Characterization of a Family of Glycoproteins Associated with the Bile Canalicular Membrane of Normal Hepatocytes but Not Expressed by Two Transplantable Rat Hepatocellular Carcinomas


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ABSTRACT

Xenoantisera were used to investigate the expression of normal cell-surface components on transplantable rat hepatocellular carcinoma (253 and 311c) cells. Cell-surface components, immunoprecipitated from non-ionic detergent extracts of 125I-labeled hepatocytes by xenoantisera against rat hepatocytes, 253 cells, or 311c cells, were analyzed by 2-dimensional polyacrylamide gel electrophoresis. Comparison of the gel patterns revealed that anti-hepatocyte antiserum was reactive with a component having an apparent molecular weight of 105,000 (gp105) that was not recognized by xenoantisera against 253 or 311c cells. This component was isolated by affinity adsorption to Sepharose-conjugated wheat germ agglutinin, reconstituted in liposomes, and used to immunize a rabbit. The resulting antiserum, anti-gp105, was reactive with a family of glycoproteins that had an apparent molecular weight of 105,000 but differed in isoelectric points. Immunohistochemical techniques revealed that gp105 was localized in the bile canalicular domain of the hepatocyte plasma membrane. Immunodepletion analysis, blocking of immunoprecipitation by extracts of tumor cells, and labeling of cells by immunohistochemical techniques indicated that gp105 was not expressed at the surface of 253 or 311c cells. These studies suggest that the altered expression of gp105 on 253 and 311c hepatocellular carcinoma cells may be associated with the abnormal tissue architecture and growth patterns exhibited by these transplantable tumors.

INTRODUCTION

In recent years, there has been increasing interest in determining the relationship between cell surface alterations and the phenotypic changes characteristic of malignant cells, e.g., loss of histotypic growth patterns, invasion of surrounding tissues, and metastasis to distant sites. Initially, considerable emphasis was placed on delineating qualitative or quantitative changes associated with the plasma membrane of malignant cells. A number of investigators reported differences in the interaction of normal and malignant cells with plant lectins (24). Others reported changes in glycolipid composition (6, 13) or in the size and structure of glycopeptides cleaved from intact cells by protease digestion (12, 31–33, 36). Cell surface changes were also detected in a number of studies by the ability of malignant cells to induce specific protective immune responses in host animals (28).

Although these types of studies have been valuable in delineating changes in the overall carbohydrate and antigenic composition of the cell surface, they give only limited insight into the nature of quantitative or qualitative changes in specific cell surface components or the functional consequences resulting from these changes. To overcome this limitation, many recent investigations have used an immunological approach in which xenoantisera or monoclonal antibodies raised against malignant cells are used as immunological probes to isolate and characterize antigenically distinct cell surface components on malignant cells. Application of this approach has allowed the identification of inappropriately expressed normal tissue antigens such as HLA-DR antigens (37) or transferrin-like receptors on human melanoma cells (5), oncospecific antigens on human pancreatic carcinoma cells (10), and most recently a clonally expressed tumor-specific antigen found on mouse lymphoma cells but not on any adult or fetal lymphoid cells (1).

In the present studies, we have used xenoantisera to investigate the expression of normal cell-surface components on 2 transplantable hepatocellular carcinomas. In this study, we describe the isolation by lectin affinity chromatography of a subset of cell-surface glycoproteins present on normal ACI rat hepatocytes. These glycoproteins have an apparent molecular weight of 105,000 by SDS-PAGE and have been collectively designated gp105. We show by immunohistological analysis, using a rabbit antiserum raised against gp105, that components antigenically related to gp105 are absent from the surface of cells of the 253 and 311c hepatocellular carcinomas, transplantable tumors that were derived from primary tumors induced in ACI rats by 2-acetylaminofluorene.

We further demonstrate, using indirect immunofluorescence and immunoferritin-labeling techniques, that this family of glycoproteins is localized in situ in the bile canalicular domain of the hepatocyte plasma membrane, suggesting that the absence of gp105 from transplantable hepatocellular carcinomas may be associated with the loss of normal tissue architecture.

MATERIALS AND METHODS

Normal Adult Hepatocytes. Hepatocytes were obtained from normal adult and regenerating ACI rat livers by a modification of the collagenase perfusion technique of Bonney et al. (4), as modified by Starling et al.

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3 The abbreviations used are: SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; gp105, glycoproteins with a molecular weight of 105,000; PBS, phosphate-buffered saline; NP40, Nonidet P-40; SACI, heat-killed, formalin-fixed Staphylococcus aureus Cowan I; SDS, sodium dodecyl sulfate; RCA, Ricinus communis agglutinin I; LCA, Lens culinaris agglutinin; WGA, wheat germ agglutinin.

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from New England Nuclear, Boston, Mass. Labeled cells were extracted
transplants. This was accomplished by mechanically expelling the liver
collected from the buffer-Ficoll:Hypaque interface and washed 3 times in
the resulting cell suspension (10^7 cells) was layered onto an equal volume
from Dr. Frederick Becker at the University of Texas M. D. Anderson
Publications Division, Bastrop, Texas.

Transplantable Hepatocellular Carcinomas. Two solid transplantable
hepatocellular carcinomas (253 and 311c) derived from primary
tumors induced in ACI rats by 2-acetylaminofluorene (2,3) were obtained
Veterinary Resources Division, Bastrop, Texas.

In some cases, 253 and 311c cells were grown as intrahepatic
transplants. This was accomplished by mechanically expelling the liver
through a lateral incision made below the xyphoid process of an anes-
ethetized rat and injecting tumor cells just under the surface of Glissen’s
capsule. After 5 to 7 days, the resulting nodules were excised and
utilized for immunofluorescence staining as described below.

Radiolabeling Procedures. Cells were surface labeled with [3H] (carrier
free; Amersham/Seearle Corp., Arlington Heights, Ill.) by the lactoperox-
ide;glucose oxidase procedure of Keski-Oja et al. (18). ^H labeling was
accomplished using the method of Liao et al. (21) as modified by
Gahlberg and Anderson (9). NaB^4H (40 to 80 Ci/mmol) was obtained from
New England Nuclear, Boston, Mass. Labeled cells were extracted
for 1 hr at 4° in a buffer containing 10 mM Tris-HCl, 150 mM NaCl, and
0.5% NP40 (Particle Data Laboratories, Ltd., Elmhurst, Ill.). Extracts
were clarified by centrifugation on 30,000 x g for 20 min and stored at
-20°. Specific activity of labeled cells was approximately 2 to 3 cpm/
cell for ^H and 5 to 9 dpm/cell for 3H.

Immunoprecipitation Analysis. Immunoprecipitation analysis was
accomplished by incubating aliquots of detergent lysates from radioiodi-
nated cells for 16 hr at 4° with 5 to 10 μl of antiserum. Immune complexes
were subsequently collected by adsorption to SACI (10% suspension;
The Enzyme Center, Inc., Boston, Mass.) for 2 hr at 4°. The SACI-bound
immune complexes were then washed in a buffer containing 10 mM Tris
150 mM NaCl, 1 mM sodium EDTA, 1 mg ovalbumin per ml, and 0.5%
NP40, pH 8.0. Immune complexes were eluted by suspending the
washed SACI for 1 hr in urea sample buffer [9.5 M urea; 2% NP40;2%
ampholites (LKB Instruments, Inc., Rockville, Md.); 1.5% β-mercaptoetha-
ol] or by heating at 100° for 5 min in SDS sample buffer (63 mM Tris:2%
SDS:2% β-mercaptoethanol:10% glycerol, pH 6.8).

Blocking of Immunoprecipitation with Unlabeled Cell Lysates. In
some cases, radioimmunoprecipitation was carried out with antiseras
that had been preincubated at 4° with an unlabeled cell lysate. After 16 hr,
an aliquot of radiiodinated cell extract representing 25% of the number
of cell equivalents in the unlabeled lysate was added. Following an
additional incubation for 16 hr at 4°, immune complexes were absorbed
on SACI and eluted as described above.

Absorption of Antiseras with Intact Cells. Absorption of antiserum
with intact cells was accomplished by incubating 100 to 200 μl of antiserum with an equal volume of packed cells for 1 to 2 hr at 23°. Cells
were then removed by centrifugation, and a fresh aliquot of cells was
added to the partially absorbed antiserum. After 3 absorptions with intact
cells, the antiserum was clarified at 100,000 X g for 5 min in a Beckman
airhge and stored at -20°.

Affinity Chromatography on Carrier-bound Lectins. Lectin affinity
chromatography was performed on Sepharose-bound RCA, LCA, or
WGA, WGA and WGA were prepared by procedures described previ-
ously (15). CA was purified by affinity chromatography on colchicine-binding affinity columns (Pharmacia). Lectins were coupled to Sepharose 4B using the cyanogen
bromide coupling method of Custances (7). Coupling was performed in
the presence of the appropriate saccharide inhibitor for each lectin
(0.2 m lactose for RCA, 0.3 m 2-acetamido-2-deoxy-o-glucose for WGA,
and 0.2 m methyl α-D-mannoside for LCA). Sepharose-conjugated lectins
contained approximately 10 mg of covalently bound lectin per ml of
packed gel. Lectin affinity chromatography was accomplished by apply-
ing components solubilized in 0.5% NP40 from 2 x 10^7 radiiodinated
cells to a column (0.6-cm internal diameter) containing 0.5 mg of Sephar-
ose-bound lectin equilibrated with PBS containing 0.5% NP40. Unbound
components were eluted with PBS containing 0.5% NP40, while material
specifically bound to the affinity column was released by elution with
PBS containing 0.5% NP40 and the appropriate monosaccharide inhibi-
tor. Fractions (0.5 ml) were collected and monitored for radioactivity
using a Packard Auto-Gamma scintillation spectrometer. Aliquots were
taken from the fractions in the bound and unbound peaks containing
the highest amount of reactivity and were analyzed by SDSPAGE.

Preparation of Liposomes Containing Components Bound to
WGA:Sepharose. Material extracted after 1 hr in 0.5% NP40 from 3 x
10^7 hepatocytes was submitted to affinity chromatography on a column
(0.5-cm internal diameter) containing 3 ml of Sepharose-bound WGA.
The column was washed with 2 bed volumes of PBS containing 0.2 m
2-acetamido-2-deoxy-o-glucose (Sigma) and 0.5% NP40, followed by 6 to
7 bed volumes of PBS containing 0.5% NP40 prior to loading of cell
extracts. Unbound components were eluted from the column in 10 mM
Tris, pH 7.8, containing 0.5% sodium deoxycholate (Fischer Scientific
Co., Pittsburgh, Pa.). Bound components were eluted in the same buffer
containing 0.3 m 2-acetamido-2-deoxy-o-glucose.

Fractions (1 ml) containing bound components were monitored for
absorbance at 280 nm. Peak fractions showing the highest absorbance
were pooled and assayed for protein using a modification of the method
of Lowry et al. (22) as modified by Peterson et al. (27). Components bound
to Sepharose-bound WGA were reconstituted into liposomes using a modifica-
tion of the procedure described by Englehard et al. (8).

For reconstitution, dimyristoylphosphatidylcholine (4 mg; Sigma), chole-
terol (3 mg; Sigma), and octyl glucoside (28 mg; Boehringer Mannheim,
Indianapolis, Ind.) were dissolved in a 2:1 mixture of chloroform:methanol
(4 ml). This solution was placed in a 200-ml round-bottomed flask and
dried under vacuum on a rotary evaporator at 37°. The resulting film
was redisolved twice in diethyl ether, and the liquid was evaporated.
Using a small stirring bar, the lipid film was dispersed into 3 ml of 0.5%
sodium deoxycholate in 10 mM Tris, pH 7.8, containing 300 μg of the
components bound to Sepharose-WGA. The contents of the film were
mixed vigorously, and the resulting suspension was submitted to dialysis
at 23° for 48 to 64 hr against one change of 4 mM Tris, pH 7.4, containing
0.05% sodium azide and 3 changes (1 liter) of the same buffer without
sodium azide. An aliquot (2 or 5 μl) of this solution was then placed onto
a Formvar-coated 300 mesh copper grid, negatively stained in 2%
ammonium molybdate, dried, and examined by electron microscopy to
confirm the presence of small unilamellar vesicles. Preparations shown
by electron microscopy to contain vesicles were concentrated against
solid polyethylene glycol (average M, 20,000) to a final volume of 1 ml.

Immunization of Rabbits. A male New Zealand White rabbit was
immunized by i.m. and s.c. injection of a mixture (equal volumes) of 300
μg of WGA-bound components reconstituted in liposomes in either
complete (first immunization) or incomplete (second and third immuni-
izations) Freund’s adjuvant. Immunizations were performed at 2-week
intervals. The rabbit was bled prior to the first injection and then 10 days
after each subsequent injection of the antigen. Antisera against normal
hepatocytes and 253 and 311c hepatocellular carcinoma antigens were
obtained from animals immunized with whole cells. Rat livers perfused
with Locke’s solution containing EDTA and 0.5 m sucrose were minced.

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and mechanically dispersed. Tumors 253 and 311c, were excised, minced, and dissociated by passage through a 6-ml syringe. Rabbits were then immunized by 2 i.p. injections 2 weeks apart with 5 × 10⁷ cells in Freund’s complete adjuvant, followed by booster injections at 2-week intervals. Cells were suspended in sterile 0.9% NaCl solution. Rabbits were bled 10 days after each injection. Serum collected after each bleeding was heat inactivated for 15 min at 55°C, divided into small aliquots, and stored at −20°C.

**Polyacrylamide Gel Electrophoresis.** SDS-PAGE was performed according to the procedure of Laemmli (20) on 7.5 or 10% slab gels. Electrophoresis was performed on 1.5-mm slabs at constant current (25 ma/gel). Two-dimensional electrophoresis was carried out essentially as described by O’Farrell (25). Immunoprecipitated components eluted in urea sample buffer were separated by isoelectric focusing for 14 hr on 4% polyacrylamide tube gels containing 9 m urea, 2% NP40, and 2% amphotelytes (LBK ampholine). After equilibration with SDS sample buffer, tube gels were mated with agarose to the stacking gel of a 10% slab gel and subjected to electrophoresis according to the procedure of Laemmli (20). Both 1- and 2-dimensional gels were stained overnight in 0.2% Coomassie Blue R-250, obtained in methanol:acetic acid:water (4:1.5, v/v) for 7 hr to 8 hr, and rehydrated in methanol:acetic acid:glycerol-water (5:10:4:81, v/v) (11). Gels were then dried under vacuum and visualized by autoradiography at −70°C on Kodak XAR-5 film with Dupont Cronex Lightning Plus intensifying screens or impregnated with Enhance (New England Nuclear) and visualized by fluorography at −70°C using Kodak XAR-5 X-ray film. Apparent molecular weights were calculated from protein standards [β-galactosidase (M, 116,000), bovine serum albumin (M, 68,000), and ovalbumin (M, 43,000) run concurrently with radioactively labeled samples].

**Indirect Immunoferritin Labeling.** Indirect immunoferritin labeling was performed on sections (6 to 8 μm thick) of ACI rat liver that had been embedded in Polyethylene Glycol 1000 (Fisher) using a modification of the procedure of Mazurkiewicz and Nakane (23). Rat livers were fixed by perfusion via the inferior vena cava with PBS containing 0.05% (v/v) glutaraldehyde (Polyscience, Warrington, Pa.) and 0.5% (v/v) acrolein (Polyscience). Following perfusion, the liver tissue was quickly excised, minced, and dissociated by passage through a 6-ml syringe. Rabbits and mechanically dispersed. Tumors 253 and 311c, were excised, minced, and dissociated by passage through a 6-ml syringe. Rabbits were then immunized by 2 i.p. injections 2 weeks apart with 5 × 10⁷ cells in Freund’s complete adjuvant, followed by booster injections at 2-week intervals. Cells were suspended in sterile 0.9% NaCl solution. Rabbits were bled 10 days after each injection. Serum collected after each bleeding was heat inactivated for 15 min at 55°C, divided into small aliquots, and stored at −20°C.

**Immunoprecipitation Analysis of Components Reactive with Xenoantisera.** Fig. 1 shows a comparison of components immunoprecipitated from extracts of normal hepatocytes with anti-normal hepatocyte and anti-253 antisera. It can be seen that, with the exception of a major component with an apparent molecular weight of 105,000, both antisera showed remarkably similar patterns of reactivity. This M, 105,000 component was not found in material immunoprecipitated from normal hepatocyte extracts with anti-311c antisera and was missing from 2-dimensional gel profiles of components immunoprecipitated from 253 and 311c cell extracts with anti-normal hepatocyte, anti-253, and anti-311c antisera (data not shown).

**Immunodepletion Analysis.** The absence of a major component or components with an apparent molecular weight of 105,000 on 253 and 311c cells was further confirmed by immunodepletion analysis. Normal hepatocyte extracts were sequentially immunoprecipitated with anti-253 antisera until all components reactive with this antisera were removed (Fig. 2, Lane b). Electrophoretic analysis of components immunoprecipitated from this immunodepleted extract with anti-normal hepatocyte antisera showed that the only component reactive with anti-normal hepatocyte antisera that was not removed by prior immunoprecipitation with anti-253 antisera was a component with an apparent molecular weight of 105,000 (Fig. 2, Lane c). An identical result was obtained with anti-311c-depleted extracts (data not shown).

**Lectin Affinity Chromatography.** Radiiodinated extracts of normal hepatocytes were submitted to affinity chromatography on Sepharose-bound LCA, RCA, or WGA. Results from affinity chromatography (Fig. 2, Lanes d to l) indicated that all 3 of the carrier-bound lectins were effective in selectively isolating a limited number of plasma membrane glycoproteins from the bulk of the radiolabeled cell-surface components. Comparison of autoradiograms of bound material also revealed that a glycoprotein with an apparent molecular weight of 105,000 was one of the major components in the bound fraction from Sepharose:RCA, and :LCA and was essentially the only component bound to Sepharose:WGA. Radioimmunoprecipitation analysis of the material bound to Sepharose:WGA (Fig. 2, Lanes j to l) revealed that this M, 105,000 component was reactive with anti-normal hepatocyte antisera but not with anti-253 or anti-311c antisera, suggesting that this component was identical to the M, 105,000 component immunoprecipitated from immunodepleted extracts.

NH₄HCO₃, and lyophilized. This lyophilized material was then dissolved in 10 to 20 μl of 10 mw acetic acid, diluted to 200 μl with PBS, pH 8.0, clarified in an airfuge at 100,000 × g, and stored frozen at −20°C.

**Immunofluorescence.** Indirect immunofluorescent staining was performed on frozen sections of ACI rat liver. Frozen sections, 4 to 6 μm thick, were mounted on glass slides and fixed for 10 min in ice-cold 100% acetone. Following fixation, sections were washed extensively in PBS and incubated for 45 min at 23°C in antiserum that had been affinity purified on intact cells. Sections were then washed in PBS for 20 min, incubated in 1% normal goat serum for 5 min, and incubated for 45 min in fluorescein-conjugated affinity-purified goat anti-rabbit immunoglobulin antibodies (Kirkegaard and Perry, Gaithersburg, Md.). Following a 20-min wash in PBS, sections were placed under coverslips in 90% glycerol, buffered to pH 9.5 with 0.5 μ Na₂CO₃, and examined in a Zeiss WL light microscope fitted with an IV Fl-epifluorescence condenser. Sections were photographed on Ilford XP-1 film at ASA 800.

**RESULTS**

**Immunoprecipitation Analysis of Components Reactive with Xenoantisera.** Fig. 1 shows a comparison of components immunoprecipitated from extracts of normal hepatocytes with anti-normal hepatocyte and anti-253 antisera. It can be seen that, with the exception of a major component with an apparent molecular weight of 105,000, both antisera showed remarkably similar patterns of reactivity. This M, 105,000 component was not found in material immunoprecipitated from normal hepatocyte extracts with anti-311c antisera and was missing from 2-dimensional gel profiles of components immunoprecipitated from 253 and 311c cell extracts with anti-normal hepatocyte, anti-253, and anti-311c antisera (data not shown).

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with anti-normal hepatocyte antiserum (Fig. 2, Lane c). This M, 105,000 component was designated gp105.

Preparation and Characterization of a Rabbit Antiserum against gp105. A rabbit was immunized with gp105 that had been isolated from detergent lysates of normal hepatocytes by affinity chromatography on Sepharose:WGA and reconstituted into phospholipid vesicles. The resulting antiserum, designated anti-gp105, was shown by immunoprecipitation analysis to be highly specific for gp105. Analysis of immunoprecipitates by 2-dimensional polyacrylamide gel electrophoresis revealed that this antiserum was reactive with a family of glycoproteins exhibiting similar apparent molecular weights but differing in isoelectric points (Fig. 3a). Affinity-purified anti-gp105 showed an identical pattern of reactivity (data not shown). An identical 2-dimensional gel pattern was also obtained from components immunoprecipitated by anti-gp105 from extracts of radiolabeled regenerating hepatocytes (Fig. 3b). When components immunoprecipitated by anti-normal hepatocyte antiserum from extracts immunodepleted with either anti-253 or anti-311c antiserum were analyzed by 2-dimensional polyacrylamide gel electrophoresis, a gel pattern essentially identical to that observed with anti-gp105 was obtained (Fig. 3c), suggesting that anti-gp105 was indeed reactive with the major component that was immunoprecipitated by anti-normal hepatocyte antiserum from immunodepleted extracts.

The apparent reduction in the quantity of the most acidic component immunoprecipitated after immunodepletion raises the possibility that this species is expressed in low amounts. How this component is related to gp105, was shown by immunoprecipitation analysis to be highly specific for gp105. Analysis of components immunoprecipitated from extracts of normal hepatocytes and 253 and 311c hepatocellular carcinoma cells. Comparison of cell surface components isolated by this approach revealed the presence of a M, 105,000 component on normal hepatocytes and 253 and 311c hepatocellular carcinoma cells. This component was subsequently isolated from detergent lysates of normal hepatocytes by preparative affinity chromatography on Sepharose-bound WGA and was reconstituted into liposomes and used for immunization of rabbits. The resulting antiserum was shown by immunoprecipitation analysis to be highly specific for a component that migrated as a single broad band (apparent M, 105,000) when analyzed by SDS-PAGE. The major component immunoprecipitated from 3H-labeled extracts with anti-gp105 antisera, no similar component was detected in immunoprecipitates from extracts of 3H-labeled 253 hepatocellular carcinoma cells (Fig. 4, Lanes m and n).

Indirect Immunofluorescence and Immunoferritin Labeling with Anti-gp105. When frozen sections (4 to 6 ¿tm) of normal adult ACI rat liver were stained by indirect immunofluorescence with anti-gp105 antibodies that had been affinity purified on intact rat hepatocytes, all of the parenchymal cells demonstrated a well-localized membrane fluorescence that was most intense in areas corresponding to bile canaliculi (Fig. 5). Although the endothelial cells and connective tissue elements of the portal areas were not stained, it was interesting to note that the bile duct cells were intensely stained by the anti-gp105 antibodies (Fig. 5). When sections of polyethylene glycol-embedded liver tissue were stained by the indirect immunoferritin technique and examined by electron microscopy, it was found that labeling of the cell surface by anti-gp105 was restricted almost exclusively to bile canaliculi and that little or no labeling was present on the rest of the surface of parenchymal cells (Fig. 6, a to d). No labeling of the cell surface was observed in normal rabbit serum controls (Fig. 6e). This more restricted distribution was also observed in frozen sections fixed with acrolein and glutaraldehyde prior to staining by indirect immunofluorescence with anti-gp105 (Fig. 5c), suggesting that differences in the distribution of gp105 in frozen, acetone-fixed sections and acrolein:glutaraldehyde:polyethylene glycol-embedded tissues resulted more from differences in the method of fixation than from any inherent differences between frozen and polyethylene glycol-embedded tissues. Frozen sections of ACI rat liver containing small transplanted nodules of 253 cells were also stained by indirect immunofluorescence with anti-gp105 antibodies (Fig. 5d). Examination revealed a sharp contrast between the tumor nodules and the surrounding parenchymal tissue with the dark, essentially nonfluorescent tumor nodules contrasted against a background of brightly stained parenchymal tissue. The negative reactivity of tumor cells by indirect immunofluorescence with anti-gp105 antibodies thus confirms the results from immunoprecipitation analysis and, in addition, demonstrates that the inability to immunoprecipitate gp105 results from its absence on the surface of 253 and 311c cells and not from differences in its solubility in NP40 on tumor cells and hepatocytes.

DISCUSSION

In this paper, we utilized 2-dimensional polyacrylamide gel electrophoresis to analyze components immunoprecipitated from radiolabeled extracts with antiserum raised in rabbits against intact normal hepatocytes and 253 and 311c hepatocellular carcinoma cells. Comparison of cell surface components isolated by this approach revealed the presence of a M, 105,000 component on the surface of normal hepatocytes that, as a result of some quantitative or qualitative alteration, was missing from 253 and 311c hepatocellular carcinoma cells. This component was subsequently isolated from detergent lysates of normal hepatocytes by preparative affinity chromatography on Sepharose-bound WGA and was reconstituted into liposomes and used for immunization of rabbits. The resulting antiserum was shown by immunoprecipitation analysis to be highly specific for a component that migrated as a single broad band (apparent M, 105,000) when analyzed by SDS-PAGE. The major component immunoprecipitated with this antiserum was shown by 2-dimensional polyacrylamide gel electrophoresis to consist of a family of glycoproteins having similar apparent molecular weights but differing in isoelectric points.

As expected, no components reactive with this antiserum were detected in radioiodinated extracts of 253 and 311c tumor cells, a finding that was consistent with results obtained with antiserum raised against intact cells. It seemed possible, therefore, that
either gp105 was missing from the surface of 253 and 311c cells or that cross-reactive components on these cells were not efficiently labeled by lactoperoxidase-catalyzed iodination and consequently were not detected by autoradiography. This latter possibility seemed unlikely, however, since the reactivity of anti-gp105 antiserum with normal hepatocyte extracts could not be removed by prior absorption with 253 and 311c tumor cells and could not be blocked by preincubation with detergent lysates from these cells. The absence of components related to gp105 on 253 and 311c cells was also suggested by the inability of rabbit antisera against these tumor cells to immunoprecipitate or immunodeplete gp105 from radioiodinated extracts of normal hepatocytes. In addition, both 253 and 311c tumor cells were unreactive when stained by indirect immunofluorescence with anti-gp105 antiserum. Furthermore, no components reactive with this antiserum were immunoprecipitated from extracts of tumor cells labeled with $^3$H by the NalO$_4$:NaB$_3$H$_4$ labeling method. Taken together, these results indicate the absence of major gp105-related components from the surface of 253 and 311c tumor cells.

The ability of these transplantable tumor lines to grow and proliferate in the absence of a subset of cell surface glycoproteins suggests that the expression of this family of glycoproteins is not essential for cell survival but may play a role in some liverspecific function that is vital to the tissue as a whole. That this may indeed be the case is suggested from results of immunofluorescence and immunoferritin-labeling studies that showed a concentration of gp105 in areas of the hepatocyte cell surface corresponding to bile canaliculi. Interestingly, other areas of the plasma membrane in addition to the bile canaliculi were labeled by indirect immunofluorescence with anti-gp105 on acetone-fixed frozen sections, but they were not labeled by the immunoferritin label technique in acrolein:glutaraldehyde-fixed sections of polyethylene glycol-embedded tissue. These apparent differences in the distribution of gp105 do not appear to result from differences in the antibody penetration of sections of frozen and polyethylene glycol-embedded tissue, since labeling of the latter preparation by ferritin-conjugated RCA$_1$ was uniform on all surfaces.

Differences in fixation by acetone and acrolein:glutaraldehyde, however, did appear to be a major factor, since the only region that was intensely stained in acrolein:glutaraldehyde-fixed frozen sections was the bile canaliculi. These results suggest that fixation with acrolein:glutaraldehyde may reduce the reactivity of gp105 with anti-gp105 antiserum, resulting in staining only in those areas where a subset of epitopes reactive with anti-gp105 are localized in high concentrations, i.e., the bile canaliculi. Alternatively, it is possible that treatment with acrolein:glutaraldehyde produces a more rapid and irreversible fixation of cell surface components than is possible with acetone, thus more accurately localizing gp105.

Several reports indicate that malignant transformation is accompanied by a loss or decrease in the expression of fibronectin, a large protease-sensitive glycoprotein that in vitro mediates cell attachment and spreading and in vivo forms part of the pericellular matrix and basement membranes (16, 35). Fibronectin exists on the surface of normal hepatocytes and many other types of cells as a dimer of 2 polypeptide chains with molecular weights of 220,000 (16). Cleavage of fibronectin with protease produces a major fragment with a molecular weight of 100,000 (29). Taken together, these observations suggested that gp105 may represent a proteolytic fragment of fibronectin that was formed by the action of protease contaminating the collagenase used during the isolation of hepatocytes. However, the fact that a rabbit antisera raised against fibronectin isolated from rat plasma did not immunoprecipitate any components from hepatocyte extracts that comigrated with gp105 would argue against this possibility. Also inconsistent with this possibility is the fact that no components with molecular weights >110,000 were immunoprecipitated by anti-gp105 antiserum from either detergent extracts of freshly isolated radioiodinated hepatocytes or $^3$H-labeled extracts of hepatocytes maintained in primary culture for 24 hr, an incubation period sufficient to allow the elaboration of extensive networks of fibronectin, networks that are readily visualized by indirect immunofluorescence using rabbit anti-rat fibronectin antiserum. Other than fibronectin, perhaps, the most intensively studied and characterized hepatocyte surface protein is the asialoglycoprotein-binding protein (17). A relationship between this membrane component and gp105, however, seems unlikely, since the asialoglycoprotein-binding protein not only has a much lower molecular weight (M, 40,000 to 48,000) (17) but also appears to be localized on the sinusoidal rather than the canalicular surface of the plasma membrane of hepatocytes in situ (38). The distribution of $\gamma$-glutamyltranspeptidase, on the other hand, appears to be very similar to gp105. Results from histochemical studies of human liver showed intense activity of this enzyme in bile canaliculi and bile duct epithelia but only limited activity along sinusoidal membranes and in the connective tissue of portal areas (30, 34). Rat liver $\gamma$-glutamyltranspeptidase, however, consists of 2 subunits, M, 46,000 and 22,000, respectively (30, 34), and thus bears little structural homology to gp105.

Rat hepatocyte membrane glycoproteins isolated by Kreisel et al. (19) and Ocklind and Obrink (25), on the other hand, are similar to gp105 in both size and lectin-binding properties. Although no function has, as yet, been defined for the glycoprotein described by Kreisel et al., the M, 105,000 glycoprotein described by Ocklind and Obrink appears to be involved in intercellular adhesion.

Previous studies using monoclonal antibodies or heteroantisera have defined oncofetal, tumor-specific, or inappropriately expressed adult antigens on malignant cells. With the exception of fibronectin, few studies have reported the loss or absence from tumor cells of components present on the surface of their normal counterparts. The absence of a major hepatocyte cell-surface component from 253 and 311c tumor cells was thus unexpected.

Several mechanisms could be proposed to explain the loss of this glycoprotein from the surface of these transplantable hepatocellular carcinomas. One possibility is that this component was present on the primary tumor from which these lines were derived but, owing to the strong selective pressures produced by transplantation, a relatively small subpopulation emerged that, as a consequence of the loss of gp105, showed enhanced cancer and growth potential. It is also possible that the altered expression of gp105 resulted from the inability of these transplantable tumor cells to display histotypic patterns of growth; e.g., the loss of the ability to form bile canaliculi resulted in a decreased expression of gp105. Alternatively, these tumor cells could be derived from stem cells blocked in an early stage of differentiation.

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4 D. C. Hixson, unpublished observation.

6 D. C. Hixson and J. E. Chesner, unpublished observation.
or from adult hepatocytes that had dedifferentiated to a point where only limited amounts of gp105-related components were expressed. These possibilities seem unlikely, however, in light of the fact that gp105 is expressed on fetal hepatocytes as early as Day 15 after conception.5 The presence of gp105 on regenerating hepatocytes would also seem to rule out the possibility that the absence of this protein is a result of rapid cell growth.

At present, we are raising monoclonal antibodies against gp105 that will allow us to examine more critically the expression of individual members of this family of glycoproteins on primary hepatocellular carcinomas as well as in normal tissues other than liver. Preliminary results suggest that at least 2 of the components recognized by anti-gp105 antisera are structurally and functionally distinct glycoproteins. One of these components appears to be liver specific, while the other is found in several other tissues, including kidney, heart muscle, and spleen.

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Fig. 1. Autoradiogram of components isolated by immunoprecipitation from detergent lysates of 125I-labeled hepatocytes and separated by 2-dimensional polyacrylamide gel electrophoresis. a, components reactive with anti-normal hepatocyte antiserum; b, components reactive with anti-253 antiserum. A prominent component (circled area) with a molecular weight of approximately 105,000 was not present in material immunoprecipitated with anti-253 antiserum. IEF, isoelectric focusing.

Fig. 2. Lanes a to c, comparison of the reactivity of anti-normal hepatocyte and anti-253 antisera by immunodepletion analysis. A sample of detergent extract from 125I-labeled hepatocytes was sequentially immunoprecipitated with anti-253 antiserum until all reactivity with this antiserum was removed. This immunodepleted extract was then immunoprecipitated with anti-normal hepatocyte antiserum. Immunoprecipitates were then analyzed by SDS-PAGE. Lane a, first anti-253 immunoprecipitate; Lane b, third anti-253 immunoprecipitate; Lane c, anti-normal hepatocyte immunoprecipitate from anti-253-depleted extract. Lanes d to l, lectin affinity chromatography. Detergent extracts of 125I-labeled hepatocytes were submitted to lectin affinity chromatography as described in "Materials and Methods," and bound and unbound components were analyzed by SDS-PAGE. Lanes d, f, and h show components not bound, and Lanes e, g, and i show components bound to Sepharose:RCA, Sepharose:WGA, and Sepharose:LCA, respectively, and eluted with monosaccharide inhibitor. Lanes j to l, reactivity of xenoantiserum with components bound to Sepharose:WGA. 125I-labeled hepatocyte extracts were submitted to lectin affinity chromatography on Sepharose:WGA. Bound components were eluted with 0.2 M 2-acetamido-2-deoxy-D-glucose, and those reactive with anti-normal hepatocyte, anti-253, or anti-311c antiserum were isolated by immunoprecipitation and analyzed by SDS-PAGE. Lane j, anti-normal hepatocyte immunoprecipitate; Lane k, anti-311c immunoprecipitate; Lane l, anti-253 immunoprecipitate.

Fig. 3. Two-dimensional gel analysis of gp105. a, gp105 immunoprecipitated from 125I-labeled hepatocyte extract with anti-gp105 antiserum; b, gp105 immunoprecipitated with anti-gp105 antiserum from an extract of 125I-labeled regenerating hepatocytes; c, gp105 immunoprecipitated with anti-normal hepatocyte antiserum from anti-253-depleted extracts of 125I-labeled hepatocytes. IEF, isoelectric focusing.

Fig. 4. Lanes a to e, SDS-PAGE analysis of anti-gp105 immunoprecipitates. Lanes a, b, and c show immunoprecipitates from 125I-labeled hepatocyte extracts of hepatocytes, 311c cells, and 253 cells, respectively. Lanes d and e show the reactivity of anti-gp105 antiserum with 125I-labeled hepatocyte extracts following exhaustive absorption with 253 cells (Lane d) and regenerating hepatocytes (Lane e). Lanes f to l, blocking of immunoprecipitation with unlabeled cell lysates. Components immunoprecipitated from 125I-labeled hepatocyte extracts by anti-gp105 antiserum that had been preincubated with unlabeled cell lysates were analyzed by SDS-PAGE. Lanes f to l show components immunoprecipitated by anti-gp105 following preincubation with PBS (Lane f), normal hepatocyte extract (Lane g), 253 cell extract (Lane h), and 311c cell extract (Lane i). Lanes j to l, SDS-PAGE profiles of components isolated from 125I-labeled hepatocyte extracts by sequential immunoprecipitation with anti-253 and anti-gp105 antisera. Lane j, first immunoprecipitation with anti-253 antiserum; Lane k, third immunoprecipitation with anti-253 antiserum; Lane l, immunoprecipitation of anti-253-depleted extract with anti-normal hepatocyte antiserum. Lanes m and n, fluorogram of components immunoprecipitated with anti-gp105 antiserum from 125I-labeled hepatocyte extracts. Lanes m and n show the SDS-PAGE profiles of immunoprecipitates from 125I-labeled hepatocyte and 253 extracts, respectively.

Fig. 5. In situ distribution of gp105 as determined by indirect immunofluorescence. a, acetone-fixed frozen section of ACI rat liver stained with anti-gp105 antiserum. Note the strong membrane fluorescence. b, portal area in an acetone-fixed frozen section of ACI rat liver. Anti-gp105 antiserum stained the cuboidal epithelium of the bile duct (B.D.) but not the surrounding connective tissue. c, acrolein-glutaraldehyde-fixed frozen section of ACI rat liver. Only areas corresponding to bile canaliculi are still reactive with anti-gp105 antiserum. d, acetone-fixed frozen section of ACI rat liver containing a small nodule of 253 tumor cells (T). Only the normal parenchymal tissue (NP) is stained. e, b, and c, × 400; d, × 120.

Fig. 6. Ultrastructural localization of gp105 as determined by indirect immunoferritin labeling. a, bile canaliculus (BC) and sinusoidal (S) domains of hepatocyte plasma membrane. × 9,000. b, higher magnification view of bile canaliculus shown in a. Note the high concentration of ferritin granules on microvilli. × 80,000 c, longitudinal section through a bile canaliculus. The plasma membrane along the entire length of the bile canaliculus is labeled. × 40,000. d, higher magnification view of the sinusoidal membrane domain shown in a. The cell surface is essentially unlabeled in this region. × 80,000. e, bile canaliculus stained by the indirect immunoferritin method using normal rabbit serum in place of anti-gp105 antiserum. × 60,000.
Characterization of a Family of Glycoproteins Associated with the Bile Canalicular Membrane of Normal Hepatocytes but Not Expressed by Two Transplantable Rat Hepatocellular Carcinomas


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