Use of prokaryotic-derived probes to identify poly(sialic acid) in neonatal neuronal membranes

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Communicated by Robert L. Hill, November 18, 1983

ABSTRACT Three prokaryotic-derived probes to identify and study the temporal expression of poly(sialosyl) units in neuronal tissue have been developed. A polyclonal antibody, a bacteriophage-derived endo-neuraminidase, and an Escherichia coli K1 sialyltransferase are all specific for either recognizing or synthesizing poly(sialic acid) containing α-2,8-ketosidic linkages. Poly(sialosyl) immunoreactivity with apparent Mr values of 180,000–240,000 was specific for developing neuronal tissue; it was not detected in neonatal liver or kidney or in adult brain tissue. The developmentally regulated disappearance in poly(sialic acid) is consistent with the probes described here recognizing the poly(sialosyl) carbohydrate units of a neuronal cell adhesion molecule (N-CAM). Treatment of brain extracts with a bacteriophage-derived endo-neuraminidase specific for α-2,8-linked poly(sialosyl) units abolished the immunoreactivity. The material solubilized by endo-neuraminidase was isolated, reduced with borotritide, and shown to contain oligomers of sialic acid with three to six sialyl units. Treatment of the 3H-labeled oligosialic acid with exo-neuraminidase quantitatively converted the radioactivity to sialitol, establishing that the brain-derived oligomers were composed solely of sialic acid. A membranous sialyltransferase from E. coli K1 that can transfer sialic acid to exogenous acceptors of oligo- or poly(sialic acid) also recognized rat brain membranes, further substantiating the presence of poly(sialic acid) in rat brain. This conclusion was confirmed by using a mutant of E. coli K1 that was defective in the synthesis of poly(sialic acid) and could only transfer sialic acid to exogenous acceptors of oligo- or poly(sialic acid). Sialyl polymer synthesis was restored in the mutant when brain membranes were added as exogenous acceptor.

Sialic acid occurs primarily as the terminal, nonreducing sugar on N-asparaginyl-linked glycoproteins often attached to galactosyl residues of β-, tri-, or tetraantennary sugar chains. Rarely, however, sialic acid exists internally to form poly(sialosyl) chains of various lengths. A well-characterized poly(sialic acid) is a capsular polysaccharide, the K1 antigen, found in certain strains of Escherichia coli (1–3). In E. coli K-235 the K1 antigen contains ca. 200 sialyl residues with the internal N-acetyleneuraminic acid (NeuNAc) units joined by α-2,8-ketosidic linkages (2). Recently, poly(sialic acid) has been found as a constituent of a neuronal cell surface glycoprotein in developing brain (4–6).

The interesting possibility that poly(sialosyl) units in neuronal cell adhesion molecule (N-CAM) participate in brain development has been suggested by Edelman and co-workers (7–9). These investigators showed that the poly(sialic acid) moiety of N-CAM was developmentally regulated with the embryonic form (high sialic acid content) undergoing a postnatal conversion to the adult form (low sialic acid content).

The developmental reduction in sialic acid was postulated to modulate cell–cell adhesive properties of neuronal cells and to mediate their specific organization into adult brain tissues. Another developmentally regulated neuronal glycoprotein, brain cell surface protein 2, appears to be identical to N-CAM (10).

The polypeptide moiety of N-CAM has been studied by using poly- and monoclonal antibodies (6, 7), with a significant amount of information accumulating about N-CAM's molecular organization (8). However, relatively little is known about the biosynthesis of the peptide moiety and nothing is known about synthesis of its poly(sialosyl) portion or the mechanism that mediates the developmentally regulated reduction of poly(sialic acid). Clearly, such studies would be aided by probes that specifically recognize poly(sialic acids). In this communication we present evidence that three prokaryotic-derived probes that specifically recognize or synthesize α-2,8-ketosidically linked poly(sialosyl) chains can be used to unambiguously identify polysialic acid in neuronal membranes. We also describe structural studies that confirm the presence of this novel sugar oligomer in membranes of embryonic rat brains.

MATERIALS AND METHODS

Antiserum containing polyclonal IgM antibodies against α-2,8-linked poly(sialic acid), referred to as H.46 (11), was kindly provided by W. Vann and J. B. Robins (National Institutes of Health). Rabbit IgG anti-horse IgM was iodinated by the chloramine-T method (12) using carrier-free Na125I to a specific activity of 18 μCi/μg of protein (1 Ci = 37 GBq). Electrophoret of antigens from polyacrylamide gels to nitrocellulose paper (NS) was carried out at 200 mA for 12–16 hr as described (13).

Eight-day-postnatal rats (male Sprague–Dawley) or 8-day-embryonic chickens (White Leghorn) were sacrificed by severing the spinal cord. Brains and other tissues were dissected immediately into ice-cold phosphate-buffered saline containing phenylmethylsulfonyl fluoride at 1 mg/ml and homogenates were prepared by disruption in a Dounce homogenizer. Membranes were isolated by differential centrifugation and resuspended to 20–40 mg of protein per ml in 10 mM TrisHCl (pH 7.6). For NaDdSO4/polyacrylamide gel electrophoresis (NaDdSO4/PAGE), samples were resuspended to 1–2 mg of protein per ml in Laemmlie sample buffer (14). Aliquots containing 100–200 μg of protein per well were electrophoresed through gradient (5–15%) NaDdSO4/polyacrylamide gels with cooling. Nonspecific sites were blocked with bovine serum albumin and the NS blots were then overlayed with a 1:25 or 1:50 dilution of H.46 antibodies. Unbound antibodies were removed as described (13).

Abbreviations: DP, degree(s) of polymerization; N-CAM, neuronal cell adhesion molecule; NeuNAc, N-acetyleneuraminic acid; NS, nitrocellulose paper.

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and bound antibodies were detected by autoradiography after incubating the blots with 125I-labeled anti-horse IgM. Exo-neuraminidase from *Vibrio cholerae* was purchased from Calbiochem-Behring. Digestions were performed in 0.1 M sodium acetate buffer/pH 5.5/5 mM CaCl₂. Endo-neuraminidase specific for α-2,8-linked polysialic acid was obtained from a bacteriophage isolated from University of California, Davis, sewage after enrichment on *E. coli* K-235. This phage, referred to as K1F, is morphologically identical to φL2 described by Kwiatkowski et al. (15). Lysates of K1F prepared on K1 strains of *E. coli* contained large amounts of enzyme not associated with infective particles. This “soluble” enzyme was enriched from lysates with 100- to 120-fold purification by standard procedures (unpublished data). Enzyme was assayed by using uniformly 14C-labeled poly(sialic acid) as substrate (16). One unit of endo-neuraminidase degraded 1% of the substrate to oligosialic acid in 1 min.

*E. coli* strain EV38 was derived from the encapsulated, polysialylated strain of *E. coli* K-235 (O1:K1:H') and is defective in poly(sialic acid) synthesis both in *vivo* and *in vitro*. EV11, a mutant derived from a hybrid of *E. coli* K-12 and a K1 antigen-expressing strain, is also defective in poly(sialic acid) production *in vivo*. EV11 is also defective *in vitro* in the transfer of NeuNAc from CMP-NeuNAc to endogenous acceptors but, in contrast to EV38, can transfer NeuNAc to exogenous acceptors containing oligosialic acid (EV38) or poly(sialic acid) in α-2,8-ketosidic linkages. Details of the isolation and characterization of these mutant strains will be published elsewhere.

The membrane-associated sialyltransferase complex was prepared from late logarithmic phase cells as described (2). Incorporation of [14C]NeuNAc from CMP-[14C]NeuNAc into polymeric products was carried out as described (16).

**RESULTS**

**Antibodies Specific for Bacterial Poly(sialic Acid) Detect Similar Antigenic Species in Neuronal Tissues.** Experiments using H.46 antibodies for immunoblot(s) of detergent-solubilized 8-day-postnatal rat brains consistently showed a major immunoreactivity at Mr 180,000–240,000. Fig. 1A (lane 1) shows that this reactivity existed as a broad band suggestive of apparent molecular weight heterogeneity in the component(s) detected by this method. Because H.46 antibodies have immunospecificity toward α-2,8-linked poly(sialic acids), these data suggested that rat brains contained multimers of sialic acid. Qualitatively identical immunoreactivity was also observed in 8-day-embryonic chicken brain (Fig. 1A, lane 3), chicken retina, and spinal cord, but not in liver, spleen, thymus, or bone marrow (data not shown). The immunoreactivity was not seen in adult rat brain, suggesting its decrease during embryonic development may be important during early development and differentiation. Further, immunoreactivity was abolished by prior treatment of brain extracts with an endo-neuraminidase that specifically cleaved α-2,8-linked poly(sialic acid) (Fig. 1A, lanes 2 and 4, and below). Additional confirmation that the brain-derived immunoreactive material contained poly(sialic acid) came from comparison with an immunoblot of bacterial K1 antigen. Fig. 1A (lane 5) shows the immunoblot obtained by using detergent-solubilized *E. coli* K-235. The similarity in apparent high molecular weight and band polydispersity to brain-derived antigen is striking and suggested that H.46 antibodies recognized molecular components in bacteria and in brain that contained poly(sialic acid) in α-2,8-ketosidic linkage.

H.46 immunoreactivity could also be detected after rocket immunoelectrophoresis of samples solubilized with Triton X-100. Fig. 1B (lane 2) shows a rocket obtained by using *E. coli* K-235 as antigen. The ability to detect immunoprecipitates was dependent on the presence of poly(sialic acid)

**FIG. 1.** Detection of poly(sialic acid) in brain and *E. coli* K-235 based on immunoreactivity to anti-poly(sialosyl) antibodies. (A) Immunoblotting using H.46 antibodies was performed as described in the text. Brain homogenate of 8-day-postnatal rat (lane 1) was treated with 12,000 units of endo-neuraminidase per mg of brain protein for 2 hr at 28°C prior to immunoblotting (lane 2). Brain homogenate of 8-day chicken embryo (lane 3) is shown after treatment as above with endo-neuraminidase (lane 4). Approximately 2 × 10⁹ cells of *E. coli* K-235 were suspended in 1 ml of Laemmli sample buffer; 50 μl was electrophoresed (lane 5). (B) Qualitative rocket immunoelectrophoresis was carried out on lantern slides coated with 11.6 ml of 1% agarose containing 1% Triton X-100, 0.02% NaN₃, and 46 μl of H.46 serum per ml in 25 mM sodium barbital buffer (pH 8.6). Samples were prepared in dilution buffer (1% Triton X-100/NaN₃/barbital buffer as above), sonicated for 5 sec, loaded into wells, and electrophoresed for 2 hr at 6 V/cm. After electrophoresis, immunoprecipitates were identified by staining with Coomassie blue R-250 as described (18). Lanes 1 and 2 are the results obtained with *E. coli* strains EV38 and K-235, respectively. The membrane fraction (21 mg of protein/ml) from an 8-day-postnatal rat brain was treated as above with endo-neuraminidase and resuspended in dilution buffer to 2.1 mg/ml prior to electrophoresis (lane 3). A control sample not treated with endo-neuraminidase is shown in lane 4.

as antigen because a mutant derivative of *E. coli* K-235 unable to synthesize poly(sialic acid) (EV38) showed essentially no reactivity (Fig. 1B, lane 1). Rat brain membranes produced an immunoprecipitate qualitatively similar to that observed with the bacterial polymer (Fig. 1B, lane 4). The ability of brain samples to form immunoprecipitates was abolished by pretreatment with endo-neuraminidase (Fig. 1B, lane 3), again suggesting that the antigen was poly(sialic acid). These results corroborated previous conclusions based on immunoblotting (Fig. 1A) and provided evidence that H.46 antibodies were able to form immunoprecipitin complexes with poly(sialic acid) from sources as disparate as rat brain and bacteria. Using K1 antigen purified from *E. coli* K-235 and quantitative rocket immunoelectrophoresis, we have detected as little as 5 ng of poly(sialic acid) This technique may, therefore, be useful for routine quantitation of low amounts of poly(sialic acid) in brain tissues from various sources.

**H.46 Immunoreactivity Is Membrane Associated.** The immunoreactivity in rat brain homogenates was associated with antigens that were membrane bound. Brain homogenate from 8-day postnatal rats was separated into membrane and soluble fractions by differential centrifugation and subjected to immunoblot analysis (Fig. 2). Brain homogenate (Fig. 2,
brane sensitivity total membranepared by The were containing were pelleted at 145,000 hr Laemmli the supernatants released 0.96, 3.04, and hr at Laemmli sample buffer, and immunoblotted as above. Membrane fractions were treated with endo-neuraminidase for 15 min (lane 4), 2 hr (lane 7), and 15 hr (lane 8). Lane 6 shows immunoreactivity after 2 hr of incubation in the absence of endo-neuraminidase. Lane 5 is a blank well. After sedimenting the membranes as described above, the supernatants were lyophilized, redissolved in 0.4 ml of H2O, and used as the source of endo-neuraminidase-releasable sialyl oligomers. Thiobarbituric acid determinations (19) after hydrolysis for 2 hr at 80°C in 0.05 M H2SO4 indicated that endo-neuraminidase released 0.96, 3.04, and 4.08 μmol of sialic acid after 15 min, 2 hr, and 15 hr of digestion, respectively. These values represent oligo(sialic acid) because sialic acid content in each sample measured without heating was subtracted. Supernatant from the 2-hr control incubation that lacked enzyme contained 0.68 μmol of sialic acid. In contrast to the endo-neuraminidase-released material, none of the sialic acid in the control appeared to be in oligomeric structures because heating with acid did not increase the sialic acid content as measured by thiobarbituric acid.

Lane 2) gave a diffuse band of immunoreactivity similar to that in Fig. 1A. The membrane fraction (Fig. 2, lane 3) contained essentially all of the immunoreactivity, in contrast to the soluble fraction that was nearly devoid of antigen (Fig. 2, lane 1). Treatment of the membrane fraction with endo-neuraminidase for 15 min, 2 hr, or 15 hr resulted in complete loss of reactivity, even at the shortest period of digestion (Fig. 2, lanes 4, 7, and 8). A 2-hr control for the stability of the H.46-reactive material under the conditions used for enzyme digestion did not result in loss of immunoreactivity (Fig. 2, lane 6).

Endo-neuraminidase Treatment of Brain Membrane Released Oligomers of Sialic Acid. Direct evidence that the products of endo-neuraminidase digestion of brain membranes were oligomers of sialic acid came from chromatographic and structural analyses of the material released by endo-neuraminidase. Fig. 3 shows the profile obtained from DEAE chromatography of a partial acid hydrolysate of colominic acid, a bacterial-derived homooligomer of sialic acid with an average degree of polymerization (DP) of 10 NeuNac residues (16, 17). Under these conditions, sialyl oligomers with larger DP eluted with increasing salt concentration. A logarithmic plot of elution versus DP gave a linear relationship (Fig. 3 Inset). Thus, the DP of an unknown sample of sialyl oligomers can be estimated from its elution volume. Endo-neuraminidase-solubilized material from the 2-hr digest of brain membranes was fractionated by this method. As shown in Fig. 4A, >80% of the total sialic acid measured by thiobarbituric acid eluted from the DEAE column with a DP of 3–5 NeuNac residues. This result suggested that the H.46-reactive, endo-neuraminidase-sensitive brain material consisted of multimers of sialic acid. Proof of the oligomeric nature of this material came from demonstrating its sensitivity to exo-neuraminidase as follows. Fractions 69, 71, and 73 in Fig. 4A were pooled and reduced with potassium borotritide, converting the reducing terminal sugar of oligo(sialic acid) to [3H]sialitol. The labeled material was then digested with exo-neuraminidase and chromatographed on paper. Fig. 5 (solid line) shows that prior to enzymatic hydrolysis, all of the radioactivity chromatographed as a slowly migrating component with a DP of >1 but <10. Colominic acid, similarly labeled in the reducing terminus (Fig. 5A, dotted line), remained at the origin, while a dimer of NeuNac-NeuNac-O2H (Fig. 5A, dashed line) migrated with the [3H]-labeled brain-derived material. Exo-neuraminidase digestion of these brain-derived oligomers quantitatively released sialitol (Fig. 5B, solid line), which was also released from [3H]colominic acid by mild acid hydrolysis (Fig. 5B, dotted line). Because exo-neuraminidase cleaves from the nonreducing terminus and because we observed quantitative release of [3H] present originally in the reducing terminus, we conclude that the brain-derived oligomers were composed solely of sialic acid.

To determine sialyl oligomers with DP of >5 were released from neural membranes at the earlier times of endo-neuraminidase treatment, the products after 15 min of digestion were reduced with borotritide prior to DEAE chromatography. As an internal control, unlabeled oligomers of 2, 3, 4, and 10 NeuNac residues were added to the radiolabeled sample. As shown in Fig. 4B, essentially all of the labeled oligomers eluted with a DP of 3 and 4. The material in peaks A–E in Fig. 4B was pooled and tested for sensitivity to exo-neuraminidase as described above. Peaks D and E, which
Fig. 4. DEAE chromatography of sialyl oligomers released from brain membranes by endo-neuraminidase. (A) Sialyl oligomers (177 μg of total sialic acid) released after 2 hr of incubation with endo-neuraminidase (see legend to Fig. 2) were fractionated and assayed for sialic acid as described in the legend to Fig. 3. The column was 0.9 × 30 cm with elution at 0.3 ml/min using 150 ml of a 0–0.4 M NaCl gradient; 2.5-ml fractions were collected. The collected fractions were lyophilized and redissolved in 0.2 ml of H2O, and thiobarbituric acid assays were performed after acid hydrolysis. (B) Sialyl oligomers (223 μg of total sialic acid) released after 15 min of incubation in the endo-neuraminidase (Fig. 2) were reduced with 10 μCi of potassium borotritide, desalted, and mixed with a tracer amount of [14C]NeuNAc and 100–200 μg each of unlabeled sialyl oligomer with DP of 2, 3, 4, and 10. The mixture was fractionated as in A with a 300-ml salt gradient at 0.4 ml/min; 3-ml fractions were collected and analyzed as indicated.

Fig. 5. Brain-derived sialyl oligomers are sensitive to exo-neuraminidase. (A) The reducing terminus of colominic acid (DP 10; dotted line) and NeuNAc-NeuNAc (dashed line) were labeled by reduction with borotritide (16). Sialyl oligomers in fractions 69, 71, and 73 (Fig. 4A) were combined and reduced with borotritide as above (solid line). Aliquots containing 3–16 × 10^6 dpm were spotted on Whatman no. 3MM paper and chromatographed for 24 hr in butyl acetate/acidic acid/H2O, 3:2:1 (vol/vol). O, origin. The arrow (↓) indicates the position to which [14C]NeuNAc migrated. (B) Colominic acid labeled in the reducing terminus was hydrolyzed for 2 hr at 80°C in 0.05 M H2SO4. The hydrolysate was neutralized with 0.2 M NaOH and chromatographed as in A (dotted line). Brain sialyl oligomers labeled with 3H in the reducing termini were digested for 5 hr at 28°C with 1 unit of V. cholerae exo-neuraminidase and chromatographed as above (solid line).

2.4-fold by 8-day-postnatal rat brain membranes. This result supported the presence of α-2,8-linked polysialosyl residues in 8-day-old rat brains. Additional evidence for this conclusion was obtained by using the mutant designated EV11, as described in the Materials and Methods. Membranes from EV11 were defective in the transfer of NeuNAc from CMP-NeuNAc to endogenous acceptor molecules (Fig. 6A). Sialyltransferase activity in EV11 membranes was restored when 8-day-old rat brain membranes were added as an exogenous acceptor (Fig. 6A). Activity was proportional to the amount of brain protein added over an 8-fold range. Under these assay conditions, essentially no NeuNAc was incorporated into brain membrane preparations alone. The possible involvement of a sialyl lipid-linked intermediate in this transference reaction has not been studied. Fig. 6A also shows that membranes similarly prepared from the liver failed to restore poly(sialic acid) synthesis, presumably because they lacked polysialosyl chains. This supposition was consistent with the lack of immunoreactivity of liver membranes with H.46 antibodies. Confirmation that the brain-dependent incorporation of NeuNAc by EV11 membranes was into α-2,8-linked poly(sialic acid) was shown by demonstrating the sensitivity of the product to endo-neuraminidase digestion. As illustrated in Fig. 6B, nearly all of the radiolabeled poly(sialic acid) synthesized after 3.5 hr by the exogenous addition of brain membrane was sensitive to endo-neuraminidase. Although structural studies on the number of sialyl residues added to the polysialosyl acceptor in brain by the bacterial sialyltransferase have not been carried out, we conclude that this system is amenable for detecting polysialosyl units in as little as 0.22 mg of rat membrane protein.

**DISCUSSION**

Polysialosyl moieties on membrane glycoproteins in developing chicken and rat brains are of current interest in neurobiology because they cap N-linked oligosaccharides of the complex type on N-CAM (5, 10). Knowledge is rapidly accumulating regarding developmentally associated changes in the protein moiety of N-CAM (6, 7). In contrast, nothing is known about the biosynthesis or factors regulating the temporal expression of the polysialosyl units in N-CAM. In anticipation of studies to examine these aspects in developing...
rat and chicken brains, we have developed three prokaryotic probes for use in detecting poly(sialic acid) in neuronal tissues. Both the H.46 antibodies and the endo-neuraminidase share the property of recognizing bacterial poly(sialic acid) whereas the membranous sialyltransferase shows specificity for transferring sialyl residues from CMP-NeuNAc to exogenous acceptors containing α-2,8-linked oligo- or poly(sialic acid). Finne has shown by mass fragmentation that the internal sialic acid residues in N-CAM are α-2,8-linked (5).

The fact that endo-neuraminidase abolished immunoreactivity to H.46 antibodies demonstrates that the antibodies specifically recognized α-2,8-linked poly(sialic acid) in brain and that the reactivity was not due to a nonspecific cross-reactivity. In addition to endo-neuraminidase sensitivity, brain immunoreactivity to H.46 antibodies was also abolished by pretreatment with V. cholerae exo-neuraminidase. This provides further evidence for the sialic acid-directed specificity of this antibody. The endo-neuraminidase has proven useful in our initial studies on the biosynthesis of poly(sialic acid) in brain for it allows us to quantitate the amount of NeuNAc incorporated into poly(sialic acid). These studies have shown that a Golgi-enriched preparation from neonatal rat brains incorporated NeuNAc from CMP-NeuNAc into polymeric products. At least 20% of the NeuNAc incorporated was sensitive to endo-neuraminidase digestion.

Although we have presented no direct evidence, we believe the poly(sialic acid) detected in brain membranes by H.46 antibodies is associated with an N-CAM. This follows from the fact that, like N-CAM, our brain-derived poly(sialic acid) was membrane-associated and that its presence was correlated with development of the central nervous system. It seems likely that the polysialosyl-specific probes described in this report will be of general use for structural and biosynthetic studies on these unusual polymers. They may also prove useful for in vivo experiments to discern the role of polysialosyl carbohydrate units in neuronal development.*

*Following submission of this manuscript, Finne et al. (20) reported that the same H.46 antiserum used in these studies formed immuno-complexes with rat and human brain tissue, thus confirming one of the conclusions reached here.

The excellent editorial and secretarial assistance of Linda Troy in preparing this manuscript is acknowledged. We are grateful to Drs. W. Vann and J. B. Robbins for the H.46 antiserum. These studies were supported in part by Research Grant AI-09352 from the National Institutes of Health.