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# Purification and Characterization of a Major Cell Surface Glycoprotein in Zajdela Ascites Hepatoma Cells Which Displays a Potent Concanavalin A Receptor Activity

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A major cell surface sialoglycoprotein with Concanavalin A receptor activity has been isolated from rat Zajdela ascites hepatoma cells.

The sialic acid residues of the plasma membrane glycoproteins were specifically labeled by oxidation with $NaIO_4$ followed by reduction with $NaB^3H_4$. Surface-labeled glycoproteins were released by short incubations with TPCK-trypsin at 37°C and then separated by gel filtration on Sepharose 6B column. The predominantly labeled fraction, GP $II_2$, was then purified by chromatography on DEAE-cellulose equilibrated with 0.05 M phosphate buffer, pH 7.5, and eluted with increasing molarities of NaCl. It was shown to be homogeneous by protein and carbohydrate staining on SDS-polyacrylamide gels, isoelectric focusing, rechromatography on DEAE-cellulose and immunoelectrophoresis. It has an apparent molecular weight of 110,000 daltons.

The location of GP $II_2$ on the cell surface was confirmed by the fact that it could be labeled metabolically with D-($^3$H) glucosamine and externally through the nonpenetrating periodate-$NaB^3H_4$ system.

GP $II_2$ could not be removed from the cell surface by high salt concentrations, chelator, or chaotropic agents but was released from the membrane by detergents. This suggests that GP $II_2$ could be an integral protein.

Analysis of the carbohydrate composition of GP $II_2$ revealed galactose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid as major constituents and mannose as a minor one. This suggests that it contains carbohydrate chains both O- and N-linked to the polypeptide chain, most of them being O-linked.

Finally, GP $II_2$ has a potent Concanavalin A receptor activity. It inhibits the interaction between Concanavalin A and hepatoma cells and suppresses its effects on hepatoma cell proliferation.

**Key words: hepatoma cells, cell surface components, membrane glycoproteins, lectin receptor, sialoglycoproteins, plasma membrane glycoproteins**

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Cell surface glycoproteins have been implicated in essential cellular functions, including ectobiological activities of cells, such as cellular adhesion, regulation of cell growth, and differentiation [1–4]. They may also serve as receptors for viruses [5], lectins, [6], etc, and mask surface antigens [7], thus playing an important role in the escape of tumors from immunosurveillance [8]. Transformation of epithelial cells to the malignant phenotype is accompanied by a modification of the biological functions that would depend upon the structural and/or topological features of plasma membrane glycoproteins [4, 9].

Despite current interest in cell surface glycoproteins, little has been reported regarding the structure, function, or configuration of any particular cell surface glycoprotein [10]. Only a few have been characterized [11]. These include the major histocompatibility antigens and Ia antigens of murine and human lymphoid cells [12], as well as epiglycanin, the major glycoprotein of $TA_3$-Ha ascites cells [13], and fibronectin, the membrane glycoprotein of normal cells, which has been found to be depleted or absent in tumor cells [14–15].

Lectins are proteins that bind to specific glycoprotein receptors on the cell surface and have a variety of biological effects [6, 16]. In particular, some lectins have differential effects on agglutination and in vitro growth of normal and transformed cells as a result of their binding to cell surface glycoproteins. We previously reported the binding of Concanavalin A to Zajdela cells [17] and showed that it inhibited the in vitro growth of these cells, but stimulated normal hepatic cell proliferation [18].

As part of study of plasma membrane glycoproteins from normal and transformed cells and in order to determine which role cell surface proteins might play in tumor growth in the system, we report here the isolation and characterization of a major cell surface glycoprotein from hepatoma ascites cells, which behaves as a Concanavalin A receptor.

## EXPERIMENTAL PROCEDURES

### Tumor Cells

Zajdela hepatoma ascites cells [19] were originally produced by dimethylaminoazobenzene injection into Sprague Dawley rats and maintained in the ascites form by intraperitoneal transplantation ($2.5 \times 10^7$ cells/0.25 ml/animal) in 7–9-week-old Sprague Dawley rats, 250 g in weight (Charles River, France). Tumor cells in suspension were harvested 7 days following transplantation and washed 4 times in 0.01 M $NaHCO_3$ pH 7.4, containing 0.15 M NaCl (Buffer A).

Particule analysis with a Coulter counter showed that 99% of the cells were tumor cells without erythrocyte or lymphocyte contamination.

### Labeling of Cell Surface Glycoproteins

**External labeling.** The outer glycoproteins on the cell surface were labeled by sodium borohydride reduction following sodium periodate oxidation as previously described [20]. Briefly, $10^7$ cells/ml were oxidized at a periodate concentration of $5 \times 10^{-4}$M for 15 min in the dark at 20°C. After washing twice, the cells were reduced with 1.5 nmoles tritiated sodium borohydride (Amersham, 280 mCi/mmole).

The identification of the labeled compound was performed by paper chromatography after hydrolysis of the cells in 0.05 N $H_2SO_4$ for 1 h at 80°C as previously

described [20]. Tritium is incorporated into an oxidation product of sialic acid. After labeling, cell viability was more than 90%.

**Metabolic labeling with D-($^3$H) glucosamine.** The cells at an initial density of 2.5 × $10^5$ cells/ml were cultured in the presence of D-($^3$H) glucosamine (Amersham 22.6 Ci/mmole) at a concentration of 1 μCi/ml for 8 h. The labeled products were identified upon hydrolysis of the cells in 2 N HCl for 2 h at 100°C followed by paper chromatography. Only D-glucosamine and D-galactosamine were labeled.

The amount of isotope incorporated into the glycoproteins was determined after precipitation of the cells with 10% trichloracetic acid and extraction with ethanol; the precipitate was solubilized with ACS (Amersham, Searle) and counted in a liquid scintillation spectrometer (Intertechnique SL 300).

## Trypsin Treatment of the Cells

Experiments were undertaken to determine the conditions for releasing cell surface glycoproteins in a soluble form without causing cell lysis.

Hepatoma cells were washed several times with Buffer A prior to trypsin treatment to ensure complete removal of serum proteins. The cells were then suspended in Buffer A at a concentration range of $10^6$ to $10^8$ cells/ml and incubated at various times and with various TPCK-trypsin concentrations (Worthington 224 units/mg) at temperatures between 4 and 37°C.

The cells were removed by centrifugation for 5 min at 200g and the supernatant concentrated and desalted by ultrafiltration over a UM 20 Amicon membrane. Cell viability was tested by trypan blue exclusion test or by recovery of ability to in vitro growth.

## Separation of Trypsin-Released Glycoproteins

The trypsin-released fraction was dialysed against 0.01 M Tris-HCl buffer, pH 7.8, containing 0.25% sodium deoxycholate (Buffer B) to ensure the solubility of macromolecules, and after centrifugation at 20,000g was applied to a column of Sepharose 6B (Pharmacia, Uppsala, Sweden) (100 × 2.5 cm), equilibrated with Buffer B. The void volume was determined with Blue Dextran 2,000 (Pharmacia) and the total volume with dinitrophenyl valine.

Fractions of 3 ml were collected and absorption at 280 nm was determined spectrophotometrically. Aliquots (0.1 ml) from each tube were monitored for radioactivity.

The fraction with the highest radioactivity and specific activity (cpm/mg protein) obtained from the Sepharose 6B chromatography was applied to a DEAE-cellulose column (10 × 2 cm) equilibrated with 0.05 M sodium phosphate buffer, pH 7.5 (Buffer C). After washing with 100 ml of the same buffer, bound glycoproteins were eluted using a discontinuous gradient of 0.1, 0.2, and 0.5 M NaCl in Buffer C. Fractions of 4 ml were collected and tested as above.

## Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to Weber and Osborn [21] on 10% acrylamide gels using a Buchler instrument.

Samples (50–100 μg protein) in 0.1 ml 0.01 M phosphate buffer, pH 7.0, containing 0.1% SDS and 0.1% 2-mercaptoethanol were heated for 5 min at 100°C and then applied to the gels with 20 μl glycerol and 25 μl bromophenol blue as marker.

Electrophoresis was run at a constant current of 6 mamp/gel for 6 h. The gels were fixed overnight in 12.5% trichloracetic acid and immersed in distilled water for 1 h. Proteins were stained for 20 min with 0.25% Coomassie brilliant blue in methanol : acetic acid : water (45 : 10 : 45 V/V) and carbohydrates by the periodic acid-Schiff (PAS) method of Zacharius et al [22].

The gels were scanned with a Vernon automatic densitometer (Paris, France) at 620 nm (protein stain) and 560 nm (carbohydrate stain).

The molecular weights of the glycoproteins were calculated from the mobilities of the macromolecules under investigation and standard proteins, which were plotted against a logarithmic scale of their molecular weights.

Molecular weight standards used to calibrate the gels were cytochrome C ($12 \times 10^3$), trypsin ($25 \times 10^3$), ovalbumin ($43 \times 10^3$), and bovine serum albumin ($68 \times 10^3$).

Radioactivity was counted after cutting the gels into 2 mm slices that were left overnight in Buffer C containing 1% SDS.

Isoelectrofocusing on tube gels was performed according to Doerr and Chrambach [23] in 5% polyacrylamide gels containing 2% ampholines (LKB) at a pH between 3.5 and 5.0. The gradient was determined by cutting the gels into 2 mm slices that were left overnight in a 0.15 M NaCl solution and the pH of each slice was measured. Radioactivity was counted as above.

## Chemical Analysis

Protein content was determined according to Lowry et al [24] with a bovine serum albumin solution (50 to 200 $\mu$g/ml) as standard. Carbohydrates were determined as described by Chambers and Clamp [25]. Trimethyl silylated carbohydrates were separated by gas-liquid chromatography (Hewlett-Packard model 5710 A equipped with a column of 3% OV-225) and using a temperature gradient of 160–230°C. Mannitol was used as internal reference and a mixture of free carbohydrates was used as standard for quantification. The sialic acid content was determined by the method of Warren [26]. Amino acid and hexosamine analysis were done by ion exchange chromatography on a Beckman Automatic Analyzer (Beckman Instruments Inc., CA) following hydrolysis of the sample in 6 N hydrochloric acid under vacuum at 105°C for 24 h and 6 h, respectively [27].

## Immunoelectrophoresis

Immunoelectrophoresis was performed according to Hirschfeld [28] for 75 min under a current of 4.5 mamp/cm. An antiserum was produced by injecting rabbits intradermally with $10^7$ hepatoma cells in Freund's complete adjuvant. The rabbits were injected intravenously with $5 \times 10^7$ cells 15 days later and bled 1 wk thereafter.

## Cell Culture

Zajdela hepatoma cells were cultured in 50 mm plastic Petri dishes in the presence of 3 ml Eagle's medium containing 10% fetal calf serum, in humidified air with 5% $CO_2$. The cells were seeded at an initial concentration of $2.5 \times 10^5$ cells/ml.

Concanavalin A (Sigma grade IV) was added to the cell culture at a final concentration of 3 $\mu$g/ml of culture medium.

A sample of each cell suspension was counted in a haemocytometer and cell viability was tested by trypan blue exclusion. Less than 5% of the cells were stained by trypan blue after 8 h of culture.

### Binding Assay [17]

The assay of the binding between Concanavalin A and hepatoma cells was done in a centrifuge tube by mixing 0.1 ml of cell suspension ($10^7$ cells/ml), 0.1 ml of $^{14}C$ Concanavalin A (specific activity : $1.4 \times 10^6$ dpm/mg) solution (100 $\mu$g/ml), and 0.1 ml of bovine serum albumin solution (5 mg/ml) in presence or absence of 0.2 M methyl $\alpha$-D-mannoside to check the specific binding. After incubation for 15 min at 4°C under shaking, the cells were harvested and washed 3 times in Buffer A by centrifugation at 200 rpm. The cells were resuspended in 10 ml ACS and counted in an Intertechnique spectrometer.

### Assay for Concanavalin A Receptor Activity

The Concanavalin A receptor activity of GP $II_2$ glycoprotein was estimated on the basis of its ability to inhibit the binding of labeled Concanavalin A to hepatoma cells and to suppress Concanavalin A–induced cell proliferation.

## RESULTS

### Release of Labeled Cell Surface Glycoproteins by Trypsin

Table I shows the effects of the different conditions of trypsin treatment on the release of labeled glycoproteins from the surface of hepatoma cells without affecting cell viability.

**TABLE I. Effects of Trypsin Treatment on the Amount of Radioactivity (External Labeling) and Protein Released From the Hepatoma Cells***

| Trypsin concentration[a] | Incubation temperature (°C) | Incubation time (min) | Protein released[a] | Radioactivity (percent) |
|---|---|---|---|---|
| 2 | 37 | 20 | 130 | 13 |
| 4 | 37 | 20 | 160 | 17 |
| 8 | 37 | 20 | 175 | 20 |
| 4 | 4 | 20 | 75 | 13 |
| 4 | 20 | 20 | 100 | 15 |
| 4 | 37 | 20 | 150 | 22 |
| 4 | 37 | 10 | 150 | 12 |
| 4 | 37 | 20 | 170 | 18 |
| 4 | 37 | 30 | 220 | 21 |
| 4 | 37 | 2 × 10[b] | 265 | 25 |

*Batches of $1 \times 10^7$ hepatoma cells/ml were oxidized with sodium periodate and labeled with $NaB^3H_4$ (see Experimental Procedures). They were treated with TPCK-trypsin under the conditions indicated in the table.

[a]Trypsin concentrations and amounts of protein released are expressed in $\mu g/10^7$ cells.

[b]After 10 min of incubation, the same amount of trypsin was added and the mixture incubated for another 10 min.

Cell number influenced the amount of material released. At 37°C and pH 7.4, there was a parallel increase between the latter and cell concentration, up to $10^7$ cells/ml, after which it decreased. A concentration of $10^7$ cells/ml was therefore used for all the experiments.

Temperature was also shown to have an effect. The proteins released increased from 75 μg at 4°C to 150 μg at 37°C without any effect on cell viability. At the same temperatures, the percentage of labeled material released also increased from 13 to 22%.

Trypsin concentration and incubation time were also studied. The most effective trypsin concentration was 4 μg/$10^7$ cells/ml, while 8 μg proved to be lethal. The amount of material released from the cell surface increased with incubation time, up to 20 min, although successive incubations improved the yield. The amount of both labeling and proteins released was found to be larger when cells were incubated twice for 10 min than with a single 20-min incubation.

The conditions selected were therefore $10^7$ cells/ml in Buffer A incubated at 37°C with 4 μg TPCK-trypsin for two 10-min periods. Under these conditions, 20 to 25% of the labeled membrane glycoproteins were removed.

Cell viability after trypsin treatment was > 95% and treated cells after 8 h culture regained the same proliferation capacity as intact cells.

SDS-polyacrylamide gel electrophoresis of the trypsinate revealed a major band with a molecular weight of about 110,000 daltons, which was strongly stained by periodate-Schiff reagent and weakly stained by Coomassie reagent. A few radioactive bands only faintly stained by periodate-Schiff and Coomassie reagents were also present.

## Purification of the Major Glycoprotein Released by Trypsin From the Hepatoma Cell Surface

The major glycoprotein found in the soluble fraction after treatment of hepatoma cells with trypsin was purified to homogeneity within only a few chromatography steps.

**Fractionation of trypsin digests on Sepharose 6B columns.** The trypsin-sensitive fraction derived from cell surface labeled by the periodate-$NaB^3H_4$ procedure was first concentrated and dialysed against Buffer B to ensure solubility of the macromolecules. The Sepharose 6B elution pattern revealed 5 peaks (Fig. 1), named GP I to V. Each fraction was analyzed for radioactivity and protein content (Table II) and subjected to polyacrylamide gel electrophoresis. The GP I fraction was exluded from the Sepharose 6B column and was weakly radioactive. Electrophoresis showed that fractions GP III, IV, and V were heterogeneous and, in addition, they had a particularly low specific radioactivity.

The GP II fraction contained 40% of the radioactivity and only 2.5% of the proteins of the trypsinate applied to the Sepharose 6B. Electrophoresis analysis revealed essentially a single radioactive band strongly stained by periodate-Schiff reagent and a few number of weakly stained bands (Fig. 2).

**Purification of the GP II fraction by DEAE-cellulose chromatography.** GP II was dialysed against 0.05 M sodium phosphate buffer, pH 7.5, and then chromatographed on a DEAE-cellulose column equilibrated with the same buffer. Elution was done with buffer containing increasing concentrations of NaCl (Fig. 3). Most of the radioactivity (70%) was recovered in fraction GP $II_2$ (0.2 M NaCl),

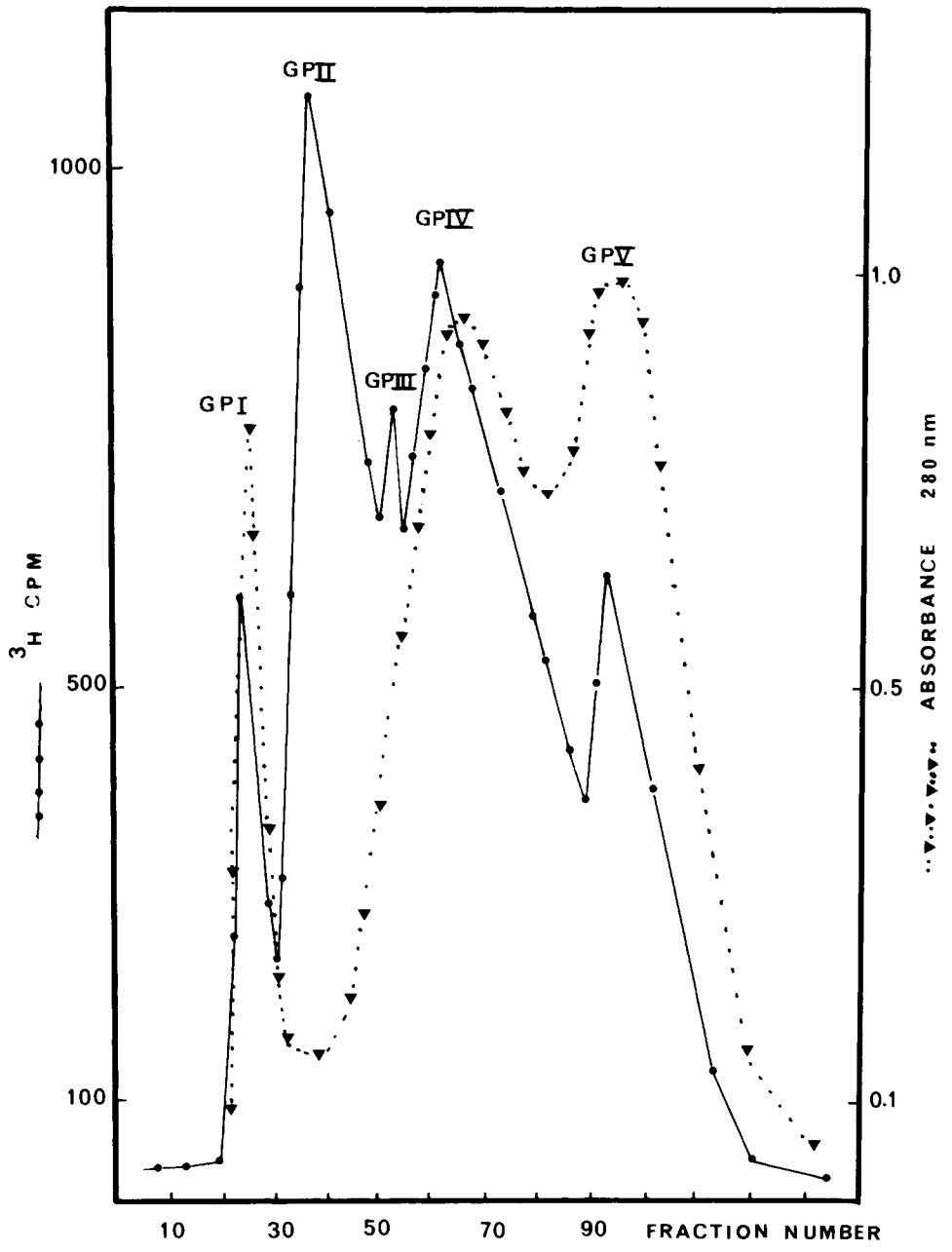


Fig. 1. Gel filtration on Sepharose 6B of trypsin digests of hepatoma cells. 10 mg of proteins in 2.0 ml 0.01 M Tris-HCl buffer, containing 0.25% sodium deoxycholate, were layered onto a Sepharose 6B column (100 × 2.5 cm). The column was then eluted with the same buffer at a flow rate of 10 ml/h and fractions of 3 ml were collected. · · · ▼ · · ▼ · ▼ · ·, absorbance at 280 nm. ●—●—●—●—●, $^3$H radioactivity.

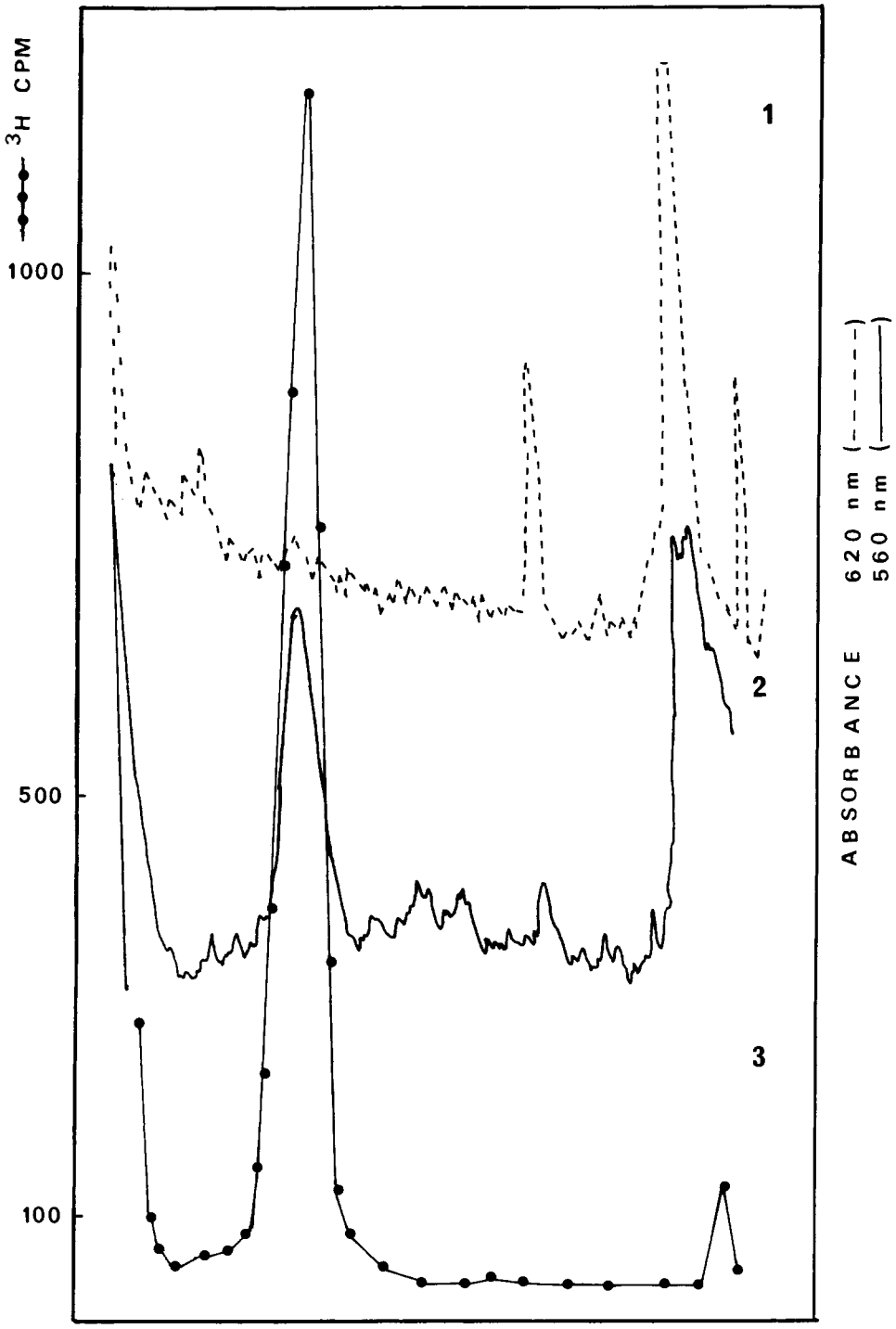


Fig. 2. SDS-polyacrylamide gel electrophoresis of the GP II fraction of the Sepharose 6B column eluate. Electrophoresis was performed in 10% acrylamide gel (10 × 0.5 cm) according to Weber and Osborn [21]. (1) After electrophoresis, one gel was stained for proteins -------- absorbance at 620 nm. (2) A second one was stained for carbohydrates ———— absorbance at 560 nm. (3) A third one was cut into 2 mm slices, incubated overnight with 0.05 M sodium phosphate buffer, pH 7.5, containing 1% SDS at room temperature, and counted —●—●—.

**TABLE II. Purification Steps of the Glycoprotein GP $II_2$ Released by Trypsin Treatment From Labeled Zajdela Hepatoma Ascites Cells***

| | Radioactivity (percent) | Protein (percent) | Specific activity Cpm/μg protein |
|---|---|---|---|
| Trypsin soluble material | 100 | 100 | 120 |
| Sepharose 6B chromatography | | | |
| GP I | 3 | 2 | 113 |
| GP II | 40 | 2.5 | 1,430 |
| GP III | 25 | 4.5 | 457 |
| GP IV | 22 | 25 | 103 |
| GP V | 10 | 66 | 12 |
| DEAE-cellulose chromatography | | | |
| GP $II_1$ | 2.7 | ND | ND |
| GP $II_2$ | 19 | 1.2 | 1,950 |
| GP $II_3$ | 5.4 | ND | ND |

*Labeled hepatoma cells were treated with TPCK-trypsin under the conditions described in the Experimental Procedures. After contrifugation, the supernatant was dialysed, lyophilized, and applied to a Sepharose 6B column equilibrated in Buffer B. The GP II fraction was dialysed and loaded on a DEAE-cellulose column equilibrated in Buffer C and eluted using a discontinuous NaCl gradient. The percentages of radioactivity and proteins are referred to the radioactivity and protein contents of the trypsin soluble material. The recoveries are 90% for Sepharose 6B column and 70% for the DEAE-cellulose column. ND: not determined.

whereas fraction GP $II_1$ (0.1 M NaCl) and GP $II_3$ (0.5 M NaCl) contained 10% and 20% of the radioactivity respectively (Table II).

**Homogeneity of the glycoprotein GP $II_2$.** The purity of GP $II_2$ after DEAE-cellulose chromatography was checked by various techniques. When rechromatographed on DEAE-cellulose column with an elution gradient of 0.1 to 0.5 M NaCl in 0.05 M phosphate buffer, pH 7.5, the glycoprotein eluted as a single and symmetrical peak at 0.2 M NaCl.

Radioactivity counting and periodate-Schiff reagent staining after SDS-polyacrylamide gel electrophoresis revealed one major band (Fig. 4a). Isoelectric focusing in a pH range of 3.5 to 5.0 showed one peak with a pI of 3.65 (Fig. 4b). The glycoprotein GP $II_2$ gave a single precipitation line by immunoelectrophoresis against a rabbit antiserum raised against Zajdela hepatoma cells (Fig. 4c).

From these data, GP $II_2$ appears homogenous and could be a major constituent released from Zajdela hepatoma ascites cell surface.

**Characterization of the major glycoprotein derived from the hepatoma cell surface.** SDS-polyacrylamide gel electrophoresis of the glycoprotein released by trypsin from intact hepatoma cells yielded a single band with an average apparent molecular weight of 110,000 daltons. Identical values for the apparent molecular weight were obtained when the glycoprotein was run on gels with higher percentages of acrylamide.

The glycoprotein released from the cells that were first treated with neuraminidase prior to the trypsin treatment had the same migration pattern.

The amino acid and carbohydrate compositions of GP $II_2$ are shown in Table III. Carbohydrates represented 32% of the molecule, which means that the molecular weight of the carbohydrate-free polypeptide would be 72,000 daltons. An in-

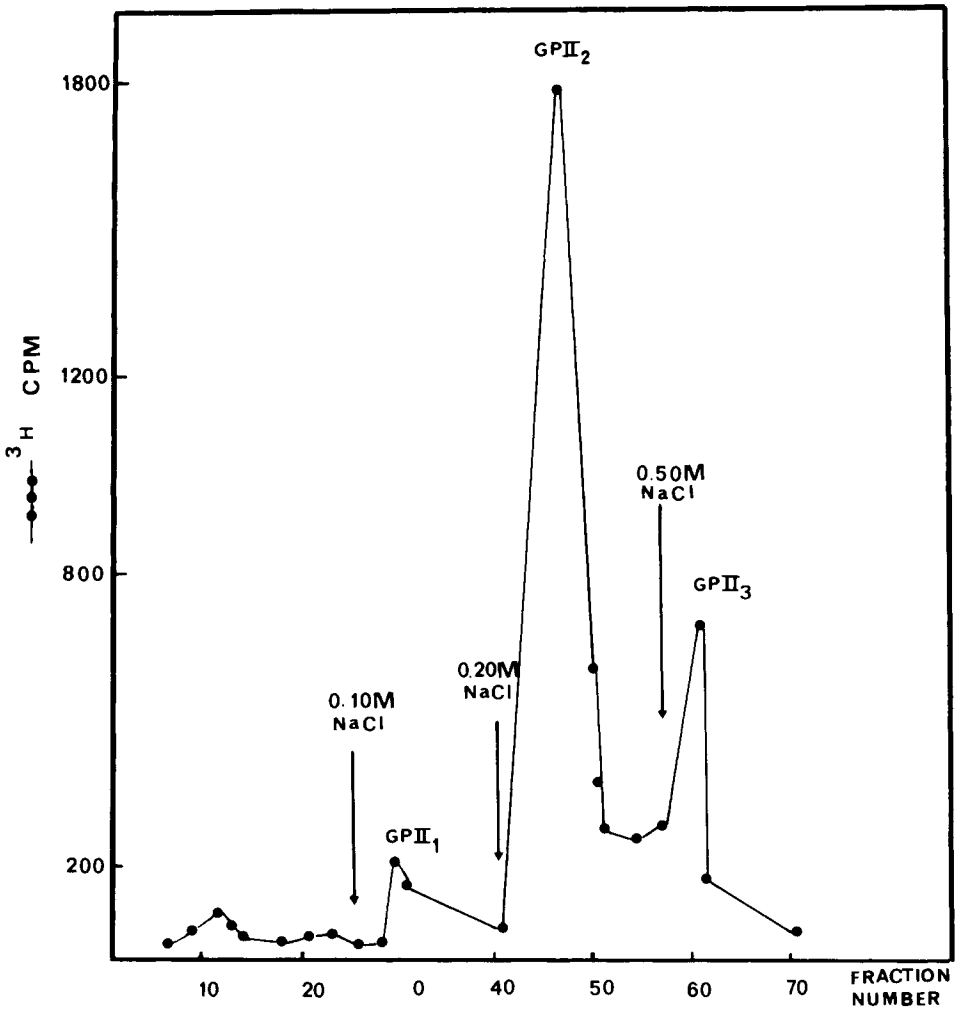


Fig. 3. DEAE-cellulose chromatography of the GP II fraction of the Sepharose 6B column eluate. GP II was dialysed against 0.05 M sodium phosphate buffer, pH 7.5. The fraction was applied to a DEAE-cellulose column (10 × 2 cm) equilibrated with the same buffer. Elution was carried out with 100 ml of phosphate buffer with different molarities of NaCl. Fractions of 4 ml were collected at a flow rate of 45 ml/h and counted for radioactivity.

teresting feature was the presence of both mannose (2.2%) and N-acetylgalactosamine (5.5%) in addition to galactose (7.0%), N-acetylglucosamine (7.0%), and sialic acid (10.0%).

This carbohydrate composition suggests that GP $II_2$ contains both N- and O-linked oligosaccharides. On the basis of the molecular weight and the N-acetylgalactosamine content, and on the assumption that this hexosamine is linked exclusively to threonine and serine residues in the polypeptide chain, it could be calculated that GP $II_2$ would contain 25 O-glycosidically linked chains per mole of glycoprotein. The estimate of the number of N-glycosidically linked chains is less certain.

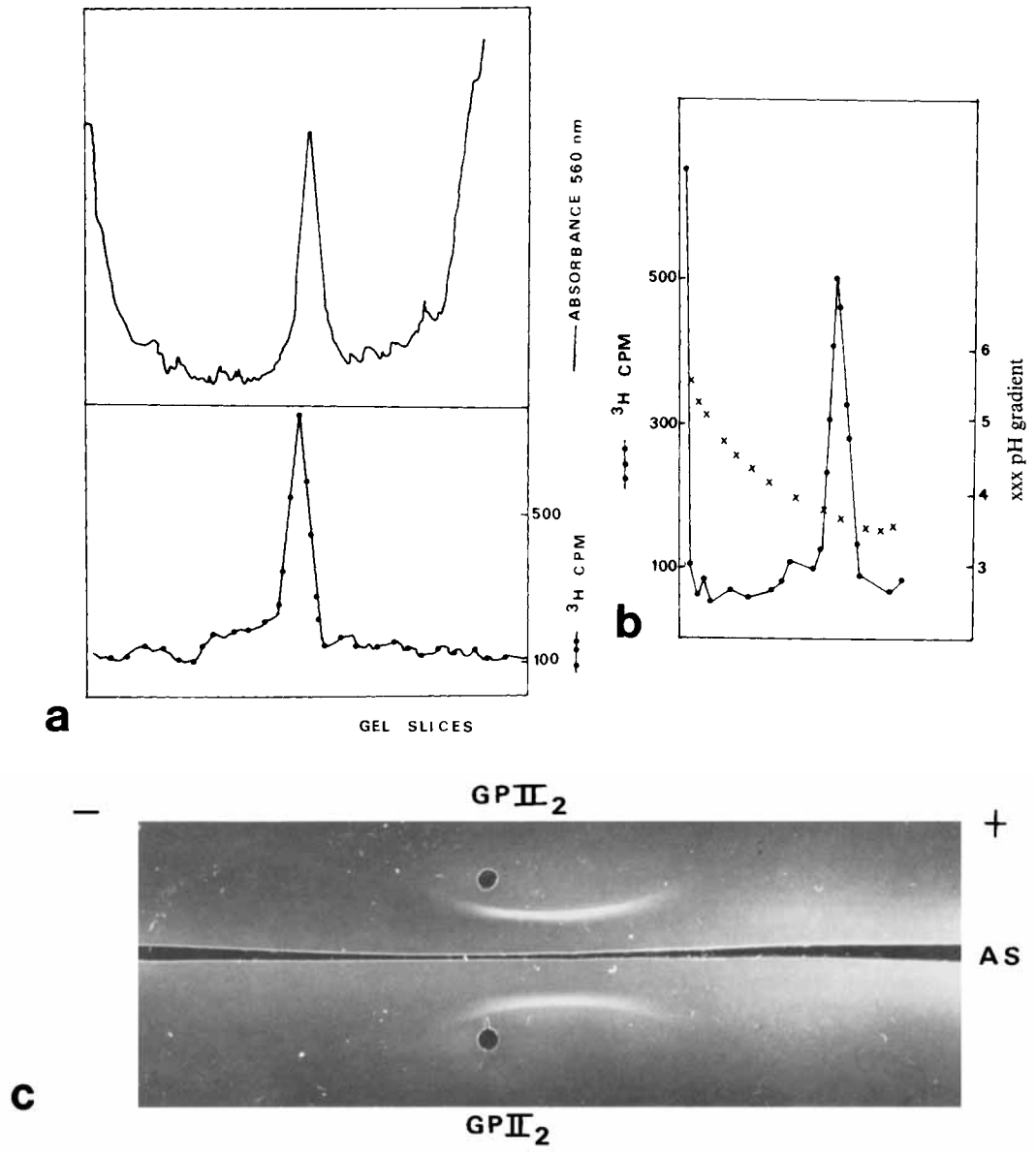


Fig. 4. Homogeneity of the GP $II_2$ glycoprotein. (a): SDS electrophoresis on 10% polyacrylamide gels. Electrophoresis was performed as described under Figure 2. ———— absorbance at 560 nm (carbohydrates). -•-•-•- radioactivity. (b): Electrofocusing was performed in 5% polyacrylamide gels containing 2% ampholines (pH 3.5 to 5.0). Radioactivity is counted in 2 mm each slice ( -•-•-•-•- ), pH gradient (xxxxx). (c): Immunoelectrophoresis : GP $II_2$ at 500 $\mu$g/ml. Center well : rabbit antiserum raised against hepatoma cells.

**TABLE III. Amino Acid and Carbohydrate Composition of the Major Plasma Membrane Glycoprotein of Zajdela Hepatoma Ascites Cells**

| Constituents | (percent) | Glycoprotein (nmole/nmole)[a] |
|---|---|---|
| Aspartic acid | 8.8 | 65.2 |
| Threonine | 6.0 | 50.0 |
| Serine | 4.9 | 46.8 |
| Glutamic acid | 11.4 | 76.8 |
| Glycine | 5.1 | 67.9 |
| Alanine | 5.6 | 62.9 |
| Valine | 4.0 | 34.2 |
| Isoleucine | 1.3 | 10.0 |
| Tyrosine | 1.2 | 7.3 |
| Leucine | 4.1 | 31.6 |
| Phenylalanine | 4.3 | 37.9 |
| Histidine | 3.6 | 18.4 |
| Lysine | 5.9 | 33.7 |
| Arginine | 1.9 | 10.5 |
| Mannose | 2.2 | 12.1 |
| Galactose | 6.9 | 38.4 |
| N-acetylglucosamine | 7.0 | 31.6 |
| N-acetylgalactosamine | 5.6 | 25.3 |
| Sialic acid | 9.8 | 31.3 |

[a]The values represent the average of 3 separate determinations. The nmoles are expressed on the basis of a molecular weight of 110,000 daltons for GP $II_2$.

**TABLE IV. Concanavalin A (Con A) Receptor Activity of the Glycoprotein GP $II_2$***

| | Cell proliferation $10^5$ cells/ml | Percent inhibition of cell growth | Con A binding Cpm/$10^6$ cells | Percent inhibition of binding |
|---|---|---|---|---|
| Control | 6.2 | 0 | ND | ND |
| Con A | 2.7 | 57 | 2.550 | 0 |
| Con A + α-MM | 5.2 | 16 | 390 | 85 |
| Con A + trypsinate | 5.9 | 5 | ND | ND |
| Con A + GP $II_2$ | 5.8 | 7 | 720 | 72 |

*The in vitro growth of hepatoma cells was measured after 8h of culture. The initial cell concentration was $2.5 \times 10^5$ cells/ml. 0.1 ml of Con A (100 μg/ml) was incubated with either 0.1 ml of medium or 0.1 of the different inhibitors for 30 min at room temperature and then the mixture was added to the cells. The binding of labeled Con A to the cells was performed under the conditions described in the Experimental Procedures. Inhibitors: 0.2 M methyl α-D-mannoside (α-MM), Crude trypsinate (900 μg/0.1 ml), GP $II_2$ (100 μg/0.1 ml). Each point represents the average value of 4 separate experiments. ND: not determined.

On the basis of 3 mannose residues per chain, 4 N-linked chains could be expected per mole of GP $II_2$.

**Location of GP $II_2$ on the hepatoma cells.** An indication of the location of GP $II_2$ was obtained from the cell viability following controlled proteolysis and by specific labeling of sialyl residues with a reagent that theoretically does not enter the cells. In addition, the metabolic labeling of GP $II_2$ with D-($^3$H)-glucosamine means

that the glycoprotein originates from the cell itself and is not an ascites ligand glycoprotein firmly bound to the plasma membrane and hence labeled by the surface labeling procedure. Furthermore, two GP $II_2$ preparations obtained from externally and metabolically labeled cells yielded two radioactive and PAS-stained components that had the same mobility in SDS-polyacrylamide gel electrophoresis.

The nature of the GP $II_2$ association with the plasma membrane was investigated through experiments releasing the glycoprotein from $^3$H-labeled hepatoma cells with a variety of reagents. None of the following procedures, known to elute peripheral membrane proteins, removed GP $II_2$ from the cells: divalent cation chelator agents; low (10 mM Tris-HCl) or high (0.05 M NaCl) ionic strength buffers; chaotropic reagents, LIS (10–20 mM) or $LiCl_2$ (up to 0.6 M). All of these solutions released less than 5% of the incorporated $^3$H label. By contrast, GP $II_2$ was easily solubilized when the cells were treated with ionic (SDS,DOC) or nonionic (Triton-X 100, NP 40) detergents. At concentrations below 0.1%, DOC Triton-X 100, NP 40 released 15–20% of the $^3$H radioactivity without altering cell viability, and increasing concentrations of detergent released increasing quantities of GP $II_2$.

## Concanavalin A Receptor Activity

It has been shown that in vitro, Concanavalin A decreased the growth of Zajdela hepatoma cells as a result of its binding to the glycoprotein receptor sites available [18].

The binding of Concanavalin A on hepatoma cells was studied in presence of methyl $\alpha$-D-mannoside (0.2 M) or GP $II_2$ (0.01 mM). Data presented in Table IV showed that this binding was inhibited at 85% and 72%, respectively.

Moreover, the inhibitory effect of Concanavalin A on cell proliferation was suppressed by both methyl $\alpha$-D-mannoside and GP $II_2$ under the conditions reported in Table IV.

Thus GP $II_2$ proved to be an extremely potent inhibitor of both Concanavalin A binding to the cell surface and its effects on cell proliferation.

## DISCUSSION

The critical role of cell surface glycoproteins in cell transformation is widely accepted [29], but very few cell surface macromolecules have been isolated from nucleated cells.

In this paper we report the isolation from hepatoma cells of a major, homogenous sialoglycoprotein that is a cell surface glycoprotein and possibly an integral protein of the membrane and that appears to be a Concanavalin A receptor site.

Most membrane glycoproteins are almost insoluble in neutral aqueous solution. Proteolytic enzymes have therefore been used to cleave the cell surface components of intact cells and release soluble glycopeptides [30–32]. The glycopeptides released can then be fractionated. The amount of material released depends on the cell type and the proteolytic conditions. In our experiments about 20–25% of the labeled glycoconjugates were released from the hepatoma cell surface under optimal conditions. This value is lower than the value obtained for TA-3H mammary adenocarcinoma ascites cells (40–50%) [33] but similar to that obtained for BHK 21 cells where, depending on the trypsin concentration, between 6 and 24% of the material was found to be released [34,35].

The fact that high cell viability was maintained and cell morphology remained unchanged after proteolysis, that exclusively sialyl residues were labeled, and that the molecules labeled externally or metabolically and released from the hepatoma cell surface were identical can be considered as a proof that GP $II_2$ is a cell surface glycoprotein.

GP $II_2$ could not be released from the cells by salt buffers, chelator, or chaotropic reagents, but increasing concentrations of detergents solubilized increasing amounts of labeled glycoprotein. Nevertheless, at concentrations above 0.1%, the detergents disrupted the lipid bilayer. It is possible that GP $II_2$ is a hydrophilic fragment of a higher molecular weight integral glycoprotein that is cleaved by trypsin. This is at present under investigation in our laboratory. Baumann and Doyle [36] described a similar phenomenon in HTC hepatoma cells. Kramer and Canellakis [37] and Cooper et al [38] have observed a discrepancy between the molecular weights of membrane glycoproteins and those released into the blood and ascites fluid.

GP $II_2$ was shown to be a Concanavalin A receptor site and it could be at least partially responsible for inhibiting the effect of Concanavalin A on the in vitro growth of hepatoma cells. It proved to be a potent inhibitor of both the interaction of the lectin with the cells and its effects on cell proliferation. As a receptor, GP II proved to be more potent than mannose. As has been noted with other lectins, glycoproteins are more effective hapten inhibitors than simple sugars [39]. It would be interesting to know how GP $II_2$ compares with other Concanavalin A receptors but these have been neither purified [40–42] nor analyzed for carbohydrate composition [43].

GP $II_2$ is clearly a major sialoglycoprotein of the hepatoma cell surface. This was borne out by the high percentage of label recovered in the GP $II_2$ fraction in comparison with the total amount of radioactivity taken up by the cells and also the results of SDS-polyacrylamide gel electrophoresis of either the cells or proteolysates. The intense PAS reaction of GP $II_2$ suggests a high degree of sialylation.

The apparent molecular weight of 110,000 daltons for GP $II_2$ is probably an overestimation in view of the irregular behavior of cell membrane glycoproteins in SDS-polyacrylamide gel electrophoresis [44]. Glycoproteins migrate at rates that are not strictly proportional to their molecular weight.

The presence of both N-acetylgalactosamine and mannose residues suggests that GP $II_2$ contains both N- and O-glycosidically linked chains. The existence of both linkages in the same molecule is not uncommon. This has been observed in soluble glycoproteins such as IgA [45], fetuin [46], and, in particular, in membrane glycoproteins [47]. The carbohydrate composition of GP $II_2$ also suggests the presence of complex O-linked glycan chains containing both N-acetylglucosamine and N-acetylgalactosamine residues. A similar composition with both hexosamines has been found in a number of membrane glycoproteins but several structures can explain this composition. The O-linked glycan chains of glycophorin, by contrast, consist of tri- or tetrasaccharide structures with only N-acetylgalactosamine [48].

For a number of reasons it has been very difficult to compare the carbohydrate compositions of the glycoconjugates released from the cell surfaces of various normal and transformed cells and, in particular, to determine with any certainty the ratios between the numbers of O- and N-linked chains. Some of these molecules have been analyzed in different systems (whole membranes, glycoproteins, single

and monolayer cells, nucleated and nonnucleated cells), which rendered the comparison difficult and often it was not known if the different oligosaccharide moieties were present in a single or in a number of glycoproteins.

GP $II_2$ appears to bear little resemblance to the major glycoprotein of either erythrocytes [11,49] or platelets [50], or to the homogeneous tumor cell surface glycoproteins the oligosaccharide chains of which are exclusively O-linked [7,51]. It also differs from the glycoprotein of normal tissues [52] and of some tumor cell surfaces [15,53,54]. Comparison with other hepatoma ascites glycoconjugates has been difficult since the analyses reported have often been of heterogeneous glycopeptide fractions. Nevertheless, some of these appear to be similar to GP $II_2$ in carbohydrate composition [36,40–42,47,55].

It is therefore impossible at this stage to say whether the presence of O-glycosidic in addition to N-glycosidic glycopeptides is characteristic of the plasma membranes of either cancer cells or free cells, such as erythrocytes, platelets, and ascites cells. This is important in determining the role of this major glycoprotein in hepatoma ascites cells. The predominance of GP $II_2$ on the cell surface suggests that it has a protective function similar to that of epiglycanin in mouse TA3-Ha mammary tumor cells [7]. It would mask membrane histocompatibility antigens, thus allowing the transplantation of the cells to noncompatible hosts. Like epiglycanin, GP $II_2$ could allow the cells to escape to immunosurveillance.

The study therefore contributes towards the characterization of a major plasma membrane glycoprotein of hepatoma cells and provides a basis for future comparison between the glycoproteins of normal liver and hepatoma cells.

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