Carbohydrate Recognition in the Peripheral Nervous System: A Calcium-dependent Membrane Binding Site for HNK-1 Reactive Glycolipids Potentially Involved in Schwann Cell Adhesion

Leila K. Needham and Ronald L. Schnaar

Departments of Pharmacology and Neuroscience, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract. The carbohydrate determinants recognized by the HNK-1 antibody are potential cell-cell recognition ligands in the peripheral nervous system (PNS). The HNK-1 reactive sulfoglucuronylneolacto (SGNL) glycolipids specifically support Schwann cell adhesion, suggesting the presence of a cell surface receptor specific for SGNL-oligosaccharides. We directly probed PNS membranes for receptors complementary to SGNL determinants using a synthetic radioligand consisting of radiiodinated serum albumin derivatized with multiple SGNL-oligosaccharides. A high-affinity, saturable, calcium-dependent binding site for this ligand was found in PNS myelin membranes. Binding activity was carbohydrate-specific (most potently inhibited by SGNL-lipids compared to other glycolipids) and PNS-specific (absent from comparable central nervous system membranes). The SGNL-specific binding activity on PNS membranes reported here may be involved in peripheral myelination or myelin stabilization.

Recently, unusual carbohydrate determinants recognized by the HNK-1 mAb and the monoclonal L2 antibodies have been proposed to function in neural recognition, perhaps as adhesive determinants (9, 26, 47). HNK-1 reactive species include a unique class of anionic glycosphingolipids, the sulfoglucuronylneolacto-lipids (SGNL-lipids), in addition to undefined, but presumably related, carbohydrate determinants on minor glycoforms of adhesive glycoproteins including NCAM, MAG, P0, P1, and J1/cytotactin (50). Consistent with a role in cell recognition, HNK-1 and L2 mAbs attenuate cell adhesion in several neural cell model systems in vitro (9, 26, 47). The observation that SGNL-lipids and SGNL-oligosaccharides perturb cell-cell and cell-substratum adhesion in vitro provides more direct evidence for a role of these HNK-1 reactive oligosaccharides as authentic adhesive ligands (29).

Further evidence for an adhesive role for SGNL-determinants comes from clinical studies. SGNL-lipids were first identified as antigens of monoclonal IgM's produced by a majority of patients with peripheral demyelinating neuropathy associated with IgM paraproteinemia (excess production of monoclonal IgM). These IgM paraproteins exhibit carbohydrate structural specificities similar to the HNK-1 and L2 antibodies (45, 62). The two major structurally characterized SGNL-lipids are closely related and consist of a nonreducing terminal glucuronic acid 3-sulfate attached (β1-3) to the terminal galactose of neolactotetraosylceramide or neolactohexaosylceramide (1, 6). SGNL-lipids are found in the adult peripheral nervous system (PNS) of many species (7, 23), but are not detected in adult cerebral cortex, although they are expressed in embryonic cortex and in adult cerebellum (8, 43). In the PNS, subcellular fractionation has localized SGNL-lipids to the axolemma-enriched fraction, as well as myelin and other Schwann cell membranes (27).

We have focused on the possible role of the SGNL-lipids as adhesive ligands recognized by Schwann cell surface receptors. The initial impetus for pursuing this hypothesis arose from the following observations. (a) The pathology and morphological changes in the subclass of patients with peripheral demyelinating neuropathy associated with a SGNL-reactive IgM paraprotein is consistent with a disruption of Schwann cell surface interactions necessary for myelin maintenance or myelination (44, 53). (b) SGNL-lipids are present in the membranes of axons and Schwann cells (27), and are therefore in the proper location to mediate axon-Schwann cell or myelin lamellae-lamellae interactions. (c) SGNL-lipids and SGNL-oligosaccharides are capable of inhibiting neural cell-cell and cell-substratum interactions in vitro (29). (d) There is a growing body of evidence that glycosphingolipids may function as determinants in cell-cell interactions involved in leukocyte and platelet adhesion to vascular endothelium and in neural cell recognition (3, 17, 51). In particular, the demonstration of a carbohydrate-specific ganglioside binding protein that is present in central nervous system (CNS) but not PNS myelin and which may be involved in oligodendrocyte-axon interactions (58, 59) raised the possibility that another class of anionic glycosphingolipids, the SGNL-lipids, may be playing a similar role in PNS myelin. More-
over, our previous studies demonstrated Schwann cell adhesion directly and selectively to SGNL-lipids, using a technique which detects adhesion of intact cells directly to chromatographically resolved lipids (57). When peripheral nerve lipids were resolved by TLC and overlaid with radio-labeled Schwann cells, the SGNL-lipids supported cell adhesion with greater potency than any other PNS lipids (38). The adhesion was cell type specific in that neither hepatocytes, fibroblasts, nor retina cells adhered to the SGNL-lipids (39). These data suggest the presence of a Schwann cell surface receptor specific for the SGNL-oligosaccharide.

To probe directly for the presence of a SGNL-oligosaccharide receptor in the PNS, we constructed a multivalent radioligand consisting of SGNL-oligosaccharides covalently attached to a radioiodinated carrier protein, BSA. Through the use of this ligand, we now report the properties of a complementary SGNL-specific binding site on PNS myelin.

**Materials and Methods**

**SGNL-lipid Purification**

SGNL-lipids were purified from dog sciatic nerve endoneurium (Pel-Freeze Biologicals, Rogers, AR), from bovine cauda equina (obtained at time of slaughter), or from human cauda equina (obtained at autopsy or kindly provided by the National Neurological Research Specimen Bank, Los Angeles, CA) using modifications of published procedures (1, 6). Tissue (~100 g wet weight, stored at ~70°C before use) was thawed, homogenized in a Polytron high-speed motor-driven homogenizer (dog and bovine tissue) or a Waring blender (human tissue), and extracted sequentially with 15 and 7.5 vol (relative to the original tissue wet weight) chloroform/methanol/water (4:8:3) (56); all solvent ratios are vol/vol. Precipitated protein was removed by centrifugation, and the supernatant evaporated to dryness. The resulting residue was extracted by incubation in 4 vol of chloroform/methanol/water (4:8:3) and a final ratio of chloroform/methanol/water of 4:8:5.6 (56). The resulting upper aqueous phase was readjusted to chloroform/methanol/water (4:8:3) and applied to a column (0.25 vol relative to original tissue weight) of DEAE-Sepharose Fast Flow (acetate form, Pharmacia LKB Biotechnology Inc., Piscataway, NJ) equilibrated in the same solvent. The column was eluted with 2 column vol of the starting solvent, 8 column vol of methanol, and 4 column vol of 0.1 M ammonium acetate in methanol, and 4 column vol of 0.1 M ammonium acetate in methanol to elute phospholipids, sulfatides, and gangliosides (18, 35). The SGNL-lipids were then eluted with 10 column vol of 0.2 M ammonium acetate in methanol. This fraction was concentrated on a rotary evaporator (Bürker, Switzerland), and dried on a vacuum centrifugal evaporator (Speedvac, Savant) to remove volatile salts. The residue was dissolved in 8 ml chloroform: methanol/water (70:30:4), and applied to a 1-ml column of Iatrobeads silicic acid (Iatron Laboratories, Tokyo, Japan). The column was washed with 6 ml chloroform/methanol/water (70:30:4), and SGNL-lipids were eluted in 2 ml chloroform/methanol/water (60:35:8) followed by 4 ml of 10:10:3. The latter two fractions were pooled, evaporated, and dissolved in chloroform/methanol/water (2:4:3:55), and applied to two prewashed C18 Sep-Pak cartridges (Waters) in tandem (60). The cartridges were washed with 16 ml water, 8 ml methanol/water (2:1), and then SGNL-lipids were eluted with 16 ml methanol/water (4:1) and 8 ml methanol. The purified SGNL-lipids were pooled, solvents evaporated, and the residue dissolved in chloroform/methanol/water (4:8:3) for storage.

The purification of the SGNL-lipids was monitored by TLC on silica gel HPTLC plates (E. Merck, Darmstadt, FRG) using chloroform/methanol/0.25% KCI (60:35:8) as developing solvent and a variety of lipid stains to detect containing glycolipids and phospholipids including resorcinol (specific for gangliosides [12, 55]), N-acetylglucosaminide dihydrochloride (specific for sugars [2]), Azure A (specific for sulfated compounds [22]), and cupric sulfate/phosphoric acid (non specific lipid stain) [61]. In the purified SGNL-lipid fraction (from all three species), two major components were detected with N-acetylglucosaminide, Azure A, and cupric sulfate/phosphoric acid: the most abundant species (60-90%) depending on the species, Rf = 0.20-0.24), and a less abundant species (10-35%, Rf = 0.09-0.14). In addition, a third species of low abundance (~5%, Rf = 0.05) was detected with these three stains in the purified SGNL-lipid fraction derived from bovine cauda equina. No resorcinol positive material was detected in these fractions. The three species were also detected with HNK-1 antibody and the two faster migrating species comigrated with authentic SGNLα- and SGNLε-lipids (kindly provided by Dr. Robert Yu, Virginia Commonwealth University, Richmond, VA). The HNK-1 immuno-overlay procedure was modified from the procedure of Magnani (35) as follows. After resolution of the lipids by HPTLC, the TLC plates were dried at 50°C for 1 h, and then treated for 30 s sequentially with hexane and with 0.01% poly(isobutylmethacrylate) (Aldrich Chem. Co., Milwaukee, WI) in hexane, after which they were dried under a stream of air. After the plates were prewet by spraying with divergent cation-free phosphor-bronzer saline (CMF-PBS) (16), they were incubated in 4°C with HNK-1 antibody (Leu-7, Becton Dickinson, Mountain View, CA) at a dilution of 1 μg/ml in CMF-PBS containing 0.2% BSA. The TLC plate was washed by transfer into CMF-PBS and bound antibody localized by incubation of the plate with biotinylated goat anti-mouse IgM and with avidin/biotinylated horseradish peroxidase complex (Vector Labs, Inc., Burlingame, CA) diluted in CMF-PBS, followed by peroxidase detection using diaminobenzidine as substrate according to the manufacturer’s instructions for processing nitrocellulose blots.

The concentration of SGNL-lipids was determined by quantitative TLC in the above solvent system and 2% Azure A in 1 mM sulfuric acid to stain sulfated compounds (22). Sulfatides were used as quantitative standards. Alternatively, the concentration of SGNL-lipids was determined by saccharide densitometric analysis on a Diamon HPLC system (see below). The two methods yielded essentially identical results. In addition, SGNL-lipids were characterized by fast atom bombardment MS (see below).

**Synthesis of [125I]SGNL-BSA**

SGNL-oligosaccharide was released from the parent lipid using ceramide glycans (V-Labs, Covington, LA) under conditions described previously (63). The optimal procedure is as follows. SGNL-lipid (2.6 μmol) isolated from dog sciatic nerve endoneurium was treated with 17 U ceramide glycans for 24 h at 37°C in 6.5 ml 50 mM sodium acetate buffer, pH 5.0, containing 0.75 mg/ml sodium cholate. The reaction was stopped by the addition of 19.5 ml of acetonitrile/water (1:2) and the solution passed over two C18 reverse phase cartridges in tandem to remove detergent and ceramide (6). The purified SGNL-oligosaccharide was conjugated to BSA by reductive amination using pyridine borane as reducing reagent (4). SGNL-oligosaccharide (~2 μmol) was added in ~50-fold molar excess to 2.7 mg BSA (essentially fatty acid- and globulin-free, Sigma Chem. Co., St. Louis, MO) in 320 μl of 0.1 M sodium phosphate buffer, pH 7.0, containing 100 mM sodium borohydride and pyridine borane (40 μl each) and the reaction was allowed to proceed for 5.5 h at 50°C under nitrogen. Analysis of the reaction mixture by silica gel HPTLC (E. Merck) using n-butanol/acetic acid/water (2:1:1) as the developing solvent and 13 mM N-(n-bnapththyl)ethylenediamine dihydrochloride in 3% sulfuric acid in methanol to visualize sugars (2) revealed the disaccharide homolog of the two oligosaccharide bands (Rf = 0.11 and 0.05), and a concomitant increase in saccharide-positive material at the origin, as expected for saccharides conjugated to protein. The reaction mixture was extracted with ethyl acetate to remove pyridine borane and SGNL-BSA was purified by gel permeation chromatography on Sephadex G-25 and anion exchange HPLC on a Beckman DEAE (50W) column, as described (58). Purified SGNL-BSA was radiolabeled using Na[125I] (Amersham Corp., Arlington Heights, IL) and iodobeads (Pierce Chem. Co. Rockford, IL) as previously described (58) to initial specific activities of 200–600 Ci/mmol.

**[125I]SGNL-BSA Characterization**

[125I]SGNL-BSA was subjected to gradient SDS-polyacrylamide electrophoresis (31) as follows. Aliquots of [125I]SGNL-BSA (~100 fmol) were boiled in sample buffer containing SDS and β-mercaptoethanol and loaded onto a 10–20% gradient polyacrylamide mini-gel (Integrated Separation Sys., Natick, MA). Electrophoresis was conducted at 40 mllamperes constant current on a Hoeffer Mighty Small II apparatus. The gel was blotted onto nitrocellulose in using a BioRad "Transblot" electrophoretic transfer apparatus. After transfer in 25 mM Tris base 192 mM glycine 20% methanol for 3 h at 70 V, the blot was dried and exposed to X-ray film to obtain an autoradiographic image, and then probed for HNK-1 reactive oligosaccharide immunolocalization. The blot was preincubated with 3% BSA in 10 mM Tris buffer, pH 7.4, containing 15 mM NaCl, and then incubated with...
Peripheral Nerve Endoneurium Subcellular Fractionation

Rat sciatic nerve myelin was prepared according to modifications of the methods of Oulton and Mezey (42) by Tiemeyer et al. (59). Rat sciatic nerve endoneurium was dissected from rat sciatic nerve (Pel-Freeze) in CMF-PBS, the endoneurium was collected by centrifugation, resuspended in 0.29 M sucrose, and stored at -70°C until use. Thawed endoneurium (~2 g, from 125 nerves) was homogenized in 20 ml of 0.29 M sucrose using a Polytron tissue disruptor and the homogenate was centrifuged at 10,000 g for 10 min to remove disruptive tissue, unbroken cells, and nuclei, yielding a crude supernatant "SI" fraction. In some experiments, a small portion of the SI fraction was centrifuged (72,000 g, 45 min), and the pellet was resuspended in 1 ml of 0.29 M sucrose, diluted 10-fold with 10 mM EGTA in 1 mM Hepes, pH 7.5, and recentrifuged. The resulting pellet was resuspended in a small volume of 0.29 M sucrose to yield the mixed membrane "P2" fraction. The remainder of SI was diluted to 24 ml with 0.29 M sucrose and 12 ml were overlaid onto each of two discontinuous sucrose gradients consisting of 10 ml 0.8 M sucrose over 10 ml 1.2 M sucrose. The gradients were centrifuged at 72,000 g for 2.5 h in a swinging bucket ultracentrifuge rotor. The material banding at the 0.29/0.8 M and 0.81/1.2 M sucrose interfaces was collected with a pasteur pipette (~6 ml wet weight of SI) in 10 mM EGTA in 1 mM Hepes, pH 7.5, and recentrifuged at 72,000 g for 45 min. The pellets from the 0.29/0.8 M sucrose interfaces and from 0.81/1.2 M sucrose interfaces were resuspended in small volumes of 0.29 M sucrose to yield a myelin enriched fraction, SIA (from the material forming a compact white band at the 0.29/0.8 M sucrose interface), and a denser mixed membrane fraction, SIB (from the material forming a diffuse white band at the 0.81/1.2 M sucrose interface). In some experiments, a portion of the SIA fraction was further subjected to discontinuous sucrose shock centrifugation as follows. As aliquot (~3 ml) of the SIA fraction was diluted with 10 vol of 10 mM EGTA in 1 mM Hepes, pH 7.5, homogenized manually (glass teflon, 5 strokes), and incubated on ice for 10 min with 10 mM EGTA at 72,000 g for 45 min, the pellet was resuspended in 6 ml of 0.29 M sucrose with trituration and brief homogenization, and resubjected to discontinuous sucrose gradient as described above, except that only the material banding at the 0.29/0.8 M sucrose interface was collected by centrifugation and resuspended in a small volume of 0.29 M sucrose to yield a purified myelin fraction, S2. All fractions were stored frozen in small aliquots before use. Protein content was determined using the biuret method (52) with BSA as a standard.

Lipid and Anionic Saccharide Inhibitors

Sulfatide and globotetraosylceramide were obtained from Matreya, Inc. (Chelmont, PA); cholesterol-3'-sulfate, galactosylceramide, and anionic saccharides (except SGNL-oigosaccharide, see above) from Sigma Chem. Co.; phospholipids were obtained from Avanti Polar Lipids (Birmingham, AL) or from Sigma Chem. Co.; and individual purified bovine brain gangliosides were obtained either from Sigma Chem. Co. or E-Y Laboratories (San Mateo, CA). Synthetic 2,3-sialyl- and 2,6-sialyl Lewis x glycolipids (25) were prepared by Dr. A. Hasegawa, Department of Applied Organic Chemistry, Gifu University, Japan, and were kindly provided by Dr. Brian Brandley, Glycomed, Inc., Alameda, CA. Sialylolactotetraosylceramide, Sialylgalactolactotetraosylceramide, and Sialylgangliotetraosylceramide were kindly provided by Paul James, Glycomed, Inc., Alameda, CA. Sialylgalactolactotetraosylceramide was prepared by chemical modification of human cauda equina SGNL-lipid by the method of Chou et al. (6). The purity and the concentrations of the products were determined by quantitative TLC on silica gel HPTLC plates (E. Merck) using chloroform/methanol/0.25% KCI as developing solvent, cupric sulfate/phosphoric acid (61) for detection, and the parent SGNL-lipid as a standard for quantitation. Each derivative appeared as a single major species which migrated distinctly from the parent SGNL-lipid. The Rf values were as follows: Parent lipid (predominantly IV3glucuronylneolactotetraosylceramide V3-sulfate), 0.35; desulfated SGNL-lipid (IV3glucuronylneolactotetraosylceramide), 0.39; and methyl esterified desulfated SGNL-lipid (IV3glucuroylneolactotetraosylceramide V3-methyl ester), 0.59. The identity of each lipid was confirmed by fast atom bombardment mass spectrometry in the negative ion mode using a Finnigan MAT 900 spectrometer with triethylamine as matrix as kindly performed by Dr. Hans Schweingruber, Glycomed, Inc., Alameda, CA. Characteristic of glycoprophingolipids (20), the
parent SGNL-lipid produced a family of molecular ions representing heterogeneity in the ceramide (lipid) moiety, with the major molecular ion (M-1) at 1593, corresponding to a ceramide composed of C18 sphingosine and a C26 fatty acid amide as previously reported (6). The major molecular ion (M-1) generated by the desulfated species was 1513, as expected, and the methyl ester generated the same molecular species (M-CH3) as well as the molecular ion (M-1) at 1527 (24).

Results

SGNL-derivatized Radioligand Synthesis and Characterization

A polyvalent SGNL-derivatized radioligand was prepared by covalently linking SGNL-oligosaccharides enzymatically released from SGNL-lipids to a carrier protein, BSA. To this end, efficient methods for the purification of SGNL-glycolipids from peripheral nerve and the production of SGNL-oligosaccharides were developed. Dog sciatic endoneurium was chosen as the tissue source because of its relatively high abundance of SGNL-lipid (23). The procedure for the purification of SGNL-lipids was based upon the unexpected observation that these highly polar lipids are differentially insoluble in chloroform/methanol (1:1). When a chloroform/methanol/water (4:8:3) lipid extract from dog sciatic endoneurium was evaporated to dryness and the residue reextracted with chloroform/methanol (1:1), ~98% of the neutral lipids, phospholipids, and glycolipids (including gangliosides) were solubilized (30), while ~70% of the SGNL-lipids were recovered in the insoluble residue. The SGNL-lipids were further purified from lipid and polypeptide contaminants by partitioning, DEAE-Sepharose chromatography, silicic acid chromatography, and reverse-phase chromatography to yield ~2.6 μmol of SGNL-lipids from 100 g (wet weight) of dog sciatic nerve endoneurium. Purified SGNL-lipids consisted of two major species, resolved by TLC, which correspond to the previously described (1,6) closely related structures IVg glucuronylneolactotetraosylceramide V1-sulfate (SGNLγ-lipid, 60%) and VIg glucuronylneolactohexaosylceramide VII-sulfate (SGNLδ-lipid, 40%) (Fig. 1). These species comigrated with known standards (kindly provided by Dr. Robert Yu, Virginia Commonwealth University, Richmond, VA) and were reactive with HNK-1 antibody (data not shown).

Figure 1. TLC analysis of SGNL-lipid purification from dog sciatic nerve endoneurium. Aliquots of lipid fractions derived from dog sciatic endoneurium were applied to high performance TLC plates, developed in chloroform/methanol/0.25% aqueous KCl (60:35:8), and visualized by CuSO4-phosphoric acid char (61). Lipids were applied in amounts relative to the indicated starting wet weight of tissue: 1 mg (lane 1) and 2.5 mg (lane 2) equivalents of total lipid extract; 2.5 mg (lane 3) and 50 mg (lane 5) equivalents of the insoluble lipid residue resulting from reextraction of total lipids with chloroform/methanol (1:1); 2.5 mg equivalents (lane 4) of the lipids soluble in the chloroform/methanol (1:1) reextraction; and 50 mg equivalents (lane 6) of the purified SGNL-lipid fraction derived from further fractionation of the chloroform/methanol (1:1) insoluble residue as detailed in Materials and Methods.

Treatment of the SGNL-lipids with leech ceramide glucanase (63) resulted in essentially complete conversion of the SGNL-lipids to the corresponding SGNL-oligosaccharides and ceramide. The oligosaccharides were purified by reverse-phase chromatography and the purity of the products was confirmed by silica gel TLC developed in n-butanol/acetic acid/water (2:1:1). The oligosaccharides were detected as two species which migrated slower than the parent lipid species (SGNLγ-lipid Rf = 0.49, SGNLδ-lipid Rf = 0.28, SGNLγ-oligosaccharide Rf = 0.11, SGNLδ-oligosaccharide Rf = 0.05) (data not shown). The SGNL-oligosaccharides were coupled to BSA by reductive amination, and the resulting SGNL-BSA conjugate was purified by DEAE-HPLC and gel filtration chromatography. The SGNL-BSA was labeled to high specific activity with Na125I for use as a radioligand (subsequent to labeling, 5 mg/ml of underivatized BSA was added to protect against radiation-induced damage).

The derivatization ratio of the SGNL-BSA conjugate was determined by hydrolysis and saccharide analysis. Since the derivatization ratio of the SGNL-BSA conjugate was determined by hydrolysis and saccharide analysis. Since the glucuronic acid–galactose glycosidic linkage is resistant to hydrolysis, the conjugate was hydrolyzed after methyl esterification and reduction of the glucuronic acid to glucose. Control hydrolysis of SGNL-lipids treated under identical conditions in the presence of underivatized BSA resulted in a galactose recovery which was 98% of theoretical. Sacccharide analysis of the hydrolyzed SGNL-BSA conjugate yielded ratios of galactose/N-acetylglycosamine (detected as glucosamine)/glucose of 2.4:1.56:1.2 (expected monosaccharide ratios for reduced SGNL-oligosaccharides (60% SGNLδ/40% SGNLγ) = 2.4:1.4:1). Based on the galactose recovery from the SGNL-BSA derivative, an average of 7.5 SGNL-oligosaccharides were attached per BSA molecule. In addition, laser desorption mass spectroscopy was performed on the parent BSA and the SGNL-BSA conjugate. The parent BSA spectrum exhibited a peak of molecular ions centered at 68,400 mass units, while the SGNL-BSA derivative spectrum exhibited a broader peak of molecular ions centered at 75,000 mass units (data not shown). The difference, 6,600 mass units, is equivalent to a derivatization ratio of 6 SGNL-oligosaccharides attached per BSA molecule. The covalent attachment of intact SGNL-oligosaccharides to BSA was confirmed by SDS-PAGE of radiiodinated SGNL-BSA and Western blot analysis with HNK-1 antibody, which requires the presence of the sulfate on the SGNL-lipid for recognition
The radioiodinated conjugate migrated as a broad band, with a major radioiodinated product comigrating with the HNK-1 reactive band at an apparent molecular weight greater than that of BSA (Fig. 2). No HNK-1 binding was detected against underivatized BSA (data not shown). The derivative is denoted (SGNL)-BSA, or simply SGNL-BSA, to be consistent with prior neoglycoconjugate terminology (28).

**125I-SGNL-BSA Binding to Rat Peripheral Nerve Myelin Membranes**

Rat PNS myelin–enriched membranes were isolated by differential centrifugation and discontinuous sucrose gradient fractionation of rat sciatic nerve endoneurium. When 125I-SGNL-BSA (0.7 nM) was incubated with increasing amounts of rat PNS myelin–enriched membranes and the bound ligand separated from the free 125I-SGNL-BSA by filtration, membrane-dependent binding was readily detected. Background binding was very low, \( \sim 0.4\% \) of the total added radioligand in the absence of added membranes. Initial experiments demonstrated that 10 \( \mu \)M SGNL-lipid reduced the binding of the 125I-SGNL-BSA to the same low background levels, as did omitting calcium from the binding buffer (see below). In subsequent experiments, specific binding was defined as binding to membranes in the presence of 20 mM CaCl\(_2\) (total) less binding in the absence of calcium or in the absence of membranes (nonspecific). Binding increased proportionally with added membranes up to 1 \( \mu \)g membrane protein (Fig. 3).

In the presence of 0.5 nM 125I-SGNL-BSA, binding to rat PNS membranes reached equilibrium within 30 min at 4°C (Fig. 4, filled circles). This incubation time was chosen for subsequent binding studies. Binding as a function of the amount of membranes added indicated that the maximum amount of radioligand bound was well below that added (see Fig. 3). This was not unexpected, since the synthetic multivalent ligand is inherently heterogeneous (see below). To determine if the 125I-SGNL-BSA which remained unbound was competent to bind to fresh membranes, membrane-bound radioligand was separated from free radioligand by filtration and a second aliquot of membranes was added to the free 125I-SGNL-BSA in the filtrate. Radioligand bound after a second incubation of 30 min was then determined (Fig. 4, open circles). The sum of the 125I-SGNL-BSA bound after the first and second incubations, expressed as a percent of the input ligand, was constant and indicated that \( \sim 6\% \) of the 125I-SGNL-BSA was competent to bind to rat PNS myelin–enriched membranes (Fig. 4, triangles). Inclusion of the protease inhibitors phenylmethylsulfonylfluoride, leupeptin, antipain, benzamidine, aprotinin, chymostatin, and pepstatin in the binding buffer did not affect the kinetics of binding nor the maximal amount of radioligand bound (data not shown). To evaluate the molecular heterogeneity of the bound and the free 125I-SGNL-BSA, membrane-bound radioligand was separated from free radioligand by centrifugation, and the two pools were analyzed by Western blot HNK-1 immunostaining and autoradiography subsequent to SDS-PAGE. Both pools were HNK-1 reactive: the free 125I-SGNL-BSA was indistinguishable from the total 125I-SGNL-BSA, while the bound 125I-SGNL-BSA exhibited enrichment for higher molecular weight HNK-1 reactive iodinated species. (No HNK-1 staining was detected against rat PNS membranes alone, data not shown.)

Binding isotherms were performed with increasing concentrations of 125I-SGNL-BSA incubated for 30 min with 0.25 \( \mu \)g membrane protein. As the concentration of 125I-SGNL-BSA was increased, nonspecific background binding of radioligand (binding to filters in the absence of membranes but presence of calcium [Fig. 5 A, triangles], or in the presence of membranes but absence of calcium [not shown]) increased in a nonsaturable, linear manner as expected. Specific binding of 125I-SGNL-BSA to rat PNS myelin–enriched membranes was saturable and exhibited high
Using a single site model resulted in a $K_0$ of 320 pM and a lower-affinity class $K_D = 460$ pmol/mg membrane protein; lower-affinity class $K_D = 460$ pmol/mg membrane protein). For determining higher-affinity subpopulation of binding sites when fit to a two site model (high-affinity class $K_0 = 17$ pM, $B_{\text{max}} = 38$ pmol/mg membrane protein; lower-affinity class $K_0 = 460$ pM, $B_{\text{max}} = 330$ pmol/mg membrane protein). For determining binding constants, the unbound (free) ligand concentration was calculated based on the proportion of total $^{125}$I-SGNL-BSA competent to bind rat PNS myelin membranes (see Fig. 4), which was experimentally determined in a parallel binding assay containing 0.5 nM ligand and an excess (10 $\mu$g) of membrane protein.

Experiments to test whether SGNL-specific antibodies (23) would block $^{125}$I-SGNL-BSA binding to membranes were inconclusive, since control immunoglobulins (mouse IgM and IgG) interfered with the binding assay as did the corresponding monoclonals HNK-1 (IgM) and F7F7 (IgG) (data not shown). The basis of the interference was not determined.

**Figure 4.** Kinetics of $^{125}$I-SGNL-BSA binding to rat PNS myelin and competency of the unbound radioligand to bind fresh membranes. Rat PNS myelin-enriched membranes (fraction SIA) were incubated with 0.5 nM $^{125}$I-SGNL-BSA under standard conditions except that the volume of each binding reaction was increased 8-fold to 2 ml and the total membrane protein added to each reaction was 60 $\mu$g. After the indicated times at 4°C, the reaction was filtered through glass fiber filters (Whatman GFC) and the filtrate collected for further use (see below). The filters were then rapidly washed, collected, and radioactivity quantitated to determine binding kinetics (o). To 225 $\mu$l of the binding assay filtrate collected at each indicated time was added fresh myelin membranes (7.5 $\mu$g in 25 $\mu$l 0.29 M sucrose). After an additional 30 min at 4°C, ligand bound to membranes after this second incubation was separated from free ligand by filtration and quantitated as in the standard assay to determine the percent of unbound ligand at each time which was competent to bind to fresh membranes (o). Nonspecific binding (in the absence of CaCl$_2$, < 0.8% of added ligand) was determined in parallel incubations and was subtracted from total binding. Data are presented as percent of added ligand specifically bound during the initial incubation (e), after the second incubation with fresh membranes (o) and the sum of these two incubations (zx). Values are the mean ± SD for replicate determinations (SD values which fall within the symbols are not shown).

**Figure 5.** Saturation isotherms for $^{125}$I-SGNL-BSA binding to rat PNS myelin. (A) Rat PNS myelin-enriched membranes (fraction SIA, 0.25 $\mu$g protein) were incubated with increasing concentrations of $^{125}$I-SGNL-BSA in 250 $\mu$l binding buffer containing 20 mM CaCl$_2$. After 30 min at 4°C, membrane-bound ligand was separated from free ligand by filtration (o, total ligand bound). Nonspecific ligand bound (zx) was determined in parallel incubations in the absence of membranes, and was subtracted from total bound to calculate specific bound (e). Values are the mean ± SD for quadruplicate (total binding) or triplicate (nonspecific binding) determinations (SD values that fall within the symbols are not shown). The abscissa is the concentration of free $^{125}$I-SGNL-BSA competent to bind PNS myelin membranes (see text). Free ligand was calculated as the amount of radioligand added times the proportion of ligand (5.3%) competent to bind (to an excess of membranes, 10 $\mu$g) less the specifically bound ligand. The solid line was generated by a nonlinear fit of the specific binding data using a single site binding model and yielded the indicated apparent dissociation constant ($K_0$) and total receptor density ($B_{\text{max}}$). (B) Scatchard transformation of specific $^{125}$I-SGNL-BSA binding to rat PNS myelin. Data for the specific binding isotherm (Fig. 5 A) was transformed by the method of Scatchard (49) (e). The dashed lines were fit to the curve using a two-site model and yielded the indicated apparent dissociation constant ($K_0$) and total receptor concentration ($B_{\text{max}}$) values.

**Divalent Cation, Ionic Strength, and pH Effects on $^{125}$I-SGNL-BSA Binding**

Ligand binding was studied as a function of divalent cation concentration, using a variety of divalent cations over a broad concentration range (Table I). Divalent cations were required for binding and calcium supported higher specific binding than other divalent cations tested. Half-maximal binding of $^{125}$I-SGNL-BSA to rat PNS myelin occurred at 5 mM Ca$^{2+}$, with maximal binding at 10 mM Ca$^{2+}$ (Fig. 6).
Table 1. Effects of Divalent Cations on 125I-SGNL-BSA Binding to PNS Myelin

| Divalent ion | Concentration (mM) | 125I-SGNL-BSA Bound* (% of control) |
|-------------|-------------------|------------------------------------|
| None        | 2.5               | 0.1 ± 0.3                           |
| SrCl2       | 2.5               | 7.9 ± 3.9                           |
|             | 5                 | 25 ± 3.3                            |
|             | 10                | 51 ± 6.9                            |
|             | 20                | 88 ± 9.4                            |
|             | 30                | 62 ± 3.2                            |
|             | 40                | 45 ± 3.9                            |
| MgCl2       | 2.5               | 11 ± 9.8                            |
|             | 10                | 21 ± 6.8                            |
|             | 20                | 38 ± 2.8                            |
|             | 30                | 35 ± 3.2                            |
| BaCl2       | 2.5               | 7.4 ± 1.0                           |
|             | 10                | 27 ± 3.0                            |
|             | 20                | 34 ± 4.5                            |
|             | 30                | 21 ± 1.9                            |
| CoCl2       | 2.5               | 9.4 ± 4.5                           |
|             | 10                | 27 ± 3.0                            |
|             | 20                | 33 ± 2.9                            |
|             | 30                | 20 ± 2.7                            |
| MnCl2       | 2.5               | 4.4 ± 2.2                           |
|             | 10                | 10 ± 3.8                            |
|             | 20                | 26 ± 10                             |
|             | 30                | 35 ± 4.4                            |
|             | 40                | 52 ± 2.7                            |

* Specific binding was determined at each salt concentration by subtracting nonspecific binding in the absence of membranes from total binding in the complete reaction. Values are the mean ± SD for triplicate determinations.

Among other divalent cations tested, Sr\(^{2+}\) supported maximal binding that was 88% of that in the presence of Ca\(^{2+}\), while other divalent cations (Mg\(^{2+}\), Ba\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\)) supported much less binding (Table I). While divalent cations were required for binding, they could be omitted from the filter wash buffer without reducing specific binding, suggesting that little dissociation occurred during the rapid (~1 min) wash. Subsequent experiments were performed with divalent cation omitted from the filter wash.

Increasing ionic strength decreased specific binding, as can be seen with higher calcium chloride concentrations (Fig. 6). Similarly, addition of MgCl\(_2\), MgSO\(_4\), NaCl, or KCl reduced radioligand binding to half-maximal levels at concentrations ranging from ~25 to 50 mM. No significant variation in binding was detected over the pH range of 6.0–8.0 (in 50 mM imidazole acetate buffer).

Subcellular and Nervous System Distribution of 125I-SGNL-BSA Binding

Peripheral nerve membrane fractions were isolated by differential centrifugation and discontinuous sucrose gradient centrifugation. Sciatic nerve endoneurium homogenate was centrifuged (low speed) to yield a crude supernatant fraction (SI), a portion of which was centrifuged at higher speed to yield a mixed membrane (P2) fraction. Most of the crude SI was subjected to discontinuous sucrose gradient centrifugation to yield myelin-enriched membranes (SI1A) and denser mixed membranes (SIB). A portion of SI1A was further subjected to osmotic shock and a second discontinuous sucrose gradient. To generate myelin fraction S2 (42), 125I-SGNL-BSA binding was clearly enriched in fractions containing PNS myelin (Fig. 7 A, Table II), while denser PNS membranes (SIB) had little binding activity.

Membrane fractions were also prepared from the CNS for comparison to PNS myelin binding of 125I-SGNL-BSA. A mixed membrane P2 fraction prepared from whole rat brain by differential centrifugation (11) and consisting of myelin membranes, synaptosomes, and mitochondria, had very low binding activity (Fig. 7 B), as did brain membranes further fractionated by discontinuous sucrose density centrifugation to yield crude myelin (P2A) and crude synaptosomal (P2B) fractions (19). Under identical conditions, PNS myelin membranes bound 20–40-fold more 125I-SGNL-BSA than did any of the CNS membrane fractions.

Among adult CNS brain regions, SGNL-lipids are found exclusively in the cerebellum (43). Therefore, cerebellar P2 membranes were prepared and tested for 125I-SGNL-BSA binding activity. Like whole brain membrane fractions, cerebellar membranes failed to bind significant ligand (Fig. 7 B).

Carbohydrate Specificity of 125I-SGNL-BSA Binding

Various compounds including SGNL-lipids and other sulfolipids, gangliosides, neutral glycosphingolipids, phospholipids, mono- and oligosaccharides, glycosaminoglycans, and other anionic polysaccharides, were tested for their ability to inhibit 125I-SGNL-BSA binding to rat PNS myelin-enriched membranes.

SGNL-lipids isolated from two sources, human and bovine cauda equina, were the most potent of the lipids tested.
The sulfated lipids sulfatide and cholesterol sulfate inhibited with moderate potency (IC₅₀'s ≈ 8 μM).

Neutral glycosphingolipids and most phospholipids failed to inhibit half-maximally even at 100 μM. Phosphatidylglycerol did inhibit, although its inhibition was dependent upon the fatty acid content. Synthetic phosphatidylglycerol having fully saturated fatty acids was noninhibitory (at 100 μM), while phosphatidylglycerol having saturated fatty acids inhibited with an IC₅₀ of 8.3 μM. The significance of this observation is unknown.

Figure 7. Binding of ¹²⁵I-SGNL-BSA to rat PNS endoneurium and brain subcellular fractions. (A) Rat peripheral nerve endoneurium homogenate was fractionated by differential centrifugation (▲, crude homogenate, S1; ▼, mixed membranes, P2) and discontinuous sucrose gradient centrifugation of S1 (●, myelin-enriched, SIA; ◦, dense membranes, SIB), or of osmotically shocked SIA (●, S2) as described in Materials and Methods. (B) Whole rat brain homogenate was fractionated by differential centrifugation (▼, mixed membranes, P2) and discontinuous sucrose gradient centrifugation (○, myelin-enriched, S1A; ◦, synaptosomes, P2B), and rat cerebellum homogenate was fractionated by differential centrifugation (●, mixed membranes, cerebellar, P2) as described in Materials and Methods. Specific binding of 0.5 nM ¹²⁵I-SGNL-BSA to aliquots of PNS or CNS fractions containing the indicated amounts of protein was determined as described in Fig. 3. Values are the mean ± SD for triplicate determinations (SD values which fall within the symbols are not shown).

Table II. ¹²⁵I-SGNL-BSA Binding Activity to Peripheral Nerve Membrane Fractions

| Fraction | Total protein (mg) | Binding activity (pmol/mg protein) | Binding recovery (%) | Binding enrichment (fold) |
|----------|-------------------|-----------------------------------|----------------------|--------------------------|
| S1       | 42.8              | 3.8 ± 1.0                         | 100                  | 1.0                      |
| P2       | 23.8              | 5.8 ± 1.0                         | 85                   | 1.5                      |
| S1A      | 17.7              | 10.7 ± 1.2                        | 116                  | 2.8                      |
| S1B      | 0.8               | 3.0 ± 1.7                         | 1.5                  | 0.8                      |
| S2       | 6.8               | 11.5 ± 2.8                        | 48                   | 3.0                      |

Specific binding of ¹²⁵I-SGNL-BSA to peripheral nerve homogenate (S1), crude membranes (P2), and fractions prepared by successive discontinuous sucrose gradient centrifugation (S1A, SIB, and S2) as detailed in Materials and Methods was determined as described in the legend to Fig. 3. The specific binding activity is reported as the mean ± SD for three determinations performed in triplicate in the linear protein range (0.1, 0.25, and 0.5 μg membrane protein, see Fig. 7).

The lipids tested were ●, SGNL-lipid (human); ▲, sulfatide; ○, cholesterol sulfate; ◦, Gα₄; and ▼, Gα₅. Non-inhibitory lipids (not shown) are listed in Table III.
Table III. Inhibition of 125I-SGNL-BSA Binding to PNS Myelin by Lipids

| Lipids (n) | IC50 (range) |
|-----------|-------------|
| SGNL-lipid, bovine (5) | 0.99 (0.55-1.2) |
| SGNL-lipid, human (7) | 0.48 (0.3-0.6) |
| Desulfated SGNL-lipid, human (2) | 1.5 (1-2) |
| Methyl esterified, desulfated SGNL-lipid, human (1) | >25 |
| sulfatide (4) | 8.2 (4-12) |
| cholesterol sulfate (2) | 10 (6.2-14) |
| sialyneolactotetraosylceramide (1) | 40 |
| 2,3 sialyl Lewis x glycolipid (1) | >10 |
| 2,6 sialyl Lewis x glycolipid (1) | >10 |
| GTb (4) | 33 (6-64) |
| GD, (2) | 58 (15->100) |
| GDib (2) | >100 |
| GMI (1) | >100 |
| globotetraosylceramide (1) | >100 |
| galactosylceramide (1) | >100 |
| phosphatidylglycerol (2)* | >100 |
| phosphatidylinositol (1) | >100 |
| phosphatidylinositolbisphosphate (1) | >100 |
| phosphatidylserine (1) | >100 |
| phosphatidic acid (1) | >100 |
| phosphatidylycholine (1) | >100 |
| phosphatidylethanolamine (1) | >100 |

Inhibition studies were performed as described in the legend to Fig. 8. The mean and range of concentrations of each inhibitor resulting in 50% inhibition (IC50) of specific 125I-SGNL-BSA binding in the absence of inhibitor was determined graphically from the indicated number (n) of independent binding experiments performed at four to seven concentrations of each inhibitor in triplicate. Control binding was comparable to that shown in Fig. 3. * Results are for phosphatidylglycerol having unsaturated fatty acid chains (C18:1). The same phospholipid having a mixture of natural fatty acids had an IC50 of 8.3 µM. Other phospholipids tested had natural fatty acids.

The molecular determinants responsible for inhibition of 125I-SGNL-BSA binding to rat PNS myelin were investigated using chemically modified SGNL-lipid. Treatment of the SGNL-lipid with methanolic HCl resulted in concomitant removal of the 3-O-sulfate from the nonreducing terminal glucuronic acid and methyl esterification of the carboxylic acid at the 6-position of the glucuronic acid (6). A portion of the methyl esterified glycolipid was converted to the desulfated parent molecule by alkali treatment (6). Desulfated SGNL-lipid remained one third as potent as the parent SGNL-lipid in inhibiting 125I-SGNL-BSA binding to rat PNS myelin. In contrast, methyl esterification of the desulfated SGNL-lipid blocked its ability to inhibit (Fig. 9, Table III).

Anionic monosaccharides (glucuronic acid and N-acetylneuraminic acid), SGNL oligosaccharides (liberated from dog SGNL-lipids by ceramide glycanase), and 3'-sialyllactose did not inhibit 125I-SGNL-BSA binding to rat PNS myelin membranes at the highest concentrations tested (5 mM). Glycosaminoglycans (polysaccharides containing uronic acids and/or sulfated saccharide residues), and dextran sulfate (a synthetic sulfated polysaccharide) inhibited binding without specificity when added in the range of 0.1 µg/ml. Colominic acid, an anionic polymer of sialic acids, was a weaker inhibitor, with an IC50 of 100 µg/ml.

Figure 9. Inhibition of 125I-SGNL-BSA binding to rat PNS myelin by SGNL-lipid derivatives. Desulfated and methyl esterified SGNL-lipid (■) and desulfated SGNL-lipid (○) were prepared from human SGNL-lipid (●) as described in Materials and Methods. Inhibition studies were performed as described in Fig. 8. 125I-SGNL-BSA specifically bound at each lipid concentration is expressed as the percent of specific control binding in the absence of inhibitor (control binding was 5.2 ± 0.3 fmol). Values are the mean ± SD for triplicate determinations (SD values which fall within the symbols are not shown).

Protease Sensitivity of 125I-SGNL-BSA Binding Activity

Preincubation of 0.25 µg of PNS myelin membrane protein with 0.01-0.5 µg trypsin and subsequent inactivation of the trypsin through the addition of soybean trypsin inhibitor resulted in a concentration dependent attenuation of 125I-SGNL-BSA binding to <50% of control. Increasing trypsin to 5 µg during the preincubation (with subsequent addition of trypsin inhibitor) did not result in a further decrease in radioligand bound, which remained at 29-51% of that bound to control membranes in various experiments. Pretreatment of membranes with trypsin inhibitor alone or with trypsin in the presence of trypsin inhibitor had little effect on the amount of radioligand bound (data not shown). Pronase pretreatment of myelin membranes (12.5 µg of membrane protein pretreated with 0.01-5 µg pronase, and the membranes recovered by centrifugation in the presence of excess BSA) also resulted in a concentration dependent, but incomplete, reduction of 125I-SGNL-BSA binding to 42% of that bound to control membranes. Binding activity on myelin membranes was heat stable, in that boiling did not reduce the amount of radioligand bound.

Discussion

The formation and maintenance of the multilamellar myelin sheath of axons depend upon coordinate cell surface recognition and interaction, both between the apposed membranes of the myelin-forming cell and the neuron, and between the scrolled myelin membranes themselves. Several coexisting families of adhesion molecules involving direct protein to protein interaction have been implicated in these cell-surface interactions of the myelin-forming cell of the PNS, the Schwann cell. We have focused on the potential role of carbohydrates as determinants recognized by Schwann cell surface receptors, and have proposed that the unusual sulfoglucuronyleneolacto-glycolipids may function as recognition...
ligands involved in myelination or myelin maintenance. Consistent with this role, our previous studies demonstrated that SGNL-lipids specifically support Schwann cell adhesion.

Our strategy to identify the hypothetical SGNL receptor on PNS myelin depended on the synthesis of a complementary high affinity radioligand. Since carbohydrate recognition often requires appropriate juxtaposition of multiple carbohydrate binding determinants (32), we constructed a multivalent SGNL-derivatized ligand that could be readily radiolabeled. This task was facilitated by a relatively rapid purification technique for SGNL-lipids and by the use of the enzyme ceramide glucanase to release SGNL-oligosaccharides, which could be linked to a BSA carrier to generate a multivalent radioliodinated ligand, 125I-SGNL-BSA. Carbohydrate analysis confirmed the multivalent nature of the ligand, and immunoblot analysis subsequent to SDS-PAGE indicated that HNK-1 reactive epitopes were covalently attached to the BSA. Since HNK-1 reactivity requires the sulfate on the glucuronol moeity and is abrogated if the glucuronol carboxyl moiety is removed (24), the immunoblot data demonstrated that (SGNL)-BSA carried intact sulfoglucuronol epitopes.

125I-SGNL-BSA was a successful ligand for identifying SGNL-directed binding activity on PNS membranes. The characteristics of this high-affinity and saturable binding activity were remarkable for a number of reasons. Within the PNS, binding was enriched on myelin rather than denser membranes. This suggests that the binding activity is a product of Schwann cells, and may be involved in their myelinating function. Our previous finding that intact primary Schwann cells bind to SGNL-lipids resolved by TLC chromatograms (38, 39) is consistent with a cell adhesion function for SGNL lipids and complementary Schwann cell receptors. The absence of 125I-SGNL-BSA binding on CNS membranes, and especially on CNS myelin, is also of note. A CNS myelin ganglioside binding activity which appears to be a product of oligodendrocytes, but which is absent from PNS myelin, may be a complementary CNS system for axon recognition (58, 59).

The possibility that the SGNL-binding site is a membrane protein is supported by the finding that preincubation of PNS myelin membranes with trypsin or pronase partially abrogates the subsequent binding of 125I-SGNL-BSA to these membranes. The observation that binding of 125I-SGNL-BSA was calcium-dependent may relate this activity to a family of vertebrate calcium–dependent carbohydrate binding proteins (15) including the selectins (54) and the glycoprotein receptors on hepatocytes and macrophages. The calcium requirement clearly distinguishes this activity from the broadly distributed galactose binding proteins which can be found in the PNS (21) as well as from the CNS myelin ganglioside binding activity mentioned above (59).

A consistent observation during these studies was that only a small portion of added radioligand (~6%) specifically bound to PNS myelin membranes, even when membranes were added in excess. The observations that recovered, unbound 125I-SGNL-BSA did not bind to fresh membranes, yet remained as macromolecular, HNK-1 reactive material, suggests that the ligand itself is structurally heterogeneous. This is not surprising, since random glycosylation of an average of 7.5 of the 60 lysines on BSA would be expected to generate a statistical variation in glycosylation number and even more variation in the relative spatial arrangement of oligosaccharides on the carrier. Studies in other carbohydrate recognition systems demonstrate that precise spatial relationships can be critical to high-affinity binding of multivalent glycoconjugates (32, 46). Our data suggest that the valence and/or the spatial arrangement of the oligosaccharides on a subpopulation (~6%) of the 125I-SGNL-BSA molecules is appropriate for high-affinity ligand binding. SGNL-lipids in natural membranes are most likely laterally mobile and may adopt the required valence and juxtaposition. Consistent with this model, monovalent SGNL-oligosaccharides failed to inhibit binding of the multivalent derivative to PNS myelin even when present at concentrations as high as 5 mM. In contrast, SGNL-lipids, added as detergent mixed micelles, specifically inhibited binding with an IC50 < 1 μM. Such marked differences between glycolipids and their monovalent oligosaccharide constituents have also been reported for other carbohydrate recognition systems (32, 58).

In performing kinetic analyses of 125I-SGNL-BSA binding, the maximum percentage of ligand competent to bind was experimentally determined to allow accurate calculation of the unbound, competent ligand concentration. Based on this analysis, the affinity is very high, with a Kd of 320 pM when fit to one site. However, Scatchard transformation of the binding isotherm was curvilinear, consistent with multiple classes of binding sites or ligands. The presence of multiple ligands with distinct binding affinities would be most consistent with our hypothesis that valence and spatial configuration of the SGNL-oligosaccharides on the carrier protein influence binding, although multiple classes of binding sites are also possible.

The higher binding affinity of the competent binding fraction of SGNL-BSA (Kd = 320 pM) compared to SGNL-lipid (Kd ~1 μM) may reflect differences in effective valence and/or presentation of the SGNL-oligosaccharides. Lipid inhibitors were added in the presence of 0.032% Triton X-100, a concentration sufficient to form micelles in which the lipids distribute, most likely at low valence. The orientation of the oligosaccharide chain may also be effected by glycolipid insertion in a detergent micelle. Nevertheless, the carbohydrate-specificity of 125I-SGNL-BSA binding was notable, in that among many anionic and neutral glycosphingolipids and phospholipids tested under identical conditions, SGNL-lipid itself was the most potent inhibitor of 125I-SGNL-BSA binding to PNS myelin (Table III). Surprisingly, the sulfate group on the glucuronic acid moiety was not required for SGNL-lipid recognition, although sulfate removal reduced SGNL-lipid inhibitory potency to one third that of the parent molecule. In contrast, esterification of the glucuronic acid (on desulfated SGNL-lipid) essentially eliminated its inhibitory activity (see Fig. 9). This is of interest, since nonsulfated forms of the SGNL-lipid are broadly distributed phylogenetically (14) and may be more ancient in evolution. Some role for the sulfate in binding was indicated by the observation that sulfatide (sulfate-3-galactosylceramide) inhibited 125I-SGNL-BSA binding (with an order of magnitude lower potency than SGNL-lipid) while its parent molecule, galactosylceramide, failed to inhibit. Gangliosides, anionic glycosphingolipid containing carboxylic acid groups on sialic acid moieties rather than on glucuronic acid, were either noninhibitory or were much less potent inhibitors than SGNL-lipid or desulfated SGNL-lipid (Table III). Of par-
ticular note is that sialylneolactotetraosylceramide, which has the same neutral saccharide core as SGNL-lipid but with a sialic acid rather than a glucuronic acid linked to the 3-position of the outermost galactose, exhibited an inhibitory potency that was <4% that of the desulfated SGNL-lipid. Although the precise carbohydrate specificity has not been defined, these data suggest that the SGNL-binding site revealed by 

In conclusion, we have identified and characterized a novel, calcium-dependent, tissue- and carbohydrate-specific SGNL-oligosaccharide-binding activity on rat PNS myelin. The prior observations (38, 39) that SGNL-lipids, which are present both in the axolemma and (in smaller amounts) in myelin and other Schwann cell membranes, support specific adhesion of rat Schwann cells suggest that the PNS myelin SGNL-lipid binding activity may be involved in Schwann cell–axon or myelin lamellae–lamellae membrane interactions necessary for myellination or myelin maintenance.

We thank Lynda Yang and Stephanie Edgar for valuable laboratory assistance; Drs. Robert Yu (Virginia Commonwealth University), Paul James and Brian Brandley (Glycomed, Inc.), and A. Hasegawa (Gifu University) for kind gifts of lipids; Dr. Richard Quailes (National Institutes of Health) for the generous gift of monoclonal antibody FTF7; Drs. Robert Cotter and Marc Chevrier for performing laser desorption mass spectroscopy analysis; and Dr. Hans Schweinberger for performing fast atom bombardment mass spectroscopy.

This work was supported in part by the National Institutes of Health grant HD14010 and the National Multiple Sclerosis Society grant RG2223. L. K. Needham was supported by training grant MH18030 from the National Institutes of Health.

Received for publication 3 November 1992 and in revised form 13 January 1993.

References

1. Ariga, T., T. Kohriyama, L. Freddo, N. Latov, M. Saito, K. Kon, S. Ando, M. Suzuki, M. E. Hemling, K. L. Rinehart, S. Kusunoki, and R. K. Yu. 1987. Characterization of sulfated glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. J. Biol. Chem. 262:848–853.

2. Bounias, M. 1980. N-(1-naphthyl)ethylenediamine dihydrochloride as a new reagent for nanomolar quantitation of sugars on thin-layer plates by a mathematical calibration process. Anal. Biochem. 106:291–295.

3. Brandley, B. K., S. J. Sweieler, and P. W. Robbins. 1990. Carbohydrate ligands of the LEC cell adhesion molecules. Cell. 63:861–863.

4. Cossu, C. J., A. I. Ahmed, and R. E. Feeney. 1982. Amine boranes as alternative reducing agents for reductive alkylation of proteins. Anal. Biochem. 124:272–278.

5. Chevrier, M. R., and R. J. Cotter. 1991. A matrix-assisted laser desorption time-of-flight mass spectrometer based on a 600 ps, 1.2 mJ nitrogen laser. Rapid Commun. Mass Spectrom. 5:611–617.

6. Chou, D. K. H., A. A. Ilyas, J. E. Evans, C. Costello, R. H. Quarles, and F. B. Jungalwala. 1987. Structure of sulfated glucuronoyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. J. Biol. Chem. 261:11717–11725.

7. Chou, D. K. H., G. A. Schwarting, J. E. Evans, and F. B. Jungalwala. 1986. Structure of sulfated glucuronoyl glycolipids in peripheral nerves reacting with HNK-1 antibody. J. Neurochem. 49:865–873.

8. Chou, D. K. H., N. Prasadarao, O. Koul, and F. B. Jungalwala. 1991. Developmental expression of HNK-1 reactive antigens in rat cerebral cortex and molecular heterogeneity of sulfoglucuronoylneolactotetraosylceramide in CNS versus PNS. J. Neurochem. 57:852–859.

9. Cole, G. J., and M. Schachner. 1987. Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (NCAM) and evidence for its role in NCAM-mediated cell adhesion. Neurosci. Lett. 78:227–232.

10. Cornbrooks, C. J., J. J. Carey, J. A. McDonald, R. Timpl, and R. P. Bunge. 1983. In vivo and in vitro observations on laminin production by Schwann cells. Proc. Natl. Acad. Sci. USA. 80:3850–3854.

11. Cotman, C. W. 1974. Isolation of synaptosomal and synaptic plasma membrane fractions. Methods Enzymol. 31:443–452.

12. Dahms, N. M., and R. L. Schnaar. 1983. Ganglioside composition is regulated during differentiation in the neuroblastoma X glioma hybrid cell line NG108-15. J. Neurosci. 3:806–817.

13. Daston, M. M., and N. Ramer. 1991. Expression of P30, a novel, calcium-dependent, tissue- and carbohydrate-specific SGNL-oligosaccharide-binding activity on rat PNS myelin. The prior observations (38, 39) that SGNL-lipids, which are present both in the axolemma and (in smaller amounts) in myelin and other Schwann cell membranes, support specific adhesion of rat Schwann cells suggest that the PNS myelin SGNL-lipid binding activity may be involved in Schwann cell–axon or myelin lamellae–lamellae membrane interactions necessary for myellination or myelin maintenance.

We thank Lynda Yang and Stephanie Edgar for valuable laboratory assistance; Drs. Robert Yu (Virginia Commonwealth University), Paul James and Brian Brandley (Glycomed, Inc.), and A. Hasegawa (Gifu University) for kind gifts of lipids; Dr. Richard Quailes (National Institutes of Health) for the generous gift of monoclonal antibody FTF7; Drs. Robert Cotter and Marc Chevrier for performing laser desorption mass spectroscopy analysis; and Dr. Hans Schweinberger for performing fast atom bombardment mass spectroscopy.

This work was supported in part by the National Institutes of Health grant HD14010 and the National Multiple Sclerosis Society grant RG2223. L. K. Needham was supported by training grant MH18030 from the National Institutes of Health.

Received for publication 3 November 1992 and in revised form 13 January 1993.

References

1. Ariga, T., T. Kohriyama, L. Freddo, N. Latov, M. Saito, K. Kon, S. Ando, M. Suzuki, M. E. Hemling, K. L. Rinehart, S. Kusunoki, and R. K. Yu. 1987. Characterization of sulfated glucuronic acid containing glycolipids reacting with IgM M-proteins in patients with neuropathy. J. Biol. Chem. 262:848–853.

2. Bounias, M. 1980. N-(1-naphthyl)ethylenediamine dihydrochloride as a new reagent for nanomolar quantitation of sugars on thin-layer plates by a mathematical calibration process. Anal. Biochem. 106:291–295.

3. Brandley, B. K., S. J. Sweieler, and P. W. Robbins. 1990. Carbohydrate ligands of the LEC cell adhesion molecules. Cell. 63:861–863.

4. Cossu, C. J., A. I. Ahmed, and R. E. Feeney. 1982. Amine boranes as alternative reducing agents for reductive alkylation of proteins. Anal. Biochem. 124:272–278.

5. Chevrier, M. R., and R. J. Cotter. 1991. A matrix-assisted laser desorption time-of-flight mass spectrometer based on a 600 ps, 1.2 mJ nitrogen laser. Rapid Commun. Mass Spectrom. 5:611–617.

6. Chou, D. K. H., A. A. Ilyas, J. E. Evans, C. Costello, R. H. Quarles, and F. B. Jungalwala. 1987. Structure of sulfated glucuronoyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. J. Biol. Chem. 261:11717–11725.

7. Chou, D. K. H., G. A. Schwarting, J. E. Evans, and F. B. Jungalwala. 1986. Structure of sulfated glucuronoyl glycolipids in peripheral nerves reacting with HNK-1 antibody. J. Neurochem. 49:865–873.

8. Chou, D. K. H., N. Prasadarao, O. Koul, and F. B. Jungalwala. 1991. Developmental expression of HNK-1 reactive antigens in rat cerebral cortex and molecular heterogeneity of sulfoglucuronoylneolactotetraosylceramide in CNS versus PNS. J. Neurochem. 57:852–859.

9. Cole, G. J., and M. Schachner. 1987. Localization of the L2 monoclonal antibody binding site on chicken neural cell adhesion molecule (NCAM) and evidence for its role in NCAM-mediated cell adhesion. Neurosci. Lett. 78:227–232.

10. Cornbrooks, C. J., J. J. Carey, J. A. McDonald, R. Timpl, and R. P. Bunge. 1983. In vivo and in vitro observations on laminin production by Schwann cells. Proc. Natl. Acad. Sci. USA. 80:3850–3854.

11. Cotman, C. W. 1974. Isolation of synaptosomal and synaptic plasma membrane fractions. Methods Enzymol. 31:443–452.

12. Dahms, N. M., and R. L. Schnaar. 1983. Ganglioside composition is regulated during differentiation in the neuroblastoma X glioma hybrid cell line NG108-15. J. Neurosci. 3:806–817.

13. Daston, M. M., and N. Ramer. 1991. Expression of P30, a novel, calcium-dependent, tissue- and carbohydrate-specific SGNL-oligosaccharide-binding activity on rat PNS myelin. The prior observations (38, 39) that SGNL-lipids, which are present both in the axolemma and (in smaller amounts) in myelin and other Schwann cell membranes, support specific adhesion of rat Schwann cells suggest that the PNS myelin SGNL-lipid binding activity may be involved in Schwann cell–axon or myelin lamellae–lamellae membrane interactions necessary for myellination or myelin maintenance.
The Journal of Cell Biology, Volume 121, 1993