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Identity of G_{A1}, G_{M1a} and G_{D1b} synthase in Golgi vesicles from rat liver

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Synthesis of ganglioside G_{D1b} from ganglioside G_{D2} was demonstrated using Golgi membranes isolated from rat liver. Competition experiments using gangliosides G_{A2}, G_{M2} and G_{D2} as substrates, and as mutual inhibitors for ganglioside galactosyltransferase activity in preparations of Golgi vesicles derived from rat liver, suggested that galactosyl transfer to these three compounds, leading to gangliosides G_{A1}, G_{M1a} and G_{D1b} respectively, is catalyzed by one enzyme. These results strengthen the hypothesis that the main site for the regulation of ganglioside biosynthesis occurs within the reaction sequence LacCer→G_{A3}→G_{D3}→G_{T3}.

Glycosphingolipid; Ganglioside; Ganglioside synthesis; Galactosyltransferase

1. INTRODUCTION

Gangliosides are a group of complex glycosphingolipids characterized by the presence of one or more sialic acid units in their oligosaccharide chain. Although gangliosides are ubiquitous plasma membrane components, little is known about their function and the regulation of their metabolism. 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

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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, Glcβ1→1Cer; LacCer, Galβ1→4Glcβ1→1Cer. In the nomenclature of Svennerholm [19] for gangliosides: G, ganglioside; M, monosialo; D, disialo; T, trisialo; and arabic numerals indicate the 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: (*N*-acetylneuraminyl)-galactosyl-glucosylceramide α2→8 sialyltransferase (EC 2.4.99.8) or G_{D3} 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_{M1a} 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_{M1a}) 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

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catalyzed by specific glycosyltransferases. Many of these enzymes, which utilize UDP-Gal, UDP-GalNAc or CMP-NeuAc as sugar donors, have been studied and partially characterized in rat liver Golgi apparatus [2–11]. Some of the glycosyltransferases involved in ganglioside biosynthesis catalyze analogous reactions differing only in the identity of the acceptor (e.g. G_{A1}, G_{M1a}, G_{D1b} synthase catalyze the transfer of galactose in β1–4 linkage to the *N*-acetylgalactosamine residue of the respective acceptor molecule). It has been proposed, but not proven, in earlier studies that these enzymes are identical [12,13]. Pohlentz et al. [10] were the first to demonstrate the identical nature of G_{A2}, G_{M2}, G_{D2} synthases as well as G_{M1b}, G_{D1a}, G_{T1b} synthases in rat liver Golgi [10]. Here, through kinetic and competition experiments, we show that G_{A1}, G_{M1a} and G_{D1b} synthases are one and the same enzyme in rat liver Golgi.

2. MATERIALS AND METHODS

2.1. *Materials*

UDP-D-[U-^{14}C]galactose were purchased from Amersham (Braunschweig) and used after dilution with the appropriate unlabelled nucleotide sugars obtained from Sigma (Deisenhofen). Sephadex G-25 superfine was obtained from Pharmacia (Freiburg). CDP-choline, Triton X-100 and bovine serum albumin were obtained from Sigma (Deisenhofen). Scintillation cocktail Pico Fluor 30 was purchased from Packard (Frankfurt) and sodium cacodylate from Fluka (Buchs, Switzerland). Male rats of the Wistar strain (300–350 g) were procured from Hagemann (Extertal, FRG). Gangliosides used in this study were available in our laboratory. G_{D2} was a gift from Professor Svennerholm (Göteborg, Sweden). All other reagents and solvents used were of analytical grade quality.

2.2. *Methods*

Golgi-rich vesicles were isolated from rat liver, essentially by the method of Sandberg et al. [14] as detailed in [15,16]. Enrichment of Golgi-specific enzymes (glycosyltransferases) was 50–80-fold. Contamination with other cellular membranes (plasma membrane, lysosomes, endoplasmic reticulum) was less than 5% [15].

2.3. *Assay of galactosyltransferases (G_{A1}, G_{M1a}, G_{D1b} synthase)*

In a total volume of 50 μl, assay solutions contained glycolipid acceptor (G_{A2}, G_{M2}, G_{D2}) up to 200 μM, 0.3% (w/v) Triton X-100, 64 mM sodium cacodylate/HCl (pH 7.35), 10 mM CDP-choline, 20 mM $MnCl_2$, 500 μM UDP-[^{14}C]Gal (130–330 GBq/mol) and 50 μg Golgi protein. Incubation was for 15 min at 37°C.

Gangliosides G_{A2}, G_{M2} and G_{D2} 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 N_2 and sonicated for 45 s in buffer before use in the enzyme assay. After incubation for 15 min the reaction was stopped by adding 1 ml chloroform/methanol (2:1, v/v). The gangliosides were separated from radioactive nucleotide sugars by Sephadex G-25 gel chromatography [15]. 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 30 min, and linear with protein concentration up to 50–75 μg per assay depending on the Golgi preparation. During the 15 min incubation period the decrease in sugar nucleotide concentration was less than 5%.

All experiments were performed 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.

2.4. *Product identification*

Eluted fractions from the Sephadex columns were dried under a stream of N_2, redissolved in 100 μl 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 [17].

3. RESULTS

The conversion of G_{A2}, G_{M2} and G_{D2} to G_{A1}, G_{M1a} and G_{D1b}, respectively, could be catalyzed by either the same or three different galactosyltransferases. In order to discriminate between these two possibilities, two of the three substrates (G_{A2}, G_{M2}, G_{D2}) were used in the enzyme assay at the same time at various relative concentrations, keeping the total acceptor concentration at 200 μM. For two independent enzymes, each recognizing only one of the 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 equations (eqn 1):

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

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 [18]:

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

The K_m and v_{max} values were determined simultaneously with the same Golgi preparation. If

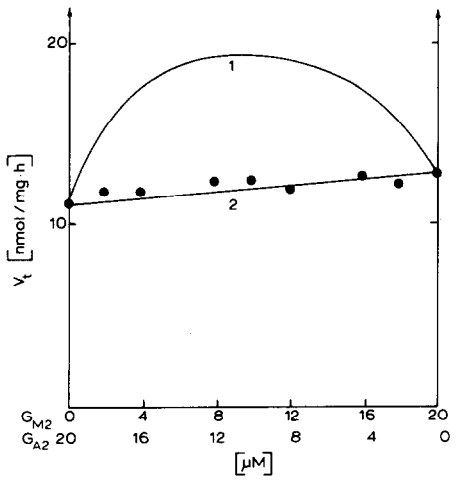

Fig.1. Competition between G_{A2} and G_{M2} in the galactosyltransferase assay. As described in section 2, G_{A2} and G_{M2} were used as acceptors for galactosyltransferase in various relative concentrations, keeping the total acceptor concentration at 20 μM. Total reaction velocities determined experimentally (●) or calculated for the different models (eqn 1 or 2) are plotted vs 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).

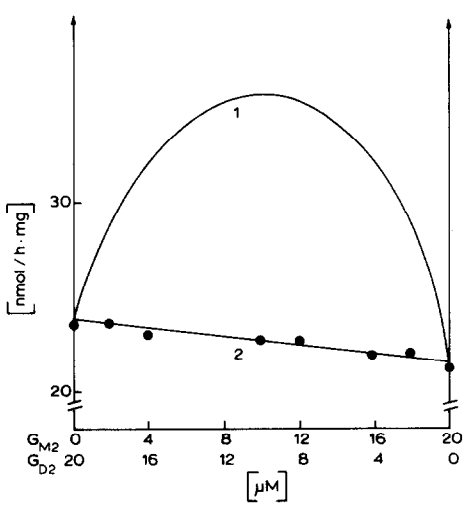

Fig.2. Competition between G_{M2} and G_{D2} in the galactosyltransferase assay. As described in section 2, G_{M2} and G_{D2} were used as acceptors for galactosyltransferase in various relative concentrations, keeping the total acceptor concentration at 20 μM. Total reaction velocities determined experimentally (●) or calculated for the different models (eqn 1 or 2) are plotted vs 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).

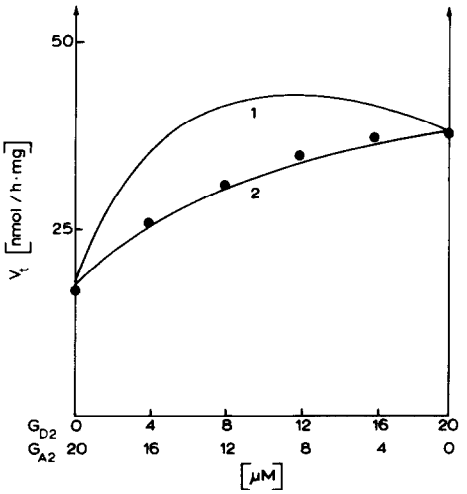

Fig.3. Competition between G_{A2} and G_{D2} in the galactosyltransferase assay. As described in section 2, G_{A2} and G_{D2} were used as acceptors for galactosyltransferase in various relative concentrations, keeping the total acceptor concentration at 20 μM. Total reaction velocities determined experimentally (●) or calculated for the different models (eqn 1 or 2) are plotted vs 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).

G_{A2} and G_{M2} were used in the galactosyltransferase assay as acceptors (G_{A2}, $K_m = 8$ μM, $v_{max} =$ 15 nmol/h per mg; G_{M2}, $K_m = 4$ μM, $v_{max} =$ 15 nmol/h per mg) the total reaction velocities could be calculated from eqns 1 and 2 (fig.1). The measured v_t values clearly fitted with those calculated using eqn 2; these results show that G_{A1} and G_{M1a} are synthesized from their respective precursors at the same active site of a single enzyme. In analogous experiments when G_{M2} and G_{D2} (G_{M2}, $K_m = 9$ μM; $v_{max} = 31$ nmol/h per mg; G_{D2}, $K_m = 6$ μM; $v_{max} = 30$ nmol/h per mg), or G_{A2} and G_{D2} (G_{A2}, $K_m = 27$ μM; $v_{max} = 40$ nmol/h per mg; G_{D2}, $K_m = 6$ μM; $v_{max} = 49$ nmol/h per mg) were used as glycolipid acceptors for synthesis of G_{M1a} and G_{D1b} or of G_{A1} and G_{D1b}, respectively, similar results were obtained (figs 2,3).

These experiments show that G_{A1}, G_{M1a} and G_{D1b} synthesis is catalyzed by a single galactosyltransferase in rat liver Golgi.

4. DISCUSSION

The identical nature of the G_{M2} and G_{D2} syn-

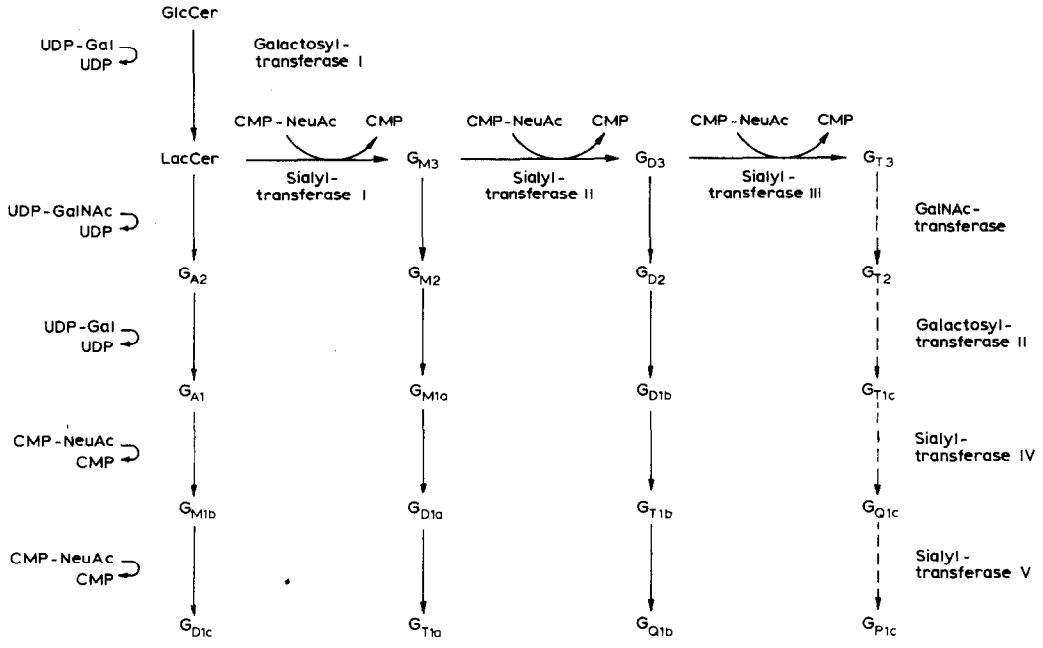

Fig.4. Suggested scheme for ganglioside biosynthesis.

thases, G_{M1a} and G_{D1b} synthases, and G_{D1a} and G_{T1b} synthases was suspected in earlier studies [12,13]. Pohlentz et al. [10] were able to demonstrate 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_{M1a} synthase they also demonstrated that this method allows one to distinguish between two different enzymes [10].

The present study demonstrates that G_{A1}, G_{M1a} and G_{D1b} are synthesized by the same galactosyltransferase in rat liver Golgi. The synthesis of G_{D1b} from G_{D2}, using Golgi membranes isolated from rat liver, is demonstrated for the first time. The results support the modified model of ganglioside biosynthesis proposed previously (fig.4) [10]. In this model, the sialyltransferases I–III seem 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.4). 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] that a major site for the regulation of ganglioside biosynthesis occurs in the reaction sequence LacCer → G_{M3} → G_{D3} → G_{T3} (fig.4).

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