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Biosynthesis of the blood group P^k and P_1 antigens by human kidney microsomes*,†

Pascal Bailly[a], Friedrich Piller[a], Baiba Gillard[b], Alain Veyrières[c], Donald Marcus[b], and Jean-Pierre Cartron[a,‡]

[a] *Laboratoire de Biochimie Génétique, Unité INSERM U76, Institut National de Transfusion Sanguine, 6 rue Alexandre Cabanel, F-75739 Paris (France)*

[b] *Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030 (U.S.A.)*

[c] *Laboratoire de Chimie, École Normale Supérieure, F-75005 Paris (France)*

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ABSTRACT

On human erythrocytes, the membrane components associated with P^k and P_1 blood-group specificity are glycosphingolipids that carry a common terminal α-D-Gal*p*-(1→4)-β-D-Gal unit, the biosynthesis of which is poorly understood. Human kidneys typed for P_1 and P_2 (non-P_1) blood-group specificity have been assayed for (1→4)-α-D-galactosyltransferase activity by use of lactosylceramide [β-D-Gal*p*-(1→4)-β-D-Glc*p*-ceramide] and paragloboside [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-ceramide] as acceptor substrates. The linkage and anomeric configuration of the galactosyl group transferred into the reaction products were established by methylation analysis before and after α- and β-D-galactosidase treatments, as well as by immunostaining using specific monoclonal antibodies directed against the P^k and P_1 antigens. The results demonstrated that the microsomal proteins from P_1 kidneys catalyze the synthesis of P^k [α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-ceramide] and P_1 [α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-ceramide] glycolipids, whereas microsomes from P_2 kidney catalyze the synthesis of the P^k glycolipid, but not of the P_1 glycolipid. Competition studies using a mixture of two oligosaccharides (methyl β-lactoside and methyl β-lacto-*N*-neotetraoside) or of two glycolipids (lactosylceramide and paragloboside) as acceptors indicated that these substrates do not compete for the same enzyme in the microsomal preparation from P_1 kidneys. The results suggested that the P^k and P_1 glycolipids are synthesized by two distinct enzymes.

INTRODUCTION

The human blood-group P systems consist of five phenotypes, P_1, P_2, P^k_1, P^k_2, and p, depending on the presence or absence of three antigens, P^k, P, and P_1, on erythrocytes[1]. These antigens have been described as glycosphingolipids[2,3] that are considered to be derived from a common precursor, lactosylceramide, by stepwise glycosylation[4]. The P antigen, globoside β-D-Gal*p*NAc-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-ceramide, is expressed on all normal red cells. The P^k antigen, α-D-Gal*p*-(1→4)-β-

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‡ To whom correspondence should be addressed.

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D-Gal*p*-(1→4)-*β*-D-Glc*p*-ceramide, is the precursor of the P antigen, and is expressed weakly on normal red cells and abundantly on P^k_1 and P^k_2 erythrocytes. These two rare phenotypes are characterized by the absence of the P antigen, like red cells of the p phenotype, which in addition lack the P^k and P_1 antigens. The P_1 antigen, α-D-Gal*p*-(1→4)-*β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*NAc-(1→3)-*β*-D-Gal*p*-(1→4)-*β*-D-Glc*p*-ceramide, is expressed on erythrocytes of 75% of Caucasoïdes. The P^k and the P_1 antigens are neutral glycosphingolipids that consist of a D-galactosyl group α-(1→4)-linked to lactosylceramide and paragloboside, respectively, and therefore share the same terminal disaccharide unit, α-D-Gal*p*-(1→4)-*β*-D-Gal.

The exact mechanism of the biosynthesis of the P_1 and the P^k antigen has not yet been elucidated. The principal biochemical and genetic questions to be answered concern the nature of the products of the P^k and the P_1 genes, *i.e.*, the (1→4)-α-D-galactosyltransferases that add the terminal immunodominant sugar unit to the P^k and the P_1 determinants. From studies on individuals lacking either one or both of these two antigens, three hypotheses have been proposed to explain the biochemistry and genetics of these antigens. The first two propose that the P^k and P_1 genes are not alleles[5]. Accordingly, two different (1→4)-α-D-galactosyltransferases should be involved in the synthesis of the two antigens, or an unidentified regulatory protein modifies the acceptor substrate specificity of one single enzyme which, subsequently, can act on both lactosylceramide and paragloboside. The third hypothesis proposes that the P^k and the P_1 genes are alleles[6,7]: the P^k-transferase should then catalyze only the synthesis of globotriaosylceramide (P^k antigen) from lactosylceramide, and the P_1-transferase should catalyze this reaction as well as the synthesis of the P_1 antigen from paragloboside. Since human kidneys contain relatively large amounts of α-D-galactosyltransferase activity[8], microsomal preparations from this tissue typed in the P/P_1 blood-group systems were used to adress this question. We report, herein, the identification in P_1 tissue extracts of (1→4)-α-D-galactosyltransferase involved in the biosynthesis of the P_1 antigen, and that of another enzyme, present in P_2 tissue extracts, which synthetizes the P^k antigen.

EXPERIMENTAL

Materials. — UDP-D-[^{14}C]galactose (12.3 GBq/mmol) and [^{3}H]$NaBH_4$ (185 GBq/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). ^{125}I-Labeled sheep anti-mouse or anti-rat Igs (740 kBq/μg and 257 kBq/μg, respectively) were obtained from the Radiochemical Centre (Amersham, U.K.). UDP-D-galactose, CDP-choline, α-lactalbumin, bovine serum albumin, phosphatidylglycerol, Triton X-100, D-galactose oxidase from *Dactylium dendroides*, *β*-D-galactosidase from jack beans (EC 3.2.1.23), and α-D-galactosidase from coffee beans (EC 3.2.1.22) were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). UDP-hexanolamine was synthesized according to the published procedure[9] and was a generous gift of Dr. C. Augé (Université Paris Sud, Orsay, France). LacCer acid–Sepharose[10] and CNBr-activated Sepharose[11] were prepared as described. Con A–Sepharose was from Pharma-

cia (Uppsala, Sweden). β-D-Gal*p*-(1→4)-β-D-Glc*p*OMe (methyl β-lactoside, LacOMe) and β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*OMe (methyl β-lacto-*N*-neotetraoside, LNnTOMe) were synthesized as described previously[12]. β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-ceramide (lactosylceramide, LacCer) (upper band 90%) and α-NeuAc-(2→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-ceramide (sialosylparagloboside) were purified from human erythrocytes. Paragloboside (PG) was obtained from desialylation of sialosylparagloboside by treatment with M formic acid for 60 min at 100°. [6-^{3}H]PG and[6-^{3}H]LacCer were prepared by oxidation with D-galactose oxidase, followed by reduction with tritiated (^{3}H)-$NaBH_4$[13]. Methyl 2,3,4,6-tetra-*O*-methyl-D-galactoside was obtained from per-*O*-methylLacCer as described[14]; methyl 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactoside were obtained similarly from per-*O*-methylgloboside.

Fresh human kidneys from unmatched organ donors were obtained by the courtesy of Dr. D. Drôz (Hôpital Necker, Paris) and from France-Transplant (Hôpital St. Louis, Paris). Microsomes from frozen human kidneys were prepared according to Yusuf *et al.*[15] as described[8]. The 38-13 monoclonal antibody (anti-P^k blood-group typing reagent) and the CLB-ery-2 monoclonal antibody (anti-P blood-group typing reagent) were generous gifts of Dr. M. Fellous (Institut Pasteur, Paris, France) and Dr. von dem Borne (University of Amsterdam, The Netherlands), respectively. The monoclonal antibody designed 154-IX-B6 used as an anti-P_1 blood-group typing reagent was from Dr. G. Vezon (Bordeaux, France). Red cells from individuals typed in the P/P_1 blood-group systems were from the Institut National de Transfusion Sanguine (Paris).

P and P_1 typing of human kidneys. — The human tissue was cut into pieces and homogenized in 15mM Tris·HCl, pH 8 (5 vols.) containing 5mM EDTA, 0.5mM phenylmethylsulfonyl fluoride (PMSF), and 20% (w/v) of glycerol. After centrifugation at 4° for 10 min at 10000*g*, the membrane pellet was resuspended and washed three times in 10mM phosphate buffer (pH 7.2)–150mM NaCl. The membranes (1 vol.) were centrifuged off (10000*g*, 10 min) and the pellet was incubated for 2 h at 4° with a serial dilution (1 vol. each) of monoclonal antibodies ranging from 80 to 640 ng/mL and 0.7 to 5.6 ng/mL for CLB-ery-2 and 154-IX-B6, respectively. After 2 h at 4°, the mixtures were centrifuged and the supernatants were tested by agglutination assays in microtiter plates at 4° against papain-treated P_1 red cells as described earlier[16]. Control assays without membranes were simultaneously analyzed.

Affinity chromatography. — Kidney microsomes were solubilized in 25mM sodium cacodylate, pH 6.8, containing 0.25% (w/v) Triton X-100 and applied to UDP-hexanolamine–Sepharose (14 μmol/mL) or LacCer acid–Sepharose (10 μmol/mL) at a flow rate of 3 mL/h at 4°. In some experiments, 25mM $MnCl_2$ or 100mM lactose were included in the loading buffer for the UDP-hexanolamine–Sepharose. Elution was attempted for both columns with 0 to 1M salt gradients or with pulses of mM UDP-D-Gal or 100mM lactose, respectively. The eluted fractions were desalted before the transferase assays.

Assay of D-galactosyltransferase activities and analysis of reaction products. — For oligosaccharide acceptors, the reaction mixture consisted of sodium cacodylate (pH

6.8, 1.25 μmol), Mn^{2+} (0.25 μmol), D-galactono-1,4-lactone (0.5 μmol), CDP-choline (0.5 μmol), α-lactalbumin (250 μg), UDP-D-[^{14}C]galactose (5 nmol; specific activity, 2 × 10^4 c.p.m./nmol), microsomal protein (100–150 μg), and various amounts of sugar substrates in a total volume of 25 μL. After 1 h at 37°, the reaction was stopped and the mixture applied to Dowex AG 1 X8 (HCO_2^-) anion-exchange resin. The flow-through was dried and analyzed by descending paper chromatography (Whatman 3MM) for 16 h in 5:5:1:3 (v/v) ethyl acetate–pyridine–acetic acid–water, and the radioactive spots were detected with a Packard Radiochromatogram Scanner (model 7201). Each peak was eluted from the paper with water, purified by gel filtration on Bio-Gel P-4, and treated with β-D-galactosidase from jack beans (0.3 unit) to remove β-D-galactosyl groups as previously described[8]. Released D-galactose was separated by descending paper chromatography as described above, and the reaction products were quantitatively determined by liquid-scintillation counting. For glycolipid acceptors (lactosylceramide and paragloboside), the incubation mixture was the same except that the glycolipid (100 nmol) was used as substrate in the presence of phosphatidylglycerol (0.1 mg) and 0.3% (w/v; final concentration) Triton X-100. The radioactively labeled glycolipid products were isolated by high-performance t.l.c (h.p.t.l.c.) as previously described[8], and incubated with or without α- or β-D-galactosidase prior to methylation[17] and hydrolysis for structure determination according to Stoffyn *et al.*[14].

The reaction products were immunologically characterized by immunostaining[18]. Since phosphatidylglycerol migrates in the same zone as the P_1 glycolipid, it was first removed by h.p.t.l.c. (Merck, Kieselgel 60) in 60:35:8 (v/v) chloroform–methanol–water (solvent *A*). The glycolipids were then separated by h.p.t.l.c. in solvent *A*, fixed, and stained with the monoclonal antibodies against P_1 glycolipid or globotriaosylceramide (P^k antigen). The plates were finally incubated with ^{125}I-labeled sheep anti-mouse Ig or ^{125}I-labeled sheep anti-rat Ig (10^6 c.p.m./mL), washed, and dried. Radioactive bands were visualized by autoradiography.

RESULTS AND DISCUSSION

Human kidney samples have been typed in the P/P_1 blood group systems with two IgM monoclonal antibodies, CLB-ery-2 and 154-IX-B6, of well defined specificity[19,20]. The CLB-ery-2 antibody is directed against the P blood group antigen (globoside) and agglutinates strongly P_1 and P_2 red cells but not P^k or p erythrocytes[19]. Absorption of this antibody with the membrane preparations from human kidneys removed completely its agglutinating properties, indicating that all samples belong to the P-positive phenotype. The 154-IX-B6 antibody is directed against the P_1 blood group antigen (IV^4-α-GalnLcOse4Cer) and agglutinates strongly P_1 and P^k_1 red cells but not P_2, P^k_2, or p erythrocytes[20]. Except one, all kidney membrane preparations examined removed the agglutinating properties of this antibody. According to the data reported above, this latter sample is P-positive and P_1-negative, and belongs to the P_2 phenotype[1]. The other kidney samples were all P-positive and P_1-positive, and belong therefore to the P_1 phenotype[1]. The tissue typings were confirmed by a parallel study of the red cells from

the kidney donors. In the following studies, the galactosyltransferase activities present in the microsomal preparations from the P_1 and P_2 kidneys were investigated by use of lactosylceramide and paragloboside as glycolipid substrates.

Biosynthesis of P^k glycolipid. — When lactosylceramide (LacCer) was used as acceptor and UDP-D-[^{14}C]Gal as sugar donor, both the P_1 and P_2 microsomal preparations catalyzed the transfer of D-[^{14}C]galactose to the glycolipid acceptor and gave reaction products migrating, on h.p.t.l.c., as authentic globotriaosylceramide (GbOse3Cer) (Fig. 1A, lanes 1 and 2). No product was detected in the presence of EDTA in the reaction mixture, or when the Mn^{2+} cations were omitted. The bands obtained with both enzyme preparations exhibited a similar intensity and appeared as doublets on the chromatogram. The upper band of each doublet, however, was more intense since a substrate preparation containing mostly the upper band of standard LacCer was used. By use of nonradioactive UDP-D-Gal as sugar donor to transfer D-galactose onto LacCer, the reaction products obtained with the P_1 and P_2 microsomal preparations were found to strongly react with the rat monoclonal antibody 38-13 (Fig. 1B, lanes 1 and 2), which specifically recognizes the P^k blood group antigen[21], GbOse3Cer, present in the mixture of neutral glycolipid standards (Fig. 1B, lane STD). When LacCer was omitted from the reaction mixture, no significant radiolabeled product could be detected (less than 100 c.p.m.), and the immunostaining was negative both with the P_1 and P_2 microsomal fractions (data not shown), indicating that no endogeneous P^k antigen was present in the preparations.

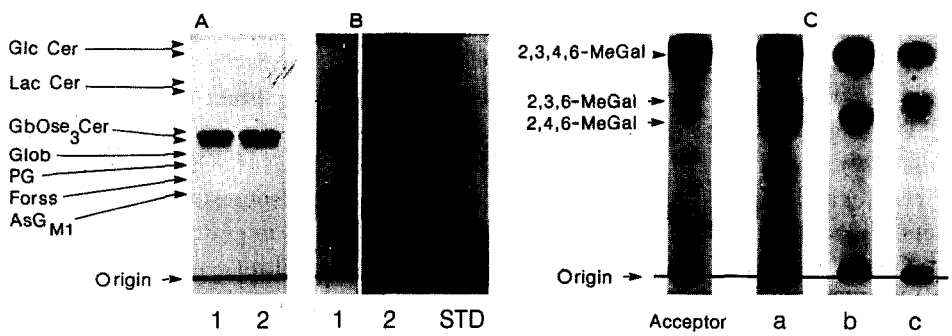

Fig. 1. Biosyntheses and characterization by h.p.t.l.c. of glycolipid products obtained by enzymic transfer of D-galactose to lactosylceramide by human kidney microsomes from P_1 and P_2 individuals: (A) Autoradiography and (B) immunostaining with the monoclonal anti-P^k reagent of the reaction products obtained with ^{14}C-labeled and unlabeled UDP-Gal, respectively, and chromatographed in solvent *A*. Numbers 1 and 2 refer to the P_1 and P_2 membrane preparations, respectively. The lane indicated STD contains all the standard neutral glycolipids whose position is shown on the left (Glc Cer, β-D-Glc*p*-ceramide; Forss, Forssman antigen, α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-ceramide). The light band seen in B, lane 2, after immunostaining of reaction products synthetized by P_2 microsomes was not constant and was not identified. (C) Autoradiography of the methylation analysis carried out with the D-[^{14}C]galactosylated products obtained from [6-^{3}H]LacCer in the presence of P_2 kidney microsomes. The labeled D-galactose derivatives were chromatographed in 500:9 acetone–5M NH_4OH; a, b, and c refer to the reaction products untreated (a) or treated with α-D-galactosidase (b), or β-D-galactosidase (c) before methylation analysis. The arrows indicate the migration position for methyl 2,3,4,6-tetra-*O*-methyl- and 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactosides, which were revealed by the aniline phthalate reagent.

The reaction products formed by the P_1 and P_2 microsomal preparations were further characterized by use of [6-^{3}H]LacCer as substrate and UDP-D-[^{14}C]Gal as sugar donor. The purified, labeled reaction products were methylated and hydrolyzed for structure determination as described by Stoffyn *et al.*[14]. The methylated, radioactive sugar derivatives were identified by h.p.t.l.c., followed by autoradiography (Fig. 1C). As described earlier for P_1 membrane preparations[8], the reaction products obtained with P_2 membranes reveal two bands, not present in the acceptor, which migrated as 2,3,6- and 2,4,6-tri-*O*-methyl-D-galactose, respectively (Fig. 1C, lane a), indicating that a D-galactosyl group had been linked to O-4′, as well as to O-3′ of [6-^{3}H]LacCer. The α/β ratio of anomers were determined by α- and β-D-galactosidase degradation before methylation analysis. Methyl 2,3,6-tri-*O*-methylgalactoside was absent from the reaction products treated with α-D-galactosidase before methylation (Fig. 1C, lane b), whereas methyl 2,4,6-tri-*O*-methylgalactoside could not be detected after β-D-galactosidase digestion (Fig. 1C, lane c). Together, these results indicated that both the P_1 and P_2 membrane preparations can transfer a D-galactopyranosyl group α-(1→4) and β-(1→3) to LacCer. The two reaction products, formed in the proportion of 30 and 70%, respectively, have a similar mobility on h.p.t.l.c. (Fig. 1A), and could not be separated even after acetylation. However, β-D-galactosidase from jack beans may be used to remove selectively the β-D-galactopyranosyl-(1→3) group formed during the reaction (Fig. 1C, lane c).

These results demonstrated that P_1 and P_2 kidneys contain a (1→4)-α-D-galactosyltransferase with a strict requirement for divalent cations (Mn^{2+}), which converts LacCer into GbOse3Cer (P^k antigen). An enzyme with a similar property has been purified from rat liver[22], and interestingly it was shown that neither lacto-*N*-neotetraose nor paragloboside was a substrate for this enzyme, suggesting that the synthesis of P^k and P_1 antigens could be directed by different enzymes.

Biosynthesis of the P_1 glycolipid. — When paragloboside (nLcOse4Cer) was used as acceptor and UDP-D-[^{14}C]Gal as sugar donor with the microsomal preparations from P_1 and P_2 kidneys, respectively, the reaction products formed migrated as asialo-GM_1 [α-D-Gal*p*-(1→3)-β-D-Gal*p*NAc-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→1)-ceramide] on h.p.t.l.c. (Fig. 2A, lanes 1 and 2), but more product was synthesized with the P_1 microsomal preparation. No products were found in the presence of EDTA or when Mn^{2+} was omitted.

When unlabeled UDP-Gal was used as sugar donor, immunostaining with a specific murine monoclonal (154-IX-B6) against the P_1 blood-group antigen[20] indicated that only the reaction products obtained with the P_1 kidney could be detected (Fig. 2B, lanes 1 and 2), thus suggesting that the P_2 kidney microsome preparation was catalyzing the synthesis of a D-galactose-containing glycolipid devoid of P_1 blood-group activity. The incorporation of D-[^{14}C]galactose and the immunostaining reaction were negative when nLcOse4Cer was omitted from the reaction mixture (data not shown), indicating that no P_1 antigen was detectable in the microsomal preparation.

The reaction products obtained from P_1 and P_2 microsomes assays were structurally characterized by use of [6-^{3}H]nLcOse4Cer as substrate and UDP-D-[^{14}C]Gal as

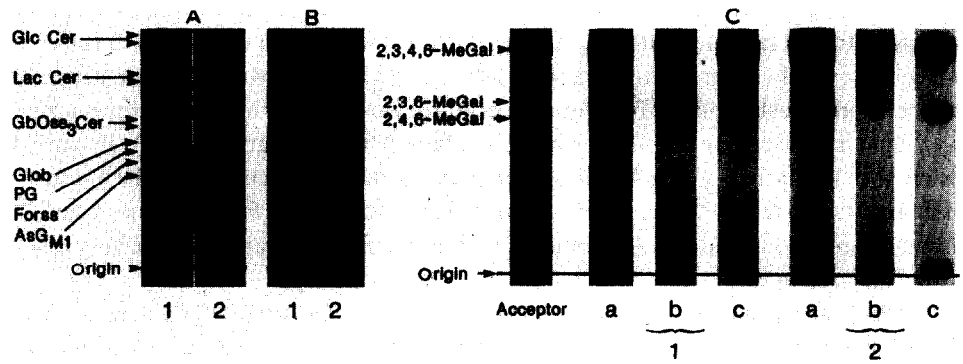

Fig. 2. Biosynthesis and characterization by h.p.t.l.c. of glycolipid products obtained by enzymic transfer of D-galactose to paragloboside by human kidney microsomes from P_1 and P_2 individuals: (A) Autoradiography and (B) immunostaining with the monoclonal anti-P_1 reagent of reaction products obtained with ^{14}C-labeled and unlabeled UDP-Gal, respectively. Other indications are as in Fig. 1. (C) Autoradiography of the methylation analysis carried out with the D-[^{14}C]galactosylated products obtained from [6-^{3}H]nLcOse4Cer in the presence of P_1 (1a, b, and c) or P_2 (2a, b, and c) kidney microsomes. Other indications are as in Fig. 1, except that b and c refer to the reaction products treated with β- and α-D-galactosidase before methylation analysis, respectively.

sugar donor according to the procedure of Stoffyn *et al.*[14], as described above for the synthesis of P^k antigen. The analysis of the methylated radioactive D-galactose derivatives (Fig. 2C, lanes 1a, b, and c), obtained from reaction products formed during the P_1 enzyme assay, indicated that α-D-galactopyranosyl-(1→4) and β-D-galactopyranosyl-(1→3) groups were incorporated into paragloboside. On the contrary, the microsomal preparation from the P_2 kidney was able to transfer only a β-D-galactopyranosyl-(1→3) (Fig. 2C, lanes 2a, b, and c). If α-lactalbumin is omitted in the incubation mixture containing nLcOse4Cer, a radioactive reaction product migrating as nLcOse4Cer was found. This is probably explained by removal of the terminal D-galactopyranosyl group from LcOse4Cer, followed by reincorporation of D-[^{14}C]galactose catalyzed by the widely tissue distributed UDP-D-Gal:*N*-acetyl-D-glucosaminide (1→4)-β-D-galactosyltransferase. In the presence of α-lactalbumin, the lactose synthetase activity is high and, therefore, the microsomes should be carefully washed to remove any glucose present in the preparation.

The results demonstrated that P_1 kidneys contain an α-D-galactosyltransferase that can transfer a α-D-galactosyl-(1→4) group to nLcOse4Cer. The reaction products formed migrated on h.p.t.l.c. as asialo-GM_1 and reacted strongly with the murine monoclonal antibody directed against the P_1 antigen. The authentic P_1 glycolipid (IV^4-α-GalnLcOse4Cer), isolated from human red cells, comigrated with asialo-GM_1 on t.l.c. developed with the same solvent[3] and, therefore, together these results strongly indicated that the P_1 glycolipid had been synthesized *in vitro* in a cell free system. Since the preparation from a P_2 kidney was unable to synthesize the P_1 glycolipid, it is proposed that a specific (1→4)-α-D-galactosyltransferase, present in kidneys from P_1 individuals only, is responsible for the synthesis of the P_1 blood-group antigen. An enzyme having such a specificity has not been described before.

As expected from earlier results[8] and those presented above, the (1→3)-β-D-galactosyltransferase present in the P_1 and P_2 kidney preparations can transfer a β-D-galactosyl-(1→3) group to paragloboside, and a glycolipid with a similar structure has been previously isolated from human erythrocytes[23]. Both the P_1 and this unusual glycolipid have the same mobility on h.p.t.l.c. plates (Fig. 2A), but only the P_1 glycolipid reacts with the anti-P_1 antibody. Since both glycolipids are synthesized by the microsomal preparation from P_1 kidneys, it is expected and was found (Fig. 2A, lanes 1 and 2) that more reaction products are obtained with P_1 membrane than in the enzyme assay with P_2 microsomes.

Presence of one or two distinct α-D-galactosyltransferases in P_1 kidneys. — The results presented above with LacCer and nLcOse4Cer as substrates suggested that P_2 kidneys have a unique (1→4)-α-D-galactosyltransferase, which can synthesize the P^k antigen but not the P_1 antigen. However, in P_1 individuals, an α-D-galactosyl-(1→4) group is transferred to both substrates. This raises the question whether one or two distinct enzymes are synthesizing these structures, since the P^k and P_1 glycolipids share an identical terminal disaccharide unit, α-D-Gal*p*-(1→4)-β-D-Gal. In order to distinguish between the presence of one or two (1→4)-α-D-galactosyltransferases in P_1 kidney, we attempted to separate the two transferase activities by affinity chromatography on various immobilized ligands. UDP-hexanolamine–Sepharose did not bind α-D-galactosyltransferase activity, either in the presence or absence of Mn^{2+} ions or lactose. However, the UDP-hexanolamine column removed about 90% of the UDP-Gal:*N*-acetyl-D-glucosaminide (1→4)-β-D-galactosyltransferase, which could be eluted easily with UDP or a salt gradient. In the β-D-galactosyltransferase preparations, only traces of α-D-galactosyltransferase activity could be detected. Likewise, an affinity support based on the acceptor substrate, LacCer acid–Sepharose, was not effective in binding the α-D-galactosyltransferase activities. A nonspecific affinity ligand, Con A–Sepharose, removed some protein and could be used to purify somewhat the glycosyltransferases, but all of the α-D-galactosyltransferase activities could be recovered in the flow-through. From these results, it became clear that no separation of the two transferase activities could be obtained with the limited amount of material available. Therefore, an indirect method was used to test whether one or two transferases are involved in the biosynthesis of the P^k and P_1 antigen in the kidney microsomes from P_1 donors. Competition experiments were carried out with LacOMe [β-D-Gal*p*-(1→4)-β-D-Glc*p*OMe] and LNnTOMe [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*OMe] as substrates, as well as lactosylceramide and paragloboside. Three different kidneys from P_1 donors were tested. Using the two oligosaccharide acceptors in a wide range of concentrations, either separate or together in the same incubation mixture, we found that the purified reaction products, α-D-Gal*p*-(1→4)-LacOMe and α-D-Gal*p*-(1→4)-LNnTOMe, were produced in similar amounts in the reaction mixture containing the two acceptor substrates, as compared to the reaction mixtures containing either one or the other substrate (Table I). These results showed that the two acceptor substrates do not compete for a single active site on one enzyme. Competition experiments carried out with lactosylceramide and paragloboside, which

TABLE I

Study of the (1→4)-α-D-galactosyltransferases from P_1 kidneys by acceptor-substrate competition[a]

Acceptor substrate		*D-[^{14}C]galactose transferred (c.p.m.)*	
Oligosaccharides (mM)			
LNnTOMe	*LacOMe*	*LNnTOMe*	*LacOMe*
	400		36 560
50		5 901	
100	400	12 138	35 147
50	400	6 240	35 128
25	400	3 270	35 217
12.5	400	1 609	37 320
Glycolipids (μg)			
nLcOse4Cer	*LacCer*	*nLcOse4Cer*	*LacCer*
50		823	
	50		2 325
50	50	800	2 490

[a] The reaction mixtures were prepared as described in the Experimental section, except for the indicated amounts of acceptors used. After incubation for 1 h at 37°, the reaction products were purified, treated with β-D-galactosidase (0.3 unit) to remove the D-[^{14}C]galactose incorporated as β-D-galactosyl-(1→3) groups, and quantitatively determined by liquid-scintillation counting. Three different kidneys from P_1 donors were tested and the data reported represent the mean value for each assay. The mean error was 5% for each determination.

are the physiological substrates of the (1→4)-α-D-galactosyltransferases, gave similar results. These results correlate well with the identification of the P_1 and P glycolipids in human kidney[24,25].

Genetics of P^k and P_1 substances. — The biosynthesis of the P_1 chemical structure has been examined in detail in kidney extracts from three donors of the P_1 phenotype. According to Graham and Williams[6], the synthesis of the P blood-group antigens is under the control of two independant loci, P^k and P. There are three possible alleles at the P^k locus (P^k, $P^k{}_1$, and p) and individuals of the P_1 phenotype (80% of Caucasians) belong to the $P^kP^k{}_1$, $P^k{}_1P^k{}_1$, or $P^k{}_1p$ genotype, whereas those of the P_2 (non-P_1) phenotype (20% of Caucasians) are either P^kP^k or P^kp. Considering that the p allele is very rare[1] and taking into account the frequency of the P_1 and P^k genes (P^k gene frequency is identical to the P_2 gene frequency defined by Race and Sanger, see ref. 1), it is calculated that among P_1 donors, 63 and 37%, respectively, belong to the $P^kP^k{}_1$ and $P^k{}_1P^k{}_1$ genotypes. Since we have investigated only the kidney samples from three P_1 donors, it cannot be decided whether the two possible genotypes are represented. If they belong to the $P^kP^k{}_1$ genotype, the presence of two (1→4)-α-D-galactosyltransferases with a different substrate specificity is expected, one which converts only the lactosylceramide in ceramide trihexoside (P^k antigen) and the other which, in addition, converts the paragloboside into P_1 blood-group structure. The substrate competition experiments

indeed indicated the presence of two (1→4)-α-D-galactosyltransferases in the three kidney extracts examined, but it is rather surprising that no detectable effect on the biosynthesis of P^k or P_1 antigen were found when large amounts of the P_1 or P^k substrates, respectively, were added to the reaction mixture, although both enzymes used lactosylceramide as substrate. It is also obvious that if one (or several) kidney extracts belong to the $P^k_1P^k_1$ genotype, the enzyme competition data would exclude the two-locus model[6], and it would be important to study whether or not regulatory proteins are involved in the biosynthesis of P^k and P_1 antigens[3]. Preliminary experiments indicated only that the amount of P_1 antigen synthesized by a mixture of P_1 and P_2 microsomal proteins (1:1, by weight) is within expected values calculated from a two-fold dilution of the P_1 enzyme preparation and, therefore, there is no obvious evidence for the presence of an inhibitory or regulatory component in the P_2 kidney microsomes, which may explain the substrate specificity of the (1→4)-α-D-galactosyltransferases.

It is known that in fibroblasts derived from p individuals the (1→3)-*N*-acetyl-β-D-galactosaminyltransferase that forms the P antigen is present[26], but it is ineffective since its glycolipid acceptor substrate (P^k glycolipid) is missing. The simultaneous lack of P^k and P_1 antigens, however, is still not understood, and conflicting results have been published concerning the presence or absence of the (1→4)-α-D-galactosyltransferase that synthesizes the P^k glycolipid in these donors[26,27]. The more recent findings, established with EBV-transformed lymphoblastoid cell lines from p donors, suggested that this transferase is normally detectable by *in vitro* assays, but might be functionally inactive *in vivo*[27]. The discovery of a P_1 glycosyltransferase now presents the opportunity to study in detail all the enzymes involved in the biosynthesis of the P/P_1-related glycolipids. Recently, the blood group ABH glycosyltransferases cloned by Yamamoto *et al.*[28] showed that the nucleotide sequences of the ABO alleles are closely related. A duplicated gene having few single-base substitutions could be the origin of the two (1→4)-α-D-galactosyltransferases, which would explain their very similar enzymic properties as well as their identical behavior in the different affinity media. The key to understanding the complex genetic control of P^k and P_1 expression is the elucidation of the nature of the genetic alterations underlying the rare p phenotype, in which the P^k, P, and P_1 antigens are lacking.

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