Specific Recognition of N-Acetyl neuraminic Acid in the G_{M2} Epitope by Human G_{M2} Activator Protein*

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G_{M2} Activator is a low molecular weight protein cofactor that stimulates the enzymatic conversion of G_{M2} into G_{M3} by human β-hexosaminidase A and also the conversion of G_{M2} into G_{A2} by clostridial sialidase (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). Among the five known activator proteins for the enzymatic hydrolysis of glycosphingolipids, only G_{M2} activator is effective in stimulating the hydrolysis of G_{M2}. However, the mechanism of action of G_{M2} activator is still not well understood. Using a unique disialosylganglioside, GalNAc-G_{D1a}, as the substrate, we were able to show that in the presence of G_{M2} activator, GalNAc-G_{D1a} was specifically converted into GalNAc-G_{M2} by clostridial sialidase, while in the presence of saposin B, a nonspecific activator protein, GalNAc-G_{D1a} was converted into both GalNAc-G_{M2} and GalNAc-G_{M1b}. Individual products generated from GalNAc-G_{D1a} by clostridial sialidase were identified by thin layer chromatography, negative secondary ion mass spectrometry, and immunostaining with a monoclonal IgM that recognizes the G_{M2} epitope. Our results clearly show that G_{M2} activator recognizes the G_{M2} epitope in GalNAc-G_{D1a}. Thus, G_{M2} activator may interact with the trisaccharide structure of the G_{M2} epitope and render the GalNAc and NeuAc residues accessible to β-hexosaminidase A and sialidase, respectively.

Sugar chains in glycosphingolipids of higher animals are catabolized by lysosomal glycosidases, and some of the hydrolytic steps have been shown to require the presence of protein cofactors called activator proteins (1-3). Among the five known activator proteins, four were derived from a common precursor, prosaposin, by partial proteolysis (4-6) and sequentially named as saposins A, B, C, and D, based on their placement in the amino-terminal end of prosaposin (3). The gene of prosaposin is located at a single locus on chromosome 10 (7, 8). Functionally, both saposins A and C can stimulate β-glucosidase to hydrolyze glucosylceramide, and saposin C was also reported to stimulate the hydrolysis of galactosylceramide (9). Saposin B, previously called nonspecific activator protein (10), has been shown to have a broad specificity toward a wide variety of glycolipid substrates and enzymes. Saposin D was shown to stimulate the hydrolysis of sphingomyelin (11) and ceramide in vivo (12). However, the true function of saposin A and D remains to be established.

The fifth activator protein is the product of a separate gene located on chromosome 5 (13) and has been named G_{M2} activator, since this activator protein was found to stimulate most efficiently the hydrolysis of G_{M2} by β-N-acetylhexosaminidase A (1-3). The fact that G_{M2} hydrolysis is not efficiently stimulated by any of the four saposins and that the deficiency of G_{M2} activator in type AB G_{M2} gangliosidosis results in massive cerebral accumulation of G_{M2} (14-16) indicate the physiological importance of this activator protein in vivo for the degradation of G_{M2}.

It has been postulated that G_{M2} activator extracts a single molecule of G_{M2} from the micelles and presents the monomeric form of G_{M2} to β-N-acetylhexosaminidase A (17). It has also been suggested that the GalNAc residue in G_{M2} should be degradable by β-N-acetylhexosaminidase A without the assistance of G_{M2} activator; however, in biological membranes, the carbohydrate head group of G_{M2} is shielded from the enzyme cleavage by the head groups of other adjacent glycosphingolipids. For β-N-acetylhexosaminidase A to reach the GalNAc residue in G_{M2}, it requires G_{M2} activator to lift the G_{M2} molecule a few angstroms out of the membrane surface (2). In contrast, we have shown that the effectiveness of G_{M2} activator in stimulating the hydrolysis of G_{M2} may be due to its ability to recognize and interact with the specific trisaccharide structure of the G_{M2} epitope and render the GalNAc and NeuAc residues accessible to β-hexosaminidase A and sialidase.

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addition of a GalNAc residue onto GD₁₂₅ alters the susceptibility of the NeuAc to clostridial sialidase. We hypothesized that GM₂ activator and saposin B might act differently toward the enzymatic hydrolysis of the two NeuAc residues in GalNAc-GD₁₂₅. We, herewith, present evidence to show that GM₂ activator clearly recognizes the specific branched trisaccharide structure in the GM₂ epitope of GalNAc-GD₁₂₅ while the stimulatory activity of saposin B does not require a specific sugar chain structure.

**EXPERIMENTAL PROCEDURES**

### Materials

GalNAc-GD₁₂₅ was isolated from the total ganglioside mixture of bovine brain (22). The chemical structure of GalNAc-GD₁₂₅ was established from HPTLC and NMR analysis as well as gas-chromatographic analysis of the fatty acid methyl esters and of the long-chain bases (22). Ga₃ was isolated from the brain of a Tay-Sachs patient (23). Asialo-GM₂ (GM₁₂) was prepared from GM₂ by mild acid hydrolysis (20). The native saposin B, also called nonspecific activator protein (specific activity, 3 × 10⁶ units/mg) (24), and the native GM₂ activator (specific activity, 2 × 10⁶ units/mg) (25) were isolated from human liver. The recombinant saposin B was produced in Escherichia coli from a cDNA construct as described below. The recombinant GM₂ activator was also produced in E. coli as previously described (18). The monoclonal IgM that recognizes the branched terminal trisaccharide in both GM₂ and GalNAc-GD₁₂₅ was obtained from a patient with neuropathy associated with gammopathy (26) and was a gift of Dr. R. H. Quarles (NINCDS, National Institutes of Health).

The following chemicals and reagents of the highest grade were purchased from commercial sources: NeuAc, clostridial sialidase type X, isopropl-1-thio-D-galactopyranoside, ampicillin, and 4-chloro-1-naphthyl, Sigma; yeast extract and trypsin, DIFCO; restriction endonucleases and T4 DNA ligase, Life Technologies, Inc.; Taq DNA polymerase, Promega; T7 sequencing kit version 2.0, U. S. Biochemicals Corp.; E. coli strain BL-21 (DE3), Novagen; peroxidase-conjugated rabbit anti-human IgM (μ chain specific), Cappel; bacteriophage λgt11 cDNA library as template. The upstream primer was 5'-TATGGATCCGGGGACGTTTGCCAGGA and the downstream primer was 5'-GCTCAACGTTTCCTCCTACTCATCACAGAACC-3', as designed from the reported nucleotide sequence of prosaposin between nucleotides 591 and 845 for saposin B (6). The cDNA fragment was verified for its sequence and then subcloned into pQE-30, QIA cloning vector, at BamHI and HindIII sites. This construct was designated as pQE-5B. The recombinant human saposin B protein was overexpressed using M15(Rep) pQE-3B according to the protocol provided for QIA expression system. The overexpressed protein was first purified by a Ni-NTA-agarose column under denaturing conditions and then refolded using a previously described method (18). The refolded recombinant saposin B was further purified through a Sephadex G-50 column (2.5 × 85 cm). The recombinant saposin B was found to be as active as the native human hepatic saposin B (24) in stimulating the hydrolysis of GM₂.

### Methods

**Enzymatic Hydrolysis of GalNAc-GD₁₂₅**—GalNAc-GD₁₂₅, 5 μg (0.25 nmol) in micellar form, was incubated with 6 units (as defined by the manufacturer) of clostridial sialidase and 5 μg (0.27 nmol) of the recombinant saposin B in 100 μl of 10 mM acetate buffer, pH 5.5, at 37 °C for 18 h. Since the molecular mass of GM₂ activator (18,871 Da), under the above conditions, the concentration of saposin B was about 6.8 times that of GM₂ activator. To ensure the observed results were independent of the activator concentrations, we also performed experiments using two levels of the same molecular concentration of GM₂ activator and saposin B (2.7 and 3.7 μM). After incubation, the reactions were stopped by heating the tubes in a bath of boiling water for 3 min, and then 10 μl of 1 N KCl and 50 μl of a slurry of Nucleosil C18 (C18 beads settled by gravity in 0.1 M KCl and 50 mM sodium citrate, pH 4.0) were added to each tube. The mixture was vortexed and left for 5 min to allow the glycosphingolipids to be adsorbed on the C18 beads. After centrifugation at 2,000 rpm using a Bedman T-6 centrifuge, the beads were washed twice with 1 ml of water, and the glycosphingolipids were then extracted from the C18 beads by the method of Williams and McCluer (27) using 0.5 ml of methanol followed by 0.5 ml of chloroform/methanol (2:1 v/v). The extracts were combined, dried, and analyzed by TLC using a precoated Silica Gel G-60 HPTLC plate, Merck (Darmstadt, Germany); and the peroxidase-conjugated anti-human IgM (μ chain specific) antibodies, Cappel.

**TLC Immunostaining**—The terminal branched trisaccharides of GalNAc-GD₁₂₅ and GalNAc-GD₁₂₅, one of the products from the action of sialidase, were identified on TLC using the monoclonal IgM (26), which recognizes the GM₂ epitopes in GM₂, GalNAc-GD₁₂₅, and GalNAc-GM₁₂₅. The TLC overlay procedure was essentially the same as that described by Magnani et al. (29), except that the binding of the ganglioside with the antibodies (1:100 dilution) was detected by a second antiserum, which was the peroxidase-conjugated rabbit anti-human IgM (μ chain specific). The antibody binding was revealed with 4-chloro-1-naphthyl, a substrate for peroxidase.

Construction of pQE-5B and Expression of Saposin B—Human saposin B cDNA was obtained by polymerase chain reaction using human liver cDNA library as template. The upstream primer was 5'-TAATGGATCCGGGGACGTTTGCCAGGA-3' and the downstream primer was 5'-GCTCAACGTTTCCTCCTACTCATCACAGAACC-3', as designed from the reported nucleotide sequence of prosaposin between nucleotides 591 and 845 for saposin B (6). This cDNA fragment was verified for its sequence and then subcloned into pQE-30, QIA expression vector, at BamHI and HindIII sites. This construct was designated as pQE-5B. The recombinant human saposin B protein was overexpressed using M15(Rep) pQE-3B according to the protocol provided for QIA expression system. The overexpressed protein was first purified by a Ni-NTA-agarose column under denaturing conditions and then refolded using a previously described method (18). The refolded recombinant saposin B was further purified through a Sephadex G-50 column (2.5 × 85 cm). The recombinant saposin B was found to be as active as the native human hepatic saposin B (24) in stimulating the hydrolysis of GM₂.

### RESULTS AND DISCUSSION

**Hydrolysis of GD₁₂₅ and GalNAc-GD₁₂₅ by Clostridial Sialidase**—Fig. 1 shows that GD₁₂₅ is readily converted to GM₂ by clostridial sialidase, while GalNAc-GD₁₂₅ was resistant to this sialidase. This indicates that the external NeuAc residue in GD₁₂₅ is easily hydrolyzed by clostridial sialidase, while the same NeuAc residue in GalNAc-GD₁₂₅ is resistant to the enzyme. Thus, the attachment of a GalNAc to GD₁₂₅ converts the sialidase-sensitive NeuAc to become sialidase resistant.
Acquotti et al. (22) reported that the external NeuAc residue in GD1α has a higher flexibility than the same NeuAc residue in GalNAc-GD1α, as the GalNAc-(NeuAc)-Gal trisaccharide is a compact unit. This may explain the differences in the susceptibility of the external NeuAc residue in GD1α and GalNAc-GD1α to clostridial sialidase in the absence of an activator protein.

Hydrolysis of GalNAc-GD1α by Clostridial Sialidase in the Presence of GM2 Activator or Saposin B—GM1α activator and saposin B have been postulated to function as biodetergents to solubilize glycosphingolipid molecules from their micellar forms in aqueous media (10, 17). However, the detergent-like mechanism cannot satisfactorily explain why GM2 activator has such a stringent specificity toward the substrate, GM2. Recently, we found that GM2 activator could stimulate not only the hydrolysis of the GalNAc residue from GM2 by β-N-acetylgalactosaminidase A but also the NeuAc residue from GM2 by clostridial sialidase (18). Previously, we have also reported that saposin B was able to stimulate the conversion of GM2 to Gα2 by clostridial sialidase (19). These observations led us to use one enzyme (clostridial sialidase) under one condition to examine the hydrolysis of the two NeuAc residues from a unique disialo-lyanglioside, GalNAc-GD1α, in the presence of GM2 activator or saposin B. Our rationale is that the external NeuAc residue in GalNAc-GD1α is part of the trisaccharide with GM2-like structure (GM2 epitope), while this is not the case for the internal NeuAc. Therefore, the two NeuAc residues in GalNAc-GD1α may behave differently toward the hydrolysis by clostridial sialidase in the presence of GM2 activator or saposin B.

When clostridial sialidase removes the NeuAc associated with the GM2 epitope from GalNAc-GD1α, the product will be GalNAc-GM1α, which no longer carries the GM2 epitope. When the sialidase removes the internal NeuAc from GalNAc-GD1α, the product will be a neutral glycosphingolipid, GalNAc-Gα1. Scheme I illustrates the cleavage of one or two NeuAc residues from GalNAc-GD1α.

Thus, using GalNAc-GD1α, it should be possible to differentiate the actions of GM2 activator and saposin B. Fig. 2A shows that in the presence of GM2 activator (lane 3), GalNAc-GD1α was converted into one major band and one very minor fast moving band, whereas in the presence of saposin B (lane 4), GalNAc-GD1α was converted into two major and one minor products. The monoclonal IgM that recognizes the GM2 epitope (26) was used for the initial identification of these products as shown in Fig. 2B. All lanes in Fig. 2B correspond to that in Fig. 2A. The monoclonal IgM stained the residual GalNAc-GD1α as shown in lanes 3, while in lane 4, a band moving faster than GalNAc-GD1α was also stained. This band corresponds to the second fast moving band in Fig. 2A, lane 4. This indicates that, in the presence of saposin B, clostridial sialidase removed the internal NeuAc residue from GalNAc-GD1α and that the product retained the GM2 epitope. The detailed structural identification of each product in lanes 3 and 4 is presented below.

The amounts of GM2 activator (5 μg) and saposin B (20 μg) used in Fig. 2A, lanes 3 and 4, were based on our prior experiences in using them for the hydrolysis of other glycosphingolipids. Since these amounts represent two different activator concentrations, we further compared the effect of these two activator proteins at two levels of concentrations: 2.7 μM (5 μg of GM2 activator or 2.5 μg of saposin B) for the low activator concentration and 10.7 μM (20 μg of GM2 activator or 10 μg of saposin B) for the high activator concentration. As shown in Fig. 2A, only in the presence of saposin B did clostridial sialidase produce the second fast moving band from GalNAc-GD1α (Fig. 2A, lanes 4 and 8). When saposin B was in a low concentration (2.7 μM), very little hydrolysis of GalNAc-GD1α was observed (Fig. 2A, lane 6). Again, the second fast moving band was stained by the monoclonal IgM that recognizes the GM2 epitope (Fig. 2B, lanes 4 and 8). In contrast, this ganglioside was practically not produced from GalNAc-GD1α in the presence of GM2 activator (Fig. 2B, lanes 3, 5, and 7). The fastest moving band in Fig. 2A, lane 4 or 8, was not stained by the monoclonal IgM, indicating the absence of the GM2 epitope in this product. Furthermore, this band was not stained by the resorcinol reagent (34) indicating the absence of NeuAc in this product and was identified to be GalNAc-Gα1 by mass spectrometry. Identical results as shown in Fig. 2 were obtained by using the native human hepatic GM2 activator (25) and saposin B (24) in place of the recombinant activator proteins (results not shown).

Analysis of the products derived from GalNAc-GD1α by negative SIMS—Two parallel plates were made for the analysis of the products. One plate (Fig. 3A) was sprayed with the diphenylamine reagent to reveal the glycosphingolipids. The glycosphingolipids produced from GalNAc-GD1α in the presence of saposin B (P1) were resolved into 4 bands, designated as P1-α,
P1-m, P1-b, and P1-c (Fig. 3A, lane 4). Among them, P1-a and P1-m were the major products. The glycosphingolipids produced from GalNAc-GD1α in the presence of GM2 activator (P2) were resolved into 3 bands, designated as P2-a, P2-b, and P2-c (Fig. 3A, lane 5). Among them, P2-a and P2-b were the major products. With the exception of P1-m, all other products were detected in both incubation mixtures. The products on the parallel plate were blotted on a PVDF membrane as described by Taki et al. (31, 32). Each band on the PVDF membrane was excised as shown in Fig. 3B and analyzed by negative SIMS, and the results are presented below.

P1-a and P2-a were identified to be GalNAc-GM1α. The deprotonated molecule and fragmentation patterns of these two glycosphingolipids corresponded to that of GalNAc-GM1α, as shown in Fig. 4, A and B.

P1-m, one of the major products in the presence of saposin B, was identified to be GalNAc-GM1α. The deprotonated molecule and the fragmentation profile of P1-m indicated that the NeuAc residue came off first, and then the other fragment ions were identical to that of GalNAc-GM1α. This band was not produced in the presence of GM2 activator (P2).

P1-b and P2-b were identified to be GalNAc-GM1α. Both mass spectra of P1-b (Fig. 6A) and P2-b (Fig. 6B) corresponded to GalNAc-GM1α, but different from that of GalNAc-GM1α, as the characteristic fragment ions corresponding to that from GM2, and GM1α were detected.

P1-s and P2-s were identified to be the residual parent GalNAc-GO₁α. The TLC mobilities of P1-s and P2-s (Fig. 3A) and their mass spectra (Fig. 7, A and B) were identical to that of the substrate GalNAc-GO₁α. Also, the mass spectrum of GalNAc-GO₁α shows that the major molecular species of the ceramide moieties were long chain base 18:1, fatty acid 18:0 (m/z 564) and long chain base 20:1, fatty acid 18:0 (m/z 592). These results agree well with the previous data on the lipid composition of GalNAc-GO₁α (22).

P1-c and P2-c were the very minor products and their exact structures were not identified. Both showed the deprotonated molecule of m/z 1886 and the fragment ion of m/z 603, which is characteristic of the ion [NeuAc-NeuAc + Na - H₂O - H]. As this ion was not detected in the parent GalNAc-GO₁α, P1-c and P2-c might be the products of the glycosyltransfering action of clostridial sialidase (the hydrolysis of the external NeuAc residue from GalNAc-GO₁α and transferring of the NeuAc to the internal NeuAc to form NeuAc-NeuAc-containing ganglioside).

Quantitative estimation of the above products was accomplished by scanning the TLC plate using a Shimadzu CS-930 TLC scanner (35). In the presence of saposin B and 6 units of clostridial sialidase (Fig. 2A, lane 4), the production of GalNAc-GM1α, GalNAc-GM1β, and GalNAc-GA1 was in a ratio of 1.5:5.6:2.9, whereas in the presence of the double amounts of the sialidase (Fig. 3A, lane P1), the ratio was 1:1.56:1.86. These results indicate that saposin B stimulated, without discrimination, the cleavage of the external and the internal NeuAc residues of GalNAc-GO₁α, and the higher sialidase concentration promoted the production of GalNAc-GA1. In contrast, in the presence of GM2 activator and 6 units of the sialidase (Fig. 2A, lane 5), the ratio of GalNAc-GM1α and GalNAc-GA1 was 8:8:1, whereas in the presence of the double amounts of the enzyme (Fig. 3A, lane P2), the ratio of these two products was 7:3. This indicates that GM2 activator specifically stimulated the hydrolysis of the external NeuAc, which is associated with the GM2 epitope, and virtually did not stimulate the hydrolysis of the internal NeuAc from GalNAc-GO₁α to produce GalNAc-GM1β (only a trace of GalNAc-GM1β was detected by immunostaining as shown in Fig. 2B, lane 5). After the removal of the external NeuAc residue, some GalNAc-GM1α was converted into
The differential hydrolysis of the external and the internal NeuAc residues in GalNAc-GD1a by one sialidase in the presence of GM2 activator or saposin B may indicate that these two NeuAc residues are distinct within their microenvironments, even though in the same molecule. Recently, Acquotti et al. (22) studied the conformational properties of GalNAc-GD1a as a free monomer in (CD$_3$)$_2$SO or as inserted in a micelle of fully deuterated dodecyl phosphocholine in D$_2$O. They concluded from the H6-C6-C7-H7 and H7-C7-C8-H8 dihedral angles that the two NeuAc conformations for both GalNAc-GD1a and GD1a were very similar to GM1 (36), GM3 (37), and GD1b (38). Moreover, the chemical shifts of the external and the internal Gal, GalNAc, and NeuAc residues were completely superimposed, and no distinction could be made between the two sets of trisaccharide structures in GalNAc-GD1a. Therefore, the physico-chemical determinations of GalNAc-GD1a could not distinguish the external and the internal NeuAc residues. These results, however, do not corroborate with the results that saposin B and GM2 activator can discriminate the two NeuAc residues. The difference may be due to the fact that the physico-chemical studies were performed in (CD$_3$)$_2$SO or dodecyl phosphocholine micelles in which the behavior of GalNAc-GD1a molecule might be different from that found in the aqueous system used for the in vitro enzymatic hydrolysis. The different specificities expressed by GM2 activator and saposin B toward the two NeuAc residues in GalNAc-GD1a clearly show the distinct functions of these two activator proteins.

The unique structural feature of GalNAc-GD1a is the presence of two GM2 epitopes, the branched trisaccharide GalNAc-(NeuAc)-Gal, linked in tandem. This ganglioside provided us with an excellent model to show for the first time the distinct mode of action of saposin B and GM2 activator. Our results strongly suggest that GM2 activator can recognize the external NeuAc residue in GalNAc-GD1a, while saposin B does not exhibit this specificity. Since GM2 activator can stimulate the hydrolysis of only one NeuAc residue between the two supposedly identical NeuAc residues in GalNAc-GD1a, it is reasonable...
to consider that the two trisaccharide units (G\textsubscript{M2} epitopes) in GalNAc-GD\textsubscript{1a} are not completely identical and they are distinguishable by G\textsubscript{M2} activator protein. Whether this difference is the result of intra-saccharide interaction or the influence by the hydrophobic ceramide is still not clear. We have used ceramide glycanase (33) to prepare the lipid-free oligosaccharide from hydrophobic ceramide is still not clear. We have used ceramide glycanase (33) to prepare the lipid-free oligosaccharide from hydrophobic ceramide is still not clear. We have used ceramide glycanase (33) to prepare the lipid-free oligosaccharide from hydrophobic ceramide is still not clear. We have used ceramide glycanase (33) to prepare the lipid-free oligosaccharide from hydrophobic ceramide is still not clear. We have used ceramide glycanase (33) to prepare the lipid-free oligosaccharide from hydrophobic ceramide.

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