Degradation of G_{M1} and G_{M2} by mammalian sialidases

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In mammalian tissues, the pathway known for the catabolism of G_{M1} [Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; where Cer is ceramide] is the conversion of this ganglioside into G_{M2} [GalNAcβ4(Neu5Acα3)Galβ4GlcβCer] by β-galactosidase followed by the conversion of G_{M2} into G_{M3} (Neu5Acα3Galβ4GlcβCer) by β-N-acetylhexosaminidase A (Hex A). However, the question of whether or not G_{M1} and G_{M2} can also be respectively converted into asialo-G_{M1} (Galβ3GalNAcβ4Galβ4GlcCer; G_{A1}) and asialo-G_{M2} (GalNAcβ4Galβ4GlcβCer, G_{A2}) by mammalian sialidases has not been resolved. This is due to the fact that sialidases purified from mammalian tissues always contained detergents that interfered with the *in vitro* hydrolysis of G_{M1} and G_{M2} in the presence of an activator protein. The mouse model of human type B Tay–Sachs disease created by the disruption of the *Hexa* gene showed no neurological abnormalities, with milder clinical symptoms than the human counterpart, and the accumulation of G_{M2} in the brains of affected mice was only limited to certain regions [Sango,

Yamanaka, Hoffmann, Okuda, Grinberg, Westphal, McDonald, Crawley, Sandhoff, Suzuki and Proia (1995) *Nat. Genet.* **11**, 170–176]. These results suggest the possible presence of an alternative catabolic pathway (the G_{A2} pathway) in mouse to convert G_{M2} into G_{A2} by sialidase. To show the existence of this pathway, we have used recombinant mammalian cytosolic sialidase and membrane-associated sialidase to study the desialylation of G_{M1} and G_{M2}. We found that the mouse membrane-bound sialidase was able to convert G_{M1} and G_{M2} into their respective asialo-derivatives in the presence of human or mouse G_{M2} activator protein. The cytosolic sialidase did not exhibit this activity. Our results suggest that, *in vivo*, the stable NeuAc of G_{M1} and G_{M2} may be removed by the mammalian membrane-associated sialidase in the presence of G_{M2} activator protein. They also support the presence of the G_{A2} pathway for the catabolism of G_{M2} in mouse.

Key words: asialo-G_{M1}, asialo-G_{M2}, G_{M2} catabolism, rat sialidase.

INTRODUCTION

Sialic acids are commonly found at the non-reducing termini of complex carbohydrate chains [1]. They also exist in Nature as oligomers or polymers in animal glycoproteins, e.g. the neural cell adhesion molecule, fish egg glycoproteins and bacterial capsular polysaccharides [2]. The NeuAc residue linked α2→3 to the internal Gal of the gangliosides G_{M1} [Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; where Cer is ceramide] or G_{M2} [GalNAcβ4(Neu5Acα3)Galβ4GlcβCer] has been regarded as a stable sialic acid because of its resistance to microbial and mammalian sialidases [3]. On the other hand, the NeuAc residues linked α2→3 to the terminal Gal of G_{M1} and G_{M3} (Neu5Acα3Galβ4GlcβCer) as well as that linked α2→8 to another NeuAc are quite susceptible to various microbial and mammalian sialidases [1]. Among several well-studied microbial sialidases, those from *Clostridium perfringens* [4] and *Vibrio cholerae* [5] have been most commonly used for *in vitro* cleavage of the NeuAc from sialoglycoconjugates. The sialidase activity in mammalian tissues was first reported by Warren and Spearing in 1960 [6] and the distribution of this enzyme in mammalian organs was surveyed by Carubelli as early as 1962 [7]. However, because of their low abundance and instability upon purification, our understanding on the specificity of mammalian sialidases was fragmentary until the genes of a number of sialidases were cloned in the 1990s. Furthermore, the existence of multiple

isoforms of sialidases in different cell organelles further complicated the studies of mammalian sialidases. Based on their substrate specificities, immunological properties and subcellular localization, mammalian sialidases have been classified into four types by Miyagi et al. [8–10]: cytosolic [8], intralysosomal [9], lysosomal-membrane-associated [10] and plasma-membrane-associated [10] sialidases. The first molecular cloning of a mammalian sialidase was reported by Miyagi et al. [11] in 1993 for rat skeletal-muscle cytosolic-type sialidase. This sialidase was shown to be specific for cleaving NeuAc from sialo-oligosaccharides, sialoglycoproteins except submaxillary mucin, and gangliosides except G_{M1} and G_{M2} [8]. Homologous cDNA from Chinese hamster ovary cells [12] showed high sequence identity within the cytosolic-type sialidases. Intralysosomal sialidase was also cloned from human [13–15] and mouse [16–18] and a high degree of homology was also observed for this sialidase between the two species [18]. Miyagi et al. [19] have subsequently cloned synaptosomal-membrane-associated sialidase from bovine brain and transiently expressed the enzyme in COS-7 cells [19]. Very recently, recombinant mouse membrane-associated sialidase (MEMMSD) has also been cloned and expressed in COS-1 cells [20]. It has been shown *in vitro* that, in the presence of a detergent, the membrane-associated sialidases (lysosomal-membrane- and plasma-membrane-associated) cleaved NeuAc efficiently from various gangliosides; however, their activities towards the hydrolysis of the NeuAc from G_{M1} or

Abbreviations used: Cer, ceramide; G_{M1}, Galβ3GalNAcβ4(Neu5Acα3)Galβ4GlcCer; G_{M2}, GalNAcβ4(Neu5Acα3)Galβ4GlcβCer; G_{A2}, GalNAcβ4Galβ4GlcβCer; G_{M3}, Neu5Acα3Galβ4GlcβCer; G_{M2}AP, G_{M2} activator protein; Hex A, β-N-acetylhexosaminidase A; MEMMSD, mouse membrane-associated sialidase; ET3dSD, rat skeletal-muscle cytosolic sialidase.

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G_{M2} were found to be extremely low [10,19]. Thus the question on the hydrolysis of stable NeuAc from G_{M1} and G_{M2} by mammalian sialidases remains to be resolved.

It has been well documented that some steps in the catabolism of glycosphingolipids by glycosidases require the assistance of a protein cofactor [21,22]. Five such protein cofactors have been described: saposins A, B, C and D [22], and G_{M2} activator protein (G_{M2} AP) [21]. The four saposins are derived from partial proteolysis of a common precursor called prosaposin, whose gene was mapped to human chromosome 10 [22]. G_{M2} AP is a separate protein whose gene is located on chromosome 5 [23]. Among these protein cofactors, only G_{M2} AP could effectively stimulate the hydrolysis of the GalNAc from G_{M2} by human β -N-acetylhexosaminidase A (Hex A) [21]. G_{M2} AP has also been shown to stimulate the cleavage of the NeuAc from G_{M2} by clostridial sialidase [24]. Further studies on the specificity of this activator protein showed that, between the two NeuAc residues in GalNAcGD1a [GalNAc β 4(Neu5Ac α 3)Gal β 3GalNAc β 4-(Neu5Ac α 3)Gal β 4Glc β Cer], G_{M2} AP only effectively stimulated the hydrolysis of the NeuAc associated with the branched trisaccharide of the G_{M2} epitope by clostridial sialidase [25]. Moreover, hydrolysis of the GalNAc and the NeuAc from 6' G_{M2} , a synthetic G_{M2} analogue with the GalNAc residue linked β 1 $\rightarrow$ 6 to Gal, did not require the presence of G_{M2} AP [26]. These results indicate that enzymic degradation of the branched trisaccharide structure in G_{M1} or G_{M2} may depend on the presence of G_{M2} AP. A study of the mouse model of human Tay–Sachs disease through the disruption of the *Hexa* gene suggested that mouse might have an alternative pathway for degrading G_{M2} by converting G_{M2} into G_{A2} (asialo- G_{M2} ; defined as GalNAc β 4Gal β 4Glc β Cer) which can be catabolized subsequently without impediment [27]. We have shown that mouse Hex B was capable of hydrolysing G_{A2} to lactosylceramide in the presence of G_{M2} AP [28]. Thus the development of a very mild neurological pathology with limited accumulation of G_{M2} in the affected mice may be attributed to the presence of this alternative catabolic pathway for G_{M2} in mice. However, the conversion of G_{M2} into G_{A2} by a mammalian sialidase without the assistance of a detergent has never been demonstrated. Recently, a mouse membrane-associated sialidase (MEmmSD) expressed in COS-1 cells [20] and a rat skeletal-muscle cytosolic sialidase (ET3dSD) expressed in *Escherichia coli* and COS-1 cells [11] became available to us. These recombinant sialidases are of high specific activity and devoid of any detergent. Therefore, we have used these recombinant mammalian sialidases to examine the hydrolysis of the stable NeuAc from G_{M1} and G_{M2} in the presence of G_{M2} AP. Our results showed that with the assistance of G_{M2} AP, only the membrane-associated sialidase, but not the cytosolic sialidase, could hydrolyse the NeuAc from G_{M1} and G_{M2} .

MATERIALS AND METHODS

Materials

G_{M2} was isolated from the brain of a Tay–Sachs patient according to the method of Svennerholm [29]. G_{M1} was a generous gift of Dr G. Toffano of Fidia Research Laboratory, Abano Terme, Italy. Recombinant ET3dSD was overexpressed in *E. coli* and another preparation of the same cytosolic sialidase, MErcSD, was expressed in COS-1 cells [11]. Recombinant MEmmSD was overexpressed in COS-1 cells [20]. For convenience, the name of the construct for a specific type of sialidase without the prefix 'p' is used to designate the overexpressed sialidases in this paper. The recombinant human and mouse G_{M2} AP were prepared as previously reported [26,28]. The pre-coated silica Gel-60 TLC

plates were obtained from Merck, Darmstadt, Germany. Clostridial sialidase type X was obtained from Sigma. Other commercially available chemicals were reagent grade.

Sialidase activity

The activity of cytosolic sialidase was determined at pH 6.5 using 4-methylumbelliferyl-NeuAc as a substrate [30]. One unit of sialidase activity was defined as the amount that liberates 1 nmol of 4-methylumbelliferone/min at 37 °C. Since the membrane-associated sialidase had a very low activity towards 4-methylumbelliferyl-NeuAc, this activity was established using bovine brain mixed ganglioside as a substrate [8]. One unit of this sialidase was defined as the amount of enzyme that catalysed the release of 1 nmol of sialic acid from bovine brain mixed gangliosides/h [8].

Enzymic hydrolyses of G_{M1} and G_{M2}

The incubation mixture (50 μ l) contained 7 nmol of G_{M1} or G_{M2} , 0.02 unit (10 μ l) of sialidase and 3 μ g (171 pmol) of G_{M2} AP in the buffer solutions specified below for different enzymes. The buffer solutions used were 10 mM acetate buffer, pH 4.6, for the membrane-associated sialidase and 10 mM acetate buffer, pH 6.0, for both the cytosolic sialidase and clostridial sialidase. After incubation at 37 °C for 5–6 h, the reaction was stopped by heating the mixture in a bath of boiling water for 3 min and the incubation mixture was dried in a SpeedVac. The material in each tube was dissolved in 20 μ l of chloroform/methanol (2:1, v/v) and spotted on to a TLC plate. The tubes were washed twice with 20 μ l of chloroform/methanol (2:1, v/v), and each wash was spotted on the TLC plates to ensure the complete transfer of material from tube to plate. The plates were developed with chloroform/methanol/12 mM $MgCl_2$ (5:4:1, by vol.), sprayed with the diphenylamine reagent [31,32] and heated at 110 °C for 15–20 min to reveal glycolipids and NeuAc. The intensity of the colour for each band was quantified using a ScanJet 2c/ADF scanner (Hewlett Packard) and the NIH Image 1.61 program.

RESULTS

Hydrolysis of NeuAc from G_{M1} and G_{M2} by MEmmSD

For the best match of the origins of sialidases and G_{M2} AP, we chose to use recombinant MEmmSD [19,20] and recombinant mouse G_{M2} AP [28] to study the hydrolysis of G_{M1} and G_{M2} . We also included the recombinant human G_{M2} AP [24] for comparison, as this activator protein was used for the hydrolysis of G_{M2} by clostridial sialidase in our previous study [24]. To show the specificity of G_{M2} AP, we also included recombinant human saposin B [33], which is known to exhibit a broad specificity in stimulating the hydrolysis of a number of glycosphingolipids, but very little activity in stimulating the hydrolysis of G_{M2} by Hex A [33]. As shown in Figure 1, both the human and mouse G_{M2} AP stimulated the conversion of G_{M2} into G_{A2} by clostridial sialidase (Figure 1, lanes 4 and 5). These results are consonant with those reported previously [24] and also served as positive controls in the subsequent analysis. Both human and mouse G_{M2} AP also stimulated the hydrolysis of the NeuAc from G_{M2} carried out by MEmmSD (Figure 1, lanes 8 and 9). The level of stimulation was estimated to be about 3-fold in repeated experiments. MEmmSD alone in the absence of G_{M2} AP showed only slight activity (Figure 1, lane 7). This represents the basal activity of this recombinant sialidase under our assay conditions. The protein preparation expressed from the vector itself (ME18S) did not show detectable activity (Figure 1, lanes 11–14) under the assay

Figure 1 Hydrolysis of the NeuAc from G_{M2} by the mammalian membrane-associated sialidase (MEMmSD)

G_{M2} (7 nmol) in 50 μ l of 10 mM acetate buffer, pH 4.6, was incubated with 0.02 units of MEMmSD at 37 °C for 10 h in the presence of 171 pmol (3 μ g) of the recombinant human G_{M2} AP (hAP) or mouse G_{M2} AP (mAP) or 269 pmol (2.42 μ g) of the recombinant human saposin B (Sap B). For comparison, G_{M2} was also incubated with 3.5 units of clostridial sialidase (Cl.SD) in the presence or absence of G_{M2} AP. The incubation mixtures were analysed by TLC as described in the Materials and methods section. Lanes 1 and 18 (from the top) show the standard G_{A2} , G_{M3} and NeuAc; lane 2, G_{M2} ; lane 3, G_{M2} + Cl.SD; lane 4, G_{M2} + Cl.SD + hAP; lane 5, G_{M2} + Cl.SD + mAP; lane 6, G_{M2} + Cl.SD + Sap B; lane 7, G_{M2} + MEMmSD; lane 8, G_{M2} + MEMmSD + hAP; lane 9, G_{M2} + MEMmSD + mAP; lane 10, G_{M2} + MEMmSD + Sap B; lane 11, G_{M2} + ME18S (vector control); lane 12, G_{M2} + ME18S + hAP; lane 13, G_{M2} + ME18S + mAP; lane 14, G_{M2} + ME18S + Sap B; lane 15, Cl.SD only; lane 16, MEMmSD only; lane 17, ME18S only.

Figure 2 Time course of hydrolysis of the NeuAc from G_{M1} and G_{M2} by recombinant MEMmSD

G_{M1} or G_{M2} (7 nmol) in 10 mM acetate buffer, pH 4.6, was incubated with 0.02 unit of MEMmSD in the presence or absence of 171 pmol of the recombinant G_{M2} AP at 37 °C for 1, 2, 3 and 4 h. The incubation mixtures were analysed by TLC as described in the Materials and methods section. The vertical bars indicate the error ranges and a.u. indicates arbitrary units.

conditions and saposin B also did not stimulate this reaction (Figure 1, lane 10). The lowest detectable levels for G_{A1} (asialo- G_{M1} ; Gal β 3GalNAc β 4Gal β 4GlcCer) and G_{A2} by this method were estimated to be 0.3 nmol for both glycosphingolipids. These results indicate that MEMmSD is able to catalyse the hydrolysis of the NeuAc from G_{M2} and this reaction is stimulated by G_{M2} AP. This membrane-associated sialidase is also able to catalyse the hydrolysis of the NeuAc from G_{M1} , as described in the next section.

Time-course analysis of G_{M1} and G_{M2} hydrolysis by mammalian membrane-associated sialidase

Time-course analyses of the hydrolyses of the NeuAc from G_{M1} and G_{M2} by MEMmSD in the presence and absence of G_{M2} AP are shown in Figure 2. For both G_{M1} and G_{M2} , the hydrolysis of NeuAc by MEMmSD was enhanced about 3-fold in the presence of G_{M2} AP. G_{M1} appeared to be a slightly better substrate than G_{M2} for MEMmSD. These results are similar to the previous observation that G_{M2} AP can enhance the hydrolysis of the G_{M2} NeuAc by clostridial sialidase [24]. Taken together, we have shown that G_{M2} AP, a natural activator protein, may be involved in the enzymic hydrolysis of the stable NeuAc from G_{M1} and G_{M2} in the mammalian system.

Hydrolysis of NeuAc from G_{M1} and G_{M2} by recombinant rat skeletal-muscle cytosolic sialidases

G_{M1} and G_{M2} were subjected to hydrolysis by two preparations of recombinant rat skeletal-muscle cytosolic sialidase (ET3dSD and MErcSD) in the presence or absence of G_{M2} AP. ET3dSD was the sialidase overexpressed in *E. coli* and MErcSD was overexpressed in COS-1 cells [11]. Part of Figure 3 shows the results using ET3dSD; identical results were obtained using MErcSD (not shown). As can be seen from Figure 3, ET3dSD did not show detectable hydrolysis of the NeuAc from G_{M2} in either the absence (Figure 3, lane 2) or presence (Figure 3, lane 3) of G_{M2} AP. Under the same conditions, using MEMmSD, the conversion of G_{M2} into G_{A2} was enhanced by the addition of G_{M2} AP (Figure 3, lanes 4 and 5). Likewise, no detectable hydrolysis of the NeuAc from G_{M1} was observed using the cytosolic sialidase.

Figure 3 Hydrolysis of the NeuAc from G_{M2} by ET3dSD

G_{M2} (7 nmol) in 50 μ l of 10 mM acetate buffer, pH 6.0, was incubated with 0.02 unit of ET3dSD in the presence of 171 pmol (3 μ g) of the recombinant G_{M2} AP at 37 °C for 10 h. For comparison, the hydrolysis of G_{M2} by MEMMDS in 10 mM acetate buffer, pH 4.6, was also included (lanes 4 and 5). The incubation mixtures were analysed using TLC as described in the Materials and methods section. Lane 1, G_{M2} ; lane 2, G_{M2} + ET3dSD; lane 3, G_{M2} + ET3dSD + G_{M2} AP; lane 4, G_{M2} + MEMMDS (pH 4.6); lane 5, G_{M2} + MEMMDS + G_{M2} AP (pH 4.6); lane 6, G_{M2} + ET3d vector control + G_{M2} AP; lane 7, G_{M2} + ME18S + G_{M2} AP; lane 8, ET3d vector control only; lane 9, standard G_{A2} .

The pH optimum for the cytosolic sialidase is pH 6.0 and that of the membrane-associated sialidases is pH 4.6. Because of this difference, hydrolyses of G_{M1} and G_{M2} by the two cytosolic sialidase preparations were also examined using buffers of both pH values and for a prolonged incubation time up to 24 h. In all cases, neither cytosolic sialidase preparation, MErcSD or ET3dSD, exhibited detectable activity towards the hydrolysis of the NeuAc from G_{M1} or G_{M2} .

DISCUSSION

G_{M2} AP was initially found to specifically stimulate the hydrolysis of the GalNAc from G_{M2} by human Hex A [21]. Subsequently, we attempted to examine whether G_{M2} AP could also stimulate the hydrolysis of the NeuAc from G_{M1} or G_{M2} by mammalian sialidases. However, before recombinant sialidases became available, sialidases prepared from mammalian tissues were always contaminated with detergents, all of which interfere with the assay of G_{M2} AP's stimulatory activity on the hydrolysis of gangliosides. In addition, mammalian sialidases are very unstable upon purification. Thus in our previous study we chose to use clostridial sialidase instead of mammalian sialidase and found that G_{M2} AP was also able to stimulate this microbial sialidase to hydrolyse the NeuAc from G_{M2} [24]. Only until recently did suitable mammalian sialidases become available for our study. The recombinant mammalian sialidases used in this study were devoid of detergents. Our results provide evidence of the conversion of G_{M2} into G_{A2} by the mammalian membrane-associated sialidase and support the existence of the G_{A2} pathway for the catabolism of G_{M2} in mouse.

Whether or not the recombinant sialidase preparations contained some activator-like substances that could stimulate the hydrolysis of G_{M1} and G_{M2} was also carefully assessed. The most sensitive method for the detection of biologically active G_{M2} AP is to check the ability of the preparation to stimulate the hydrolysis of the GalNAc from G_{M2} carried out by Hex A [21]. Using this method, we did not find any stimulatory activity in MEMMDS, indicating the absence of activator-like material in the sialidase preparation. Moreover, the protein preparation expressed by the empty plasmid, pME18S, also did not react with the polyclonal antibodies against human G_{M2} AP in Western-blotting analysis, indicating no endogenous G_{M2} AP-like material

in the preparation. However, the product of pME18S reacted with the polyclonal antibodies against human saposin B. The concentration of the saposin B-like material was estimated to be about 1 μ g/ μ l. This concentration of saposin B-like material in the amount of preparation used in the experiment had no effect on the hydrolysis of G_{M2} , as shown in Figure 1 (lanes 11–14).

The substrate specificity and immunological properties of the mammalian cytosolic sialidase [8] were found to be distinct from those of the membrane-associated sialidase [10]. In assays performed *in vitro* using bile salts as stimulants, the cytosolic sialidase was found to be active towards sialo-oligosaccharides, sialoglycoproteins and some gangliosides, except G_{M1} and G_{M2} [8]. The membrane-associated sialidase [10], on the other hand, was found to preferentially hydrolyse gangliosides in the presence of a detergent, but had only very low activity towards G_{M1} and G_{M2} [10]. In this study, we have found that G_{M2} AP, a natural protein cofactor known for stimulating the conversion of G_{M2} into G_{M3} , was also able to stimulate the hydrolysis of the stable NeuAc from both G_{M1} and G_{M2} by MEMMDS. The cytosolic sialidase was devoid of this activity towards both substrates in the presence or absence of G_{M2} AP. Our results therefore suggest that G_{M2} AP may assist the catabolism of the branched trisaccharide in G_{M1} and G_{M2} in the mammalian system. Our results agree with the observations of Miyagi et al. [8,10,11,19,20] that, of the four types of mammalian sialidase, only the membrane-associated sialidases (sialidases I and II) showed any activity towards the hydrolysis of G_{M1} and G_{M2} using detergent as a stimulant. Although the recombinant intralysosomal sialidase is still not available to us, Miyagi et al. [9] have shown that this type of sialidase did not cleave gangliosides. It was reported that the degradation of polysialogangliosides by human placental lysosomal sialidase was enhanced by saposin B [34]. However, this study did not examine the removal of the stable NeuAc from G_{M1} and G_{M2} . The present results support our previous hypothesis that G_{M2} AP may be needed for the enzymic cleavage of the GalNAc and NeuAc from the rigid trisaccharide core of G_{M1} and G_{M2} [24–26,28]. It is possible that the interaction of G_{M2} AP with G_{M1} or G_{M2} may induce conformational changes of the branched trisaccharide and render the GalNAc and NeuAc residues accessible to Hex A and sialidase, respectively. A crystallographic study of human G_{M2} AP [35] suggested that the novel β -cup structure of G_{M2} AP could accommodate the ceramide residue of a glycosphingolipid inside the hydrophobic cavity of the cup and leave the head group of the glycolipid around the rim of the cup. Thus the branched trisaccharide may assume a new conformation that is accessible by the enzyme. Future studies of the co-crystallization of G_{M2} AP with G_{M2} may be able to shed light on the mechanism by which G_{M2} AP enhances the enzymic hydrolysis of the NeuAc from G_{M1} and G_{M2} .

This research was supported by National Institutes of Health grant NS 09626 to Y.-T.L. and Grants in Aid to T.M. for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports, Culture and Technology, Japan

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