Biochimica et Biophysica Acta, 599 (1980) 493—504

BBA 78781

ACCESSIBILITY OF PLASMA MEMBRANE SIALOGLYCOCONJUGATES OF NOVIKOFF TUMOR CELLS TO EXOGENOUS NEURAMINIDASE AND PROTEASES

JOHN R. GLENNEY, Jr. [a] and EARL F. WALBORG, Jr. [a,b,*]

[a] *The University of Texas Health Science Center at Houston, Graduate School of Biomedical Sciences, Houston, TX 77030 and* [b] *The University of Texas System Cancer Center, Science Park, Research Division, Smithville, TX 78957 (U.S.A.)*

(Received October 3rd, 1979)

Key words: Glycoconjugate; Surface labeling; Neuraminidase; Papain; Trypsin; (Novikoff tumor cell)

Summary

Plasma membrane glycoconjugates of Novikoff tumor cells were radioactively labeled by oxidation with $NaIO_4$ followed by reduction with NaB^3H_4 Submission of the radioactively labeled glycoconjugates to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate followed by fluorography revealed the presence of at least ten major glycoproteins and a glycolipid fraction. The glycolipid fraction contained 34% of the cell-surface radioactive label. Pretreatment of cells with neuraminidase from *Vibrio cholerae* reduced radioactive labeling of the glycoproteins by 71% and that of the glycolipids by 39%. Sequential treatment of cells with papain and neuraminidase further reduced radioactive labeling of the glycolipid fraction, indicating that resistance of this fraction to the hydrolytic action of neuraminidase was determined, at least in part, by steric factors. Incubation of cells with papain resulted in extensive degradation of most of the radioactively labeled glycoproteins with the exception of a subset of glycoproteins having apparent molecular weights of 48 000 ± 5000. Trypsin was more selective, degrading three glycoproteins having apparent molecular weights of 200 000, 140 000 and 37 000.

Introduction

Transformation of epithelial cells to the malignant phenotype is accompanied by the acquisition of new surface properties that influence cell-cell

* To whom correspondence should be addressed.
Abbreviation: SDS, sodium dodecyl sulfate.

adhesion and communication [1]. These aberrant functional properties are associated with alterations in the composition, structure, topography, and/or dynamics of cell-surface glycoconjugates [2—4]. Studies in this laboratory revealed differences in the plasma membrane glycoproteins of normal and malignant rat liver cells; e.g., Novikoff tumor cells exhibited increased lability of their cell-surface receptors for the lectin concanavalin A to degradation by papain [4,5], and the glycopeptides cleaved from the surface of hepatocellular carcinoma cells by papain contained significantly higher quantities of macrosialoglycopeptides [5].

Although controlled proteolysis yielded valuable information regarding the plasma membrane glycoproteins of normal and malignant rat liver cells, it precluded the characterization of individual glycoprotein components. Recently, surface labeling techniques, combined with polyacrylamide gel electrophoresis and fluorography, allowed identification and resolution of the major plasma membrane glycoproteins of Novikoff tumor cells [6,7]. Affinity chromatography of surface-labeled glycoproteins on Sepharose-conjugated lectins permitted assignment of lectin receptor properties to the major sialoglycoproteins. This report describes the accessibility of the major plasma membrane glycoconjugates of Novikoff cells to proteases and neuraminidase.

Materials

Chemicals. $NaIO_4$, toluene, chloroform, and methanol were reagent grade chemicals purchased from Fisher Scientific Co., Pittsburgh, PA. NaB^3H_4 (spec. act., 48 Ci/mmol) was obtained from New England Nuclear, Boston, MA. Triton X-100, $NaBH_4$, *N*-acetylneuraminic acid and hen ovalbumin were purchased from Sigma Chemical Co., St. Louis, MO.

Enzymes. Protease-free neuraminidase from *Vibrio cholerae* was obtained from Calbiochem, La Jolla, CA. Papain (twice crystallized, 16.6 units/mg protein), trypsin (three times crystallized, 200 units/mg protein), chymotrypsin (three times crystallized, 45—70 units/mg protein), and collagenase from *Clostridium histolyticum* (type III) were obtained from Worthington Biochemical Corp., Freehold, NJ. Deoxyribonuclease I (DNAase) from bovine pancreas (3800 Kunitz units/mg protein) was obtained from Sigma Chemical Co.

Reagents for polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). The source and quality of chemicals used for electrophoresis, including those used in the sample buffer and the proteins that served as molecular weight standards, were reported previously [6,7]. Samples in 2% SDS, 1% β-mercaptoethanol, 10% glycerol and 62.5 mM Tris, pH 6.8, were heated at 100°C for 3 min prior to application to the gel.

Materials used for fluorography. Reagents used for fluorography, performed according to the method of Bonner and Laskey [8], have been described previously [6].

Methods

Radioactive labeling of cell-surface glycoconjugates. The Novikoff hepatocellular carcinoma [9] was maintained in the ascitic form in Sprague-Dawley

rats as described previously [6]. Tumor cells were harvested and washed in phosphate-buffered saline, pH 7.4 [6], as described previously [6]. Radioactive labeling of cell-surface glycoconjugates was performed using periodate oxidation followed by reduction with NaB^3H_4 [10,11], as described previously [6]. Briefly, cells were treated with 2 mM $NaIO_4$ in an ice bath for 10 min at a concentration of $2.5 \cdot 10^6$ cells/ml.

Treatment of cells with neuraminidase. Cells were incubated with neuraminidase prior to or immediately following radiolabeling. Cells ($2.5 \cdot 10^6$) in 1 ml phosphate-buffered saline, pH 7.0, containing 1 mM $CaCl_2$, were incubated with various concentrations of neuraminidase for 1 h at 37°C. Enzyme treatment was performed in 1 ml plastic tubes (Fisher Scientific Co., Cat. No. 4-978-145). Agitation of the samples was achieved using a tube rotator (Scientific Equipment Products, Baltimore, MD, Cat. No. 60448) at 20 rev./min. The cells were washed twice, suspended in phosphate-buffered saline, pH 7.0, at 4°C, and radioactive labeled as described above. Alternatively, cells were radioactive labeled, washed three times in phosphate-buffered saline, pH 7.0, and incubated with neuraminidase as described above.

Treatment of radioactive labeled cells with trypsin, chymotrypsin, and papain. Radioactive labeled cells were washed four times in phosphate-buffered saline, pH 7.4, and suspended at a concentration of $2.5 \cdot 10^6$ cells/ml in the same buffer containing trypsin or chymotrypsin. 100 μg/ml DNAase I was included in the incubation mixture. The cells were incubated for 1 h at 37°C on the tube rotator as described above. Following incubation the cells were washed three times with phosphate-buffered saline, pH 7.4, and aliquots taken for determination of cell viability and cell number, as well as for SDS-polyacrylamide gel electrophoresis. Cell viability was assayed by exclusion of trypan blue [12]. Cell counts were performed using an electronic particle counter or a hemocytometer.

Cells, suspended in a buffer containing cysteine and EDTA [13], pH 6.5, were incubated with papain for 40 min at 37°C. Prior to digestion, the cells were washed once with this same buffer.

Treatment of cells with collagenase. Radioactive labeled cells were washed once and suspended in Ca^{2+}-free Hanks' buffer containing bovine serum albumin and 0.05% collagenase [5] at a concentration of $2.5 \cdot 10^6$ cells/ml. The cell suspension was incubated for 20 min at 37°C on the tube rotator. Following incubation, the cells were washed three times in phosphate-buffered saline, pH 7.4, and aliquots were taken for analysis as above.

Sequential treatment of cells with papain and neuraminidase. Cells were washed twice with phosphate-buffered saline, pH 7.4, and once with the papain incubation buffer prior to incubation with papain as described above. Following incubation with papain, the cells were washed three times with phosphate-buffered saline, pH 7.0, containing 1 mM $CaCl_2$ and incubated with neuraminidase as described above.

Determination of the radioactivity insoluble in trichloroacetic acid. To 25 μl of radioactive labeled cell suspension was added 25 μl of 1% Nonidet P-40 (NP-40) (Particle Data Laboratories, Ltd., Elmhurst, IL), and the mixture was allowed to stand 30 min in an ice-bath. To this mixture was added 100 μl of a solution containing 10 mg ovalbumin/ml phosphate-buffered saline, pH 7.4,

followed by 750 μl of ice-cold 10% trichloroacetic acid. After vigorous mixing, the suspension was placed at 4°C for 12—18 h to complete the precipitation. The precipitate was collected by centrifugation at 15 500 × *g* for 5 min, washed twice with 750 μl of ice-cold 10% trichloroacetic acid, and dissolved in 0.2 N NaOH containing 2% SDS. The radioactivity of the dissolved precipitates was measured in a Packard Tri-Carb Liquid Scintillation Spectrometer, using a scintillation counting mixture of toluene/Triton X-100 (2 : 1, v/v), containing 4 g Omnifluor (New England Nuclear)/l. Efficiency of counting was calculated using a channels ratio method by comparison to quenched ^3H standard purchased from New England Nuclear.

Identification and quantitation of the ^3H-labeled sialic acid derivatives. Radioactive labeled Novikoff cells (10^5) in 25 μl of phosphate-buffered saline, pH 7.4, were centrifuged at 15 500 × *g* for 1 min. The supernatant buffer was removed and replaced with 100 μl of 0.1 N HCl. After mixing, the sample was incubated at 80°C for 1 h, the optimum conditions for hydrolysis of glycosidic bonds involving sialic acid [14]. Following incubation, the solution was neutralized with dilute NaOH and solubilized in 1% SDS. A 5 μl aliquot of this solution was subjected to descending chromatography on Whatman No. 1 paper using a solvent consisting of 1-butanol/1-propanol/0.1 N HCl/H_2O (1 : 2 : 1, v/v) [11]. *N*-Acetylneuraminic acid, used as a standard, was visualized by staining with alkaline $AgNO_3$ [15]. The distribution of ^3H in the chromatogram was determined by slicing each lane into 1 cm sections, incubating these in 1 ml of H_2O for 1 h, and counting in the scintillation counter.

Resolution and visualization of radioactive labeled glycoconjugates. SDS-polyacrylamide gel electrophoresis in 7.5% gels and fluorography [8] of the dried gels was performed as described previously [6,7]. X-ray film was preflashed to be a background fog of 0.15 absorbance unit (540 nm) as described by Laskey and Mills [16]. Densitometry of the X-ray film was performed on a Beckman Model 35 Spectrophotometer with a linear transport scanning attachment. Quantitation was accomplished by planimetry of the densitometry scans. The relationship between ^3H incorporation and film grain density was examined. Cells, radioactive labeled to high specific activity, were solubilized in sample buffer, and serial dilutions of the sample were submitted to SDS-polyacrylamide gel electrophoresis, fluorography, and quantitative densitometry. It was confirmed that a linear relationship existed between ^3H incorporation and grain density up to 1.1 absorbance units, the maximum density present in the gels of interest. Apparent molecular weights of the radioactive labeled glycoproteins were assigned on the basis of their mobility in 7.5% gels.

Results

Radioactive labeling of Novikoff tumor cells by $NaIO_4/NaB^3H_4$

Incorporation of ^3H into the cell-surface glycoconjugates of Novikoff cells was dependent on the concentration of $NaIO_4$ used for oxidation. As found for other cell lines [11], maximal oxidation of Novikoff cells by 2 mM $NaIO_4$ at 0—4°C was attained at 10 min. Under these conditions cell viability was >90%. Oxidation for 10 min with 10 mM $NaIO_4$ or oxidation with 2 mM $NaIO_4$ for periods between 10 and 30 min resulted in comparable oxidation, but was

accompanied by loss of cell viability. Greater than 90% of the ^3H incorporated into Novikoff cells was precipitated by trichloroacetic acid, while 34% of the radioactive label was soluble in $CHCl_3/CH_3OH$ (2 : 1, v/v). When radioactive labeled cells were subjected to mild acid hydrolysis, more than 97% of the ^3H was solubilized as a component that chromatographed as a single symmetrical peak, having a mobility of 1.4 relative to *N*-acetylneuraminic acid (Table I). Such a mobility is consistent with the product of $NaIO_4$ oxidation of sialic acids [11].

As demonstrated previously [6], cell-surface glycoproteins, radioactive labeled using the $NaIO_4/NaB^3H_4$ method, are effectively resolved into at least ten components by SDS-polyacrylamide gel electrophoresis (see lane A of Figs. 2, 3 and 5). In addition to glycoproteins having molecular weights of at least 25 000, a radioactive labeled component migrating in front of the tracking dye was observed (Figs. 2, 3 and 5). This latter component was completely soluble in $CHCl_3/CH_3OH$ (Fig. 1), presumptive evidence that this fraction is glycolipid.

Effect of proteases on cell-surface glycoproteins of Novikoff tumor cells

When radioactive labeled Novikoff cells were treated with trypsin, only a subset of cell-surface glycoproteins was sensitive to degradation by this enzyme (Fig. 2). Degradation of these particular glycoproteins was dependent on trypsin concentration with individual glycoprotein bands exhibiting differential lability. A glycoprotein band, having an apparent molecular weigh of 200 000, completely disappeared upon incubation of the cells with 5 μg trypsin/ml; whereas glycoprotein bands with apparent molecular weights of 140 000 and 37 000 required a 10-fold higher concentration of trypsin for degradation. The majority of glycoproteins were not significantly degraded by trypsin, even at concentrations of 100 μg/ml or less. Although DNAase was included in the incubation mixture to prevent cell aggregation, this enzyme alone had no effect on the electrophoretic profile of the glycoproteins (Fig. 2A and B).

The radioactive labeled glycoproteins were more sensitive to degradation by

TABLE I

PAPER CHROMATOGRAPHY OF ACID HYDROLYZATES OF CELLS RADIOACTIVE LABELED USING $NaIO_4/NaB^3H_4$

Conditions for acid hydrolysis: 0.1 N HCl, 1 h, 80°C. Novikoff tumor cells were radioactive labeled using $NaIO_4/NaB^3H_4$ with or without pretreatment with neuraminidase. As a control, cells were labeled with NaB^3H_4 alone. Radioactive labeled cells (10^5 in 25 μl phosphate-buffered saline, pH 7.4) were centrifuged and the supernatant buffer removed. The cells were suspended in 100 μl of 0.1 N HCl and incubated for 1 h at 80°C. The hydrolyzate was neutralized with dilute NaOH, solubilized in 1% SDS, and a 5 μl aliquot was subjected to paper chromatography and assay of radioactivity as described in Methods. The radioactivity resolved by chromatography was present in a single symmetrical peak, having a mobility of 1.4 ± 0.1 relative to *N*-acetylneuraminic acid.

Treatment	Radioactivity at origin (dpm)	Radioactivity in peak corresponding to $NaIO_4$ oxidation product of sialic acid (dpm)
NaB^3H_4	600	800
$NaIO_4/NaB^3H_4$	2000	58 000
Neuraminidase + $NaIO_4/NaB^3H_4$	1700	24 000

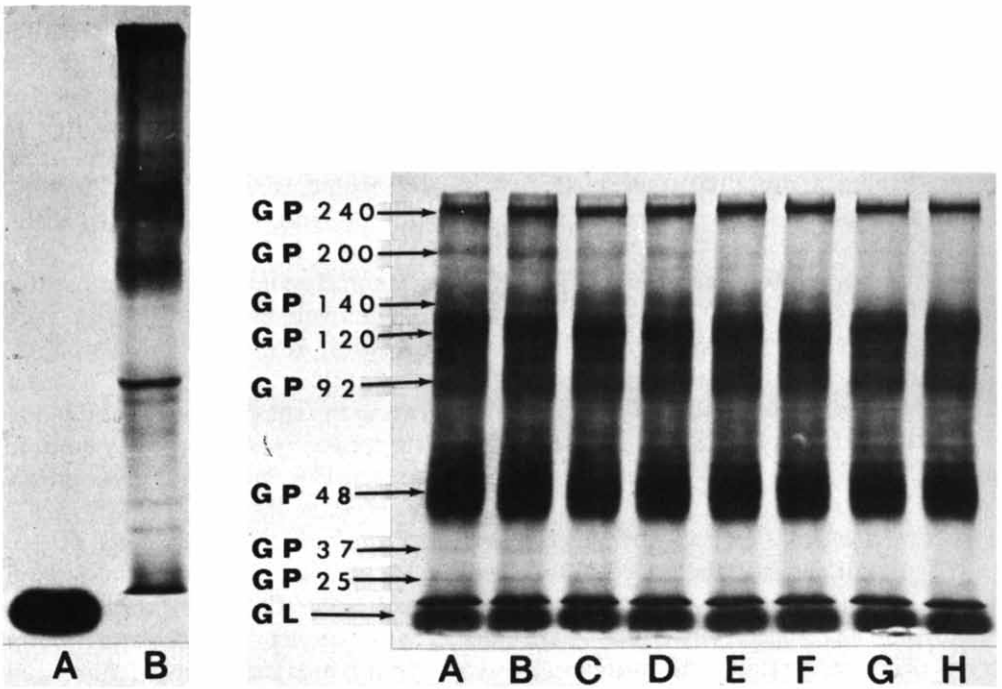

Fig. 1. Resolution of radioactive labeled cell surface glycoconjugates fractionated by extraction with $CHCl_3/CH_3OH$. Novikoff tumor cells were radioactive labeled using $NaIO_4/NaB^3H_4$ and washed five times with phosphate-buffered saline, pH 7.4. The cells were then extracted three times (15 min each) with 20 vols. of $CHCl_3/CH_3OH$ (2 : 1, v/v). The pooled extracts were counted for radioactivity, dried under a stream of N_2, and the residue resuspended in electrophoresis sample buffer. The components insoluble to $CHCl_3/CH_3OH$ were solubilized directly in sample buffer. The samples were submitted to SDS-polyacrylamide gel electrophoresis in 7.5% gels and the radioactive labeled components visualized by fluorography. Each lane contained radioactive labeled components from $6 \cdot 10^4$ cells. Components soluble (lane A) or insoluble (lane B) in $CHCl_3/CH_3OH$. The proteins precipitated in $CHCl_3/CH_3OH$ were not adequately solubilized in SDS-containing sample buffer, as evidenced by the large amount of radioactive labeled material that did not enter the gel. For this reason the protein profile of lane B cannot be compared to those shown in Figs. 2, 3 and 5.

Fig. 2. Resolution of radioactive labeled cell surface glycoconjugates derived from cells treated with trypsin. Novikoff tumor cells, radioactive labeled using $NaIO_4/NaB^3H_4$, were incubated with trypsin for 1 h at 37°C. Following incubation, the cells were washed three times in phosphate-buffered saline, pH 7.4, and aliquots submitted to SDS-polyacrylamide gel electrophoresis in 7.5% gels. The radioactive labeled components were visualized by fluorography. Controls included cells incubated without enzyme (lane A) and cells incubated with only 100 μg/ml DNAase (lane B). Cells were treated with the following concentrations of trypsin in the presence of 100 μg/ml DNAase: 0.5, 1, 5, 10, 50 or 100 μg/ml (lanes C—H, respectively). Each lane contained radioactive labeled components from $6 \cdot 10^4$ cells. The major glycoproteins (GP) were assigned numerical designations based on their apparent molecular weights ($X10^{-3}$), estimated by their mobility in 7.5% gels. The glycolipid fraction (GL) migrated ahead of the tracking dye.

papain than trypsin (Figs. 2 and 3). As in the case of trypsin, degradation of particular glycoproteins was dependent on enzyme concentration (Fig. 3). At higher papain concentrations all the radioactive labeled glycoproteins were degraded to some extent; however, a subset of glycoproteins that had apparent molecular weights of 48 000 ± 5000 exhibited significant resistance to this enzyme. Quantitative densitometry revealed that only 20% of this subset of glycoproteins was degraded by treatment with 1 mg papain/ml, while the

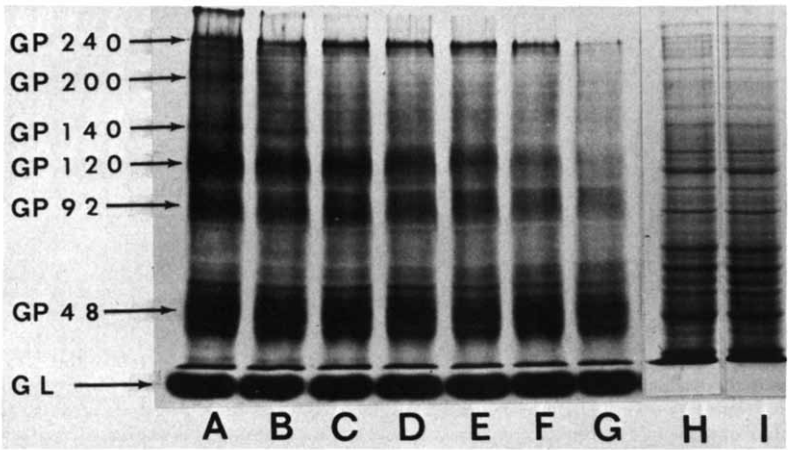

Fig. 3. Resolution of radioactive labeled cell surface glycoconjugates derived from cells treated with papain. Novikoff tumor cells, radioactive labeled using $NaIO_4/NaB^3H_4$, were incubated with papain for 40 min at 37°C. Following incubation, the cells were washed three times in phosphate-buffered saline, pH 7.4, and aliquots submitted to SDS-polyacrylamide gel electrophoresis in 7.5% gels. The radioactive labeled components were visualized by fluorography (lanes A—G), or proteins were stained by Coomassie blue (lanes H and I). As a control, cells were incubated in the absence of enzyme (lanes A and H). Cells were treated with the following concentrations of papain: 3, 10, 30, 100 or 1000 μg/ml (lanes B—G, respectively). Coomassie blue-stained proteins derived from cells treated with 1 mg papain/ml are shown in lane I. Each lane contained components derived from $6 \cdot 10^4$ cells.

remainder of the glycoproteins were 78% degraded. The electrophoresis profile of Coomassie blue-stained proteins, derived from cells treated with 1 mg papain/ml, was not significantly different from that of proteins derived from control cells incubated in the absence of enzyme (Fig. 3). Since the stained proteins are predominantly cytoplasmic components, their lack of degradation to lower molecular weight products indicates that the action of papain was restricted to the cell surface. Collagenase treatment of Novikoff cells had no effect on the electrophoretic profile of the radioactive labeled glycoconjugates (data not shown).

Incubation of radioactive labeled Novikoff cells with trypsin, papain, or collagenase was achieved under conditions that maintained cell viability at least 90% and minimized cell loss to less than 25%, including loss due to sample manipulation. Treatment of cells with 5 μg/ml chymotrypsin resulted in less than 50% cell viability; therefore the effect of this enzyme was not pursued further.

Effect of neuraminidase on radioactive labeling of cell-surface glycoconjugates

Treatment of Novikoff cells with neuraminidase prior to radioactive labeling decreased incorporation of ^3H into cell-surface glycoconjugates (Figs. 4 and 5). The magnitude of the reduction, determined as radioactivity precipitated by trichloroacetic acid, was 55% (Fig. 4). Pretreatment of the cells with neuraminidase reduced radioactive labeling of glycoproteins more than the glycolipids (Figs. 4 and 5). Quantitative densitometry of the fluorograms revealed that incorporation of ^3H into glycolipid components was reduced 39%, while incorporation of ^3H into the glycoproteins was reduced 71% (Table II). Paper

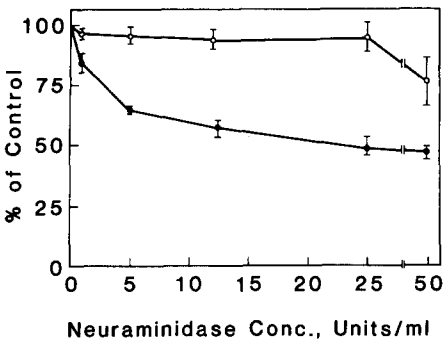

Fig. 4. Effect of exogenous neuraminidase on radioactive labeling of Novikoff cells using $NaIO_4/NaB^3H_4$. Novikoff cells were treated with neuraminidase prior to radioactive labeling (●——●) or immediately following radioactive labeling (○——○) by $NaIO_4/NaB^3H_4$. Each concentration point represents ^3H incorporation into trichloroacetic acid-insoluble components, and ^3H incorporation is expressed as mean percent of the control cells incubated without enzyme. The S.D. indicated by the limit bars were calculated using four separate labeling experiments. The viability of cells treated with neuraminidase, even at concentrations as high as 50 units/ml, was greater than 95%.

chromatography of mild acid hydrolyzates of the glycoconjugates radioactive labeled after treatment of the cells with neuraminidase indicated that the ^3H was present in an $NaIO_4$ oxidation product of sialic acid (Table I). When cells were radioactive labeled first and then treated with neuraminidase (1—25 units/

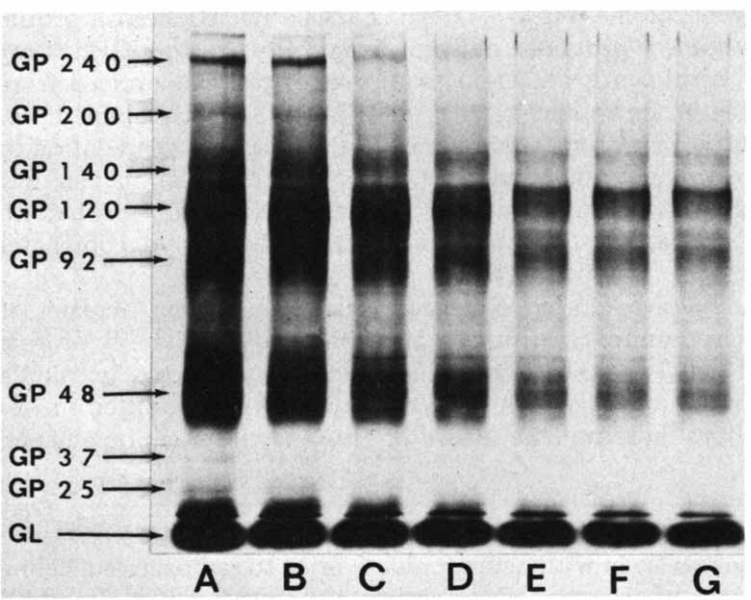

Fig. 5. Effect of pretreatment of the cells with neuraminidase on the labeling of cell surface glycoconjugates by $NaIO_4/NaB^3H_4$. Novikoff cells were labeled by $NaIO_4/NaB^3H_4$, solubilized immediately in SDS-containing sample buffer (lane A) or incubated in the absence of neuraminidase (lane B) or incubated in the presence of 1, 5, 12.5, 25 or 50 units of neuraminidase/ml (lanes C—G, respectively). Following incubation for 1 h at 37°C, the cells were washed twice with phosphate-buffered saline, pH 7.4, radioactive labeled using $NaIO_4/NaB^3H_4$, and aliquots taken for SDS-polyacrylamide gel electrophoresis in 7.5% gels. The radioactive labeled components were visualized by fluorography. Each lane contained radioactive labeled components from $6 \cdot 10^4$ cells.

TABLE II

EFFECT OF NEURAMINIDASE AND/OR PAPAIN ON RADIOACTIVE LABELING OF NOVIKOFF TUMOR CELLS USING $NaIO_4/NaB^3H_4$

Cells were washed twice with phosphate-buffered saline, pH 7.4, and once with Earle's buffer containing cysteine and EDTA [13], pH 6.5. The cells, suspended in the same buffer, were incubated for 40 min at 37°C with or without 200 μg papain/ml. After incubation the cells were washed three times with phosphate-buffered saline, pH 7.0, containing 1 mM $CaCl_2$. The cells, suspended in the same buffer, were incubated for 1 h at 37°C with or without 25 units neuraminidase/ml. The cells were washed twice in phosphate-buffered saline, pH 7.4, and radioactive labeled using $NaIO_4/NaB^3H_4$. Cells ($6 \cdot 10^4$) from each treatment were subjected to SDS-polyacrylamide gel electrophoresis. The radioactive labeled components were visualized by fluorography. The amount of labeling in the glycoproteins (area of the gel between the tracking dye and the top of the gel) and the glycolipids (band migrating in front of the tracking dye) was quantitated by planimetry of densitometric scans of fluorograms.

First incubation	Second incubation	Glycoprotein labeling (% of control)	Glycolipid labeling (% of control)
Earle's buffer	phosphate-buffered saline	100	100
	25 units neuraminidase/ml	29	61
200 μg papain/ml	phosphate-buffered saline	58	80
	25 units neuraminidase/ml	23	22

ml), less than 5% of the incorporated ^3H was released from the cell surface (Fig. 4).

Sequential treatment of cells with papain and neuraminidase increased the accessibility of the glycolipids to neuraminidase. Whereas incorporation of ^3H into glycolipids was reduced 39% by neuraminidase alone, sequential treatment of the cells with papain and neuraminidase reduced radioactive labeling of the glycolipids by 78% (Table II).

Discussion

Cell-surface glycoconjugates can be radioactive labeled using mild periodate oxidation, followed by reduction with NaB^3H_4 [10,11,14]. Combination of this radioactive labeling technique with SDS-polyacrylamide gel electrophoresis and fluorography represents a valuable approach to investigate the composition and chemical properties of plasma membrane glycoconjugates. Using this experimental approach, some of the molecular consequences of the hydrolytic action of neuraminidase on cell-surface glycoconjugates of Novikoff tumor cells have been identified. For example, treatment of Novikoff cells with neuraminidase prior to radioactive labeling reduced incorporation of ^3H into cell-surface glycoconjugates by only 55% (Figs. 4 and 5), indicating that not all the cell-surface sialic acid was cleaved by neuraminidase. The resistance of some cell-surface sialic acid residues to cleavage by *V. cholerae* neuraminidase may result from structural differences in certain sialooligosaccharide moieties or to steric factors, including proximity to the hydrophobic region of the plasma membrane or masking by other membrane components or moieties. Consistent with observations on other cell lines [17—19], the sialic acid residues associated with the glycolipid fraction were more resistant to neuraminidase than those associated with the plasma membrane glycoproteins (Table II). Since treatment of

Novikoff cells with papain increased the lability of the sialoglycolipid fraction to neuraminidase, steric factors must be at least partially responsible for the resistance of this fraction to hydrolysis by neuraminidase. When Novikoff cells were radioactive labeled prior to treatment with neuraminidase at concentrations no greater than 25 units/ml, less than 5% of the ^{3}H was cleaved from the cell surface, an observation consistent with the low affinity of *V. cholerae* neuraminidase for the periodate oxidation product of sialic acid [20].

Previous studies in this laboratory [4,5,21] demonstrated that papain degraded the plasma membrane glycoproteins of Novikoff tumor cells, cleaving sialoglycopeptides possessing concanavalin A and/or wheat germ agglutinin receptor activity from the cell surface. The present study provides new information regarding the identity of glycoproteins from which these sialoglycopeptides are derived. Papain-labile glycoproteins, having apparent molecular weights between 80 000 and 150 000, possess the major portion of the concanavalin A and wheat germ agglutinin receptor activity [7]. It should be noted, however, that incubation of Novikoff cells with papain at concentrations as high as 1 mg/ml did not completely degrade most glycoproteins, indicating their inherent resistance to degradation by papain. Previous investigations indicated the existence of two classes of lectin receptors at the surface of Novikoff cells: papain-labile receptors that yield sialoglycopeptides bearing lectin receptor activity and papain-resistant receptors that function in the lectin-induced cytoagglutination of papain-treated cells [22]. As proposed earlier [22], these papain-resistant lectin receptors could reside on plasma membrane glycolipids or glycoproteins. From the present studies it is apparent that some of these papain-resistant receptors are glycoproteins.

The distribution of the radiolabel in fluorograms of glycoproteins derived from papain-treated cells provides information concerning the mechanism by which plasma membrane glycoproteins are degraded by papain. Although the amount of radioactive labeled glycoproteins decreased as a function of papain concentration, concomitant appearance of lower molecular weight radioactive labeled products of proteolysis was not observed (Figs. 3 and 4), suggesting that plasma membrane glycoproteins are degraded by cleavage of preferred site(s) that allow release of glycopeptides containing essentially all the radioactive labeled sialic acid residues rather than by cleavages within the peptide domain bearing the sialooligosaccharide moieties. A similar situation exists for the papain-catalyzed degradation of histocompatibility antigens on the surface of human lymphoid cells [23]. The general resistance of the plasma membrane glycoproteins to the hydrolytic action of papain may be explained on the basis of the proximity of the preferred cleavage site(s) to the hydrophobic region of the membrane or the association of the plasma membrane glycoproteins into multimeric units that shield the preferred cleavage site(s). In addition, the degree of substitution of the glycoproteins with heterosaccharide moieties may prevent access of papain to the peripheral portions of the peptide chains. Consistent with this latter possibility is the fact that papain resulted in cleavage of macrosialoglycopeptides from the surface of Novikoff and AS-30D tumor cells [21,22,24].

A group of glycoproteins having apparent molecular weights of 48 000 ± 5000 contains a major portion of the radioactive label, indicating that they are

present in relatively high concentration in the plasma membrane or that they contain a high number of sialooligosaccharide moieties. This group of glycoproteins, previously shown to possess receptor activity for *Ricinus communis* agglutinin I [6], is very resistant to degradation by papain (Figs. 3 and 4). This group of glycoproteins is labeled poorly, if at all, by lactoperoxidase-catalyzed iodination of intact cells, a method specific for exposed tyrosine residues, further evidence for the shielding of the peptide moiety from exogenous enzymes. On the other hand, the carbohydrate moieties of these glycoproteins are accessible to exogenous enzymes as evidenced by the fact that neuraminidase treatment of Novikoff cells produced an 80% reduction in labeling of these glycoproteins by $NaIO_4/NaB^3H_4$. Also their carbohydrate moieties are labeled by galactose oxidase/NaB^3H_4 following treatment of the cells with neuraminidase [6].

In contrast to papain, incubation of Novikoff cells with trypsin was accompanied by degradation of a more restricted number of the radioactive labeled cell-surface glycoproteins, e.g., components with apparent molecular weights of 200 000, 140 000, and 37 000. Resistance of membrane glycoproteins to the action of trypsin has also been reported for other cell types [25—27]. Bauman and Doyle [27] demonstrated that trypsin preferentially degraded several plasma membrane glycoproteins of a rat hepatoma cell line, one of which yielded a macrosialoglycopeptide having an apparent molecular weight of 55 000. The degradation of this glycoprotein was accompanied by cell aggregation. Trypsin-induced aggregation of Novikoff cells was also observed; however, when DNAase was included in the incubation mixture, cell aggregation was abolished. This effect was not due simply to the presence of exogenous protein, since inclusion of 100 μg/ml bovine serum albumin or 100 μg/ml ovalbumin in the incubation mixture did not inhibit trypsin-induced cell aggregation. Thus, in the case of Novikoff cells, trypsin-induced cell aggregation was associated with the presence of DNA rather than the degradation of specific cell-surface proteins.

Treatment of Novikoff cells with collagenase under conditions similar to those used to disperse rat hepatocytes [5] produced no significant effects on the radioactive labeled cell-surface glycoconjugates. 0.5% bovine serum albumin was included in the incubation to minimize the action of contaminating proteases present in collagenase preparations [5]. These data reinforce previous investigations [5] indicating that incubation of Novikoff tumor cells with collagenase in the presence of bovine serum albumin has no demonstrable deleterious effects on the cell-surface glycoconjugates.

This study contributes to the characterization of the major plasma membrane glycoproteins of Novikoff hepatocellular carcinoma cells and lays the groundwork for future comparison to the glycoproteins of cells derived from other hepatocellular carcinomas and normal hepatocytes.

Acknowledgements

This research was supported by grants from The National Cancer Institute (CA 18829), The Paul and Mary Haas Foundation, and The George and Mary Josephine Hamman Foundation. J.R.G. was the recipient of Predoctoral Fellow-

ships from The Rosalie B. Hite Foundation and The J.S. Abercrombie Foundation. The authors acknowledge the able technical assistance of Timothy E. Hayes in certain aspects of this research.

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