed. The GST-sialidase did not cleave Neu5Ac at position II of the Gg_4Cer backbone, neither without nor after supplementation of the enzyme buffer with 0.5 mg/mL sodium taurodeoxycholate (data not shown).

Desialylation of neolacto-series gangliosides.—Terminally α(2-3)- and α(2-6)-sialylated neolacto-series gangliosides (IV^3-α-Neu5Ac-nLc_4Cer, IV^6-α-Neu5Ac-nLc_4Cer, and VI^3-α-Neu5Ac-nLc_6Cer) are sensitive towards *V. cholerae* sialidase degradation and have been used as references (see Table 1). As shown by resorcinol stain in Fig. 6 (lane g), each ganglioside migrates as a double band on HPTLC plate due to substitution with C24- (upper band) and C16-fatty acid (lower band). After incubation of 2 μg of HGG with 100 μU/mL and 1.6 mU/mL *V. cholerae* sialidase, respective asialogangliosides (nLc_4Cer and nLc_6Cer) were visualized with the polyclonal anti-nLc_4Cer (anti-Galβ(1-4)GlcNAc-R) antibody as shown in Fig. 6 (lanes a and b, respectively). Both, IV^3-α-Neu5Ac-nLc_4Cer and VI^3-α-Neu5Ac-nLc_6Cer, were converted into nLc_4Cer and nLc_6Cer, respectively, by *V. cholerae* sialidase in a concentration dependent manner. Without sialidase treatment, two positive IV^6-α-Neu5Ac-nLc_4Cer bands were detected (Fig. 6, lanes c and f) since sialylation at position 6 to terminal galactose does not

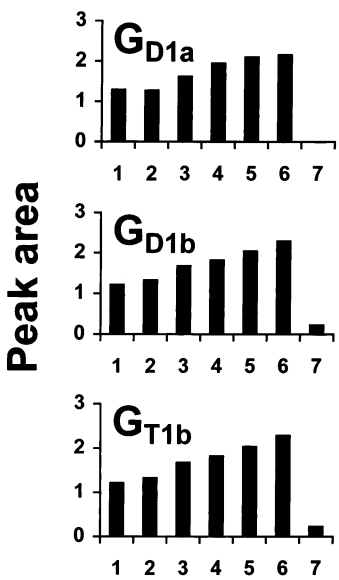

Fig. 4. TLC overlay kinetic: *V. cholerae* sialidase treatment of human brain gangliosides (HBG) and subsequent immunodetection of G_{M1} (hydrolysis product of G_{D1a}, G_{D1b}, and G_{T1b}) with choleragenoid and anti-choleragenoid antibody. In lanes 1–7, 0.2 μg of HBG were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/mL, lane 1; 100 μU/mL, lane 2; 200 μU/mL, lane 3; 400 μU/mL, lane 4; 800 μU/mL, lane 5; 1.6 mU/mL, lane 6; *V. cholerae* sialidase buffer as control, lane 7. Immunostained bands were quantified by quintuple TLC scanning and corresponding peak area columns are shown.

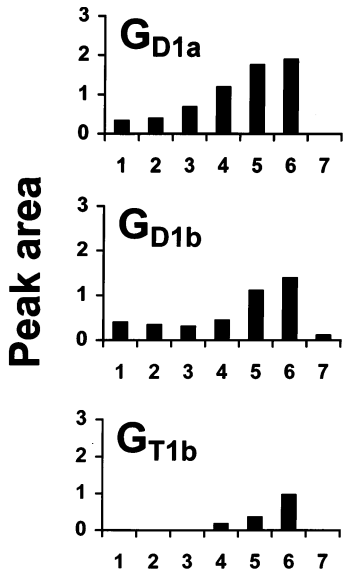

Fig. 5. TLC overlay kinetic: GST-sialidase treatment of human brain gangliosides (HBG) and subsequent immunodetection of G_{M1} (hydrolysis product of G_{D1a}, G_{D1b}, and G_{T1b}) with choleragenoid and anti-choleragenoid antibody. In lanes 1–7, 0.2 μg of HBG were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/mL, lane 1; 100 μU/mL, lane 2; 200 μU/mL, lane 3; 400 μU/mL, lane 4; 800 μU/mL, lane 5; 1.6 mU/mL, lane 6; sialidase buffer as control, lane 7. Immunostained bands were quantified by quintuple TLC scanning and corresponding peak area columns are shown.

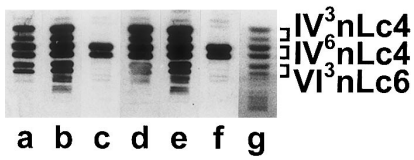

Fig. 6. Sialidase treatment of human granulocyte gangliosides (HGG) and subsequent immunostaining with polyclonal anti-nLc_4Cer antibody. In lanes a–f, 2 μg of HGG were chromatographed; lane g shows the resorcinol stain of 20 μg HGG. The HPTLC plates were fixed and incubated for 2 h with increasing activities of *V. cholerae* sialidase (100 μU/mL, lane a; 1.6 mU/mL, lane b; *V. cholerae* sialidase buffer as control, lane c) and GST-sialidase (100 μU/mL, lane d; 1.6 mU/mL, lane e; GST-sialidase buffer as control, lane f). The entire TLC kinetics of *V. cholerae* and GST-sialidase are shown in Figs. 7 and 8, respectively.

hinder binding of the polyclonal antibody.[25–27] Therefore, no conclusions concerning α(2-6) desialylation can be drawn from this data. At the low enzyme concentration (100 μU/mL),

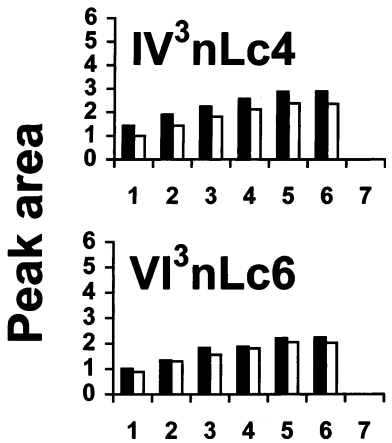

Fig. 7. TLC overlay kinetic: *V. cholerae* sialidase treatment of human granulocyte gangliosides (HGG) and subsequent immunostaining of desialylated IV^3-α-Neu5Ac-nLc$_4$Cer and VI^3-α-Neu5Ac-nLc$_6$Cer with polyclonal anti-nLc$_4$Cer antibody. In lanes 1–7, 2 μg of HGG were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/mL, lane 1; 100 μU/mL, lane 2; 200 μU/mL, lane 3; 400 μU/mL, lane 4; 800 μU/mL, lane 5; 1.6 mU/mL, lane 6; *V. cholerae* sialidase buffer as control, lane 7. Immunostained bands were quantified by quintuple TLC scanning and corresponding peak area columns are shown.

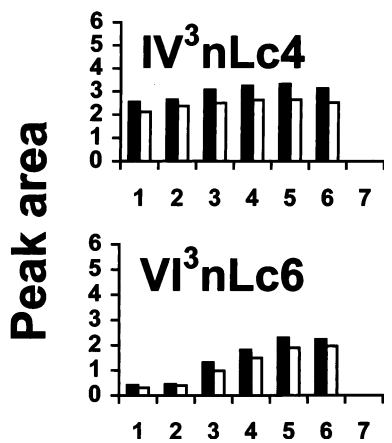

Fig. 8. TLC overlay kinetic: GST-sialidase treatment of human granulocyte gangliosides (HGG) and subsequent immunostaining of desialylated IV^3-α-Neu5Ac-nLc$_4$Cer and VI^3-α-Neu5Ac-nLc$_6$Cer with polyclonal anti-nLc$_4$Cer antibody. In lanes 1–7, 2 μg of HGG were chromatographed. The HPTLC plates were fixed and incubated for 2 h with increasing activities of sialidase: 50 μU/mL, lane 1; 100 μU/mL, lane 2; 200 μU/mL, lane 3; 400 μU/mL, lane 4; 800 μU/mL, lane 5; 1.6 mU/mL, lane 6; sialidase buffer as control, lane 7. Immunostained bands were quantified by quintuple TLC scanning and corresponding peak area columns are shown.

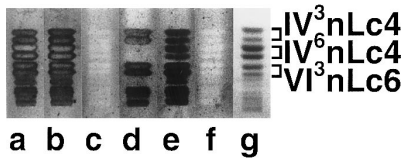

Fig. 9. Sialidase treatment of human granulocyte gangliosides (HGG) and subsequent immunostaining with monoclonal 1B2–1B7 anti-Galβ(1-4)GlcNAc-R antibody. In lanes a–f, 5 μg of HGG were chromatographed; lane g shows the resorcinol stain of 20 μg HGG. The HPTLC plates were fixed and incubated for 2 h with increasing activities of *V. cholerae* sialidase (800 μU/mL, lane a; 3.2 mU/mL, lane b; *V. cholerae* sialidase buffer as control, lane c) and GST-sialidase (800 μU/mL, lane d; 3.2 mU/mL, lane e; GST-sialidase buffer as control, lane f).

both the GST-sialidase (Fig. 6, lane d) and the *V. cholerae* sialidase (Fig. 6, lane a) exhibited IV^3-α-Neu5Ac-nLc$_4$Cer and VI^3-α-Neu5Ac-nLc$_6$Cer hydrolyzing activity. Using 1.6 mU/mL, both enzymes were undistinguishable in the TLC overlay assay and showed identical immunostained asialoganglioside bands (Fig. 6, lanes b and e). The complete TLC kinetics of IV^3-α-Neu5Ac-nLc$_4$Cer and VI^3-α-Neu5Ac-nLc$_6$Cer desialylation by *V. cholerae* and CHO cell sialidase are shown in Figs. 7 and 8, respectively. The direct comparison of both kinetics demonstrates almost identical substrate specificities of both sialidases. Because the polyclonal antibody binds to nLc$_4$Cer as well as to IV^6-α-Neu5Ac-nLc$_4$Cer, parallel TLC kinetics were performed using the monoclonal antibody 1B2-1B7 which specifically binds to Galβ(1-4)GlcNAc-R without any cross reactivity towards sialylated neolacto-series gangliosides. In these assays, both enzymes were found to hydrolyze IV^6-α-Neu5Ac-nLc$_4$Cer in considerable quantities. After incubation of 5 μg of HGG with 800 μU/mL and 3.2 mU/mL *V. cholerae* sialidase, respective asialogangliosides of α(2-3)- and α(2-6)-sialylated neolacto-series gangliosides were detectable with the monoclonal antibody 1B2–1B7 (Fig. 9, lanes a and b) and no cross reactivity towards any gangliosides was observed in the controls without enzyme (Fig. 9, lanes c and f). The preferential desialylation of α(2-3)-sialylated over α(2-6)-sialylated neolacto-series gangliosides by the GST-sialidase is obvious from Fig. 9 (lanes d and e), where no hydrolysis of α(2-6)-sialylated gangliosides was observed in case of 800

μU/mL (Fig. 9, lane d) and moderate desialylation occurred after increasing the enzyme activity to 3.2 mU/mL (Fig. 9, lane e). Unfortunately, no comparative quantification of the bands could be performed by TLC scanning because the monoclonal antibody gave heterogeneous immunostained bands with uncolored spots in the inner core of GSL bands (Fig. 9, lanes a and b).

3. Discussion

Membrane-associated sialidases (plasma membrane sialidase I and lysosomal membrane sialidase II) require detergents for solubilization and hydrolyse gangliosides preferentially. Membrane sialidase I hydrolyses gangliosides only,[8,9] whereas membrane sialidase II also acts on oligosaccharides and glycoproteins.[4] Concerning their biological function, membranous cell surface ganglioside sialidase has been suggested to be involved in growth control of human fibroblasts.[28] In murine neuroblastoma and cerebellar granule cells, ganglioside sialidase activity was found to increase with cell density and considered to be associated with cell differentiation.[29–31] Human neuroblastoma cells exhibited two ganglioside-degrading sialidase activities: one that was associated with the plasma membrane and whose specific activity increased drastically during cell proliferation, and a lysosomal activity that showed no such activity increase during cell growth.[9,32] Further evidence for this hypothesis was provided, indicating that ganglioside sialidase on the cell surface is not only responsible for growth control but also involved in differentiation of neuronal cells.[33] Moreover, surface sialidase (redistributed after activation from secondary granule-enriched fractions to the plasma membrane) was shown to be associated with neutrophile activation and adhesion towards nylon and plastic surfaces.[34] Compared to this functional evidence of surface ganglioside-hydrolyzing sialidase, the physiological function of sialidase in the cytosol is entirely unknown. Sialoglycoproteins and gangliosides, suitable substrates for cytosolic sialidase, are not expected to be present in the cytosol. However, throughout the last years evidence has accumulated that GSLs are not only located in the outer leaflet of the plasma membrane, but are also found associated with intracellular organelles.[35] The formation of cytosolic ganglioside–protein complexes has been determined in fibroblasts[36] and in skeletal and cardiac muscle.[37] Moreover, the association of GSLs with microtubules and intermediate filaments of a variety of cell types has been well documented.[38–40] In this study we could show that the cytosolic sialidase, beside its hydrolyzing activity towards MUF-Neu5Ac and sialoglycoproteins,[41,42] is capable to desialylate a wide spectrum of different types of gangliosides in a concentration dependent manner by use of a TLC overlay kinetic assay. Similar to *V. cholerae* sialidase, GST-sialidase does not require any detergents or solubilizing agents for the degradation of gangliosides to asialogangliosides on the TLC plate, reflecting to the non-membranous location of the water-soluble enzyme in the cytosol. The enzyme exhibited almost identical hydrolysis activity in degradation of G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc) compared with reference *V. cholerae* sialidase. CHO cells exhibit a simple double band ganglioside pattern composed of about 99% G_{M3} which is characteristic for many epithelial cells.[43] G_{M3}(Neu5Ac) represents the major and G_{M3}(Neu5Gc) a minor constituent of the ganglioside fraction of CHO cells.[44] Thus, G_{M3} should be the major ganglioside target for CHO cell sialidase which has been shown in this study to hydrolyze this ganglioside with same efficiency as *V. cholerae* sialidase. Considering more complex gangliosides, CHO cell sialidase exhibited slightly enhanced activity towards terminally α(2-3)-sialylated neolacto-series gangliosides IV^3-α-Neu5Ac-nLc_4Cer and VI^3-α-Neu5Ac-nLc_6Cer compared to reference *V. cholerae* sialidase. The ganglio-series gangliosides G_{D1a}, G_{D1b}, and G_{T1b}, predominantly expressed in neuronal cells and the preferential substrates of *V. cholerae* sialidase, were less suitable targets for the CHO cell sialidase. Taken together, the combination of TLC separation, conventional chemical staining of GSL bands, and in situ sialidase treatment of gangliosides followed by overlay binding with specific anti-asialogan-

glioside antibodies can rapidly generate detailed information on enzyme activity and specificity without large investments in instrumentation. The TLC overlay kinetic assay is easy to perform and provides particular enzyme hydrolysis data requiring only microgram quantities of ganglioside mixtures as substrates.

An increasing body of biochemical and histochemical data on the colocalization of gangliosides and sialidase in the cytosol, and the data presented in this study strongly suggest the involvement of the cytosolic sialidase in ganglioside metabolism on intracellular level. The concept that membrane gangliosides may regulate cell growth and induce differentiation has developed during the past two decades (for reviews see Refs. 45–48). However, a body of evidence suggests that GSLs might act not only as cell surface membrane embedded compounds but also, at least in parts, in a 'protein-associated' configuration of yet unknown nature on subcellular level by yet unknown mechanisms.

4. Experimental

Reference gangliosides.—G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), both substituted with C24- and C16-fatty acids (double bands in TLC runs),[49] were isolated from CHO and murine hybridoma cells, respectively, as previously described.[44] A preparation of human brain gangliosides (HBG), composed of G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, was purchased from Supelco Inc. (Bellefonte, PA, USA).

Human granulocyte gangliosides (HGG) were isolated and purified by Iatrobeads 6RS-8060 chromatography (Macherey–Nagel, Düren, Germany) as previously described.[50,51] A G_{M3}-depleted ganglioside fraction, comprising as major compounds IV^3-α-Neu5Ac-nLc$_4$Cer, IV^6-α-Neu5Ac-nLc$_4$Cer, and VI^3-α-Neu5Ac-nLc$_6$Cer (all substituted with C24- and C16-fatty acids, resulting in double bands in TLC runs), was obtained in the (1:2, v/v) $CHCl_3$–MeOH eluate.[25]

High-performance thin-layer chromatography.—Gangliosides were separated on Silica Gel F 60 precoated glass-backed high-performance thin-layer chromatography plates (HPTLC plates, size 10 × 10 cm, thickness 0.2 mm, Art. no. 5633, E. Merck, Darmstadt, Germany) in (120:85:20, each by vol.) $CHCl_3$–MeOH–water, supplemented with 2 mM $CaCl_2$. Gangliosides were visualized with resorcinol.[52]

Monoclonal and polyclonal antibodies; cholera toxin B subunit.—The polyclonal rabbit anti-Gg$_4$Cer antibody has been originally described by Müthing and Mühlradt.[53] The polyclonal chicken anti-LacCer and anti-nLc$_4$Cer antibodies have been characterized in earlier publications.[25,54,55] The anti-Galβ(1-4)GlcNAc-R monoclonal mouse IgM antibody producing hybridoma 1B2–1B7[56] was from the American Type Culture Collection (ATCC, Rockville, MD, USA; TIB-189). Cholera toxin B subunit (= choleragenoid) specific for ganglioside G_{M1} was from Sigma (Deisenhofen, Germany, no. C-7771) and goat anti-choleragenoid antiserum from Calbiochem (Frankfurt a.M., Germany, no. 227040).

Sialidase treatment and TLC immunostaining (TLC overlay kinetic assay).—Two reviews, concerning the details of the TLC immunostaining procedure, have been published.[22,23] Briefly, gangliosides were chromatographed and the silica gel was fixed with 0.5% polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany) in hexane. The plates were then washed with *V. cholerae* sialidase buffer (0.05 M AcONa, 9 mM $CaCl_2$, pH 5.5) or GST-sialidase buffer (0.1 M AcONa, pH 5.5). TLC overlay kinetics were performed at rt with increasing activities of reference *V. cholerae* sialidase (Behring–Werke GmbH, Marburg, Germany) or GST-sialidase: 50, 100, 200, 400, 800 μU/mL, and 1.6 mU/mL. After 2 h incubation, the reaction was stopped by washing with phosphate buffered saline (PBS), and unspecific protein binding was blocked by 15 min incubation of the plate with solution A (1% bovine serum albumin (BSA) in PBS). Desialylated G_{M3} and neolacto-type gangliosides were detected with polyclonal chicken anti-LacCer and anti-nLc$_4$Cer antibodies, respectively, both diluted 1:1000 in solution A. Alternatively, the latter gangliosides were detected after sialidase treat-

ment with 1B2–B7 monoclonal mouse IgM antibody, 1:20 in solution A diluted hybridoma supernatant.

The degradation of G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} to Gg_4Cer by the GST-sialidase was tested with and without sodium taurodeoxycholate supplemented buffer. For that purpose 5 μg HBG aliquots were separated on TLC plates. After silica gel fixation, the plates were incubated with 5 mU/mL enzyme for 3 h at rt without and in the presence of 0.5 mg/mL sodium taurodeoxycholate. The reaction was stopped and the overlay assay was performed with 1:1000 diluted anti-Gg_4Cer antibody as described above.

The immunodetection of G_{M1} (hydrolysis product of G_{D1a}, G_{D1b}, and G_{T1b}) was performed with choleragenoid (250 ng/mL in solution A) and goat anti-choleragenoid antibody (1:4000 dilution in solution A). Choleragenoid and the antibodies were incubated for 1 h, followed by threefold washing with solution B (0.05% Tween 10 in PBS). Secondary rabbit anti-chicken IgG, goat anti-mouse IgG and IgM, goat anti-rabbit IgG, and rabbit anti-goat antisera, all affinity chromatography-purified and labeled with alkaline phosphatase, were from Dianova (Hamburg, Germany) and used as 1:2000 dilutions in solution A. After 1 h incubation with secondary antibodies, the plates were washed three times with solution B, followed by twofold rinsing with glycine buffer (0.1 M glycine, 1 mM $ZnCl_2$, 1 mM $MgCl_2$, pH 10.4), to remove phosphate. Bound antibodies were visualized with 0.05% (w/v) 5-bromo-4-chloro-3-indolylphosphate (Biomol, Hamburg, Germany) in glycine buffer. Immunostained chromatograms were quantified with a CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM compatible personal computer and densitometric software. Bands were measured in quintuplicate in reflectance mode at 630 nm with a light beam slit of 0.1×2 mm and their intensities were calculated as integrals of peak areas.

5. Abbreviations

Enzymes: sialidase or neuraminidase or *N*-acylneuraminosyl glycohydrolase (EC 3.2.1.18). BSA, bovine serum albumin; CHO, Chinese hamster ovary; GSL(s), glycosphingolipid(s); GST, glutathione S-transferase; HBG, human brain gangliosides; HGG, human granulocyte gangliosides; HPTLC, high-performance thin-layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PBS, phosphate buffered saline. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations[57] and the nomenclature of Svennerholm.[58] Lactosylceramide or LacCer, Galβ(1-4)Glcβ1Cer; gangliotriaosylceramide or Gg3Cer, GalNAcβ(1-4)Galβ-(1-4)Glcβ(1-1)Cer; gangliotetraosylceramide or Gg_4Cer, Galβ(1-3)GalNAcβ(1-4)Galβ-(1-4)Glcβ(1-1)Cer; neolactotetraosylceramide or nLc_4Cer, Galβ(1-4)GlcNAcβ(1-3)Galβ-(1-4)Glcβ(1-1)Cer; neolactohexaosylceramide or nLc_6Cer, Galβ(1-4)GlcNAcβ(1-3)Galβ-(1-4)GlcNAcβ(1 - 3)Galβ(1 - 4)Glcβ(1 - 1)Cer; G_{M3}, II^3-α-Neu5Ac-LacCer; G_{M1}, II^3-α-Neu5Ac-Gg_4Cer; G_{D1a}, IV^3-α-Neu5Ac, II^3-α-Neu5Ac-Gg_4Cer; G_{D1b}, II^3-α-$(Neu5Ac)_2$-Gg_4Cer; G_{T1b}, IV^3-α-Neu5Ac, II^3-α-$(Neu5Ac)_2$-Gg_4Cer; G_{Q1b}, IV^3-α-$(Neu5Ac)_2$, II^3-α-$(Neu5Ac)_2$-Gg_4Cer; IV^3-α-Neu5Ac-nLc_4Cer or IV^3nLc4; IV^6-α-Neu5Ac-nLc_4Cer or IV^6nLc4; VI^3-α-Neu5Ac-nLc_6Cer or VI^3nLc6. Only Neu5Ac-substituted gangliosides are presented in this list of abbreviations.

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