FEBS Letters 351 (1994) 291–294

FEBS 14496

Biosynthetic pathway for a new series of gangliosides, GT1aα and GQ1bα

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Received 18 July 1994; revised version received 24 July 1994

Abstract A new class of gangliosides, GT1aα and GQ1bα, were initially identified as cholinergic neuron-specific antigens in bovine brain. These gangliosides have in common α2-6 NeuAc linked to the GalNAc residue in the gangliotetraose core structure. In this study, we have determined the biosynthetic pathways of GT1aα and GQ1bα using rat liver Golgi fraction. The results showed that GT1aα and GQ1bα were synthesized from GD1a and GT1b, respectively, by the action of a GalNAc α2-6 sialyltransferase. It was also demonstrated that these two gangliosides were found to exist as extremely minor components in rat liver.

Key words: Ganglioside; Sialyltransferase; Ganglioside biosynthesis; Cholinergic neuron-specific antigen

1. Introduction

Gangliosides, sialic acid-containing glycosphingolipids, have been shown to be involved in a variety of biological phenomena, such as cell–cell interactions, growth, differentiation, cell transformation, and signal transduction pathways [1–4]. We have found that bovine brain contained more than 100 species of gangliosides including extremely minor components [5]. In fact, we have isolated and characterized several new species of gangliosides such as α-series gangliosides [6], de-*N*-acetylated GM1 [7], hybrid type gangliosides containing GM2 epitope [8], and cholinergic neuron-specific gangliosides [9,10].

The occurrence of cholinergic neuron-specific gangliosides (Chol-1) was first described by Richardson et al. using a polyclonal antibody (anti-Chol-1) against synaptic plasma membrane of electric organ from *Torpedo marmorata* [11]. Immunohistochemical staining with polyclonal and monoclonal antibody (GGR-41) revealed that the antigens were characteristically distributed on the cell bodies and nerve terminals of the cholinergic neurons in the central and peripheral neurons [12,13]. Although Chol-1 gangliosides were expected to be functionally important molecules in the cholinergic system, their chemical structures have not been identified because they are extremely minor components. Recently, we have determined the chemical structures of two novel Chol-1 gangliosides as GT1aα and GQ1bα [9,10]. It is of interest to note that both gangliosides contained NeuAc attached to GalNAc through an α2-6 linkage (Fig. 1).

We have attempted to study the sialyltransferase activities responsible for the synthesis of Chol-1 gangliosides in microsomal fraction prepared from rat brain. However, the enzymatic activity was too low to be detected. Subsequently we found that rat liver Golgi fraction contained sialyltransferase activity capable of synthesizing Chol-1 gangliosides. This communication describes the pathway for the biosynthesis of unique gangliosides, GT1aα and GQ1bα, using a Golgi fraction prepared from rat liver. The natural occurrence of these two gangliosides in rat liver is also described.

2. Materials and methods

2.1. Materials

Donryu rats (female, 4-week-old) were purchased from SLC Inc. (Hamamatsu, Japan). CMP-[^{14}C]NeuAc (11.3 GBq/mmol) was obtained from NEN Research Products (Boston, USA). CMP-NeuAc was from Sigma Chemical Company (St. Louis, USA). Gangliosides from bovine brain were prepared as described previously [6,9,10]. GD1aα and GT1bα were prepared by the treatment of GT1aα and GQ1bα, respectively, with sialidase L which specifically hydrolyzes the NeuAc α2-3Gal linkage [14]. Sialidase L was isolated and purified as described [15]. Peroxidase-labeled goat anti-mouse IgG and IgM were purchased from Jackson Immunoresearch Lab. (Avondale, PA). Mouse monoclonal antibody specific for cholinergic gangliosides, GT1aα and GQ1bα, was prepared as described in detail elsewhere [16]. All other chemicals were of highest purity available.

2.2. TLC

Analytical TLC was carried out on precoated HPTLC plates (Silica Gel 60, E. Merck) using the following solvent systems: chloroform/methanol/12 mM $MgCl_2$ (5:4:1, by volume; solvent A), chloroform/methanol/12 mM $MgCl_2$ (4:5:1, by volume; solvent B), and chloroform/methanol/12 mM $MgCl_2$/15 M NH_4OH (50:40:7:3, by volume; solvent C). Gangliosides were visualized with resorcinol/HCl reagent.

2.3. TLC-immunostaining

TLC-immunostaining was performed by the method of Kusunoki et al. [17]. Gangliosides were applied onto a plastic plate (Poligram Sil G, Nagel, Germany) and developed with a combination of solvent system A and C. The plate was subjected to immunostaining with a monoclonal antibody against GT1aα and GQ1bα (GGR-41, mouse IgG2a) [16]. Densitometric analysis was carried out by dual-wavelength densitometric scanner, Shimadzu CS-9000 (Shimadzu, Kyoto, Japan) for quantification of the gangliosides.

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Abbreviations: The nomenclature used for gangliosides is based on the system of Svennerhorm and Hirabayashi et al. [9,24]. Cer, ceramide; Gal, galactose; Glc, glucose; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; TLC, thin layer chromatography; HPTLC, high performance thin layer chromatography.

SSDI 0014-5793(94)00883-3

2.4. Sialyltransferase assay using rat liver Golgi fraction and identification of the reaction products

Rat liver Golgi fraction was prepared by the method of Sandberg et al. [18]. Sialyltransferase activity was measured by the method of Hidari et al. [19] with a slight modification. In brief, the reaction mixture contained 15 nmol GD1a or GT1b, 3.2 mM CMP-[^{14}C]NeuAc (155 MBq/mmol), 0.4% Triton CF-54, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 150 mM Na cacodylate/HCl buffer (pH 6.5), and 50 μg of Golgi protein in a total volume of 50 μl. The mixture was incubated for 2 h at 37°C. The reaction was terminated by addition of 0.5 ml of water. The reaction products were desalted by ASPEK pak tC18 CARTRIGE column chromatography (M&S Instruments Trading Inc., Tokyo, Japan). Subsequently, the products were dissolved in 200 μl of methanol and applied onto a Sephadex LH-60 column (0.8 × 5 cm) which had been equilibrated with methanol. Fractions of 50 μl of the effluent were collected, and fraction number 14–17 containing the radioactive ganglioside products were pooled and analyzed by TLC and TLC-immunostaining. Amounts of reaction products were calculated by densitometric analysis with BAS 2000 (Fuji Film, Tokyo, Japan) based on the calibration curve of CMP-[^{14}C]NeuAc. For identification of their chemical structures, the reaction products were further purified by preparative TLC and analyzed by TLC-immunostaining with GGR-41.

2.5. Preparation of ganglioside fraction from rat liver

Fresh rat tissues of brain and liver (4-week-old) were dissected and lyophilized. Ganglioside was obtained by sequential extraction with 10 vol. of chloroform/methanol (2:1, v/v) and chloroform/methanol/water (5:5:1, by volume). The extracts were combined, treated with 0.5 M KOH in methanol for 30 min at 37°C [20], dialyzed against water and lyophilized.

3. Results and discussion

3.1. Presence of sialyltransferase activity capable of synthesizing GT1aα and GQ1bα in rat liver

In order to determine the biosynthetic pathways of Chol-1 gangliosides, several species of gangliosides were examined for their ability to serve as substrate for sialyltransferases in rat liver Golgi fraction. As shown in Table 1, among the gangliosides tested, only products derived from GD1a and GT1b reacted with monoclonal antibody GGR-41 against GT1aα and GQ1bα. Putative precursors such as GD1aα and GT1bα, which were isolated as another type of Chol-1 ganglioside (unpublished results), have no potency to serve as substrates for Gal α2-3 sialyltransferase.

To confirm the chemical structures of the synthesized products, they were characterized by TLC (Fig. 2) and TLC-immunostaining with GGR-41 which is specific for GT1aα and GQ1bα (Fig. 3). As shown in Fig. 2, the TLC mobilities of two major products synthesized from GD1a were identical to those of GT1a and GT1aα, respectively. Similarly, when GT1b was used, the two major products migrated to the positions identical to those of GQ1b and GQ1bα, respectively. Previously, Iber et al. reported that a single α2-8 sialyltransferase in rat liver synthesized GT1a and GQ1b from GD1a and GT1b, respectively [21]. In the present study, we identified two additional products, GT1aα and GQ1bα.

As expected, the gangliosides gave positive reaction with GGR-41, indicating that the products from GD1a and GT1b are GT1aα and GQ1bα, respectively (Fig. 3). The amounts of radiolabeled products, GT1aα and GQ1bα, were approximately 2.6 and 2.9 pmol, respectively, under our incubation conditions, and agreed with the amounts obtained from densitometric analysis of TLC-immunostaining.

For maximum enzyme activity, divalent metal ions, Mg^{2+} and Mn^{2+} were shown not to be required (Table 2). Similar to other sialyltransferases for ganglioside biosynthesis, addition of the detergent Triton CF-54 was effective.

Recently, GD1α synthase (GalNAc α2-6 sialyltransferase)

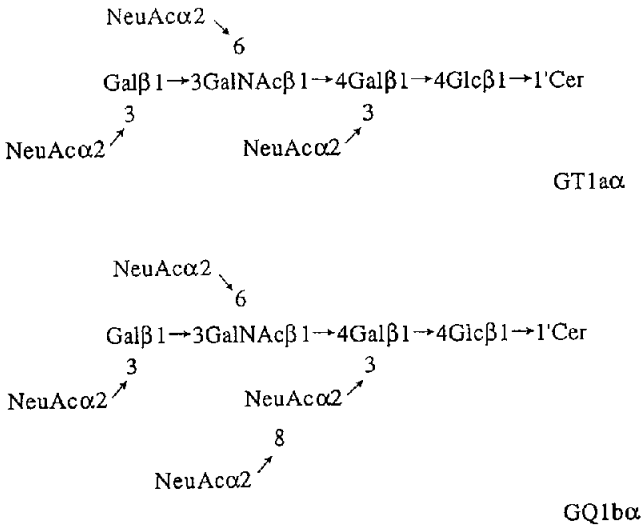
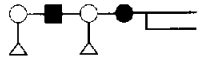
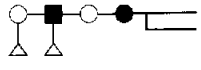
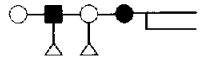
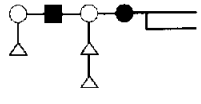
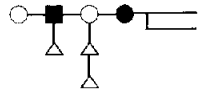
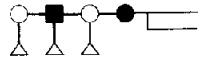


Fig. 1. Chemical structures of GT1aα and GQ1bα.

Table 1, Biosynthesis of GT1aα and GQ1bα in rat liver

Acceptor		Immunoreactive product with GGR-41	Activity (pmol/mg protein/h)
GD1a		GT1aα	26.0 [a] 25.7 [b]
GD1α		n.d.	n.d.
GD1aα		n.d.	n.d.
GT1b		GQ1bα	28.6 [a] 29.0 [b]
GT1bα		n.d.	n.d.
GT1aα		n.d.	n.d.

The sialyltransferase activities were measured by [a]BAS2000 and [b]TLC-immunostaining with GGR-41. n. d., Not detected.

● : Glc, ○ : Gal, ■ : GalNAc, △ : NeuAc, ⊢ : Cer

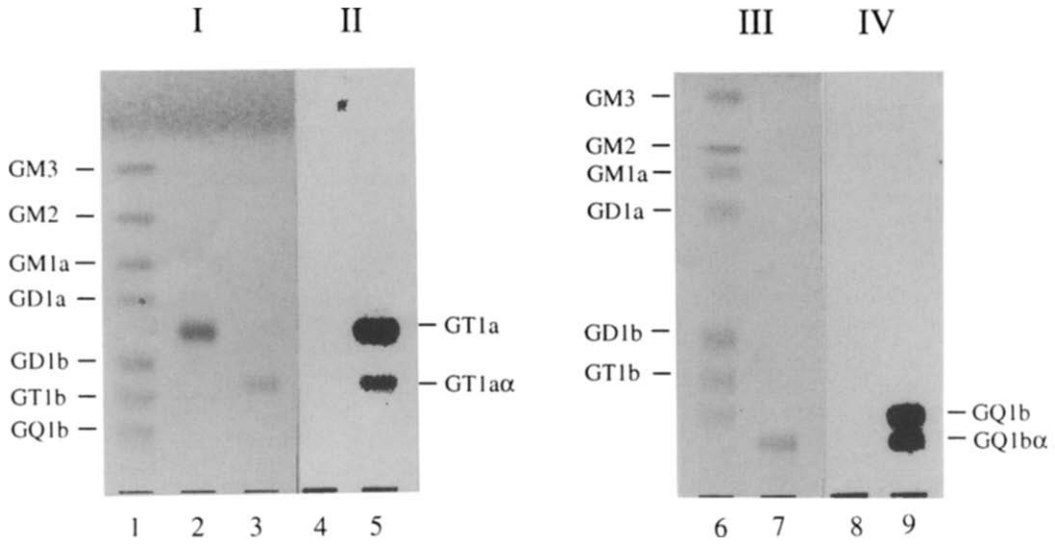


Fig. 2. Reaction products from GD1a or GT1b by rat liver sialyltransferase. Reaction products from GD1a and GT1b (panel II and IV, respectively) were analyzed with FUJIX BAS 2000. Standard gangliosides were visualized by resorcinol/HCl reagent (panel I and III). Lane 1 and lane 6, standard ganglioside mixture (GM3, GM2, GM1a, GD1a, GD1b, GT1b, and GQ1b); lane 2, standard GT1a; lane 3, standard GT1aα; lane 4, reaction products without any acceptors; lane 5, reaction products from GD1a; lane 7, standard GQ1bα; lane 8, reaction products without any acceptors; lane 9, reaction products from GT1b. TLC plate was developed first with solvent C and then solvent A in the same direction (panel I and II). For panel III and IV, the plate was developed by the same procedure except solvent B as the second solvent.

was found in rat liver [19] and brain [22]. It is not clear whether or not the formation of Chol-1 gangliosides and GD1α is performed by a single GalNAc α2-6 sialyltransferase. When GT1aα and GQ1bα were used as substrates, no radiolabeled ganglioside products could be detected (data not shown), suggesting that the two gangliosides are final products of ganglioside metabolism, and the GalNAc α2-6 sialyltransferase may be responsible for the termination of ganglioside synthesis.

3.2. Detection of Chol-1 gangliosides in normal rat liver

Since rat liver contained enzyme activity for the synthesis of Chol-1 gangliosides, we examined whether the same tissue possesses Chol-1 gangliosides by sensitive TLC-immunostaining with GGR-41. As shown in Fig. 4, the lipid fraction from rat liver gave two immunoreactive spots corresponding to GT1aα and GQ1bα. Densitometric analysis of the immunostained spots showed that the amount of GT1aα and GQ1bα were 0.75 and 1.5 μg per g dry tissue weight, respectively. The two major immunoreactive compounds moved slightly ahead of the corresponding standard gangliosides isolated from bovine brain tissues. This must be due to the compositional differences in their ceramide moiety. Ceramides from rat liver gangliosides have been shown to contain C22:0 and C24:0 as the predominant fatty acids [23].

In conclusion, rat liver contained an GalNAc α2-6 sialyltransferase, which transfers a sialic acid to the C6 position of the penultimate GalNAc residue in the gangliotetraose chain. This enzyme is of particular importance since the action of this enzyme may terminate further sialylation of ganglioseries gangliosides.

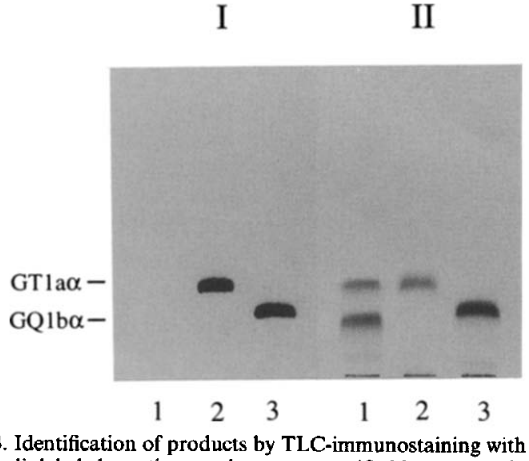


Fig. 3. Identification of products by TLC-immunostaining with GGR-41. Radiolabeled reaction products were purified by preparative TLC. The purified reaction products were analyzed by FUJIX BAS 2000 (panel I) and immunostaining with GGR-41 (panel II). Lane 1, standard GT1aα and GQ1bα; lane 2, GT1aα synthesized from GD1a; lane 3, GQ1bα synthesized from GT1b. The plate was developed first with solvent C and then solvent A in the same direction.

Table 2
Effect of incubation condition on GT1aα and GQ1bα synthase

Conditions	Synthase activity (pmol/mg protein/h)[a]	
	GT1aα	GQ1bα
Complete	25.7	29.0
$-Mg^{2+}$	26.1	29.3
$-Mn^{2+}$	25.9	28.8
$-Mg^{2+}$, Mn^{2+}	25.6	29.2
−Triton CF-54	11.7	14.4
−Acceptor	n.d.	n.d.
−CMP-NeuAc	n.d.	n.d.
−Enzyme	n.d.	n.d.

[a]The measurement of sialyltransferase activity was carried out by densitmetric analysis of TLC-immunostaining with GGR-41. n.d., not detected.

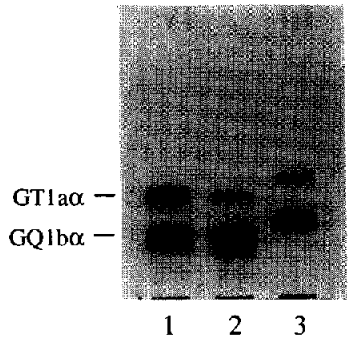


Fig. 4. The presence of GT1aα and GQ1bα in rat liver. GT1aα and GQ1bα in rat liver were detected by TLC-immunostaining with GGR-41. Lane 1, standard GT1aα (40 ng) and GQ1bα (25 ng) from bovine brain; lane 2, ganglioside fraction from 2 mg of dried rat brain tissue; lane 3, ganglioside fraction from 20 mg of dried rat liver tissue. TLC plate was developed first with solvent C and then solvent A in the same direction.

Acknowledgements: This work was supported by a Grant-in Aid for Scientific Research on Priority Area, to Y.H., No.05274106, from the Ministry of Education, Science and Culture of Japan, an NIH Grant NS 09626 to Y.-T. Li, and Fellowships of the Japan Society for the Promotion of Science for Japanese Junior Scientists to F.I.

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