Characterization of an Alternatively Spliced G\textsubscript{M2} Activator Protein, G\textsubscript{M2A} Protein

AN ACTIVATOR PROTEIN WHICH STIMULATES THE ENZYMATIC HYDROLYSIS OF N-ACETYLNEURAMINIC ACID, BUT NOT N-ACETYLGLALACTOSAMINE, FROM G\textsubscript{M2}*

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GM\textsubscript{2} activator protein is a protein cofactor which stimulates the enzymatic hydrolysis of both Gal\textsubscript{N}Ac and NeuAc from GM\textsubscript{2}. We have previously isolated two cDNA clones, GM\textsubscript{2} activator cDNA and GM\textsubscript{M2A} cDNA, for human GM\textsubscript{2} activator protein (Nagarajan, S., Chen, H.-C., Li, S.-C., Li, Y.-T., and Locker, J. M. (1992) Biochem. J. 282, 807-813). GM\textsubscript{M2A} mRNA is an RNA alternative splicing product that contains exons 1, 2, 3, and intron 3 of the genomic DNA sequence of GM\textsubscript{2} activator protein (Klima, H., Tanaka, A., Schnabel, D., Nakano, T., Schröder, M., Suzuki, K., and Sandhoff, K. (1991) FEBS Lett. 289, 260-264). GM\textsubscript{M2A} cDNA encodes a protein (GM\textsubscript{M2A} protein) containing 1-109 of the 160 amino acids of human GM\textsubscript{2} activator protein, plus a tripeptide (VST) encoded by intron 3 at the COOH terminus. Thus, GM\textsubscript{M2A} protein can be regarded as a form (truncated version) of GM\textsubscript{2} activator protein. We have expressed GM\textsubscript{M2A} cDNA in Escherichia coli using pT7-7 as the vector. The recombinant GM\textsubscript{M2A} protein was purified to an electrophoretically homogeneous form and was found to stimulate the hydrolysis of NeuAc from GM\textsubscript{2} by clostridial sialidase, but not the hydrolysis of Gal\textsubscript{N}Ac from GM\textsubscript{2} by \textit{β}-hexosaminidase A. Like GM\textsubscript{2} activator protein, GM\textsubscript{M2A} protein also specifically recognized the terminal GM\textsubscript{2} epitope in Gal\textsubscript{N}Ac-C6 GD1a and stimulated the hydrolysis of only the external NeuAc from this ganglioside by clostridial sialidase. These results enabled us to discern the enzymatic hydrolyses of Gal\textsubscript{N}Ac and NeuAc from GM\textsubscript{2} and establish that the NeuAc recognition domain of GM\textsubscript{2} activator protein is located within amino acids 1-109. The presence of GM\textsubscript{M2A} mRNA in human tissues and the selective stimulation of NeuAc hydrolysis by GM\textsubscript{M2A} protein indicate that this activator protein may be involved in the catabolism of GM\textsubscript{2} through the asialo-GM\textsubscript{2} pathway.

It has been well established that the catabolism of ganglioside GM\textsubscript{2} requires the assistance of a protein cofactor called GM\textsubscript{2} activator protein (1, 2). The physiological importance of GM\textsubscript{2} activator protein has been shown by the presence of an autosomal recessive genetic disease, the AB variant of Tay-Sachs disease, which is caused by the deficiency or the defect of GM\textsubscript{2} activator protein (3-5). In addition to GM\textsubscript{2} activator protein, four other activator proteins for the catabolism of glycosphingolipids have been reported. These four activator proteins are derived from the proteolytic processing of a single precursor protein, prosaposin (6-8), and have been named saposin A, B, C, and D according to their placements from the amino terminus of the prosaposin (9). Saposin B is also known as a nonspecific activator protein and has been found to have a detergent-like activity which stimulates the hydrolyses of various glycolipids by different glycosidases (10). Among the five activator proteins, only GM\textsubscript{2} activator protein is derived from a separate gene (11). We have isolated two distinct cDNA clones for human GM\textsubscript{2} activator protein (12). One of them, GM\textsubscript{2} activator cDNA, which has also been isolated by others (13, 14), encodes almost the entire amino acid sequence of the native GM\textsubscript{2} activator protein isolated from human kidney (15). The other clone, GM\textsubscript{M2A} cDNA, which was reported only by us, has an identical 5'-terminal sequence as that of GM\textsubscript{2} activator cDNA from nucleotides 1 to 302, but different for the next 346 nucleotides toward the 3' end. Klima et al. (16) isolated the genomic DNA which covered 94% of GM\textsubscript{2} activator cDNA, and identified the presence of three introns and four exons. The last exon, exon 4, spanned the segment coding for the carboxyl terminus of GM\textsubscript{2} activator protein and the entire 3'-untranslated region of the GM\textsubscript{2} activator cDNA. Comparing the sequence of GM\textsubscript{M2A} cDNA with this genomic DNA, we found that the last 346 nucleotides of GM\textsubscript{M2A} cDNA were identical to the sequence of 5' end of intron 3 (the exons and the introns are defined based on GM\textsubscript{2} activator mRNA). Thus, GM\textsubscript{M2A} mRNA is an alternative splicing product of GM\textsubscript{2} activator RNA in which the potential 5' splicing site between exon 3 and intron 3 is not subjected to the splicing process. As shown in Fig. 1, the coding region of GM\textsubscript{2} activator cDNA contains the end portion of exon 1, all of exons 2 and 3, and the front portion of exon 4. While the coding region of GM\textsubscript{M2A} cDNA contains the identical exon 1, 2, and 3 as in GM\textsubscript{2} activator cDNA, and a stretch of 9 nucleotides encoding a tripeptide, VST, at the COOH terminus which is derived from intron 3. This 9-nucleotide sequence is immediately followed by a stop codon.

It has been postulated that the function of GM\textsubscript{2} activator protein is to extract a single GM\textsubscript{2} molecule from the micelles and to present the substrate-activator complex to \textit{β}-hexosaminidase A (17), or to lift GM\textsubscript{2} from biological membranes where the sugar chain of GM\textsubscript{2} molecules may be shielded by other complex lipids with larger headgroups (18, 2). In contrast, we have shown that the action of GM\textsubscript{2} activator protein in stimulating the hydrolysis of GM\textsubscript{2} by \textit{β}-hexosaminidase A may

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1 The abbreviations used are: GM\textsubscript{2}, \textit{II}-NeuAcGgOse\textsubscript{Cer}; GM\textsubscript{A}, \textit{II}-NeuA\textsubscript{C}Lac\textsubscript{C}er; Gal\textsubscript{N}Ac-GD\textsubscript{1a}, IV\textsubscript{3}Gal\textsubscript{N}Ac, IV\textsubscript{3}NeuAc, II\textsubscript{3}NeuAcLac\textsubscript{C}er; PAGE, polyacrylamide gel electrophoresis.
There, the recombinant GM2A protein was expressed and purified according to the method described previously (19). The production of GM2A protein (0.2 μg) Lane 3, the recombinant GM2A, activator protein (0.2 μg). The gel was stained with silver staining.

Enzymatic Hydrolysis of NeuAc from GM2—For the hydrolysis of GM2 from T. fasei (21) and radioactive GM2 (22; 23) was incubated with the appropriate enzyme and the designated activator protein in a final volume of 100 μl at 37 °C. For the conversion of GM2 into GM2A, 40 μM GM2 was incubated with 10 units of galactosidase in 10 mM acetate buffer, pH 5.5, in the presence of activator protein. For the hydrolysis of NeuAc from GalNAc-GM1a, 20 μM GalNAc-GM1a was used. For quantitative analysis of the hydrolysis of NeuAc from GM2, the incubation conditions were identical to that described above, except that the indicated concentrations of [1H]GalNAc were used.

Enzymatic Hydrolysis of GalNAc from GM2A—For the hydrolysis of GalNAc from GM2A, 20 μM GM2A was incubated at 37 °C with 0.2 units of β-hexosaminidase A in 10 mM acetate buffer, pH 4.6, in the presence of the indicated amount of an activator protein. The quantitative analysis of the conversion of GM2A to GM2A was carried out by using [1H]GalNAc as a substrate at the indicated concentrations and measuring the release of [1H]GalNAc.

**Materials**
Gm2, from T. fasei brain (21) and the radioactive Gm2 (22; 23) were prepared as previously reported. The recombinant GM2A activator protein and the recombinant saposin B were produced in Escherichia coli as described previously (19, 20). β-Hexosaminidase A (specific activity, 33.3 units/mg) (24) was isolated from human liver. GalNAc-GD1a was isolated from the total ganglioside mixture of bovine brain (25). The following reagents of the highest grade were obtained from commercial sources: clostridial sialidase Type X, isopropyl thio-galactoside, ampicillin, and glutathione, Sigma; yeast extract and tryptone, Difco; b-galactoside, am-

**Methods**
Expression of GM2A Protein—The fragment of GM2A cDNA, which encodes amino acids 1-109 of the human GM2 activator protein plus the tripeptide VST at the COOH terminus, was obtained by polymerase chain reaction using GM2A-KS Bluescript as template. The upstream primer was: 5′-CGC-TCT-AGA-CCG-CTC-TGG-CTT-3′ and the downstream primer was 5′-TCA-

**RESULTS AND DISCUSSION**
Expression and Purification of GM2A Protein—As in the case of the recombinant GM2A activator protein (19), the recombinant GM2A protein produced in E. coli was also found to accumulate in the inclusion bodies. The same scheme used for the extraction, refolding, and purification of the recombinant GM2A activator protein (19) was used for the preparation of the recombinant GM2A protein. As shown in the SDS-PAGE profile (Fig. 2), the purified recombinant GM2A protein (lane 2) moved at the position corresponding to 13.5 kDa, the calculated molecular size of GM2A protein. It is considerably smaller than the recombinant GM2A activator protein (Fig. 2, lane 3). The identity of GM2A protein was also verified by the microsequencing of the
GM2A protein was also recognized by the polyclonal antibodies against GM2A activator protein (5).

Hydrolysis of NeuAc from GM2 by Clostridial Sialidase in the Presence of GM2 Activator Protein or GM2A Protein—Our previous results indicate that GM2 activator protein can stimulate the hydrolysis of both GaINAc and NeuAc from GM2 by β-hexosaminidase A and dostridial sialidase, respectively, and that it may be able to recognize the branched trisaccharide structure GalNAcα1-4(NeuAcα2-3)Gal, the GM2 epitope (19). Since GM2A protein contains only the NH₂-terminal 109 amino acids of GM2 activator protein without the COOH terminus 110–160 amino acids which were derived from exon 4, it would be important to examine whether or not this short version of the activator protein possesses the two known biological activities expressed by GM2 activator protein. The TLC analysis of the conversion of GM2 to GM1 in Fig. 3A) showed that, as in the case of GM2 activator protein (lane 4), GM2A protein (lane 5) also stimulated the hydrolysis of NeuAc from GM2 by dostridial sialidase. However, GM2A protein was found to be slightly less effective than GM2 activator protein. In order to compare the stimulatory potency of these two activator proteins, quantitative analysis was performed using [³H]GM2 at three different substrate concentrations instead of triplicates of one concentration. As shown in Fig. 3B, at all three substrate concentrations tested, the conversion of GM2 to GM1 was greatly enhanced by the presence of 2.5 mM of either GM2 activator protein or GM2A protein. GM2A protein was about 20% less effective than GM2 activator protein.

Hydrolysis of NeuAc from GaINAc-GD1a by Clostridial Sialidase in the Presence of Activator Proteins—Previously we have shown that GM2 activator protein specifically recognized and

stimulated the hydrolysis of the NeuAc residue in the GM2 epitope of GaINAc-GD1a by clostridial sialidase (20). We, therefore, examined the possible recognition of the same NeuAc residue in GaINAc-GD1a by GM2A protein. As shown in Fig. 4, GM2A protein did preferentially stimulate the hydrolysis of the external NeuAc in GaINAc-GD1a. In the presence of 2.5 mM GM2A protein, the sialidase hydrolysis produced GalNAc-GM1a as the major product and GalNAc-GA1 as the minor product from GaINAc-GD1a (Fig. 4A, lane 6). The same products were produced from GaINAc-GD1a by the clostridial sialidase in the presence of 2.5 mM GM2 activator protein as seen in Fig. 4A, lane 5. The products GaINAc-GM1a and GaINAc-GM1b and gaINAc-GA1 were analyzed by secondary ion mass spectrometry as described previously (20). The strict specificity of GM2 activator protein and GM2A protein toward the hydrolysis of the terminal NeuAc from GaINAc-GD1a was further demonstrated by the comparison of this result with that of the parallel experiments carried out in the presence of 10 or 20 mM saposin B. The concentrations of saposin B were chosen according to our previous experience (20). Our results clearly showed that in addition to GalNAc-GM1a and GaINAc-Ga1, GaINAc-GM1b was also produced from GaINAc-GD1a in the presence of saposin B (Fig. 4B, 20 mM in lane 7 and 10 mM in lane 8). This indicates that saposin B can stimulate the hydrolyses of both NeuAc residues from GaINAc-GD1a. Since the concentrations of saposin B used in Fig. 4A were much higher than that of the two other activator proteins, we repeated the experiment using 20 mM of each activator protein. As shown in Fig. 4B, in the presence of GM2A protein (lane 2) or GM2 activator protein (lane 3'), only GaINAc-GM1a and GaINAc-GA1 were produced. However, in the presence of saposin B (lane 1'), GalNAc-GM1b was also produced in addition to GalNAc-GM1a and GaINAc-GA1. These results suggest that GM2 activator protein and GM2A protein have the same specificity in recognizing the external NeuAc residue of GaINAc-GD1a. It is of interest to note that in the presence of 10 mM saposin B (Fig. 4A, lane 8), slightly more GalNAc-GM1a was produced than GalNAc-GM1b, while in the presence of 20 mM saposin B (Fig. 4A, lane 7), the reverse was observed. Thus, the concentration of saposin B appeared to influence the ratio of GaINAc-GM1a to GalNAc-GM1b produced from GaINAc-GD1a. In contrast, the production of only GalNAc-GM1a in the presence of GM2 activator protein or GM2A protein.
Hydrolysis of GalNAc from GM2 (0.5 activator protein was an effective activator for the enzymatic GM2 and GalNAc-GD1a, it is logical to assign the NeuAc recognition domain of GM2 activator protein to be within amino acids 1–109. The functional importance of the COOH-terminal segment of GM2 activator protein is supported by the fact that a case of type AB Tay-Sachs disease was found to be caused by a Arg→Pro mutation at the exon 4 coding region (29). The Arg→Pro mutation may disrupt the tertiary structure of GM2 activator protein which is vital for its activity and/or stability. Although our results suggest that amino acids 1–109 of GM2 activator protein is sufficient for the stimulation of the cleavage of NeuAc from GM2 by clostridial sialidase and the 51 amino acids at the COOH terminus is essential for the stimulation of GalNAc cleavage from GM2 by β-hexosaminidase A, it is not possible at this point to ascertain if GM2 activator protein requires a simultaneous interaction with both GalNAc and NeuAc residues in the GM2 epitope for the stimulation of hydrolysis of GalNAc from GM2. The fact that GM2 activator protein is not as effective in stimulating the hydrolysis of GalNAc from GM2 as that of GM2A by β-hexosaminidase A (19, 30) suggests that the binding of both the NeuAc and the GalNAc residues in the GM2 epitope may be necessary for the action of GM2 activator protein on the hydrolysis of GalNAc from GM2.

We have synthesized a 23-mer peptide (see “Experimental Procedures”) which covers amino acids 106 through 128 of the GM2 activator protein. This segment of the peptide is encoded mostly by exon 4. The synthetic 23-mer peptide showed neither the stimulatory activity for the hydrolysis of GalNAc nor for the hydrolysis of NeuAc from GM2. When this peptide was mixed with GM2A protein, it did not enable GM2A protein to stimulate the hydrolysis of GalNAc from GM2A. Taken together, our results indicate that the recognition of both the NeuAc and GalNAc residues in the GM2 epitope is a unique function of GM2A activator protein. As in the case of GM2 activator protein, GM2A protein also requires the hydrophobic lipid moiety of the substrate to express the stimulatory activity, since the oligosaccharide derived from GM2A was not hydrolyzed by β-hexosaminidase A or clostridial sialidase in the presence of GM2A activator protein. As reported previously, GM2A mRNA was found in both human placenta and fibroblasts, although in a much lower abundance than the mRNA of GM2 activator protein (12). This suggests that the existence of an alternative splicing for GM2 activator RNA may be physiologically important. Nature may use the alternative splicing of GM2 activator RNA to direct the catabolism of GM2. The production of GM2A activator mRNA or GM2A mRNA should lead to the production of GM2 activator protein or GM2A protein, respectively. In the presence of GM2A activator protein, the catabolism of GM2A may preferentially go through the cleavage of GalNAc residue, which is a well established pathway for the catabolism of GM2A. This pathway explains the biochemical bases of Tay-Sachs diseases caused by the deficiency or the defect of β-hexosaminidase A, or GM2 activator protein. However, in the presence of GM2A mRNA the catabolism of GM2A might shift to a possible alternate pathway, GM2A→Gm2A, since GM2A protein can only stimulate the hydrolysis of NeuAc from GM2A by clostridial sialidase, but not the hydrolysis of GalNAc. This would mean that the control of the production of GM2A activator mRNA and GM2A mRNA could be the branching point to direct the GM2A hydrolysis to GM2A→Gm2A or GM2A→Gm3A pathways. The possible in vivo pathway for the conversion of GM2A to Gm2A has been proposed by Riboni et al. (31) in studies on the Neuro2a cell line. The authors suggested that the Gm2A pathway was carried out by a specific sialidase which could convert GM2A to Gm2A. It has also been suggested by Fin-
gerhut et al. (32) that the degradation of gangliosides by lysosomal sialidase also required an activator protein. The question of the physiological role of GM2A protein will remain unanswered until the native GM2A protein is isolated. By SDS-PAGE and Western blotting analysis, we have observed the presence of a protein band corresponding to 13 kDa which reacted with the antibody against GM2 activator protein in the partially purified placental GM2 activator protein preparation. At the present time, the isolation of GM2A protein is hampered by the lack of an assay method which can distinguish GM2A protein from GM2 activator protein.

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