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COMPARISON OF ISOLATED PEANUT AGGLUTININ RECEPTOR GLYCOPROTEINS FROM HUMAN, BOVINE AND PORCINE ERYTHROCYTE MEMBRANES

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Summary

Affinity chromatography has been used to isolate and compare the peanut agglutinin receptors from neuraminidase-treated human, bovine and porcine erythrocyte membranes. Passage of Triton X-100-solubilised membrane material through either Sepharose- or acrylamide-based affinity columns resulted in the reversible binding of receptor molecules to the immobilised lectin. Elution with 0.2 M galactose released specifically bound glycoprotein fractions, the composition and molecular weights of which were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.

Carbohydrate analysis by gas chromatography identified these bound glycoprotein fractions as the major sources of the *O*-glycosidic-linked disaccharide galactosyl-β-(1 → 3)-*N*-acetylgalactosamine in these membranes. It is suggested that these isolated fractions represent a discrete population of glycoproteins within the membranes studied, which possess both *O*-glycosidic- and *N*-glycosidic-linked carbohydrates.

Introduction

The use of lectins as probes for the investigation of cell membrane glycoprotein carbohydrates is well established [1]. Lectins can be grouped into subclasses which bind *O*-glycosidic- or *N*-glycosidic-linked saccharide chains [2] and from their known binding specificities, sugar structures and sequence on cell surfaces can be inferred [3]. Fluorescent lectin derivatives as well as derivatives containing heavy metals have been used to demonstrate directly the distribution of receptors on cell surfaces [4], while radioactive labelling

has allowed the estimation of their numbers [5].

There is, however, little information available about the distribution of carbohydrate structures within individual species of glycoproteins from cell membranes. Affinity chromatography, using immobilised lectins, has proved a valuable tool in the isolation of glycoconjugates [4] and has in recent years been increasingly applied to the isolation of membrane glycoproteins [6]. One particularly interesting lectin is peanut agglutinin which has a reported high affinity for α- and β-linked terminal galactose and in particular for the disaccharide structure, galactosyl-β-(1 → 3)-*N*-acetylgalactosamine (Gal-β-(1 → 3)-GalNAc) [7]. This disaccharide is the reported immunodominant structure of the Thomsen Friedenreich antigen [8] which appears to be ubiquitous in nature and is normally found in the cryptic form substituted by one or two sialic acid moieties [9]. After neuraminidase treatment, this structure was determined chemically and serologically in glycoprotein mixtures derived from several membrane sources [10]. Carter and Sharon [11] used immobilised peanut agglutinin to isolate a receptor molecule from solubilised neuraminidase-treated human erythrocyte membranes which was tentatively identified as asialoglycophorin. Recently, it has been shown by the use of an affinity cross-linking reagent, that on intact neuraminidase-treated human red cell surface, peanut agglutinin reacts with more than one glycoprotein species [12].

This study is a comparison of the isolated and purified peanut agglutinin receptor glycoproteins from the plasma membranes of human, bovine and porcine erythrocytes and a demonstration that these molecules represent a major source of protein-bound Gal-β-(1 → 3)-GalNAc in these membranes.

Materials and Methods

Peanut agglutinin was extracted from fresh peanuts [13] and purified by affinity chromatography on acid-treated Sepharose [14]. The lectins from *Helix pomatia* and *Canavalia ensiformis* (concanavalin A) were obtained commercially (Medac GmbH, Hamburg), and the agglutinin from *Tridacna squamosa* (clam) was purified by the method previously described for *Tridacna maxima* [14]. A sialoglycoprotein-rich fraction [15], prepared from human erythrocyte ghosts using lithium diiodosalicyclic acid (Eastman Kodak), was a generous gift from Mr. F. Hanisch of this department. Without further purification, the glycoprotein fraction was desialylated (50 mM H_2SO_4 at 80°C for 1 h), and after extensive dialysis against 0.1 M acetic acid at 4°C the glycoproteins were freeze-dried.

Analytical procedures

Protein was determined by using the method of Lowry et al. [16] or, in the presence of 1% non-ionic detergent, by the modification described by Dulley and Grieve [17]. Free sialic acid was assayed by using the method of Aminoff [18] after release from glycoproteins by hydrolysis in 50 mM H_2SO_4 at 80°C for 1 h. The monosaccharide composition of glycoprotein samples was determined by gas chromatography after total acid hydrolysis (0.6 M HCl at 100°C for 16 h), reduction and formation of the alditol acetate derivatives [19]. The disaccharide, Gal-β-(1 → 3)-GalNAc was determined by gas chromatogra-

phy after alkaline reductive cleavage (50 mM NaOH, 1 M $NaBH_4$ at 50°C for 20 h) as its trimethylsilyl derivative, comparison being made with an authentic reduced standard derived from a desialylated milk fat globule membrane glycopeptide [19]. Ouchterlony double-diffusion experiments were performed in a Tris/Ca^{2+} buffer system (10 mM Tris, 1 mM $CaCl_2$, 0.9% (w/v) NaCl, pH 7.3) containing 1.0% (v/v) Triton X-100 (BDH, Poole) and were allowed to proceed at 4°C for 24 h.

Preparation of immobilised peanut agglutinin derivatives

Peanut agglutinin was immobilised on CNBr-activated Sepharose 4B by using the method of March et al. [20] in the presence of 1.0 M D-galactose to protect the sugar binding site. A concentration of 2 mg peanut agglutinin/ml of swollen gel was found to give optimum binding and recovery in a test system using asialofetuin. Alternatively, the lectin was directly bound to glutaraldehyde-treated polyacrylamide hydrazide. Hydrazide Bio-Gel P-150 (Bio-Rad laboratories) (10 ml swollen gel) was incubated overnight at 37°C with 5% (v/v) glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.4, 100 ml). The gel was repeatedly washed, by resuspension and gentle centrifugation (1500 × *g* for 10 min), with degassed buffer at 4°C until the odour of glutaraldehyde had disappeared. Finally, the sedimented gel was resuspended in buffer (4 vol.) containing 1.0 M D-galactose and peanut agglutinin (4.0 mg protein/ml of packed gel) and incubated, with gentle mixing, at 22°C for 4 h. Unbound protein (45—50% of the original) was removed by washing in 0.9% (w/v) NaCl, 1.0 M D-galactose and excess aldehyde groups blocked by incubating the gel in 10 vol. of 100 mM Tris, 0.9% (w/v) NaCl, 1.0 M D-galactose buffer (pH 7.3) at 22°C for 4 h. Finally, the derivative was reduced with Tris/galactose/saline buffer (pH 7.3) containing 10 mM KBH_4 at 22°C for 30 min. Sepharose- and acrylamide-based affinity gels (approx. 10 ml) were packed in columns of 0.75 cm internal diameter and washed extensively with the Tris/Ca^{2+}/saline buffer (pH 7.3). When not in use, derivative-coated gels could be stored in Tris/Ca^{2+}/saline buffer, 0.5 M in D-galactose and 0.02% NaN_3, at 4°C for several weeks without significant loss of activity.

Isolation and solubilisation of erythrocyte membranes

In all experiments fresh citrated blood was used and the buffy coat was removed by aspiration after centrifugation (1500 × *g*, 10 min). Neuraminidase-treated erythrocytes were prepared by incubating phosphate-buffered saline-washed packed cells (10 ml) with 1 ml (1 U) of neuraminidase (Behringwerke AG) in 2 vol. of phosphate-buffered saline, pH 5.6, containing 1 mM $CaCl_2$ at 37°C for 2 h. Parallel controls were run, in which cells were incubated in buffer without neuraminidase. After treatment, cells were thoroughly washed in 0.9% (w/v) NaCl and ghosts prepared principally by using the method of Dodge et al. [21] except that a Tris/saline buffer system (pH 7.3) was used throughout. Isolated membrane pellets (approx. 2 ml) were dispersed in 10 ml of buffer containing 10 mM Tris, 1 mM $CaCl_2$, 0.9% NaCl, 1% Triton X-100, 2 mM phenylmethanesulphonyl fluoride and 1% ethanol (buffer A) and homogenised by ultrasonic sound (five bursts of 30 s) at 0°C. The turbid suspension was centrifuged (100 000 × *g*, 60 min) and the clear super-

natant used immediately for affinity chromatography or frozen (−20°C) until required.

Affinity chromatography and isolation of bound glycoproteins

Sepharose- or polyacrylamide-based peanut agglutinin affinity columns (bed volume approx. 10 ml) were pre-equilibrated in buffer A at 4°C and solubilised membrane extracts (30 ml) were applied. A flow rate of 6 ml/h was maintained and the effluent was continually recycled at 4°C for 16 h. Unbound material was then removed by washing with buffer A, after which the same buffer containing 0.2 M D-galactose was applied. Fractions (2 ml) were automatically collected and aliquots (100 μl) assayed for protein. The protein-containing fractions which eluted after the application of D-galactose were pooled (approx. 20 ml) and extensively dialysed against 0.1 M acetic acid at 4°C. The dialysates were freeze-dried and the resulting oily residues extracted three times with $CHCl_3/CH_3OH$ (2 : 1, v/v) to remove residual detergent and lipids. The air-dried protein residues were used directly for further analysis. Samples of solubilised membrane not subjected to affinity chromatography were treated identically.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Before electrophoresis, delipidated proteins (1—2 mg), dissolved in a sample buffer system containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol (1 ml), were treated for 10 min in boiling water. Flat bed electrophoresis was performed according to the method of Laemmli [22] in 7.5% acrylamide gel, 0.1% in SDS. Gels were fixed and stained with Serva blue G (Serva, Heidelberg), formally called Coomassie blue G250, or alternatively with periodic acid Schiff reagent by the method described by Maurer [23]. Apparent molecular weights were estimated by comparison of electrophoretic mobilities with protein standards of known molecular weight.

Results and Discussion

The binding of peanut agglutinin to either Sepharose 4B or polyacrylamide beads (Materials and Methods) proved equally effective in producing affinity chromatography media suitable for the isolation of detergent-solubilised membrane-derived receptor glycoproteins. The preparation of these affinity gels is rapid, and bound peanut agglutinin shows no loss of specificity or binding potential in the presence of the non-ionic detergent Triton X-100, comparing favourably with the reported properties of this lectin immobilised by other methods [24]. Affinity chromatography of solubilised erythrocyte membranes, after neuraminidase treatment, enabled the isolation of membrane components by specific lectin-receptor complex formation. Prolonged washing with starting buffer, high salt concentration (2.0 M) and changes in pH (4.0—8.0) failed to dissociate these complexes. Only with dilute solutions (0.2 M) of D-galactose or D-lactose were the bound components eluted from the peanut agglutinin affinity column (Fig. 1). Specifically bound material was extensively dialysed and prepared as described in Materials and Methods; the defatted protein residues were analysed by flat bed polyacrylamide gel electrophoresis

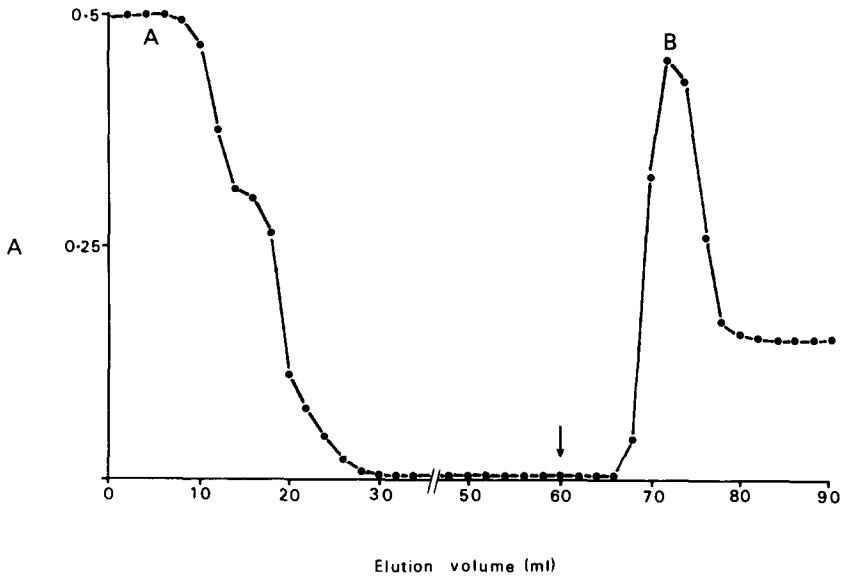

Fig. 1. Fractionation of Triton X-100-solubilised neuraminidase-treated erythrocyte ghosts by affinity chromatography using immobilised peanut agglutinin. Solubilised membrane proteins (Materials and Methods) were applied to columns (approx. 10 ml) of Sepharose 4B or Hydrazide Bio-Gel P-150 containing 1—2 mg bound peanut agglutinin/ml bed volume. Unbound membrane proteins were removed by washing with starting buffer and bound material was subsequently eluted by the addition of 0.2 M D-galactose (arrow) to the elution buffer. Aliquots (100 μl) of fractions (2 ml) were individually assayed for protein using the procedure of Lowry et al. [16] as modified by Dulley and Grieve [17].

in the presence of SDS and after acid hydrolysis or alkaline reduction for carbohydrate composition by gas chromatography.

Treatment of neuraminidase-treated human erythrocyte ghosts with buffer containing 1% (v/v) Triton X-100 (Materials and Methods) solubilised 55—60% of the membrane proteins. Affinity chromatography of this material resulted in the retention and subsequent elution from the column of 4.5% of the detergent-extracted proteins. Parallel treatment of native erythrocyte membrane resulted in the same amount of protein being dissolved but no subsequent binding to the column. Electrophoretic comparisons of starting and affinity-isolated materials are shown in Fig. 2A and B (lanes 1a and 1b), stained for protein and carbohydrate, respectively. A major component, with an apparent mean molecular weight of 80 000, and a minor component, with an apparent molecular weight of 100 000, show a high degree of purification and relative concentration compared to the starting material. By comparison with an authentic standard (Materials and Methods), the major component was tentatively identified as an asialoglycophorin A dimer (Fig. 3A and B, lanes 2 and 3). The discrepancy observed between the migration of the affinity-isolated component and the acid-desialylated standard probably results from the differences in sialic acid content, as not all sialic acid residues on membrane-integrated glycoproteins are accessible to neuraminidase [25]. Previously, the asialo derivative of glycophorin isolated by the method of Marchesi and Andrews [15] has been shown, in the absence of detergent, to bind to immobilised peanut agglutinin [26]. Asialoglycophorin A has also been identified as

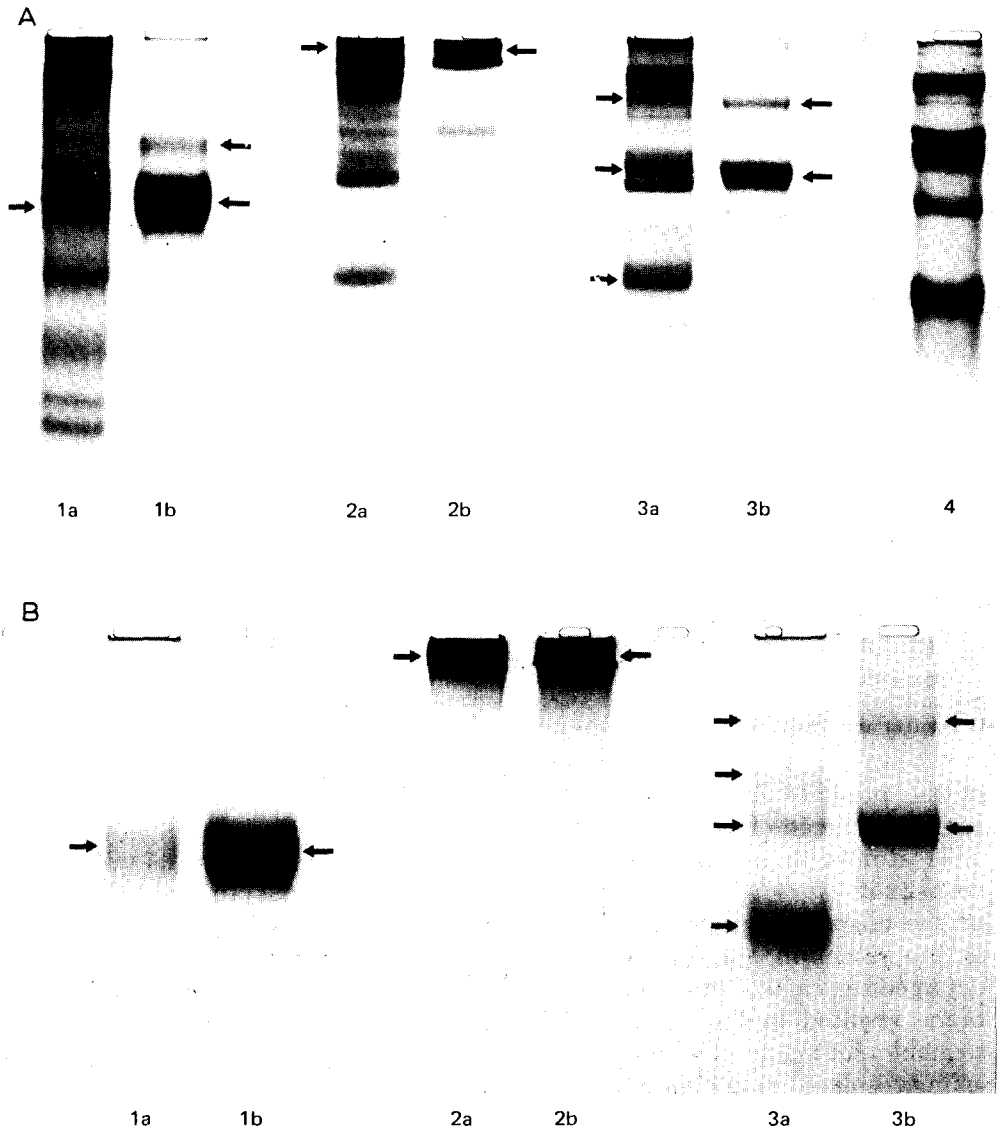

Fig. 2. (A) Polyacrylamide gel electrophoresis of solubilised membrane proteins from neuraminidase-treated human (1), bovine (2) and porcine (3) erythrocyte ghosts, comparing starting material (a) and affinity-isolated components (b). Lane 4 shows molecular weight standards (from the top; myosin, 200 000 daltons; β-galactosidase, 130 000 daltons; phosphorylase B, 95 000 daltons; bovine serum albumin, 68 000 daltons; and ovalbumin, 43 000 daltons). For affinity-isolated fractions, 10 μg of protein were used, for complex mixtures of proteins 20—30 μg of protein were used. The 7.5% acrylamide gel (containing 0.1% SDS) was stained for protein with Serva blue G. Bands marked by arrows correspond to periodic acid Schiff positive bands in B. (B) Polyacrylamide gel electrophoresis of solubilised membrane proteins from neuraminidase-treated human (1), bovine (2) and porcine (3) erythrocyte ghosts, comparing starting material (a) and affinity-isolated components (b). Conditions were as described for A except that approximately twice as much protein was applied to the gel and after fixation gels were stained for glycoproteins with periodic acid Schiff reagent. Bands marked by arrows correspond to marked protein components in A.

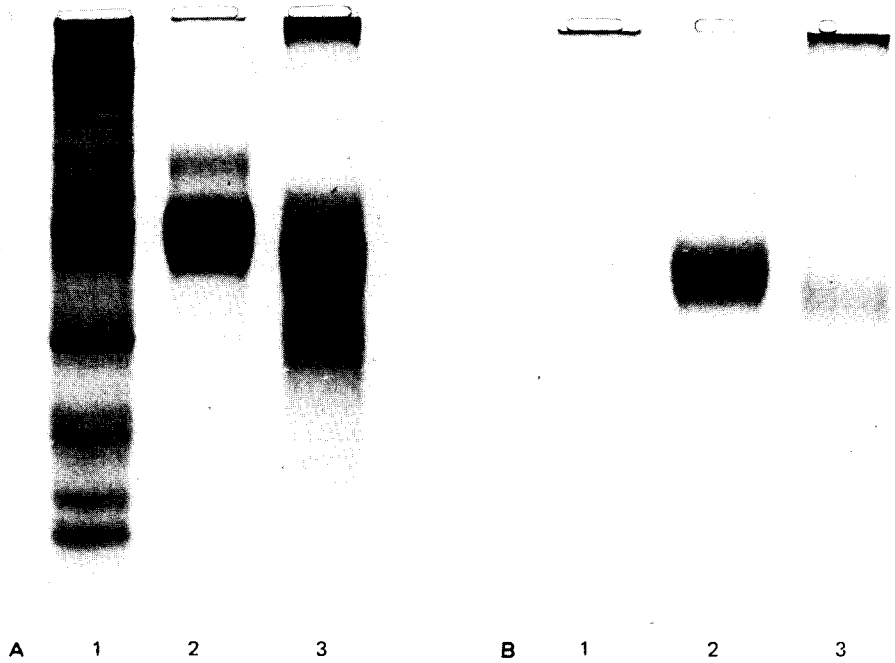

Fig. 3. (A) Polyacrylamide gel electrophoresis of solubilised neuraminidase-treated human erythrocyte ghosts (lane 1), peanut agglutinin affinity-isolated components (lane 2), and acid-desialylated glycophorin A (lane 3). Conditions were as described for Fig. 2A. 7.5% acrylamide gels containing 0.1% SDS were stained for protein with Serva blue G. (B) Polyacrylamide gel electrophoresis of solubilised neuraminidase-treated human erythrocyte ghosts (lane 1), peanut agglutinin affinity-isolated components (lane 2), and acid-desialylated glycophorin A (lane 3). Conditions were as described for Fig. 2A except that twice as much protein was applied to the gel. Gels were stained with periodic acid Schiff reagent.

one of the peanut agglutinin receptors isolated from solubilised neuraminidase-treated human erythrocyte ghosts by affinity chromatography [11], and was subsequently shown to be the major molecular species to which the lectin binds on the surface [12]. In contrast to previous findings [11,12], the monomeric form of asialoglycophorin A (mol. wt. 40 000—41 000) was not detected in this study, even in polyacrylamide gels containing 0.4% SDS (results not shown). This may be attributed to a greater tendency for the asialo form of glycophorin A to aggregate under the conditions of isolation and subsequent treatment employed.

The minor glycoprotein species (apparent mol. wt. 100 000) has not previously been demonstrated as a peanut agglutinin receptor glycoprotein from this membrane [11,12]. The possibility exists that this molecular species is a contaminant resulting from incomplete dissociation of protein-protein interactions by the detergent. However, the relatively large amounts obtained and the relative lack of other contaminating proteins (Fig. 2A and B, lane 1b), plus the fact that the molecule is a glycoprotein (see Fig. 3B, lane 2) suggests its validity as a true alternative receptor molecule. Although the use of high concentrations of SDS in the gels failed to promote detectable dissociation of the 100 000 dalton band (results not shown), it could still represent a stable

TABLE I

DISTRIBUTION OF CARBOHYDRATE RESIDUES BETWEEN UNBOUND AND BOUND (AFFINITY-ISOLATED) GLYCOPROTEINS DERIVED FROM NEURAMINIDASE-TREATED SOLUBILISED ERYTHROCYTE MEMBRANES

Gal-β-(1 → 3)-GalNHol, galactosyl-β-(1 → 3)-*N*-acetylgalactosaminitol; N.D., not detected.Values expressed as μmol/mg protein. Each result is the average of at least two determinations, reproducibility was within 10%.

Sugar residues	Human erythrocyte		Bovine erythrocyte		Porcine erythrocyte	
	Unbound	Bound	Unbound	Bound	Unbound	Bound
Sialic acid	0.005	0.02	0.07	0.04	trace	0.02
Fucose	0.041	n.d.	0.036	trace	0.021	n.d.
Galactose	0.08	0.310	0.100	0.851	0.094	0.082
Glucose	trace	n.d.	0.023	n.d.	trace	n.d.
Mannose	0.031	0.093	0.024	0.101	0.021	0.064
N-Acetylgalactosamine	0.071	0.267	0.01	0.310	0.021	0.177
N-Acetylglucosamine	0.051	0.142	0.04	0.296	0.028	0.075
N-Acetylgalactosaminitol	n.d.	0.019	n.d.	n.d.	n.d.	n.d.
Gal-β-(1 → 3)-GalNHol	trace	0.220	trace	0.151	trace	0.096

aggregate. For example, this protein may be a tetrameric form of asialoglycophorin B, the native monomeric form of which has a reported molecular weight of approx. 25 000 [27]. Like glycophorin A, the N-terminus of glycophorin B is rich in carbohydrates, the overall composition of which appears to be similar to that of glycophorin A [28].

Monosaccharide analyses, by gas chromatography, of bound and unbound material derived from human erythrocyte membranes are shown in Table I. The affinity-isolated receptor molecules possess monosaccharides characteristic of both *O*-glycosidic-linked and *N*-glycosidic-linked carbohydrate units, whereas the monosaccharide composition of the unbound material indicates the presence of predominantly alkaline stable units. Alkaline borohydride treatment of the two fractions released *N*-acetyl-D-galactosaminitol and the reduced disaccharide, Gal-β-(1 → 3)-GalNAc from the affinity-isolated glycoprotein

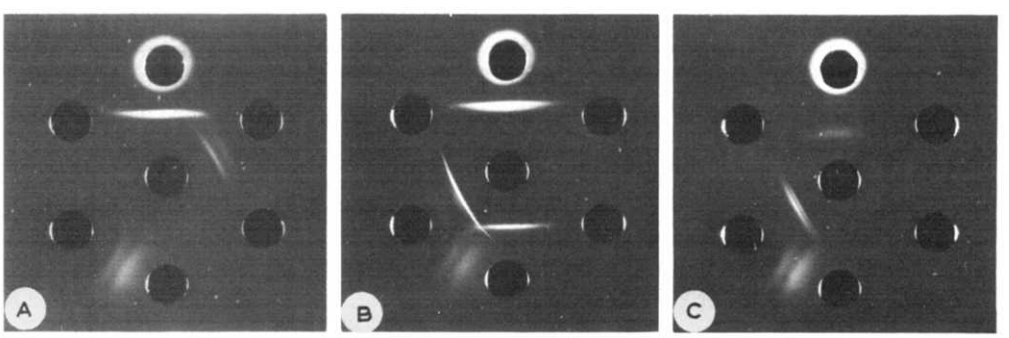

Fig. 4. Double-diffusion experiments in 1% (w/v) agarose gels containing 1% (v/v) Triton X-100. Peanut agglutinin affinity-isolated membrane glycoproteins (1 mg/ml) from (A) human, (B) bovine and (C) porcine erythrocytes (centre wells) were tested for receptor activity against different lectins. In clockwise order (12 o'clock = No. 1), the lectins (2 mg/ml) were peanut agglutinin (1), *Helix pomatia* (2), concanavalin A (4) and *Tridacna squamosa* (5).

fraction, whereas only small amounts of the latter sugar were released from unbound material after similar treatment (Table I), indicating that the peanut agglutinin-isolated fraction represents a major source of Gal-β-(1 → 3)-GalNAc in the human erythrocyte membrane.

Interaction between the affinity-isolated glycoprotein and the lectins from the peanut and *H. pomatia* were detected by double-diffusion experiments (Fig. 4A), corroborating the results of gas chromatographic analysis after alkaline reduction.

Glycopeptides derived from the MN blood group active glycoprotein of the human erythrocyte membrane are known to possess the *O*-glycosidic-linked disaccharide Gal-β-(1 → 3)-GalNAc substituted by sialic acid [29,30], but the presence of *O*-glycosidic-linked *N*-acetyl-D-galactosamine was not conclusively demonstrated. After neuraminidase treatment of a sialoglycoprotein-rich fraction derived from these membranes, Glöckner et al. [10] were able serologically to detect *H. pomatia* receptors, but on the basis of chemical evidence concluded that these were *N*-glycosidic-linked. The absence of a reaction between the isolated glycoproteins and the lectin, concanavalin A (Fig. 4A), although mannose was detected by gas chromatography, may be explained in terms of steric hindrance from the *O*-glycosidic-linked disaccharide substituents on the glycoproteins. Fukuda and Osawa [31] have previously reported an enhancement of reactivity between concanavalin A and isolated glycophorin after alkaline borohydride treatment. Alternatively, the substitution patterns of the mannosyl units present in the core region of the *N*-glycosidic-linked carbohydrate groups do not produce the bi-antennary structure required for the strongest binding with concanavalin A [32,33].

The lectin from *T. squamosa* reportedly binds galactose β-(1 → 4)- and (1 → 6)-linked to *N*-acetylglucosamine [34]. Consequently, the absence of a precipitate between this lectin and the glycoprotein fraction suggests the presence in the latter of galactose-β-(1 → 3) linked to *N*-acetylglucosamine at the non-reducing termini of alkali-stable carbohydrate groups, as has been suggested to exist on a glycopeptide fragment derived from glycophorin A [35].

Treatment of native and neuraminidase-treated bovine erythrocyte ghosts with buffers containing 1.0% Triton X-100 resulted in solubilisation of 50—55% of the total membrane proteins in both cases, but only with the neuraminidase-treated sample was 5.0% of the solubilised protein retained and eluted from the peanut agglutinin affinity column with dilute sugar solutions. The bound material represented a partially purified high molecular weight glycoconjugate (apparent molecular weight greater than 200 000) which was excluded from 7.5% polyacrylamide gels (Fig. 2A and B, lanes 2a and b). Some co-chromatographing contaminants were evident in the bound material, but their relative concentrations were low. Electrophoresis in gels of lower acrylamide concentration (4.0%) and in the presence of buffer containing higher concentrations of SDS (0.4%) failed to allow the bound glycoprotein fraction to migrate into the gel or to induce dissociation of the material into subunits, respectively. Similar properties have been reported previously [36] for a major sialoglycoprotein extracted from native bovine erythrocyte membranes.

The distribution of carbohydrate between the bound and unbound material derived after peanut agglutinin affinity chromatography of solubilised, neuraminidase-treated bovine erythrocyte membranes is shown in Table I. As demonstrated with the human system, the affinity-isolated glycoprotein fraction contains a significant proportion of the glycoprotein-bound Gal-β-(1 → 3)-GalNAc. Emerson and Kornfeld [36] failed to detect the sialic acid substituted form of this carbohydrate in a highly purified sialoglycoprotein extracted with lithium diiodosalicyclic acid from native bovine erythrocytes, but concluded from monosaccharide analysis of the total alkaline borohydride-released oligosaccharide fraction, that in this glycoprotein the *O*-glycosidic-linked carbohydrates were in the form of more complex structures comprising galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine. It is of interest, however, that the same investigators were able to identify this disaccharide indirectly on the intact bovine erythrocyte membrane by binding studies using the I^{125}-labelled lectin from *Agaricus bisporus* which requires galactose-β-(1 → 3)-*N*-acetylgalactosamine → serine (threonine) for binding [37]. Direct chemical detection of *O*-glycosidic-linked Gal-β-(1 → 3)-GalNAc was made by gas chromatography after desialylation of a crude glycoprotein fraction extracted from bovine erythrocytes [10]. Probably, the peanut agglutinin affinity-isolated glycoprotein fraction described in this report represents the partially desialylated derivative of the purified sialoglycoprotein of Emerson and Kornfeld [36]. The fact that no direct analytical procedure for the detection of desialylated reduced saccharides was employed by these authors might explain their failure to recognise galactosyl-β-(1 → 3)-*N*-acetylgalactosaminitol in the alkali-labile carbohydrate fraction.

The affinity-isolated glycoprotein fraction from bovine erythrocyte membranes reacts with peanut agglutinin but not with the lectin from *H. pomatia*, which requires *O*-glycosidic-linked *N*-acetylgalactosamine (Fig. 4B). The desialylated glycoprotein fraction gave a strong precipitate with concanavalin A, complementing the gas chromatographic analysis and indicating that the possible factors preventing the binding of concanavalin A, which are suggested to exist in the case of the human erythrocyte peanut agglutinin receptor, are absent in this system. The reactivity between the bovine-peanut agglutinin receptor fraction and the lectin from *T. squamosa* contrasts with the behaviour of the human receptors, indicating that, unlike the latter, the bovine alkali-stable oligosaccharide structures have more reducing termini carrying galactose-β-(1 → 4) or -(1 → 6) linked to *N*-acetylglucosamine.

A treatment identical to that described for human and bovine systems, of erythrocyte ghosts prepared from fresh porcine blood, resulted in 60—65% solubilisation of the total membrane protein and the isolation, by peanut agglutinin affinity chromatography, of 5—6% of the solubilised material derived from neuraminidase-treated erythrocytes. Electrophoresis (Fig. 2A and B, lanes 3a and b), comparing starting material with bound, demonstrates that two glycoprotein species with apparent molecular weights of 80 000—85 000 and 160 000—170 000 are partially purified and concentrated by the affinity chromatography step. The 80 000—85 000 dalton and 160 000—170 000 dalton bands probably represent monomers and dimers, respectively, of a single glycoprotein species. As observed with the affinity-isolated glycoproteins

from human erythrocyte membranes, high (0.4%) SDS concentrations did not induce dissociation of the dimeric form (results not shown). A third, and possibly major, glycoprotein species which is clearly visible in the starting material (Fig. 2B, lane 3a) with an apparent molecular weight of 45 000–50 000 is not isolated by affinity chromatography. The molecular weight data suggest this may be a monomeric species of the 80 000—85 000 dalton component, but it is equally probable, in view of its absence in the affinity-isolated fraction, that this glycoprotein is a distinct molecular species lacking the peanut lectin receptor carbohydrate.

The carbohydrate analysis of porcine erythrocyte membrane peanut agglutinin receptors (Table I) reveals a generally lower level of sugar substitution than in the human and bovine cases. As with the previous two systems, the affinity-isolated fraction represents a major source of Gal-β-(1 → 3)-GalNAc when compared with the unbound material.

Positive reactions were obtained in double-diffusion experiments (Fig. 4C) with the lectins from peanut and *T. squamosa*, the latter showing two distinct precipitates, and negative reactions with *H. pomatia* lectin and concanavalin A, confirming the absence of *O*-glycosidic-linked *N*-acetylgalactosamine and suggesting that the mannosyl residues detected by gas chromatography are unsuitable as lectin receptors.

We report here that pre-treatment of erythrocyte membranes with neuraminidase is essential for the exposure of peanut agglutinin receptor sites on membrane-integrated glycoprotein molecules, which after solubilisation can be specifically isolated by affinity chromatography with the immobilised lectin. The demonstration that these bound glycoproteins possess a large part of the total *O*-glycosidic-linked galactosyl-β-(1 → 3)-*N*-acetylgalactosamine of the membrane is in agreement with the finding that the presence of the unsubstituted disaccharide structure on a glycoprotein is obligatory before binding to immobilised peanut agglutinin is possible (unpublished observations). Although the properties of the bound glycoprotein fraction from the three erythrocyte membranes vary considerably, gel electrophoresis and carbohydrate analysis indicate that they may represent a partially purified subgroup, within these membrane glycoprotein populations, which are substituted with both *N*- and *O*-glycosidic-linked carbohydrates. The traces of alkali-labile disaccharide identifiable in unbound material from all erythrocyte sources (Table I) may represent residual receptor molecules not complexed by the immobilised peanut agglutinin, however, it is possible that it resides on different, sparingly substituted glycoproteins. Parallel controls without neuraminidase pre-treatment showed no binding of solubilised glycoprotein material to the affinity column, suggesting the absence of unmasked Gal-β-(1 → 3)-GalNAc. This result is in contrast to those reported by Glöckner et al. [10] who were able to demonstrate exposed Gal-β-(1 → 3)-GalNAc chemically on a sialoglycoprotein-rich fraction prepared from phenol extracts of these membranes.

The biological relevance of this carbohydrate group on a subpopulation of erythrocyte membrane glycoproteins is not clear. The disaccharide is the reported immunodominant structure of the Thomsen Friedenreich antigen [8] the natural antibodies against which are responsible for the observed

agglutination of neuraminidase-treated red cells when they are returned to the serum from which they were derived. These antibodies presumably represent a sub-population of a larger family of naturally circulating anti-carbohydrate antibodies found in the sera of vertebrates [38]. Accordingly, the demonstration of the cryptic nature of this carbohydrate structure on solubilised glycoproteins extracted from native erythrocyte membranes is important because substitution by sialic acid theoretically affords protection against spontaneous complex formation with circulating serum antibodies. Human erythrocyte membrane glycoproteins carrying sialic acid-substituted Gal-β-(1 → 3)-GalNAc are involved in the designation of blood group M and N specificities [39] and may perform similar functions in animal systems. Springer et al. [40] have suggested that the T-antigen (synonymous with the Thomsen Friedenreich antigen) is a human breast carcinoma-associated antigen and it may be assumed that this serologically demonstrable change reflects differences in carbohydrates of the transformed epithelium cell membrane glycoproteins. Generally, transformation is associated with changes in the overall composition and properties of cell membrane glycoproteins [41]. Andersson and co-workers [42,43] have shown that there are distinct differences in the SDS gel patterns of leukaemic lymphocyte membrane glycoproteins dependent on the type and the level of differentiation of the cell line. More specifically, peanut agglutinin has been shown to be a marker for undifferentiated T lymphocyte sub-populations in mice [44]; to distinguish between mouse embryonal carcinoma cells and their differentiated derivatives [45] and to differentiate between normal and leukaemic cells [46].

We propose that the isolation of a sub-population of plasma membrane glycoproteins substituted with both *N*- and *O*-glycosidic-linked carbohydrate, as has been described in this report using immobilised peanut agglutinin, may prove a useful approach with scientific as well as clinical significance for the understanding of transformation-related changes in membrane glycoproteins.

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