Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11427–11431, November 1994
Biochemistry

Polysialic acid engineering: Synthesis of polysialylated neoglycosphingolipids by using the polysialyltransferase from neuroinvasive *Escherichia coli* K1

(neural cell adhesion molecules/oncodevelopmental antigens/meningitis/metastasis/glycobiology)

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Communicated by William J. Lennarz, August 8, 1994 (received for review June 9, 1994)

ABSTRACT The CMP-sialic acid:polyα2,8sialosyl sialyltransferase (polyST) in neurotropic *Escherichia coli* K1 inner membranes catalyzes synthesis of the α2,8-linked polysialic acid capsule. The capsule is a neurovirulent determinant associated with neonatal meningitis in humans. A functionally similar polyST in human neuroblastomas polysialylates neural cell adhesion molecules. While bacteria do not synthesize glycosphingolipids (GSLs), we report here that the *E. coli* K1 polyST can selectively polysialylate several structurally related GSLs, when added as exogenous sialyl acceptors. A structural feature common to the preferred sialyl acceptors ($G_{D3} > G_{T1a} > G_{Q1b} = G_{T1b} > G_{D2} = G_{D1b} = G_{D1a} > G_{M1}$) was the disialyl glycotope, Siaα2,8Sia, α2,3-linked to galactose (Sia is sialic acid). A linear tetrasaccharide with a terminal Sia residue (e.g., G_{D3}) was the minimum length oligosaccharide recognized by the polyST. Endo-*N*-acylneuraminidase was used to confirm the α2,8-specific polysialylation of GSL. Ceramide glycanase was used to release the polysialyllactose chains from the ceramide moiety. Size analysis of these chains showed that 60–80 Sia residues were transferred to the disialyllactose moiety of G_{D3}. The significance of these findings is two-fold. (*i*) The *E. coli* K1 polyST can be used as a synthetic reagent to enzymatically engineer the glycosyl moiety of GSL, thus creating oligo- or polysialylated GSLs. Such "designer" GSLs may have potentially important biological and pharmacological properties. (*ii*) The use of GSLs as exogenous sialyl acceptors increases the sensitivity of detecting polyST activity. The practical advantage of this finding is that polyST activity can be identified and studied in those eukaryotic cells that express low levels of this developmentally regulated enzyme and/or its acceptor.

Polysialic acids (polySias) are a structurally unique group of carbohydrate chains that covalently modify cell surface glycoconjugates on cells that range in evolutionary diversity from microbes to humans (for recent reviews, see refs. 1–6). PolySia expression can influence neuronal development and the neuroinvasive potential of neurotropic bacteria and some human cancers (1, 6–10). The α2,8-linked polySia capsule in neuropathogenic *Escherichia coli* K1 and *Neisseria meningitidis* group B, for example, is a virulence determinant associated with neonatal meningitis in humans (11). The α2,8-polySia chains that cap N-linked oligosaccharides of the complex type on the embryonic form of neural cell adhesion molecules (N-CAMs) and on sodium channel proteins are structurally and immunologically identical to the polySia capsule in *E. coli* K1 and *N. meningitidis* group B (12, 13). A structurally diverse group of polySia chains also occur in fish egg polysialoglycoproteins (2, 3) and in the jelly coat of sea urchin eggs (14). Polysialylated N-CAMs are oncodevelopmental antigens that are reexpressed on the surface of diverse human tumors including neuroblastoma (9), nephroblastoma (Wilms tumor, ref. 15), myeloma (L. Hanneman, J. Guptil, J. J. Hemperly, E. Hersh, F. Tompson, J. J. Moore, J. A. M. Richter-Barbuto, J. Ye, J. J. McDonald, F.A.T., and T. M. Grogan, unpublished results), lymphoma (16), and leukemia (17). Consequently, polysialylation is an area of glycobiology that is having an important impact on studies in molecular microbiology, neurobiology, oncology, and cell and developmental biology.

Our studies have focused on the unresolved problem of how synthesis and surface expression of the polySia glycotope is regulated in *E. coli* K1 (1), human neuroblastoma (18), and trout ovaries during oogenesis (19). Our aim has been to identify and ultimately characterize the specific proteins and their genes to provide a molecular basis for studying reactions relevant to expression and regulation of polySia biosynthetic genes.

The genetic organization of the *kps* gene cluster that controls polySia capsule expression in neuroinvasive *E. coli* K1 has turned out to be unexpectedly complex. The multigenic cluster is encoded in ≈17 kb of DNA that consists of three coordinately regulated regions (1, 20, 21). At least a dozen proteins have been identified that are required for the synthesis, activation, and polymerization of sialic acid (Sia) (region 2); the energetics and translocation of the polySia chains across the inner membrane (region 3); and export of the polySia chains from the periplasmic surface of the inner membrane to the outer face of the outer membrane (region 1). Also complex is the overall biosynthetic pathway that was mostly characterized before *kps* had been cloned and sequenced (22–27). These latter studies showed that initiation and polymerization of polySia-chain synthesis in *E. coli* K1 is catalyzed by a CMP-sialic acid:polyα2,8sialosyl sialyltransferase complex, designated polyST. This complex is postulated to carry out the following reactions:

$$\text{(1) CMP-Sia} + P\text{-C}_{55} \overset{\text{polyST}}{\rightleftharpoons} \text{Sia-}P\text{-C}_{55} + \text{CMP}$$

$$\text{(2) } n\text{(Sia-}P\text{-C}_{55}) \xrightarrow{\text{polyST}} (\rightarrow 8\text{Sia}\alpha 2\rightarrow)_n\text{-}P\text{-C}_{55} + (n-1)P\text{-C}_{55}$$

$$\text{(3) } (\rightarrow 8\text{Sia}\alpha 2\rightarrow)_n\text{-}P\text{-C}_{55} \xrightarrow[\text{+ endogenous acceptor}]{\text{polyST}} (\rightarrow 8\text{Sia}\alpha 2\rightarrow)_n\text{-acceptor} + P\text{-C}_{55},$$

Abbreviations: Sia, sialic acid; Neu5Ac, *N*-acetylneuraminic acid; polySia, α2,8-linked polysialic acid; GSL, glycosphingolipid; Cer, ceramide; polyST, CMP-sialic acid:polyα2,8sialosyl sialyltransferase; endo N, endo-*N*-acylneuraminidase; DP, degree of polymerization; Lac Cer, lactosylceramide; $GbOse_3$ Cer, globotriaosylceramide.

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where P-C_{55} is the glycosyl carrier lipid undecaprenyl phosphate. Biosynthesis thus involves preassembly of Sia residues on a polyisoprenyl phosphate carrier lipid (22), an assembly pathway analogous to the dolichyl phosphate pathway for synthesis of N-linked oligosaccharides on eukaryotic glycoproteins (28, 29).

The functional domain of the polyST in *E. coli* K1 is located on the inner surface of the cytoplasmic membrane (1). This means that inside-out membrane vesicles can be used to study the catalytic site of the polyST because enzymes normally located on the inner face of the inner (cytoplasmic) membrane appear on the exterior side of the vesicles. As such, glycoconjugates of defined structure and conformation can be tested as exogenous sialyl acceptors. For example, α2,8-linked sialyl oligomers can be polysialylated by the polyST on inside-out vesicles (30). In the present investigation, we have extended this observation to follow-up on an unexpected finding that a trisialoganglioside was an effective exogenous acceptor. While bacteria do not synthesize glycosphingolipids (GSLs), data are presented that show that the polyST exposed on the cytoplasmic surface of inside-out vesicles from *E. coli* K1 can be used as a synthetic reagent to construct a class of structurally defined polysialylated GSLs. By using endo-*N*-acylneuraminidase (endo N), these glycolipids can be further tailored to create polySia chains of variable lengths. Further, treatment of polysialylated GSLs with ceramide glycanase (Cer glycanase) can be used to prepare a glycoconjugate, polysialyllactose, also with short, intermediate, or full-length polySia chains.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled CMP-Neu5Ac (or CMP-Sia) and G_{D3} were gifts from Snow Brand (Tokyo). CMP-[^{14}C]Neu5Ac (300 mCi/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. All gangliosides, including the neutral glycolipids lactosylceramide (Lac Cer), globotriaosylceramide ($GbOse_3$ Cer), and globoside, were purchased from either BioCarb (Lund, Sweden) or Calbiochem. They were all >95–98% pure (by NMR or TLC) except for Lac Cer and $GbOse_3$ Cer, which were >90% pure (by NMR). Cer glycanase was purchased from Boehringer Mannheim, and endo N was purified from the *E. coli* K1 bacteriophage, K1F, as described (31).

Bacterial Strain and Growth Conditions. The *E. coli* K-12/K1 hybrid derivative designated EV11, whose genetic construction has been described (32), was used throughout this study. EV11 is defective in catalyzing the endogenous synthesis of polySia because of a presumed defect in either the synthesis or proper assembly of the endogenous acceptor (27). Inside-out vesicles prepared from EV11 can, however, polysialylate exogenous acceptors (13). Thus, [^{14}C]polySia-chain synthesis in EV11 inside-out vesicles, dependent on the addition of an exogenous acceptor, is a sensitive indicator for detecting polysialylation because the signal-to-noise (background) ratio is so high (13). EV11 was grown in 250 ml of trypticase soy broth (Becton Dickinson) at 37°C on a rotary shaker. Cells were harvested at late exponential–early stationary phase by centrifugation at 4°C.

Preparation of Inside-Out Membrane Vesicles Containing the PolyST Complex from *E. coli* K1 (EV11). All procedures for preparing inside-out vesicles containing the polyST complex in *E. coli* K1 (EV11) were carried out at 4°C. Vesicles were prepared as described, with minor modifications (13, 24). EV11 cells [2 g (wet weight)] were washed twice with ice-cold TM buffer (50 mM Tris·HCl, pH 7.8/25 mM $MgCl_2$). Washed cells were resuspended in 5 ml of ice-cold TMD buffer (TM buffer/1 mM dithiothreitol) and disrupted by passage through an ice-cold French pressure cell at 8000 psi (1 psi = 6.9 kPa). DNase I (40–50 Kunitz units) was added, and the cell homogenate was incubated for 10 min on ice. Unbroken cells were sedimented by centrifugation for 10 min at 3000 × *g*, using a DuPont Sorvall F-28/36 rotor. The inverted membranes, designated inside-out membrane vesicles, were sedimented by ultracentrifugation in a Beckman type 60 Ti rotor for 1 hr at 250,700 × *g*. The pellet was resuspended in 3 ml of TMD buffer using a Dounce homogenizer.

PolyST Assay Using GSLs as Exogenous Acceptors. Incorporation of [^{14}C]Neu5Ac from CMP-[^{14}C]Neu5Ac into GSLs, added as exogenous acceptors, was carried out essentially as described when using sialyl oligomers as acceptors (30). Each 200-μl incubation mixture contained 10 μmol of Tris·HCl (pH 7.8), 5 μmol of $MgCl_2$, 0.2 μmol of dithiothreitol, 112 nmol of CMP-[^{14}C]Neu5Ac (2.7 mCi/mmol), EV11 inside-out membrane vesicles (1 mg of protein), and 0.1 mg of GSLs. The Sia-containing GSLs were resuspended in the Tris buffer before being added to the incubation mixture. The neutral gangliosides were resuspended in the same buffer containing Triton X-100, such that the final concentration of Triton X-100 in the incubation mixture was 0.017%. As shown (22), 0.017% Triton X-100 has no effect on the *E. coli* K1 polyST activity. To ensure that the GSLs were incorporated into the membrane vesicles, GSLs were first preincubated with vesicles for 20 min at 33°C and then CMP-[^{14}C]Sia was added. Incubations were carried out at 33°C and after 0, 30, 60, and 120 min, 20-μl aliquots were removed and spotted on Whatman 3MM paper. To determine the endo N sensitivity of the [^{14}C]Sia incorporated onto the GSL acceptors, 50×10^{-3} units of endo N was added to the remaining 120-μl incubation mixture after 2 hr. After an additional 10 min and 60 min of incubation with endo N, 20 μl of sample was spotted on Whatman 3MM, and the papers were chromatographed overnight in ethanol/1 M ammonium acetate, pH 7.5, 7:3 (vol/vol). This procedure separates CMP-[^{14}C]Neu5Ac from [^{14}C]polysialylated GSL, which remained at the origin. Radioactivity at the origin was quantitated by scintillation counting and expressed as specific activity (nmol of [^{14}C]Neu5Ac incorporated per mg of protein).

Cer Glycanase and the Polyacrylamide Gel Electrophoresis Method to Determine the Extent of Polysialylation of G_{D3}. The number of Sia residues transferred by the *E. coli* K1 (EV11) polyST to G_{D3} was determined by polyacrylamide gel electrophoresis after the polysialylated sialyllactose chains were released from the ganglioside by Cer glycanase (33, 34). Gel electrophoresis was carried out as described by Pelkonen *et al.* (35).

The polysialylated G_{D3} samples were prepared for gel electrophoresis after a 2-hr incubation with CMP-[^{14}C]Neu5Ac as follows. A 90-μl sample was removed from the standard incubation mixture and centrifuged in a Beckman TL-100 rotor at 250,000 × *g* for 30 min. The pellet, containing polysialylated G_{D3}, was resuspended in 54 μl of 50 mM sodium acetate (pH 6.0) containing 17 μl of 1% sodium cholate (in sodium acetate buffer). The suspension was divided into two aliquots. Cer glycanase (15 μl; 0.75×10^{-3} units) was added to one aliquot, and 15 μl of sodium acetate buffer was added to the other as a control for Cer glycanase. After incubation at 37°C for 18 hr, the samples were electrophoresed. The mobility of each dye in the 25% polyacrylamide gel was empirically related to the number of Sia residues or the degree of polymerization (DP) as described by Pelkonen *et al.* (35) as follows: xylene cyanole, DP52; bromphenol blue, DP19; bromcresol purple, DP11.5; phenol red, DP4. Autoradiography was carried out at −70°C on Kodak X-Omat AR film (25).

RESULTS

Enzymatic Synthesis of Polysialylated GSLs by the *E. coli* K1 PolyST. As shown in Fig. 1, the *E. coli* K1 polyST could recognize and polysialylate the glycosyl chains of several structurally related GSLs, when added as exogenous sialyl acceptors. Acidic GSLs that were tested and found to be polysialylated included G_{D3}, G_{T1a}, G_{D2}, G_{T1b}, G_{Q1b}, G_{D1b}, G_{D1a}, and G_{M1}. A structural motif common to the preferred GSL acceptors (G_{D3}, G_{T1a}, G_{Q1b}, G_{T1b}, G_{D2}, and G_{D1b}) was the disialyl glycotope, Siaα2,8Sia α2,3-linked to Gal. There was not an obligatory requirement for this sialotope, however, since G_{D1a} and G_{M1}, both of which contained only a single Sia residue (Siaα2,3Gal), showed lower, yet clearly detectable, acceptor activity. To our knowledge, this is the first *in vitro* demonstration of α2,8-polysialylation in the absence of a preexisting α2,8-Sia linkage. The *E. coli* K1 polyST could not polysialylate the neutral glycolipids globoside, lactosylceramide, or globotriaosylceramide or the gangliosides G_{M3} or G_{M2} (Fig. 1).

Efficacy of GSLs as Exogenous Sialyl Acceptors for Polysialylation. The kinetics of polysialylation of the various acceptor lipids was determined using saturating amounts of GSLs (Fig. 1). The specific activity of Sia incorporated into each GSL shown in Fig. 1 was calculated on the basis that the same weight of GSLs was used in each experiment. To compare the efficacy of each GSL as an exogenous sialyl acceptor, however, the specific activity was recalculated based on the linear period of Sia incorporation (30 min) by using the equivalent molar concentration of each glycolipid. As summarized in Table 1, G_{D3} and G_{T1a}, both of which contained the distal α2,8-disialyl structural motif, were the most effective acceptors. These data also show that a linear tetrasaccharide containing a terminal Sia residue (G_{D3}) was

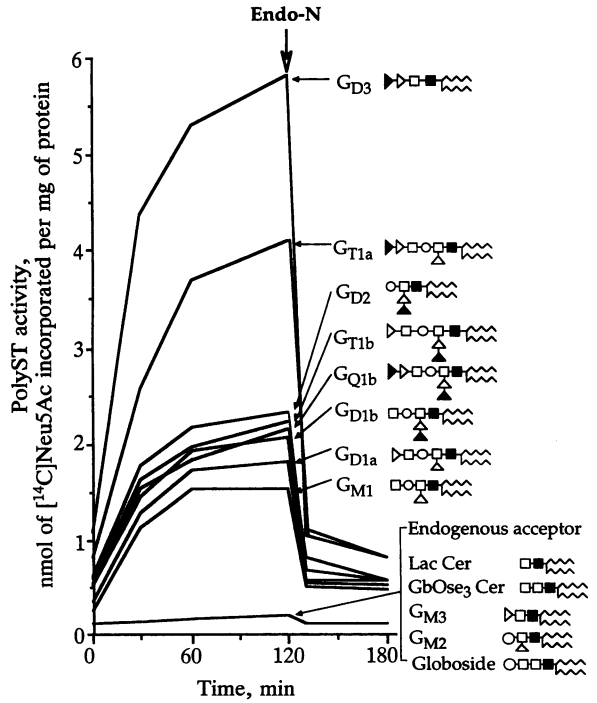

FIG. 1. Enzymatic synthesis of polysialylated GSLs. The *E. coli* K1 (EV11) polyST was used to determine which GSLs could be polysialylated, when added as exogenous sialyl acceptors. Inside-out vesicles containing the polyST were prepared and the polysialylation assay was carried out. The α2,8-Sia linkages in the polysialylated GSLs were confirmed by showing their sensitivity to endo N, which catalyzes the specific depolymerization of α2,8-Sia linkages (see Fig. 2, lane 3). ▶, Neu5Acα2,8; ▷, Neu5Acα2,3; ○, GalNAc; □, Gal; ■, glucose; ≈, cer.

Table 1. Quantitative summary of the selective polysialylation of GSLs by the *E. coli* K1 (EV11) polyST

Exogenous GSL acceptor	Structure	PolyST activity: Specific activity	PolyST activity: Relative activity, %
G_{D3}		1360	100
G_{T1a}		1107	81
G_{Q1b}		775	57
G_{T1b}		698	51
G_{D2}		603	44
G_{D1b}		544	40
G_{D1a}		485	36
G_{M1}		365	27
G_{M2}		0	0
G_{M3}		0	0
Lac Cer		0	0
$GbOse_3$ Cer		0	0
Globoside		0	0
Endogenous Acceptor		0	0

▶ Neu5Acα2,8 ■ Glucose ≈ Ceramide
▷ Neu5Acα2,3 □ Galactose ○ GalNAc

Specific activity is nmol of [^{14}C]Neu5Ac incorporated per μmol of GSL per mg of protein per 30 min (minus background).

the minimum oligosaccharide chain length required for polysialylation, since a linear trisaccharide with a nonreducing Sia residue (G_{M3}) was not polysialylated. Other GSLs that contained the α2,8-disialyl glycotope (e.g., G_{Q1b}, G_{D2}, or G_{D1b}) showed 40–60% of the acceptor activity of G_{D3}. Thus, the bisialylated oligosaccharide chain containing two Sia residues linked to the proximal Gal residue (G_{T1b} or G_{Q1b}) were poorer acceptors than those containing a single Sia residue linked to the same proximal Gal (G_{T1a}), or with the proximal Gal residue unsubstituted (G_{D3}). This may result because of steric hindrance caused by the branched chains or because of competitive inhibition between the two sialylated oligosaccharide chains. On the basis of these results, we conclude that the preferred hierarchy of GSLs as exogenous sialyl acceptors for polysialylation by the *E. coli* K1 polyST is $G_{D3} > G_{T1a} > G_{Q1b} = G_{T1b} > G_{D2} = G_{D1b} = G_{D1a} > G_{M1}$.

Proof of α2,8-Polysialylation of GSLs. Endo N was used to confirm that the GSL-dependent incorporation of [^{14}C]Sia was into α2,8-linked polySia chains. As shown in Fig. 1, endo N catalyzed the depolymerization of the ^{14}C-sialylated GSL products, when added at 2 hr. Concomitant with this depolymerization was the formation of [^{14}C]sialyl oligomers (Fig. 2, lane 3). In contrast to polysialylated GSLs, the sialyl oligomers were chromatographically mobile and, therefore, did not remain at the origin (Fig. 1). Endo N is a key enzyme for identifying polySia chains because it is highly specific for hydrolyzing α2,8-linked polySia chains (13, 31). Based on the sensitivity of the ^{14}C-sialylated GSL acceptors to endo N (Fig. 1), we conclude that they contained α2,8-polySia chains.

Synthesis of Polysialyllactose and Characterization of the DP of G_{D3}. Proof that the [^{14}C]polySia chains were attached to

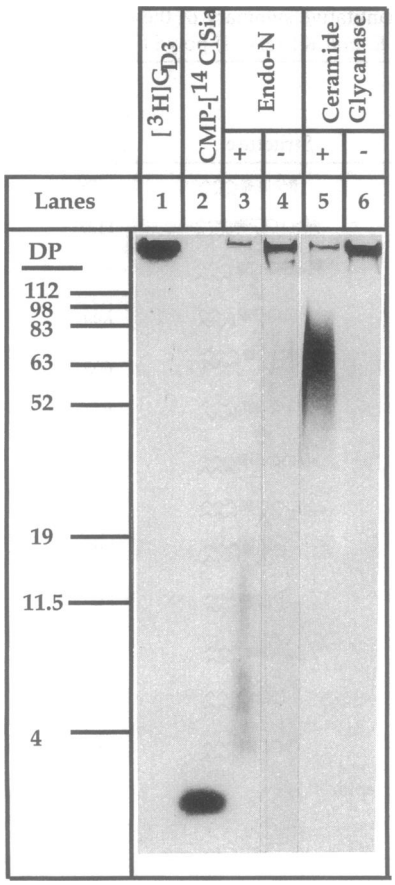

FIG. 2. Synthesis of polysialylated G_{D3} and polysialyllactose and determination of the DP. The extent of polysialylation of G_{D3} was determined by polyacrylamide gel electrophoresis after the polysialyllactose chains were released from the ganglioside by Cer glycanase. Approximately 60–80 Sia residues were transferred to G_{D3} (lane 5). The oligoSia products resulting from the endo N-catalyzed depolymerization of polysialylated G_{D3} are shown in lane 3. The controls show that neither G_{D3} (lane 1) nor polysialylated G_{D3} (lanes 4 and 6) entered the gel. Inside-out vesicles containing the polysialylated G_{D3} were isolated by ultracentrifugation and the samples were prepared for electrophoresis.

the glycosyl moiety of the G_{D3} was provided by showing that they were released by Cer glycanase (Fig. 2). This enzyme cleaves the glycosidic bond linking the disialyllactose moiety of G_{D3} to Cer (33, 34). Using this fact, we were able to generate the sugar molecule [^{14}C]polysialyllactose (Fig. 2, lane 5) and to also determine the DP or approximate number of Sia residues transferred to G_{D3} by the *E. coli* K1 polyST. As shown in Fig. 2, neither [^{3}H]G_{D3} (lane 1) nor ^{14}C-polysialylated G_{D3} (lanes 4 and 6) penetrated a nonNaDodSO$_4$/polyacrylamide gel. In contrast, the ^{14}C-polysialylated G_{D3} migrated into the gel after treatment with Cer glycanase (lane 5). Cer glycanase sensitivity of the ^{14}C-polysialylated G_{D3} (lane 5) and the release of [^{14}C]sialyl oligomers from the molecule by endo N (lane 3) verified that ^{14}C-polysialylation of the sialyllactose moiety of G_{D3} had occurred. The result in Fig. 2 (lane 5) also shows that 60–80 Sia residues were transferred to the disialyllactose moiety of G_{D3}, after a 2-hr incubation at 33°C, creating ^{14}C-labeled polysialyllactose. On the basis of these results, we conclude that the structure of the newly synthesized polysialoganglioside from G_{D3} is Siaα2 - (8Siaα2)$_{60\text{-}80}$ - 8Siaα2 - 3Galβ1 - 4Glcβ1 - 1ceramide. Results in accord with the hypothesis that the polySia chains are transferred *en bloc* to G_{D3} from a high-energy "activated" intermediate, possibly undecaprenyl phosphate, have been reported (36). Approximately 70% of the [^{14}C]polySia-G_{D3} molecules (DP60–80) that were synthesized after a 2-hr incubation remained bound to the inside-out membrane vesicles after ultracentrifugation. This finding corroborates the report of Felgner *et al.* (37) that gangliosides are rapidly incorporated into the membrane bilayer when added to preformed phospholipid vesicles.

DISCUSSION

These studies show that the polyST from neuroinvasive *E. coli* K1 can be used as a synthetic reagent to synthesize structurally unique GSLs, in which the glycosyl moiety of the gangliosides are ^{14}C-polysialylated. To our knowledge, such long-chain polysialylated GSLs have not been previously described, although "polysialogangliosides" have been reported in embryonic chicken brain (38) and in the brains of fish and lower vertebrates (39). In chicken brain, however, the seven Sia residues are distributed between the two nonreducing terminal branches of the sugar chain. The length of the Sia chains in the fish brain gangliosides has not been determined. Given the remarkable structural diversity in α2,8-linked polySia chains that have been described recently in other glycoconjugates (1–4), it is probable that GSLs with polySia chains may occur naturally. The difficulty in isolating such hydrophilic lipids has likely precluded their identification. Thus, while the significance of our present findings with respect to naturally occurring polysialylated gangliosides awaits further study, it seems likely that such polysialylated GSLs may have important biological and pharmacological functions. This relates particularly to the number of GSLs that are developmentally regulated heterophile antigens that function as surface receptors for bacteria, viruses, and toxins (40–42). Some GSLs are also tumor-associated antigens that have been implicated in transmembrane signaling and as mediators for cell–cell interactions (41–45). Some cell adhesion processes also appear to involve a complementary GSL–GSL interaction that is mediated by specific carbohydrate residues in each GSL (45, 46).

These studies were initiated after our serendipitous discovery that a trisialoganglioside functioned as an exogenous sialyl acceptor in the *E. coli* K1 (EV11) polyST assay. Our follow-up studies were made possible by three key findings. (*i*) The isolation and characterization of an *E. coli* K1 mutant (EV11) that was defective in catalyzing transfer of polySia chains to the endogenous acceptor (31) allowed us to prepare inside-out membrane vesicles from EV11 that contained the polyST complex and could polysialylate exogenous sialyl acceptors. As shown in Fig. 1, the nearly complete absence of endogenous polyST activity makes this a very sensitive assay to detect polysialylation and to study the structural requirements of exogenous acceptors. (*ii*) The use of endo N, an enzyme that is selective for catalyzing the depolymerization of α2,8-linked polySia chains, provided a facile procedure to confirm that the exogenous acceptor-dependent incorporation of [^{14}C]Sia was into [^{14}C]polySia chains (Fig. 1). This conclusion was further verified by showing that the products of endo N hydrolysis of ^{14}C-polysialylated G_{D3} were [^{14}C]sialyl oligomers (Fig. 2). (*iii*) The sensitivity of ^{14}C-polysialylated G_{D3} to Cer glycanase made it possible to confirm that the glycosyl chains of the ganglioside were polysialylated. Cer glycanase also made it possible to determine that 60–80 Sia residues had been transferred to the disialyllactose moiety of G_{D3}, by releasing [^{14}C]polysialyllactose chains from ^{14}C-polysialylated G_{D3} (Fig. 2).

It is interesting to note that disialyllactose, the tetrasaccharide moiety of G_{D3}, was the shortest oligosaccharide chain on a GSL that was polysialylated (Fig. 1). In contrast, nonlipidated short sialyl oligomers that do not attach to the membrane are not polysialylated by the *E. coli* K1 polyST but rather serve as an acceptor for the addition of only a few Sia

residues. Therefore, conclusions regarding the molecular mechanism of glycoconjugate chain polymerization that are based on studies using low molecular weight exogenous acceptors that are not polymerized may be of dubious significance.

In summary, while bacteria do not contain GSL, these studies demonstrate that the polyST from neuroinvasive *E. coli* K1 can be used to create a class of polysialylated GSLs and a sugar molecule, polysialyllactose. A second practical advantage of this finding is that the exogenous addition of GSLs (e.g., G_{D3}) to eukaryotic membranes can be used to identify polyST activities that are normally expressed at such low levels as to preclude study. Indeed, using this strategy, we have recently identified and characterized a polyST activity in the sea urchin *Lytichinus pictus*. The discovery, developmental expression, and study of this enzyme were made possible because it also polysialylated G_{D3}, when added as an exogenous sialyl acceptor (J.-W.C., F.A.T., and W. J. Lennarz, unpublished results). These results thus predict that polyST activities in other species will likely be discovered by using this experiment protocol.

We thank Ann Douglass for expert secretarial assistance. This research was supported by National Institutes of Health Grant AI-09352 (F.A.T.).

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