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Lectin Affinity Chromatography of Cell Surface Proteins of Novikoff Tumor Cells

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Novikoff hepatocellular carcinoma cells were radioiodinated by a cell surface-specific method using lactoperoxidase/^{125}I. The iodinated proteins were solubilized in 0.5% Nonidet P-40 and subjected to affinity chromatography on Sepharose-conjugated lectins (Ricinus communis agglutinins I or II, soybean agglutinin, concanavalin A, or wheat germ agglutinin) and analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Almost all the iodinated proteins bound to one or more of the Sepharose-conjugated lectins, presumptive evidence that these peptides are glycosylated. Lectin affinity chromatography resolved defined subsets of iodinated glycoproteins and suggested that certain glycoproteins could be fractionated on the basis of heterogeneity of their heterosaccharide moieties. Incubation of the iodinated cells with neuraminidase resulted in increased binding of iodinated proteins to Sepharose-conjugated Ricinus communis agglutinins I and II and soybean agglutinin and decreased binding to Sepharose-conjugated wheat germ agglutinin. Binding of iodinated proteins to concanavalin A was unaffected by neuraminidase treatment of the cells. These studies demonstrate the utility of lectins for the multicomponent analysis of plasma membrane proteins.

Key words: **cell surface, plasma membrane, glycoproteins, affinity chromatography, lectins, Novikoff hepatocellular carcinoma, neuraminidase**

Transformation of epithelial cells to the malignant phenotype is accompanied by the acquisition of new surface properties that influence cell-cell adhesion and communication [1, 2]. These altered functional properties of the plasma membrane presumably result from alterations in the composition, structure, topography, and/or dynamics of cell surface components, including the plasma membrane glycoproteins [3, 4]. Lectins, sugar-binding

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proteins, have been used to probe the properties of cell surface glycoproteins of normal and malignant epithelial cells [5, 6]; for example, studies in this laboratory, using rat hepatocytes and Novikoff rat ascites hepatocellular carcinoma cells, showed that cell surface receptors for the lectin concanavalin A (Con A) exhibited differential lability to cleavage from the cell surface by papain, the Con A receptors of Novikoff cells being more labile [2, 7]. Although controlled proteolysis yielded valuable information regarding cell surface heterosaccharide moieties, it precluded the characterization of individual plasma membrane glycoproteins. The investigations reported here have been designed to identify and characterize the plasma membrane glycoproteins of Novikoff tumor cells. Cell surface proteins have been radiolabeled using a lactoperoxidase/^{125}I method and the glycoproteins resolved by affinity chromatography on carrier-bound lectins and by polyacrylamide gel electrophoresis.

METHODS

Tumor Cells

Novikoff hepatocellular carcinoma [8] cells were propagated in the ascitic form by transplantation in 6- to 9-week-old female Sprague Dawley rats (A. R. Schmidt, Inc., Madison, Wisconsin). Tumor cells were harvested 5–8 days following inoculation of 1 ml of ascitic fluid. Cells were washed (55g, 5 min) three times in phosphate-buffered saline (PBS), pH 7.4.

Lactoperoxidase-Catalyzed Iodination

Cells were iodinated by a modification of the method of Keski-Oja et al [9]. Briefly, cells were washed (50g, 5 min) with PBS containing 10^{-6} M KI. To the cell suspension (10^7 cells in 0.5 ml PBS, containing 10^{-6} M KI) in a 1-ml plastic tube (Fisher Scientific Co., Houston, Texas, Cat. No. 4-978-145) was added 10 μl of lactoperoxidase in PBS (1 mg/ml), 5 μl of D-glucose (D-Glc) oxidase in PBS (40 units/ml), 0.5 ml PBS containing 10 mM D-Glc and 1.5 mCi Na^{125}I. The tube was capped and placed on a tube rotator (Scientific Equipment Products, Baltimore, Maryland, Cat. No. 60448) at 20 rpm for 10 min at 23°C. The cells were washed (500g, 1 min) three times with PBS containing 5×10^{-3} M KI. Chemicals were obtained from the following sources: lactoperoxidase and D-Glc oxidase from Sigma Chemical Co., St. Louis, and Na^{125}I (17 Ci/mg) from New England Nuclear, Boston.

In some cases iodinated cells were treated with Vibrio cholera neuraminidase, a protease-free preparation obtained from Calbiochem, La Jolla, California. In such cases cells were washed two additional times with PBS containing 1 mM $CaCl_2$ and resuspended in 1 ml of the same buffer. To the iodinated cell suspension was added 0.1 ml of neuraminidase (500 units/ml in 0.05 M Na acetate buffer containing 0.9% NaCl and 0.1% $CaCl_2$, pH 5.5) or buffer alone. The tubes were capped and incubated at 37°C for 1 h on a tube rotator. Following incubation, the cells were washed (500g, 1 min) three times with PBS.

Following iodination cells were analyzed for viability by exclusion of trypan blue [10] and loss of cells by counting in a hemocytometer. At the conclusion of the labeling procedure cell viability was >90% and cell loss was <25%.

Preparation of Carrier-Bound Lectins

Ricinus communis agglutinins I and II (RCA_I and RCA_{II}) were prepared essentially by the method of Nicolson and Blaustein [11]. Soybean agglutinin (SBA) was prepared using the methods of Liener [12] and Lis et al [13]. Wheat germ agglutinin (WGA) was

prepared by the method of Nagata and Burger [14] as described previously [15]. The lectins were coupled to Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, New Jersey) using the method of Cuatrecasas [16]. Coupling was performed in the presence of 0.2 M saccharide inhibitors: lactose (Eastman Kodak Co., Rochester, New York) for RCA_I and RCA_{II}, 2-acetamido-2-deoxy-D-galactose(D-GalNAc) for SBA and 2-acetamido-2-deoxy-D-glucose (D-GlcNAC) (Sigma) for WGA. Con A coupled to Sepharose was purchased from Pharmacia. The Sepharose-conjugated lectins contained 7–10 mg of covalently bound lectin per milliliter of settled gel. Carrier-bound lectins were washed three times with PBS containing 0.5% Nonidet P-40 (NP-40) and stored at 4°C in PBS containing 0.5% NP-40 and 0.02% NaN_3.

Affinity Chromatography of Solubilized Plasma Membrane Glycoproteins on Sepharose-Bound Lectins

To the radiolabeled cell suspension (10^7 cells per 0.5 ml PBS) was added an equal volume of PBS containing 1% NP-40. The suspension was vortexed for 15 sec and after standing 30 min the suspension was centrifuged at 15,500g for 30 min. Solubilization of the glycoproteins was performed at 4°C.

Affinity chromatography was performed on columns (0.6 cm internal diameter) containing 0.25 ml of carrier-bound lectin equilibrated with PBS containing 0.5% NP-40. The NP-40-solubilized cell components from 2×10^6 cells were applied to the column at a flow rate of 0.5 ml/h. The column was then eluted with 7 ml of PBS containing 0.5% NP-40 at a flow rate of 1.5 ml/h. Material bound to the affinity column was eluted with PBS, containing either 0.2 M lactose for carrier-bound RCA_I and RCA_{II}, 0.1 M D-GalNAc for carrier-bound SBA, 0.2 M D-GlcNAc for carrier-bound WGA, or 0.2 M methyl α-D-mannoside (Sigma) for carrier-bound Con A, at a flow rate of 5 ml/h. Fractions (0.5 ml) were collected and monitored for radioactivity using a Packard Auto Gamma Scintillation Spectrometer. Affinity chromatography was performed at 4°C. The three fractions from each peak containing the highest amounts of radioactivity were pooled for subsequent electrophoretic analysis.

Resolution and Visualization of Radiolabeled Glycoproteins

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed according to the procedure of Laemmli [17], using a Model 221 slab gel electrophoresis apparatus (BioRad Laboratories, Richmond, California). Samples adjusted to 2% SDS, 1% β-mercaptoethanol, 10% glycerol, 62.5 mM Tris, pH 6.8, were heated at 100°C for 3 min prior to application to the gel. Electrophoresis was performed in 1.5-mm slabs at constant current (25 mA per gel). The gels were stained overnight in 0.2% Coomassie Blue R250 in methanol:acetic acid:water [23:4:23 (v/v)]. The gels were destained for 5–7 h in the above solvent.

Molecular weight standards used to calibrate the gels were myosin (a gift from Dr. D. Via), β-galactosidase from Escherichia coli (Worthington Biochemical Corp., Freehold, New Jersey), rabbit muscle phosphorylase A (Worthington), bovine serum albumin (Sigma), hen ovalbumin (Sigma), Con A (a highly purified preparation obtained from Dr. D. C. Hixson) and bovine hemoglobulin (Pentex, Inc., Kankakee, Illinois). These proteins or their monomeric peptides have molecular weights of 200,000, 130,000, 98,000, 68,000, 43,000, 25,000, and 15,500, respectively. Other chemicals used for polyacrylamide gel electrophoresis were electrophoresis grade obtained from BioRad Laboratories.

The gels were swollen in methanol:acetic acid:glycerol:water [5:7.5:2:85.5 (v/v)] and dried under vacuum. Autoradiography was performed using calcium tungstate intensify-

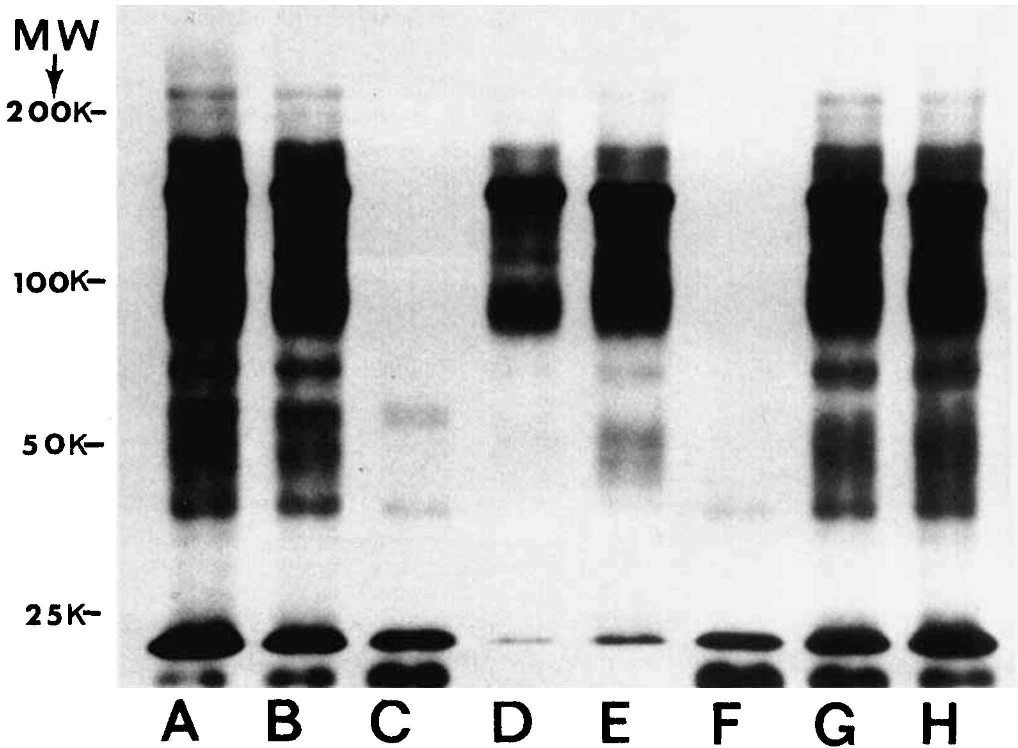

Fig. 1. SDS polyacrylamide gel electrophoresis of ^{125}I-labeled plasma membrane proteins subjected to affinity chromatography on Sepharose-RCA$_I$. Radiolabeled proteins were visualized by autoradiography. Each lane contains radiolabeled components derived from the same number of cells, ie, (2.5–3) × 10^4 cells. Lanes A–D: cells not treated with neuraminidase; lanes E–H: cells treated with neuraminidase; lanes A, H: SDS-solubilized whole cells; lanes B, G: components solubilized by 0.5% NP-40; lanes C, F: components not retained on Sepharose-RCA$_I$; lanes D, E: components retained on Sepharose-RCA$_I$.

ing screens and incubation at –70°C according to the procedure of Swanstrom and Shank [18]. X-ray film (RP-5 Xomat) was purchased from Kodak and X-ray intensifying screens (Cronex, Lightning Plus) were obtained from DuPont, Wilmington, Delaware.

RESULTS

Radioiodination and Solubilization of Cell Surface Proteins

Novikoff cells were effectively labeled by the lactoperoxidase/^{125}I method. At least ten major components having apparent molecular weights of 35,000–250,000 could be distinguished (Fig. 1). In addition, a component which migrated in front of the tracking dye was also resolved. The specificity of the reaction was demonstrated by controls in which lactoperoxidase or D-Glc oxidase were omitted in which no radiolabeled components were observed (data not shown).

As demonstrated by Swanstrom and Shank [18] the use of calcium tungstate intensifying screens and low temperature (–70°C) greatly enhanced the detection of ^{125}I by autoradiography. This was accomplished without loss of resolution of the radiolabeled components.

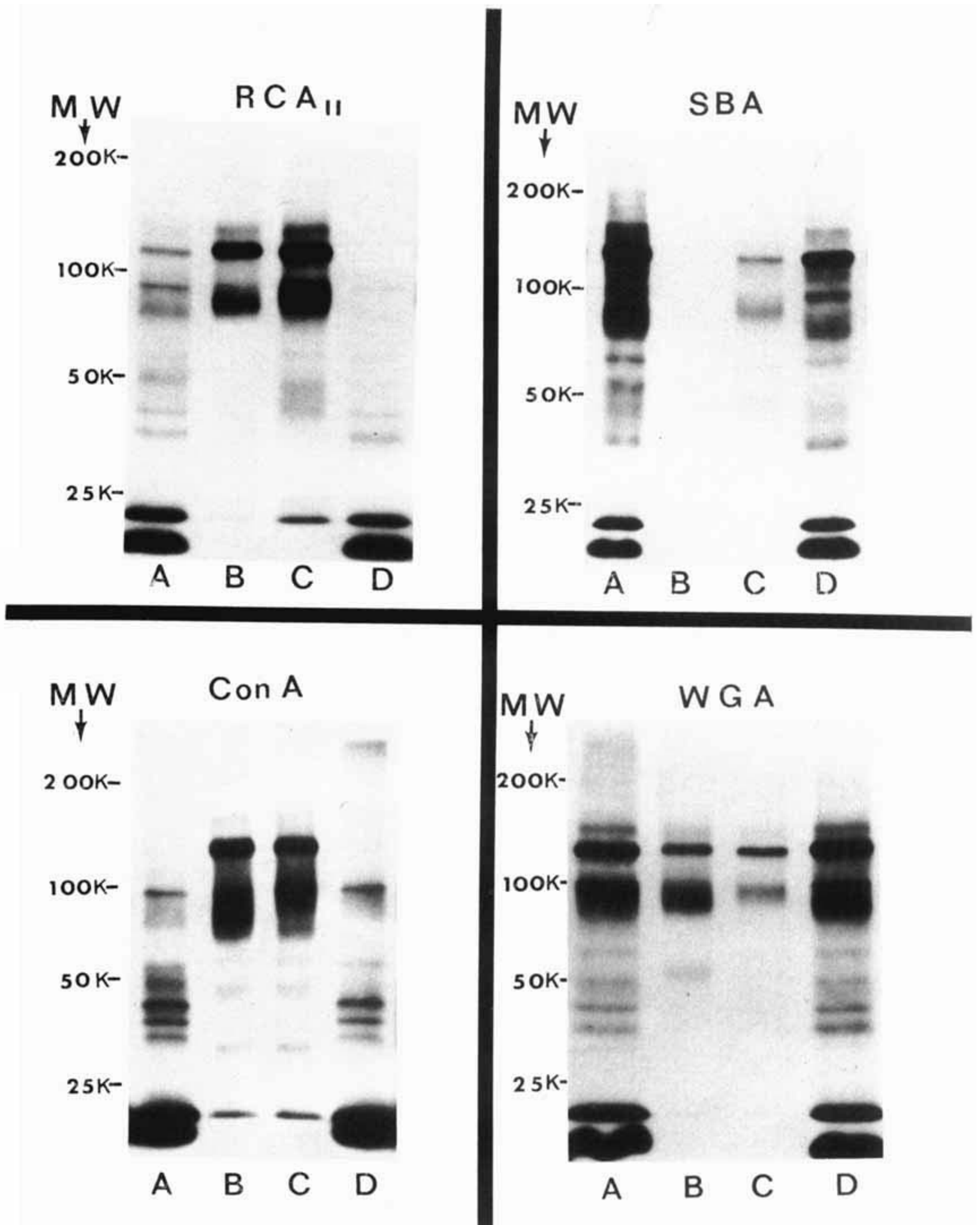

Fig. 2. SDS polyacrylamide gel electrophoresis of ^{125}I-labeled proteins subjected to affinity chromatography on Sepharose-bound RCA_{II}, SBA, Con A, and WGA. Each lane contains radiolabeled components derived from the same number of cells, ie, $(2.5-3) \times 10^4$ cells. Radiolabeled proteins were visualized by autoradiography. Lane A: unbound components; lane B: bound components; lane C: bound components; neuraminidase-treated cells; lane D: unbound components; neuraminidase-treated cells.

Over 85% of the ^{125}I-labeled cell surface components were solubilized in 0.5% NP-40. Furthermore, the autoradiographic pattern of SDS polyacrylamide gels of iodinated cells solubilized in sample buffer containing 2% SDS immediately following iodination was identical to the pattern of the 0.5% NP-40 extracts (Fig. 1), indicating that proteolysis during solubilization did not introduce artifacts.

Affinity Chromatography of Radioiodinated Components on Carrier-Bound Lectins

Affinity chromatography of the components solubilized in 0.5% NP-40 resulted in fractionation of the radiolabeled components (Figs. 1 and 2). Furthermore, affinity chromatography on carrier-bound lectins resolved the radiolabeled components from the bulk of the Coomassie Blue-stained peptides. This suggests that the vast majority of Coomassie-stained peptides are not glycoproteins, as demonstrated previously [20]. Recovery of radioactivity from the carrier-bound lectin columns was 80–85% and duplicate columns always agreed to within ±2%.

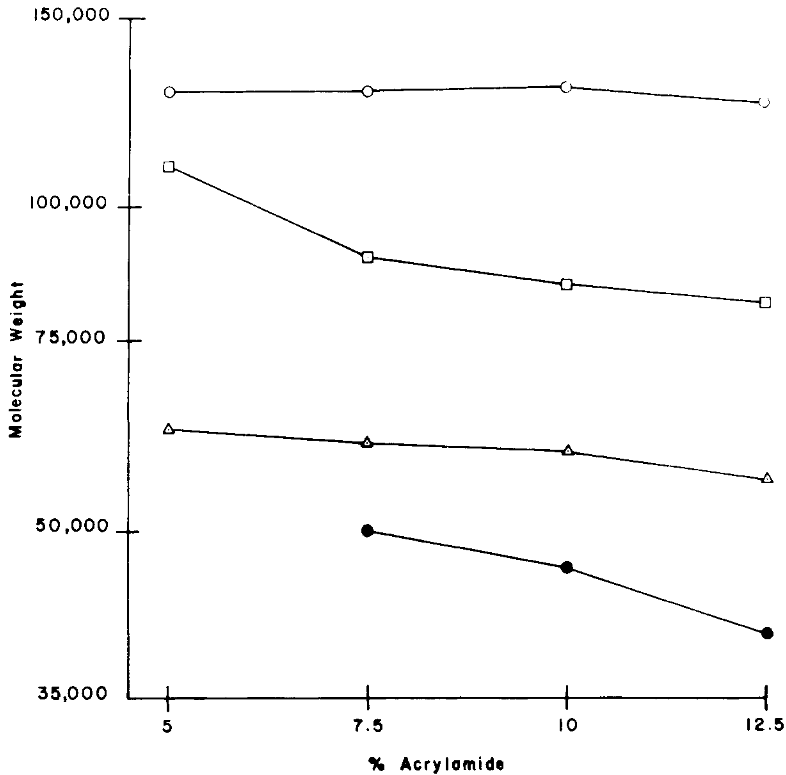

Fig. 3. The effect of acrylamide concentration on apparent molecular weight of peptides that bind to Sepharose-conjugated SBA after neuraminidase treatment. Cells were labeled within ^{125}I, treated with neuraminidase, solubilized in PBS containing 0.5% NP-40, and subjected to affinity chromatography on Sepharose-SBA. The iodinated components eluted with 0.1 M GalNAc were submitted to SDS polyacrylamide gel electrophoresis. Five to seven peptides of known molecular weights were used to estimate the molecular weights of the iodinated peptides. The data represent the average of three separate determinations. The apparent molecular weights agreed within ± 4%. Individual lines are peptides having apparent molecular weights of 128,000 (○–○), 90,000 (□–□), 61,000 (△–△), and 50,000 (●–●) in 7.5% gels (see Fig. 3).

Estimation of the Molecular Weight of the Major Peptides That Bind to Sepharose-SBA

Glycoproteins that contain more than 10% carbohydrate behave anomalously on SDS polyacrylamide gel electrophoresis, the degree of anomaly being some direct function of its percent carbohydrate [19]. Segrest and Jackson [19] have demonstrated that a more accurate estimate of the molecular weight may be obtained by using several gels of different acrylamide concentrations. Such an analysis has been performed on four iodinated peptides derived from neuraminidase-treated cells. These glycoproteins were bound to Sepharose-SBA and eluted with D-GalNAc (Fig. 3). Exposure of the X-ray film to the gel for longer periods of time still showed only the four radiolabeled bands seen in Figure 3 (SBA, lane C). Five to seven molecular weight standards were used to calibrate the gels. An accurate estimate of the molecular weight is approached using gels of higher acrylamide concentration; thus the SBA-binding components have molecular weights of 124,000 (±3,000), 80,000 (±2,000), 55,000 (±2,000), and 40,000 (±1,000) in 12.5% gels. Furthermore, whereas the molecular weight of the largest of these glycoproteins did not vary appreciably between 5% and 12.5% acrylamide, the molecular weight of the next largest component varied from 109,000 (±3,000) in 5% gels to 80,000 (±2,000) in 12.5% gels.

Effect of Neuraminidase Treatment of Radioiodinated Cells on Lectin Binding of Cell Surface Proteins

Previous investigations [15] indicated that lectin binding to the cell surface was altered by treatment of Novikoff cells with neuraminidase; therefore the effect of neuraminidase treatment on the lectin binding of the iodinated peptides was investigated (Table I, Figs. 1 and 2). The incubation conditions employed for neuraminidase treatment of Novikoff cells yield maximal release of cell surface sialic acid [15]. Whereas there was no effect of neuraminidase treatment on binding of the iodinated proteins to Sepharose-Con A, significant alterations in binding to Sepharose-conjugated RCA_I, RCA_{II}, SBA, and WGA were observed. Neuraminidase treatment of the cells was accompanied by increased binding to RCA_I, RCA_{II}, and SBA, as evidenced by the percentage of total label bound to the Sepharose-con-

TABLE I. Effect of Neuraminidase on the Binding of ^{125}I-Labeled Plasma Membrane Proteins to Sepharose-Conjugated Lectins

	% ^{125}I bound to Sepharose-conjugated lectins	
Lectin	Without neuraminidase	With neuraminidase
RCA_I	58	73
RCA_{II}	29	60
SBA	<0.1	13
Con A	26	26
WGA	19	14

Novikoff cells were labeled with ^{125}I and treated with neuraminidase or buffer alone as described in Methods. Cells were then solubilized in PBS containing 0.5% NP-40 and subjected to lectin affinity chromatography on Sepharose-conjugated lectins. Equivalent amounts of trichloroacetic acid-precipitable radioactivity from control or neuraminidase-treated cells were loaded on duplicate lectin affinity columns and eluted under identical conditions. The column fractions (0.5 ml) were assayed for radioactivity. The amount of ^{125}I bound to the column and eluted with saccharide is expressed as a percentage of the total trichloroacetic acid-precipitable radioactivity recovered from the column.

jugated lectins and the disappearance or diminution of iodinated components from fractions not bound to the lectin affinity columns. Particularly striking was the increase in binding of iodinated components to RCA_{II} and SBA. On the other hand, neuraminidase treatment of the cells was accompanied by decreased binding to WGA.

DISCUSSION

Cell surface radiolabeling techniques, coupled with lectin affinity chromatography, represent a powerful tool to study the composition and structure of plasma membrane glycoconjugates. By use of lectins possessing different saccharide specificities information can be gained concerning the structure and heterogeneity of the heterosaccharide moieties of the plasma membrane glycoproteins. Our previous studies [20] using saccharide-specific labeling techniques to radiolabel plasma membrane glycoconjugates, ie, reduction with NaB^3H_4 following oxidation by $NaIO_4$ or D-galactose oxidase, have now been extended to a cell surface labeling method using radioiodination. In addition the number of lectins used for affinity chromatography has been increased to include lectins specific for many of the saccharide structures present in glycoproteins, including D-Gal by RCA_I [11], D-Gal/D-GalNAc by RCA_{II} [11], D-GalNAc by SBA [13], α-D-manno- or α-D-glucopyranosyl residues by Con A [21], and D-GlcNAc or sialic acid by WGA [14, 22].

Binding of almost all major iodinated proteins to one or more of the Sepharose-conjugated lectin columns is strong evidence that these components are glycoproteins. This conclusion is corroborated by previous studies in which most of these components were labeled by saccharide-specific cell surface labeling techniques [20]; however, since noncovalent multimeric associations of plasma membrane proteins and glycoproteins resistant to disruption by nonionic detergents have been reported [23], this conclusion must be considered tentative. Comparative analysis of the iodinated proteins and the plasma membrane glycoproteins labeled using the $NaIO_4/NaB^3H_4$ or D-Gal oxidase/NaB^3H_4 method must await analysis by high-resolution two-dimensional gel electrophoresis [24]. One major iodinated protein did not bind to any of the Sepharose-conjugated lectins and therefore may be a nonglycosylated protein. This protein had a molecular weight of 40,000 in 7.5% gels.

Juliano and Li [25] demonstrated that plasma membrane glycoproteins can be resolved on the basis of their binding to lectins. They used immunoprecipitation by lectins to resolve two of the major plasma membrane glycoproteins of CHO cells. Plasma membrane glycoproteins of Novikoff tumor cells were fractionated using five lectins of differing saccharide specificities: RCA_I, RCA_{II}, SBA, Con A, WGA. For example, a glycoprotein having an apparent molecular weight of 125,000 in 7.5% gels bound completely to Sepharose-conjugated RCA_I and Con A but did not bind to Sepharose-conjugated SBA. This same glycoprotein was fractionated into bound and unbound forms by affinity chromatography on carrier-bound RCA_{II} and WGA, suggesting heterogeneity in its heterosaccharide moiety or moieties. Such heterogeneity could not be detected using the ^{125}I-lectin overlay technique [26] or precipitin formation in Ouchterlony-type double diffusion [27].

Treatment of cells with neuraminidase affects the surface properties of tumor cells, including alteration of cell surface charge [28], exposure of new saccharide moieties [29, 30], and enhancement of immunogenicity [31]. For example, Nicolson [30] reported that neuraminidase treatment of erythrocytes, lymphoma cells, and fibroblasts was accompanied by an increase in RCA_I-induced cytoagglutination and binding of labeled lectin to the cell surface. Previous studies in this laboratory demonstrated that the immunogenicity of Novikoff tumor cells was not affected by neuraminidase [32], even though this treatment

resulted in a loss of cell surface charge [33], and the exposure of D-Gal and/or D-GalNAc residues on cell surface heterosaccharide moieties [15]. The significant increase in the binding of iodinated proteins to Sepharose-conjugated RCA_I, RCA_{II}, and SBA confirms these previous findings. Lectin affinity chromatography and SDS polyacrylamide gel electrophoresis of the glycoproteins bound to Sepharose-conjugated RCA_I, RCA_{II}, and SBA allowed assignment of this increased lectin receptor activity to specific glycoprotein species. It should be noted, however, that this experimental approach only allows identification of new lectin-binding species of glycoproteins. For example, neuraminidase may expose new RCA_I binding sites on particular glycoproteins, but if these glycoproteins already contain RCA_I binding sites, the new binding sites will not be detected.

These studies demonstrate the utility of lectins as saccharide-specific probes for the multicomponent analysis of plasma membrane glycoproteins and suggest methodology for the resolution and partial structural characterization of glycoproteins present at the surface of other normal and malignant cells.

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