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IDENTIFICATION OF THE PRODUCT FORMED BY HUMAN ERYTHROCYTE GALACTOSYLTRANSFERASE

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Summary

Sepharose 4B-immobilized desialylated ovine submaxillary mucin was used as an acceptor for galactose transfer from UDP-galactose, catalyzed by a Triton X-100-solubilized galactosyltransferase from human erythrocyte ghosts. The product could be cleaved from the insoluble acceptor substrate by alkaline borohydride treatment and identified on Bio-Gel P-2 as a disaccharide. The nature of the glycosidic bond of the isolated material was elucidated by periodate oxidation/NaB[^{3}H]$_4$ reduction/acid hydrolysis and subsequent identification of the aminopolyol formed as L-threosaminitol. Specific cleavage of the enzymatic product by β-galactosidase indicated a β-configuration for incorporated galactose. These data permit classification of the enzyme as a UDP-galactose: α-D-*N*-acetylgalactosaminyl-protein $\beta(1 \rightarrow 3)$ transferase.

Furthermore, in the presence of Triton X-100, the enzyme from normal erythrocytes catalyzed transfer of galactose to the glycan moieties of asialo-agalacto-glycophorin in Tn-erythrocytes from a patient with permanent mixed-field polyagglutinability.

Introduction

An erythrocyte galactosyltransferase, transferring galactose from uridine-5'-diphosphogalactose to desialylated ovine submaxillary mucin (asialo-mucin), has previously been reported from this laboratory [1].

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Galactosyltransferase activity has been shown to be absent in the Tn-transformed erythrocytes from patients affected by permanent mixed-field polyagglutinability [1—3]. The molecular basis of the Tn-transformation consists in the specific absence of sialic acid and galactose in the *O*-glycosyl carbohydrate side-chains of glycophorin [4,5]. Upon sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis, the glycoprotein bands corresponding to the major erythrocyte membrane sialoglycoprotein, glycophorin, show increased mobility [5]. These findings suggested that glycophorin is the physiological substrate for the described galactosyltransferase.

In previous studies, however, the linkage type formed by this galactosyltransferase has not been established. Erythrocytes may contain different glycosyltransferase activities [1,6,7], which may interfere in kinetic analysis, especially if acceptor substrates such as asialo-agalacto-fetuin, containing heterogeneous acceptor sites, are used [8]. Work from this laboratory revealed substantial incorporation of galactose into asialo-mucin by purified human milk galactosyltransferase specific for GlcNAc acceptor sites [9]. Therefore, a formal identification of the linkage type catalyzed by any glycosyltransferase must be considered as a prerequisite for further work aimed at purification and kinetic analysis.

The present study was carried out in order to elucidate the linkage type catalyzed by erythrocyte galactosyltransferase, using an approach which is applicable to crude enzyme preparations. Furthermore, the biological significance of this enzyme activity was defined by authenticating Tn-glycophorin as acceptor substrate.

Materials and Methods

Chemicals were of analytical reagent grade from Merck, Darmstadt, F.R.G., or from Fluka, Buchs, Switzerland. Uridine diphospho-D-[6-^{3}H]galactose, ammonium salt (spec. act. 13.1 Ci/mmol), uridine diphospho-D-[U-^{14}C]galactose, lithium salt (spec. act. 343 mCi/mmol) and sodium boro[^{3}H]hydride (spec. act. 15.5 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, U.K. The sodium boro[^{3}H]hydride used after the periodate oxidation step was from New England Nuclear and had a specific radioactivity of 250 Ci/mol. Butyl-PBD scintillator was from Ciba-Geigy, Switzerland, Sepharose 4B from Pharmacia, Sweden, and Bio-Gel P-2 from Bio-Rad Laboratories, U.S.A. *Escherichia coli* β-galactosidase Grade IV and galactose oxidase (*Dactylium dendroides*) were obtained from Sigma.

Ovine submaxillary mucin was prepared according to Carlson et al. [10], desialylated by acid hydrolysis and attached to Sepharose 4B as described previously [11].

Biological material: Tn-transformed erythrocytes were obtained from R.R., a subject with permanent mixed-field polyagglutinability (93% Tn-transformed erythrocytes), as described previously [1] and were used without prior separation from the small proportion of normal cells. Blood from healthy blood group A or O individuals was used exclusively as source of the galactosyltransferase activity. Erythrocyte membranes were prepared as follows: approx. 45 ml blood were withdrawn by venipuncture into a syringe containing 15 glass

beads of 3—4 mm diameter and the syringe rocked gently for 5 min to induce defibrination [12]. The clot was removed and the defibrinated blood washed with 0.172 M Tris-HCl buffer (pH 7.6) and filtered through two layers of standard cellulose filter paper, to give a final volume of 200 ml. This procedure removed essentially all of the leukocytes. Membranes were then prepared according to Hanahan and Ekholm [13]. The final suspension in 0.011 M Tris-HCl buffer (pH 7.6) was made 0.02% in sodium azide and stored at 4°C.

Incorporation of [^{14}C] and [^{3}H]galactose into Sepharose 4B-immobilized asialo-mucin and identification of the product

1-ml aliquots of erythrocyte membranes (5 mg/ml) were solubilized by addition of 0.5 ml 3% (w/v) Triton X-100/0.18% sodium azide and vortex mixing. After centrifugation at 100 000 × *g* for 1 h, the supernatants were pooled. 1 ml supernatant was then added to 2 ml of packed immobilized asialo-mucin, followed by 100 μl 1 M $MnCl_2$, 400 μl 1 M sodium cacodylate (pH 7.2) and 500 μl 1 mM UDP-[^{14}C]galactose (2 mCi/mmol). The resulting mixture was incubated for 4 h at 37°C with paddle stirring, then an additional 1 ml solubilized erythrocyte membranes was added before incubating again for 4 h. The supernatant was then aspirated and the beads washed with 5 × 10 ml of a solution containing 10% Triton X-100/0.5 M NaCl/2 M urea. Identical washing procedures were carried out with the same solution excluding urea and finally with distilled water. 2 × 100 μl aliquots of the final suspension in 4 ml water were transferred to glass fibre filters, dried at 100°C for 10 min and counted after addition of 10 ml 8% (w/v) Butyl-PBD/toluene. Alkaline borohydride treatment of the galactosylated mucin product and the isolation of the released oligosaccharides was carried out as described previously [11]. The reduced oligosaccharide mixture was then applied to a Bio-Gel P-2 column in order to isolate the ^{14}C-labelled product. Linkage determination of the isolated disaccharide was performed by the periodate oxidation/NaB[^{3}H]$_4$ reduction/hydrolysis method as described previously [14]. The resulting ^{3}H-labelled aminopolyol was identified by TLC [15] and high-voltage electrophoresis [14]. The anomeric configuration of the galactose incorporated was studied on the product chain which had been obtained from [^{3}H]galactosylated mucin product, synthesized enzymatically as above for the ^{14}C-labelled product except that 500 μl 1 mM UDP-[^{3}H]galactose (40 mCi/mmol) were used. This was also then followed by alkaline borohydride treatment, as above. Digestion of the isolated ^{3}H-labelled reduced disaccharide with *E. coli* β-galactosidase was accomplished essentially as described before [14].

Incorporation of [^{3}H]galactose into the glycophorin of Tn-membranes

Blood group A erythrocyte membranes were solubilized as for the asialo-mucin incorporation experiments. The incubation mixture for incorporation of [^{3}H]galactose into Tn-glycophorin was as follows: 390 μl solubilized membranes/60 μl 0.3 M $MnCl_2$/60 μl 1 M sodium cacodylate (pH 7.2)/90 μl 1% Triton X-100/180 μl Tn-erythrocyte membrane suspension (3.5 mg protein/ml)/120 μl 2 mM UDP-[^{3}H]galactose (50 mCi/mmol) were mixed and incubated for 17 h at 25°C. The incubation mixture was then exhaustively dialyzed against 2 × 5 l 65 mM Tris-HCl (pH 6.8) and finally adjusted to the sample

buffer concentrations given by Laemmli [16], including 2 mM EDTA. The mixture was divided into 0.1 ml portions and frozen at −20°C. Labelled reference materials were treated in like manner. Samples were heated at 100°C for 5 min prior to SDS-polyacrylamide gel electrophoresis.

Acid hydrolysis of Tn-membrane product

Material dialyzed against distilled water was heated for 2 h at 100°C in the presence of 2 M HCl. The supernatant after centrifugation at 100 000 × *g* for 1 h was deionized on Bio-Rad RG 501-X8 mixed-bed ion-exchange resin and lyophilized.

Results

Ghosts prepared from normal human erythrocytes were incubated with immobilized asialo-mucin in the presence of UDP-[^{14}C]galactose, Mn^{2+} and Triton for 8 h at 37°C as described in Materials and Methods. After incubation, asialo-mucin agarose was extensively washed and subjected to alkaline borohydride treatment which removed all of the ^{14}C label. This indicated incorporation of galactose into the alkali-labile carbohydrate moieties of asialo-mucin. After removal of cations and boric acid, the resulting material was lyophilized and analyzed as follows: more than 95% of the radioactivity applied to a Bio-Gel P-2 column was recovered as one peak, eluting in fractions corresponding to authentic Gal(1 → 3)GalNacol in calibration experiments (Fig. 1). The material in this peak was lyophilized and subjected to periodate oxidation/NaB[^{3}H]$_4$ reduction/hydrolysis [14]. The resulting ^{3}H-labelled aminopolyol could be identified as L-threosaminitol by TLC (data not shown) and high-voltage electrophoresis (Fig. 2), indicating that galactose had been linked to position C-3 of *N*-acetylgalactosaminyl residues on asialo-mucin.

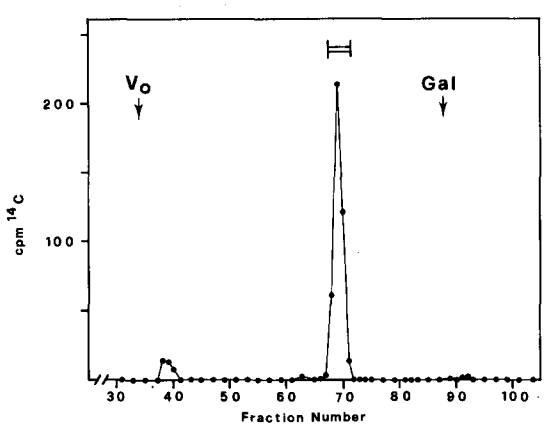

Fig. 1. Gel filtration on Bio-Gel P-2 of the reduced oligosaccharide obtained from in vitro [^{14}C]galactosylated asialo-mucin-agarose. The oligosaccharide sample was subjected to gel filtration on Bio-Gel P-2, 200—400 mesh. Column dimensions: 1.6 × 200 cm. Buffer: 0.05 M ammonium acetate (pH 5.2). Flow rate: 12 ml/h. Fraction volume: 4 ml. 200 µl aliquots were assayed for ^{14}C-labelled radioactivity. The fractions indicated by the bar (□), 68—71, containing the product disaccharide chain, were pooled and lyophilized. V_0: column void volume. Gal: elution position of galactose.

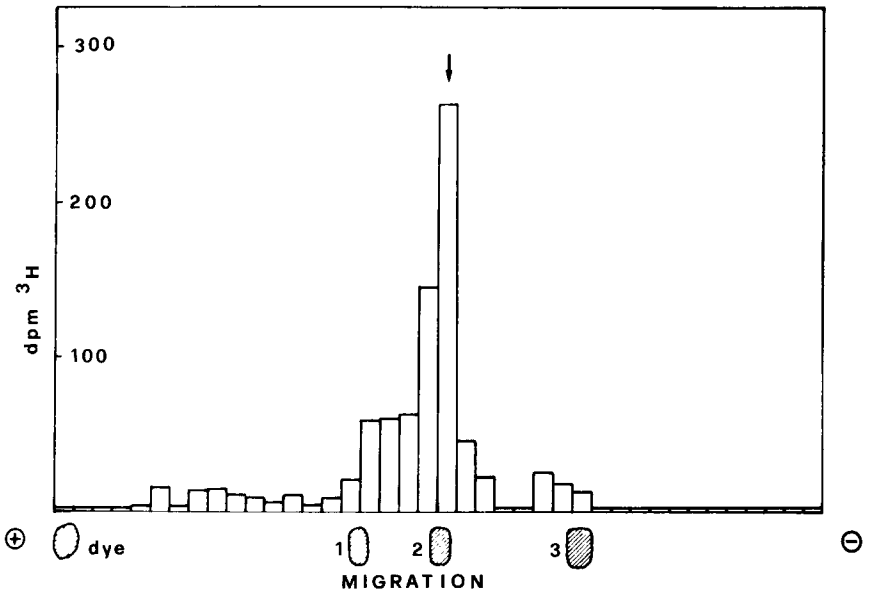

Fig. 2. Identification of [^{3}H]aminopolyol by high-voltage electrophoresis. A sample (7 nCi) of the [^{3}H]-aminopolyol obtained as described in the text was applied together with (1) L-arabinosaminitol; (2) L-threosaminitol; (3) serinol (as reference aminopolyols) and dye, Bromophenol blue, to a Whatman 3 MM paper strip. Electrophoresis was carried out in 1% sodium tetraborate at 10°C for 1 h at 65 V/cm. After detection of the standards with ninhydrin the strip was cut into 1.5 cm segments and counted by liquid scintillation. Arrow shows point of application of sample and standard.

The anomeric configuration of [^{3}H]galactose incorporated in a similar experiment was determined by specific cleavage of the reduced disaccharide obtained by alkaline borohydride treatment as described above, using *E. coli* β-galactosidase: fractions containing [^{3}H]Gal(1 → 3)GalNacol from a Bio-Gel P-2 column were pooled and lyophilized. The material obtained was incubated in a small volume (100 μl) with 4.3 units *E. coli* β-galactosidase for 96 h at 37°C, in the presence of 0.02% NaN_3. The incubation mixture was then subjected to Bio-Gel P-2 chromatography which resolved two peaks of radioactivity. The first peak eluted corresponded in position to unchanged [^{3}H]Gal β(1 → 3)GalNacol and contained 20% of the original radioactivity, whereas the second peak, containing 80% of the original radioactivity, corresponded in position to galactose. The two peaks could be identified by high-voltage electrophoresis and TLC [14] as [^{3}H]Gal β(1 → 3)GalNacol and [^{3}H]galactose, respectively (data not shown), indicating the β-anomeric configuration for the enzyme product. From this we conclude that erythrocyte galactosyltransferase forms a linkage Gal β(1 → 3)GalNac-protein.

Information concerning its biological function was obtained by using Tn-membranes deficient in enzyme and carrying possible acceptor sites. Erythrocyte ghosts were prepared from the blood of patient R.R. [1], a subject with permanent mixed-field polyagglutinability. In order to confirm the presence of asialo-agalacto-glycophorin, the ghosts (93% Tn-transformed) were analyzed by SDS-polyacrylamide gel electrophoresis. Protein and glycoprotein staining patterns are depicted in Fig. 3. As indicated by arrows, only minor differences

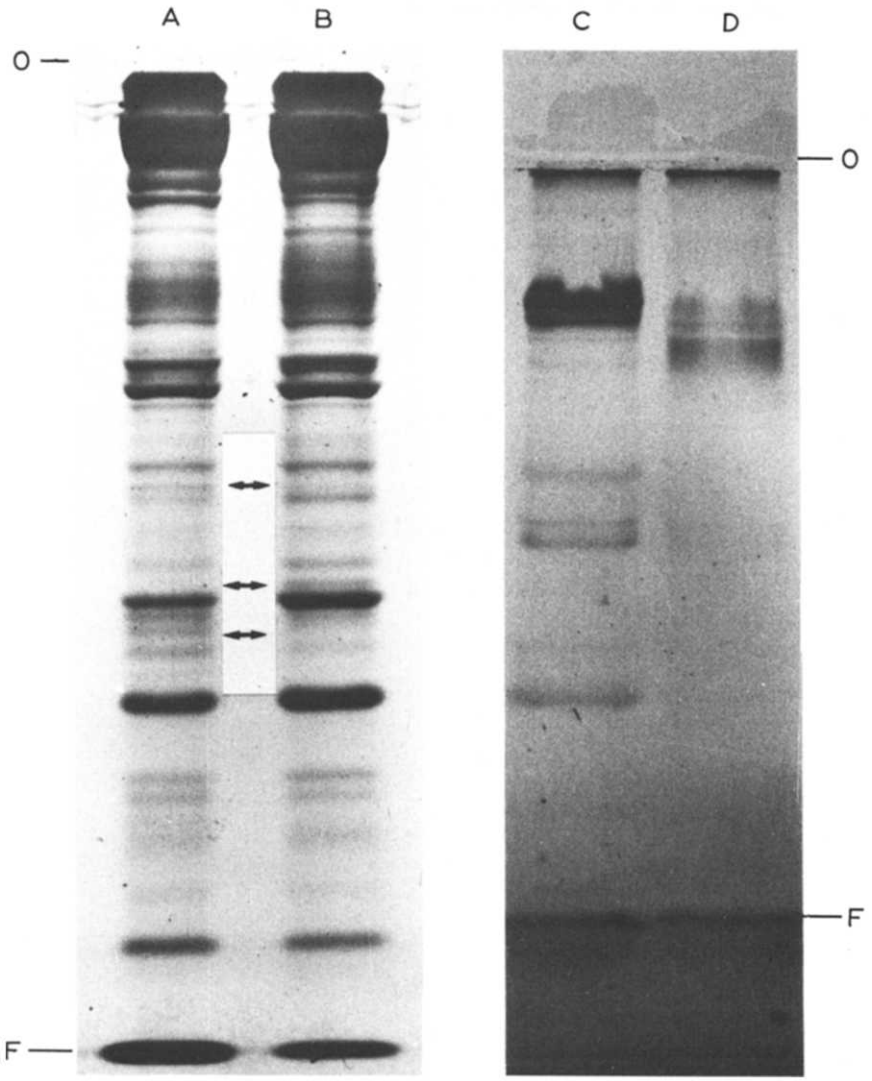

Fig. 3. Comparison of normal (A, C) and Tn-transformed (B, D) erythrocyte membrane proteins and glycoproteins analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli [16]; 12% acrylamide gels. Gels were stained for protein (A, B) using Coomassie brillant blue, and for glycoprotein (C, D) using periodate/Schiff's reagent [5]. O = gel origin; F = Bromophenol blue dye front. Double-headed arrows indicate minor differences in protein-staining patterns between normal and Tn-transformed erythrocytes from a patient with permanent mixed-field polyagglutinability.

could be detected between normal and Tn-erythrocyte membrane protein patterns. On the other hand, major differences can be seen in the glycoprotein patterns, essentially in agreement with those previously described by Dahr et al. [5]. The increased electrophoretic mobility of Tn-glycophorin was attributed to lack of sialic acid and galactose residues [5].

Non-dialyzable ^{3}H label was found to be present after incubation of normal membranes (as source of transferase) and Tn-membranes (as source of acceptor

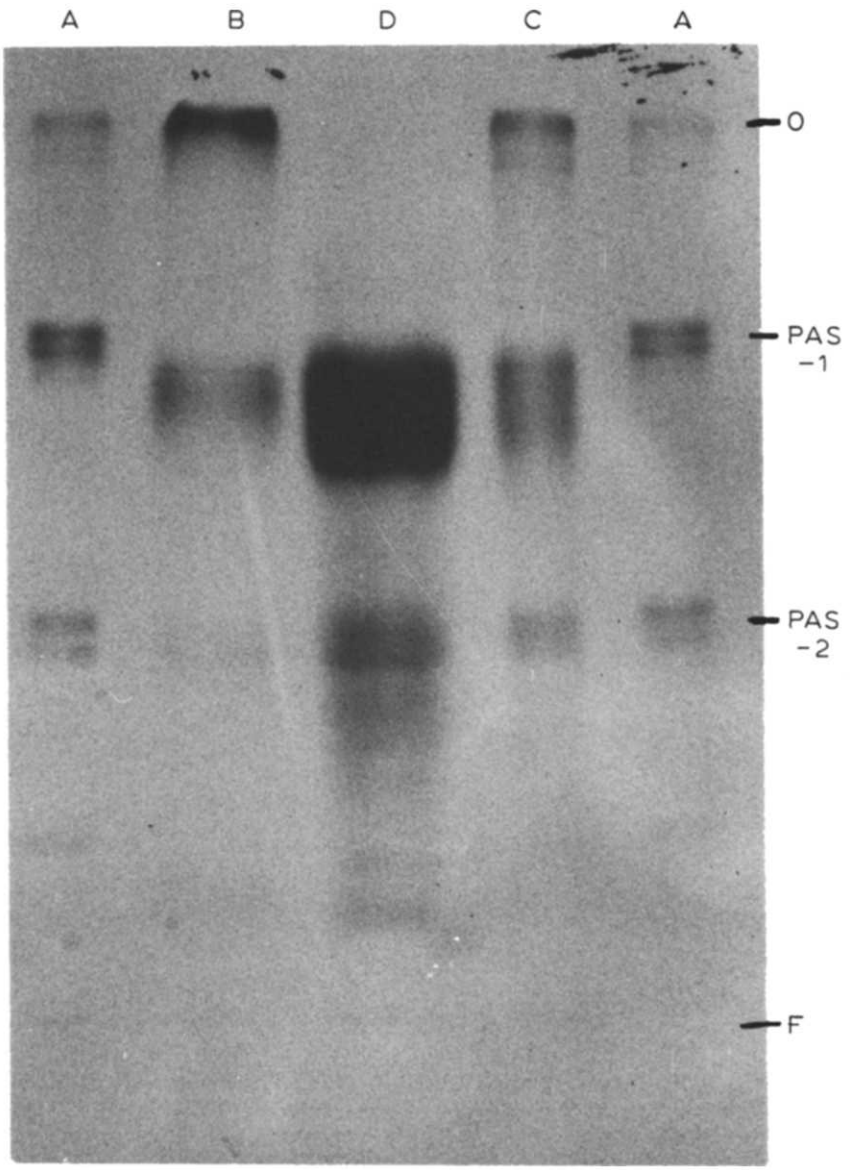

Fig. 4. Incorporation of [^{3}H]galactose into Tn-glycophorin: samples were analyzed by SDS-polyacrylamide gel electrophoresis according to Laemmli [16]; 12% acrylamide gels. The gel was developed by fluorography [27]. A: Normal erythrocyte membranes labelled by the periodate/NaB[^{3}H]$_4$ method B: Tn-transformed erythrocyte membranes labelled by the periodate/NaB[^{3}H]$_4$ method [28]. C: Tn-transformed erythrocyte membranes labelled by the galactose oxidase/NaB[^{3}H]$_4$ method [29]. D: Mixture of Tn-transformed and normal erythrocyte membranes incubated under appropriate conditions for the enzymatic transfer of [^{3}H]galactose from UDP-[^{3}H]galactose to potential acceptors (see Materials and Methods). ^{3}H incorporation is seen mainly in the region of Tn-glycophorin, labelled by other methods in lanes B and C. The same treatment with Tn-transformed or normal erythrocyte membranes alone did not lead to bands of radioactivity detectable by this method (not shown). O = gel origin; F = Bromophenol blue dye front. PAS-1, PAS-2 indicate positions of periodate/Schiff's reagent-staining sialoglycoproteins [30].

substrate) with UDP-[^{3}H]galactose and Mn^{2+} in the presence of Triton X-100. This material was subjected to SDS-polyacrylamide gel electrophoresis together with normal and Tn-membranes which had been labelled with ^{3}H in their sugar moieties by the periodate/NaB[^{3}H]$_4$ or galactose oxidase/NaB[^{3}H]$_4$ procedures as reference materials, as shown in Fig. 4. Incorporation of [^{3}H]galactose into electrophoretic bands with similar mobilities to those of the asialo-agalacto-glycophorin bands of Tn-erythrocytes labelled by the galactose oxidase/NaB[^{3}H]$_4$ method can be seen. The position of the incorporated label corresponds to the dimer and monomer of glycophorin. Incubation of equivalent amounts (protein) of normal membranes alone or Tn-membranes alone, under identical conditions, did not lead to detectable incorporation of label using these methods. Confirmation was needed that the incorporated label was indeed galactose and not glucose, since erythrocytes contain UDP-galactose 4′-epimerase [17]. Labelled Tn-glycophorin was therefore subjected to acid hydrolysis. 90% of the label could be recovered and identified as galactose by TLC and high-voltage electrophoresis [14] (data not shown), demonstrating incorporation of galactose into Tn-glycophorin.

Discussion

This work demonstrates the presence of an *N*-acetylgalactosaminide $\beta(1 \rightarrow 3)$ galactosyltransferase in human erythrocyte ghosts. The data confirm the assumption that a galactosyltransferase present in human erythrocytes can unequivocally be assayed using asialo-mucin as an acceptor substrate. Cartron et al. [2] used *p*-nitrophenyl-α-*N*-acetylgalactosaminide as acceptor for a human erythrocyte galactosyltransferase. This enzyme activity was also shown to be deficient in Tn-transformed cells [2]. From this evidence we assume that the two transferases assayed with asialo-mucin and *p*-nitrophenyl-α-*N*-acetyl-galactosaminide, respectively, are identical. It is not clear from the data of Cartron et al. whether the use of the latter substrate permits accurate measurement of initial velocities, as is possible using asialo-mucin [1]. Comparative studies using both acceptor substrates are currently being carried out in order to define the specificity for the acceptor substrate.

Asialo-mucin has found widespread application in the measurement of the mucin-type galactosyltransferase [18—20]. However, previous results obtained in our laboratory showed that an *N*-acetylglucosaminide $\beta(1 \rightarrow 4)$galactosyl-transferase in a partially purified form from human serum [2] or in a highly purified form from human milk [9] incorporated galactose into asialo-mucin to a sizable extent. It is, therefore, impossible to define the proportions of activities of these two galactosyltransferases in crude systems as long as asialo-mucin is used as acceptor substrate. Addition of free GlcNAc to the assay system is necessary in order to inhibit the GlcNAc $\beta(1 \rightarrow 4)$galactosyltransferase [12,21]. This limitation does not apply to the studies described here, since the product was isolated and chemically identified.

Since this galactosyltransferase catalyzed the formation of a Gal $\beta(1 \rightarrow 3)$ GalNAc linkage in glycoproteins, it is not surprising to observe the lack of this structure in enzyme deficient cells. Indeed, human blood cells including erythrocytes [1,2], leukocytes [3] and platelets [22] deficient in galactosyl-

transferase activity carry the Tn-antigen. This cryptantigen which can be identified serologically with anti-Tn-antibodies or Salvia sclarea lectin [23,24] consists of terminal GalNAc [5]. On erythrocytes the carrier of this antigen is glycophorin [5]. Asialo-agalacto-glycophorin of Tn-erythrocytes, therefore, should act as acceptor substrate for an enzyme preparation which is able to form a Gal $\beta(1 \rightarrow 3)$GalNAc linkage. As shown in Fig. 4, conclusive evidence is presented that a galactosyltransferase obtained from normal erythrocyte ghosts indeed transfers galactose to asialo-agalacto-glycophorin from Tn-transformed erythrocytes. The incubation conditions necessary for this transfer included non-ionic detergent. It is not known at present whether asialo-agalacto-glycophorin in situ could act as acceptor substrate, since galactosyltransferase is not measurable in the absence of detergent [1]. Whether or not an assay system avoiding the use of detergent can be devised is presently under investigation.

The data permit the conclusion that the galactosyltransferase activity described in this paper is involved in the biosynthesis of the heteroglycan moiety of a membrane glycoprotein glycophorin. Recently, Rapoport et al. [25] described a cytosolic protein, lipoxygenase, which appears to be a glycoprotein synthesized at a late stage of reticulocyte maturation [26]. The mechanism of glycosylation of this cytosolic protein is unclear; the involvement of glycosyltransferases as described in this paper is an attractive hypothesis.

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