Interaction of G\textsubscript{M2} Activator Protein with Glycosphingolipids*

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G\textsubscript{M2} activator protein is a protein cofactor that has been shown to stimulate the enzymatic hydrolysis of both GalNAC and NeuAc from G\textsubscript{M2} (Wu, Y. Y., Lockyer, J. M., Sugiyama, E., Pavlova, N. V., Li, Y.-T., and Li, S.-C. (1994) J. Biol. Chem. 269, 16276–16283). To understand the mechanism by which G\textsubscript{M2} activator stimulates the hydrolysis of G\textsubscript{M2}, we examined the interaction of this activator protein with G\textsubscript{M2} as well as with other glycosphingolipids by TLC overlay and Sephadryl S-200 gel filtration. The TLC overlay analysis unveiled the binding specificity of G\textsubscript{M2} activator, which was not previously revealed. Under the conditions optimal for the activator protein to stimulate the hydrolysis of G\textsubscript{M2} by β-hexosaminidase A, G\textsubscript{M2} activator was found to bind avidly to acidic glycosphingolipids, including gangliosides and sulfated glycosphingolipids, but not to neutral glycosphingolipids. The gangliosides devoid of sialic acids, such as asialo-G\textsubscript{M1} and asialo-G\textsubscript{M2}, and the G\textsubscript{M2} derivatives whose carboxyl function in the NeuAc had been modified by methyl esterification or reduction, were only very weakly bound to G\textsubscript{M2} activator. These results indicate that the negatively charged sugar residue or sulfate group in gangliosides is one of the important sites recognized by G\textsubscript{M2} activator. For comparison, we also studied in parallel the complex formation between glycosphingolipids and saposin B, a separate activator protein with broad specificity to stimulate the hydrolysis of various glycosphingolipids. We found that saposin B bound to neutral glycosphingolipids and gangliosides equally well, and there was an exceptionally strong binding to sulfatide. In contrast to previous reports, we found that G\textsubscript{M2} activator formed complexes with G\textsubscript{M2} and other gangliosides in different proportions depending on the ratio between the activator protein and the ganglioside in the incubation mixture prior to gel filtration. We were not able to detect the specific binding of G\textsubscript{M2} activator to G\textsubscript{M2} when G\textsubscript{M2} was mixed with G\textsubscript{M1} or G\textsubscript{M3}. Thus, the specificity or the mode of action of G\textsubscript{M2} activator cannot be simply explained by its interaction with glycosphingolipids based on complex formation. The binding of G\textsubscript{M2} activator to a wide variety of negatively charged glycosphingolipids may indicate that this activator protein has functions other than assisting the enzymatic hydrolysis of G\textsubscript{M2}.

In higher animals, the sugar chains of glycosphingolipids are catabolized by the sequential action of lysosomal exoglycosidases (1). It has been shown that, in addition to β-hexosaminidase A, the conversion of G\textsubscript{M2} into G\textsubscript{M3} requires the assistance of G\textsubscript{M2} activator, a low molecular weight protein cofactor (2–4). The physiological significance of G\textsubscript{M2} activator has been demonstrated by the fact that the congenital defect of this activator protein leads to cerebral accumulation of G\textsubscript{M2} in type AB Tay-Sachs disease (5, 6).

Human G\textsubscript{M2} activator has been isolated from kidney (4), brain (6), and liver (7). This activator has been shown to be very specific in stimulating the hydrolysis of GalNAC from G\textsubscript{M2} by β-hexosaminidase A (1, 4, 7). This activator protein was also shown to assist the hydrolysis of NeuAc from G\textsubscript{M2} by clostripidial sialidase (8) and to recognize the branched trisaccharide (G\textsubscript{M2}-epitope) in G\textsubscript{M2} (9). This activator, however, is not required for the hydrolysis of water-soluble synthetic substrates such as 4-methylumbelliferyl-β-GlcNAc or p-nitrophenyl-β-GlcNAc by β-hexosaminidase A. The mode of action of G\textsubscript{M2} activator is still not well understood. Through the studies of complex formation between G\textsubscript{M2} activator and glycosphingolipids using electrophoresis, isoelectric focusing, and ultracentrifugation (4, 10), Conzelmann and Sandhoff (4) postulated that the action of G\textsubscript{M2} activator is to extract a single G\textsubscript{M2} molecule from its micelles to form a water-soluble protein-lipid complex (1:1 ratio), which serves as the true substrate for β-hexosaminidase A. This hypothesis, however, is not supported by two simple facts: (a) The water-soluble tetrasaccharide derived from G\textsubscript{M2} cannot be hydrolyzed by β-hexosaminidase A in the presence or absence of the activator (8) and (b) saposin B, another activator protein whose action is to solubilize glycosphingolipids, does not stimulate the hydrolysis of G\textsubscript{M2} by β-hexosaminidase A.

The results of previous studies on the interaction between glycosphingolipids and the activator proteins isolated from human tissues might have been complicated by the possible presence of contaminated proteins. Recently, we have cloned the cDNA encoding human G\textsubscript{M2} activator (11) and also expressed the cDNA in Escherichia coli (8). The availability of pure recombinant human G\textsubscript{M2} activator in large quantities made the re-examination of the interactions between G\textsubscript{M2} activator and glycosphingolipids possible. To understand the role of the G\textsubscript{M2} activator, we have studied the interaction of G\textsubscript{M2} activator with various glycosphingolipids by TLC overlay and Sephadryl S-200 gel filtration. For comparison, we have also studied in parallel the interaction of glycosphingolipids with saposin B, a nonspecific activator protein that has been reported to stimulate the enzymatic hydrolysis of a wide variety of glycosphingolipids (12). We found that in aqueous medium, such as gel filtration, one molecule of G\textsubscript{M2} activator was able to associate with multiple molecules of gangliosides. By TLC overlay, G\textsubscript{M2} activator was found to bind to various negatively charged gangliosides (12).
charged glycosphingolipids without showing preference to any particular sugar chain.

**EXPERIMENTAL PROCEDURES**

**Materials—**GM2-activator or saposin B were prepared from the brain of a Tay-Sachs patient (13). Gα1 and Gα2 were prepared from Gm1 and Gm2, respectively, by mild acid hydrolysis (14). The following glycosphingolipids were the generous gifts: Gm1, from Drs. G. Kischner and G. Toffano (Fidia Research Laboratory, Italy); GaLcαc-Gm3, and Neu-Gcα2 (15), from Dr. S. Sommo (University of Milan, Milan, Italy); the chemically modified Me-Gm2 (the carboxyl group of NeuAc in Gm2 was methyl esterified) and HO-Gm2 (the carboxyl group of NeuAc in Gm2 was reduced to alcohol) (16, 17), from Dr. S. Handa (Tokyo Medical and Dental University, Tokyo); Sα3 from Dr. T. Ishizuka (Teikyo University, Tokyo); and the chemically synthesized gangliosides, KDN-Gm3, IVKDNLaOse3-Cer, and IVKDNLoOse3-Cer, from Dr. A. Hasagawa (Gifu University, Gifu, Japan). Oligo-Gm2 was prepared from Gm2, using ceramide glucanase (18). PE-Gm2, the neoglycolipid, was prepared by conjugating 1′-NeuAcGgOse3 that was derived from GM2 to dipalmitylphosphatidylethanolamine by reductive amination (19). 3H-Labeled lyophilized. The stimulatory activities of the 14C-labeled GM2 activator were measured in one of the above-mentioned buffer solutions, which contained 1% each of bovine serum albumin, Sigma; [14C]formaldehyde (specific activity, 55 mCi/mmol), American Radiolabeled Chemicals (St. Louis, MO); Polygram SIL G TLC plate, Machery-Nagel (Duren, Germany); dimethylamine borane complex, ammonium acetate, Aldrich; Sephacryl S-200 gel filtration, 25 mM ammonium acetate buffer, pH 7.0. To this solution, 88.4 μl of dimethylamine borane (2.2 μmol) was added. After addition of 5 μl (2.2 μmol) of aqueous 1% formaldehyde, the mixture was left at room temperature for 6 h. Then, the 14C-labeled protein was separated from the reagents by gel filtration on a Bio Gel P-6 column (0.9 × 30 cm) connected to an absorbance monitor by the absorbance at 280 nm (Waters 490E UV-VIS detector). Fractions of 0.5 ml (2 ml) were collected through the entire run, and each fraction was analyzed for the content of the activator protein and the glycosphingolipid.

**Hydrolysis of Gm2—**The fractions that contained the protein-lipid complex eluted from the Sephacryl S-200 column were incubated with 0.5 units of β-hexosaminidase A at 37°C for 3 h. Each incubated fraction was evaporated to dryness, dissolved in 20 μl of chloroform/methanol (2/1, v/v), and analyzed by Silica gel 60 TLC plate using chloroform:methanol:water (60/35/8, v/v/v) as the developing solvent. Gangliosides were visualized by spraying the plate with diphenylamine reagent (25) followed by heating at 110–120°C for 15–20 min.

**Analytical Methods—**When the activator protein was incubated with only 14C-lgm1 or 14C-lgm3, the amount of the 14C-lgm1 in the protein-lipid complex was determined as follows: a 20-μl aliquot of each fraction obtained from the Sephacryl S-200 column was mixed with 5 ml of Universol, and the radioactivity was measured by a Tri-Carb model liquid scintillation counter (Packard Instrument Co., IL). When the activator protein was incubated with Gm2, which was not radiolabeled, the amounts of Gm2 in the protein-lipid complexes were determined by TLC analysis using the radioactive fraction that was then quantitated by Scan Jett II CX (Hewlett Packard, Boise, ID) and NIH image 1.55. When the activator protein was incubated with both 14CH1gM1 and 14CH1gM3, the two gangliosides in the protein-lipid complex were first separated from each other by TLC using chloroform:methanol:water (60/35/8, v/v/v) as the developing solvent. The 14CH1gM1 and 14CH1gM3 on the plate were first revealed by primulin reagent (27) and then individually scraped off the plate and mixed with Universol; the radioactivity was then measured by a scintillation counter. When the activator protein was incubated with both Gm2 and Gm3, the amounts of Gm2 and Gm3 in the protein-lipid complex were determined as follows: an aliquot of each fraction was evaporated to dryness, dissolved in 20 μl of chloroform/methanol (2/1, v/v), and applied onto a TLC plate. The plate was developed with the solvent system as described above for separating Gm1 and Gm2, and the gangliosides were visualized with the diphenylamine reagent (25). The amounts of Gm2 and Gm3 were quantitated by scanning the TLC plate with a Scan Jet IICX and analyzed by NIH image 1.55.

**Determination of Protein—**Protein was determined by the method of Lowry et al. (28) using bovine serum albumin as a standard.

**RESULTS**

**Interaction of Gm2 Activator with Glycosphingolipids on a TLC Plate—**The interactions between Gm2 activator and glyco- sphingolipids were examined by TLC overlay on plates where glyco- sphingolipids were associated with silica gel. Fig. 1A shows the representative common acidic and neutral glycosphingolipids on the plate that were stained by the diphenylamine reagent (25). While the same amount (15 nmol) of each glycosphingolipid was applied on the plate, GlcCer and GalCer showed weaker staining than Gm1, since the color intensity produced by the diphenylamine reagent depends on the sugar content of the glycosphingolipids. The same TLC plate prior to the chemical staining was overlaid with the radiolabeled Gm2 activator as described under “Experimental Procedures,” and the results are shown in Fig. 1B. The conditions for the overlay were first chosen to use the low ionic strength acidic buffer (25 mM ammonium acetate buffer, pH 4.0), which is the optimal condition for Gm2 activator to stimulate the hydrolysis of Gm2 by β-hexosaminidase A. Under this condition, Gm2 activator protein binds only to gangliosides Gm1, Gm2, and Gm3 (lanes 1, 2, and 3, respectively) but very weakly to the neutral glycosphingolipids, LacCer, GalCer, and GlcCer (lanes 4, 5, and 6, respectively). The bindings of Gm2 activator to 14 other glycosphingolipids were further examined under the same conditions. As summarized in Table I, Gm2 activator binds to several other acidic glycolipids such as Gm4, the synthesized PE-Gm2 (8) which contains the oligosaccharide of Gm2 linked to phosphati-
ability of the two modified GM2 derivatives to interact with GM2 buffer solution used for TLC overlay was 25 mM ammonium acetate, pH 4.0. The binding detected in a high ionic strength acidic buffer (250 mM ammonium acetate, pH 4.0) was considerably reduced in the presence of GM2 activator prior to the chemical staining as shown in A. Glycosphingolipids (15 nmol each) were applied onto a TLC plate: lane 1, GM1; lane 2, GM2; lane 3, GM3; lane 4, LacCer; lane 5, GalCer; and lane 6, GlcCer. Each lane in A corresponds to that of B. The buffer solution used for TLC overlay was 25 mM ammonium acetate, pH 4.0. Detailed conditions are described in the text.

Fig. 1. Detection of the complex formation between GM2 activator and the common glycosphingolipids GM2, GM3, GM3', LacCer, GlcCer, and GalCer by TLC overlay. A, the TLC plate with the indicated glycosphingolipids was stained with the diphenylamine reagent. B, the radioautogram of the same TLC plate that was overlaid with the 3H-labeled GM2 activator prior to the chemical staining as shown in A. Glycosphingolipids (15 nmol each) were applied onto a TLC plate: lane 1, GM1; lane 2, GM2; lane 3, GM3; lane 4, LacCer; lane 5, GalCer; and lane 6, GlcCer. Each lane in A corresponds to that of B. The buffer solution used for TLC overlay was 25 mM ammonium acetate buffer, pH 4.0. The detailed conditions for the experiment are described in the text.

The extent of the bindings of GM2 activator to the glycosphingolipids was significantly reduced by raising the pH and the ionic strength of the buffer (pH 6.8), almost no bindings between GM2 activator and glycosphingolipids were detected (Fig. 3, A and A'). Even sulfatide, which usually binds strongly to GM2 activator, was only very weakly bound to the activator protein under the neutral pH (lane 5). The binding detected in a high ionic strength acidic buffer (250 mM ammonium acetate, pH 4.0) was also considerably reduced in the presence of GM2 activator prior to the chemical staining as shown in A. Glycosphingolipids (15 nmol each) were applied onto a TLC plate: lane 1, GM1; lane 2, GM2; lane 3, GM3; lane 4, LacCer; lane 5, GalCer; and lane 6, GlcCer. Each lane in A corresponds to that of B. The buffer solution used for TLC overlay was 25 mM ammonium acetate, pH 4.0. Detailed conditions are described in the text.

Interaction between Saposin B and Glycosphingolipids—Saposin B is a nonspecific activator protein that stimulates the enzymatic hydrolysis of a number of glycosphingolipids catalyzed by different glycosidases (12). This activator protein was reported to bind glycosphingolipids to form lipid-protein complexes (29, 30). Therefore, the interactions between saposin B and glycosphingolipids were detected (Fig. 3B'). The extent of the bindings of GM2 activator to the glycosphingolipids was significantly reduced by raising the pH and the ionic strength of the buffer (pH 6.8), almost no bindings between GM2 activator and glycosphingolipids were detected (Fig. 3, A and A'). Even sulfatide, which usually binds strongly to GM2 activator, was only very weakly bound to the activator protein under the neutral pH (lane 5). The binding detected in a high ionic strength acidic buffer (250 mM ammonium acetate, pH 4.0) was also considerably reduced in the presence of GM2 activator prior to the chemical staining as shown in A. Glycosphingolipids (15 nmol each) were applied onto a TLC plate: lane 1, GM1; lane 2, GM2; lane 3, GM3; lane 4, LacCer; lane 5, GalCer; and lane 6, GlcCer. Each lane in A corresponds to that of B. The buffer solution used for TLC overlay was 25 mM ammonium acetate, pH 4.0. Detailed conditions are described in the text.

Positive
- Galβ1-3GalNAcβ1-4(Galα1-3Galβ1-4Glcβ1-1'Cer (GM1)
- GalNAcβ1-4(NeuAcβ2-3Galβ1-4Glcβ1-1'Cer (GM2)
- NeuAcα2-3Galβ1-4Glcβ1-1'Cer (GM3)
- NeuAcα2-3Galβ1-1'Cer (GM4)
- GalNAcβ1-4(NeuAcβ2-3Galβ1-4Glcβ1-1'Cer (GM1)
- GalNAcβ1-4(Glcβ1-1'Cer (GalNAc-Gm)
- GalNAcβ1-4(NeuAcβ2-3Galβ1-4Glcβ1-1'Cer (GM3)
- GalNAcβ1-1'Cer (GalNAc-Gm)
- HSO3-3Galβ1-1'Cer (sulfatide)
- HSO3-3Galβ1-4Glcβ1-1'Cer (Sas)
- Galβ1-3GalNAcβ1-4(NeuGcα2-3Galβ1-4Glcβ1-1'Cer (NeuGc-Gm)
- KDNα2-3Galβ1-4Glcβ1-1'Cer (KDN-Gm)
- KDNα2-6Galβ1-3GlcNAcβ1-4Glcβ1-1'Cer (IVKDNL-Ose4Cer)
- KDNα2-6Galβ1-3GlcNAcβ1-4Glcβ1-1'Cer (IVKDNL-Ose4Cer)
- GalNAcβ1-4(NeuAcα2-3Galβ1-4Glcβ1-1'Cer (PE-Gm)

Negative
- Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1'Cer (Galα)
- Galβ1-4Galβ1-4Glcβ1-1'Cer (Galβ)
- GalNAcβ1-4(NeuAcα2-3Galβ1-4Glcβ1-1'Cer (HO-Gm)
- GalNAcβ1-4(NeuAcα2-3Galβ1-4Glcβ1-1'Cer (Me-Gm)
- Galβ1-1'Cer (GalCer)
- Glcβ1-1'Cer (GlcCer)
- Galβ1-4Glcβ1-1'Cer (LacCer)
and glycosphingolipids were also examined in the same manner for comparison. As shown in Fig. 4, saposin B was found to bind not only to gangliosides and sulfatide but also to GM2 and LacCer. Compared with GM2 activator, saposin B bound to glycosphingolipids better at the neutral pH (pH 6.8) (Fig. 4B), and the general behavior of binding was not greatly affected by the acidic pH (Fig. 4A) or the high ionic strength of the buffer solution (Fig. 4C).

Interaction of GM2 Activator with Gangliosides in Micellar Forms—Since the results of the TLC overlay experiment showed the preferential binding of GM2 activator to the anionic glycosphingolipids, we subsequently examined the interactions between the GM2 activator and the gangliosides in aqueous medium using Sephacryl S-200 gel filtration to separate the protein-lipid complexes. Sephacryl S-200 column offers a special advantage for this analysis because this column adsorbs the free gangliosides but not the protein-lipid complexes. This enabled us to isolate and analyze the content in the complexes. When GM2 activator was applied alone to the column, the protein was not adsorbed and eluted from the column at the retention time of 28 min (Fig. 5A), whereas applying [3H]GM2 alone, the ganglioside was retained by the Sephacryl S-200 gel. When an incubation mixture containing [3H]GM2 and GM2 activator in a molar ratio of 1:1 or 50:1 was applied to the column, a peak containing both GM2 activator and [3H]GM2 was eluted (Fig. 5, B and C). This peak was confirmed to be the protein-lipid complex by two separate analyses: (a) rechromatography of this complex did not result in the separation of the activator protein from [3H]GM2 and (b) incubation of the complex with β-hexosaminidase A resulted in the conversion of [3H]GM2 into [3H]GM3 (Fig. 6). In these experiments, the recoveries of the activator protein and the gangliosides were determined to be in the range of 57–72% and 69–87%, respectively. As shown in Fig. 5, B and C, the complex derived from the incubation mixture that contained [3H]GM2 and GM2 activator in a molar ratio of 50:1 (Fig. 5C) had a slightly shorter retention time and a broader peak area than that derived from the mixture that contained [3H]GM2 and GM2 activator in an equimolar ratio (Fig. 5B). Similar chromatographic profiles were obtained when GM2 activator was incubated with either GM1 or GM3.
However, no complex formation was detected when GM2 activator was incubated with oligo-GM2 (data not shown) indicating that, in addition to the negative charge, the lipid moiety of the glycolipid is also essential for binding. The complexes formed between GM2 activator and the different molar ratios of gangliosides were individually isolated from the Sephacryl S-200 column and analyzed for the ratio between the ganglioside and the activator protein. As shown in Table II, when GM2 activator was incubated with an equimolar ratio of either GM1 or GM2, the molar ratio between GM2 activator and the respective ganglioside in the complex was found to be approximately 1:1. However, when the activator protein was incubated with 50 molar excess of either GM1 or GM2, the molar ratio between the ganglioside and GM2 activator in the complex was found to be about 50:1 in both cases. When the bindings between GM2 activator and a 50-fold molar excess of GM2 was examined, we found that the ratio of GM3 to the activator protein was about 80:1. It is well documented that in an aqueous medium GM3 exists as vesicles that are larger than micelles (31). Therefore, it is not surprising to find that the ratio of GM3/activator protein to be larger than that of GM2/activator protein or GM1/activator protein. As also shown in Table II, the association of saposin B to GM2 was very similar to that of GM2 activator protein. Thus, the interactions between the activator and the gangliosides detected in aqueous medium are similar for saposin B and GM2 activator. Whereas, the bindings on TLC overlay showed that saposin B bound to all glycosphingolipids, and GM2 activator bound preferentially to the anionic glycosphingolipids.

The Binding of GM2 Activator to the Mixture of GM1 and GM2 or GM2 and G_M3—We have reported that GM2 activator was able to recognize the branched trisaccharide epitope of GM2 (8, 9). We, therefore, examined whether GM2 activator can specifically bind only to GM2 when GM2 was mixed with GM1 or GM3. GM2 activator was first mixed with GM1 or GM3 in chloroform:methanol (2/1, v/v), dried, and redispersed in an aqueous buffer solution. The aqueous ganglioside mixture was then incubated with GM2 activator and subjected to Sephacryl S-200 gel filtration as described under “Experimental Procedures.” The complexes were isolated, and the amounts of GM2, GM1, and the activator protein (or GM2, GM3, and the activator protein) were determined. As shown in Table III, GM2 activator did not appear to bind preferentially to GM2 to form the activator protein-GM2 complex in 1:1 ratio. Rather, it associated with the mixture of gangliosides in the proportion similar to that in the original ganglioside mixture. For example, when GM2 activator was incubated with a mixture containing an equimolar ratio of GM1 and GM2, the molar ratio of GM2 activator, GM1, and GM2 in the complex was found to be close to 1:1:1. However, when GM2 activator was incubated with a mixture containing 25-fold excess of GM1 and GM2, the detected ratio of the activator protein to GM1 and GM2 in the complex was 1:17.3:17.0. No preferential extraction of GM2 from the two ganglioside mixtures was observed. A similar result was obtained from the incubation of GM2 activator with a mixture of GM2 and GM3. These results indicate that the composition of the complexes formed under the micellar form of ganglioside was determined by the pre-existing status of the ganglioside micelles.

**DISCUSSION**

Among the five activator proteins that stimulate the enzymatic hydrolysis of glycosphingolipids, saposin B and G_M3 activator have been shown to interact and affect the glycosphingolipid substrates (29, 30). Several methods have been used to demonstrate the complex formation between the activator proteins and glycosphingolipids, and in some studies the molar ratios between the protein and the lipid were also determined. For example, Fischer and Jatzkewitz (32) studied the complex formation between saposin B and sulfatide using electrophoresis and reported that the ratio of these two components in the complex was 1:1. Also using electrophoresis, Wenger and Inui (33) reported the ratio of the two compounds in the saposin B: sulfatide complexes to be 1:4 and 1:2.6, respectively. Vogel et al. (34) studied the binding of saposin B to the individual gangliosides, such as G_M1, G_M2, G_M3, and G_D1a as well as sulfatide by centrifugation and determined the molar ratios between saposin B and each of these gangliosides in the protein-lipid complexes to be almost 1:1. For GM2 activator, Conzemmann et al. (4, 10) concluded from their studies using ultracentrifugation and isoelectric focusing, and electrophoresis that GM2 activator can form the activator protein-GM2 complex in 1:1 ratio. These experiments were carried out under the conditions required for the specific methodology used (for example, high sucrose density for ultracentrifugation and high pH for electrophoresis). Using TLC overlay, we have shown clearly that the high ionic strength or high pH of the buffer

**TABLE II**

| Mixture (mol/mol) | Ganglioside/ GM2-Act* found in the complex |
|------------------|-------------------------------------------|
| GM1:GM2:GM2-Act | 1:1 | 1.08 |
| GM1:GM2:GM2-Act | 50:1 | 48.2 |
| GM2:GM2-Act | 1:1 | 1.03 |
| GM1:GM2-Act | 50:1 | 51.0 |
| GM2:Saposin B | 50:1 | 83.9 |
| GM2-Act, GM2 activator protein. |

**TABLE III**

| Mixture (mol/mol) | Determined ratio of ganglioside/GM2-Act* in the complex |
|------------------|----------------------------------------------------------|
| GM1 | GM2 | GM3 | GM2-Act |
| G_M1:G_M2:G_M2-Act | 1:1:1 | 0.81 | 0.84 | 1.0 |
| 25:25:1 | 17.3 | 17.0 | 1.0 |
| G_M2:GM2-Act | 25:25:1 | 23.2 | 25.8 | 1.0 |
| GM2-Act, GM2 activator protein. |
solution inhibited the interactions between G_M2 activator and the glycosphingolipid substrates. Therefore, we chose to analyze the complex formation between G_M2 activator and gangliosides using 25 mM ammonium acetate buffer, pH 4.0, which is optimal for the enzymatic hydrolysis of G_M2 in the presence of G_M2 activator.

By TLC overlay, G_M2 activator was found to bind to various anionic glycosphingolipids without showing preference to any particular sugar chain. Thus, G_M2 activator does not behave like lectins, which display the recognition of specific saccharide structure. The involvement of an anionic residue of a glycosphingolipid in the complex formation with an activator protein has been suggested. We have reported that the carboxylic function of the NeuAc in G_M2 was important for the binding of saposin B to the affinity column packed with the immobilized sulfatide or its derivatives as ligands. In the present studies using TLC overlay, we have clearly demonstrated that the anionic group in glycosphingolipids is vital for the complex formation with G_M2 activator but not with saposin B. While the bindings of G_M2 activator to gangliosides and sulfatides are greatly affected by the assay conditions, such as the pH and the ionic strength of the buffer solutions (Figs. 1, 3, and 4), no such effects were found for saposin B. These results, again, support the importance of the negative charge in a glycosphingolipid to form the glycolipid-G_M2 activator complex.

Wynn (36) proposed the triple binding domain theory of a glycosphingolipid to saposin B based on the conformational studies of the glycosphingolipids. He predicted that there are three possible interactions between a glycosphingolipid and the protein: (a) the hydrophobic interaction of the hydrocarbon chains of the ceramide moiety and a complementary hydrophobic domain in the protein molecule; (b) the electrostatic interaction between saccharide acid or sulfate group and a positively charged group of the protein; and (c) the hydrophilic interaction between a hydroxyl group in a sugar moiety and a complementary plane of the protein. Wynn (36) also pointed out that the glycolipid which has at least two of these structural features will strongly bind to saposin B. Our results on the binding behavior of both saposin B and G_M2 activator toward glycosphingolipids agree well with this model, since we have shown that G_M2 activator was not able to distinguish the saccharide backbone, the number of sugar residues, and the position or the nature of sialic acid (Table 1). It is evident that G_M2 activator is not specific to bind only G_M2. As both saposin B and G_M2 activator were shown to be able to transport glycosphingolipids from the donor to the acceptor liposomes (10), G_M2 activator may have a specific role in vivo to transport the acidic glycosphingolipids.

Our results on the complex formation between G_M2 activator and the micellar forms of gangliosides agree well with the studies of Cantu et al. (37). They studied the micelle formation in mixed gangliosides using light scattering and neutron scattering and reported that when G_M2 and G_T11 were mixed in different molar ratios in aqueous solution, the two gangliosides formed a single family of mixed micelles rather than that of two families of unmixed micelles, and the ratio of each ganglioside in the mixed micelles depended on the molar concentration of each ganglioside (37).

In contrast to previous reports (4, 10), we were not able to explain the mode of action of G_M2 activator based on our studies on the complex formation between this activator protein and glycosphingolipids, especially G_M2. The fact that G_M2 activator interacts with a wide variety of anionic glycosphingolipids indicates that this activator protein may have functions other than assisting the enzymatic hydrolysis of G_M2.

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