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Solubilization of a membrane-associated protein from rat nervous system tissues which binds anionic glycolipids and phospholipids

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Abstract

An anionic glycolipid and phospholipid binding protein was characterized in detergent-solubilized rat brain synaptosomes using a synthetic, polyvalent radiolabeled ganglioside-protein conjugate as radioligand. Gangliosides are prominent cell surface glycoconjugates in vertebrate brain, where they may function in membrane protein regulation or in cell-cell recognition. The neoganglioprotein $(G_{T1b})_{13}$BSA was radioiodinated and used to probe solubilized synaptosomal proteins for ganglioside binding activity using a receptor-ligand precipitation assay. Binding data revealed a high affinity (K_D = 1 nM), saturable (B_{max} = 173 pmol/mg protein) binding activity that was proteinase sensitive, calcium independent and maximal at neutral pH. Size exclusion chromatography of the synaptosomal $(G_{T1b})_{13}$BSA binding activity indicated a M_r of ≈ 28 kDa. Binding activity with similar characteristics was solubilized from other rat tissues, with activity from sciatic nerve = muscle > synaptosomes > central nervous system myelin = liver. Gangliosides added as mixed detergent-lipid micelles inhibited $(G_{T1b})_{13}$BSA binding: G_{T1b}, G_{D1a} and G_{D1b} were the most effective inhibitors (IC_{50} ≈ 200 nM), while G_{M1} and G_{M3} were 5-fold less effective. In addition, the sphingolipids sulfatide and sialylneolactotetraosylceramide were effective inhibitors, with IC_{50} values of 300 nM and 200 nM, respectively. The neutral sphingolipid G_{A1} did not block $(G_{T1b})_{13}$BSA binding. Phosphatidylcholine and phosphatidylethanolamine were non-inhibitory, however phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol inhibited half-maximally at 200–300 nM. Inhibition by both gangliosides and anionic phospholipids was competitive with $(G_{T1b})_{13}$BSA. We conclude that a binding protein for anionic glycolipids and phospholipids is distributed on nerve and muscle membranes.

Keywords: Lipid binding; Ganglioside; Brain membrane; Carbohydrate receptor

1. Introduction

On the cell surface, proteins, lipids, and their associated carbohydrates interact in concert to mediate cell-cell adhesion, cell-cell communication, and transmembrane signal transduction. Our research has been directed toward identification of glycolipid binding proteins in brain membranes and elucidation of the roles they play in lipid-mediated cellular processes. In particular, we are interested in the roles of gangliosides, a class of sialylated glycosphingolipids which are found in all vertebrate tissues and which occur in relatively high concentration in the brain [1,2]. They exist primarily in the outer leaflet of the plasma membrane, where their carbohydrate groups extend beyond the cell surface into the extracellular environment. Because of their location on the cell surface and the variety of structures encoded by their carbohydrate moieties, it has been suggested that gangliosides serve as cell-surface recognition molecules [3,4]. The important role that gangliosides play in cellular functions is increasingly supported by evidence from a number of laboratories suggesting that gangliosides have roles in physiological cell processes such as cell adhesion, neurite outgrowth and signal transduction [5–7].

Using polyvalent synthetic ganglioside-protein conjugates, neogangliproteins, as high affinity radioligands for ganglioside receptors, we previously characterized a ganglioside binding activity specific to central nervous system myelin [8,9]. The myelin ganglioside receptor binds selectively to the '1b' family of gangliosides (G_{D1b}, G_{T1b}, G_{Q1b}), and may be involved in interactions between oligodendroglia and axons during processes of myelination. In the current report we describe a lipid binding protein

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identified due to its ability to bind $(G_{T1b})_{13}$BSA with high affinity. This protein was initially characterized in preparations of proteins solubilized from rat brain synaptosomes and is distinct from the myelin ganglioside receptor.

A preliminary report of this work was presented [10].

2. Experimental procedures

2.1. Membrane preparation

Fractions enriched in either synaptosomes or myelin were prepared from crude brain membranes by differential centrifugation followed by sucrose density centrifugation using a modification of the procedure of Gray and Whittaker [11] as described previously [9]. Briefly, whole brains were removed from rats and homogenized in 10 volumes of ice-cold 0.32 M sucrose using a Potter-Elvehjem glass-Teflon homogenizer with motor-driven pestle. The homogenate was centrifuged at $1000 \times g$ for 10 min, at which time the pellet (P1) was discarded. Supernatant was transferred to clean tubes and centrifuged at $17\,000 \times g$ for 30 min. The resulting pellet (P2) was resuspended in 0.32 M sucrose and layered onto a discontinuous sucrose gradient composed of 10 ml 1.2 M sucrose under 10 ml 0.8 M sucrose. The gradient was centrifuged at $53\,000 \times g$ for 2 h. A white band of material at the 0.32/0.8 M sucrose interface was collected as myelin and a broader, light brown band at the 0.8/1.2 M sucrose interface was collected as synaptosomes. The myelin and synaptosome fractions were each diluted to 20 ml with 0.32 M sucrose and centrifuged at $100\,000 \times g$ for 30 min. The resulting pellets were resuspended in 0.32 M sucrose and stored at −20°C if not used immediately. Sciatic nerve membranes [9,12], liver plasma membranes [13], and muscle sarcolemma [14,15] were prepared as described previously.

2.2. Solubilization of synaptosomal proteins and CM-Sepharose ion exchange chromatography of the detergent extract

In a typical preparation, 10 ml of synaptosome fraction containing 70 mg synaptosomal protein were added to 30 ml of aqueous solution such that the final solubilization buffer contained 10 mM CHAPS detergent, 10 mM in 2-(*N*-moropholino)ethanesulfonic acid (Mes), pH 6.0, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 1.25 μg/ml leupeptin, 2.5 μg/ml antipain, 12.5 μg/ml benzamidine, 10 units/ml aprotinin, 1.25 μg/ml chymostatin, and 1.25 μg/ml pepstatin. Following incubation with gentle mixing at 4°C for 1 h, the membrane suspension was centrifuged at $100\,000 \times g$ for 1 h, the supernatant collected and the pellet discarded. Protein determination revealed extraction of 21% of the synaptosomal proteins (15 mg) under these conditions. The supernatant was immediately subjected to ion exchange chromatography.

Step gradient CM-Sepharose ion-exchange chromatography was performed on solubilized proteins to remove endogenous anionic lipids that might serve as inhibitors of binding. Unless indicated, 40 ml of solubilized synaptosomal protein (prepared as described above) containing approximately 15 mg protein, was applied to a 1 × 8 cm CM-Sepharose column equilibrated in 10 mM Mes, 10 mM CHAPS, pH 6.0, and washed with three column volumes of the same buffer. ^{125}I-$(G_{T1b})_{13}$BSA binding activity was eluted, along with 20% of the applied protein, in a step composed of 10 mM Mes, 10 mM CHAPS, 0.2 M NaCl, pH 6.0. Fractions containing protein were detected by UV absorbance at 280 nm, pooled and stored in aliquots at −20°C.

2.3. Tissue distribution of ^{125}I-$(G_{T1b})_{13}$BSA binding activity

In experiments to assess the tissue distribution of the ^{125}I-$(G_{T1b})_{13}$BSA binding activity, various tissue membrane fractions were extracted in a total of 5 ml of the CHAPS-Mes buffer described above. Membranes from the following sources were extracted at the indicated concentrations (in mg/ml protein): synaptosomes (0.5 and 2.0); liver (0.5); sciatic nerve (0.25); myelin (2.0); and muscle sarcolemma (2.0). Samples were incubated with gentle shaking at 4°C for 1 h. Following centrifugation at $100\,000 \times g$ for 1 h, samples were subjected to ion-exchange chromatography: 1–2 ml aliquots of solubilized membrane protein, prepared as described above, were applied to a 1 ml column of CM-Sepharose which was equilibrated, washed, and eluted as described for synaptosomal membranes above.

2.4. ^{125}I-$(G_{T1b})_{13}$BSA binding to solubilized membrane proteins

Ganglioside G_{T1b} was covalently linked, thorough its ceramide moiety, to the carrier protein bovine serum albumin (BSA) using a maleimide/sulfhydryl crosslinking scheme [16]. The conjugate was purified by ion exchange HPLC and size exclusion chromatography, and characterized by quantitative carbohydrate composition analysis and SDS-PAGE [8]. On average, the conjugate used in these studies carried 13 covalently bound G_{T1b} molecules per BSA molecule. The conjugate (typically 50 μg) was radioiodinated using Na^{125}I (1 mCi) and Iodobeads (Pierce, Rockford, IL) and collected by gel filtration chromatography. Specific activities varied from 0.074 to 1.3 mCi/nmol, as indicated in figure and table legends. Radioligand binding was determined using a polyethyleneglycol precipitation assay, which selectively precipitates the larger receptor-ligand complex without precipitating ^{125}I-$(G_{T1b})_{13}$BSA [9,17]. Aliquots of solubilized protein (as indicated, typically ≈ 1 μg protein per assay) were incubated with ^{125}I-$(G_{T1b})_{13}$BSA (as indicated, typically 50–

250 pM) in 0.5 ml of a buffer containing 50 mM Hepes and 0.01% Triton X-100, pH 7.4. Following a 30 minute incubation at 4°C, 0.5 ml of ice-cold 30% polyethyleneglycol in 50 mM Hepes buffer (pH 7.4) was added with rapid mixing, and the solution was incubated for 15 min at 4°C. Precipitated receptor-ligand complex was collected on glass fiber filters using a Brandel Cell Harvester. Filters were washed rapidly with 15% polyethyleneglycol in 50 mM Hepes buffer, pH 7.4, collected, and radioactivity determined using a γ-radiation counter. Non-specific binding was determined in parallel incubations containing 10–20 μM G_{T1b} as specific inhibitor.

2.5. G-100 gel filtration chromatography of ^{125}I-$(G_{T1b})_{13}$BSA binding activity

A 1 × 45 cm Sephadex G-100 column was equilibrated at 4°C in 50 mM Hepes, 10 mM CHAPS, 150 mM NaCl, pH 7.4. The elution volume of molecular weight standards and of ^{125}I-$(G_{T1b})_{13}$BSA-binding activity were obtained in separate runs. For standardization, the column was loaded with 275 μl of running buffer containing 0.5 mg cytochrome *c*, 2 mg soybean trypsin inhibitor, 2 mg bovine serum albumin, and 1 mg of catalase. Flow rate was maintained at 4 ml/h and 0.75 ml fractions were collected. Elution of standards was monitored by A_{280} (proteins) and A_{800} (cytochrome *c* and catalase). A subsequent standard solution composed only of 2 mg each of bovine serum albumin and soybean trypsin inhibitor was run under the same conditions to confirm elution volumes for these standards. Finally, ^{125}I-$(G_{T1b})_{13}$BSA binding activity in a preparation of solubilized synaptosomal proteins was applied and eluted as described above and in the legend to Fig. 5. Each fraction was assayed for radioligand binding and protein concentration.

2.6. Proteinase treatment of ^{125}I-$(G_{T1b})_{13}$BSA binding activity

Trypsin (Worthington Biochemicals Cat. #TRL3) was prepared as a 1 mg/ml solution in 1 mM HCl and added to detergent-solubilized proteins in 1.5 ml microfuge tubes at the indicated concentrations. Control samples received an equivalent aliquot of 1 mM HCl without trypsin. Pronase (Calbiochem Cat. #537088) was prepared as a 10 mg/ml solution in 50 mM Hepes, pH 7.4. For pronase digestion experiments, 1 ml of Hepes, pH 7.4, was added to 1 ml of synaptosomal membrane fraction (5.4 mg protein) in each of two tubes. One tube received 0.2 ml of the pronase solution (final concentration 0.9 mg/ml pronase) while the other tube received 0.2 ml of 50 mM Hepes, pH 7.4. Tubes were incubated for 30 min at 37°C with gentle shaking then were centrifuged at 100 000 × *g* for 30 min. The resulting pellets were washed and resuspended in 2 ml of solubilization buffer (see above) via brief manual homogenization using a glass-Teflon homogenizer. The resulting membrane suspensions were incubated with gentle shaking for 1 h at 4°C, then centrifuged at 100 000 × *g* for 1 h. The resulting supernatant was passed over a 1 ml CM-Sepharose column equilibrated with 10 mM Mes, 10 mM CHAPS, pH 6.0, and washed with two column volumes of the same buffer. Bound protein was eluted in a step of the same buffer containing 0.2 M NaCl. Binding activity in the salt-eluted protein was determined as described above.

2.7. Protein determinations

Protein concentrations were routinely determined using the BCA assay (Pierce) using conditions supplied by the manufacturer. For fractions obtained following G-100 chromatography, protein concentration was determined using a slot-blot dye binding assay described previously [18].

3. Results

3.1. Neoganglioprotein binding to solubilized rat brain synaptosomal proteins

Previous studies demonstrated the presence of a ganglioside receptor in central nervous system myelin membranes which is specific for the structurally related '1b' family of gangliosides (G_{D1b}, G_{T1b}, and G_{Q1b}). In light of the broad distribution of gangliosides in the brain, it seemed likely that the myelin specific receptor might represent one of a family of receptors potentially involved in mediating cellular functions via interactions with gangliosides. In an effort to identify additional receptors, other central nervous system membrane fractions were analyzed for the presence of ^{125}I-$(G_{T1b})_{13}$BSA binding activity. This report describes a ^{125}I-$(G_{T1b})_{13}$BSA binding activity readily solubilized from rat brain synaptosomes which is distinct from the myelin ganglioside receptor.

Following extraction of ≈ 20% of the proteins from synaptosomes using the detergent CHAPS, the solubilized proteins were subjected to CM-Sepharose ion-exchange chromatography as described in Experimental procedures. This procedure removed anionic lipids, including gangliosides, which might act as endogenous inhibitors in the binding assay. Anionic lipids (and some proteins) eluted from the resin unretarded (data not shown), while a portion of the proteins were retained and eluted with a higher salt concentration. Following chromatography, ^{125}I-$(G_{T1b})_{13}$BSA binding assays were performed using polyethyleneglycol precipitation to separate bound from free radioligand. Significant levels of binding activity in the solubilized membrane fraction were detected (Fig. 1). Binding increased with increasing amount of membrane protein added, and was reduced to low levels by addition of the inhibitor, underivatized G_{T1b}. Subsequently, specific binding was defined as binding in the absence of G_{T1b} (total) less binding in the presence of 10–20 μM G_{T1b}. A

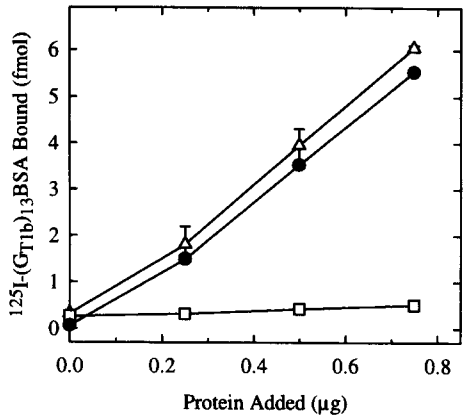

Fig. 1. ^{125}I-(G$_{T1b}$)$_{13}$BSA binding to CHAPS-solubilized synaptosomal membrane proteins. Synaptosomes were solubilized with CHAPS, the extract was subjected to CM-Sepharose ion-exchange chromatography, and binding activity of the salt-eluted protein fraction was determined as described under Experimental procedures using the indicated amounts of membrane protein and 35 pM ^{125}I-(G$_{T1b}$)$_{13}$BSA labeled at 1.0 mCi/nmol. Specific binding (filled circles) was obtained by subtracting background binding (open squares) from total binding (open triangles). Background binding was determined in the presence of 20 μM G$_{T1b}$. Data are presented as mean ± S.D. for duplicate determinations.

pH profile of the binding activity (Fig. 2) indicated that binding was optimum at a neutral pH, and subsequent binding assays were performed using Hepes buffer (pH 7.4). The binding activity was not reduced by addition of up to 100 mM NaCl, nor was it affected by addition of 5 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$. However, binding was significantly reduced by addition of 250 mM NaCl, 5 mM

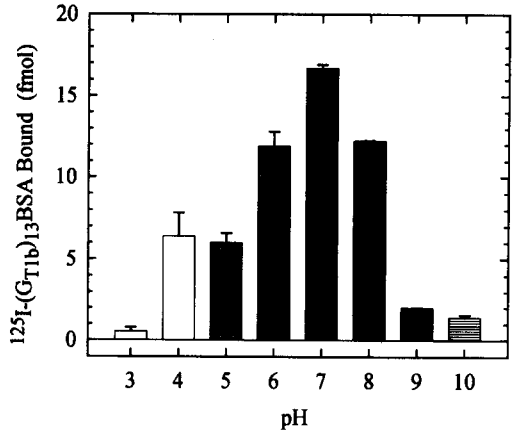

Fig. 2. Effect of pH on ^{125}I-(G$_{T1b}$)$_{13}$BSA binding to solubilized synaptosomal proteins. Binding assays were performed as described in the text, with the exception that 50 mM Hepes buffer (pH 7.4) was replaced with 50 mM of the following (each buffer type is represented by a different column fill pattern): citrate, pH 3 and pH 4; Mes, pH 5 and pH 6; Hepes, pH 7; Tris (tris-(hydroxymethyl)aminomethane), pH 8 and pH 9; and CAPS (3-(cyclohexylamino)propanesulfonic acid), pH 10. In addition, the PEG solution used for precipitation of receptor-ligand complex was prepared in water rather than 50 mM Hepes. Each assay contained 0.67 μg synaptosomal protein and 75 pM ^{125}I-(G$_{T1b}$)$_{13}$BSA labeled at 0.93 mCi/nmol. Data are presented as mean ± S.D. for duplicate determinations.

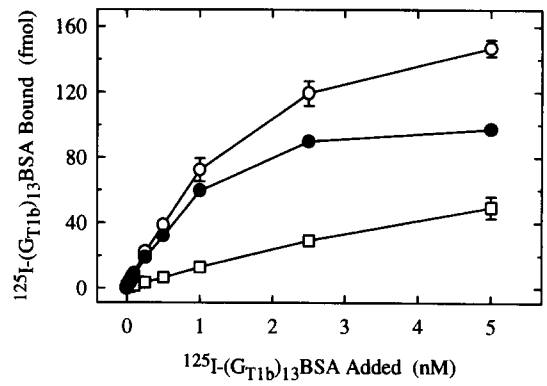

Fig. 3. Saturation isotherms for ^{125}I-(G$_{T1b}$)$_{13}$BSA binding to solubilized synaptosomal membranes. Solubilized synaptosomal membrane protein was incubated with the indicated concentrations of ^{125}I-(G$_{T1b}$)$_{13}$BSA (0.68 mCi/nmol) and assayed as described under Experimental procedures using 0.73 μg protein/assay. Specific binding (filled circles) is defined as total binding (open circles) minus non-specific binding (open squares, binding in the presence of 10 μM G$_{T1b}$). Data are presented as mean ± S.D. for triplicate determinations.

$CoCl_2$ or 20 mM $CaCl_2$, $MgCl_2$, or $MnCl_2$ (data not shown).

Binding isotherms performed with increasing concentrations of ^{125}I-(G$_{T1b}$)$_{13}$BSA incubated with equivalent amounts of protein solubilized from rat brain synaptosomes revealed high affinity, saturable binding (Fig. 3). Non-specific binding was non-saturable, increasing linearly with increasing concentrations of radioligand. Scatchard transformation of specific binding data was linear and revealed a K_D of 1.0 nM and a B_{max} of 173 pmol/mg protein added (Fig. 4). Addition of an excess of bovine serum albumin (1 mg/ml, 1.5 μM) in the assay did not reduce ^{125}I-(G$_{T1b}$)$_{13}$BSA binding, indicating that recognition of the ganglioside moiety was responsible for the high binding affinity of the conjugate.

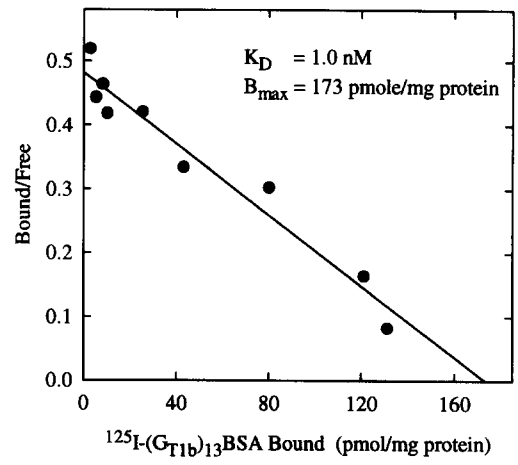

Fig. 4. Scatchard transformation of ^{125}I-(G$_{T1b}$)$_{13}$BSA binding to solubilized synaptosomal protein. Data from saturation isotherms in Fig. 3 were transformed by the method of Scatchard [28]. B_{max}, total receptor concentration; K_D, apparent dissociation constant.

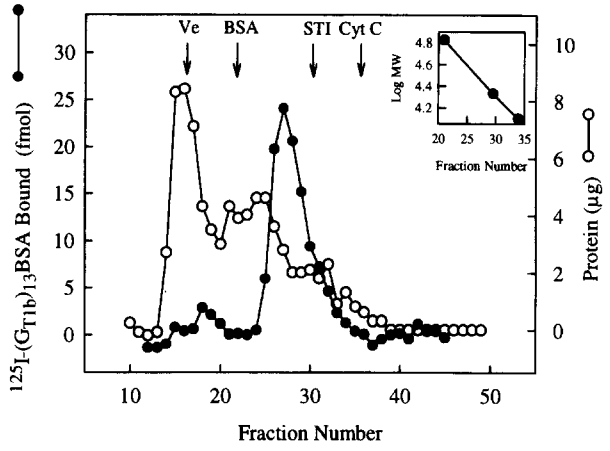

Fig. 5. Gel filtration chromatography of ^{125}I-(G$_{T1b}$)$_{13}$BSA binding activity solubilized from synaptosomes. CHAPS-solubilized synaptosomal protein (≈ 140 μg in 200 μl) was loaded onto a 1.0×45 cm Sephadex G-100 gel filtration column equilibrated with a buffer composed of 50 mM Hepes, pH 7.4, 10 mM CHAPS, and 150 mM NaCl. The flow rate was maintained at approximately 4 ml/h with collection of 0.75 ml fractions. Protein (open symbols) and radioligand binding (filled symbols) were determined as described under Experimental procedures. Binding assays contained 200 μl of each column fraction and 250 pM ^{125}I-(G$_{T1b}$)$_{13}$BSA labeled at 0.074 mCi/nmol. Elution positions of the standards BSA, soybean trypsin inhibitor (STI), and cytochrome *c* (cyt c), run separately under identical conditions, are indicated with arrows at the top of the figure, where V_e represents exclusion volume. (Inset) Log molecular weight of standards vs. fraction number, indicating a linear relationship between size and time of elution.

3.2. Molecular properties of the synaptosomal ganglioside binding protein

Gel exclusion chromatography was performed to characterize the relative molecular weight of the binding protein solubilized from rat brain synaptosomes (Fig. 5). Based on comparison to standards run under identical conditions, these data indicate that the ^{125}I-(G$_{T1b}$)$_{13}$BSA binding activity solubilized from synaptosomes has a M_r of ≈ 28 000 Da.

To determine if the binding activity was a protein, solubilized synaptosomal proteins were treated with trypsin prior to performing a binding assay. Proteolysis was inhibited by the addition of soybean trypsin inhibitor. Control samples were treated with soybean trypsin inhibitor only, or trypsin in the presence of soybean trypsin inhibitor. Results consistently demonstrated significant (> 40%), although incomplete loss of binding activity when samples were treated with trypsin (Table 1). In contrast, binding was reduced by less than 5% when samples were treated with trypsin in the presence of soybean trypsin inhibitor, or treated with soybean trypsin inhibitor alone.

Because trypsin is a relatively specific proteinase, it was possible that partial proteolysis of the binding activity results in a functional protein with reduced binding activity. Therefore, membranes were treated with pronase, a preparation containing several proteinases. The synaptosome fraction was treated with pronase for 30 min and treated membranes were pelleted by centrifugation, washed, resuspended in CHAPS solubilization buffer, and solubilized protein was subjected to CM-Sepharose ion-exchange chromatography prior to assay (see Experimental procedures). Control samples were treated in an identical manner, except that the initial incubation was in the absence of pronase. To control for small amounts of pronase that might be carried into the assay from the initial incubation, assays were also performed on samples in which equal amounts of treated and control protein were combined. Pronase treatment of synaptosomal membranes resulted in 93% loss of binding activity (Table 1). Controls in which pronase treated and untreated samples were mixed indicate that pronase contamination of the binding assay was not

Table 1
Proteinase sensitivity of ^{125}I-(G$_{T1b}$)$_{13}$BSA binding to proteins solubilized from brain membranes

Treatment	^{125}I-(G$_{T1b}$)$_{13}$BSA binding activity (% of control)
Synaptosomal membrane proteins treated after solubilization [a]	
control	100
+ trypsin (50 μg/ml)	58 ± 4
+ soybean trypsin inhibitor (250 μg/ml)	96 ± 1
+ trypsin and soybean trypsin inhibitor	103 ± 2
Synaptosomal membranes pretreated before solubilization [b]	
control membranes	100
pronase-treated membranes	7 ± 1
control membranes + pronase-treated membranes	90 ± 2

[a] Solubilized synaptosomal proteins (30 μg/ml) were incubated at room temperature for 30 min in 200 μl of 50 mM Hepes, 10 mM CHAPS, 150 mM NaCl, pH 7.4, containing 50 μg/ml trypsin. Proteolysis was stopped by the addition of a 5-fold weight excess of soybean trypsin inhibitor. In control samples, solubilized protein was incubated in the presence of trypsin inhibitor alone, or with trypsin premixed with trypsin inhibitor. Samples were assayed for binding activity as described under Experimental procedures using 1.2 μg synaptosomal protein per assay, and 200 pM ^{125}I-(G$_{T1b}$)$_{13}$BSA labeled at 0.18 mCi/nmol.

[b] Synaptosomal membranes (5.4 mg membrane protein) were incubated in 2.2 ml of 50 mM Hepes, pH 7.4, in the presence or absence (control) of 0.9 mg/ml pronase. Proteolysis was allowed to proceed for 30 min at 37°C, at which time membranes were centrifuged at 100 000 × *g*, and the supernatant discarded. Pellets were washed three times with 1 ml of 50 mM Hepes, pH 7.4, and subsequently solubilized and subjected to ion-exchange chromatography as described under Experimental procedures. Samples were assayed for binding activity as described under Experimental procedures using 0.27–0.37 μg synaptosomal protein per assay, and 30 pM ^{125}I-(G$_{T1b}$)$_{13}$BSA labeled at 1.3 mCi/nmol.

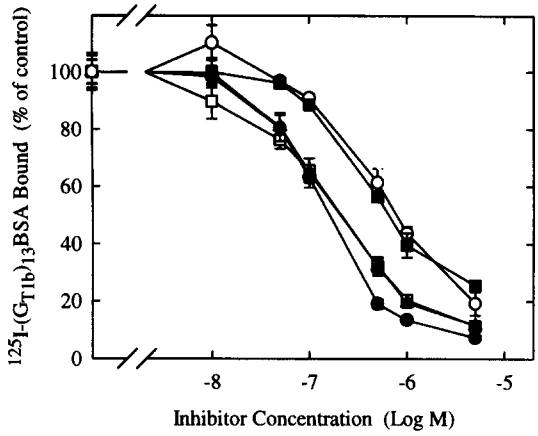

Fig. 6. Inhibition of ^{125}I-(G_{T1b})$_{13}$BSA binding to CHAPS-solubilized synaptosomal protein by gangliosides. Binding assays were performed in the presence of the indicated concentrations of ganglioside using 40 pM ^{125}I-(G_{T1b})$_{13}$BSA (1.2 mCi/nmol) as radioligand and 1.3 μg solubilized synaptosomal protein as receptor. Binding is expressed as a percent of that measured in the absence of added inhibitor, and represents the mean ± S.D. for triplicate determinations. The gangliosides tested were: ●, G_{T1b}; ▲, G_{D1a}; □, G_{D1b}; ○, G_{M1}; and ■, G_{M3}.

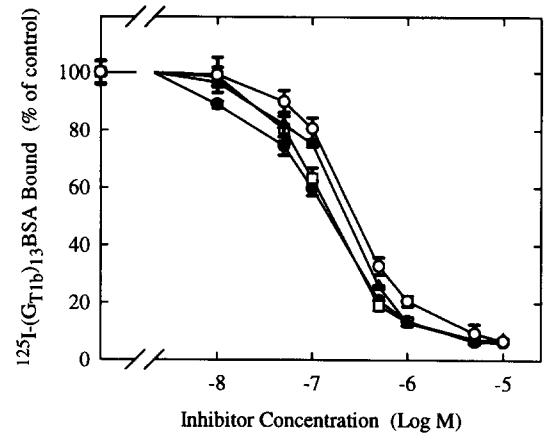

Fig. 7. Inhibition of ^{125}I-(G_{T1b})$_{13}$BSA binding to CHAPS-solubilized synaptosomal protein by sphingolipids and phospholipids. Binding assays were performed in the presence of the indicated concentrations of lipids using 50 pM ^{125}I-(G_{T1b})$_{13}$BSA (0.8 mCi/nmol) as radioligand and 1.0 μg solubilized synaptosomal protein as receptor. Binding is expressed as a percent of that measured in the absence of added inhibitor, and represents the mean ± S.D. for triplicate determinations. The lipids tested in this experiment were: ●, G_{T1b}; □, sialylneolactotetraosylceramide; ○, phosphatidylserine; and ▲, phosphatidylglycerol.

responsible for the observed loss of binding activity in the pronase treated samples.

3.3. Structural specificity of ^{125}I-(G_{T1b})$_{13}$BSA binding to solubilized synaptosomal proteins

The structural specificity of the solubilized binding protein was determined using a variety of lipids and glycoconjugates as inhibitors of ^{125}I-(G_{T1b})$_{13}$BSA binding to the CHAPS solubilized proteins from synaptosomes.

The di- and trisialogangliosides, G_{T1b}, G_{D1a} and G_{D1b} were effective inhibitors of neoganglioprotein binding, with IC_{50} values of ≈ 200 nM (Fig. 6, Table 2). The monosialogangliosides, G_{M1} and G_{M3}, were ≈ 5-fold less effective. The neutral glycosphingolipid asialo-G_{M1} was ineffective at inhibiting neoganglioprotein binding at concentrations up to 10 μM, while the anionic sphingolipids sulfatide and sialylneolactotetraosylceramide were effective inhibitors of ^{125}I-(G_{T1b})$_{13}$BSA binding, having IC_{50} values of 300 nM and 200 nM, respectively (Fig. 7, Table 2). The phospholipids phosphatidylethanolamine and phosphatidylcholine did not inhibit neoganglioprotein binding at concentrations up to 10 μM (Table 2). However, anionic phospholipids, including phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol, were relatively effective inhibitors of binding, having IC_{50} values comparable to those observed for the di- and trisialogangliosides (Fig. 7, Table 2).

To further characterize inhibition by the most effective ganglioside and phospholipid inhibitors, kinetic analyses of ^{125}I-(G_{T1b})$_{13}$BSA inhibition by G_{T1b} and phosphatidyl-

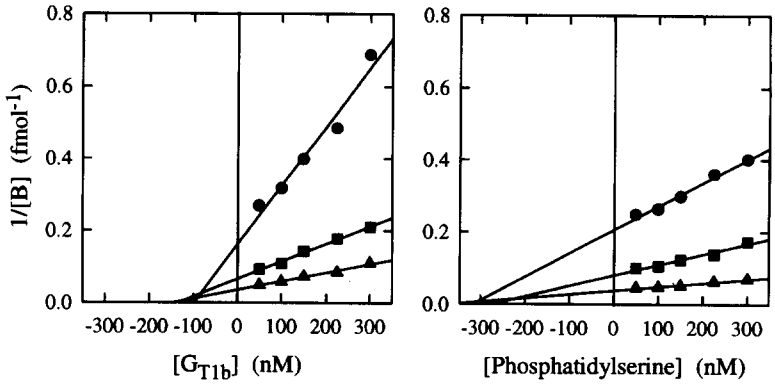

Fig. 8. Dixon plots for inhibition of ^{125}I-(G_{T1b})$_{13}$BSA binding by G_{T1b} and phosphatidylserine. Inhibition studies were performed as described in Experimental procedures, except that binding was measured at three different concentrations of ^{125}I-(G_{T1b})$_{13}$BSA (20 pM, 50 pM, and 100 pM all labeled at 1.0 mCi/nmol) with varying amounts of either G_{T1b} (left panel) or phosphatidylserine (right panel) added as inhibitor. Each assay tube contained 1.5 μg of synaptosomal protein. Symbols correspond to averages of triplicate determinations. Lines correspond to least squares linear regressions for each set of data points.

Table 2
Inhibition of ^{125}I-$(G_{T1b})_{13}$BSA binding to solubilized brain protein by soluble lipids, oligosaccharides, and soluble sugars

Inhibitor	IC_{50} [a] (nM)
Gangliosides	
G_{T1b}	200
G_{D1a}	200
G_{D1b}	200
G_{M1}	1000
G_{M3}	1000
G_{A1}	> 10000
Other sphingolipids	
sulfatide	300
sialylneolactotetraosylceramide	200
Phospholipids	
phosphatidylglycerol	300
phosphatidylserine	300
phosphatidylinositol	600
phosphatidylethanolamine	> 100000
phosphatidylcholine	> 100000
Soluble sugars	
G_{T1b}-oligosaccharide	> 50000
G_{M1}-oligosaccharide	> 50000
2,6-sialyllactose	> 50000
2,3-sialyllactose	> 50000
lactose	> 100000
cellobiose	> 100000
sialic acid	> 500000
glucuronic acid	> 500000

[a] Inhibition studies were performed as described in the legend to Fig. 6 using the binding assay described in Experimental procedures. The concentration of each inhibitor resulting in 50% inhibition (IC_{50}) of ^{125}I-$(G_{T1b})_{13}$BSA binding was determined graphically from inhibition curves performed in triplicate at eight concentrations of each inhibitor.

serine were performed. Single-reciprocal Dixon plots [19] for inhibition of binding by G_{T1b} at three different concentrations of ^{125}I-$(G_{T1b})_{13}$BSA were linear and consistent with competitive inhibition with a K_i of 120 nM (Fig. 8). Dixon analysis of phosphatidylserine inhibition under identical conditions indicated that this lipid also inhibited ^{125}I-$(G_{T1b})_{13}$BSA binding in a competitive manner, with a K_i of 275 nM (Fig. 8). The K_i values agree reasonably well with IC_{50} values obtained from inhibition profiles shown in Figs. 6 and 7.

Oligosaccharides prepared from G_{M1} and G_{T1b} did not inhibit ^{125}I-$(G_{T1b})_{13}$BSA binding at concentrations up to 50 μM. The oligosaccharides sialyllactose, lactose, and cellobiose were also ineffective as inhibitors, as were the monosaccharides sialic acid and glucuronic acid at concentrations up to 0.5 mM (Table 2).

3.4. Tissue specificity of ^{125}I-$(G_{T1b})_{13}$BSA binding

^{125}I-$(G_{T1b})_{13}$BSA binding to CHAPS solubilized proteins from a variety of tissue membranes was determined. Isolated membranes from the central nervous system, peripheral nerve, muscle and liver were treated in a manner identical to rat brain synaptosomes (see Experimental procedures), then were screened for binding activity. For each membrane type, different proportions of membrane protein were extracted and recovered after CM-Sepharose chromatography. Therefore, binding activity was normalized to the amount of membrane protein subjected to extraction. When compared to the activity measured using the synaptosomal extract, which was included in each experiment, the following results were obtained (% compared to synaptosomal extract ± S.D.): skeletal muscle membranes, 188 ± 3%, sciatic nerve membranes, 187 ± 5%, CNS myelin membranes, 50 ± 3%, and liver membranes, 46 ± 2%. Proteins solubilized from sciatic nerve and muscle membranes had > 4-fold higher specific binding than those solubilized from liver membranes.

4. Discussion

The polyvalent ganglioside-protein conjugate, ^{125}I-$(G_{T1b})_{13}$BSA was used to detect a novel lipid binding protein in detergent extracts from synaptosomes. Binding activity only became apparent after extracted proteins were resolved from anionic lipids on a cation exchange column, presumably due to removal of endogenous gangliosides and other inhibitory anionic lipids. A distinct ganglioside binding activity on myelin membranes [8,9] was only revealed after removal of endogenous lipids by extraction with buffer containing 0.3% deoxycholate, which left the ganglioside binding activity on the residual myelin membranes. The same treatment of synaptosomal membranes strips them of ganglioside binding activity, although no activity is measurable in the deoxycholate (or cholate) extracts (data not shown). Treatment of synaptosomes with either 10 mM CHAPS or 0.1% Triton X-100 provided soluble synaptosomal protein preparations which bound ^{125}I-$(G_{T1b})_{13}$BSA after CM-Sepharose chromatography (data not shown). This is in contrast to the ganglioside binding activity on myelin membranes [9], which is resistant to detergent solubilization (up to 1% Triton X-100) unless high salt concentrations are used (400 mM KCl).

The relatively small size of the synaptosomal binding protein (28000 Da, Fig. 5) indicates that it is not among a group of high molecular weight proteins which have previously been characterized as ganglioside binding proteins, including fibronectin [20], and some growth factor receptors, such as the EGF receptor [21]. Given that the activity was solubilized from synaptosomes, we considered whether it was membrane bound or released from the intrasynaptosomal space. Two lines of evidence support a membrane localization. First, osmotic shock of the synaptosomes and collection of membranes by centrifugation prior to extraction and CM-Sepharose resolution led to ^{125}I-$(G_{T1b})_{13}$BSA binding activity with properties like those extracted from intact synaptosomes (data not shown). Second, pre-incubation of synaptosomes with and without pronase under mild osmotic conditions resulted in retention of the binding activity in the control and loss of binding activity in the

proteinase treated sample. This suggests that the activity is not 'cryptic' but is exposed at the synaptosomal surface.

Comparison of the inhibition profiles of a number of different gangliosides (Fig. 6, Table 2) demonstrates a ^{125}I-$(G_{T1b})_{13}$BSA binding specificity distinct from that previously characterized on CNS myelin membranes [8]. Whereas the myelin activity demonstrated a specificity for the '1b' series of gangliosides (having a NeuAc2–8NeuAc2–3 group on the internal galactose of the gangliotetraose chain) the activity solubilized from synaptosomes was equally inhibited by G_{D1b} and G_{D1a} (the latter having two NeuAc2–3 residues, one on each galactose). Although there was not a direct relationship between blocking efficiency and the number of sialic residues on a ganglioside (G_{T1b} is not more effective than G_{D1b}), the monosialogangliosides G_{M1} and G_{M3} were 5-fold less effective at blocking ^{125}I-$(G_{T1b})_{13}$BSA binding than the polysialogangliosides tested. The inability of G_{T1b} oligosaccharide to effectively inhibit ^{125}I-$(G_{T1b})_{13}$BSA binding suggests that the ceramide moiety of the intact lipid may be important for binding. Alternatively, the ability of the intact lipids to insert into the detergent micelles present in the assay may allow them to be presented to the binding protein in a more potent multivalent array. The importance of carbohydrate valency has been demonstrated for vertebrate lectins [22], and may also be critical for high-affinity binding in this system.

The ability of some phospholipids to competitively inhibit ^{125}I-$(G_{T1b})_{13}$BSA binding to solubilized synaptosomal membrane proteins distinguishes this activity from that previously characterized on myelin membranes, on which anionic phospholipids exerted less potent, non-competitive inhibition [8].

Tissue distribution data further highlighted significant differences between the ganglioside binding activity solubilized from synaptosomes and that on myelin membranes. The ganglioside binding activity previously described was limited to central nervous system myelin, and was not detected at significant levels on sciatic nerve or liver membranes [8,9]. In contrast, binding activity similar to that initially solubilized from synaptosomes was solubilized from the membranes of sciatic nerve, muscle, and a smaller amount from liver and CNS myelin.

The role of this newly characterized binding protein remains to be determined. A variety of small, broadly distributed lipid binding proteins have been described which mediate the transfer of lipids between intracellular membranes [23]. The specificities of the lipid transfer proteins that have been described, however, do not correspond to those demonstrated for the lipid binding protein reported here. Glycolipid transfer proteins bind both charged and neutral glycosphingolipids, and do not bind phospholipids. Non-specific phospholipid transfer proteins have been characterized which bind both phospholipids and glycolipids, but they do not discriminate among phospholipid structures. Furthermore, lipid transfer proteins are cytosolic, and the cytosolic fraction obtained from whole rat brain did not demonstrate significant ^{125}I-$(G_{T1b})_{13}$BSA binding activity (data not shown), making lipid transfer between intracellular membranes an unlikely function.

The membrane location of the ganglioside binding protein reported here puts it in an ideal location to mediate regulatory effects that involve lipid interaction. Second messenger systems involving the breakdown products of phospholipids [24–26] and sphingolipids [27] are the focus of current research, and intact gangliosides have been implicated in a variety of signal transduction events including kinase activation and cell-cell recognition [3,4]. In light of the emerging role of lipids as regulatory components at the cell surface, both as cell recognition molecules and regulators of membrane protein function, the binding protein described here may be among a variety of cell surface proteins involved in lipid-mediated cell regulation.

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