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UDP-galactose:lactosylceramide α-galactosyltransferase activity in human placenta

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The activity of UDP-Gal:LacCer galactosyltransferase in human placenta was studied by using crude homogenate and Triton CF-54 extract as the source of enzyme. Transfer of galactose to lactosylceramide was optimal in the presence of 0.1% Triton CF-54 and Mn^{2+} at pH 6.3, and the reaction product was susceptible to α-galactosidase.

Keywords: glycolipid biosynthesis, p blood group, globotriaosylceramide, P^k antigen

Abbreviations: LacCer, lactosylceramide (Galβ1-4Glcβ1-1Cer); Gb_3, globotriaosylceramide (Galα1-4Galβ1-4Glcβ1-1Cer); Gb_4, globoside (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1Cer); TLC, thin-layer chromatography; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance; EDTA, ethylenediamine tetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

Introduction

In normal human erythrocytes, the major neutral glycosphingolipid is globoside, the P antigen [1]. However, there are rare individuals who lack globoside, and these people belong to the p or P^k blood groups. 'Little' p individuals have a hereditary deficiency of all P blood group active glycolipids, namely globoside, globotriaosylceramide (the P^k antigen), and P_1 glycolipid. Thus, the precursor of globo-series glycosphingolipids, lactosylceramide, is the major glycolipid of p erythrocytes [2].

Antibodies against the P blood group antigens might cause abortions early in pregnancy, when the mother belongs to the p blood group but the father is of P_1 or P_2 phenotype. Rydberg *et al.* [3] found that anti-PP_1P^k antibodies from a p woman with repeated abortions bound to glycolipid antigens isolated from an aborted placenta. The placental tissue is, however, rich in blood vessels, and red-cell glycolipids may be present in such preparations. Moreover, a P-like antigen activity has been found in p individuals, and the structure has been identified as GalNAcβ1-3Galβ1-4GlcNAc [4, 5]. It is therefore important to study the biosynthesis of P blood group active glycolipids in human placenta. In the present work, we have used normal full-term placenta as a source of the enzyme, and measured UDP-galactose:lactosylceramide galactosyltransferase activity in crude homogenate and in Triton CF-54 extract.

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Materials and methods

Materials

UDP-[^{14}C]galactose (325 mCi mmol^{-1}) and NaB[^{3}H]$_4$ (7 Ci mmol^{-1}) were purchased from Amersham International (Buckinghamshire, UK). Triton CF-54, Triton X-100, CHAPS, Nonidet P-40, sodium cholate, sodium taurocholate, UDP-galactose, CDP-choline, galactonolactone, galactose oxidase (from *Dactylium dendroides*, 170 units per mg protein), α-galactosidase (from green coffee beans, 10 units per mg protein) and β-galactosidase (from *Aspergillus niger*, 27 units per mg protein) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Silica gel 60 TLC plates were obtained from E. Merck (Darmstadt, Germany). Standard glycolipids were isolated from human erythrocytes.

Triton extraction

Fresh placental tissue was extensively washed with phosphate buffered saline (PBS: 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4) and trimmed off larger arteries and membranes. Part of the tissue (124 g) was frozen (−70 °C), and the rest (80 g) was homogenized in five parts (w/v) of cold water. The homogenate was then passed through a cheesecloth and centrifuged for 30 min at 20 000 × **g**. The supernatants were discarded, and the pellets were resuspended in two parts of water. The suspension was centrifuged as before, the supernatants were discarded and the pellets once again suspended in water. Triton CF-54 and solid NaCl were then added to the suspension to

concentrations of 0.5% (w/v) and 0.2 M, respectively. After stirring for 60 min, the suspension was centrifuged at 20000 × **g** for 60 min to yield a Triton extract [6]. All operations were carried out at 0–4° C. Protein was measured by the method of Lowry *et al.* [7].

Assay for UDP-Gal:LacCer galactosyltransferase

The reaction mixture consisted of 30 μg lactosylceramide, 12.5 nmol UDP-galactose containing 40 nCi UDP-[^{14}C]-Gal, 50–100 μg Triton CF-54, 1.0 μmol $MnCl_2$, 4 μmol sodium cacodylate, pH 6.3, 0.25 μmol galactonolactone, 0.25 μmol CDP-choline, and an enzyme preparation in a total volume of 50 μl. After 1 h at 37 °C, the reaction was stopped by the addition of 50 μl 0.05 M EDTA. Six parts of chloroform:methanol (2:1) were then added to the mixture, and the reaction products were purified by Folch partition. The solvent was evaporated, lipids were dissolved in 30 μl of chloroform:methanol and separated on TLC with a solvent system of chloroform:methanol:water (60:30:5 by vol). Red-cell globotriaosylceramide was co-applied as a standard. The lipid bands were visualized with iodine vapour, and the silica gel corresponding to globotriaosylceramide was scraped off the plate. The samples were counted in a Triton X-114 based scintillation fluid using a Wallac LKB 1210 Ultrobeta liquid scintillation counter.

Treatment of the reaction product with galactosidases

The reaction product was characterized by galactosidase digestion. In these experiments, the specific activity of UDP-galactose used was increased fourfold, and the radiolabelled glycolipid was subjected to Folch partition after extraction from the TLC plate. For treatment with α-galactosidase, approximately 5 pmol of the purified product was pipetted into each tube. The glycolipid was incubated with 30 mU enzyme in 100 μl 0.1 M sodium citrate buffer, pH 5.0, containing 10% sodium taurocholate [8]. For treatment with β-galactosidase, the same amount of the radioactive product was incubated with 30 mU enzyme in 100 μl 50 mM sodium citrate buffer, pH 4.3, containing 0.3% sodium taurocholate [9]. In control experiments, the enzyme was omitted. After incubation at 37 °C for up to 20 h, the reaction mixtures were subjected to Folch partitioning. The extent of the degradation of the product by the enzyme was calculated by measurement of the radioactivity in the lower phase [10].

Isolation of glycolipids from placenta

Frozen human placenta (26.3 g) was cut into thin slices with a scalpel, and the tissue was homogenized in two volumes (w/v) of water with a Dounce homogenizer. The homogenate was poured into 795 ml of chloroform:methanol (2:1 by vol) and stirred overnight. The solvent was removed in a rotary evaporator, and the residue was further dried by lyophilization. Glycosphingolipids were isolated from the extract by the method of Saito and Hakomori [11] and purified by Bio-Sil A chromatography using a chloroform:methanol:water system [12].

Preparation of [^{3}H]globotriaosylceramide

Globotriaosylceramide was isolated from human erythrocytes and labelled with the galactose oxidase/NaB[^{3}H]$_4$ procedure. The labelled glycolipid was purified by solvent partition. Bio-Sil A chromatography, and preparative thin-layer chromatography [13].

Fatty acid analysis

Fatty acid methyl esters were extracted from methanolysed glycolipid samples with several portions of hexane [14] and analysed by gas-liquid chromatography on an HP-1 capillary column (Hewlett-Packard, Avondale, PA, USA). The starting temperature was 150 °C, and a temperature gradient of 8 °C min^{-1} was used up to 270 °C. The final time was 10 min.

Nuclear magnetic resonance spectroscopy

^{1}H-NMR spectra at 500 MHz were recorded at 338 K on a Varian Unity 500 spectrometer operating in the Fourier transform mode using quadrature detection. The samples were dissolved in 600 μl [$^2H_6$]DMSO containing 2% ^2H_2O. Chemical shifts were measured in ppm by reference to internal TMS (0 ppm, 338 K). The chemical shift values were rounded to 0.01 ppm because of their strong temperature dependence.

Results

Solubilization of UDP-Gal:LacCer galactosyltransferase activity

UDP-Gal:LacCer galactosyltransferase activity was solubilized from crude placenta homogenate by the procedure of Joziasse *et al.* [6]. Triton CF-54 was used instead of Triton X-100. The specific activity of the extract was increased twofold compared with the starting material, with a recovery of 18% of total activity. Unless otherwise specified, assays were conducted with the Triton CF-54 extract.

Effect of pH and detergent on enzyme activity

The pH dependence of the UDP-Gal:LacCer galactosyltransferase was examined with sodium cacodylate buffer. As shown in Fig. 1, the enzyme was active in the whole pH range studied, and maximal activity was obtained at pH 6.3.

The ability of various detergents to stimulate enzyme activity was tested using crude homogenate as the source of enzyme. The maximal activity was found in the presence of Triton CF-54, and the optimal concentration was about 0.1% (w/v) (Fig. 2). Other detergents, Triton X-100, Nonidet P-40, CHAPS, and sodium cholate, also stimulated the enzyme activity but less effectively (data not shown). These experiments were performed at a concentration of 0.1% (w/v).

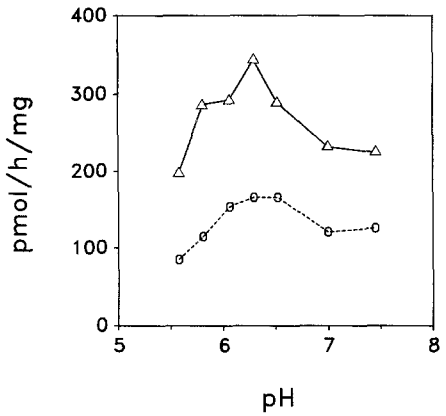

Figure 1. Effects of varying pH on the UDP-Gal:LacCer galactosyltransferase activity: △, Triton CF-54 extract; ○, crude homogenate.

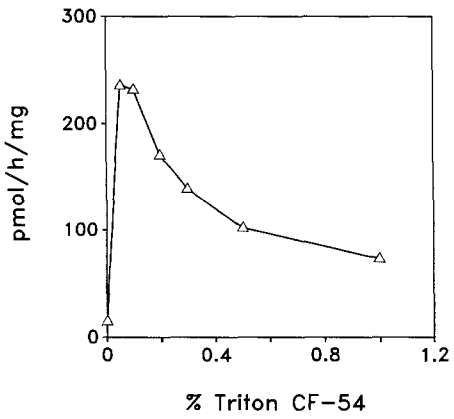

Figure 2. Effect of Triton CF-54 concentration on the UDP-Gal:LacCer galactosyltransferase activity. In this experiment, the homogenate was used as a source of the enzyme.

Requirement for additives for optical activity

The effect of additives on the transfer of galactose to lactosylceramide is shown in Table 1. Mn^{2+} was essential for maximal activity. Of the other divalent cations tested, only Co^{2+} stimulated enzyme activity to some extent (data not shown). Endogenous acceptor activity in the absence of LacCer was less than 5%. In contrast, removal of CDP–choline or galactonolactone from the reaction mixture did not result in a marked loss of activity. The rate of reaction in optimal conditions as a function of time is shown in Fig. 3.

Table 1. Reaction requirements for UDP-Gal: LacCer galactosyltransferase activity.

Condition	*% Complete activity*
Complete	100
−LacCer	3
$-MnCl_2$	6
−Galactonolactone	97
−CDP–choline	94

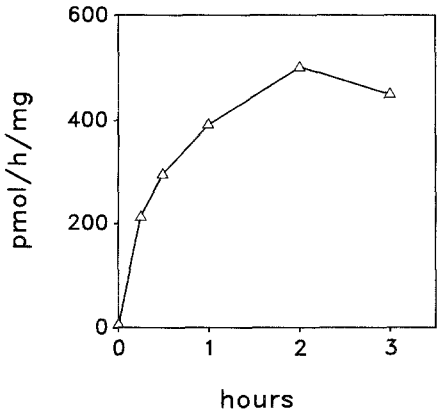

Figure 3. Effect of incubation time on the UDP-Gal:LacCer galactosyltransferase activity.

Characterization of the reaction product

The reaction product was purified by thin-layer chromatography and treated with galactosidases. As expected, the linkage of radiolabelled galactose was susceptible to α-galactosidase; however, the β-galactosidase preparation was able to liberate some of the terminal sugar residue (Table 2). Therefore we tested the specificity of the enzyme preparations by incubating [^{3}H]globotriaosylceramide (38 000 counts min^{-1}) under the same conditions. Of this glycolipid, 94% was hydrolysed with α-galactosidase and 16% with β-galactosidase in 20 h.

Isolation and characterization of globotriaosylceramide from human placenta

To estimate the amount of globotriaosylceramide in human placenta, carefully rinsed tissue was used as a starting material for glycolipid isolation. Figure 4 shows a chromatogram of glycolipid fractions from the placenta and human erythrocytes after Florisil chromatography. The lipids are from the lower phase of Folch partition; the major band below globoside in lane A comigrates with GM_3.

Glycolipids of the placenta were separated on a Bio-Sil A column, and purified globotriaosylceramide (about 1 mg) was characterized by fatty acid analysis and by NMR

Table 2. Susceptibility of the reaction product to α- and β-galactosidases.

Enzyme	*Incubation time (h)*	*Degradation of the product (%)*
α-Galactosidase	0.5	29
	3	73
	20	92
β-Galactosidase	0.5	2
	3	9
	20	18

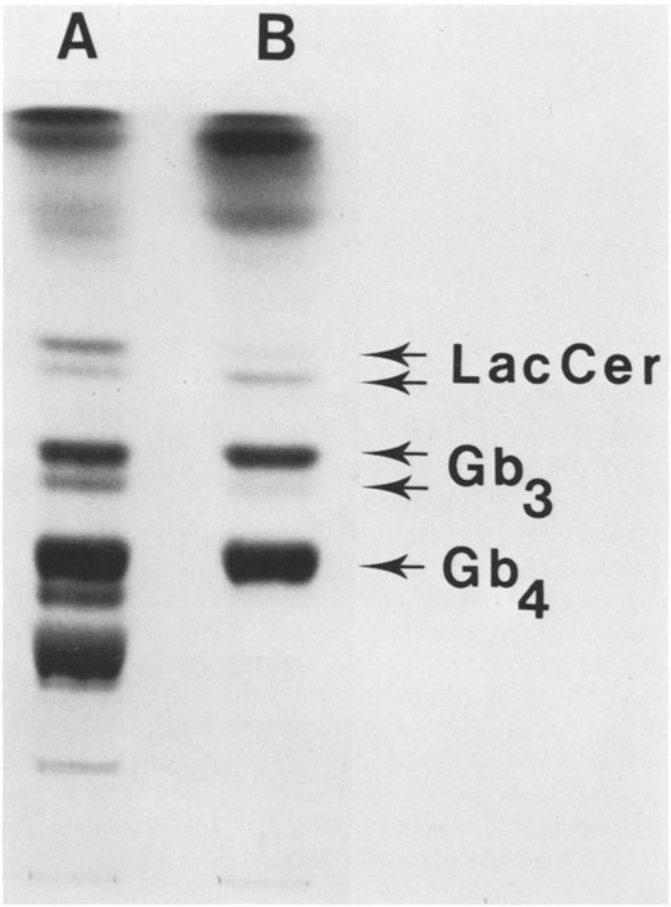

Figure 4. Thin-layer chromatogram of the glycolipids from human erythrocytes and placenta. A, Glycolipid fraction from the placenta; the amount of lipid corresponds to 0.13 g of tissue. B, Glycolipid fraction from erythrocytes; the amount of lipid corresponds to 0.6 ml whole blood. The plate was stained with the orcinol/sulfuric acid reagent. Positions of major neutral glycolipids are indicated: Gb_3, globotriaosylceramide; Gb_4, globoside.

spectroscopy. Long chain C24:0 and C24:1 fatty acids were not as dominating in placental globotriaosylceramide (44% of total fatty acids) as in red-cell globotriaosylceramide (77% of total fatty acids). Shorter chain fatty acids were enriched in the lower band of the Gb_3 doublet, but no α-hydroxy fatty acids were detected. The carbohydrate H-1 chemical shifts of globotriaosylceramides from the placenta and erythrocytes were identical (Table 3) and comparable with those obtained by Dabrowski *et al.* for globotriaosylceramide from human plasma [15]. The intensities of carbohydrate H-1 signals of the placental glycolipid were similar (Fig. 5), indicating that Galα1-4Galβ1-4Glcβ1-1Cer was the only carbohydrate structure in the sample.

Discussion

Human placenta has been shown by GC/MS studies to contain P blood group active glycolipids and acidic

Table 3. Carbohydrate H-1 chemical shifts (ppm from TMS) of globotriaosylceramides of human placenta (HP) and erythrocytes (E).

	Galα1-4	*Galβ1-4*	*Glcβ1-1Cer*
HP	4.81	4.27	4.17
E	4.81	4.27	4.17

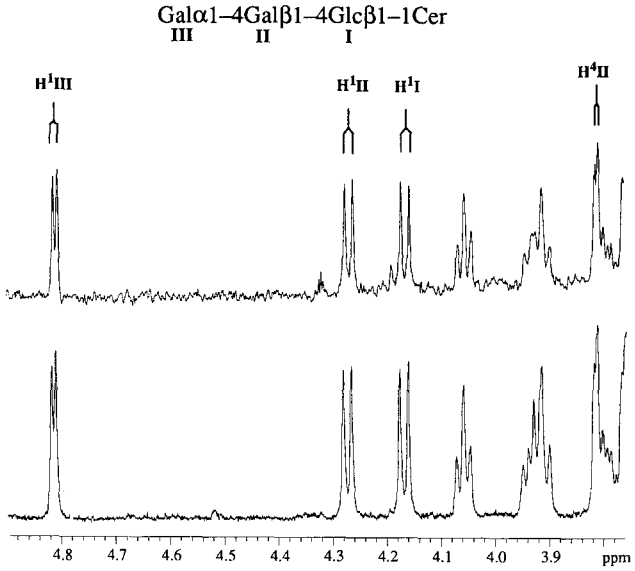

Figure 5. The anomeric regions of ^{1}H-NMR spectra of globotriaosylceramides from human placenta (upper) and erythrocytes (lower).

glycolipids of the lactoseries [16–18]. No glycosphingolipids belonging to ganglioseries were detected. By using a monoclonal anti-P antibody, globoside was localized in endothelial cells, trophoblast, and interstitial cells in mature human placenta [19]. Our results show the P^k antigen is synthesized by placental cells, and only a small portion of the glycolipid may originate from erythrocytes.

Bailly *et al.* [20] have studied biosynthesis of the blood group P^k and P_1 antigens by human kidney microsomes. The microsome preparations could transfer the D-galactopyranosyl group α(1-4) and β(1-3) to lactosylceramide, and the reaction products were formed in the proportions of 30% and 70%, respectively. According to our galactosidase digestion experiments, there is very little or no β-galactosyltransferase activity in human placenta. Moreover, NMR spectra of placental and red-cell globotriaosylceramides were identical. The natural acceptor for the β(1-3)-galactosyltransferase is obviously some other glycoconjugate than lactosylceramide. A good candidate is paragloboside [20], and a pentaglycosylceramide with the structure Galβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc1-1Cer has been isolated previously from human erythrocytes [21].

Isolation of membrane bound glycosyltransferases is

hampered by the small amount of these enzymes in tissues and by the lability of detergent-solubilized enzyme preparations. Only a few glycosyltransferases acting on neutral glycolipids have so far been purified [22–25]. The placenta is the only human tissue which can be obtained easily in large amounts, and therefore it might be a suitable starting material for isolation of various glycolipid glycosyltransferases. Iizuka *et al.* have detected normal *in vitro* UDP-Gal: LacCer galactosyltransferase activity in lymphoblastoid cells of the p type, despite the lack of Gb_3 [26]. This finding indicates that the defect in biosynthesis of the P^k antigen may be a more complex phenomenon than a mere mutation in the α-galactosyltransferase gene. To learn more about the factors affecting P^k antigen expression, the enzyme should be purified and characterized at the molecular level.

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