A unique biosynthetic pathway for gangliosides exists in *Xenopus laevis* oocytes

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Abstract It was previously reported that monosialosylgangliopentaosyl ceramide (GalNAc-GM1b) was a major ganglioside in *Xenopus laevis* oocytes. Here we determined biosynthetic pathways for the ganglioside by detailed measurements of glycosyltransferase activities. CMP-NeuAc:asialo-GM1 α2-3 sialyltransferase (α2-3 ST) and UDP-GalNAc:GM1b β1-4 N-acetylgalactosaminyltransferase (β1-4 GalNAcT) exhibited much higher activity than CMP-NeuAc:GalNAc-GA1 α2-3 ST and UDP-GalNAc:asialo-GM1 β1-4 GalNAcT, respectively. These observations indicated the existence of a unique biosynthetic pathway in the oocytes as follows; asialo-GM1 → GM1b → GalNAc-GM1b.

Key words: Xenopus laevis; Ganglioside biosynthesis; Glycosyltransferase

1. Introduction

It is well known that gangliosides are one of the most ubiquitous components of the plasma membrane. In gangliosides, more than 100 molecular species have been identified on the basis of the diversity of their carbohydrate structures [1]. The structural diversity is caused by specific glycosyltransferases which sequentially transfer monosaccharides [2]. Previous studies have suggested that gangliosides may play important roles in a variety of biological phenomena, such as cell–cell and cell–substratum interactions, growth and differentiation, cell transformation, and signal transduction pathways (for reviews see [3–6]).

Xenopus laevis oocytes are a suitable system for exploring molecules involved in development because of the large amount of information available on embryonic morphogenesis [7]. However, detailed studies have not been carried out concerning gangliosides in *Xenopus laevis* oocytes. Before this system is adopted for investigation of the effect of active gangliosides on development, it is essential to obtain fundamental information, such as the composition of gangliosides and the enzymatic property of glycosyltransferases in the oocytes.

Previously we determined the acidic glycosphingolipid components and the complete structure of a major ganglioside in *Xenopus laevis* oocytes [8]. In this study, we characterized en-

dogenous glycosyltransferases responsible for the synthesis of gangliosides in the oocytes and found a unique biosynthetic pathway for gangliosides.

2. Materials and methods

2.1. Animals
Xenopus laevis females were purchased from the Nippon Bio-Supp. Center (Tokyo, Japan).

2.2. Materials
CMP-[9-³H]NeuAc (862.1 GBq/mmol), UDP-[1-³H]Gal (477.3 GBq/mmol), and UDP-[1-³H]GalNAc (307.1 GBq/mmol) were purchased from Du Pont/NEN Research Products (Boston, USA). GM3 was purified from dog erythrocytes. GalNAc-GM1b was isolated from *Xenopus laevis* oocytes [8]. GalNAc-GA1 was obtained by desialylation of GalNAc-GM1b with sialidase (EC 3.2.1.18) from *Arthrobacter ureafaciens* (Nacalai Tesque Inc., Tokyo, Japan). GM1b was synthesized from asialo-GM1 by α2-3 ST using a rat liver Golgi fraction [9] according to the method of Hidari et al. [10]. All other chemicals were of the highest purity available.

2.3. Preparation of the enzyme fraction from Xenopus laevis oocytes
Immature oocytes without any hormone treatment were dissociated from the ovaries. The oocytes (8.0 g, wet weight) were homogenized in 3 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose and 0.1 mM EDTA, using a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 10 min at 5,000 × g in a Hitachi RPR 20-2 rotor (Tokyo, Japan). The supernatant was further centrifuged for 1 h at 200,000 × g in a Beckman 60 Ti rotor (CA, USA). After the supernatant was removed, the pellet was suspended in 150 mM sodium cacodylate/hydrochloric acid buffer (pH 6.5) containing 1 mM 2-mercaptoethanol. This preparation was used as the enzyme source throughout the experiments. All steps were performed at 0–4°C. Protein concentrations of the enzyme fraction were determined with the Micro BCA Protein Assay Reagent (Pierce, IL, USA), using bovine serum albumin as a standard [11].

2.4. Glycosyltransferase assays
All the enzyme reactions were carried out in a total volume of 50 ml at 37°C for 2 h. (i) The assay of ST was performed by the procedure of Pohlentz et al. [12] with a slight modification. The reaction mixture comprised 0.3 mM acceptor-substrate, 3.2 mM CMP-[³H]NeuAc (155 MBq/mmol), 0.3% (w/v) Triton CF-54, 10 mM MnCl₂, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, 150 mM sodium cacodylate/HCl buffer (pH 6.5), and 280 μg of enzyme protein. (ii) The assay of GalT was performed following the procedure of Wilkinson et al. [13] with a slight modification. The mixture comprised 0.5 mM acceptor-substrate,

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Abbreviations: The nomenclature used for gangliosides is based on the system of Svennerholm [24] and the IUPAC-IUB [25]. Cer, ceramide; GalNAc, N-acetylgalactosamine; NeuAc, N-acetylneuraminic acid; CMP-NeuAc, cytidine-5′-phosphate-N-acetylneuraminic acid; lactosylceramide, LacCer; asialo-GM2, Gg3Cer; asialo-GM1, Gg4Cer; GalNAc-GA1, IV⁴GalNAcGg4Cer; GalNAc-GM1a, IV⁴Gal-NAcII³NeuAcGg4Cer; GalNAc-GM1b, IV⁴GalNAcIV³NeuAcGg4Cer; ST(s), sialyltransferase(s); GalNAcT(s), N-acetylgalactosaminyltransferase(s); GalT(s), galactosyltransferase(s); GM3 synthase, CMP-NeuAc: lactosylceramide α2-3 sialyltransferase; GM1b synthase, CMP-NeuAc: asialo-GM1 α2-3 sialyltransferase; TDC, sodium taurodeoxycholate; SDS, sodium dedecylsulfate.

0.5 mM UDP-[^{3}H]Gal (185 MBq/mmol), 0.5% (w/v) Triton X-100, 10 mM MnCl$_2$, 150 mM sodium cacodylate/HCl buffer (pH 6.0), and 280 μg of enzyme protein. (iii) The assay of GalNAcT was performed by the procedure of Senn et al. [14] with a slight modification. The mixture comprised 0.3 mM acceptor-substrate, 0.1 mM UDP-[^{3}H]GalNAc (155 MBq/mmol), 0.5% (w/v) Triton X-100, 10 mM MnCl$_2$, 5 mM CDP-choline, 64 mM sodium cacodylate/HCl buffer (pH 7.0), and 280 μg of enzyme protein. The activity of each glycosyltransferase increased linearly with increasing enzyme protein concentration and with increasing incubation time under the experimental conditions (data not shown).

2.5. Quantification and identification of reaction products

The enzyme reaction was terminated with 450 ml of distilled water. The reaction product was separated by a C$_{18}$ reverse-phase column (BOND ELUT, size 3 ml; Varian Sample Preparation Products, USA) according to the method of Kundu and Suzuki [15]. The enzyme activity was calculated from the incorporation of radiolabeled sugar into an acceptor-substrate by measurement with a liquid scintillation counter. For identification of products, the products were spotted on an HPTLC or plastic TLC plate that was developed with chloroform/methanol/0.5% CaCl$_2$ (55:45:10, v/v/v), and then exposed to an X-ray film or immunostained using the procedure of Higashi et al. [16].

3. Results

3.1. Activity of endogenous glycosyltransferases in Xenopus oocytes

Three kinds of glycosyltransferases, α2-3 ST, β1-4 GalNAcT and β1-3 GalT were investigated in the oocytes (Table 1). The activity of GM1b synthase was over 4-fold higher than that of CMP-NeuAc:GalNAc-GA1 α2-3 ST. GalNAc-GM1b synthase (UDP-GalNAc:GM1bβ1-4 GalNAcT) was also detected with potent activity. However, GalNAc-GA1 synthase was not detected significantly. These findings showed that GalNAc-GM1b was formed from asialo-GM1 followed by GM1b synthesis (Fig. 1). Furthermore, several ganglioside formations were conspicuously found as follows; asialo-GM2→asialo-GM1, lactosylceramide→asialo-GM2, GM2→GM1a, and GM3→GM2. These results also indicated that biosynthetic pathways of a-series and asialo-series gangliosides

predominantly existed in Xenopus laevis oocytes (Fig. 1). Indeed, these gangliosides are mainly found in the tissue [8].

3.2. Characterization of sialyltransferases in Xenopus oocytes

The activity of glycosyltransferases including STs, GalTs and GalNAcTs was recovered in a fraction of 200,000 × g pellets. GM3 and GM1b synthases were here enzymatically characterized because of their importance in the metabolic flow of the ganglioside biosynthesis. Both sialyltransferases had a similar optimal pH (6.5–6.7) for their activities: their properties are summarized in Table 2. In divalent cations, GM3 synthase activity was enhanced more than twice in the presence of Mg^{2+} or Mn^{2+}, and reduced by Cu^{2+} or Zn^{2+}. In particular, Zn^{2+} completely inhibited the activity. On the other hand, GM1b synthase activity was reduced in the presence of Fe^{2+}. The divalent cations examined were not essential for the activity of GM1b synthase. Among the detergents tested, Triton CF-54
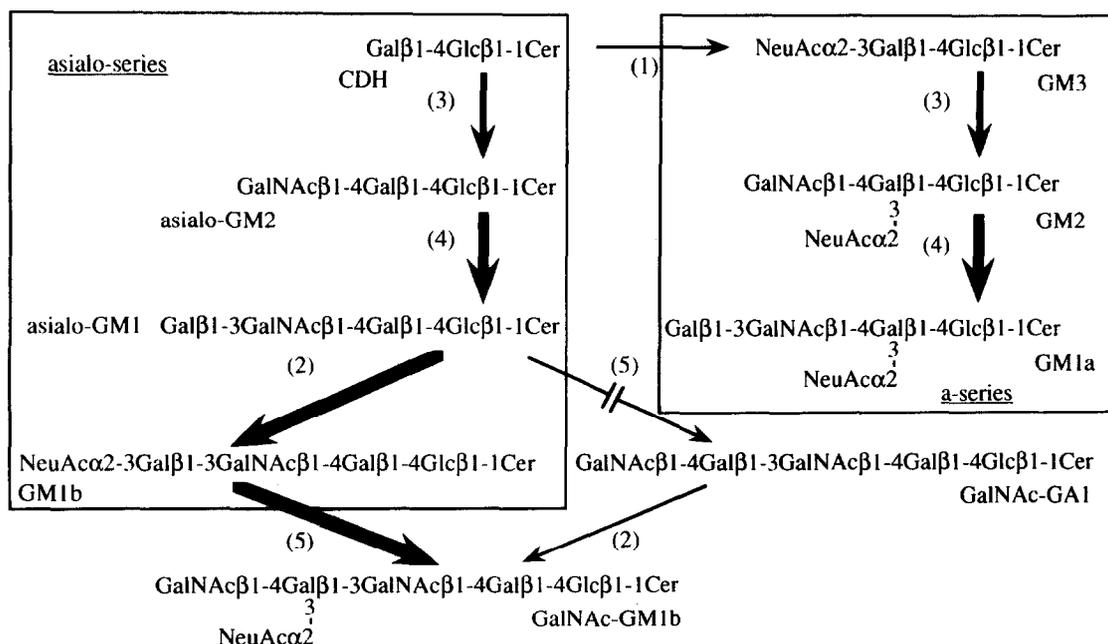
Table 1
Activities of glycosultransferase in Xenopus laevis oocytes

Glycosyltransferase	Substrate	Product	Activity (pmol/h, mg protein)
α2-3ST	Lactosylceramide	GM3	30.2 ± 4.0
	Asialo-GM1	GM1b	109.7 ± 8.9
	GalNAc-GA1	GalNAc-GM1b	27.5 ± 9.1
β-4 GalNAcT	Lactosylceramide	Asialo-GM2	41.8 ± 10.9
	Asialo-GM1	GalNAc-GA1	N.D.
	GM3	GM2	58.5 ± 11.8
	GM1b	GalNAc-GM1b	78.6 ± 4.6
β1-3 GalT	Asialo-GM2	Asialo-GM1	83.3 ± 2.6
	GM2	GM1a	106.2 ± 2.7

The values indicated are averages ± 2 S.D. for three independent experiments.
N.D., not detected.

Fig. 1. The biosynthetic pathway for gangliosides in Xenopus laevis oocytes. (1) α2-3 ST; (2) α2-3 ST; (3) β1-4 GalNAcT; (4) β1-3 GalT; (5) β1-4 GalNAcT. Bold arrows indicate high activities of glycosyltransferases.
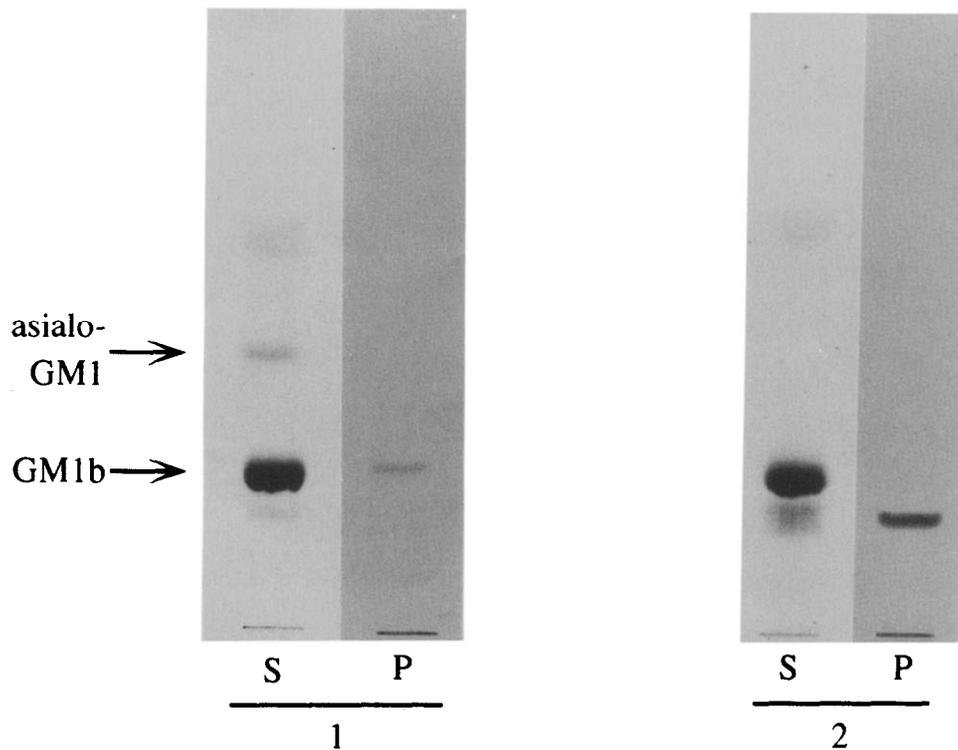
Fig. 2. Identification of reaction products by autoradiography. Lane S, standard glycosphingolipids stained with orcinol reagent; lane P, reaction product with glycosyltransferase in *Xenopus laevis* oocytes on autoradiography. Panel 1, product from asialo-GM1 (0.3 mM) by ST. Panel 2, product from GM1b (0.3 mM) by GalNAcT.

effectively activated both synthases. The optimal concentration of Triton CF-54 was commonly 0.2–0.4% (w/v) (data not shown). Without detergents the enzyme activities were not detected, indicating that both STs in the oocytes are membrane-bound enzymes similar to other glycosyltransferases from various tissues previously reported (for a review see [17]). Under the optimal assay conditions for GM1b synthase defined above, the apparent K_m values for asialo-GM1 and CMP-NeuAc were 63.6 and 159.5 mM, respectively. The K_m value for CMP-NeuAc of *Xenopus* GM1b synthase was over 10-fold higher

Table 2
Enzymatic properties of sialyltransferases from *Xenopus* oocytes

Effectors	Relative activities(%)	
	GM3 synthase	GM1b synthase
Divalent cations[a]		
Without cation	100	100
Mg^{2+}	213	94
Mn^{2+}	234	96
Ca^{2+}	120	98
Cu^{2+}	32	18
Fe^{2+}	102	64
Zn^{2+}	0	23
Detergents[a]		
Triton CF-54	100	100
Triton X-100	115	38
Tween80	13	0
TDC	0	0
SDS	0	0

[a]The sialyltransferase assay conditions are described in section 2 without each effector. The concentration of effectors were 10 mM for divalent cations and 0.4% for detergents.

than that of α2-3 sialyltransferases (GM3 synthase or GM1b synthase) from the rat liver, whereas the affinity with glycolipid-acceptor was to the same extent [12,18].

3.3. Identification of reaction products

The reaction products by glycosyltransferases involved in GalNAc-GM1b biosynthesis were determined by autoradiography (Fig. 2). In panel 1, a product from asialo-GM1 by ST co-migrated with standard GM1b on a HPTLC plate. In panel 2, a product from GM1b by GalNAcT exhibited identical TLC mobility to standard GalNAc-GM1b isolated from *Xenopus* oocytes. These results indicated that the products were GM1b and GalNAc-GM1b, respectively. Furthermore, the product by ST was confirmed to be GM1b by the TLC-immunostaining method with a mouse monoclonal antibody, M2590, that reacted with the terminal non-reducing sugar sequence, NeuAcα2-3Galβ1-R [19] (Fig. 3).

4. Discussion

GalNAc-GM1b was previously determined as a major ganglioside in the oocytes [8]. In this study, we found two different biosynthetic pathways for gangliosides in *Xenopus* oocytes (Fig. 1). One was a unique pathway through which GalNAc-GM1b was formed as a major ganglioside. The other was a ubiquitous pathway in the mammalian neural system, in which GM1a was predominantly formed [20].

Previous studies demonstrated the existence of several kinds of GalNAcTs from different species and tissues [12,21–23]. Our study shows high activity of both GM2 and GalNAc-GM1b
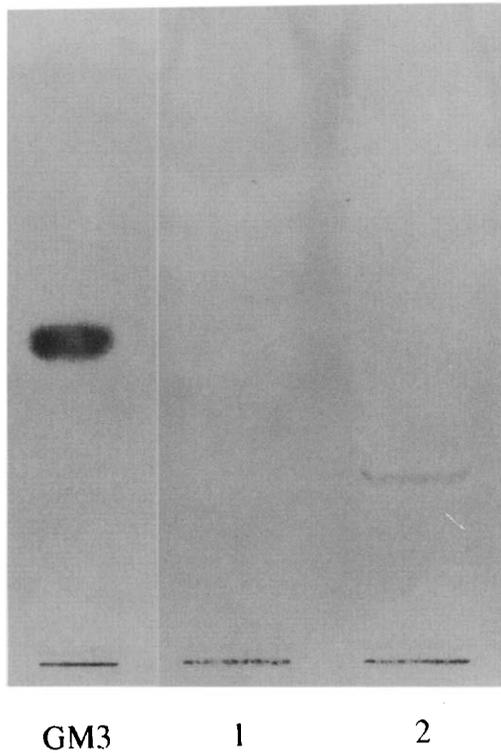
GM3   1   2

Fig. 3. Identification of a reaction product by TLC-immunostaining with the M2590 antibody. Lane 1, products without any substrates by ST in *Xenopus laevis* oocytes. Lane 2, products from asialo-GM1 (0.3 mM) by ST in *Xenopus laevis* oocytes.

synthase. Since crude homogenates from the oocytes were used as the enzymatic source, it is impossible to distinguish whether two synthetic steps are catalyzed by a single GalNacT or not. Further study is required to elucidate this question.

We have obtained information on the enzymatic properties of glycosyltransferases and the biosynthesis of gangliosides in *Xenopus laevis* oocytes for the first time. These observations will serve for studying the biological functions of gangliosides on development and differentiation.

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