Conversion of cellular sialic acid expression from N-acetyl- to N-glycolyneuraminic acid using a synthetic precursor, N-glycolymannosamine pentaacetate: inhibition of myelin-associated glycoprotein binding to neural cells

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Sialic acids are prominent termini of mammalian glycoconjugates and are key binding determinants for cell–cell recognition lectins. Binding of the sialic acid–dependent lectin, myelin-associated glycoprotein (MAG), to nerve cells is implicated in the inhibition of nerve regeneration after injury. Therefore, blocking MAG binding to nerve cell sialoglycoconjugates might enhance nerve regeneration. Previously, we reported that certain sialoglycoconjugates bearing N-acetylneuraminic acid (NeuAc) but not N-glycolyneuraminic acid (NeuGc) support MAG binding (Collins et al., 1997a). We now report highly efficient conversion of sialic acids on living neural cells from exclusively NeuAc to predominantly NeuGc using a novel synthetic metabolite precursor, N-glycolymannosamine pentaacetate (ManNGcPA). When NG108–15 neuroblastoma-glioma hybrid cells, which normally express only NeuAc (and bind to MAG), were cultured in the presence of 1 mM ManNGcPA, they expressed 80–90% of their sialic acid precursor pool as NeuGc within 24 h. Within 5 days, 80% of their ganglioside-associated sialic acids and 70% of their glycoprotein-associated sialic acids were converted to NeuGc. Consistent with this result, treatment of NG108–15 cells with ManNGcPA resulted in nearly complete abrogation of MAG binding. These results demonstrate that ManNGcPA treatment efficiently alters the sialic acid structures on living cells, with a commensurate change in recognition by a physiologically important lectin.

Key words: sialic acid biosynthesis/N-acetylmannosamine/N-acetylnueraminic acid/N-glycolyneuraminic acid/siglec recognition

Introduction

Sialic acids are abundant nonreducing terminal sugars on mammalian glycoconjugates. They differ from other mammalian monosaccharides in their complexity, bearing a carboxylic acid group, an N-acyl substituent, and an exocyclic glycerol side chain (Schauer, 1982; Variki, 1992). Variety in sialic acid linkages as well as N- and O-acyl substituents results in a large number of unique structural determinants. Cell–cell recognition proteins, as well as pathogens and toxins, take advantage of the structural diversity and cell surface disposition of sialic acids for highly specific recognition and binding (Rogers et al., 1986; Weis et al., 1988; Schengrund et al., 1991; Holmgren, 1994; Kelm et al., 1996; Sharon, 1996; McEver, 1997; Variki, 1997; Miller-Podraza et al., 1997).

N-Acetylnueraminic acid (NeuAc), the predominant sialic acid in nature, is synthesized in vivo by a multistep pathway (Roseman, 1970) beginning with the conversion of N-acetylgalactosamine to N-acetylmannosamine-6-phosphate by a bifunctional epimerase/kinase (Kundig et al., 1966; Hinderlich et al., 1997). ManNAc-6-P is converted to NeuAc-9-P by condensation with phosphoenol pyruvate, and then to CMP-NeuAc which is the activated NeuAc donor for glycolipid and glycoprotein oligosaccharide biosynthesis (Kean and Roseman, 1966; Watson et al., 1966). Hydroxylation of NeuAc (in the CMP-NeuAc form) by a specific hydroxylase converts NeuAc to N-glycolyneuraminic acid (NeuGc), a member of the sialic acid family which is rare (or absent) in humans, but is common in non-neural tissues of many other species (Kawano et al., 1995; Chou et al., 1998).

To experimentally introduce modified sialic acids on intact cells and tissues, this pathway has been short-circuited by addition of unnatural N-acylmannosamines, including N-propanoyl-, N-butanoyl-, N-pentanoyl-, and N-levulinoyl-mannosamine, among others (Angelino et al., 1995; Keppler et al., 1995; Yarema et al., 1998). These precursors are taken up, converted to the corresponding sialic acids, and expressed on cell surface glycoconjugates. Cells engineered to display modified sialic acids on intact cells and tissues is this pathway has been short-circuited by addition of unnatural N-acylmannosamines, including N-propanoyl-, N-butanoyl-, N-pentanoyl-, and N-levulinoyl-mannosamine, among others (Angelino et al., 1995; Keppler et al., 1995; Yarema et al., 1998).

We and others have recently reported the carbohydrate binding specificity of a sialic acid binding lectin, myelin-associated glycoprotein (MAG), a member of the “siglec” family of immunoglobulin-like lectins (Kelm et al., 1994a; Collins et al., 1997a,b; Crocker et al., 1998; Kelm et al., 1998). In the nervous system, MAG functions in the stabilization of the myelin sheath surrounding axons (Fruttiger et al., 1995; Bartsch et al.,
Fig. 1. N-Acyl-2-deoxymannosamine precursors and their corresponding sialic acids. ManNAc (top panel) and ManNPr (center panel) are converted intracellularly to NeuAc and NeuPr respectively (Keppler et al., 1995). ManNGc (bottom panel) was synthesized as the monoacetate (ManNGcMA, R1 = H) or pentaacetate (ManNGcPA, R1 = CO(2)CH(3)). Each is presumably deacylated intracellularly by non-specific esterases, forming ManNGc, which is then converted to NeuGc. Cellular sialic acids are found as the free saccharide (R = H), as the activated sugar donor (R = CMP), or in glycoconjugates (R = any of the oligosaccharide structures to which sialic acid is attached).

1997; Lassmann et al., 1997; Sheik et al., 1999), and in the control of axon cytoarchitecture (Yin et al., 1998). In addition, MAG inhibits nerve regeneration, and has been proposed to contribute to the limited functional recovery typical of central nervous system (e.g., spinal cord) injury (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Schnaar et al., 1998). Presumably, MAG binds to sialoglycoconjugate targets on nerve cells to initiate its physiological and pathological effects. MAG is highly restrictive in its requirements for sialic acid substructure, in that it requires the N-acetyl group, the glycerol side chain, and the carboxylic acid group for recognition (Collins et al., 1997a,b; Kelm et al., 1998). Given the sensitivity of MAG binding to even minor changes in sialic acid substructure, the goal of the experiments described here was to develop a highly efficient N-acetylmannosamine precursor which would result in modification of a large proportion of the nerve cell sialic acid, rendering the cells resistant to MAG binding.

Results

NeuGc biosynthesis from ManNGc monoacetate

The goal of these studies was to convert sialic acids on live neuronal cells from NeuAc, which is a key determinant for MAG binding, to NeuGc, which is nonpermissive for MAG binding (Collins et al., 1997a). A potential NeuGc precursor, N-glycolytmannosamine monoacetate (ManNGcMA, Figure 1, R1 = H) was tested for its ability to generate altered sialic acids in neuronal and non-neuronal cells. A previously reported synthetic sialic acid precursor, N-propanoylmannosamine (ManNPr), was used as a positive control (Kepler et al., 1995). NG108–15 cells (rodent neuroblastoma/glioma hybrid cells (Hamprecht, 1977)) were incubated in 20 mM synthetic precursor or control precursor (N-acetylmannosamine, ManNAc) for 48 h, and then harvested, fractionated and sialic acids quantified as described in Materials and methods. Sialic acid precursor pools were quite elastic, in that treatment with 20 mM ManNAc increased intracellular sialic acid as much as 14-fold (Figure 2, top panel). Similarly, treatment with 20 mM ManNPr resulted in a 4-fold increase in sialic acid, with >95% as N-propanoylneuraminic acid (NeuPr). In contrast, treatment with 20 mM ManNGcMA resulted in a markedly smaller sialic acid precursor pool compared to the untreated control, although most (>70%) was in the NeuGc form. The differential effect on pool size was cell type or species specific, in that treatment of a human T-cell-related cell line (Jurkat) with 20 mM ManNGcMA resulted in an increase in total sialic acid precursor pool size (comparable to treatment with ManNPr) which was nearly all NeuGc (data not shown).

Although ManNPr and ManNGcMA had very different effects on the sialic acid precursor pool size in NG108–15 cells, the size of the ganglioside pool remained relatively constant (Figure 2, center panel). Treatment with 20 mM ManNPr converted ≈70% of the ganglioside sialic acid to the NeuPr form within 48 h, whereas treatment with 20 mM ManNGcMA resulted in less (≈30%) conversion to the NeuGc form.

The glycoprotein sialic acid concentration was more sensitive to the precursor pool size, with total glycoprotein sialic acid increasing ≈2-fold in cells treated with ManNAc, increasing ≈1.4-fold in cells treated with ManNPr, and decreasing by ≈40% in cells treated with ManNGcMA (Figure 2, bottom panel). As with the gangliosides, treatment with 20 mM ManNPr converted ≈70% of the glycoprotein sialic acid to the NeuPr form within 48 h, whereas treatment with 20 mM ManNGcMA resulted in ≈30% conversion of glycoprotein sialic acids to the NeuGc form.

Although ManNPr was efficiently incorporated into gangliosides and glycoproteins as NeuPr, it was not used to modify MAG binding, since NeuPr is reported to support MAG (Kelm et al., 1998), whereas NeuGc does not support MAG binding (Collins et al., 1997a; Kelm et al., 1998). Therefore, improved methods to deliver ManNGc into cells were tested.

NeuGc biosynthesis from ManNGc pentaacetate

Peracetylation has been shown to greatly increase uptake and anabolic utilization of carbohydrates (Sarkar et al., 1995, 1997). Therefore, ManNGcMA was peracetylated to give N-glycolytmannosamine pentaacetate (ManNGcPA, Figure 1, R1 = Ac) and tested as a precursor for NeuGc biosynthesis. NG108–15 cells were incubated with 0.1 or 1.0 mM ManNGcPA (or 5 mM ManNGcMA for comparison) for 48 h. Peracetylated sugars were routinely dissolved in dimethylsulfoxide (DMSO), and delivered to the cells in medium containing a final concentration of 0.5% DMSO, which was non-toxic to cells (see below). Whereas none of these treatments significantly altered the sialic acid precursor pool size, treatment with 1 mM ManNGcPA resulted in 82% conversion from NeuAc to NeuGc (Figure 3, top panel). Treatment with 0.1 mM ManNGcPA resulted in ≈2-fold greater conversion to the
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NeuGc form than did treatment with 5 mM ManNGcMA, indicating an increase in potency of 100-fold due to peracetylation. Incorporation of NeuGc into gangliosides and glycoproteins was related to the precursor pool composition, resulting in ≈50% NeuGc within 48 h when cells were treated with 1 mM ManNGcPA (Figure 3, center and bottom panels) and much less when cells were treated with 5 mM ManNGcMA.
ganglioside and glycoprotein sialic acid levels were not significantly altered by treatment with the peracetylated precursor.

If incorporation of NeuGc into gangliosides and glycoproteins occurs during glycoconjugate turnover or via desialylation/resialylation, higher levels of conversion (above 50%) might require longer incubation times. To test this, NG108–15 cells were treated with 1 mM ManNGcPA for up to 144 h. The sialic acid precursor pool was >80% in the NeuGc form within 24 h, and remained high throughout the experiment (Figure 4, top panel). Consistent with prior experiments, half of the ganglioside and glycoprotein sialic acids were converted to the NeuGc form within 48 h. Longer incubation resulted in increased incorporation of NeuGc, with gangliosides expressing ≈80% of their sialic acid as NeuGc and glycoproteins expressing 60–70% as NeuGc as the experiment progressed (Figure 4, center and bottom panels).

To ensure that the per-O-acetylated derivative ManNGcPA did not result in significant production of O-acetylated forms of NeuGc, an aliquot of the extracted polar phase containing free sialic acids (see Materials and methods) from cells treated for 48 h with 1 mM ManNGcPA was subjected to thin layer chromatography in propanol-water (7:3) to resolve O-acetylated from non-O-acetylated sialic acids (Schauer, 1987). O-Acetylated sialic acids from bovine submaxillary mucin served as standards (Varki and Diaz, 1984). The results in Figure 5 reveal that there is no significant proportion of O-acetylated NeuGc in the precursor pool, confirming apparently complete de-O-acetylation of ManNGcPA prior to or coordinate with its metabolism to sialic acid.

Effects of ManNGcPA-treatment on cell viability and growth

Treatment of NG108–15 cells with 1 mM ManNGcPA sharply slowed growth and led to a modest decrease in cell viability after 70 h (from 91% (control) to 76% (1 mM ManNGcPA),
to NeuGc results in a loss of MAG binding, cells were treated whether conversion of NG108–15 cell sialic acids from NeuAc to NeuGc form (data not shown). In nearly 50% conversion of ganglioside sialic acids to the NeuGc form (data not shown). Incorporation of NeuGc into glycoconjugates of NG108–15 cells with a sub-cytostatic concentration ManNGcPA (0.25 mM). Incorporation of NeuGc into gangliosides (solid bars) and glycoproteins (gray bars) after growth in the presence of 0.25 mM ManNGcPA for the indicated times was determined as described in Materials and methods. At 96 h one flask of rapidly growing cells was split and equal portions replated in the same medium or in the absence of the precursor (for an additional 24 h) as indicated to determine the rate of reversibility of incorporation. Over the course of the experiment, total ganglioside sialic acid averaged 3.2 ± 0.7 nmol/mg total cell protein (mean ± SD) and protein sialic acid averaged 3.8 ± 1.2 nmol/mg protein.

Discussion

As the major nonreducing terminal saccharide on mammalian glycoconjugates, sialic acids are key determinants for binding many lectins, toxins, and pathogens (Varki, 1997). Sialic acids each have a carboxylic acid group, N-acyl group, and an exocyclic glycerol chain, making them the most complex of mammalian monosaccharides. Variations in the N-acyl group and in O-acetylation provide a high degree of sialic acid structural diversity (Schauer, 1982; Varki, 1992). Although some sialic acid binding proteins are relatively indifferent to sialic acid fine structure (e.g., the selectins; Tyrrell et al., 1991; Brandley et al., 1993; Norgard et al., 1993), others bind with great specificity. Among the most highly specific sialic acid binding lectins are those of the siglec family, which are sensitive to modification of the carboxylic acid, the glycerol chain and/or the N-acyl moiety (Kelm et al., 1994b, 1998; Sjoberg et al.,

see Figure 6). To address the possible basis for the toxic effects of the precursor, growth and cell viability were compared in cultures treated with glucose pentacetate as a control. The glucose pentacetate-treated cells demonstrated more profound decreases in cell growth and viability than the ManNGcPA-treated cells, indicating that uptake and deacetylation of peracetylated precursors, even glucose, is toxic to these cells at 1 mM concentrations. In contrast, treatment with 0.25 mM ManNGcPA resulted in no significant decrease in either cell growth or viability. Subsequent kinetic studies revealed that 0.25 mM ManNGcPA treatment resulted in >70% conversion of NeuAc to NeuGc in NG108–15 cell gangliosides after 48 h, and a similar conversion in glycoproteins at 96 h (Figure 7). Withdrawal of the precursor results in partial reversal of the conversion after 24 h of chase. Growth of the cells in as low as 0.02 mM ManNGcPA for 48 h resulted in nearly 50% conversion of ganglioside sialic acids to the NeuGc form (data not shown).

Effect of ManNGcPA-treatment on myelin-associated glycoprotein binding

NG108–15 cells, a mouse neuroblastoma/rat glioma hybrid, display many qualities of cholinergic neurons, including neurite outgrowth, synthesis, storage and release of acetylcholine, and functional synapse formation with muscle cells in vitro (Hamprecht, 1977; Nelson et al., 1978). Neurite outgrowth from NG108–15 cells is inhibited by MAG (McKerracher et al., 1994), an interaction which is reversed by pretreatment of the cells with neuraminidase (Schnaar et al., 1998). To test whether conversion of NG108–15 cell sialic acids from NeuAc to NeuGc results in a loss of MAG binding, cells were treated with 1 mM ManNGcPA for 144 h (at which time 80% of the glycoconjugate sialic acid was in the NeuGc form), collected and incubated with a soluble chimeric protein consisting of the extracellular portion of MAG fused with human Fc as an immunochemical tag. Binding was quantified by flow cytometry. Compared to control cells, specific binding of MAG-Fc to cells treated with 1 mM ManNGcPA was inhibited 95% (Figure 8). Treatment with either 0.25 mM or 1 mM ManNGcPA for 96 h (at which time >70% of the glycoconjugate sialic acid was in the NeuGc form, see Figures 4, 7) resulted in >70% inhibition of MAG-Fc binding, whereas treatment with 1 mM ManNAc-tetraacetate had no significant effect on binding (Figure 9).

Discussion

As the major nonreducing terminal saccharide on mammalian glycoconjugates, sialic acids are key determinants for binding many lectins, toxins, and pathogens (Varki, 1997). Sialic acids each have a carboxylic acid group, N-acyl group, and an exocyclic glycerol chain, making them the most complex of mammalian monosaccharides. Variations in the N-acyl group and in O-acetylation provide a high degree of sialic acid structural diversity (Schauer, 1982; Varki, 1992). Although some sialic acid binding proteins are relatively indifferent to sialic acid fine structure (e.g., the selectins; Tyrrell et al., 1991; Brandley et al., 1993; Norgard et al., 1993), others bind with great specificity. Among the most highly specific sialic acid binding lectins are those of the siglec family, which are sensitive to modification of the carboxylic acid, the glycerol chain and/or the N-acyl moiety (Kelm et al., 1994b, 1998; Sjoberg et al.,
Most relevant to the current study is binding selectivity based on the N-acyl moiety. MAG and sialoadhesin bind NeuAc but not NeuGc on sialoglycoconjugate targets (Kelm et al., 1994b, 1998; Collins et al., 1997a). In contrast, murine CD22 requires NeuGc, whereas human CD22 binds either NeuAc or NeuGc (Kelm et al., 1994b). The crystal structure of the N-terminal Ig-like domain of sialoadhesin complexed with 3′-sialyllactose demonstrates that the acetamido methyl group is in van der Waals contact with the indole ring of a tryptophan (Trp-2), the mutation of which abrogates binding (May et al., 1998). However, since the same Trp is found in murine and human CD22 as well as in MAG, the molecular basis for their varying NeuAc/NeuGc selectivity awaits resolution of other siglec crystal structures to allow direct comparisons.

The high selectivity of MAG for NeuAc rather than NeuGc offers an opportunity to subtly modify nerve cell sialic acids and thereby block MAG binding. Since MAG is implicated in maintaining stable myelin–axon interactions (Fruttiger et al., 1995; Bartsch et al., 1997; Lassmann et al., 1997; Sheikh et al., 1999), directing nerve regeneration after injury (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Schäfer et al., 1996; Schnaar et al., 1998), such modification has implications both for studying and possibly modulating important neural cell–cell interactions.

Modification of sialic acids in live cells or animals using synthetic N-acylmannosamines was pioneered by the Reutter laboratory (Kayser et al., 1992a). The incorporation of synthetic N-acylmannosamines into sialic acid precursor pool, glycolipids (gangliosides) and glycoproteins in the same cell population. The observation that the sialic acid precursor pool size is highly elastic is consistent with a prior report on the effect of ManNAc treatment on sialic acid synthesis (Thomas et al., 1985). Although our analytical conditions did not routinely distinguish between the free and nucleotide sugar forms of sialic acid, it has been reported that the precursor pool is predominantly free sialic acid (Thomas et al., 1985), a contention supported by thin layer chromatography analysis in this study (Figure 5). Large changes in the precursor pool size did not quantitatively impact sialic acid incorporation into gangliosides, and only modestly affected incorporation into glycoproteins. However, conversion of the precursor pool from exclusively NeuAc to largely NeuPr or NeuGc resulted in incorporation of the modified sialic acids into both gangliosides and sialoglycoproteins. The delay of incorporation of modified sialic acids into glycoconjugates compared to their appearance in the precursor pool (e.g.,

Fig. 8. Flow cytometric analysis of MAG-Fc binding to NG108–15 cells following treatment with 1 mM ManNGc. NG108–15 cells were treated for 144 h with 1 mM ManNGcPA or ManNAc (control). All culture media contained 0.5% (v/v) DMSO. Cells were collected, incubated with MAG-Fc chimera, stained with fluorescent anti-Fc secondary antibody, and subjected to flow cytometry on an Epics Profile II cytometer as described in Materials and methods. Black bars, MAG-Fc binding; gray bars, secondary antibody only (control).

Fig. 9. Quantifying MAG-Fc binding to NG108–15 cells treated with different sialic acid precursors. NG108–15 cells were cultured for 96 h in medium containing 0.5% DMSO (control) or in the same medium containing the indicated concentrations of ManNGcPA or the peracetylated NeuAc precursor, ManNAc tetra-O-acetate (ManNAcTA). Binding of MAG-Fc chimera was determined by flow cytometry as described in Materials and methods. The mean fluorescence intensity of each cell population was normalized to that of the control cells.
Figure 4) may reflect the rate of turnover of gangliosides and sialoglycoproteins in these cells.

Our finding of equivalent incorporation of precursor NeuPr or NeuGc into both gangliosides and sialoglycoproteins in living cells suggests that various sialyltransferases readily use the modified sialic acid precursors. These results are consistent with previous reports demonstrating insensitivity of sialyltransferases to chemically modified CMP-sialic acid derivatives in vitro (Higa and Paulson, 1985; Kelm et al., 1998), but do not rule out the possibility that some of the >15 known sialyltransferases (Tsuji, 1996) may distinguish between natural and synthetic precursors, resulting in differential incorporation into different specific sialic acid glycoforms. Our data are consistent with prior reports of modified sialic acid incorporation into glycolipids (Kayer et al., 1992b) and glycoproteins (Keppler et al., 1995), but conflict with a recent report indicating no incorporation of NeuPr into gangliosides in ManNPr-treated neural cells (Schmidt et al., 1998). This issue is worthy of further study to determine the basis for differences in precursor utilization.

As with other N-acylmannosamine precursors, treatment with ManNGc (as the monoacetate or pentaacetate) led NG108–15 cells to synthesize and incorporate NeuGc, a major sialic acid found in non-neural tissues of many nonhuman species. Our testing of peracetylation to enhance uptake and anabolic utilization of carbohydrates was based on the work of Sarkar et al. (Sarkar et al., 1995, 1997). As in their system, peracetylation greatly increased utilization, and this simple modification is likely to be useful for other synthetic sialic acid precursors. It is assumed that the peracetylated species enter the cells more rapidly, where they are fully de-O-acetylated (perhaps by nonspecific esterases) prior to or concurrent with their conversion to the corresponding sialic acids, as shown in Figure 5.

The demonstration that ManNGcPA is an effective NeuGc precursor, and that conversion of neural NeuAc to NeuGc abrogates MAG binding (Figures 8, 9), opens the way for future animal testing. A prior study showed little incorporation of intraperitoneally injected ManNPr into sialic acids in the brains of rats, even though it was incorporated into liver and serum glycoproteins (Kayer et al., 1992a). It is hoped that peracetylation, or modification of the route of delivery, will result in higher nervous system conversion from NeuAc to NeuGc using ManNGcPA.

Animals which express NeuGc as a major sialic acid outside of the nervous system express only minor amounts within the nervous system (Chow et al., 1998; Mikami et al., 1998). Although the mechanism of NeuGc exclusion in the brain has yet to be determined, we demonstrate that cultured rodent neural cells readily convert ManNGcPA to NeuGc, which is effectively incorporated into both gangliosides and sialoglycoproteins.

Materials and methods

Mannosamine precursors and sialic acid derivatives

D-Mannosamine•HCl, ManNAc, NeuAc, and NeuGc were purchased from Sigma Chemical Co. (St. Louis). N-Glycolylmannosamine monoacetate (ManNGcMA, Figure 1) was synthesized using the method of Kuboki et al. (Kuboki et al., 1997). Briefly, D-mannosamine•HCl (1 g) and sodium bicarbonate (7.5 g) were added to 40 ml water. After chilling the mixture on ice, 2.6 ml of acetoxyacetyl chloride (Aldrich Chemical Co., Milwaukee, WI) were added dropwise and the reaction stirred for 3 h on ice. Formation of the desired product was confirmed by silica gel thin layer chromatography, using ethyl acetate-acetic acid-water (3:2:1) as developing solvent, ninhydrin to detect unreacted starting material, and orcinol-sulfuric acid reagent (Schnaar and Needham, 1994) to detect sugars (product Rf = 0.63). After filtering through Celite, the filtrate was neutralized with 2 M HCl and concentrated. The product was purified by silica gel column chromatography using ethyl acetate-isopropanol-water (27:8:4) as the eluant. The product, whose identity was confirmed by 1H NMR (Kuboki et al., 1997), was dissolved in water and stored at −20°C. Product concentration was determined by quantitative high performance liquid chromatography on a Dionex Carbotop column with pulsed amperometric detection (Hardy et al., 1988) using ManNac as a quantitative standard.

N-Glycolylmannosamine pentaacetate (ManNGcPA) was prepared by treating ManNGcMA (0.3 g) with acetyl chloride (2 ml) in pyridine (2 ml) for 1 h at ambient temperature. The reaction was monitored by silica gel thin layer chromatography, using toluene-ethanol (10:1) as the developing solvent and orcinol-sulfuric-acid reagent to visualize sugars (product Rf = 0.32). The reaction mixture was concentrated and the product purified by silica gel column chromatography using step-wise elution with toluene, toluene-ethanol (50:1), and toluene-ethanol (20:1) as the eluants. Fractions containing the desired product were evaporated, resuspended in DMSO and the concentration determined by quantitative high pressure liquid chromatography. The structure of the peracetylated compound was confirmed by 1H NMR, which revealed full O-acetylation and an α:β ratio of 2:1.

N-Propanoylmannosamine (ManNPr) was synthesized according to the method of Keppler et al. (Keppler et al., 1995). D-Mannosamine•HCl (3 mmol), sodium methoxide (3.3 mmol), and propionic anhydride (3.6 mmol) in 300 ml of methanol were stirred for 2 h at 0°C. The desired product was isolated by silica gel column chromatography using ethyl acetate–methanol–water (5:2:1) as the eluant. Fractions were analyzed by thin layer chromatography using the same solvent for development and orcinol-sulfuric acid reagent for detection (Rf = 0.59). Fractions containing product were combined, evaporated to dryness, the residue dissolved in water, and stored at −20°C. N-Propanoylneuraminic acid (NeuPr) standard was prepared enzymatically (Comb and Rosenman, 1960) by reacting ManNPr with pyruvate using N-acetyleneuraminic acid aldolase (Sigma).

Cell culture and treatment with sialic acid precursors

NG108–15 cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose formulation) containing 5% iron-enriched calf serum, 100 µM hypoxanthine, 16 µM thymidine, and 5 µM aminopterin. Cells were cultured at 37°C in a humidified atmosphere of 90% air/10% CO2. For treatment with sialic acid precursors, mannosamine derivatives were diluted into the appropriate medium and filter sterilized. The growth medium was then replaced and cells were cultured in the presence of the precursor for 24–144 h. In experiments test-
ing ManNGcPA, all cells (control and experimental) were grown in the presence of 0.5% (v/v) DMSO, the carrier for ManNGcPA.

**Sialic acid analyses**

Cells were harvested using hypertonic Ca\(^{2+}\)- and Mg\(^{2+}\)-free phosphate-buffered saline containing 1 mM EDTA (Yang et al., 1996a), collected by centrifugation, and homogenized in ice-cold water using a Potter-Elvehjem glass/Teflon homogenizer. Methanol (2.6 volumes) was added, the suspension was mixed and brought to ambient temperature, and then chloroform (1.3 volumes, based on the original homogenate) was added and the suspension mixed vigorously. The suspension was centrifuged 30 min at 2000 × g. After collecting the supernatant, the pellet (containing precipitated proteins) was suspended in water or concentrated ammonium hydroxide and a portion was used for protein assay (BCA, Pierce, Rockford, IL). The supernatant was partitioned by adjusting the chloroform–methanol–water ratio to 4:8:5 by addition of water, mixing thoroughly, and centrifuging as above. The upper phase was collected and a portion was subjected to reverse phase chromatography, using Sep-Pak C18 cartridges (Waters, Milford, MA), to isolate gangliosides (Schnaar, 1994).

To quantify and identify sialic acids, a portion of resolubilized protein, organic/aqueous soluble pool, or reverse-phase purified gangliosides was evaporated to dryness in a 500-µl polypropylene tube and 20 µl of 0.1 M HCl/0.25 M NaCl was added. One AmpliWax bead (Perkin Elmer Corp, Foster City, CA) per reaction was added to block evaporation, and the sample hydrolyzed for 3 h at 80°C. Released sialic acids were analyzed by injecting an aliquot (1–10 µl) onto a Dionex high pressure liquid chromatography system (Dionex Corporation, Sunnyvale, CA) using a HPIEC-AS6 column and a pulsed amperometric detector (Manzi et al., 1990). Elution solutions were: A, 0.75 mM NaOH; B, 200 mM NaOH; and C, 400 mM sodium acetate in 50 mM NaOH. NeuAc and NeuPr were resolved by elution with 40% A, 50% B, 10% C for 15 min at 1 ml/min. NeuGc was resolved in separate injections using step gradient elution: 0–1.8 min, 18% A, 50% B, 32% C; 2–10 min, 40% B, 60% C. Sialic acids were identified by comparison of their elution time with those of standard NeuAc, NeuPr and NeuGc and were quantified in comparison to a standard curve of commercial NeuAc (for NeuAc and NeuPr) and NeuGc.

To confirm that the quantified peaks represented sialic acids, 10 µl of acid hydrolysates were treated with 1 µl of 1 M sodium phosphate (pH 7.2) and 10 µl of 0.1 M NaOH. Neutralized samples were incubated at 37°C for 2 h with or without 0.9 U of N-acetylneuraminic acid aldolase (Sigma), 32 µM NADH and 0.1 µg lactate dehydrogenase. Enzyme-dependent disappearance of the presumed sialic acid peak (Comb and Roseman, 1960; Manzi et al., 1990) was taken as evidence for its identity (data not shown). Sialic acid content in the ganglioside and glycoprotein fractions is expressed per milligram of total cell protein. Precursor pool sialic acid (sialic acid plus CMP-sialic acid) was calculated by subtracting the ganglioside value from the organic/aqueous pool value, both expressed per milligram of cell protein.

**MAG binding**

Flow cytometry was used to test binding of a soluble MAG-Fc chimera to precursor-treated and control NG108–15 cells. A chimeric protein consisting of the entire extracellular domain of MAG fused via a three amino acid bridge (TGK) to the human Fc domain was produced using the "pIgPlus" vector (Novagen, Madison, WI). A PCR fragment coding for the extracellular domain of MAG was prepared using MAG in pCDM8 as template (Yang et al., 1996a), the T7 5’ primer (TAATACGACTCCTATAGGG) and GATCGGATCCCTTA-CCTGTGTGGCCACATCGTCGTTGTC as the 3’ primer. The fragment was cut with BamHI and HindIII and directionally cloned into the pIgPlus vector. The resulting construct was sequenced to confirm its identity, transfected into COS cells, and the resulting MAG-Fc chimera was purified from the culture medium using Protein G affinity chromatography.

For MAG binding studies, NG108–15 cells were cultured in the presence of sialic acid precursors for the indicated times, then were released from culture dishes using hypertonic Ca\(^{2+}\)/Mg\(^{2+}\)-free phosphate-buffered saline (Yang et al., 1996a) and resuspended (at 2 × 10^5 cells/ml) in 25 mM Heps-buffered Hanks’ balanced salt solution (Bashor, 1979) containing 5 mg/ml bovine serum albumin. MAG-Fc (6 µg) was added to 100 µl of the same buffer containing 6 µg fluorescein isothiocyanate-labeled goat anti-human Fc (Jackson Immunoresearch, West Grove, PA) and incubated on ice for 4 h 45 min with frequent mixing. Cell suspension (100 µl containing 50,000 cells) was added to the premixed antibody solution and the mixture incubated on ice for 45 min. The cells were then centrifuged for 4 sec at 16,000 × g and washed three times by resuspension in 200 µl of buffer with bovine serum albumin and centrifugation. The resulting cell pellet was resuspended in 100 µl buffer with bovine serum albumin and flow cytometry was performed on an Epics Profile II cytometer (Coulter Corp, Hialeah, FL).

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**Abbreviations**

NeuAc, N-acetylneuraminic acid; MAG, myelin-associated glycoprotein; ManNAc, N-acetylmannosamine; ManNgc, N-glycolylmannosamine; ManNGcPA, N-glycolylmannosamine...
References


