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Identity of G_{D1C}, G_{T1a} and G_{Q1b} synthase in Golgi vesicles from rat liver

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Competition experiments using G_{M1b}, G_{D1a} and G_{T1b} as substrates, and as mutual inhibitors for ganglioside sialyltransferase activity in preparations of Golgi vesicles derived form rat liver, suggested that sialyl transfer to these three respective compounds, leading to gangliosides G_{D1C}, G_{T1a} and G_{Q1b}, respectively, is catalyzed by one enzyme. These results are incorporated into a model for ganglioside biosynthesis and its regulation.

Glycosphingolipid; Ganglioside; Biosynthesis; Sialyltransferase

1. INTRODUCTION

Gangliosides are complex, ubiquitous, plasma membrane components, which are characterized by the presence of one or more sialic acid units in their oligosaccharide chain. These reactions are catalyzed by specific glycosyltransferases.

Ganglioside biosynthesis takes place in the Golgi apparatus where, starting with glucosylceramide, it progresses through the sequential addition of galactose, *N*-acetylgalactosamine and *N*-acetylneuraminic acid to the growing oligosaccharide chain [1]. These reactions are catalyzed by specific glycosyltransferases, which utilize UDP-

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Abbreviations: Cer, ceramide (*N*-acylsphingosine); Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; NeuAc, *N*-acetylneuraminic acid; UDP-Gal, uridine 5′-diphosphogalactose; UDP-GalNAc, uridine 5′-diphospho-*N*-acetylgalactosamine; CDP-choline, cytidine 5′-diphosphocholine; CMP-NeuAc, cytidine 5′-monophospho-*N*-acetylneuraminic acid; GlcCer, glucosylceramide, Glc1→1Cer; LacCer, Galβ1→4Glcβ1→1Cer; In Svennerholm nomenclature [20] for gangliosides: G, ganglioside; M, monosialo; D, disialo; T, trisialo; and arabic numerals indicate sequence of migration in thin-layer chromatograms.

Enzymes: UDP-*N*-acetylgalactosamine:galactosyl-glucosylceramide β1→4 *N*-acetylgalactosaminyltransferase (EC 2.4.1.-) or G_{A2} synthase; UDP-*N*-acetylgalactosamine:(*N*-acetylneuraminyl)-galactosyl-glucosylceramide β1→4 *N*-acetylgalactosaminyltransferase (EC 2.4.1.92) or G_{M2}-synthase; UDP-*N*-acetylgalactosamine:(*N*-acetylneuraminyl-*N*-acetylneuraminyl)-galactosyl-glucosylceramide β1→4 *N*-acetylgalactosaminyltransferase (EC 2.4.1.-) or G_{D2}-synthase; CMP-*N*-acetylneuraminate:galactosyl-glucosylceramide α2→3 sialyltrans-ferase (EC 2.4.99.-) or G_{M3}-synthase; CMP-*N*-acetylneuraminate:(*N*-acetylneuraminyl)-galactosyl-glucosylceramide α2→8 sialyltransferase (EC 2.4.99.8) or G_{D3}-synthase, CMP-*N*- acetylneuraminate:(*N*-acetylneuraminyl-*N*-acetyl-neuraminyl)-galactosyl-glucosylceramide α2→8 sialyl-transferase (EC 2.4.99-) or G_{T3}-synthase; UDP-galactose:*N*-acetylgalactosaminyl-galactosyl-glucosylceramide β1→3 galactosyltransferase (EC 2.4.1.-) or G_{A1}-synthase; UDP-galactose:*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosyl-glucosylceramide β1→3 galactosyltransferase (EC 2.4.1.62) or G_{M1}-synthase; UDP-galactose:*N* - acetylgalactosaminyl - (*N* - acetylneuraminyl - *N* - acetylneuraminyl)-galactosyl-glucosylceramide β1→3 galactosyltransferase (EC 2.4.1.-) or G_{D1b}-synthase; CMP-*N*-acetylneuraminate:asialoganglioside (G_{A1}) α2→3 sialyltransferase (EC 2.4.99.-) or G_{M1b}-synthase; CMP-*N*-acetylneuraminate:monosialoganglioside (G_{M1}) α2→3 sialyltransferase (EC 2.4.99.2) or G_{D1a}-synthase; CMP-*N*-acetylneuraminate:disialoganglioside (G_{D1b}) α2→3 sialyltransferase (EC 2.4.99.-) or G_{T1b}-synthase; CMP-*N*-acetylneuraminate:monosialoganglioside (G_{M1b}) α2→8 sialyltransferase (EC 2.4.99.-) or G_{D1c}-synthase; CMP-*N*-acetylneuraminate:disialoganglioside (G_{D1a}) α2→8 sialyltransferase (EC 2.4.99.-) or G_{T1a}-synthase; CMP-*N*-acetylneuraminate:trisialoganglioside (G_{T1b}) α2→8 sialyltransferase (EC 2.4.99.-) or G_{Q1b}-synthase

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Gal, UDP-GalNAc or CMP-NeuAc as sugar donors. Many of these enzymes have been studied and partially characterized in rat liver Golgi apparatus [2–12]. Earlier studies demonstrated that some of these glycosyltransferases (namely *N*-acetylgalactosaminyltransferase, galactosyltransferase II and sialyltransferase IV) (fig.1), are one and the same enzyme. [10,12]. The analogous acceptors in these studies differed only in the quantity of their neuraminic acid residues, bound to the inner galactose of these glycosphingolipids.

In the present study we show, using kinetic and competition experiments, that sialyltransferase V, catalyzing the binding reaction of an *N*-acetylneuraminic acid in α2–8 linkage to the sialic acid residue of G_{M1b}-, G_{D1a}- and G_{T1b} acceptors, is one and the same enzyme in rat liver Golgi.

2. MATERIALS AND METHODS

2.1. *Materials*

Cytidine-5′-monophospho-*N*-acetyl [4,5,6,7,8,9-^{14}C]-neuraminic acid (CMP-[^{14}C]NeuAc, 10.77 GBq/nmol) were purchased from Amersham and used after dilution with non-radioactive nucleotide sugars obtained from Sigma. Sephadex G-25 superfine and DEAE-Sepharose Cl-6B were from Pharmacia. Triton CF-54 and serum albumin were from Sigma. Scintillation cocktail Pico Fluor 30 was from Packard and cacodylate was from Fluka. Male rats from the Wistar strain (250–300 g) were procured from Hagemann (Extertal, FRG). All other reagents and solvents used were of analytical grade.

2.2. *Methods*

2.2.1. Preparation of Golgi vesicles

Golgi-rich vesicles were isolated from rat liver, essentially by a method of Sandberg et al. [13] as described in detail [14,15]. Enrichments of Golgi-specific enzymes (glycosyltransferases) were 50–80-fold. Contamination with other cellular membranes (plasma membrane, lysosomes, endoplasmatic reticulum) was <5% [14].

2.2.2. Assay of *N*-acetylneuraminyltransferase (sialyltransferase) (G_{M1b}-, G_{D1a}-, G_{T1b} synthase)

In a total volume of 50 μl, the assay contained 80–120 μM glycolipid acceptor (G_{M1b}, G_{D1a} or G_{T1b}), 0.3% (w/v) Triton CF-54, 150 mM sodium cacodylate/HCl buffer, pH 6.4, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM CMP-[^{14}C]NeuAc (4000–6000 cpm/nmol) and 50 μg of Golgi protein. Incubation was for 30 min at 37°C.

Gangliosides G_{M1b}, G_{D1a} and G_{T1b} and the detergent Triton X-100 were in chloroform/methanol (2:1, v/v) solution. The substances in the organic solvents were first dried under a stream of nitrogen and sonicated for 60 s in buffer before use in the enzyme assay. After incubation for 30 min the reaction was stopped by adding 1 ml chloroform/methanol (2:1 v/v). The gangliosides were separated from the radioactive nucleotide sugars by Sephadex G-25 gel chromatography [14]. The radioactivity of the products was determined in a liquid scintillation counter. Rates for all the reactions described in this paper were linear with time, for at least to 30 min, and linear with a protein concentration up to 50–75 μg per assay depending on the Golgi preparation. During the 30 min incubation period, the decrease in sugar nucleotide concentration was less than 5%.

All experiments were done at least twice and mean values are presented in the figures. The K_M values presented are apparent K_M values determined in detergent containing assays.

2.2.3. Product identification

Eluted fractions from the Sephadex columns were dried under a stream of nitrogen, redissolved in 100 μl of chloroform/methanol (2:1, v/v) and applied to silica gel G 60 tlc plates. Chromatograms were developed in chloroform/methanol/0.2% $CaCl_2$ in water (60:35:8, v/v). Radiolabelled material was visualized by fluorography as described previously [16].

3. RESULTS

The conversion of G_{M1b}, G_{D1a} and G_{T1b} to G_{D1c}, G_{T1a} and G_{Q1b}, respectively, could be catalyzed either by the same or by three different sialyltransferases. In order to discriminate between these two possibilities, two of the three substrates (G_{M1b}, G_{D1a}, G_{T1b}) were used in the enzyme assay at the same time at various relative concentrations, keeping the total acceptor concentration at 120 μM. For two independent enzymes, each recognizing only one of substrates a and b, the total reaction velocity v_t can be calculated as the sum of two partial velocities v_a and v_b given by their respective Michaelis eqn 1:

$$v_t = v_a + v_b = \frac{V_a}{(1 + K_a/[a])} + \frac{V_b}{(1 + K_b/[b])} . \quad (1)$$

If both substrates are accepted by the same enzyme (i.e. the same active site) and each substrate acts as a competitive inhibitor of the other, the inhibitor constant (K_i) of either substrate would be equal to its K_M value ($K_M = K_i$). The total velocity is given by eqn 2 as described in [17]:

$$v_t = v_a + v_b = \frac{V_a}{1 + (K_a/[a])[1 + ([b]/K_b)]} + \frac{V_b}{1 + (K_b/[b])[1 + ([a]/K_a)]} . \quad (2)$$

The K_M and V_{max} values were determined simultaneously with the same Golgi preparation. If G_{D1a} and G_{T1b} were used in the sialyltransferase assay as acceptors (G_{D1a}:$K_M = 16$ μM, $V_{max} =$ 0.5 nmol/h·mg; G_{T1b}:$K_M = 10$ μM, $V_{max} =$ 0.4 nmol/h·mg), the total reaction velocities could be calculated from eqns 1 and 2 (fig.1). The measured v_t values clearly fit with those calculated using eqn 2; these results show that G_{D1c} and G_{T1a} are synthesized from their respective precursors at the same active site of a single enzyme.

In analogous experiments when G_{M1b} and G_{D1a} (G_{M1b}:$K_M = 519$ μM; $V_{max} = 8$ nmol/h·mg; G_{D1a}: $K_M = 33$ μM; $V_{max} = 1$ nmol/h·mg) were used as glycolipid acceptors for G_{D1c} and G_{T1a} synthesis similar results were obtained (fig. 2). These experiments show that G_{D1c}, G_{T1a} and G_{Q1b} synthesis is catalyzed by a single sialyltransferase V in rat liver Golgi.

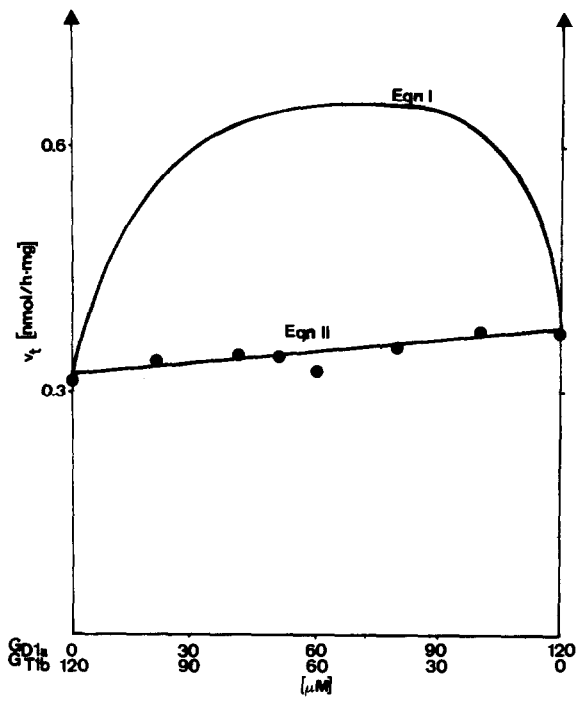

Fig.1. Competition between G_{D1a} and G_{T1b} in the sialyltransferase assay. As described in section 2.2. G_{D1a} and G_{T1b} were used as acceptors for sialyltransferase in various relative concentrations, keeping the total acceptor concentration at 120 μM. Total reaction velocities determined experimentally (●), or calculated for the different models (eqn 1 or 2) are plotted against the substrate concentration. The kinetic constants used were those given in section 3. Upper curve, v_t as calculated from eqn 1 (two different enzymes); lower curve, v_t as calculated from eqn 2 (one enzyme).

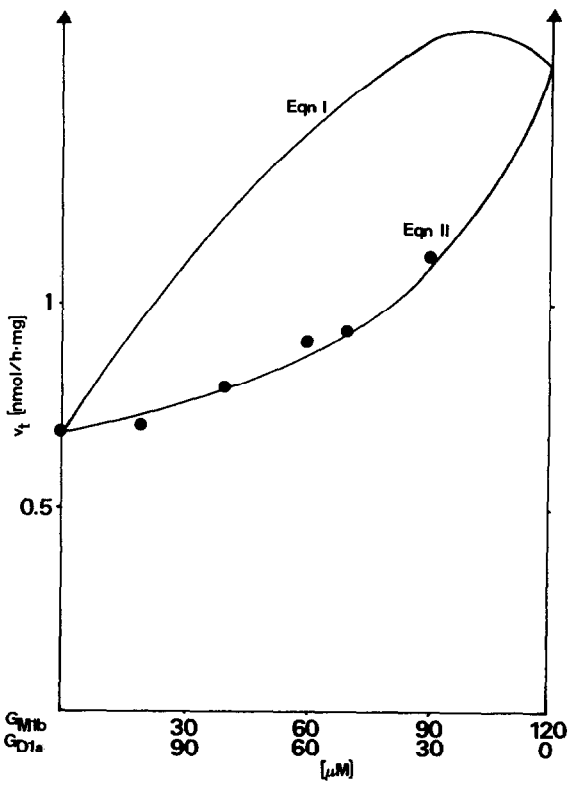

Fig. 2. Competition between G_{M1b} and G_{D1a} in the sialyltransferase assay. As described in section 2.2. G_{M1b} and G_{D1a} were used as acceptors for sialyltransferase in various relative concentrations, keeping the total acceptor concentration at 120 μM. Total reaction velocities determined experimentally (●), or calculated for the different models (eqn 1 or 2) are plotted against the substrate concentration. The kinetic constants used were those given in section 3. Upper curve, v_t as calculated from eqn 1 (two different enzymes); lower curve, v_t as calculated from eqn 2 (one enzyme).

4. DISCUSSION

The identical nature of some glycosyltransferases was suspected in earlier studies [18,19]. Pohlentz et al. [10] was the first who demonstrated that the synthesis of G_{A2}, G_{M2} and G_{D2} from their respective precursors is catalyzed by the same *N*-acetylgalactosaminyltransferase in rat liver Golgi, as is the synthesis of G_{M1b}, G_{D1a} and G_{T1b} by the same sialyltransferase [10]. By proving the non-identity of G_{D3}- and G_{D1a}-synthase they also

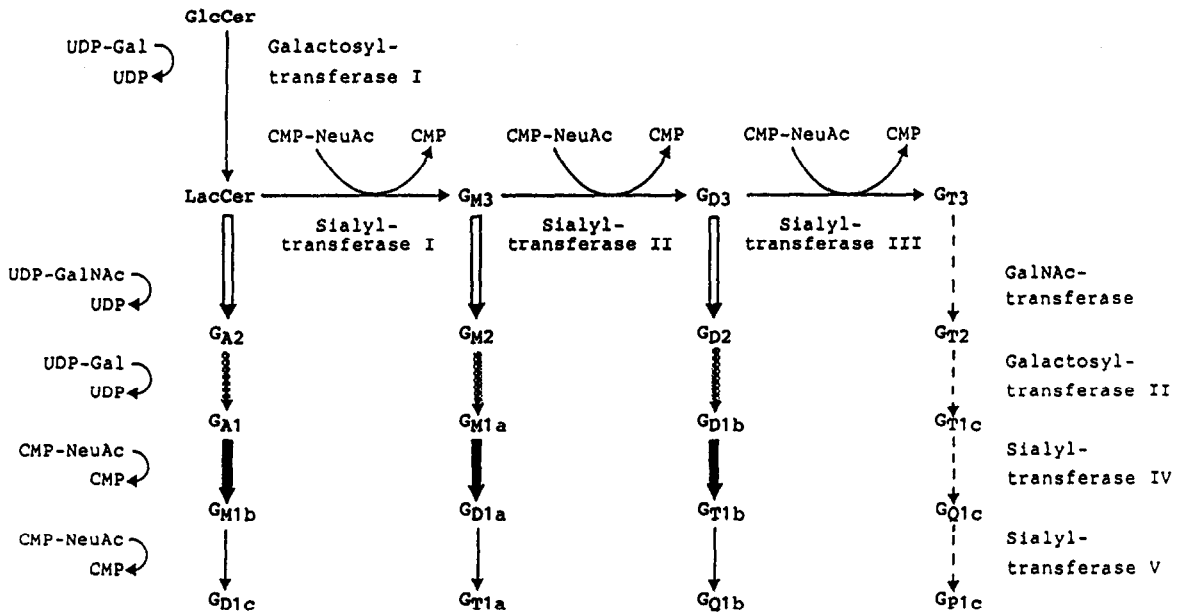

Fig.3. Suggested scheme for ganglioside biosynthesis [10,12].

demonstrated that this method allows one to distinguish between two different enzymes [10]. In antoher study we showed that one galactosyltransferase catalyzes the conversion from G_{A2}, G_{M2} and G_{D2} to G_{A1}, G_{M1a} and G_{D1b} [12].

The present study demonstrates that G_{D1c}, G_{T1a} and G_{Q1b} are synthesized by the same sialyltransferase V in rat liver Golgi. The results support the modified model of ganglioside biosynthesis proposed previously (fig. 3) [10,12]. In this model, the sialyltransferase I–III seems to determine to which series a certain ganglioside molecule is directed (asialo, a, b or c). Starting with LacCer, G_{M3} or G_{D3}, and also possibly with G_{T3}, further biosynthesis of gangliosides belonging to the three, and possibly four, different series (i.e. asialo, a, b or c) is catalyzed by the same set of enzymes namely *N*-acetylgalactosaminyltransferase, galactosyltransferase II, sialyltransferase IV and sialyltransferase V (fig. 3). These four enzymes seem to recognize the common neutral carbohydrate 'backbones' of their respective acceptors. The different number of sialic acid residues bound to the inner galactose of the carbohydrate backbone has an influence on the kinetic properties of these glycosyltransferases. The present results also support our hypothesis [10,12] that a major site for the regulation of ganglioside biosynthesis occurs in the reaction sequence $LacCer \rightarrow G_{M3} \rightarrow G_{D3} \rightarrow G_{T3}$ (fig. 3).

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