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# Multivalent ganglioside and sphingosine conjugates modulate myelin protein kinases

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**Abstract**

Gangliosides, added exogenously at concentrations of 10–100 μM, inhibit intrinsic protein kinase activities in purified rat brain myelin. Multivalent neogangliproteins – gangliosides covalently attached, via their lipid moieties, to bovine serum albumin – were much more potent, inhibiting myelin protein phosphorylation half-maximally at a concentration of 100 nM. Different ganglioside conjugates varied 10-fold in inhibitory potency; GT1b-conjugates being the most potent and GM3-conjugates being the least. Conjugates of ganglioside oligosaccharides, lacking the lipid moiety, did not inhibit myelin protein phosphorylation, whereas conjugates of sphingosine inhibited nearly as potently as GT1b conjugates. Conjugate-mediated inhibition of myelin protein phosphorylation was due to inhibition of a protein serine kinase activity rather than activation of a phosphatase activity. We conclude that (i) clustered gangliosides or sphingosine are potent myelin protein kinase inhibitors, and (ii) sphingolipid metabolism is not required for myelin protein kinase inhibition. In contrast to their effects on myelin protein phosphorylation, ganglioside conjugates stimulated phosphorylation of a presumptive axon membrane protein. The data support the conclusion that gangliosides and other sphingolipids, when appropriately clustered, are potent modulators of central nervous system protein phosphorylation. © 1997 Elsevier Science B.V.

*Keywords:* Glycosphingolipid; GT1b; Neoglycoprotein; Protein serine kinase

## 1. Introduction

Gangliosides are a diverse class of negatively charged glycosphingolipids having a hydrophobic ceramide moiety (consisting of a fatty acid amide of sphingosine) and a hydrophilic carbohydrate chain containing one or more sialic acids. They are expressed by all vertebrate tissues, but predominate in the nervous system [2]. Gangliosides are primarily expressed on the outer leaflet of the plasma membrane [3], ideally located for involvement in cell-cell interactions. Increasing evidence indicates that gangliosides act as modulators of cell functions, including cell growth [4,5]; apoptosis [6]; neuritogenesis [7]; and differentiation of hematopoetic cells [8,9], keratinocytes [10], neuronal cells [11], and oligodendrocytes [12]. Many of these actions [4–7,10–12] involve ganglioside modulation of protein kinases.

Abbreviations: MBP, myelin basic protein; PKC, $Ca^{2+}$/phospholipid-dependent protein kinase; BSA, bovine serum albumin; Sph, sphingosine; GlcSph, glucosylsphingosine; LacSph, lactosylsphingosine; LBP, long myelin basic protein; SBP, short myelin basic protein; Ganglioside nomenclature is according to Svennerholm [1]

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Moreover, less complex sphingolipids including sphingosine [13], sphingosine-1-phosphate [14,15], and ceramide [16,17] are important intracellular modulators of protein kinases.

The myelin membrane is a multilamellar structure deposited around many axons in the central and peripheral nervous systems [18]. Although a primary function of myelin is to provide insulation required for efficient nerve conduction, there are many myelin-associated enzymes, such as phospholipases and protein kinases, which appear to be involved in signal transduction [19]. In particular, myelin basic protein (MBP), a major phosphate acceptor, has a half-life of several months, whereas the half-life of an MBP phosphate group is only minutes [20]. The function of MBP phosphate turnover is not known.

Exogenously added gangliosides modulate myelin protein kinase activities. Kim et al. [21] and Goldenring et al. [22], reported that different gangliosides, of which GT1b was the most potent, inhibited $Ca^{2+}$/phospholipid-dependent protein kinase (PKC) in purified rat central nervous system myelin. Subsequently Chan [23–25] reported that gangliosides inhibited the calcium-independent phosphorylation of some proteins, but stimulated phosphorylation of others in guinea pig myelin. Recently, ganglioside GM3 was reported to modulate protein kinase activity in O2A cells, the precursors of the myelin-producing oligodendrocytes [12].

In most cases, high concentrations of ganglioside (30–250 μM) have been used to elicit changes in myelin protein kinases. It is difficult to establish whether the observed effects were biophysical – due to the detergent-like properties of amphipathic gangliosides – or due to specific biochemical effects of the ganglioside structures. Gangliosides also chelate calcium through their sialic acid residues [26], and many of the reported ganglioside-inhibited protein kinases are calcium-dependent.

We postulated that synthetic multivalent arrays of gangliosides, covalently linked through their lipid moiety to a protein carrier (see Fig. 1 for schematic structures), may be potent modulators of myelin protein phosphorylation. These conjugates, termed *neogangliosproteins*, have proven valuable in studies of ganglioside binding proteins in the brain [27–29]. Since the gangliosides are covalently linked to a hydrophilic protein, presumably with the carbohydrate portion oriented outward, the problems of detergent-like membrane perturbation are reduced. The stable chemistry of their covalent conjugation makes release, uptake, and metabolism unlikely. Moreover, since the gangliosides are synthetically clustered in multivalent arrays, more potent effects may be expected [30]. Low concentrations of neogangliosproteins are less likely to cause the membrane perturbation or calcium chelation effects associated with high concentrations of unconjugated gangliosides. We report here that neogangliosproteins (and other sphingolipid conjugates) are potent modulators

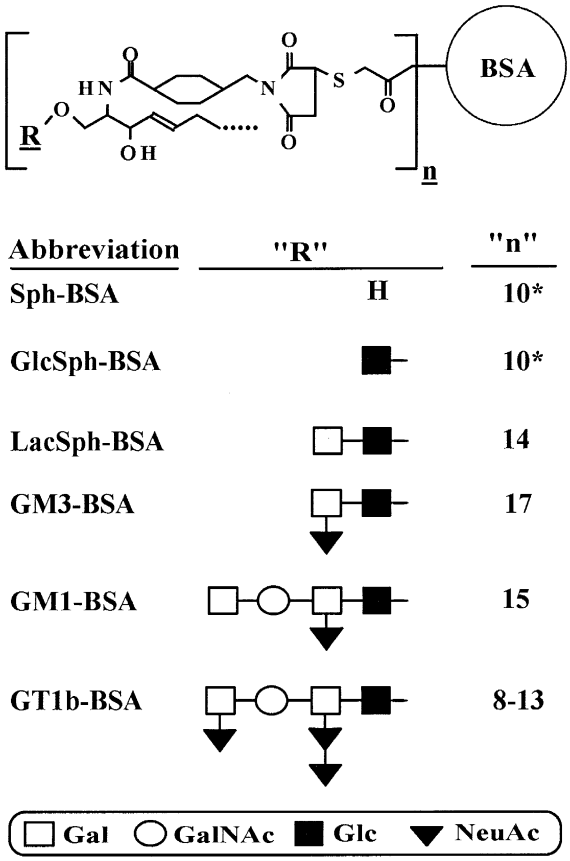


Fig. 1. Sphingolipid-BSA conjugates. The structures of six sphingolipid-BSA conjugates used in these studies are shown in increasing order of complexity. The average density of derivatization was determined by using Dionex sialic acid and/or neutral sugar analysis [32], except those marked with an *asterisk*, which were estimated by a shift of average molecular weight as measured by mass spectrometry [36]. GT1b conjugates having different densities of derivatization were used as indicated in the text.

of intrinsic myelin and putative axolemmal protein phosphorylation.

## 2. Materials and methods

### 2.1. Materials

[$\gamma$-$^{32}$P]ATP was from Dupont NEN (Boston, MA, USA). Protein kinase inhibitors chelerythrine, H-89, and KN-62 were from Biomol (Plymouth Meeting, PA, USA). Bio-Rex RG 501-X8 mixed bed resin and protein molecular weight markers were from BioRad (Hercules, CA, USA). Gangliosides were from Matreya (Pleasant Gap, PA, USA) unless otherwise indicated. Bovine brain sphingosine (Sph) was from Miles Laboratories (Elkart, IN, USA). Glucosylsphingosine (GlcSph, human Gaucher spleen) and lactosylsphingosine (LacSph) were the kind gifts of S. Chatterjee, The Johns Hopkins School of Medicine, and monosaccharide-conjugated BSA (neoglycoproteins) were the kind gifts of Y.C. Lee and R.T. Lee, The Johns Hopkins University. BSA conjugated with oligosaccharides released from gangliosides were synthesized by reductive amination, as described [31]. All other reagents were from standard suppliers.

### 2.2. Synthesis of sphingolipid-BSA conjugates

Neoganglioproteins and other sphingolipid-BSA conjugates used in this study are shown schematically in Fig. 1. Conjugates were synthesized as described previously for GT1b-BSA [32]. Briefly, the lysoganglioside forms of GT1b, GM1, and GM3 (bovine spleen) were prepared from their parent gangliosides by alkaline methanolysis [33]. The lysogangliosides, Sph, GlcSph, or LacSph were incubated with a tenfold molar excess of the heterobifunctional crosslinker, succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane 1-carboxylate (SMCC, Pierce, Rockford, IL, USA) in anhydrous dimethylformamide. After 1–2 days at 45°C, the resulting sulfhydryl-reactive maleimidyl intermediates were purified from starting materials by normal phase silica gel column chromatography using Iatrobeads (Iatron Labs, Tokyo).

Additional sulfhydryl groups were introduced to BSA (fatty acid and globulin free, Sigma) in the form of *N*-acetylthioacetate esters. The protein was incubated in 50 mM HEPES buffer pH 8 with a 20-fold molar excess of *N*-succinimidyl *S*-acetylthioacetate (SATA, Pierce) for 16 h at 4°C. The product, SATA-BSA, was purified by gel filtration on a Sephadex G-25 column. After deblocking (see below), SATA-BSA was assayed for sulfhydryl content colorimetrically [34], and typically contained 12–15 mol of free sulfhydryl per mol BSA.

Immediately before the coupling reaction, SATA-BSA was deblocked by incubation with 50 mM hydroxylamine HCl, 2.5 mM EDTA, 4 mM sodium phosphate pH 7.5. After 2 h at room temperature, dithiothreitol (2 mM final) was added. After 15 min at room temperature, the deblocked SATA-BSA was purified by Sephadex G-25 chromatography in 10 mM sodium phosphate buffer pH 7. Protein-containing fractions were pooled and quantified by absorbance at 280 nm. Purified SATA-BSA (50 nmol) was added to the dried, maleimide-derivatized sphingolipid (1 $\mu$mol). The progress of the subsequent coupling reaction was assessed by silica gel TLC, and was typically maximal after 1–2 days at 4°C.

Sphingolipid-BSA conjugates were purified on a TSK-gel DEAE-5PW HPLC column (Tosohaas, Montgomeryville, PA, USA) as described [35]. Excess free sulfhydryl groups were then reduced and alkylated as follows. Pooled DEAE fractions were incubated with 0.6 mM dithiothreitol for 1 h at 37°C, followed by 3 mM iodoacetamide for 1 h at 37°C, followed by 15 mM dithiothreitol for 1 h at 37°C, before desalting on a Sephadex G-25 column eluted with 10 mM sodium phosphate buffer pH 7. The degree of derivatization of the conjugates, with the exception of GlcSph-BSA and Sph-BSA, was measured by acid hydrolysis of an aliquot of the conjugate, followed by sugar analysis as previously described [32]. Sph-BSA and GlcSph-BSA were analyzed by matrix-assisted laser desorption mass spectrometry [36], kindly performed by Drs Kathleen Walker and Robert Cotter, The Johns Hopkins School of Medicine. The molecular weight of these two conjugates was estimated, and their valency calculated based on the increase in molecular weight compared to the parent BSA.

GT1b-neoganglioproteins with valencies between 1 and 4 were synthesized by an alternate linkage procedure utilizing bis(sulfosuccinimidyl)suberate as

crosslinker [35]. One preparation of sphingosine-BSA was synthesized by direct coupling with disuccinimidyl adipate (Fig. 4 only, as indicated).

### 2.3. Myelin membranes

Male Sprague-Dawley rats were sacrificed by decapitation, and their brains rapidly removed and rinsed in ice cold 0.32 M sucrose. Purified myelin membranes were prepared as described by Norton and Poduslo [37], except that protease inhibitors (0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM EDTA) were added to all solutions. Membranes were stored at −20°C in small aliquots. To ensure removal of EDTA from the membranes prior to use, aliquots were water-washed twice by centrifugation at 14 000 rpm in a microcentrifuge. Rat brain crude homogenate, and crude myelin (P2A), synaptosomal (P2B), and mitochondrial (P2C) fractions were prepared as described [27,38], using the same protease inhibitors as above.

### 2.4. Myelin protein kinase activity

Protein kinase reactions were prepared in 500 μl microcentrifuge tubes in a total volume of 20 μl containing kinase buffer (50 mM Hepes pH 7.4, 10 mM $MgCl_2$, 0.03% Triton X-100, 300 μM $CaCl_2$, 200 μM EGTA [final free $Ca^{2+}$ concentration = 100 μM]), purified myelin membranes (3 μg protein), 36 μM [γ-$^{32}$P]ATP (typically 1–2 Ci/mmol, or approximately 1 μCi per reaction), and potential effectors as indicated. Some experiments were performed at different divalent cation concentrations, as noted. Neoganglioproteins were added from concentrated stocks in 10 mM sodium phosphate pH 7. Unconjugated gangliosides were evaporated from stocks stored in chloroform-methanol-water (4:8:3) and dissolved by sonication in water. Reactions were initiated by addition of [γ-$^{32}$P]ATP, and incubated for 5 min (or as indicated) at 30°C. The reactions were terminated by addition of 6.7 μl of 4X SDS sample buffer, to give final concentrations of 2% SDS, 0.5% dithiothreitol, 10% glycerol, 60 mM Tris HCl pH 6.8, and 0.005% bromophenol blue.

Samples were electrophoresed on 14 × 16 cm SDS-PAGE gels with a linear acrylamide gradient from 12 to 18%, stained with Coomassie Brilliant Blue R-250, and dried between sheets of porous cellophane. Incorporation of $^{32}$P was detected by autoradiography on pre-flashed Kodak X-Omat AR film at −70°C with an intensifying screen. Alternatively, $^{32}$P was detected using a Fuji BAS-IIIs imaging plate and Fuji BAS1000 phosphorimage system for quantification of radiolabel incorporation into individual SDS-PAGE bands.

### 2.5. Phosphoamino acid analysis

Phosphoamino acids were analyzed using thin layer electrophoresis [39,40]. Myelin protein kinase reactions were performed as described above, using twice the volume and four times higher [$^{32}$P]ATP specific activity. After SDS-PAGE, the gel was soaked in water containing Bio-Rex 501-X 8 mixed bed resin for 30 min, dried, and subjected to autoradiography. Phosphoprotein bands were excised, rehydrated (50 mM $(NH_4)HCO_3$, 10 min), forced through a wire mesh mounted in a syringe barrel, and digested with 40 μg of trypsin (Worthington, Freehold, NJ, USA) at room temperature overnight. The suspension was filtered through glass fiber filters, lyophilized, resuspended in 100 μl of 5.7 N HCl, sealed in a 200 μl capillary tube, and hydrolyzed at 110°C for 90 min. The hydrolyzed sample was lyophilized, resuspended in 5 μl of electrophoresis buffer (pyridine-acetic acid-formic acid-water (1:18:14:267), pH to 2.5 with pyridine), mixed with phosphoamino acid standards, spotted on a 20 × 20 cm cellulose TLC plate and electrophoresed at 1000 V for 45 min. Phosphoamino acids were subsequently detected with ninhydrin spray reagent and $^{32}$P by autoradiography.

### 2.6. Protein phosphatase assay

Two identical 100 μl myelin protein kinase reactions were performed as described above. After 5 min at 30°C, 50 μl of 12.5 mM unlabeled ATP (≈ 200-fold excess) in kinase buffer was added (to stop incorporation of radioactive phosphate) either with or without 3 μM $(GT1b)_8$BSA (1 μM final). At timed intervals from 0 to 30 min, 10 μl aliquots were removed, mixed with 10 μl 2X SDS sample buffer, resolved by SDS-PAGE, and $^{32}$P incorporation into proteins quantified using a phosphorimager. In parallel control reactions, addition of phosphatase in-

hibitors (17 mM EDTA and 40 mM NaF), resulted in no decrease in protein-associated radioactivity over 30 min (data not shown).

## 3. Results

### *3.1. Neoganglioproteins modulate myelin protein kinase activity*

Highly purified myelin membranes were incubated with [$\gamma$-$^{32}$P]-ATP as described in Section 2. No exogenous substrate was added, therefore, protein phosphorylation was dependent on both endogenous protein kinases and endogenous protein substrates. When parallel reactions were performed in the presence or absence of 500 nM $(GT1b)_8$BSA, marked effects on protein phosphorylation were noted (Fig. 2). The most striking effect was a sharp decrease in the rate of phosphorylation of a protein doublet with apparent molecular weight of 25 kDa (pp25) and a protein with apparent molecular weight of 20 kDa (pp20). In contrast, the phosphorylation of proteins with apparent molecular weights of 21 and 17 kDa were unaffected or enhanced under these experimental conditions. Based on their comigration with known myelin proteins, the 21 and 17 kDa phosphoproteins are designated as myelin basic proteins (long (LBP) and short (SBP)). The identities of pp25 and pp20 are not known, although they may be splice variants of the myelin basic proteins [41]. In some experiments pp25 appeared to be a doublet (Fig. 2), the upper band of which migrated near myelin proteolipid protein. However, the lower band of the pp25 doublet did not comigrate with any major myelin protein, and there have been no published reports of phosphoryla-

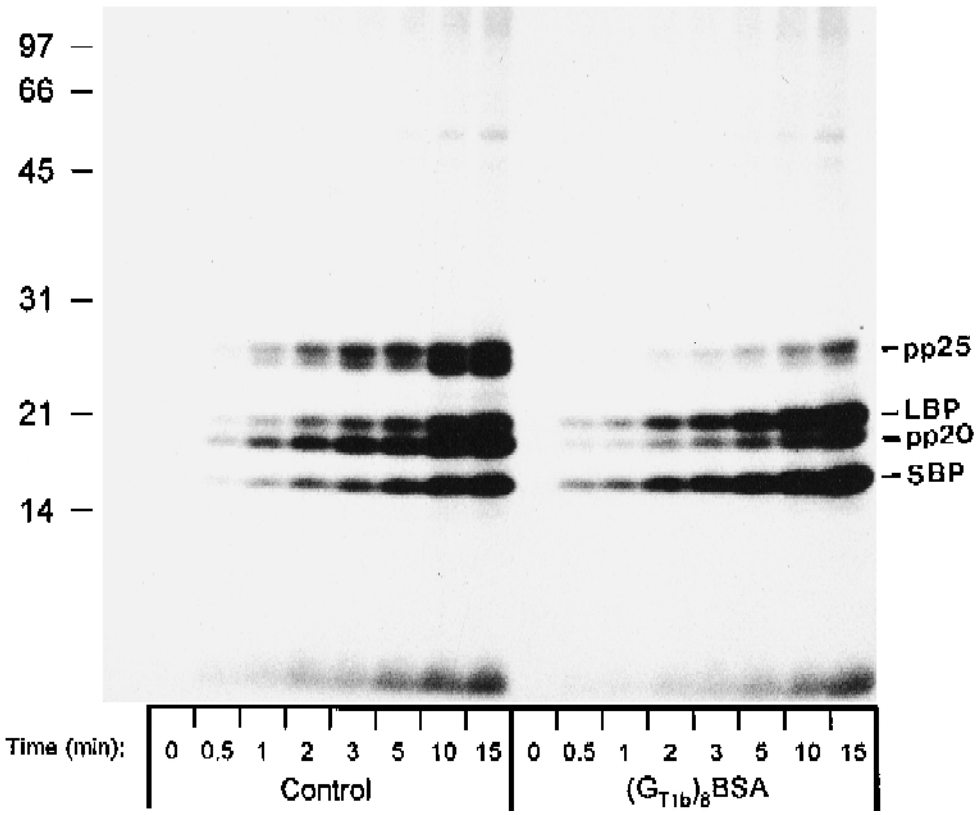


Fig. 2. Time course of myelin protein phosphorylation: effect of $(GT1b)_8$BSA. Purified myelin membranes were incubated in kinase buffer containing [$\gamma$-$^{32}$P]ATP at 30°C for the indicated times ($[Mg^{2+}] = 40$ mM). The reactions in the *right panel* contained 500 nM $(GT1b)_8$BSA. The reactions were stopped by addition of SDS sample buffer and subjected to SDS-PAGE, followed by autoradiography. Molecular mass markers (in kDa) are indicated at the left; major phosphoprotein designations are indicated at the right (LBP, long myelin basic protein; SBP, short myelin basic protein).

tion of proteolipid protein. Since the doublet bands of pp25 were not well resolved, they are treated as a single species for quantification.

To determine the divalent cation dependence of phosphorylation, kinase reactions were performed with varying amounts of $Mg^{2+}$ and $Ca^{2+}$ (data not shown). Omission of $Ca^{2+}$ had no significant effect on the incorporation of radiolabel or inhibition by $(GT1b)_8BSA$. The effects of $Mg^{2+}$ were complex. Radiolabel incorporation into pp25 and pp20 was maximal at 20 mM $Mg^{2+}$, and $(GT1b)_8BSA$ inhibited their phosphorylation at all $Mg^{+2}$ concentrations tested. In contrast, incorporation of radiolabel into LBP and SBP was maximum at 10 mM $Mg^{2+}$. At this $Mg^{2+}$ concentration, $(GT1b)_8BSA$ inhibited phosphorylation of these proteins, whereas at higher concentrations (40 mM, see Fig. 2) phosphorylation was either unchanged or slightly enhanced. Since we observed striking inhibition of pp25 and pp20 phosphorylation by neogangliosproteins over a broad range of $Mg^{2+}$ concentrations, subsequent studies focused on these species, using 10 mM $Mg^{2+}$.

Many protein-carbohydrate interactions have relatively low single-site affinities, but show markedly increased affinity when multiple copies of the carbohydrate are appropriately clustered [42]. To determine whether neoganglioprotein inhibition of myelin protein kinase activity exhibits this 'cluster effect', myelin protein kinase assays were performed in the presence of unconjugated GT1b and a series of GT1b neogangliosproteins (Fig. 3). The clustered presentation of ganglioside determinants on the neoganglioprotein resulted in a 1000-fold increase in inhibitory potency (comparing GT1b ($IC_{50} \approx 100$ μM) and $(GT1b)_8BSA$ ($IC_{50} \approx 100$ nM)). Kinase inhibitory potency correlated with derivatization density: $(GT1b)_8BSA \gg (GT1b)_4BSA = (GT1b)_2BSA > (GT1b)_1BSA$. These data also indicate that the linkage chemistry of the ganglioside to BSA was not responsible for the inhibition, in that $(GT1b)_4BSA$ was a highly potent inhibitor, although the GT1b was linked via an alternate synthetic scheme which did not utilize thioesters [35]. The increased inhibitory potency of $(GT1b)_1BSA$ over unconjugated GT1b ($\approx$ 10-fold) even though both are 'monovalent', may be due to the presence of higher order derivatives in the $(GT1b)_1BSA$ preparation, which has an *average* of one GT1b per BSA molecule. Intermediate valency conjugates were not tested at concentrations greater than 10 μM due to limited availability.

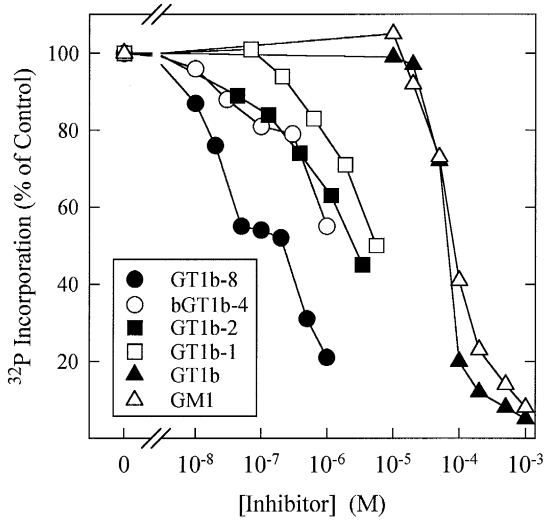


Fig. 3. Effect of multivalency on the inhibitory potency of GT1b-neogangliosproteins. Myelin membranes were incubated with [$\gamma$-$^{32}$P]ATP under standard assay conditions in the presence of various concentrations of unconjugated gangliosides GT1b and GM1, or with the following GT1b-neogangliosproteins: GT1b-8, $(GT1b)_8BSA$; bGT1b-4, biotinylated $(GT1b)_4BSA$; GT1b-2, $(GT1b)_2BSA$; GT1b-1, $(GT1b)_1BSA$. The reactions were stopped, and $^{32}$P incorporation into pp25 was analyzed by SDS-PAGE and phosphorimage analysis. Data are the means of duplicate determinations.

### *3.2. Structural specificity of neoganglioprotein inhibition*

The specificity of neoganglioprotein inhibition of myelin protein phosphorylation was investigated by comparing the effects of structurally related BSA conjugates. Neither BSA alone, nor BSA derivatized with monosaccharides (Gal, GalNAc, Glc, or Man) had any inhibitory activity when tested at 2.5 μM, a value 25-fold higher than the $IC_{50}$ for $(GT1b)_8BSA$ (data not shown, [43]). Notably, when the oligosaccharide portion was released from of GT1b, and covalently attached to BSA, the resulting conjugate ($(oligoGT1b)_{12}BSA$) did not inhibit phosphorylation (Fig. 4). In contrast, a conjugate having sphingosine covalently linked to BSA (via disuccinimidyl adipate) inhibited myelin protein phosphorylation. These data indicated that the clustered sphingosine moiety was necessary and perhaps sufficient for ganglioside modulation of myelin protein phosphorylation.

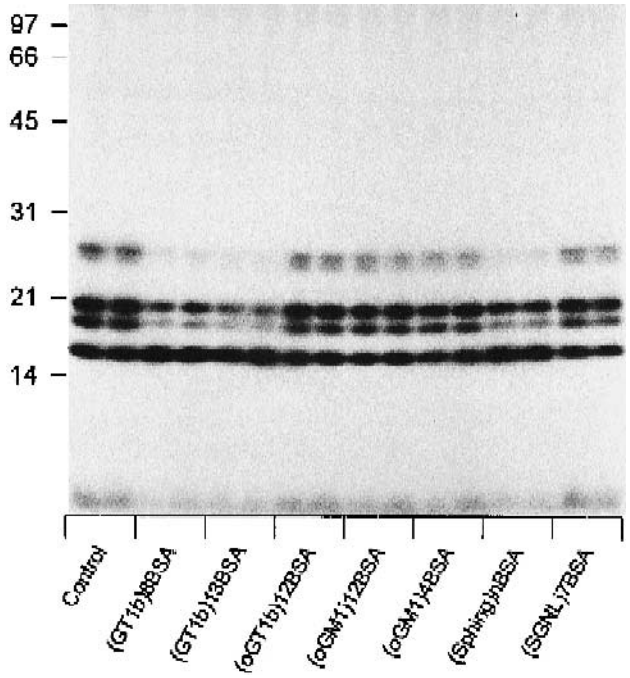


Fig. 4. Structural specificity of neoganglioprotein inhibition of myelin protein kinase activity. Myelin protein kinase reactions were performed under standard conditions in the presence of 500 nM of the indicated BSA conjugates. Reactions were stopped, and subjected to SDS-PAGE and autoradiography. Assays were performed in duplicate, and electrophoresed in adjacent lanes on the gel. Molecular mass markers (in kDa) are shown at the left. Ganglioside designations preceded by an 'o' indicate conjugates in which the oligosaccharide released from the respective ganglioside was linked to BSA by reductive amination [31]. SGNL designates the oligosaccharide 3-sulfo-glucuronylneolactotetrose linked to BSA by reductive amination [52]. The valency of the disuccinimidyl adipate-linked sphingosine-BSA ($(Sphing)_n$BSA) used in this particular experiment (unlike subsequent experiments) was not determined.

Based on these data, a series of multivalent ligands, containing sphingolipids of varying complexity were constructed (Fig. 1). All lipids were linked using the same chemistry, and the conjugates contained 8 to 17 lipid molecules per BSA. Conjugates were tested for their ability to inhibit radiolabeled phosphate incorporation into myelin proteins (Fig. 5). The most striking result from these studies was that all of the conjugates were inhibitory, including $(Sph)_{10}$BSA, which contains no carbohydrate. However, conjugates containing different oligosaccharide structures exhibited a range of potencies: $(GT1b)_8$BSA and $(GT1b)_{13}$BSA were 6–10-fold more potent inhibitors of phosphorylation than $(GM3)_{17}$BSA.

### *3.3. Characterization of the neoganglioprotein-modulated myelin protein kinase activity*

Decreased phosphorylation of a protein may result from inhibition of protein kinases, stimulation of protein phosphatases, or both. The effect of $(GT1b)_8$BSA on protein phosphatase activity in myelin membranes was measured as described in Section 2. Phosphatase activity was assayed as the time-dependent loss of radioactivity from prelabeled phosphoproteins, performed either in the presence or absence of the neoganglioprotein. Addition of $(GT1b)_8$BSA did not stimulate myelin protein phosphatase activity (data not shown), indicating that the decrease in protein phosphorylation caused by neogangliproteins resulted from a decrease in kinase activity.

Phosphoamino acid analysis of the major labeled phosphoproteins in the myelin membranes showed

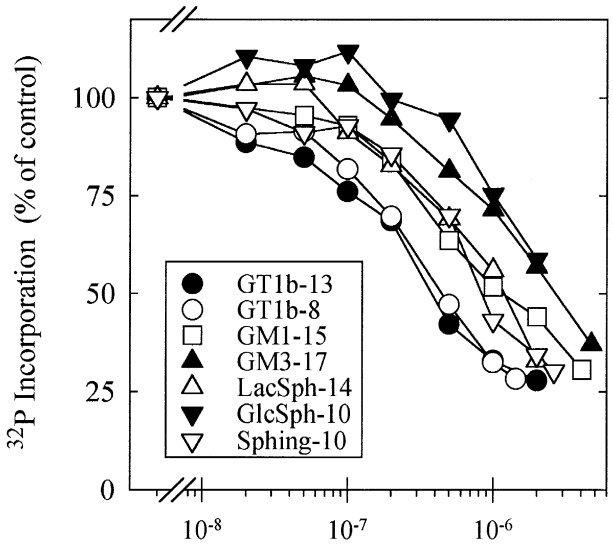


Fig. 5. Inhibition of myelin protein kinase activity by sphingolipid-BSA conjugates. Protein kinase reactions were performed under standard conditions in the presence of the indicated concentrations of various sphingolipid-BSA conjugates. The valency of each conjugate is listed after the dash, i.e. GT1b-8 is $(GT1b)_8$BSA. $^{32}$P incorporation into pp25 was analyzed by SDS-PAGE and phosphorimage analysis. Data are the means of two experiments, each performed in duplicate.

that phosphorylation occurred exclusively on serine residues under the conditions used (data not shown). However, the serine protein kinase inhibitors chelerythrine, H-89, and KN-62 did not block phosphorylation (when used at appropriate concentrations), indicating that the neoganglioprotein-modulated activity was not among the major classes of serine protein kinases (PKC, cAMP- or cGMP-stimulated protein kinases, or $Ca^{+2}$/calmodulin-stimulated protein kinase respectively). Moreover, kinase activity was not stimulated by dibutyryl cAMP, dibutyryl cGMP, or calcium/calmodulin (data not shown).

### *3.4. Brain membrane distribution of ganglioside-modulated protein kinases*

To determine whether the neoganglioprotein-modulated protein kinase was found in other brain membranes, crude rat brain homogenate was fractionated by sucrose density centrifugation. The resulting fractions, denoted P2A, P2B, and P2C, represent crude myelin membranes, synaptosomes, and mitochondria, respectively. These three membrane fractions, along with crude brain homogenate and the highly purified myelin membranes used in all other experiments were assayed for kinase activity, in the presence or absence of 500 nM $(GT1b)_8$BSA. The gel autoradiogram, shown in Fig. 6, was overexposed to better visualize minor bands. Using either crude (P2A) or purified myelin membranes, the same four major species were radiolabeled, and $(GT1b)_8$BSA inhibited phosphorylation of pp25 and pp20. Phosphorylated myelin basic proteins were also visible in P2B and P2C, presumably due to a small amount of contaminating myelin in those fractions. There were many higher molecular weight bands labeled in the

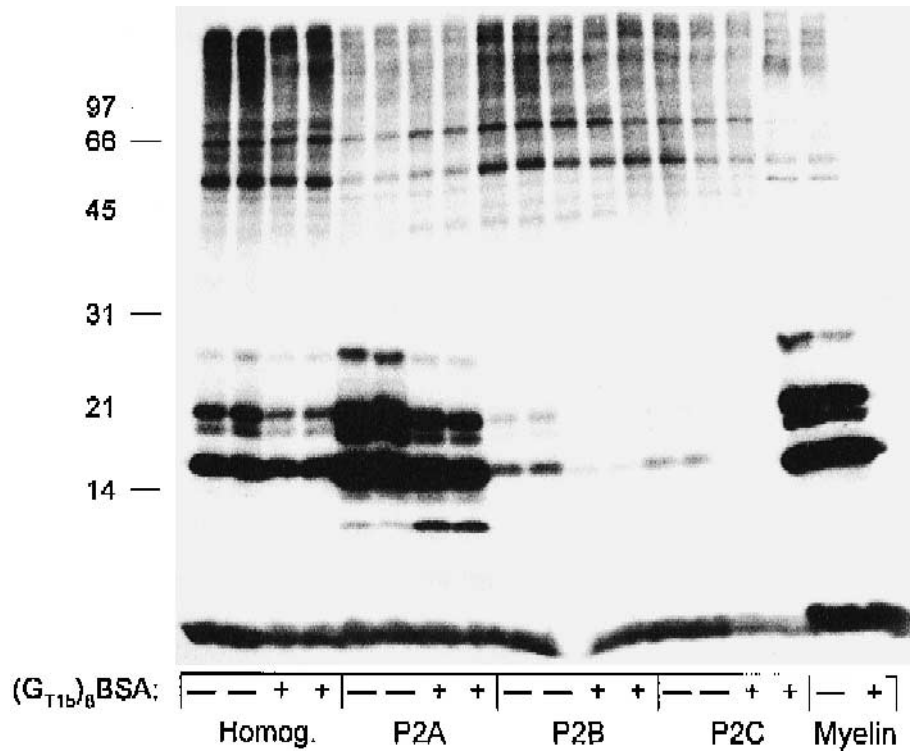


Fig. 6. Brain membrane specificity of neoganglioprotein-modulated protein kinase activity. The indicated brain membrane fractions were assayed for myelin protein kinase activity, as described in Section 2, in the presence or absence of 500 nM $(GT1b)_8$BSA as indicated. *Homog*., crude brain homogenate; *P2A*, crude myelin fraction; *P2B*, synaptosome fraction; *P2C*, mitochondrial fraction; *Myelin*, highly purified myelin membranes. Reactions were performed in duplicate and electrophoresed in adjacent lanes (except single lanes for purified myelin). Molecular mass markers (in kDa) are shown at the left.

crude homogenate and non-myelin fractions, but none was markedly or reproducibly modulated by neoganglioprotein.

The P2A reaction produced an unexpected result: phosphorylation of a small protein ($\approx$ 12 kDa), which was not apparent in reactions using purified myelin, was *stimulated* by neoganglioprotein (phosphorylation of 40 and 66 kDa proteins was less dramatically stimulated). Half-maximal stimulation occurred at a $(GT1b)_8BSA$ concentration of 300 nM (data not shown). The 12 kDa phosphoprotein (pp12) was not detected in any reaction using the more highly purified Norton-Poduslo [37] myelin, with or without neoganglioprotein present. The Norton-Poduslo procedure includes hypo-osmotic shock to disrupt myelin whorls into small fragments, releasing the axonal plasma membrane (axolemma) which is then removed by discontinuous sucrose gradient centrifugation. Since osmotic shock is not part of the P2A preparation, it is reasonable to speculate that pp12 is localized to the axolemma.

The structural specificity of stimulation of pp12 phosphorylation with sphingolipid conjugates was similar to that for inhibition of phosphorylation of myelin-specific proteins: $(GT1b)_8BSA$ and $(Sph)_{10}BSA$ were the most effective, whereas $(GlcSph)_{10}BSA$ was the least effective (data not shown). However, unconjugated GT1b, at high concentrations (100 $\mu$M) modestly inhibited pp12 phosphorylation (45%), whereas $(GT1b)_8BSA$ (300 nM) stimulated phosphorylation. These data indicate that exogenously added unconjugated gangliosides, at high concentrations, may have biophysical or biochemical effects distinct from those of the more potent ganglioside conjugates.

## 4. Discussion

Although control of myelin protein kinase activity by gangliosides has been reported previously [21–23], the covalent ganglioside conjugates used in the current study demonstrate the following additional major points. (i) Clustered gangliosides are potent kinase modulators. Their ability to inhibit myelin protein kinase activity at nanomolar concentrations is indicative of a direct biochemical mechanism, rather than an alteration in 'bulk' membrane properties or indirect chelation of divalent cations. (ii) Metabolism of gangliosides is not required for kinase modulation. Synthetic ganglioside conjugates, in which the gangliosides are linked to BSA via stable thioether and amide linkages, are not likely to be readily metabolized. Yet they are 1000-fold more potent than unconjugated gangliosides in modulating myelin protein kinase activity, indicating that metabolism to active species such as ceramide and sphingosine is an unlikely mechanism for ganglioside-mediated kinase modulation. The observation that GT1b and sphingosine derivatives synthesized using alternative chemistries had similar effects, combined with the lack of effect by BSA alone, indicates that the carrier or coupling chemistry is not responsible for kinase modulation. Therefore, data presented here support the hypothesis that gangliosides and other sphingolipids play important regulatory roles in the enzymatic activity of central nervous system myelin.

It is unknown whether the ganglioside conjugates described here act primarily on the kinase(s) present in myelin or on kinase substrates. Inhibition of multiple distinct myelin polypeptide substrates suggests that the ganglioside conjugates may act on the enzyme, either directly or through a signaling pathway. Ganglioside GM3 inhibits epidermal growth factor receptor autophosphorylation through direct interaction of the ganglioside with the enzyme. [5,44]. In contrast, GM1 appears to stimulate phosphorylation of tyrosine hydroxylase in PC12 cells indirectly, by modulating voltage-sensitive $Ca^{2+}$ channels [45]. These studies indicate that gangliosides utilize multiple mechanisms to modulate protein kinases.

The identity of the ganglioside-modulated protein kinase responsible for myelin protein phosphorylation in the current study was not determined, although it does not appear to be one of the major known protein kinases. For instance, although sphingolipids modulate PKC [46], and PKC is abundant in myelin [47], the major form of calcium-dependent PKC was not the ganglioside-modulated protein kinase responsible for phosphorylation in the current study since: (i) $Ca^{2+}$ was not required for activity, nor was activity significantly stimulated by the addition of $Ca^{2+}$ or $Ca^{2+}$/phosphatidylserine (not shown), and (ii) the PKC-specific inhibitor, chelerythrine, did not block the activity when used at concentrations which typically block PKC. In previous reports on ganglioside

modulation of myelin protein kinase activities the properties of the kinases under study varied. In the work of Chan [23], as in the present study, $Ca^{2+}$ was not required for kinase activity, whereas Kim et al. [21] reported that the ganglioside-inhibited myelin protein kinase activity in myelin was PKC, based on its $Ca^{2+}$ dependence (under their conditions) and the reported abundance of PKC in myelin [47].

Myelin protein kinase activity was potently inhibited by all of the sphingolipid-BSA conjugates tested, the best inhibitor being $(GT1b)_8$BSA. This result, and the weak inhibition by $(oligoGT1b)_{12}$BSA, suggest that the sphingosine moiety, alone or as part of a ganglioside structure, is necessary and sufficient for inhibition of the myelin protein kinase activity detected under our conditions. However, the 10-fold range of potencies observed for conjugates with different oligosaccharide structures indicates that the carbohydrate moieties contribute to the specificity of the interaction. The data support the interpretation that a myelin protein kinase interacts with the sphingosine moiety in each conjugate, but that the oligosaccharide structure can either enhance or diminish the interaction. Although 'sphingosines' exist in many molecular forms [48], only two are relevant to the current study, since $>95\%$ of the long chain bases on the gangliosides used are either sphingosine (C18 : 1) or C-20 sphingosine (C20 : 1). The spleen glycosphingolipids (GlcCer, LacCer, GM3) carry exclusively sphingosine [49], whereas the bovine brain gangliosides (and semi-synthetic bovine brain sphingosine) carry about equal amounts of the C18 : 1 and C20 : 1 forms (based on mass spectrometry and gas-liquid chromatography, data not shown). Whether the two forms vary in their ability to modulate myelin protein phosphorylation was not determined.

The enhanced potency of ganglioside conjugates compared to their unconjugated counterparts (Fig. 3) may be due to specific spatial clustering. Spatial clustering of unconjugated gangliosides also occurs, via micelle formation. However, the critical micelle concentration (e.g. $\approx 10^{-8}$ M for GM1 [50]) is far below the effective concentration for inhibition of myelin protein phosphorylation ($>10^{-5}$ M, Fig. 3). It is possible that synthetic clustering of the sphingolipids on BSA generates intermolecular distances which are either particularly well suited to binding to target sites on the myelin membrane, or which impose artificial stress on target structures after binding, thereby altering their activities.

Fractionation of brain membranes (Fig. 6) indicated that the ganglioside-inhibited kinase activity was primarily or exclusively found on myelin. Under the assay conditions employed, the four myelin phosphoproteins were the major phosphorylated species, even in crude brain homogenates. Furthermore, myelin phosphoproteins were the only species which were notably inhibited by ganglioside conjugates. Therefore, it is reasonable to speculate that ganglioside regulation of protein phosphorylation is involved in myelin-specific membrane functions.

Fractionation of brain membranes also led to the discovery of a protein (pp12) whose phosphorylation was markedly stimulated by ganglioside conjugates. Based on its appearance in partially purified myelin but in no other membrane fraction (including purified myelin), this protein may be localized to axolemmal membranes, which are the neuronal target of myelination. Axolemma and myelin have different ganglioside expression [51]. In the rat, axolemma gangliosides are mostly polysialylated (GT1b, GD1b), whereas myelin gangliosides are mostly monosialylated (GM1). Whether the differential distribution has a functional impact on protein phosphorylation in these membranes has not been determined.

Further elucidation of the mechanisms of ganglioside-modulated protein kinase activities await molecular identification on the endogenous enzyme(s) and substrates involved. The high potency of the ganglioside and other sphingolipid conjugates synthesized for the current study make them powerful tools for further elucidation of ganglioside-modulated protein kinase activities.

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