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Binding and transport of gangliosides by prosaposin

(saposins/sphingolipid activator proteins/ganglioside binding protein/ganglioside transport protein)

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ABSTRACT **Prosaposin, the precursor of saposins A, B, C, and D, which activate lysosomal hydrolysis of sphingolipids, exists in various tissues and body fluids and is especially abundant in the nervous system. Prosaposin and saposins A, B, C, and D formed stable complexes with 13 different gangliosides as measured by an assay using column chromatography. Gangliosides of the gangliotetraose type (*a* series) were bound with high affinity, whereas *b* series gangliosides, O-acetylated gangliosides, and gangliosides with shorter carbohydrate chains, were bound with lower affinity. Prosaposin and saposins transferred gangliosides from donor liposomes to erythrocyte ghost membranes. Prosaposin also stimulated ganglioside GM1 β-galactosidase more than mature saposins. Prosaposin exists as a secretory protein and as an integral membrane protein, and we propose that prosaposin is active as a ganglioside binding and transport protein *in vivo*.**

Gangliosides are enriched in neural membranes and have been implicated in a wide range of processes including neuronal differentiation, the outgrowth and extension of neurites (neuritogenesis), synaptogenesis, cell recognition, nervous system repair, and protection against excitotoxic damage in the nervous system (1–5) presumably acting as modular regulators (6, 7). Ganglioside insertion into membranes (8, 9) may be mediated by surface-located membrane-bound (receptor) proteins and several candidate proteins for ganglioside binding and transfer have been inferred (9–13) but not identified. In this report, we demonstrate that prosaposin, the precursor of saposins A, B, C, and D (14), binds tightly to gangliosides, forms stable ganglioside–prosaposin complexes, and transfers gangliosides from donor liposomes to acceptor membranes. Prosaposin exists as a secretory form and an integral membrane form in addition to serving as precursor for the lysosomal saposin proteins (15). We propose that prosaposin may act as a ganglioside binding and transport protein *in vivo*.

MATERIALS AND METHODS

Materials. Ganglioside GQ1b was a gift from Yoshio Hirabayashi (Riken, Japan) and [^{35}S]sulfatide was a gift of Arvan Fluharty (UCLA). Mixtures of gangliosides enriched with *O*-acetyl GT1c and *O*-acetyl GQ1c, respectively, were obtained by Fractogel DEAE column chromatography as described (16), and mixtures enriched with nonacetylated GT1c and GQ1c, respectively, were also obtained (16). All other gangliosides were isolated and purified as described (17).

Saposins A, C, and D were all isolated in pure form from the spleen of a patient with Gaucher disease as described (18). Saposin B was purified from a heat-treated extract of Gaucher disease spleen by a slightly modified procedure involving reverse-phase HPLC on a C4 column and isoelectric focusing with the Rotofor cell as described elsewhere. Each purified protein gave a single band on SDS/PAGE, and no cross-contamination of one saposin with another was detected after immunoblotting using monospecific antibodies against saposins A, B, C, and D, respectively.

Prosaposin was purified from the spent media of *Spodoptera frugiperda* (Sf9) cells infected with a baculovirus expression vector (pAC 610) containing a full-length cDNA for human prosaposin as described elsewhere (19); purification steps included column chromatography on Con A-Sepharose and reverse-phase HPLC on a C4 column. The purity of the final purified preparation was assessed by SDS/PAGE, immunoblotting, and N-terminal analysis. Naturally occurring prosaposin was also isolated by the same method from human seminal plasma (19).

Sodium boro[^{3}H]hydride and [^{14}C]formaldehyde were obtained from NEN. Bio-Lyte (Ampholyte) and Bio-Gel P-30 were purchased from Bio-Rad. Con A-Sepharose and S Sepharose Fast Flow were obtained from Pharmacia. Dimethylamine borane and ovalbumin were obtained from Aldrich and Sigma, respectively.

Assays for Stimulation of Enzymatic Activities. Ganglioside GM1 β-galactosidase assays were carried out as described (20) using human liver β-galactosidase purified to near homogeneity (21) as the enzyme source. A partially purified preparation of human placental sialidase prepared as described by Hiraiwa *et al.* (22) was used as the source of sialidase. Sialidase assays were carried out as described (22) using gangliosides GM3 and GD1a as substrates, measuring released sialic acid by a sensitive fluorescence assay (23).

Radiolabeling. Ganglioside GM1 was labeled in the terminal galactose moiety (24) and saposins A, B, C, and D and prosaposin were radiolabeled by reductive formylation (25) using 200 μg of individual saposins or prosaposin.

Binding Assays. [^{14}C]Saposin or prosaposin (0.1–0.5 nmol; 2000–5000 cpm) and various gangliosides (0.1–20 nmol; 10,000–100,000 cpm) were mixed and incubated in 100 μl of 50 mM sodium acetate buffer (pH 4.0) at 37°C for 30 min unless otherwise specified. The incubation mixture was then applied to a small column of S Sepharose (0.6 × 1.0 cm), which was prewashed with 50 mM sodium acetate buffer (pH 4.0) (buffer I). The column was first eluted with 2 ml of buffer I followed by 2 ml of the same buffer containing 1.0 M sodium chloride (buffer II). The first fraction contained prosaposin or saposins A, B, C, and D bound to gangliosides, and the second fraction contained unbound prosaposin or saposins. Analysis of saturation curves by Lineweaver–Burk plots was performed with the computer program STATVIEW 512 (Brain Power, Calabasas, CA). [^{35}S]Sulfatide behaved in a manner similar to gangliosides in this system.

Analysis of Saposin–Ganglioside Complexes. Isoelectric focusing was carried out with a Rotofor cell apparatus (Bio-Rad). Rotofor runs were performed with 2% ampholyte (pH 2–5) in a vol of 50 ml. A 200-μl sample was diluted 1:2 with 4% Bio-Lyte 2/5 and injected at the middle of the cell chamber. Focusing of samples was performed at constant

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power (12 W) for 5 hr at 5°C. After fractionation, the pH and the radioactivity of each fraction were measured.

Gel filtration analysis was carried out by HPLC on a Shimadzu Shim-pack Diol-150 column (0.7 × 25 cm). The column was eluted with 10 mM sodium acetate buffer (pH 5.2) containing 0.1 M sodium chloride, with a flow rate of 0.5 ml/min; 1-ml fractions were collected and assayed for radioactivity.

Assay for Ganglioside Transport. Donor liposomes containing [^{3}H]ganglioside GM1 were prepared with egg phosphatidylcholine (12 μmol), cholesterol (6 μmol), dicetylphosphate (0.5 μmol), and [^{3}H]ganglioside GM1 (0.5 μmol) using a modified procedure of Radin and Metz (26). These lipids were dissolved in chloroform/methanol (1:1) and evaporated to dryness; then 2.5 ml of 10 mM sodium acetate buffer (pH 4.0) containing 0.15 M sodium chloride was added. The solution was Vortex mixed for 1 min and then sonicated under an N_2 stream for 1 hr on ice. After sonication, liposomes were purified by gel filtration on a Sepharose CL-4B column (1.7 × 15 cm), which was equilibrated with 10 mM sodium acetate buffer (pH 4.0) containing 0.15 M sodium chloride. The liposomes containing ganglioside GM1 were eluted immediately after the void volume. The volume of this fraction was adjusted by the addition of elution buffer to contain 5 nmol of [^{3}H]ganglioside GM1 in 70 μl and used for lipid transfer assays. Human erythrocytes were obtained fresh from a volunteer and erythrocyte ghosts were prepared according to Radin and Metz (26) and used as acceptor membranes.

The donor liposomes (70 μl) were incubated with 50 μl of the ghost membrane suspension (200 μg of protein) in a total vol of 500 μl in 10 mM sodium acetate buffer (pH 4.0) containing 0.15 M sodium chloride at room temperature in the presence of 50 μg of ovalbumin and various amounts of saposins or prosaposin. After 1 hr of incubation, the samples were centrifuged and the membrane precipitates were washed three times with 1 ml of ice-cold 10 mM sodium acetate buffer (pH 4.0) containing 0.15 M sodium chloride. The precipitates were suspended in 1 ml of the same buffer, and ^{3}H radioactivity (900-μl portions) and protein contents (50-μl portions) were measured by liquid scintillation counting and the method of Lowry *et al.* (27), respectively.

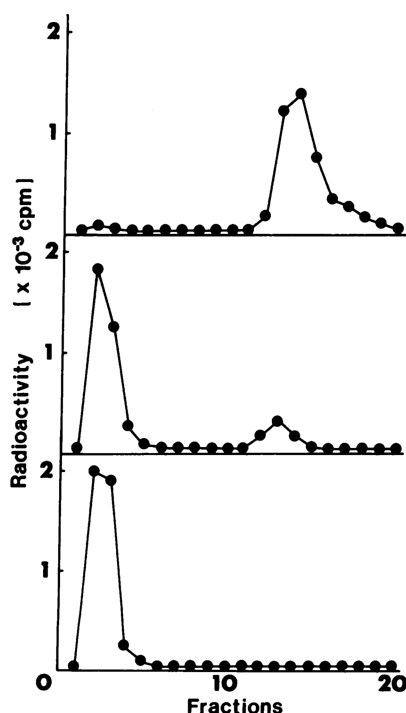

FIG. 1. Ion-exchange column chromatography of saposin B, ganglioside GM1, and saposin B–ganglioside GM1 complexes. (*A*) [^{14}C]Saposin B (0.5 nmol; 5000 cpm) without incubation with ganglioside was applied to an S Sepharose column. Two-hundred-microliter fractions were collected and the radioactivity in each fraction was measured. (*B*) The same amount of [^{14}C]saposin B was incubated with 1 nmol of ganglioside GM1 and analyzed. (*C*) The same amount of [^{14}C]saposin B was incubated with 5 nmol of ganglioside GM1 and analyzed. ●, ^{14}C radioactivity.

RESULTS

Optimal binding of ganglioside GM1 to prosaposin as well as to saposins A, C, and D was found at pH 4.0 with a marked reduction above pH 5.0. Binding occurred over a wide temperature range (4°C–80°C) and was very rapid, nearing completion within 3 min of incubation. The binding increased by increasing the ratio of ganglioside to each protein (Fig. 1). Prosaposin and saposins A, B, C, and D were all incubated with various amounts of different gangliosides and the fraction that became bound to each ganglioside was determined. Seminal plasma prosaposin was compared to recombinant prosaposin in the initial binding studies; binding was the same for both proteins. In all later studies, recombinant prosaposin was used because of the limited availability of seminal plasma prosaposin.

Kinetic analyses suggested that saposins A and B bound to ganglioside GM1 with high affinity (Fig. 2), whereas saposins C and D appeared to bind with lower affinity. There were also indications of high-affinity binding with much lower capacity by saposins C and D. The binding by prosaposin was intermediate between these two groups of saposins. Similar results, high-affinity binding by saposins A and B and low-affinity binding by saposins C and D, were obtained with 12 additional gangliosides. These binding curves suggest that the binding between saposins and gangliosides may be a cooperative process, which may reflect conformational changes of saposins or ganglioside aggregation and micelle formation. With the assay method used, estimation of bound and unbound ganglioside for calculation of actual affinity constants by Scatchard plots was not possible. However, plotting the binding data by Lineweaver–Burk analysis made it possible to calculate half-effect values (concentrations of gangliosides necessary to bind one-half the capacity of each saposin) (see Fig. 2). Although these values are not directly translatable into actual binding constants, they allowed for comparison of relative binding affinities of prosaposin and the four saposins for 13 different gangliosides and sulfatide (Table 1).

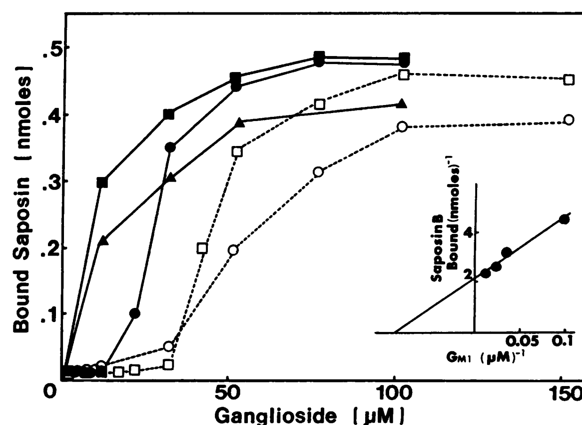

FIG. 2. Binding of prosaposin and saposins to ganglioside. [^{14}C]Prosaposin (●), saposin A (■), saposin B (▲), saposin C (○), and saposin D (□) (0.5 nmol; 5000 cpm) were incubated with increasing amounts of ganglioside GM1 and the amounts of saposin–ganglioside complexes formed were determined. (*Inset*) Lineweaver–Burk plot of binding between saposin B and ganglioside GM1.

Table 1. Binding of gangliosides to prosaposin and saposins A, B, C, and D

	Structure	Prosaposin	Sap A	Sap B	Sap C	Sap D
GM1	Gal-GalNAc-Gal-Glc-Cer	(1.0)	7.1	11.9	(8.3)	(8.0)
	SA	2.8			55.6	24.6
GM2	GalNAc-Gal-Glc-Cer		9.5	17.3	(10.0)	(5.1)
	SA				72.0	74.0
GM3	Gal-Glc-Cer		12.9	25.0	(12.0)	(8.1)
	SA				74.0	64.0
GD1a	Gal-GalNAc-Gal-Glc-Cer	(<2.0)	8.0	6.4	(<1.0)	(<2.0)
	SA SA	8.0			33.3	55.8
GD1b	Gal-GalNAc-Gal-Glc-Cer		13.8	19.2	(7.6)	(14.3)
	SA				90.0	95.2
	SA					
GT1b	Gal-GalNAc-Gal-Glc-Cer	(<2.0)	6.3	5.8	(<2.0)	(<1.0)
	SA SA	8.7			24.3	11.7
	SA					
GT1c	Gal-GalNAc-Gal-Glc-Cer		16.5	28.6	(0.6)	(2.3)
	SA				35.7	100
	SA					
	SA					
Acetyl-GT1c			35.1	58.8	(0.7)	(1.0)
					35.7	62.5
GQ1b	Gal-GalNAc-Gal-Glc-Cer	(1.0)	18.8	16.6	(6.6)	(<1.0)
	SA SA	8.0			34.4	30.7
	SA SA					
GQ1c	Gal-GalNAc-Gal-Glc-Cer		10.7	12.8	(<1.0)	(<1.0)
	SA SA				16.7	30.7
	SA					
	SA					
Acetyl-GQ1c			19.8	22.3	(3.4)	(2.7)
					28.7	55.5
GP1c	Gal-GalNAc-Gal-Glc-Cer		11.7	19.4	(3.3)	(1.5)
	SA SA				10.3	14.2
	SA SA					
	SA					
Acetyl-GP1c			21.7	28.9	(1.0)	(1.2)
					24.7	37.7
Sulfatide		NB		64.5		

Half-effect concentrations (μM) are listed in two categories—higher capacity and lower capacity (in parentheses). NB, no binding detected; SA, *N*-acetylneuraminic acid; Sap, saposin.

From these data, some structural rules were apparent. These rules include (*i*) gangliosides with shorter carbohydrate chains, such as gangliosides GM2 and GM3, were bound with lower affinity than those with longer carbohydrate chains; (*ii*) gangliosides of the gangliotetraose type with the greatest number of sialic acid residues had the highest binding affinity; (*iii*) gangliosides of the *a* series (with terminal sialic acid residues) were bound more avidly than gangliosides of the *b* series (without terminal sialic acids) by approximately a factor of 3; and (*iv*) when sialic acid residues were acetylated, binding affinity was reduced.

Of the five proteins, prosaposin had the highest apparent affinity, especially for complex gangliosides, such as ganglioside GQ1b. The binding affinity of saposin B for sulfatide was reduced compared to the affinity for gangliosides and prosaposin did not bind sulfatide.

Stable complex formation between gangliosides and saposins was demonstrated by gel-filtration analysis and isoelectric focusing. For gel-filtration analysis, the complex formed by [^{3}H]ganglioside GM1 and [^{14}C]saposin B yielded a single peak (Fig. 3). The ratio of radioactivities in the peak demonstrated that the peak contained a mixture of ganglioside GM1 and saposin B in a molar ratio of 1:1.

In the Rotofor isoelectric focusing experiments, mixtures of [^{14}C]saposin B with [^{3}H]ganglioside GM1 or [^{3}H]ganglioside GM2 and mixtures of [^{14}C]saposin C with [^{3}H]ganglioside GM1 were analyzed. When saposin B or C (without ganglioside) was analyzed, the proteins focused in the pH 4.5 area (Fig. 4). After incubation with ganglioside GM1 at pH 4.0 in a 1:1 ratio, ganglioside GM1–saposin B complexes migrated in the area of pH 3.5. Determination of the ratio of ^{3}H and ^{14}C in the peak gave a ganglioside GM1/saposin B ratio

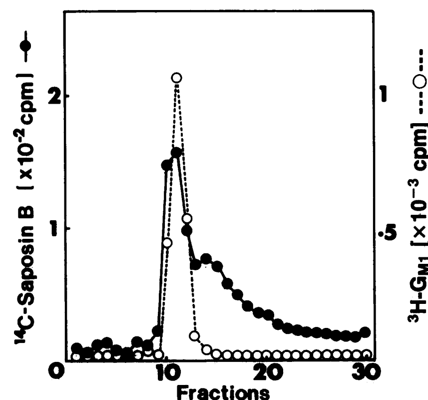

FIG. 3. Gel filtration of saposin B–ganglioside GM1 complexes. [^{14}C]Saposin B (4 nmol; 10,000 cpm) was incubated with [^{3}H]ganglioside GM1 (40 nmol; 240,000 cpm) and fractionated on an S Sepharose column. A portion of the fraction that passed through the column (400 μl) was analyzed by gel filtration. ●, [^{14}C]Saposin B; ○, [^{3}H]ganglioside GM1.

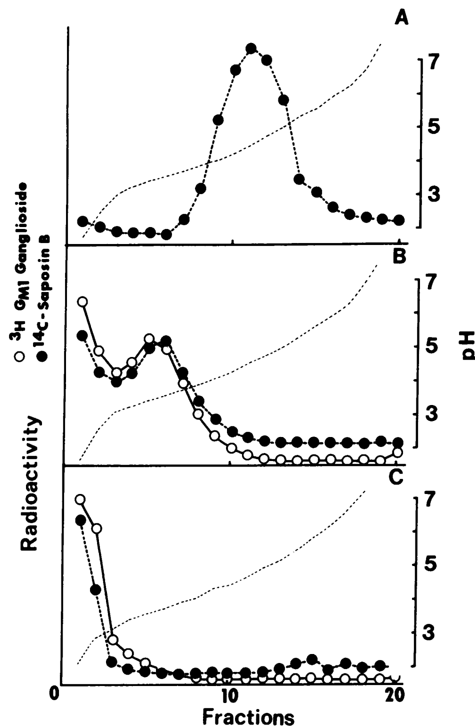

FIG. 4. Isoelectric focusing of saposin B–ganglioside GM1 complexes. [^{14}C]Saposin B (4 nmol; 10,000 cpm) was incubated with and without [^{3}H]ganglioside GM1 and then analyzed on the Rotofor cell apparatus. (*A*) [^{14}C]Saposin B incubated without [^{3}H]ganglioside GM1. (*B*) [^{14}C]Saposin B incubated with 4 nmol of [^{3}H]ganglioside (100,000 cpm). (*C*) [^{14}C]Saposin B incubated with 40 nmol of [^{3}H]ganglioside GM1 (100,000 cpm). ●, [^{14}C]Saposin B; ○, [^{3}H]ganglioside GM1. pH values are indicated by dashed line.

of 2:1. Similar results were obtained with ganglioside GM1 complexed to saposin C. When ganglioside GM1 was mixed with saposin B in a ratio of 10:1 the complex migrated at a more acidic pH; in the peak the ganglioside/saposin ratio was ≈10:1.

Prosaposin and saposins A, B, C, and D promoted the transfer of ganglioside GM1 from donor liposomes to acceptor erythrocyte ghosts. Transfer rates were obtained by subtracting the low rate found when only ovalbumin was present. Transfer rates were concentration dependent (Fig. 5); after 60 min, prosaposin, saposin A, and saposin B transferred ≈10% of the ganglioside, whereas saposin D and saposin C transferred 33% and 54%, respectively. When ^{14}C-labeled prosaposin and saposins A, B, C, and D were used to assess binding of each protein to erythrocyte ghosts under conditions in which ganglioside transfer occurred, prosaposin and saposins A, B, and D were not bound to the ghost membranes, whereas 13% of saposin C was bound.

To assess the relationship between binding and transport and stimulation of ganglioside GM1 hydrolysis, prosaposin and saposins A, B, C, and D were tested for stimulatory activity in the presence of acid β-galactosidase. Prosaposin and saposin D, in addition to saposin B, stimulated hydrolysis of ganglioside GM1 by acid β-galactosidase; saposins A and C were minimally active (Fig. 6*A*). On a molar basis, the stimulation by prosaposin was greater than that by saposin B, whereas saposin D stimulated activity at about one-half the concentration of saposin B. When Michaelis–Menten kinetics were analyzed, the K_m values for β-galactosidase when saposin B or prosaposin was present were the same; when saposin D was present, a lower K_m for stimulation was obtained (Fig. 6*B*). In contrast, saposins A, B, C, and D did not stimulate ganglioside sialidase activity at concentrations

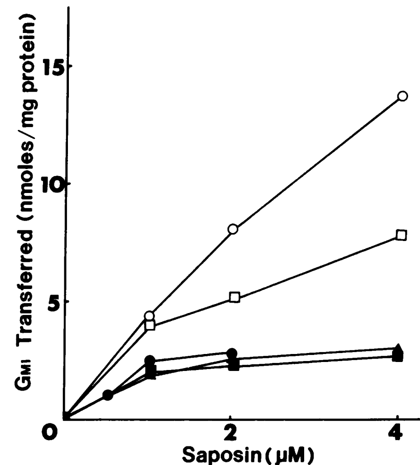

FIG. 5. Ganglioside transfer activity. Donor liposomes containing 5 nmol of [^{3}H]ganglioside GM1 were incubated with acceptor erythrocyte membranes (0.2 mg of protein) in the presence of increasing amounts of prosaposin or saposins. Acceptor membranes were collected and the ^{3}H radioactivity and protein content were determined in the washed membranes. Symbols are as in Fig. 2.

of saposins where stimulation of ganglioside GM1 β-galactosidase activity was substantial.

DISCUSSION

These experiments demonstrate that prosaposin as well as saposins A, B, C, and D bind and transport gangliosides *in vitro*, which leads us to propose that prosaposin may play a significant role in the cellular movement and distribution of gangliosides *in vivo*. Current evidence suggests that prosaposin is the only member of the saposin family with an extralysosomal location as a secretory protein (28–30) and as an

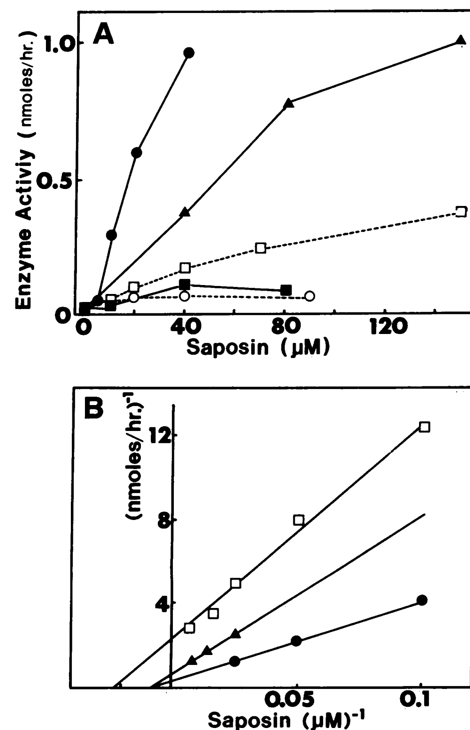

FIG. 6. (*A*) Effects of prosaposin and saposins on the activity of ganglioside GM1 β-galactosidase. Stimulation of ganglioside GM1 β-galactosidase activity by various amounts of prosaposin and saposins. Symbols are as in Fig. 2. (*B*) Lineweaver-Burk plots of activated ganglioside GM1 β-galactosidase in the presence of prosaposin (●), saposin B (▲), and saposin D (□).

integral membrane protein bound by a mannose-6-phosphate-independent mechanism (15). We postulate that secretory prosaposin could transfer gangliosides to the plasma membrane and that integral membrane prosaposin could transfer gangliosides from one cytoplasmic location to another.

It was previously demonstrated that saposin B binds ganglioside GM1 and forms tight complexes (31). Saposin B was also reported to bind complex gangliosides (such as GM1 and GD1a) and transport them from donor to acceptor liposomes (32). It is not surprising that saposin B possesses ganglioside transfer activity since it was shown earlier that saposin B acts by extracting sphingolipids from micelles or membranes to give water-soluble activator–lipid complexes (33), and such transfer should be reversible. However, it is unexpected to discover that prosaposin and the remaining saposins also possess ganglioside binding and transfer properties.

Theoretical models of each saposin indicate a compact rigidly cross-linked disulfide-bridged polypeptide containing a common hydrophobic pocket (14), suggesting that they should all act as lipid binding proteins. The fact that all four saposins are now demonstrated to actively bind and transfer gangliosides *in vitro* suggests that in prosaposin all four domains are active. However, our kinetic analysis indicated that the binding capacity of prosaposin is about equal to that of the individual mature saposins. Perhaps only one domain is open and the other three domains are sterically hindered in prosaposin.

Analysis of ganglioside–saposin complexes gave variable amounts of ganglioside GM1 bound to each saposin depending on the initial concentration of the ganglioside in the incubation. Vogel *et al.* (32) analyzed the interaction of saposin B with ganglioside GM1 by centrifugation; they obtained a molar ratio of 0.5:1 for binding of ganglioside GM1 to saposin B; a somewhat higher ratio was found for more complex gangliosides. Wenger and Inui (31) found bound gangliosides/saposin B ratios of 2–4:1 after gel electrophoresis of the complexes. Thus, the amount of ganglioside bound appears to be variable. The amounts of ganglioside bound *in vivo* may depend on many factors, including the state of the ganglioside (micellar or membrane bound), the type of ganglioside, the pH of binding, the presence of divalent cations, other lipids, and other binding proteins.

Previous investigators have searched for ganglioside binding proteins especially in brain or cultured neuronal cell lines. In rat brain, Tiemeyer *et al.* (12) reported the presence of a membrane-associated protein that specifically bound gangliosides of the *b* series with high affinity. This protein was later localized to myelin membrane preparations (13); it differs from prosaposin in both specificity and localization. In cultured cerebral granular cells, Sonnino *et al.* (9) reported that several membrane proteins were labeled after incubation with a photoreactive ganglioside GM1 derivative. Two prominent proteins so-labeled were released by trypsin treatment and their molecular masses (46 and 31 kDa) were similar to the initial products of proteolysis of prosaposin, which we have identified (19). However, additional binding proteins were apparent in the study cited (9), varying from 12 to 112 kDa, making any comparison tenuous. Other reports of ganglioside binding proteins describe the presence of proteins (10, 11) that have basic isoelectric points.

Our data indicate that the highest affinity of prosaposin (and saposins A, B, C, and D) is for the *a* series gangliotetraose-type gangliosides possessing terminal sialic acid residues. It has previously been reported that the presence of gangliotetraose-type gangliosides correlated with neuronal differentiation and synaptogenesis; a pronounced increase in their concentrations occurred with that phase of neuronal development (34–36). Tsuji *et al.* (37) have reported that ganglioside GQ1b has a unique potency to enhance neuroblastoma cell proliferation and to increase the number and total length of neurites (neuritogenesis) *in vitro*. This ganglioside is bound tightly by prosaposin. In summary, the *in vitro* data presented here and the *in vivo* studies on localization of prosaposin lead us to propose that prosaposin is an important ganglioside binding and transfer protein *in vivo*.

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