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CARBOHYDRATE-CHAIN ANALYSIS BY LECTIN BINDING TO MIXTURES OF GLYCOPROTEINS, SEPARATED BY POLYACRYLAMIDE SLAB-GEL ELECTROPHORESIS, WITH *IN SITU* CHEMICAL MODIFICATIONS

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ABSTRACT

The interactions of five ^{125}I-lectins of differing binding-specificities with seven glycoproteins containing known carbohydrate chains were investigated on polyacrylamide gels after electrophoretic separation. The glycoproteins were also chemically modified in the gels in order to gather additional information. The glycoproteins used were: porcine thyroglobulin (PTG); bovine-serum fibronectin (BFN); human-serum transferrin (HTF), bovine fetuin (BFE); subfractionated, hen ovalbumins having galactosylated, hybrid-type, carbohydrate chains (OVG); agalactosyl, hybrid-type-carbohydrate chains (OVH); and high-mannose-type-carbohydrate chains (OVM). Wheat-germ agglutinin (WGA) stained BFE, OVG, and OVH, but removal of sialic acid from BFE resulted in loss of WGA binding. After desialosylation and Smith degradation *in situ*, BFE and HTF reacted strongly, and PTG slightly with WGA. *Ricinus communis* agglutinin I stained PTG, BFN, HTF, and BFE after removal of sialic acid. *Arachis hypogaea* (peanut) agglutinin reacted only with BFN after removal of sialic acid. *Lens culinaris* hemagglutinin bound only to PTG, and concanavalin A bound to PTG, BFE, HTF, OVH, and OVM. The results were consistent with previously proposed lectin–carbohydrate-binding specificities, indicating that lectins may be used to determine the carbohydrate-chain structures of particular glycoproteins, even in complex mixtures.

INTRODUCTION

The heterogeneous nature and relatively small quantities of cell-surface glycoproteins have been a major hindrance to elucidating the structure and function of these molecules, and only a few structures of cell-membrane glycoprotein-carbohydrate chains have been determined[1–5]. In order to overcome the difficulties of carbohydrate analysis using small quantities of membrane glycoproteins, it has been necessary to develop methods of examining the carbohydrate-chain structures of individual glycoproteins in heterogeneous mixtures. The detection by direct, radiolabeled-lectin staining of the carbohydrate chains of individual glycoprotein, after

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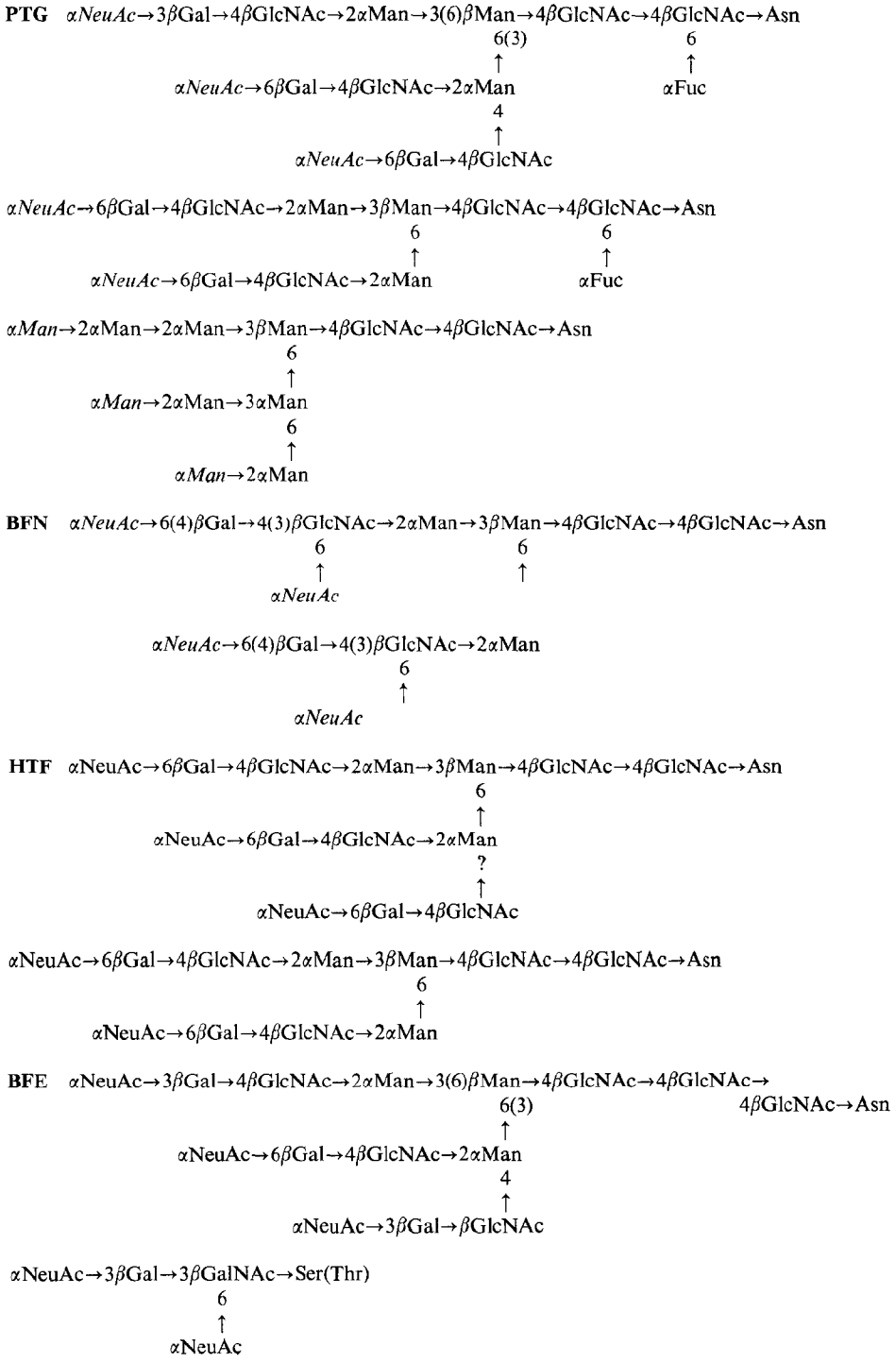
their separation by sodium dodecyl sulfate ($NaDodSO_4$)-polyacrylamide, slab-gel electrophoresis has been used for cellular, lectin-binding-component analysis[9–11]. This technique has now become more useful, since recent studies on the interactions of lectins with oligosaccharides or glycopeptides have demonstrated that certain lectins are capable of recognizing specific classes of saccharide chains[12–16]. Although the interactions of lectins with glycoproteins may be affected by many factors[17], accumulating evidence on lectin-binding-site specificities has enabled us to estimate some of the structural features of carbohydrate chains of glycoproteins by investigating their interactions with several, specific lectins. We report herein the binding of five lectins to seven different glycoproteins bearing known carbohydrate structures, after their separation by electrophoresis on $NaDodSO_4$-polyacrylamide gels. We also combined this technique with specific carbohydrate-chain chemical modifications *in situ*, in order to determine some carbohydrate structural characteristics of glycoproteins.

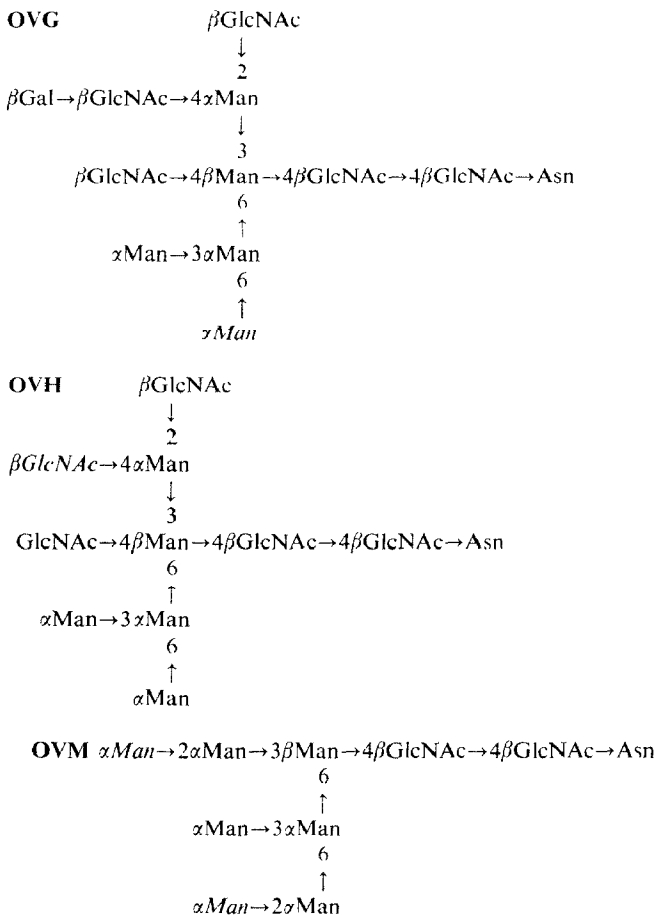
EXPERIMENTAL

Glycoproteins. — Porcine thyroglobulin (PTG), purchased from Sigma Chemicals Co. (St. Louis, MO 63178), was further purified by gel filtration on Sephacryl S-300 (Pharmacia, Uppsala, Sweden) in the presence of 0.5% $NaDodSO_4$. The major peak-fraction was pooled and stored at −20°. Bovine-serum fibronectin (BFN) or cold, insoluble globulin was purified according to Engvall and Ruoslahti[18] from calf serum (Irvine Scientific, Santa Ana, CA 92705). Human transferrin (HTF) and bovine fetuin (BFE) were purchased from Sigma Chemicals and used directly. Ovalbumin (Sigma, Grade V) was further subfractionated into four fractions on Con A–Sepharose (Pharmacia) according to Iwase *et al.*[19]. The fraction containing galactosylated, hybrid-type carbohydrate chains (denoted OA by Iwase *et al.*[19] and OVG in this paper), agalactosyl, hybrid-type carbohydrate chains (denoted OC by Iwase *et al.*[19] and OVH in this paper), and high-mannose-type carbohydrate chains (denoted OD by Iwase *et al.*[19] and OVM in this paper) were used. The structures of the carbohydrate constituents of these standard glycoproteins, as determined by Yamamoto *et al.*[20], Tsuji *et al.*[21], Takasaki *et al.*[22], Spik *et al.*[23], Montreuil[24], Nilsson *et al.*[25], Tai *et al.*[26,27], and Yamashita *et al.*[28] are given in Scheme 1.

Lectins. — Wheat-germ agglutinin (WGA) was purified according to Bloch and Burger[29]. *Ricinus communis* agglutinin I[30] (RCA_I) and *Arachis hypogaea* (peanut) agglutinin[31] (PNA) were purified as described previously, except that acid-treated Sepharose 6B was used instead of[32] Sepharose 4B or Sepharose 6B. *Lens culinaris* hemagglutinin (LCH), purified according to Sage and Green[33], was kindly provided by Dr. C. L. Reading of our department. Concanavalin A (con A) was purified according to Agrawal and Goldstein[34]. All lectins were iodinated with $Na^{125}I$ (New England Nuclear, Boston, MA 02118) and chloramine-T according to Burridge[9], and then purified on specific-affinity columns. Specific radioactivities of the ^{125}I-lectin ranged from 1 to 5 c.p.m./pmol. The carbohydrate-binding specifici-


PTG αNeuAc→3βGal→4βGlcNAc→2αMan→3(6)βMan→4βGlcNAc→4βGlcNAc→Asn
6(3)
6
αNeuAc→6βGal→4βGlcNAc→2αMan
αFuc
4
αNeuAc→6βGal→4βGlcNAc
αNeuAc→6βGal→4βGlcNAc→2αMan→3βMan→4βGlcNAc→4βGlcNAc→Asn
6
6
αNeuAc→6βGal→4βGlcNAc→2αMan
αFuc
αMan→2αMan→2αMan→3βMan→4βGlcNAc→4βGlcNAc→Asn
6
αMan→2αMan→3αMan
6
αMan→2αMan
BFN αNeuAc→6(4)βGal→4(3)βGlcNAc→2αMan→3βMan→4βGlcNAc→4βGlcNAc→Asn
6
6
αNeuAc
αNeuAc→6(4)βGal→4(3)βGlcNAc→2αMan
6
αNeuAc
HTF αNeuAc→6βGal→4βGlcNAc→2αMan→3βMan→4βGlcNAc→4βGlcNAc→Asn
6
αNeuAc→6βGal→4βGlcNAc→2αMan
?
αNeuAc→6βGal→4βGlcNAc
αNeuAc→6βGal→4βGlcNAc→2αMan→3βMan→4βGlcNAc→4βGlcNAc→Asn
6
αNeuAc→6βGal→4βGlcNAc→2αMan
BFE αNeuAc→3βGal→4βGlcNAc→2αMan→3(6)βMan→4βGlcNAc→4βGlcNAc→
6(3)
4βGlcNAc→Asn
αNeuAc→6βGal→4βGlcNAc→2αMan
4
αNeuAc→3βGal→βGlcNAc
αNeuAc→3βGal→3βGalNAc→Ser(Thr)
6
αNeuAc

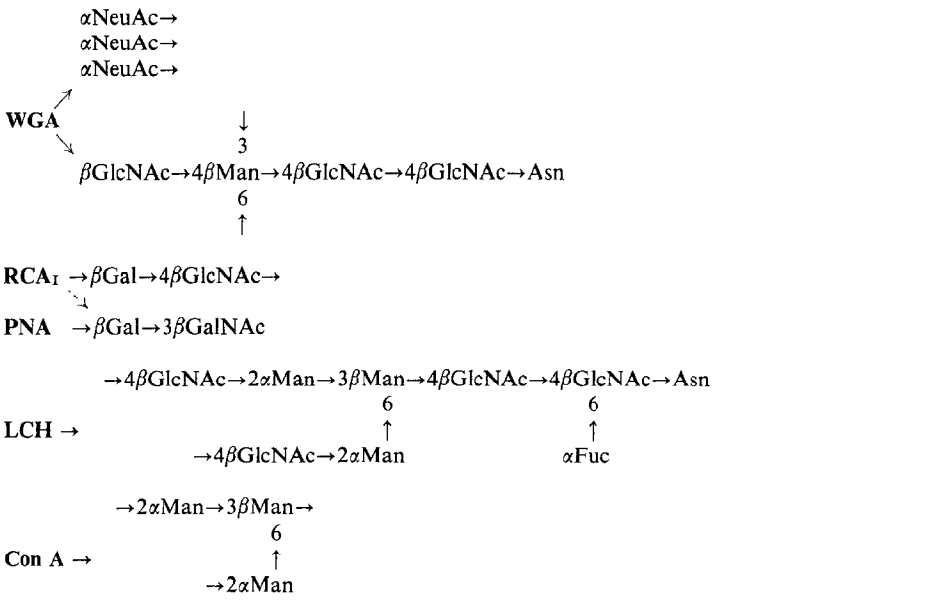
Scheme 1 (*continued*)



Scheme 1. Structures of the carbohydrate chains of the glycoproteins used in this study. There are minor differences due to incomplete glycosylation at the nonreducing, terminal ends (possible missing residues indicated in italics), and also some alternative linkage positions (indicated by parentheses). For further details, see original references.

ties of these lectins are presented in Scheme 2. Further details are described in the Results section.

Polyacrylamide-gel electrophoresis. — Electrophoresis was performed in $NaDodSO_4$ according to Laemmli[35] on 1.5-mm thick, 7% polyacrylamide, running gels with 4% stacking gels. Each standard glycoprotein was dissolved in 2% of $NaDodSO_4$, 1% of 2-mercaptoethanol, 0.5mM EDTA, 10% of glycerol, and 62.5mM tris-phosphate buffer (pH 6.8, sample buffer), at a final concentration of 50 μg/mL. Three glycoprotein mixtures containing 1 μg each of PTG, HTF, and OVG; BFN and OVH; BFE and OVM, respectively, were loaded onto the slab-gel along with a mixture of molecular-weight standards (myosin, M_r ~200 000; β-D-galactosidase,



Scheme 2. Interpretation of the binding specificities of lectins used in this study[12–17,40].

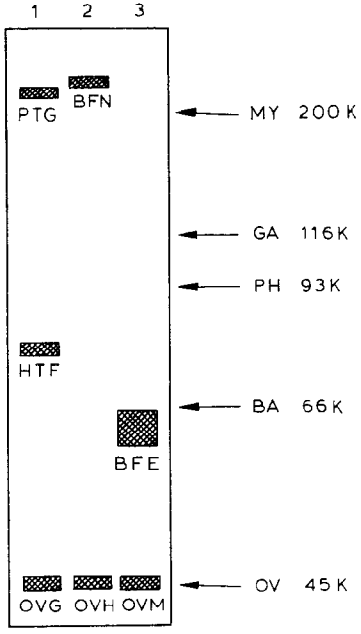


Fig. 1. Positions of migration of OVG, HTF, and PTG (lane 1); OVH and BFN (lane 2); and OVM and BFE (lane 3) after electrophoresis on 7% polyacrylamide gels in the presence of 1% $NaDodSO_4$: M_r markers are: myosin (MY), β-D-galactosidase (GA), phosphorylase b (PH), bovine-serum albumin (BA), and ovalbumin (OV).

M_r ~116 250; phosphorylase b, M_r ~92 500; bovine-serum albumin, M_r ~66 200; and ovalbumin, M_r ~45 000; Bio-Rad Laboratories, Richmond, CA 94804). Electrophoresis was performed under a constant current of 40 mA for ~4 h. The gels were stained with Coomassie Brilliant Blue R-250 (0.25 g/L in 10% acetic acid and 25% 2-propanol) overnight and destained with 10% acetic acid–10% 2-propanol at room temperature. The positions of these glycoproteins after electrophoresis on 7% gels are schematized in Fig. 1.

Chemical treatment of glycoprotein carbohydrate-chains in polyacrylamide gels.— Sialic acid was removed *in situ* by mild acid hydrolysis as described previously[36]. Briefly, a destained slab-gel was heated[37] in 50mM sulfuric acid (1 L) for 1 h at 80°, and washed repeatedly with a large excess of 50mM Tris·hydrochloride in 130mM sodium chloride buffer, pH 7.3. Smith degradation was performed under the conditions described by Irimura *et al.*[2] as follows: after electrophoresis, staining, destaining, and mild acid-hydrolysis, the polyacrylamide slab-gel was incubated in 75mM sodium periodate (150 mL) in 50mM sodium acetate buffer, pH 4.0, for 48 h at 4° in the dark. The reaction was stopped by washing the gel with 1% 1,2-ethanediol for 1 h. The gel was then treated with 0.1M sodium borohydride in 0.1M sodium borate buffer, pH 8.0, for 4 h at room temperature, stained with Coomassie Brilliant Blue R-250 (as the former stain had been bleached), destained, and processed for mild acid-hydrolysis under the same conditions as used for removal of sialic acid.

Reaction of polyacrylamide slab-gels with ^{125}I-labeled lectins. Polyacrylamide slab-gels, soaked in 50mM Tris·hydrochloride in 130 mM sodium chloride buffer, pH 7.4, were overlaied with a ^{125}I-labeled lectin solution (40 mL) in the same buffer containing 0.5% bovine-serum albumin (fraction V, Sigma). The mixture was incubated on a rocker platform (Bellco-Glass, Vineland, NJ 08360) for 2 h. In the case of con A, bovine-serum albumin was omitted. The concentrations of lectins used for staining were 0.1 μM for WGA and RCA$_I$, and 0.2 μM for PNA, LCH, and con A. The activity of the lectin solutions was assessed in each experiment by taking aliquots of ^{125}I-labeled lectin solution (20 μL) and counting the radioactivity bound to washed, packed human erythrocytes (150 μL); for PNA, neuraminidase-treated human erythrocytes were used[38]. The gels were repeatedly washed with 50mM Tris·hydrochloride in 130mM sodium chloride buffer, pH 7.4 (100 mL), until the supernatant solution contained < 100 c.p.m./mL. The gels were dried on filter paper (Whatman 3MM), and autoradiography was performed with Kodak X-O-Mat AR-5 X-ray film and intensifying screens at −70°.

RESULTS

Binding of WGA to standard glycoproteins. – Binding of ^{125}I-labeled WGA to the standard glycoproteins, after electrophoresis in 7% polyacrylamide gels, indicated that BFE, OVG, and OVH reacted with this lectin, whereas PTG, BFN, HTF, and OVM failed to react (Fig. 2). After sialic acid had been hydrolyzed from

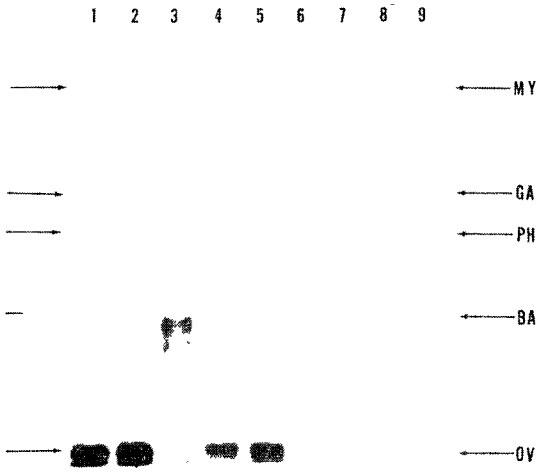


Fig. 2. Autoradiograph of $NaDodSO_4$–polyacrylamide gel stained with ^{125}I-WGA. Each lane contained 1 μg each of the same glycoproteins as described in legend to Fig. 1. Lanes 1, 4, and 7: OVG, HTF, and PTG; lanes 2, 5, and 8: OVH and BFN; and lanes 3, 6, and 9: OVM and BFE. Lanes 1–3, untreated and treated with ^{125}I-WGA; lanes 4-6, desialylated by mild acid-hydrolysis and treated with 125I-WGA; and lanes 7–9, desialylated, Smith degraded, and treated with ^{125}I-WGA. Molecular weight markers are the same as in Fig. 1.

the glycoproteins by heating the gels in the presence of mild acid, followed by washing and staining with ^{125}I-WGA, binding to BFE could no longer be detected, whereas the ovalbumins having hybrid-type-carbohydrate chains retained their WGA-binding properties (Fig. 2, Lanes 4–6). These results demonstrated that WGA binding to fetuin was mediated by sialic acid-containing carbohydrate chains, as suggested by Monsigny *et al.*[39] and Bhavanandan and Katlic[17] (Scheme 2). On the other hand, sialic acid was not involved in the interactions of ^{125}I-WGA with OVG and OVH. In this case, the terminal 2-acetamido-2-deoxy-β-D-glucopyranosyl group that is linked to a β-D-mannopyranosyl residue appeared to be important, as shown by Yamamoto *et al.*[15] using glycopeptides of known structures. Thus, comparing WGA-binding to the glycoproteins separated by gel electrophoresis, before and after mild acid-hydrolysis, enables distinction of the two different, outer carbohydrate chains that may form the combining sites for WGA.

In order to obtain further information on the carbohydrate core-structures, glycoproteins in polyacrylamide gels were desialosylated, subjected to Smith degradation, and tested with ^{125}I-WGA. The degradation products of HTF and BFE bound to ^{125}I-WGA, and the Smith degradation product of PTG bound slightly to ^{125}I-WGA (Fig. 2, Lanes 7–9), but ^{125}I-WGA did not bind to any of the other glycoproteins after these treatments. The three glycoproteins binding to ^{125}I-WGA share the common feature of having complex-type sugar chains with three outer branches (see Scheme 1). Although PTG contains this type of complex-sugar chain, its content is relatively low[20]. Smith periodate-degradation of the carbohydrate chains, after

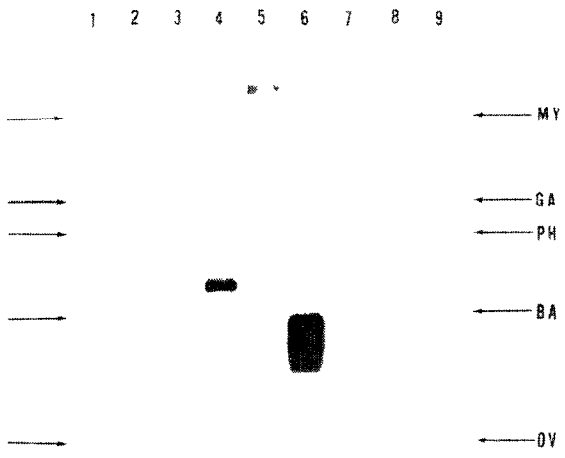


Fig. 3. Autoradiograph of NaDodSO$_4$–polyacrylamide gel stained with ^{125}I-RCA$_I$ or with ^{125}I-PNA. For content of lanes, see legend to Fig. 2. Lanes 1–3: untreated and treated with ^{125}I-RCA$_I$; lanes 4–6: desialylated by mild acid-hydrolysis and treated with ^{125}I-RCA$_I$; and lanes 7–9: desialylated by mild acid hydrolysis and treated with ^{125}I-PNA. Molecular weight markers are the same as in Fig. 1.

removal of sialic acid, is believed to produce a β-D-Glc*p*NAc-(1→2)-[β-D-Glc*p*NAc-(1→4)]-α-D-Man*p*-(1→6)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn structure that presumably interacts strongly with WGA. Conversely, Smith periodate-degradation of bibranched, complex-type, carbohydrate residues results in a β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn structure that does not interact strongly with WGA. OVH and OVM were not bound by WGA after Smith degradation. This result is consistent with the previous findings[15] that the trimannosylchitobiose core-structure of the asparagine-linked, carbohydrate chain is not bound to WGA. Smith degradation of OVG gave a β-D-Glc*p*NAc-(1→4)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→4)-Asn structure that did not react with WGA, as suggested by Yamamoto *et al.*[15].

Binding of RCA$_I$ and PNA to standard glycoproteins. — Prior to removal of sialic acid, PTG, BFN, and HTF bound to RCA$_I$ only weakly (Fig. 3, lanes 1–3). The nature of the binding of ^{125}I-RCA$_I$ to these sialic acid-containing glycoproteins is unclear; it may be explained either by the linkage between sialic acid and D-galactose residues[40], or by the existence of a small proportion of carbohydrate chains devoid of sialic acid. RCA$_I$ bound strongly to the standard glycoproteins containing complex-type, carbohydrate chains, such as PTG, BFN, HTF, and BFE, after sialic acid had been removed (Fig. 3, lanes 4–6). OVG showed the same low binding of ^{125}I-RCA$_I$ before and after mild acid hydrolysis. These results are consistent with our earlier finding[12] that RCA$_I$ is useful in the detection of complex-type, carbohydrate chains having a terminal Gal→GlcNAc sequence (Scheme 2). Although this experiment did not provide evidence for the precise specificity of RCA$_I$ and its apparent, lower binding-constant to Gal→GalNAc sequences[40], this

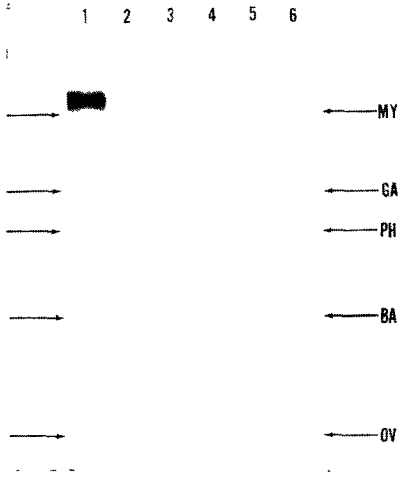


Fig. 4. Autoradiograph of NaDodSO4–polyacrylamide gel stained with ^{125}I-LCH or with ^{125}I-con A. For content of lanes, see legend to Fig. 2. Lanes 1–3: untreated and treated with ^{125}I-LCH; lanes 4–6: untreated and treated with ^{125}I-con A. Molecular weight markers are the same as in Fig. 1.

difference in specificity has been confirmed by Tsao and Kim[41]. Of the standard glycoproteins, PNA interacted only with BFE after removal of sialic acid (Fig. 3, lanes 7–9), which is consistent with our previous results[12] showing that PNA binds preferentially to the Gal→GalNAc sequence (Scheme 2), a conclusion confirmed by others[42]. Binding of this lectin to any of the standard glycoproteins, before removal of sialic acid, was not observed. Thus, the complementary labeling-profiles of RCA_I and PNA appear to be useful for determining whether the galactose-containing carbohydrate chains belong to the mucin- or complex-type as described earlier[12].

Binding of LCH and con A to standard glycoproteins. — LCH bound only to PTG among the standard glycoproteins (Fig. 4, lanes 1–3). This result is consistent with the finding of Kornfeld *et al.*[16] who showed that LCH binds to glycopeptides having two α-D-mannopyranosyl residues with free OH-3 and -4 groups, and an α-L-fucopyranosyl group linked to the 2-acetamido-2-deoxy-D-glucose residue linked to asparagine. As shown in Scheme 1, BFN and HTF contain such complex-type, carbohydrate chains, but do not contain an L-fucopyranosyl group linked to the core. BFE has an L-fucosyl-containing carbohydrate core, but with three outer branches. Con A interacted with PTG, HTF, OVH, and OVM (Fig. 4, lanes 4–6). From previous observations of binding to glycopeptides[13,14], con A is known to bind to high-mannose-type, agalactosyl hybrid-type, and complex-type carbohydrate chains having two outer branches that possess two α-D-mannopyranosyl residues with unsubstituted OH-3 and -4 groups (Scheme 2). Our results are consistent with these previous reports, except for BFN which bound con A only weakly. The reason for this behavior is unknown, but it is possibly due to the unusual content of sialic acid residues of the BFN carbohydrate chains[28].

DISCUSSION

Lectins have been widely used for the study of the structures and functions of cellular glycoproteins, but only until recently has the exact nature of the binding specificities of some lectins been elucidated. This report describes a procedure for estimating the structure of carbohydrate chains of individual glycoprotein-components, after separation by $NaDodSO_4$–polyacrylamide gel-electrophoresis. The technique presented herein is based on procedures described by others[6–9], but it is more refined, because (a) the staining conditions were carefully controlled to avoid nonspecific or low-affinity binding of ^{125}I-lectins, (b) lectin binding was with chemical modifications of the carbohydrate chains, and (c) the labeling of each glycoprotein was compared to the labeling of the same glycoprotein with ^{125}I-lectins having related carbohydrate-binding specificities. Thus, when concentrations of ^{125}I-con A 10 times higher than usual were used, not only glycoproteins not usually stained but simple proteins, loaded as molecular-weight markers, were weakly stained. It was also important to monitor the concentration and activity of the labeled lectins by measuring the binding to erythrocytes. The methods described herein have been useful for identifying the lectin-reactive molecules produced by cells, and for determining the types of carbohydrate chains of individual glycoprotein-components while in polyacrylamide gels. The major advantages of the procedure are that (a) it is applicable to virtually any mixture of glycoproteins that is soluble in $NaDodSO_4$; (b) the analysis may be performed in the presence of a large excess of nonglycosylated proteins; and (c) the chemical modifications are performed after electrophoresis, and the modified glycoproteins are identified at the position of electrophoretic migration. The technique has been applied successfully to the analysis of complex mixtures of glycoproteins from murine-melanoma cells that possess differing metastatic abilities[4,5].

When applying the lectin-labeling to mixtures of cellular glycoproteins, the following precautions must be heeded: (a) in the experiments described herein, the amount of standard glycoproteins was fixed at 1 μg, but the actual amounts of cellular glycoproteins may not be known. Thus, weakly stained bands in gels could result from either a low amount of a particular component, or a lower lectin-affinity for the carbohydrate chains. In this respect, it is extremely important to use relatively low concentrations of lectins in the staining procedures, in order to avoid binding to low-affinity receptors. Since the association constants of the interactions of known lectins with glycopeptide receptors were considered[14,44] $> 5 \times 10^6 M^{-1}$, lectin concentrations as low as 0.1–0.2 μM were used. However, even with such staining conditions, large amounts of low-affinity, glycoprotein-lectin receptors could be detected as positive binding. In this regard, comparison between the labelings by lectins having related specificities provided additional information. (b) The comigration of two different glycoproteins cannot be differentiated from the presence of a single glycoprotein containing multiple, heterogeneous carbohydrate chains. (c) The effect, on lectin binding, of multiple carbohydrate chains linked to a single polypeptide, is not yet fully understood. Although, in this study, only removal of sialic acid and Smith

degradation were applied, other chemical modifications are possible, such as removal of L-fucose and sialic acid by acid hydrolysis[12]. Alkaline degradation would be difficult owing to the instability of the polyacrylamide gel under alkaline conditions, but electrotransferring the glycoproteins to another membrane may be feasible.

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