Glycoproteins from Human Colonic Adenocarcinoma

ISOLATION AND CHARACTERIZATION OF CELL SURFACE CARCINOEMBRYONIC ANTIGEN FROM A CULTURED TUMOR CELL LINE*

DEAN TSAO AND YOUNG S. KIM

From the Gastrointestinal Research Laboratory (151M2), Veterans Administration Hospital, San Francisco, California 94121 and the University of California, Department of Medicine, San Francisco, California 94143

Alterations in cell surface glycoproteins have been implicated in malignancy. We examined surface membrane proteins of a cultured cell line, SKCO-1, which had been derived from a human colonic adenocarcinoma. Cell surface labeling of SKCO-1 cells with galactose oxidase, followed by reduction with sodium borotritide, revealed five major labeled glycoproteins upon sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. At least three additional labeled glycoproteins could be detected if galactose oxidase treatment was preceded by neuraminidase treatment. Some, but not all, of the glycoproteins could be iodinated by lactoperoxidase. The predominantly labeled glycoprotein (GPI) had a molecular weight of 200,000 and co-migrated in SDS gel with carcinoembryonic antigen (CEA). GPI was not removed from the cell surface by EDTA, hypertonic saline, or sonication but was released from the membrane by detergents. This glycoprotein was subsequently purified using lectin-agarose columns and gel filtration. GPI was judged homogenous by protein- and carbohydrate-stained SDS-polyacrylamide gels and had an amino acid composition similar to that of CEA. The carbohydrate composition of GPI was qualitatively similar to CEA but quantitatively distinct. GPI had a greater proportion of sialic acid and galactosamine and less fucose and glucosamine than CEA. Immunological studies, however, demonstrated identity between GPI and CEA. A study of the turnover rate of GPI showed it to have a half-life of 5 days.

Since cell surface structures and their attendant biological properties have been strongly implicated in tumorigenesis, an understanding of cell surface architecture and metabolism may aid in elucidating the mechanism of tumor development and metastasis. Although cell surface changes associated with neoplastic transformation have been reported by various investigators, much of these data were obtained from virally transformed murine or avian fibroblasts (l-3). Relatively little use of these data were obtained from virally transformed murine or avian fibroblasts (l-3). Relatively little

The purpose of the present study, using these techniques, was to examine surface glycoproteins of SKCO-1 cells, an epithelial line established from a human colonic adenocarcinoma.

MATERIALS AND METHODS

Chemicals—CEA'-ROCHE, carcinoembryonic antigen assay kit, was purchased from Hoffman-La Roche, Inc. (Nutley, N. J.); purified CEA from liver metastases of carcinoma of the colon and goat anti-CEA antisera were kindly provided by Dr. Hansen of Hoffman-La Roche, Inc. carrier-free [‘H] (15 mCi/mug) as the sodium salt and tritiated sodium borohydride (10.5 Ci/mmol) were products of Amer- sham/Searle (Arlington Heights, Ill.). Concanavalin A-Sepharose, Sepharose 4B, and Sephadex G-200 were obtained from Pharmacia. (Uppsala, Sweden). Affi-Gel 10 was purchased from Bio-Rad (Rich- mond, Calif.). Nonidet P-40 (NP-40) was from Particle Data Labora- tories (Elmhurst, Ill.). Tertitol NFX (NFX), lactose, α-methyl-β- mannoside, and galactose oxidase were obtained from Sigma (St. Louis, Mo.). Lactoperoxidase was obtained from Calbiochem (La Jolla, Calif.). Sodium dodecyl sulfate was purchased from BDH Chemicals, England. Ricinus communis agglutinin was purified from castor beans according to a modification of a method of Nicolson

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and Blausner (7), but no further separation of the lectin of molecular weight 120,000 (RCA) from that of molecular weight 60,000 (RCA) was attempted. All other chemicals were of the highest quality commercially available.

Cell Culture—The human colonic cancer cell line, SKCO-1, was developed at the Sloan Kettering Institute for Cancer Research and was generously given to us by Dr. Pagli. By histological examination the cells were of epithelial origin. They were maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin and were harvested at confluency.

Surface Labelling Techniques—Isolated cells, cultured for 22 h at 5% CO2 for the surface glycopeptides of SKCO-1 cells with galactose oxidase and tritated sodium borohydride was performed by a method similar to that described by Juhano and Shehar-Banniler (8). Confluent cultures in 75-cm² tissue culture flasks were labeled in situ after washing three times with NaCl/P, pH 7.4. Washed cells were treated with 15 μg/ml of galactose oxidase in 4 ml of Hanks’ balanced salt solution (Pacific Biological Co.), pH 7.0, at 37°C for 60 min. The cells were then washed once and incubated with 0.1 M Tris of tritated sodium borohydride for 5 min at 25°C in 2 ml of Hanks’ balanced salt solution, pH 8.0. Crystals of solid tritiated sodium borohydride were dissolved in 0.1 M NaOH just prior to use. Labeled cells were washed four times with NaCl/P, and used immediately.

Lectin, Protein A-Sepharose—Cells were solubilized in 2% NP-40, 2% NPX in NaCl/P, pH 7.4. Membranes were solubilized with 0.1% SDS, 0.1% periodic acid-Schiff reagent according to Fairbanks et al. (12). The gels containing tritium were sliced at 2-mm intervals, added to toluene scintillation mixture containing 9% NCS solubilizer (Amersham/Searle) and 0.4% Omnifluor (New England Nuclear) and counted after incubation at 50°C overnight. Gels containing tritium were sliced similarly but were counted directly in a 3-spectrometer.

Ionization of CEA—Immobilization of lactoperoxidase on Affi-Gel 10 was performed according to the instructions provided by Bio-Rad. It was found that 0.946 mCi of enzyme (0.1 mCi of total protein) was coupled to 1 ml of agarose gel using A⁺⁺⁺ = 114 (13) for lactoperoxidase. Agarose gel was suspended in an equal volume of NaCl/P, pH 7.4. Ionization of CEA with 131I was carried out as follows. To 100 μl of CEA solution (1 mg/ml in NaCl/P,) were added 5 μl of carrier-free 131I (0.5 mCi), 20 μl of the lactoperoxidase-agarose suspension (3 μg/ml A⁺⁺⁺ and 1.1 mg/ml H2O2) at a concentration of 5 × 106 cells/ml. The reaction was carried out for 45 min. Gel filtration of the reaction mixture on a Sephadex G-25 column after removal of the lactoperoxidase-agarose gel by centrifugation. Radioactively labeled CEA was collected at the void volume of the column and was stored at −20°C until used. A specific activity of 5 × 106 cpm/μg was obtained for CEA before enzymatic radioiodination.

Gas-Liquid Chromatography for Sugars—Acid-catalyzed methanolysis and gas liquid chromatography analysis was carried out as described by Esselman et al. (14).

Protein Measurement—Protein was estimated by the method of Lowry et al. (15) using crystalline bovine serum albumin as a standard.

Amino Acid Analyses—Quantitative amino acid analyses were carried out with a Beckman model 120C amino acid analyzer. The samples were hydrolyzed in 6 N HCl for 22 h at 110°C in evacuated tubes sealed under nitrogen. No correction was made for any destruction which may have occurred during hydrolysis.

Autoradiography—Autoradiography was performed according to the methods of Bonner and Rice (21).

Turnover Study—Two methods were used to study the turnover of GPI in SKCO-1 cells. Cells at 50% confluency in 25-cm² tissue culture flasks were labeled with galactose oxidase and NaB³H₄ as described above. Labeled cells were washed and incubated at 10 ml of growth medium (Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, penicillin, and streptomycin) overnight. Less than 10% of the cells were found dead or detached. The culture medium was replaced with 10 ml of fresh medium. Labeled cells were harvested at this point as a zero-time control and at 1-day intervals up to 6 days with one change of medium at Day 3. Two to four flasks of cells were used for each time point.

An alternate approach was to label confluent cells in 75-cm² tissue culture flasks. Labeled cells were detached from plates by 15-min incubation at 37°C with 27 mM citrate/isotonic phosphate buffer, pH 7.6, 10 mM rivanol with water and 0.1% SDS, 0.1% periodic acid-Schiff reagent according to Fairbanks et al. (12). The gels containing tritium were sliced at 2-mm intervals, added to toluene scintillation mixture containing 9% NCS solubilizer (Amersham/Searle) and 0.4% Omnifluor (New England Nuclear) and counted after incubation at 50°C overnight. Gels containing tritium were sliced similarly but were counted directly in a 3-spectrometer.

Direct Z-gel assay method as suggested by Hoffman-La Roche, Inc.
Cell Surface Proteins

For these studies, monolayer SKCO-I cells in culture flasks at confluency were washed and incubated with enzymes (neuraminidase, galactose oxidase) in Hanks' balanced salt solution. Properties of cell surface proteins were examined with enzymes which modify the surface proteins of intact cells and could be monitored by the incorporation of isotopes into the membrane proteins or by the change of protein patterns after gel electrophoresis. The radioactively labeled membranes obtained by ultracentrifugation of sonicated cells were solubilized in SDS, and the membrane proteins were analyzed by SDS-polyacrylamide gel electrophoresis.

A scintillant (2,5-diphenyloxazole) was introduced into the gel to speed the visualization of tritium-labeled protein in polyacrylamide gels by autoradiography. Membranes from cells labeled with galactose oxidase treatment showed the presence of a large number of labeled polypeptides of molecular weights > 500,000 (GPN1), 280,000 (GPN2), and 200,000 (GPN3), 115,000 (GPII), 100,000 (GPIII), 78,000 (GPIV), 49,000 (GPV), and 42,000 (GPVI). GPI was the major labeled component, accounting for more than 50% of the total radioactivity incorporated into cell membrane (Fig. 1).

When galactose oxidase treatment was preceded by neuraminidase treatment, labeling of bands corresponding to GPI, GPV, and GPVI was enhanced. Also, additional bands of molecular weights > 500,000 (GPN1), 280,000 (GPN2), and 62,000 (GPN3) were detected. Trypsin treatment of labeled cells produced a diminution of GPII, GPIII, and GPN1. None of the other bands showed any significant change after trypsin treatment. GPN1 was found to be radioiodinated by lactoperoxidase and H2O2, with subsequent trypsin treatment.

Isolation and Purification of GPI—Preliminary data showed that GPI remained associated with the membrane fraction after sonication, extraction with 0.1 M NaCl, 0.1 M EDTA, or treatment with 3 M KCl. However, it could be solubilized readily with detergents. Results of affinity chromatography of solubilized tritium-labeled membrane glycoproteins on a column of RCA-Sepharose are shown in Fig. 2. A small amount of labeled material emerged with the effluent (Peak I), while most of the radioactivity bound to the column and could be eluted with lactose (Peak II). Peak I contained most of the membrane proteins judging from the large quantity of nondialyzable, lyophilized material (protein determinations of this fraction were greatly affected by NP-40 and NPX, even after extensive dialysis). Autoradiography of Peak I after SDS-gel electrophoresis showed that GPI remained associated with the membrane fraction after sonication, extraction with 0.1 M NaCl, 0.1 M EDTA, or treatment with 3 M KCl.
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Characterizations of GPI—Our previous studies of CEA in cancerous and fetal intestinal cells indicated that SKCO-1 cells had the highest quantity of CEA both in the cells and in the medium of the cells examined. Over 90% of the CEA of SKCO-1 cells was found to be associated with cellular membranes. Similarity in molecular weights between GPI and CEA prompted us to compare their chemical properties. An identical migration of $^3$H-labeled GPI and $^{125}$I-labeled CEA was found using SDS-polyacrylamide gel electrophoresis as shown in Fig. 5. The results of immunodiffusion studies with goat antisera prepared against CEA are shown in Fig. 6. GPI

*Manuscript in preparation.*
mined by adding 6 ml of toluene mixture containing 5% NCS and was calculated and divided by the total radioactivity added to the antibody. Anti-CEA antiserum, A-Sepharose (A-A) for 30 min at 45° in a volume of 50 μl. The purified GPI or galactoproteins which did not bind to Con A-Sepharose with tubes were centrifuged at 1100 x g for 15 min, and the radioactivity was measured in the supernatant fraction. Control incubations of normal goat serum were also carried out. Radioactivity was determined by adding 6 ml of toluene mixture containing 5% NCS and 0.4% Omnifluor to 25 μl of supernatant which was carefully removed from the top of each tube. The radioactivity remaining in the pellet was calculated and divided by the total radioactivity added to the tube to obtain the per cent of total radioactivity precipitated.

**Table I**
Amino acid composition of GPI
Lyophilized galactoprotein I was hydrolyzed under N₂ in 6 m HCl at 110° for 22 h. Dried hydrolysates were dissolved in 0.2 m citrate buffer (pH 2.2) and analyzed on a Beckman 120 C amino acid analyzer. The values were not adjusted for any losses of amino acid caused by hydrolysis. Results shown are means of two analyses.

| Amino acid       | Residues/1000 residues |
|------------------|------------------------|
| Aspartic acid    | 110.4                  |
| Glutamic acid    | 96.3                   |
| Serine           | 129.2                  |
| Threonine        | 85.3                   |
| Isoleucine       | 38.7                   |
| Leucine          | 75.5                   |
| Proline          | 65.1                   |
| Glycine          | 75.8                   |
| Alanine          | 70.1                   |
| Valine           | 54.5                   |
| Tyrosine         | 34.9                   |
| Phenylalanine    | 28.8                   |
| Lysine           | 58.6                   |
| Histidine        | 25.4                   |
| Arginine         | 38.3                   |
| Cysteine         | 8.4                    |
| Methionine       | Trace                  |
| Tryptophan       | N.D.*                  |

* N.D., not determined.

appears to be immunologically identical to CEA since fusion of the precipitin lines without apparent spurring occurred. No precipitation line was formed between anti-CEA antiserum and the other galactoproteins (Con A-Sepharose, Peak I). The results of quantitative precipitation of GPI and other galactoproteins (Con A-Sepharose, Peak I) by CEA antiserum are shown in Fig. 7. GPI could be completely precipitated by CEA antiserum. None of the other glycoproteins which bound to RCA-Sepharose contained appreciable CEA activity. As shown in Table I, the amino acid composition of GPI is similar to those reported previously for CEA by Banjo et al. (17), Kupchik et al. (18), Terry et al. (20), and Westwood et al. (21). However, GPI was slightly higher in the basic amino acids lysine and histidine than was CEA. The carbohydrate compositions of GPI and other CEA preparations are compared in Table II. GPI is much lower in fucose and N-acetylglucosamine and higher in sialic acid and N-acetylgalactosamine than the CEA preparations isolated from liver.

**Turnover Studies of GPI** — Disappearance from cell membranes of radioactivity associated with GPI after galactose oxidase and tritiated sodium borohydride labeling was followed as a function of time. The results are shown in Fig. 8. These turnover rate studies showed a half-life for GPI of 5 days. Protein assay of SKCO-1 cells used in the turnover study indicated cells were growing during the experiment. Although the data is not shown here, we have examined labeled GPI
have used these techniques to study membrane proteins of oriented proteins of mammalian cell membranes (24). We syl- and N-acetylgalactosaminyl residues with galactose oxidase consistently produced five major and several minor differences in viability of cells which is crucial in ensuring specific labeling of cell surface proteins. Cells of low viability was found when using cell suspensions. This may be due to effects could be the result of direct blocking of galactose and other components may provide a charge barrier to normally reactive galactosyl groups.

Some of the glycoproteins, GPN1, GPI, GPV, and GPVI, could be labeled both by galactose oxidase and lactoperoxidase, while the others were not susceptible to lactoperoxidase iodination. This is interpreted to mean that some of the surface membrane glycoproteins do not have exposed tyrosine groups in their amino acid sequences.

Singer has classified membrane proteins into two groups, peripheral and integral, based on the requirements of their dissociation from membranes (29). The former are weakly bound, while the later are intercalated into the lipid bilayer. GPI could only be solubilized with detergents, suggesting that GPI is tightly bound to the membrane and apparently should be considered an integral membrane protein. GPI is apparently exposed at the cell surface and oriented toward the extracellular environment since: (a) GPI was labeled by galactose oxidase; (b) GPI was also labeled by lactoperoxidase and; (c) labeling was enhanced by prior treatment with neuraminidase.

Nonionic detergents, NPX and NP-40, were used to extract labeled cell surface glycoproteins and after centrifugation at 105,000 x g for 60 min, more than 80% of the radioactivity was found in the supernatant. An identical labeling pattern after SDS-polyacrylamide gel electrophoresis was found in the supernatant as that of the original membrane solubilized directly in SDS. Analysis by SDS-polyacrylamide gel electrophoresis of radioactive components in fractions obtained after lectin affinity chromatography indicated that Peak I from RCA-Sepharose column contained only the radioactive components which migrated in the dye-front region while all other high molecular weight labeled glycoproteins were bound to the column. No radioactivity which corresponded to GPI was found in the Peak I fraction from the Con A-Sepharose.
column. Chromatography on RCA-Sepharose, Con A-Sepha-
rose, and Sephadex G-200 resulted in the purification of GPI
to apparent homogeneity.

Carcinoembryonic antigen, one of the most extensively
studied tumor-associated antigens found in adenocarcinomas
of the human digestive tract, has been isolated in pure form
from liver metastases of colonic carcinoma, and its molecular
weight is estimated to be 200,000 (30). The similarity of the
molecular weight of GPI to that of CEA and the large amount
of CEA produced by the SKCO-1 line prompted us to compare
these two glycoproteins. GPI and CEA showed identical mo-
bilites in SDS-polyacrylamide gel electrophoresis, GPI was
completely precipitated by anti-CEA antiserum, both proteins
formed a single immunoprecipitin line on immunodiffusion,
and both had nearly identical amino acid compositions. From
these criteria it indicated that GPI is CEA. However, the
carbohydrate composition of GPI differed from that of CEA
preparations isolated by others. GPI contained the same sugar
activity recovered as GPI in the medium accounted for 60%; of
showed identical mobilities in SDS-gel electrophoresis. Radio-
medium in an intact form was demonstrated by the fact that
Release of GPI from the membrane surface into the culture
medium was allowed to flow through anti-CEA Sepharose
chromatography and identified by SDS-gel electrophoresis.
GPI from SKCO-1 cells into the culture medium. GPI was
labeled with galactose oxidase and tritiated sodium borohy-
dride. Labeled cells were allowed to grow in culture medium,
which GPI was obtained, were derived from a primary colonic
tumor. The differences in carbohydrate compositions may be a
difference between tissue and cultured cells.

Immunodiffusion and immunoprecipitation studies showed
immunological cross-reactivity of GPI with CEA, suggesting
that GPI bears antigenic sites identical to those of CEA. The
presence of CEA molecules on colonic cancer cell surfaces has
demonstrated by immunofluorescence using anti-CEA
antibody (31). It was not clear, however, whether nonsecretive
cell surface adsorption of secreted CEA contributed to the
immunofluorescence seen on the cell surface. Using a radioim-
unoassay for CEA more than 90% of the total cellular CEA
activity was found to be tightly associated with the membrane
fraction of SKCO-1 cells. This implies that at least for this
cell CEA is an integral membrane protein.

There may be several ways in which immune responses
toward tumors may be modified. Thomson (32) has proposed
that the release of tumor antigen from the cell surface of a
growing tumor is of fundamental importance for its escape
from destruction by the immune system of the host. Other
reports (33, 34) also indicate that escape may be brought about
by shedding of antigens or antigen-antibody complexes from the
cell surface. We investigated the turnover and shedding of
GPI from SKCO-1 cells into the culture medium. GPI was
labeled with galactose oxidase and tritiated sodium borohy-
dride. Labeled cells were allowed to grow in culture medium,
and labeled GPI was isolated by Con A-Sepharose affinity
chromatography and identified by SDS-gel electrophoresis.
Release of GPI from the membrane surface into the culture
medium in an intact form was demonstrated by the fact that
GPI isolated from the membrane and from the medium showed
identical mobilities in SDS-gel electrophoresis. Radio-
activity recovered as GPI in the medium accounted for 80% of
the radioactivity that disappeared from the membrane in the
form of labeled GPI. These data suggest that most of the GPI
is shed from the surface of the SKCO-1 cells in a relatively
intact form and not lost to the medium as a consequence of
extensive hydrolytic degradation. It is possible that carbohy-
drate residues of shed GPI were partially degraded by glyco-
sidases present in the medium, resulting in the release of
some of the labeled sugars from the glycoprotein. Some of the
labeled GPI in the medium also may be hydrolyzed by proteo-
ytic enzymes into small fragments which were lost during
dialysis before Con A-Sepharose chromatography. The biological
half-life of GPI, as measured by the disappearance of labeled
GPI from the cell membrane, was determined to be 5 days.
A half-life of approximately 6 days was estimated by Drewinko
and Yang (33) for CEA in another human colonic carcinoma cell line, LoVo.

The functional role of GPI remains to be determined. Our
observation that this protein is sterically available on the
external surface of the cell, that it shares chemical and
immunological properties with CEA, and that it is shed from
the cell surface into the surrounding environment suggests
that it may play a part in the tumorigenic processes of colonic
carcinoma.

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