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IDENTIFICATION OF UDP-GALACTOSE : LACTOSE (LACTOSYLCERAMIDE) α-4 AND β-3 GALACTOSYLTRANSFERASES IN HUMAN KIDNEY

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SUMMARY: Two galactosyltransferases were identified in human kidney microsomes which both transfer galactose from UDP Gal to lactose as well as to lactosylceramide. Using a solubilized and a partially purified enzyme preparation sufficient product could be obtained for detailed structural analysis. The trisaccharide products were isolated by gel permeation chromatography and separated by preparative high performance thin layer chromatography. The anomeric configuration of the transferred galactose was determined by specific glycosidase digestion and the linkage was identified by methylation and gas-liquid-chromatography. The glycolipid products were not separated but analyzed directly, before and after α or β galactosidase digestion, by methylation, hydrolysis and thin layer chromatography. Into both acceptor substrates galactose was incorporated in α1-4 (30%) and β 1-3 (70%) linkages. The α 1-4 galactosyltransferase is responsible for the synthesis of the P^k antigen Galα1-4Galβ1-4Glc-ceramide in human kidney. The β 1-3 galactosyltransferase has not previously been identified .

INTRODUCTION: Galactose residues are the immunodominant structures of several human blood group antigens like B, T, P^k and P_1 (1). The biosynthesis of the B and T antigens has been studied in detail. The P^k (Galα1-4Galβ1-4Glc-Cer) and the P_1 (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) antigens are glycolipids which carry both the Galα1-4Gal determinant (2). From studies on individuals which lack either one or both of the antigens two biosynthetic models have been proposed: (A) two different α 1-4 galactosyltransferases are responsible for the synthesis of the two antigens or (B) an unidentified

Abbreviations: LacCer, lactosylceramide, Galβ1-4Glc-ceramide; GbOse3Cer, globotriaosylceramide, Galα1-4Galβ1-4Glc-ceramide (P^k antigen); PMSF, phenylmethylsulfonylfluoride; HPLC, high performance liquid chromatography; HPTLC, high performance thin layer chromatography; GLC, gas liquid chromatography; BSA, bovine serum albumin.

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regulatory protein modifies the acceptor substrate specificity of one single enzyme (1,2).

Human kidney is known to contain both the P^k (3) as well as the P_1 antigen (4) and therefore is one of the possible sources for studying this question. In this paper we present the characterization by specific glycosidase digestion and methylation analysis of the products obtained by incubating enzyme preparations from human kidney with lactose and lactosylceramide as acceptor substrates and UDP Gal as the sugar donor.

MATERIAL AND METHODS: UDP [^{14}C] Gal (327 mCi/mmol) and [^{3}H] $NaBH_4$ (5 Ci/mmol) were purchased from New England Nuclear, Boston, MA. UDP-Gal, CDP-chloline, Gal, Glc, lactosylceramide, phosphatidylglycerol, galactonolactone, BSA, Triton X-100, β-galactosidase from jack beans, α-galactosidase from coffee beans, and galactose oxidase from Dactylium dendroides were obtained from Sigma chemical Co., St. Louis, MO. Lactose was from Koch-Light, U.K. Galα1-4Galβ1-4Glc was synthesized and donated by Prof. P. Sinay and Dr. J.C. Jacquinet (Orléans, France). Fresh human kidneys from unmatched organ donors were obtained through the courtesy of Dr. D. Droz (Hôpital Necker, Paris) and stored at -80°C until used.

Enzyme preparation.

All steps were carried out at 4°C. Microsomes from frozen human kidneys were prepared as described (5) in 15mM Tris-HCl pH 8.0, containing 5mM EDTA, 0.5mM PMSF and 20 % (w/v) glycerol (buffer A). The enzymes were extracted from the microsome suspension (5mg protein/ml) by constant stirring for 2 h at 4°C in buffer A containing 0.25 % (w/v) Triton X-100 (buffer B). After centrifugation (70,000 x g, 1 h), the clear supernatant was loaded on a DEAE-cellulose column (0.5 x 5 cm) equilibrated in buffer B and the column was rinsed until the eluate was essentially free of protein. Contaminating proteins were eluted in the absence of detergent with 10 ml of 0.5 M NaCl in buffer A whereas the galactosyltransferases eluted only when 0.25% Triton was included in the elution buffer. The active fractions were pooled, dialyzed for 24 h against 25mM sodium cacodylate buffer pH 6.8 containing 25 % (w/v) glycerol, and the concentration of $MnCl_2$ was ajusted to 25 mM. This preparation was used as source of galactosyltransferases without further purification. Protein concentrations were determined by the method of Lowry et al (6).

Galactosyltransferase assay systems.

Method 1 : The incubation mixture contained in a total volume of 25 µl : lactose, 5 µmol ; sodium cacodylate (pH 6.8), 1.25 µmol ; Mn^{2+}, 0.25 µmol ; galactonolactone, 0.5 µmol; CDP-choline, 0.5 µmol ; α-lactalbumin, 250 µg ; UDP [^{14}C] Gal, 5 nmol (specific activity, 2 x 10^4 cpm/nmol) ; and 5-100 µg of microsomal protein. After incubation at 37°C for 60 min, the samples were processed and counted as described (7).

Method 2 : The reaction mixture was as in method 1, except that LacCer (100 nmole) was used as acceptor in the presence of 0.1 mg of phosphatidylglycerol and the specific activity of UDP [^{14}C] Gal was increased to 3 x 10^5 cpm/nmol. After 3 h at 37°C, the reaction was stopped with 10 vol. $CHCL_3/CH_3OH$ (2 : 1 v/v) and the whole mixture was applied to Whatman 3 MM paper and chromatographed in water overnight. The glycolipids remain at the origin and were extracted from the dried paper with 3 x 5 ml $CHCl_3/CH_3OH/H_2O$ (20 : 10 : 2, by vol.), dried and rechromatographed on HPTLC plates in $CHCl_3/CH_3OH/H_2O$ (60: 40: 9, by vol; solvent A). The labelled glycolipids were localized by autoradiography (Fuji X-ray film, Japan), scraped from the plates and quantified by scintillation counting. For methylation

analysis the radioactive products were eluted from the silica gel as above and concentrated.

Large scale preparation of reaction products.

A reaction mixture (assay method 1) was scaled up to 7.5 ml (2.25 mg protein corresponding to a preparation from 20 g of wet tissue) with a specific activity of UDP [^{14}C] Gal of 10^5 cpm/nmol. After 8 hours at 37°C the reaction was stopped by addition of an equal volume of ethanol and the supernatant was applied successively to Dowex AG 1X8 (formate form) and Dowex AG 50X8 (H^+) columns (2x6 cm) equilibrated in water. The flow through was concentrated and fractionated on a Sephadex G 25 (superfine) column (1.5x120 cm) also equilibrated in water. The peak of labeled oligosaccharides (Ve/Vo =0.44) still contained large amounts of lactose, as revealed by HPTLC analysis in ethanol/water/n-butanol/pyridine/acetic acid (100 : 30 : 10 : 10 : 3, by vol ; solvent B), and was rechromatographed on Sephadex G25 as above.

The two trisaccharides present in this fraction were separated by preparative HPTLC in solvent A. Before methylation analysis the purity of the isolated compounds was checked by HPLC on a lichrosorb-NH_2 column (4 x 250 mm ; 5 µm ; Merck, Darmstadt, W. Germany) in 70 % acetonitrile at a flow rate of 2ml/min. The effluents were monitored by a differential refractometer (Spectra Physics 6040) and by an UV detector at 190 nm (Spectra Physiscs 8773R).

Treatment with galactosidases.

The purified trisaccharides (2 nmol) were digested overnight at 37°C with one unit of α-galactosidase from coffee beans in 50mM sodium citrate buffer pH 4.5, 1 % (w/v) BSA, 2mM EDTA and one unit of β-galactosidase from jack beans in 50mM phosphate citrate buffer pH 5.4, 1 % (w/v) BSA (total volume 25 µl). When the glycolipid products were digested, 125 µg sodium taurodeoxycholate was added to the incubation medium.

Methylation analysis

The purified and reduced trisaccharide products were methylated and analyzed according to the published procedures (8, 9). The glycolipid products were analyzed following the strategy developped by Stoffyn et al (10).[6- ^{3}H] -Gal labeled LacCer was used as acceptor substrate and UDP [^{14}C] - Gal as donor substrate in a reaction mixture described for assay method 2. The labeled products were isolated and aliquots were treated with α-or β-galactosidase. Treated and untreated products were methylated (8), hydrolysed and analyzed by HPTLC in acetone/5M NH_4OH (50 : 0.9, by vol; solvent C).

RESULTS: Microsomal preparations from human kidney were found to contain enzymatic activity capable to transfer Gal from UDP Gal to lactose and lactosylceramide. The transfer rate was dependent on the incubation time, the protein concentration and the amount of donor or acceptor substrate added. The enzymatic activities measured under standard conditions were 2.4 nmol/h/mg protein for lactose and 2.7 nmol/h/mg for LacCer. Among the divalent metal ions tested only Mn^{2+} was an effective cofactor. Maximal transfer rate was obtained at 25 mM and EDTA completely inhibited the reaction. Preliminary attempts to isolate the reaction products as well as glycosidase digestion experiments indicated that with both acceptors, lactose and LacCer, two products had been formed in a ratio of about 2 to 1. The specific activity and the ratio of the two products formed did not vary significantly among the five kidney samples analyzed. However, due to the low enzyme activities and the

instability of the enzymes during incubation the amount of products obtained was insufficient for structural studies.

Partial purification of galactosyltransferases : In order to obtain a more active and more stable enzyme preparation attempts were made to solubilize and purify the galactosyltransferases. In spite of protease inhibitors, PMSF and EDTA and 20% glycerol as a stabilizing agent, the solubilized membrane preparations rapidly lost their activity regardless of the detergent used. Triton X-100 at a detergent to protein ratio of 2 : 1 proved most useful.

The rapid binding to DEAE-Cellulose followed by extensive washing with low and high ionic strength buffers and the elution with 0.5 M NaCl in 0.25% Triton gave an enzyme preparation which was purified about 20 fold with a yield of 50% and was stable at 4°C for several weeks in the presence of 20% glycerol. In the enzyme assay mixture the incorporation of Gal into lactose or LacCer was linear for more than 5 h compared to about 1 h for the crude microsome preparation.

Identification of the products formed with lactose as acceptor :

Using the partially purified and concentrated enzyme preparation and lactose as acceptor about 500 nmol of products were synthesized corresponding to 35% of the added UDPGal. The reaction products were isolated by gel filtration and separated by preparative thin layer chromatography in solvent B (Fig 1,A). Digestion with specific galactosidases (Fig. 1, B, C) revealed that the faster moving band contained a β linked terminal galactose whereas the transferred galactose in the slower moving spot was released only with a α galactosidase. The slower moving band showed the same Rf value as the chemically synthesized P^k trisaccharide (Galα1-4Galβ1-4Glc).

Before methylation the purity of the two trisaccharides was checked by HPLC on a Lichrosorb-NH_2 column. Both preprations proved homogeneous with only a slight (<5%) contamination by lactose in the upper band. Again, the slower migrating product (HPTLC) eluted from the column with the same retention time (33 min) as the authentic P^k trisaccharide. The linkages of the transferred galactose residues were determined by methylation analysis. The GLC pattern

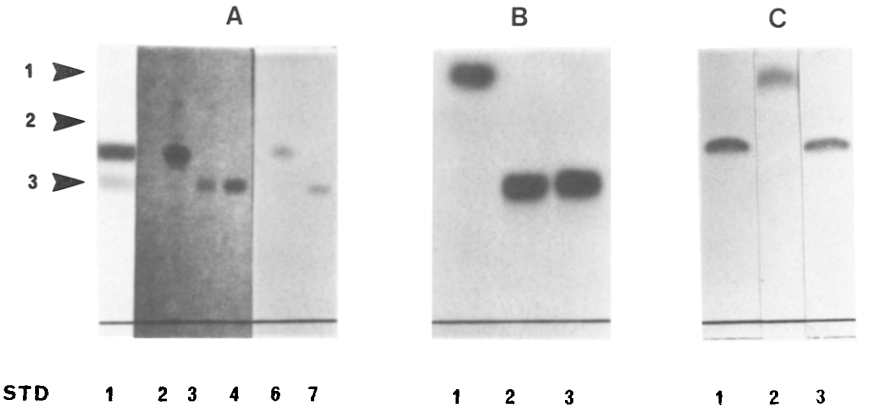

Fig. 1. Thin-layer chromatography of biosynthesized trisaccharides (Solvent B).
A) : Lanes 1, 6 and 7 : Autoradiographies of mixture and separated trisaccharide products. Lanes 2 and 3 : Orcinol staining of separated products and lane 4 the authentic Galα1-4 Galβ1-4Glc trisaccharide . B) and C) : Galactosidase digestion of slow (B) and fast (C) migrating bands (Autoradiographies). Lanes : 1, α-galactosidase ; 2, β-galactosidase ; 3, no-enzyme.
The arrows on the left indicate the position of 1, Gal ; 2, lactose ; and 3, Galα1-4Galβ1-4Glc.The origin is indicated by the straight line.

of the methylated and acetylated sugar derivatives (Fig. 2) indicates the presence of a 1, 2, 4, 6-tetra-O Me 3-0 Ac Gal for the band migrating faster in the HPTLC system. Since labeled Gal is released from this compound only by the β galactosidase, Galβ1-3Galβ1-4Glc may be proposed as the structure of

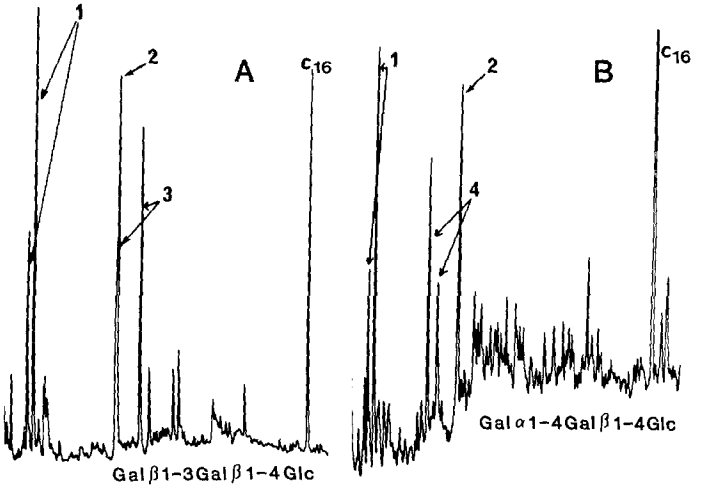

Fig. 2. Identification of methylated sugar derivatives by GLC on OV-110 (9).
A) Fast and B) Slow migrating trisaccharide. 1, Per-Me-Gal ; 2, 1,2,3,5,6, penta O-Me 4,O-Ac-Glc^{itol} ; 3, 1,2,4,6, tetra O-Me 3,O-Ac-Gal ; 4, 1,2,3,6, tetra O-Me 4,O-Ac-Gal.

the upper band. In the slower moving substance 1, 2, 3, 6-tetra-O Me, 4-O-Ac-Gal was found (Fig. 2,B) which together with the results from the galactosidase digestion suggests the structure Galα1-4Gal β 1-4Glc for the lower band. This finding is consistent with the comigration of the lower band with the authentic P^k trisaccharide in the HPTLC system as well as in the HPLC analysis.

Identification of the glycolipid reaction products : When LacCer was incubated with UDP Gal and the purified enzyme preparation a product was formed which migrated as a single band on HPTLC in solvent A with a R_f identical to $GbOse_3Cer$. However, digestion with both α-and β-galactosidases released labeled galactose indicating the presence of two products comigrating in this solvent. For the structure determination, [6-^{3}H] labeled LacCer was used as acceptor and the mixture of the two reaction products was separated from unreacted [6-^{3}H] LacCer. Aliquots of the products were treated with α-or β-galactosidases, the glycolipids extracted, methylated and hydrolysed. The methylated radioactive galactose derivatives were separated by HPTLC in solvent C and visualized by autoradiography (Fig. 3). The untreated reaction products reveal two spots not present in the acceptor which migrate as 2,3,6 -

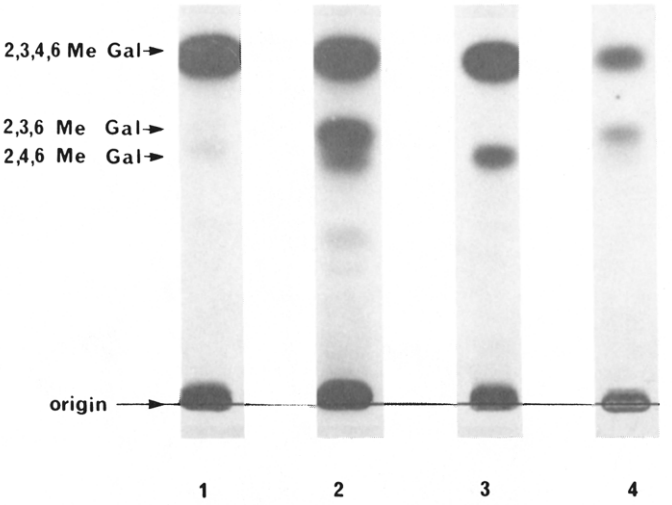

Fig. 3. Methylation analysis of double labeled glycolipids (9,10).
The reaction products were methylated, hydrolysed and chromatographed in solvent C. Labeled galactose derivatives are revealed by autoradiography. Lanes : 1, acceptor [6-^{3}H]Gal labeled LacCer) ; 2, products ; 3, products treated with α-galactosidase ; 4, treated with β-galactosidase. Arrows indicate reference compounds.

tri-OMe and 2,4,6-tri-OMeGal (Fig. 3, lanes 1 and 2) indicating that two products had been formed. In one product the [6-^{3}H] labeled galactose from the acceptor substrate had been substituted in position 4 and in the second product the galactose had been substituted in position 3. The 2,3,6 tri-O-MeGal is absent from the product mixture which had been treated with α galactosidase before methylation whereas the 2,4,6-tri-OMeGal can not be detected after β galactosidase treatment. Together, these results show that again galactose had been transferred in α 1-4 as well as β 1-3 linkage to LacCer. With both acceptors, lactose and LacCer, in about 70% of the product formed the Gal was linked in β 1-3 and in 30% in α 1-4. This ratio remained constant during purification and storage of the solubilized and enriched enzyme preparation.

DISCUSSION: Human kidney microsomes were found to contain two galactosyltransferases which both use lactose or lactosylceramide as acceptor substrates. One enzyme was found to be the α 1-4 galactosyltransferase involved in the synthesis of the P^k and P antigenic structures. The second enzyme is a β1-3 galactosyltransferase which might be involved in the biosynthesis of the core structure of the mucoseries glycolipids. Using specific galactosidase digestion and methylation analysis the anomeric and positional linkages have been determined unambiguously. The ratio of the two products formed as well as the specific enzymatic activity in the standard assays was the same for oligosaccharide and glycolipid acceptors as well as for all kidney samples tested.

The activity of the α 1-4 galactosyltransferase was found to be much higher in human kidney than in other tissues or cells tested (11). This high α 1-4 galactosyltransferase activity makes human kidney an excellent tissue to study the biosynthesis of the P^k and P_1 antigens. However, the presence of the β 1-3 galactosyltransferase which uses the same acceptor substrates reduces the usefulness of human kidney for this type of studies. Therefore work is in progress to develop an assay system which allows the rapid separation of the two reaction products and to develop a specific test for each of the two galactosyltransferases.

The β 1-3 galactosyltransferase described here has not previously been identified. Although Galβ1-3Galβ1-4Glc(NAc) structures have been reported on glycoproteins (12-15) and glycolipids (16) from several tissues they have not yet been found in human kidney. However, patients suffering from the rare neurological disease, myoclonic epileptic encephalopathy, secrete into their urine important amounts of the oligosaccharide Galβ1-3Galβ1-4Glc (17). A lactose free diet completely abolished the secretion of this trisaccharide (J.C. Michalski, personal communication) indicating that in these patients a hyperactive β 1-3 galactosyltransferase is responsible for the synthesis of the unusual structures. Further studies are necessary to elucidate the role of the β1-3 galactosyltransferase in this disease as well as the presence and the role of Galβ1-3Galβ1-4Glc(NAc) structures in human kidney and other tissues.

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