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Role of Cell Membrane Galactosyltransferase in Concanavalin A Agglutination of Erythrocytes

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It has been previously observed that rabbit erythrocyte cell surface galactosyltransferase appears to play a role in concanavalin A agglutination of these erythrocytes (Podolsky *et al.*, 1974). Further, a correlation between the occurrence or level of cell surface galactosyltransferase and concanavalin A agglutinability of other cell types has also been observed. The mechanism by which rabbit erythrocyte galactosyltransferase participates in concanavalin A agglutination has now been further defined. The enzyme was solubilized and purified. Characterization of the enzyme properties has shown them to be similar to those reported for other purified galactosyltransferases. Amino acid and carbohydrate analysis showed a high asparagine content and the presence of D-mannose. Specific α-mannosidase treatment of the enzyme showed that some of these D-mannose residues were terminal sugars. The purified enzyme also conferred concanavalin A agglutinability to non-agglutinable human erythrocytes. However, the ability to confer concanavalin A agglutinability was unrelated to the enzyme activity *per se* (as measured with fetuin acceptor) but appeared to be entirely dependent on the presence of terminal α-linked D-mannosyl residues in the enzyme structure. These findings suggest that the presence of terminal α-mannosidyl residues on cell surface glycoproteins such as galactosyltransferase may be the determining factor in agglutination of cells by concanavalin A.

Much attention has been focused on the mechanism of concanavalin A agglutination (Podolsky *et al.*, 1974), and especially on the ability of this lectin to preferentially agglutinate transformed and mitotically active cells (Moscona, 1971; Podolsky & Weiser, 1973; Sharon & Lis, 1972). However, past studies have been unable to correlate agglutination with the presence of a specific cell surface glycoprotein receptor. Studies to isolate a concanavalin A-specific cell surface receptor have not attempted to demonstrate a difference between agglutinable and non-agglutinable cell types (Allan *et al.*, 1972; Jansons *et al.*, 1973; Wray & Walborg, 1971), in spite of evidence for the specificity of concanavalin A for D-mannosyl and D-glucosyl residues in binding activity (Goldstein *et al.*, 1965; So & Goldstein, 1968). The problem has been further complicated by reports that show apparent equivalent binding of conjugated lectin to agglutinable and non-agglutinable cell types (Cline & Livingston, 1971; Ozanne & Sambrook, 1971).

In the present study the process of concanavalin A agglutination of rabbit erythrocytes was examined. It has been possible to isolate a cell surface glycoprotein from rabbit erythrocytes which binds concanavalin A and is able to confer agglutinability to non-agglutinable erythrocytes (Podolsky *et al.*, 1974). This glycoprotein has been found to have galactosyltransferase–exogenous acceptor (UDP-galactose–*N*-acetylglucosamine β-4-galactosyltransferase) activity (Podolsky *et al.*, 1974). The ability to enhance concanavalin A agglutination by this enzyme was found to be dependent on terminal D-mannosyl residues in the enzyme structure and not on its enzymic activity.

Materials and Methods

Chromatography and electrophoresis

Descending paper chromatography was done on Whatman 3MM paper with the following solvent systems: A, pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by vol.; 24h); B, butanol–ethanol–water (10:1:2, by vol.; 116h); C, butanol–pyridine–water (12:5:4, by vol.; 16h). Reducing sugars were detected on the chromatograms by the alkaline–$AgNO_3$ reaction (Trevelyan *et al.*, 1950).

Paper electrophoresis was conducted with 1% (w/v) sodium tetraborate buffer on Whatman 3MM paper. Electrophoresis was carried out for 90min with a current of 150–250mA and a voltage of 1000V.

Analytical polyacrylamide-gel electrophoresis was carried out by using 4.5% (w/v) polyacrylamide in

1.5M-Tris–HCl buffer, pH8.9. The buffer chamber contained 5mM-Tris–glycine buffer, pH8.3. Samples of volume 50μl were applied to the gels and then the gels were subjected to electrophoresis for 190min at a current of 4mA/gel. Protein was stained with Coomassie Blue, and carbohydrate was detected by using the periodic acid–Schiff reagent (Steck *et al.*, 1970).

Preparation of erythrocytes

Human erythrocytes (types A, B, AB and O) and rabbit erythrocytes (New Zealand White rabbits) were collected in 0.1M-Tris–HCl–0.154M-NaCl, pH8.0 (buffer A) to which EDTA was added to a final concentration of 2mM. Erythrocytes were washed twice in buffer A and then three times in 0.1M-sodium cacodylate–0.154M-NaCl, pH7.4 (buffer B).

Purification of rabbit erythrocyte galactosyltransferase

Live New Zealand White rabbits (approx. 2.3kg each) were bled by cardiac puncture after being rendered unconscious by intraperitoneal injection of 10% (w/v) sodium thiamylal (Surital; Parke, Davis, Detroit, Mich., U.S.A.). Blood was collected in buffer B+2mM-EDTA and membrane 'ghosts' were prepared as described by Steck *et al.* (1970). The membranes were washed three times in 0.1M-Tris–HCl buffer, pH7.9, containing 25mM-KCl, 20mM-$MgCl_2$ and 0.1mM-UDP-galactose and suspended finally in 10ml of this buffer. The suspension was sonicated in nine 15s bursts by using a Branson S-75 Sonifier at a power setting of 6. At no time did the temperature of the suspension exceed 10°C. After sonication this material was centrifuged at 105000*g* for 60min. The supernatant, termed 'soluble enzyme', contained approx. 50% of the total detected enzyme activity.

A column (2.5cm×15cm) of concanavalin A–Sepharose (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was poured and washed with 50 bed volumes of buffer A containing 5mM-$MnCl_2$. The solubilized enzyme prepared from 250ml of packed rabbit erythrocytes was then passed through the column twice in a volume of 100ml of buffer A containing 5mM-$MnCl_2$ at a flow rate of 30ml/h. Subsequently, the column was washed with 25 bed volumes of buffer A containing 5mM-$MnCl_2$ to remove non-specifically bound protein. When no more protein could be eluted with this buffer as detected by u.v. absorption at 280nm the adherent material was eluted with buffer A containing 1.5mM-EDTA–0.1M-α-methyl D-mannoside. When no more material could be eluted, the eluate was made to 10mM-$MnCl_2$ and then dialysed against buffer A at 4°C for 24h.

UDP-Sepharose was a gift from Dr. Robert Hill of Duke University (Durham, N.C., U.S.A.). The gel was stored at 4°C in 25mM-sodium cacodylate, pH7.4, containing 5mM-EDTA, 1mM-2-mercaptoethanol and 0.02% NaN_3. The gel was regenerated by washing with 10 bed volumes each of 1M-NaCl, 6M-urea, water and finally buffer A+25mM-$MnCl_2$. The non-diffusable material eluted from the concanavalin A–Sepharose column was applied twice to the column (1cm×5cm) of UDP-Sepharose in a volume of 45ml at a flow rate of 40ml/h. The column was subsequently washed with 25ml of the application buffer and adherent material was then eluted in 40ml of buffer containing 25mM-sodium cacodylate–0.04M-NaCl, pH7.4, 1.5M-urea, 10mM-2-mercaptoethanol, 5mM-*N*-acetyl-D-glucosamine and 25mM-EDTA. The eluted material was then dialysed against buffer A at 4°C for 24h and concentrated by using an Amicon ultrafiltration system with an AM-30 membrane (Amicon Corp., Lexington, Mass., U.S.A.).

Assay of galactosyltransferase activity

Endogenous activity was measured by incubating 0.1ml of enzyme source in buffer A with 0.01ml of 112μM-UDP-D-[1-^{3}H]galactose (New England Nuclear Corp., Boston, Mass., U.S.A.; 0.71mCi/mmol) or 0.01ml of 87.6μM-UDP-D-[U-^{14}C]galactose (New England Nuclear Corp.; 227mCi/mmol). The assay mixture was made to 20mM-$MnCl_2$ and incubated at 37°C for 60min. The reaction was terminated by addition of 5% (w/v) phosphotungstic acid–2M-HCl. The precipitate was collected on a glass-fibre filter, washed twice with 5% phosphotungstic acid–2M-HCl and subsequently washed three times with ethanol. The retained material was placed in a vial containing 10ml of a toluene-based scintillation-counting solution [4g of 2,5-diphenyloxazole, 50mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene, made with Liquiflour (New England Nuclear Corp.)] and counted for radioactivity in a liquid-scintillation spectrometer. Counting efficiency was checked with appropriate standards as well as against an external standard to correct for quenching. Efficiency for counting ^{3}H was 33% and that for ^{14}C was 90%.

Exogenous activity was defined as the ability to transfer galactose from UDP-galactose to an exogenously supplied acceptor. Three exogenous acceptors were used: *N*-acetyl-D-glucosamine, galactose-free thyroglobulin glycopeptide, and fetuin from which terminal sialic acid and penultimate galactose residues had been removed.

Galactosyltransferase–exogenous acceptor assay mixtures were identical with the endogenous acceptor assay above except as follows: when *N*-acetyl-D-glucosamine was used as acceptor, 0.2μmol of this sugar was added to the reaction mixture and the product

was isolated from the acid-precipitated supernatant by means of high-voltage paper electrophoresis. The product, *N*-acetyl-D-[*galactosyl*-^{14}C]lactosamine, was detected by using a Packard model 7201 radiochromatogram scanner. It co-chromatographed with *N*-acetyl-D-lactosamine obtained as a gift from Dr. Roger Jeanloz (Massachusetts General Hospital, Boston, Mass., U.S.A.). The product was further identified by acid hydrolysis and chromatography (Weiser, 1973). When thyroglobulin glycopeptide was used as an exogenous acceptor, 25μl of the glycopeptide in buffer A containing 0.2μmol of acceptor sites was added to the incubation mixture. In most assays, fetuin minus terminal sialic acid and penultimate galactose residues (SGF-fetuin) was used to assay exogenous acceptor–galactosyltransferase activity and, except where otherwise noted, all values for exogenous acceptor assays refer to this activity. When used, SGF-fetuin was added in a volume of 25μl containing 0.2μmol of acceptor sites.

Preparation of exogenous acceptors

Thyroglobulin was obtained from Nutritional Biochemical Co. (Cleveland, Ohio, U.S.A.) and glycopeptide acceptor was prepared as described by Spiro & Spiro (1968) from the unit B glycopeptide of thyroglobulin.

Terminal sialic acid and penultimate galactose residues were removed from fetuin by a modification of the method of Kim *et al.* (1971). Fetuin (1g; Gibco, Grand Island, N.Y., U.S.A.) was suspended in 50ml of 0.1M-H_2SO_4 and incubated at 80°C for 50min. The hydrolysate was neutralized by the addition of 1M-NaOH and then dialysed against water at 2°C. The sialic acid-free fetuin was then suspended in 65ml of 0.01M-sodium metaperiodate in 0.05M-sodium acetate buffer, pH4.5, and incubated for 24h in the dark at 2°C. The reaction was stopped by the addition of 5ml of glycerol and the suspension was then dialysed against water. The non-diffusible material was freeze-dried and then suspended in 0.15M-$NaBH_4$ and 0.15M-potassium tetraborate, pH8.0, for 13h at 4°C. The reaction was stopped by adjusting the pH to 5.0 by addition of 1M-acetic acid and the material was then dialysed against water. The non-diffusible, galactose-free fetuin was then freeze-dried and the amount of galactose released was determined by assaying for liberated neutral hexose. The treated fetuin is termed SGF-fetuin.

Carbohydrate and amino acid composition of rabbit erythrocyte galactosyltransferase

Determinations of the carbohydrate and amino acid composition of the purified rabbit galactosyltransferase were made by g.l.c. Purified enzyme (400μg) was dialysed against water. Inositol (25μg) was added as an internal standard and the mixture was then freeze-dried. The freeze-dried material was suspended in 1.0ml of methanolic 1M-hydrochloride and incubated at 85°C for 60min. The material was then taken to dryness under N_2 and washed. Subsequently 0.1ml of stock solution containing trimethylsialylating agents (Syland HRP; Supelco Co., Bellefonte, Pa., U.S.A.) was added and the suspension was left at room temperature (24°C) for 60min. After being dried, material was extracted into 50μl of hexane for 30min, and 1μl of this extracted material was applied to a column (152.4cm×0.3cm) of OV-17 (0.1%; g.l.c. 110 support; mesh, 120–140; Supelco Co.); a Perkin–Elmer 900g.l.c. programmed over 75–250°C, with a temperature increment of 10°C/min was used. The results of this study were compared with measurements of sugar composition made by assaying the hydrolysed enzyme (6M-HCl; 90°C, 120min) for hexose and hexosamine as described below. Sialic acid composition was determined after hydrolysis of the intact enzyme with 0.1M-H_2SO_4 at 80°C for 60min.

Amino acid analysis was carried out on the residual material from the preparation made for carbohydrate determination by modification of the method of Zumwalt *et al.* (1970). After volatilization of the hexane, 0.5ml of 6M-HCl was added and the solution was then incubated at 110°C for 24h. Subsequently the hydrolysate was dried under N_2 at 50°C. The residue was incubated with 1ml of 3M-butyl hydrochloride at 100°C for 60min. After drying, 0.1ml of 25% (w/v) trifluoroacetic anhydride in dichloromethane was added and the reactants were heated to 100°C for 60min. After the reaction mixture was cooled, 1.5μl of the mixture was applied to a column (163.2cm×0.3cm) of ethylene glycol adipate (Tabsorb; Regis Chemical Co., Morton Grove, Ill., U.S.A.), programmed over 75–250°C with a temperature rise of 4°C/min. Also, a portion was chromatographed on a column (152.4cm×0.3cm) of OV-225 (0.3% on g.l.c. 110 support; mesh 100–120; Applied Science Laboratories Inc., State College, Pa., U.S.A.) programmed over 125–225°C with a temperature rise of 4°C/min.

Inactivation of rabbit galactosyltransferase

The purified enzyme was irreversibly inactivated by two methods. The protein was denatured by boiling the enzyme suspended in buffer A for 3min. The enzyme could also be inactivated by the addition of 5.0mg of *p*-chloromercuribenzoate (Sigma Chemical Co.) to 100μg of enzyme suspended in 2ml of buffer A, pH8.0. After leaving the mixture for 10min at room temperature the reactants were dialysed against buffer A. There was no detectable galactosyltransferase–fetuin acceptor activity after either of these treatments.

α-Mannosidase treatment of rabbit galactosyltransferase

Purified α-mannosidase from jack-bean meal was kindly donated by Dr. Yu-Teh Li of Tulane University (New Orleans, La., U.S.A.); the preparation was shown to be free of other glycosidase activity. This α-mannosidase was incubated with both intact rabbit galactosyltransferase and rabbit galactosyltransferase from which terminal sialic acid had been removed as described above. In both instances, 100μg of galactosyltransferase was suspended in 0.2ml of 0.05M-sodium citrate buffer, pH4.5, and 25μl of α-mannosidase (5.45 units, i.e. 5.45μmol of *p*-nitrophenyl α-D-mannoside/min at 24°C) in this buffer was added. Also 10μl of 0.025mM-$ZnSO_4$ and 50μg of cycloheximide (Upjohn Co., Kalamazoo, Mich., U.S.A.) were included and the mixture was incubated at 37°C for 68h. Over that incubation period samples (25μl) were removed from the incubation mixture and assayed for free D-mannose.

Identification of the released carbohydrate was determined by paper chromatography by using solvent systems B and C. Quantification of the released hexose was made by using a modification of the technique of Tarentino *et al.* (1970). This method involves a coupled hexokinase–pyruvate kinase–lactate dehydrogenase system with the oxidation of NADH (equivalent to the amount of free hexose) measured by the decrease in E_{340}. D-Mannose, obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A., was used as a standard.

Preincubation of human erythrocytes

Human erythrocytes, suspended 1:40 (v/v) in buffer A, were incubated at 37°C with rabbit galactosyltransferase or other glycoproteins for 15min in the presence of 5mM-$MnCl_2$. The erythrocytes were used immediately for agglutination studies after being washed several times with buffer A. Enzyme was shown to adhere to the human erythrocytes by assaying for galactosyltransferase–exogenous acceptor activity on the erythrocyte membrane after being washed with buffer A.

Assay for concanavalin A agglutination

Incubation mixtures included 0.1ml of cells suspended in buffer A (5×10^8 cells/ml for erythrocytes; 5×10^6 cells/ml for all others) and 0.01ml of 0.1M-$MnCl_2$. Concanavalin A (obtained as a thrice-crystallized powder from Miles–Yeda Ltd., Rehovoth, Israel), was present in a final concentration of 133μg/ml. Controls were carried out by using an equal volume of buffer without lectin. Mixtures were incubated at 37°C and scored at different time-intervals. Agglutination assays were scored from ± to ++++, the latter indicating total agglutination characterized by large clumps of cells with no single cells identifiable microscopically, as previously described (Moscona, 1971; Nicolson, 1972; Noonan *et al.*, 1973).

General assay techniques

Protein was assayed by the method of Lowry *et al.* (1951) with bovine serum albumin as standard (Sigma Chemical Co.). Neutral hexose was assayed by the phenol–H_2SO_4 method (Dubois *et al.*, 1956), and hexosamine was determined by using the Elson–Morgan assay (Blix, 1948). Ovalbumin and D-glucosamine were used as standards for the respective assays. Free sialic acid was determined by the thiobarbituric assay by using an *N*-acetylneuraminic acid standard (Warren, 1959).

Results

Enzyme purification

Rabbit erythrocyte galactosyltransferase was purified according to two schemes. In the first scheme the enzyme was solubilized by vigorous sonication and then further purified by preparative polyacrylamide-gel electrophoresis (scheme I). The results of this purification have been published previously (Podolsky *et al.*, 1974). All of the enzymic activity was localized in the erythrocyte membrane and sonication released approx. 40–50% of the measurable activity.

In the purification scheme reported here (scheme II) rabbit erythrocyte membrane galactosyltransferase was again solubilized by vigorous sonication. Subsequently the solubilized material was chromatographed on a column of concanavalin A–Sepharose and then on UDP-Sepharose (as described in the Materials and Methods section). The first three steps were similar to purification by scheme I and were repeatedly consistent in degree of purification and yield. It was found that 80% of the solubilized enzyme adhered to concanavalin A–Sepharose, resulting in minimal loss of enzyme with this step. Total yield at this step amounted to 37% of the total activity detected on whole cells. UDP-Sepharose chromatography yielded an enzyme preparation purified 620-fold relative to the membrane-bound enzyme, which agrees favourably with the results of purification scheme I (Podolsky *et al.*, 1974). A final yield of 33% was obtained. However, scheme II was found preferable, as it allowed the simultaneous purification of larger amounts of enzyme (maximum 400μg against approx. 30μg/gel for scheme I). The purified enzyme ran as a single (diffuse) band on analytical polyacrylamide gels ($R_F = 0.13$–0.18) and stained positive for either protein (Coomassie Blue) or carbohydrate (periodic acid–Schiff reagent).

Table 1. *Specificity of soluble galactosyltransferase*

The effect of the addition or elimination of various components of the galactosyltransferase–SGF fetuin acceptor activity was examined by using crude soluble enzyme (i.e. after sonication). Complete assay mixture included 100μl of crude soluble enzyme, 25μl of 0.1M-$MnCl_2$ and 10μl of 112μM-UDP-D-[1-^{3}H]galactose (0.71Ci/mmol). Final assay volume was 160μl containing 0.1M-sodium cacodylate+0.154M-NaCl, pH7.4.

Incubation conditions	Galactosyltransferase activity: Radioactivity (c.p.m.)	Galactosyltransferase activity: Activity remaining (%)
Complete assay mixture	1760	100.0
–$MnCl_2$	160	0.0
–Enzyme source	160	0.0
–Fetuin	140	0.0
+Galactose-free thyroglobulin glycopeptide	—	0.0
+Sialic acid-free fetuin	—	0.0
+Native fetuin	—	0.0
+*N*-Acetyl-D-glucosamine	1310	73.0
+25μl of Triton X-100 (0.33mg)	1680	95.5
+25μl of concanavalin A (25μg)	890	50.7

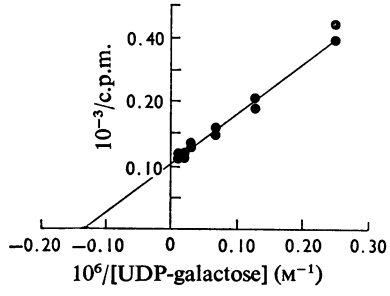


Fig. 1. *Lineweaver–Burk plot of galactosyltransferase–SGF-fetuin acceptor activity with various concentrations of UDP-galactose*

Complete assay mixture was as described for Table 1. The enzyme preparation after step 4 (concanavalin A–Sepharose) was used (40μg/0.1ml per assay). Nucleotide sugar was added in a volume of 0.01ml. For concentrations higher than 0.65μM, UDP-D-[1-^{3}H]galactose was diluted with non-radioactive UDP-galactose (Sigma Chemical Co.) to yield the appropriate concentrations. K_m was calculated to be 7.5×10^{-6}M.

Properties of the purified enzyme

Various kinetic characteristics of the purified enzyme from scheme II were determined. As with the previous purification (Podolsky *et al.*, 1974) a broad pH optimum (pH7.4–7.7) was observed. Again, the enzyme was found to have an absolute

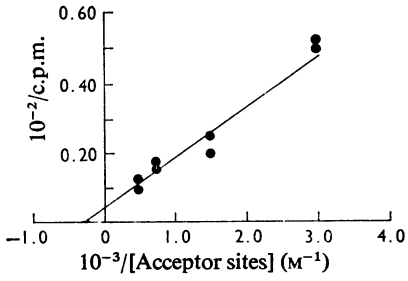


Fig. 2. *Lineweaver–Burk plot of galactosyltransferase–SGF-fetuin acceptor activity with various concentrations of acceptor*

Conditions were as described in Fig. 1 and Table 1. SGF-fetuin acceptor was added in a volume of 25μl containing the appropriate concentration of acceptor sites. K_m was calculated to be 4.0×10^{-3}M.

Table 2. *Amino acid analysis of rabbit erythrocyte galactosyltransferase*

Chromatography of amino acid derivatives (see the Materials and Methods section) was carried out by use of a Perkin–Elmer 900 g.l.c. with an injection-port temperature of 200°C and a flame-ionization detector at 250°C. N_2 was used as carrier with a flow rate of 30–40ml/min and a pressure of 345kPa. Quantification was accomplished by using a chromatodigital integrator. No internal standard was included in the derivatization reaction. Values have therefore been determined relative to total protein as determined by protein assay. Values were determined by chromatography on ethylene glycol adipate column as described in the Materials and Methods section, except where shown.

Amino acid	Residues/1000 amino acids
Glycine	91.0
Valine	81.0
Alanine	90.0
Isoleucine	37.5
Leucine	106.5
Proline	66.0
Threonine	65.9
Serine	83.0
Phenylalanine	35.3
Asparagine	133.0*
Glutamic acid	100.5*
Lysine	57.3*
Tyrosine	0.2*
Arginine	—
Methionine	—
Hydroxyproline	—

* Values were determined by chromatography on columns of both ethylene glycol adipate and OV-225 as described in the Materials and Methods section.

requirement for Mn^{2+} (Table 1), with maximum activity being achieved at 5mM-Mn^{2+}. The enzyme was found to use both SGF-fetuin and *N*-acetyl-D-glucosamine as acceptor, but was inactive towards prepared thyroglobulin glycopeptides (Table 1). No endogenous acceptor activity could be detected. Triton X-100 did not affect the activity, but concanavalin A caused a 50% inhibition of enzyme activity (Table 1). Lineweaver–Burk (1934) plots were used to determine the K_m for both UDP-galactose and exogenous acceptor sites (SGF-fetuin). These studies showed a K_m (UDP-galactose) of 7.5×10^{-6}M and K_m (SGF-fetuin) of 4.0×10^{-3}M (Figs. 1 and 2). These values are in good agreement with those obtained for other galactosyltransferases (Kim *et al.*, 1972; Caccam & Eylar, 1970). All the radioactivity incorporated into fetuin acceptor by the enzyme co-chromatographed with genuine galactose in two solvent systems (A and B).

Enzyme structural analysis

Amino acid and carbohydrate composition were determined by g.l.c. Results of the amino acid analysis are given in Table 2. No internal amino acid analysis was included in the hydrolysate, so that values are calculated on the basis of an overall amount of protein of 400μg. As indicated, there was a large amount of asparagine in the protein structure (14%), consistent with the suggestion of asparagine-type glycoprotein linkages.

Also consistent with an asparagine-type linkage is the finding of significant amounts of *N*-acetyl-D-glucosamine by g.l.c. (Table 3). The enzyme was found to also contain significant amounts of D-mannose, D-galactose and lesser amounts of *N*-acetylneuraminic acid and L-fucose. No *N*-acetylgalactosamine was detected by g.l.c. (D-Glucose was detected by g.l.c., but was subsequently shown to be a contaminant derived from the dialysis tubing.) Neutral hexose, hexosamine and sialic acid content were also determined by chemical assay (Table 3). Values obtained by these methods agreed to within 15% with those obtained by g.l.c. These observations suggest that galactosyltransferase contains approx. 15% carbohydrate by weight.

The ability of the enzyme to adhere to concanavalin A–Sepharose and the finding that it contains D-mannose suggested that the enzyme interacts directly with concanavalin A, possibly through a D-mannosyl residue. Direct interaction of the enzyme with concanavalin A was also suggested by the ability of concanavalin A to inhibit enzymic activity when rabbit erythrocyte galactosyltransferase was incubated with the lectin before assay for galactosyltransferase–SGF-fetuin acceptor activity (Table 1). The latter observation, however, could also have been due to lectin binding the fetuin substrate.

Factors responsible for concanavalin A agglutination

Rabbit erythrocyte galactosyltransferase, purified according to scheme II, still demonstrated an apparent direct interaction with concanavalin A as previously described for the other enzyme preparation (Podolsky *et al.*, 1974). In accordance with the report of Sumner & Howell (1936) human erythrocytes, type O, were found to be non-agglutinable with concanavalin A. However, after preincubation with the rabbit galactosyltransferase purified by scheme II the cells showed strong agglutination with concanavalin A, as shown in Plate 1. The enzyme was shown, both by direct assay of the human

Table 3. *Carbohydrate composition of rabbit erythrocyte galactosyltransferase*

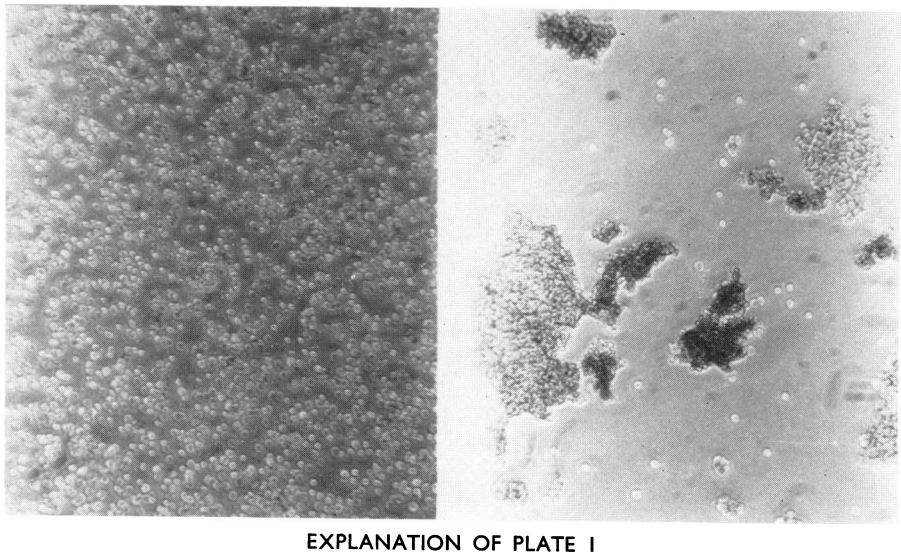
G.l.c. of purified galactosyltransferase was carried out as described in the Materials and Methods section and in the legend of Table 2. G.l.c. estimations were made relative to inositol as internal standard. Neutral hexose was determined by the phenol–H_2SO_4 method (Dubois *et al.*, 1956), hexosamine by the Elson–Morgan reaction (Blix, 1948) and sialic acid by the thiobarbituric acid assay (Warren, 1959).

Carbohydrate	Amount (μg/mg of protein)
L-Fucose	1.6
N-Acetylneuraminic acid	22.3
D-Galactose	71.0
N-Acetyl-D-glucosamine	42.5
N-Acetyl-D-galactosamine	0.0
D-Mannose	66.0
Neutral hexose	137.0
Hexosamine	51.0
Sialic acid	20.0

Table 4. *Inactivation of rabbit erythrocyte galactosyltransferase*

Galactosyltransferase (25μg) (prepared as described in scheme II) was treated by heat (100°C, 3min) or with 7M-*p*-chloromercuribenzoate (24°C, 10min). The inactivated enzyme was then dialysed overnight against 0.1M-sodium cacodylate+0.154M-NaCl, pH7.4, at 4°C. Enzymic activity was assayed by using SGF-fetuin (0.2μM acceptor in 25μl) acceptor as described in the Materials and Methods section.

	Galactosyltransferase activity (c.p.m.)
Purified enzyme (untreated)	12800
p-Chloromercuribenzoate-treated enzyme	140
Heat-treated enzyme	210
–Enzyme	160



EXPLANATION OF PLATE 1

Concanavalin A agglutination of galactosyltransferase-coated human erythrocytes

Human erythrocytes (5×10^8 cells/ml) were incubated with concanavalin A (133 μg/ml) at 37°C for 30min; 1.5mM-$MgCl_2$ was included in the incubation mixture. (*a*) Cells preincubated with buffer only; (*b*) cells preincubated with purified rabbit erythrocyte galactosyltransferase enzyme.

(*Facing p.* 218)

Table 5. *Effect of enzyme inactivation on the ability of rabbit erythrocyte galactosyltransferase to confer concanavalin A agglutinability to human erythrocytes*

Galactosyltransferase was prepared and inactivated as described in Table 4. Cells were preincubated with either unheated or treated enzymes as described in the Materials and Methods section and concanavalin A agglutinability was subsequently assayed. Scoring of agglutination was from ±, no agglutination, to ++++, total agglutination (large clumps of 100–200 cells), and was always compared with a control mixture containing suspensions of similar cell concentrations (1×10^8 cells/ml). The purified enzyme alone did not agglutinate cells.

Preincubation conditions	Incubation time (min) ...	Concanavalin A agglutination				
		15	30	60	90	120
Purified enzyme (untreated)		+++	+++	++++	++++	++++
p-Chloromercuribenzoate-treated enzyme		++	+++	++++	++++	++++
Heat-inactivated enzyme		++	+++	++++	++++	++++

Table 6. *Effect of α-mannosidase treatment on ability of rabbit erythrocyte galactosyltransferase to confer concanavalin A agglutinability*

Purified galactosyltransferase (prepared as described in Table 1) was incubated with α-mannosidase as described in the Materials and Methods section. After 68h incubation the galactosyltransferase was dialysed, preincubated (15min) with human erythrocytes, and concanavalin A agglutinability was determined. Scoring of agglutination is described in the Materials and Methods section as well as in Table 5.

Preincubation conditions	Incubation time (min) ...	Concanavalin A agglutination				
		15	30	60	90	120
Purified enzyme (untreated)		+++	+++	++++	++++	++++
Purified enzyme after α-mannosidase treatment		±	±	±	±	±
Purified enzyme+heat-inactivated α-mannosidase		+++	+++	++++	++++	++++

erythrocytes and by disappearance of galactosyltransferase activity from the supernatant, to adhere to the cell surface. The enzyme alone did not cause erythrocyte agglutination except in the presence of concanavalin A.

As shown in Table 1, treatment with concanavalin A decreased rabbit erythrocyte galactosyltransferase activity. We therefore sought to determine whether enzymic activity *per se* was required for the enzyme protein to confer agglutinability. Purified galactosyltransferase was inactivated either by heat treatment or by incubation with *p*-chloromercuribenzoate (Table 4). However, enzyme inactivated by either treatment was still able to confer agglutinability to erythrocytes in the presence of concanavalin A (Table 5). Although the presence of the inactivated galactosyltransferase on the human erythrocyte membrane could not be demonstrated directly by enzymic assay, this seemed evident, since the inactivated treated enzyme conferred erythrocyte agglutinability in the presence of concanavalin A.

These results suggested that the ability of the rabbit erythrocyte galactosyltransferase to confer concanavalin A agglutinability was in some manner related to the carbohydrate content of the enzyme. As noted above the enzyme was observed to contain significant amounts of D-mannose, a sugar which binds strongly to concanavalin A when present in a terminal α-glycosidic linkage. The existence of terminal α-linked mannose was confirmed by hydrolysis of the intact enzyme by a specific α-mannosidase, an enzyme capable of hydrolysing only α-linked terminal mannose residues. Incubation with α-mannosidase for 68h liberated 0.75 μmol of D-mannose/μmol of rabbit galactosyltransferase. The material released by α-mannosidase moved as a single spot which co-chromatographed with mannose in two solvent systems (solvent systems B and C). The control, incubation of α-mannosidase alone, demonstrated that no free hexose was found to be liberated from α-mannosidase itself after 68h of incubation.

The effect of α-mannosidase treatment on the ability of rabbit erythrocyte galactosyltransferase to confer concanavalin A agglutinability was investigated. As indicated in Table 6, removal of terminal α-mannose residues eliminated the ability of the galactosyltransferase enzyme to confer concanavalin A agglutinability. However, transferase incubated with heat-inactivated α-mannosidase still retained the ability to confer agglutinability. Together these findings suggest that the ability of rabbit erythrocyte galactosyltransferase enzyme to confer concanavalin A agglutinability is due to the presence of terminal α-linked mannose in its structure.

Discussion

The present observations attempt to explain the mechanism for the direct participation of galactosyltransferase–SGF-fetuin acceptor enzyme in concanavalin A agglutination of rabbit erythrocytes. In a previous communication it was shown that the purified enzyme alone could confer concanavalin A agglutinability to previously non-agglutinable human erythrocytes (Podolsky *et al.*, 1974). However, it is noteworthy that other glycoproteins extracted from the rabbit erythrocyte membrane were not able to confer concanavalin A agglutinability (Podolsky *et al.*, 1974). It was also shown that human immunoglobulin M (IgM), a glycoprotein that binds to concanavalin A, did not promote concanavalin A agglutination even though IgM could be shown to adhere to the surface of human erythrocytes.

The experiments described in the present paper demonstrate that the ability of galactosyltransferase to confer concanavalin A agglutinability was not related to enzymic activity, but required the presence of terminal α-linked mannose residues. The present results could indicate that the occurrence of glycoproteins with terminal α-linked mannose residues is evidence of incomplete glycoproteins on the cell surface of mature cells. Alternatively, these observations may indicate that mannose can be present as a terminal sugar on completed glycoproteins. The possibility that the enzyme is a strongly adherent glycoprotein from plasma cannot be excluded; however, repeated washings did not release enzyme activity from the rabbit erythrocyte and only vigorous sonication led to release of the enzyme. The observation that only 40–50% of the detected enzymic activity could be released after repeated sonication may be due to the localization of the enzyme within the cell membrane or related to a decrease in enzyme activity after the removal of other cell membrane components.

The finding of terminal α-linked mannose residues in a glycoprotein which can confer concanavalin A agglutinability is consistent with the known binding characteristics of concanavalin A. Indeed such a glycoside is found to be the most potent inhibitor of concanavalin A binding in the hapten inhibition test (Goldstein *et al.*, 1965). However, the observation that the existence of these residues is integrally related to concanavalin A agglutinability has several implications with regard to the various theories of concanavalin A agglutination.

To reconcile the present results with the topographic explanation of concanavalin A agglutination (Nicolson, 1972), the rabbit galactosyltransferase would be required to adhere to the human erythrocyte in some specific clustered distribution on the cell surface. The observation that neither IgM nor the α-mannosidase-treated enzyme was found to confer agglutinability, in contrast with the inactivated enzyme, indicates that the distribution of the concanavalin A receptor on the cell surface may not be a determining factor for concanavalin A agglutination. It is possible that the ability of the rabbit galactosyltransferase to confer concanavalin A agglutinability is related instead to its ability to induce some change in inherent concanavalin A-binding sites on the human erythrocyte membrane. However, this latter explanation does not seem likely since mere removal of terminal mannose residues renders the glycoprotein enzyme incapable of inducing erythrocyte agglutination.

The present results do not support the hypothesis of concanavalin A agglutination of cells based solely on quantitative differences in concanavalin A-binding sites. Presumably Concanavalin A binds to many cell surface glycoproteins, as noted above. Recent reports indicating quantitative differences of concanavalin A-binding sites based on temperature give evidence for a distinction in types of binding (Noonan & Burger, 1973*a*). Noonan & Burger (1973*b*) and Noonan *et al.* (1973) reported increased binding of concanavalin A at 0°C by agglutinable cells relative to their non-agglutinable counterparts. At higher temperatures (37°C) other workers have found little difference in concanavalin A binding by agglutinable and non-agglutinable cells (Cline & Livingston, 1971; Malluci, 1971; Ozanne & Sambrook, 1971). Perhaps the binding studies carried out at 37°C include types of concanavalin A binding (e.g. 'loose binding' to several cell surface glycoproteins), which because of their nature cannot lead to agglutination. The residual binding at 0°C may represent 'strong binding' of concanavalin A to special cell surface glycoproteins containing terminal mannose and it is possible that such binding may account for agglutination.

The present results suggest that a primary or major difference between concanavalin A-agglutinable and concanavalin A-non-agglutinable cells may be the occurrence of peculiar glycoproteins which contain terminal α-linked mannose residues. Although other glycoproteins exist on the cell surface which can potentially bind concanavalin A, only glycoproteins with terminal α-mannose residues bind the lectin firmly enough to permit cell agglutination. In the agglutination process cell–cell adhesion would presumably involve the cross-linking of concanavalin A with glycoproteins containing terminal mannose. Such a model is consistent with the present data and provides the simplest experimentally testable explanation for the process of concanavalin A agglutination. Whether the present findings are applicable to cells other than rabbit erythrocytes remains to be determined. Previous work from this laboratory (Podolsky *et al.*, 1974) and other preliminary experiments suggest that this may be the case.

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