ISOLATION AND CHARACTERIZATION OF SURFACE GLYCOPROTEINS FROM L-1210, P-388 AND HeLa CELLS

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Summary

A method is described that permits the rapid extraction of the cell surface glycoproteins of two murine leukemic cells, the P-388 and the L-1210 cells as well as those of the human adenocarcinoma cells, the HeLa cells.

Proof of the surface location of these glycoproteins is provided by labeling the intact cells; (a) with $^{125}$I by the lactoperoxidase iodination technique; (b) with $^{3}H$ by the galactose oxidase-reductive tritiation method. Most of these glycoproteins were also shown to incorporate radioactive glucosamine and fucose. By these criteria as well as by the distribution of molecular weights, the surface glycoproteins of the two murine cells are indistinguishable; however, they differ markedly from the surface glycoproteins of HeLa cells. The extracts of the murine cells were shown to contain lectin receptor activity as determined by their ability to inhibit the lectin-induced agglutination of the intact cells.

Introduction

It has become increasingly appreciated that plasma membranes play a crucial role in growth regulation of mammalian cells [1]. Although a considerable amount of work has been done on the isolation and characterization of membranes from many different cell lines, very little is known about the function and properties of each individual membrane protein. On the basis of much circumstantial evidence, for example cellular recognition [2], homing of lymphocytes [3], cellular growth regulation [4], platelet-collagen interaction [5], it has been suggested that surface glycoproteins may be involved in mediating the "social behavior" of mammalian cells. The discovery that glycoprotein A, the
major glycoprotein of the erythrocyte membranes [9], is a transmembrane pro-
tein which the gold content of glycoproteins may serve as the vehicle for the
transmission of environmental information from the exterior to the interior of
cells [7,8].

We have shown that antisemur prepared against plasma membrane proteins
can inhibit cell growth in the absence of complement. Therefore, we proposed
that anti-membrane antisemur or antibody directed against a specific surface
 glycoprotein should constitute a unique system for the study of the regulation
of cellular growth by plasma membrane [9].

We pursued this purpose by purifying plasma membranes from L-1210, P-388 and
HeLa cells [9,10], followed by the isolation of glycoprotein from plasma membranes.
Among various surface glycoprotein isolation methods [11-14], the lithium diiodosalicylate-phenol extraction method [14] yields the best results. The method is found to be generally useful for a number of
different cell types and can be used to extract the same glycoproteins either
from normal melanin preparations or, with increased yields, from the post-
nuclear fraction of the cell homogenate. The glycoproteins extracted from
leukemic cells were shown to inhibit the cell agglutination induced by plant
lectins and by antisera produced against leukemic plasma membranes.

Materials and Methods

Radioactive 111I- or 125I-glucosamine, L-[1,5,6-3H]fucose, NaB3H4, were
purchased from New England Nuclear, Boston, Mass. Glucose oxidase (EC
1.1.3.4) (200 units/ml), lactoperoxidase (EC 1.11.1.7) (80 units/mg), galactose
oxidase (EC 1.1.3.9) (100 units/mg) and phenyl methyl sulfonyl fluoride
(PMSF) were obtained from the Sigma Chemical Co., St. Louis, Mo. Fischer’s
medium, Joklick modified minimal essential medium, fetal calf serum and
horse serum were from Grand Island Biological Co., Grand Island, N. Y. NBA/2
mice bearing L-1210 tumor cells were from Arthur D. Little, Inc., Cambridge,
Mass. Lithium 3,5-diodosalicylate was prepared from 3,5-diodosalicylic acid
(Eastman Kodak, 2166) and LiOH (Mallinckrodt, Co.); diiodosalicylic acid was
recrystallized twice from anhydrous methanol. Concanavalin A and wheat germ
agglutinin were from Miles Labs., Elkhart, Ind., phytohemagglutinin A was
from Difco Lab., Detroit, Mich. All other chemicals were of standard reagent
grade.

Cell culture. L-1210 and P-388 cells were cultured as a suspension culture in
Fischer’s medium supplemented with 10% horse serum and harvested as
described [9,10]. We wish to thank Dr. F.H. Ruddle, Yale University, for the
HeLa-S3 monolayer cells; these were cultured in Eagle’s medium containing
essential medium supplemented with 10% fetal calf serum. The HeLa cells were disso-
ciated from the culture dish with 2 mM EDTA in phosphate-buffered saline
(10 mM Na2HPO4, 0.9% NaCl, pH 7.2) and grown as suspension cultures in
spinner flasks at 2.10^{-2}^{-6} 10^{-1} /ml in Joklick minimum essential medium plus
10% fetal calf serum. Leukemic cells L-1210 and P-388 were also maintained in
DBA/2 mice and harvested 6 days after the inoculation of 1·10^6 cells per
mouse. Cells obtained from ascites fluid were washed twice with Fischer’s
medium and suspended in 10 volumes 0.2% NaCl at 4°C for 3 min, the suspen-
sion was then made isotonically and washed two more times.

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Natrium dodecyl sulfat

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ion was then made isotonic by adding an equal volume of 1.6% NaCl; the cells were washed twice more times with phosphate-buffered saline.

**Idionation.** L-1210, P-388 and HeLa cells were iodinated by the Phillips-Morrison lactoperoxidase-catalyzed idionation method essentially as previously described [9,10]. The final concentrations of the reaction mixture were: 1 × 10^2−2 × 10^3/ml leukemic cells or 2 × 10^3−4 × 10^4/ml HeLa cells, 50–100 μg/ml lactoperoxidase, 20–100 μCi/ml carrier free [125]I, 20 mM glucose, 50 mM Tris/10 mM glucose oxidase in phosphate-buffered saline (58 mM NaCl, 57 mM NaHPO₄, and 18 mM KH₂PO₄ pH 7.2). The reaction was carried out at 4°C for 20 min. The reaction was terminated by dilution of the suspension with 10 volumes of cold Fischer's medium, the cells were washed three times with ice-cold phosphate-buffered saline.

**Galectose oxidase-catalyzed reductive titration.** We used Gahmberg's procedure [11] with slight modifications. Cells (1 × 10^6 cells/ml, without neuraminidase pretreatment), were suspended in phosphate-buffered saline containing 15 units/ml of galeactose oxidase, incubated at room temperature for 1 h with occasional mixing and then washed twice with phosphate-buffered saline. Titration was carried out at room temperature for 30 min with 0.1 μCi/ml of Na^252Hg (100 Ci/mmol) in phosphate-buffered saline at a cell density of 3 × 10^7/ml. The cells were then washed three times with ice-cold phosphate-buffered saline.

**Metabolic labeling.** Both radioactive glucosamine and fucose are precursors which can be incorporated specifically into surface glycoproteins [16,17]. Cells were grown in the presence of 1−5 μCi/ml of D-[3H]glucosamine or L-[3H]fucose for 48 h and harvested as described above.

**Isolation of plasma membrane.** Plasma membranes of L-1210 and P-388 cells were prepared essentially as described by Atkins and Summers [18].

**Lithium 3,5-diodo-4-hydroxyphenylate phenol extraction.** Purified plasma membranes were resuspended in 50 mM Tris−HCl (pH 7.5 with 2 mM PMSE present) at about 10 mg protein/ml concentration; alternatively, a cell homogenate which had been freed of nuclei by centrifugation at 2000 × g for 1 min was used. An equal volume of 0.6 M lithium diiodo-phenylate in 50 mM Tris buffer was added to the suspension. After 2 h the mixture was stirred at room temperature for 20 min, two volumes of cold distilled water were added and mixed thoroughly at 4°C for another 20 min. The mixture was centrifuged at 105,000 × g for 1 h at 4°C; the clear supernatant fluid was then mixed with an equal volume of cold 50% phenol (w/v) and vigorously stirred at 4°C for 20 min. The aqueous phase after centrifugation at 4000 × g for 1 h was a swinging bucket rotor was exhaustive ly dialed against water and the dialyzed material was lyophilized; the product was washed three times with absolute ethanol (−20°C). Finally, the residue was suspended in water and dialyzed overnight. Any insoluble material was removed by centrifugation at 105,000 × g for 1 h.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.** Samples to be analyzed by SDS-polyacrylamide gel electrophoresis were solubilized in 50 mM Tris·HCL, pH 8.9, 2% SDS, 5% β-mercaptoethanol and 5 mM EDTA at 100°C for 3 min. The discontinuous SDS-polyacrylamide gel electrophoresis system was used as described by Laemmli [19] or the linear gradient slab gel system as...
we previously described [9]. The gels were stained overnight with 0.025% Coomassie Blue in 25% 2-propanol and 10% acetic acid. Destaining was achieved by washing first in 0.0025% Coomassie Blue in 10% 2-propanol/10% acetic acid for 3 h and then by incubating in 10% acetic acid. Periodic acid-Schiff base staining method was used as described by Farthing and Marchesi [20]. Apparent molecular weights were determined according to the procedure of Laemmli [19]. For autoradiography, the destained gel was vacuum dried on a Whatman filter paper and exposed to X-ray film (RX-Omat film, Eastman Kodak Co., Rochester, N.Y.). The fluorographic method of Bonner and Laskey [21] was used to detect tritium or ¹³⁵I-labeled components on the gel.

Results

General approach

The lithium diiodosalicylate-phenol extraction procedure is a modification of the original method introduced by Marchesi and Andrews [14]. Originally we applied this procedure to highly purified plasma membranes. Due to the low yield of plasma membrane (approx. 1 mg from 10⁷ cells), the resulting product obtained from the lithium diiodosalicylate-phenol extraction was also low (approx. 50 μg/10⁷ cells). In order to increase the yields, we attempted the lithium diiodosalicylate-phenol extraction on intact cells or on cell homogenates. Unfortunately, due to the release of DNA and the subsequent formation of a viscous gel, this approach was not successful. However, removal of the nuclei and extraction of the post-nuclear supernatant yielded essentially the same polypeptides as obtained when purified plasma membranes were used as starting material (see below). The yield of protein in the extract was increased approximately 5-fold. This was true for both the leukemia cells and HeLa cells.

The Coomassie Blue staining pattern of lithium diiodosalicylate-phenol extracts

Purified plasma membranes, when analyzed by SDS-polyacrylamide gel electrophoresis are usually composed of more than 50 polypeptides [9,10] (Fig. 1(a) and d)). Application of the lithium diiodosalicylate-phenol extraction method to the isolated plasma membranes of leukemia cells (both L-1210 and P-388 cell lines) or of HeLa cells, yields three major peptides stainable with Coomassie Blue. They have apparent molecular weights of 60,000, 88,000 and 96,000. It might be expected that extracts from L-1210 and P-388 would be similar (Fig. 1c and f)) since both are murine leukemia cell lines derived from DBA mice [22]. It was found, however, that the lithium diiodosalicylate-phenol extraction of HeLa cells, a human adenocarcinoma tumor cell line, yields a similar, if not identical, Coomassie Blue staining gel pattern (Fig. 1(b) and c)).

Lactoperoxidase-catalyzed iodination labeling pattern

We have previously established that the lactoperoxidase-catalyzed iodination pattern of the cell surface membrane polypeptides of the L-1210 and the P-388 are very similar but differ from that obtained with HeLa cells [9].

For both the L-1210 and the P-388 cells, the major iodinated membrane polypeptides have apparent molecular weights of 220,000, 180,000, 135,000, 96,000, 88,000, 80,000, 75,000, 66,000, 60,000, 50,000, 45,000, and 42,000, and 13,000 within an estimated iodinated band has a reported its molecular weight, six are extra- dure, and have molecular weight 88,000 and 60,000 (Fig. 1(b)), 60,000 polypeptides are detected.

When the HeLa cell iodinated gel electrophoresis pattern, in molecular weight between 120,000 and 140,000 with 125I-labeled band has a 125I-labeled band with 125I-labeled band in the lithium diiodosalicylate-solubilized label in the final product of polyacrylamide gel electrophoresis.
13,000 within an estimated accuracy of ±10%. We now find that the predominant iodinated band has a molecular weight of 80,000 for which we previously reported its molecular weight at 85,000 ± 10%. Of these thirteen iodinated components, six are extractable by the lithium diiodosalicylate-phenol procedure, and have molecular weights of 220,000, 180,000, 135,000, 96,000, 88,000 and 60,000 (Fig. 2a and b). Of these, only the 96,000, 88,000 and 60,000 polypeptides are detectable with Coomassie Blue staining.

When the HeLa cell surface is iodinated and analyzed by SDS-polyacrylamide gel electrophoresis, fourteen major polypeptides are labeled. They range in molecular weight between 10,000 and approx. 250,000, with a prominent \( ^{125}I \)-labeled band at 180,000. Extraction of the iodinated plasma membranes with 0.1 M lithium diiodosalicylate solubilized between 40 and 70% of the \( ^{125}I \) label. All of the iodinated membrane proteins were equally solubilized from the membrane at this concentration of lithium diiodosalicylate as assessed by SDS-polyacrylamide gel electrophoresis. Upon extraction of the lithium diiodosalicylate-solubilized membrane with cold phenol, approx. 30% of the \( ^{125}I \) label in the lithium diiodosalicylate supernatant was found partitioned in the aqueous phase. The remainder of the radioactivity was found at the interface or in the phenol phase. Approx. 1% of the initial membrane protein was obtained in the final product derived from the aqueous phase. The SDS-polyacrylamide gel electrophoresis pattern of the \( ^{125}I \)-labeled proteins of the iso-
lated HeLa membranes and of the lithium diiodosalicylate-phenol extract is shown in Fig. 2(e) and (f). The major iodinated peptides in lithium diiodosalicylate-phenol extract have molecular weights of 230 000, 165 000, 140 000, 127 000, 88 000, 72 000 and 65 000. Except for the 88 000 polypeptide, the remainder of the lithium diiodosalicylate-phenol extractable 125I-labeled polypeptides were weakly, if at all, stainable with Coomassie Blue.

Carbohydrate labeling

We have applied four carbohydrate labeling methods in order to distinguish which components in the lithium diiodosalicylate-phenol extract are glycosylated. These include metabolic labeling with [3H]glucosamine or [3H]fucose, the galactose oxidase-reductase titration and the periodate-Schiff base staining.

Glucosamine is a ubiquitous carbohydrate moiety found in plasma membrane glycoproteins [17] and was found to be a useful indicator of glycosylation in P-388, L-1210 and HeLa cells. In Fig. 3 we have presented typical fluorograms comparing the distribution of the [3H]glucosamine-labeled species isolated in the lithium diiodosalicylate-phenol extract of P-388, L-1210 and of HeLa cells.

In the case of lithium diiodosalicylate-phenol extract of the L1210 cells and P-388 cells it can be seen that all of the iodinated polypeptides are also glucosamine labeled. The most heavily iodinated polypeptide, the 135 000 dalton species, also has the highest amount of the [3H]glucosamine label. [3H]Glucos-
The lithium diodosalicylate-phenol extract obtained from the HeLa cell
### Table 1

<table>
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<th>Band No.</th>
<th>Apparent molecular weight (× 10⁴)</th>
<th>Comassie blue stain</th>
<th>125I (1H)IGlucoammine</th>
<th>125I (1H)IFucose</th>
<th>Galactose oxidase-NaBH₄</th>
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Plasma membranes labeled with [1H]glucosamine was found to contain seven major glycoproteins ranging in molecular weight from 60,000 to 350,000. One component with an apparent molecular weight of 165,000 is the predominant [1H]glucosamine-labeled glycoprotein in either the purified plasma membrane or in the lithium diiodosalicylate-phenol extracts of plasma membranes. This species is exposed on the cell surface as indicated by its uptake of 125I by lactoperoxidase-catalyzed iodination and by the galactose oxidase-reductive titration. It is also the major periodic acid-Schiff base staining band and is in addition, labeled with [1H]fucose. A summary of the components found in the lithium diiodosalicylate-phenol extract of HeLa cells is presented in Table II. It is of interest to note that a majority (seven out of nine) of the iodinated polypeptides found in the lithium diiodosalicylate-phenol extract were also labeled with [1H]glucosamine. The molecular weight of 350,000 and gave a position of this component in the reductive titration.

**Lithium diiodosalicylate-lectins**

Lithium diiodosalicylate inhibits physiologically relevant glycoprotein cell agglutination. They also sensitized antisera prepared with protein A labeled with glutelin. A can be introduced in the extract from L-1210 cells.

**Discussion**

One of the most fruitful areas of research at the moment is the search for soluble lectins. In the case of the lectin from mouse liver, it is likely that the soluble lectin might be a component of the surface of normal cells and that the presence of such lectins could be detected by using aqueous suspensions of normal cells and the position of the lectin as determined by the specific activity of the lectin in the extract.

Two strategies have been used in this work: (1) the use of monosaccharides and (2) the use of oligosaccharides as inhibitors of the lectin activity of normal cells and the lectin activity of the extract.

### Table 2

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<th>Band No.</th>
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With [1H]glucosamine, the molecular weight of 350,000 and gave a position of this component in the reductive titration.
with \(^{[3]H}\)glucosamine. One high molecular weight band with an approximate molecular weight of 300,000 was not indicated but was labeled with \(^{[3]H}\)glucosamine and gave a positive periodic acid-Schiff reaction. The surface localization of this component is indicated by its labeling with the galactose oxidase-directed trititation.

**Lithium diiodosalicylate-phenol extracts inhibit cell agglutination induced by lectins**

Lithium diiodosalicylate-phenol extracts from both L-1210 and P-388 cells inhibit phytohemagglutinin, concanavalin A and wheat germ agglutinin induced cell agglutination. They also inhibit the agglutination induced by heat-inactivated antisemum prepared against the purified cell membranes. For example, phytohemagglutinin A induced P-388 cell agglutination (70 ng/ml phytohemagglutinin A) can be inhibited by 12 \(\mu\)g/ml lithium diiodosalicylate-phenol extract from L-1210 cells.

**Discussion**

In developing the methods, we found that when we worked with a large number of cells (\(10^6\) cells), removal of intact nuclei was absolutely critical in order to reduce the total volume of lithium diiodosalicylate solution that is necessary for the solubilization of cellular membranes. On the other hand, Hart et al. [22] recently reported the isolation of a single concanavalin A receptor from the mouse L-929 cells by first dissolving whole L-cells in 0.3 M lithium diiodosalicylate. The lithium diiodosalicylate to cell number ratio in their case was 1 mmol per \(10^6\) cells, i.e. approx. 300 ml 0.3 M lithium diido-
salicylate solution should be used to solubilize \(10^6\) cells.

Two strategies have been used to study the surface glycoproteins of mamma-
lian cells, namely: (1) the protease digestion method, and (2) the glycoprotein solubilization method. By using the former method, only cleaved glycopeptide fractions are obtained. Wallberg et al. [24,55] used papain digestion to release glycopeptides from several ascites tumor cells and showed that they inhibit agglutination by several plant lectins. Warren and his coworkers [20,27] used the trypsin and pronase digestion methods to compare the released glycopep-
tides of normal cells and transformed cells. However, in order to understand the disposition [28] as well as the physiological and biochemical functions [29, 30] of glycoproteins on the cell surface, it seems necessary to isolate the intact surface glycoproteins. Solubilization of cell surface glycoproteins has been achieved by using aqueous phenol [11], aqueous pyridine [12], detergent [31] and lithium diiodosalicylate [14]. Among these methods, the lithium diido-
salicylate solubilization coupled with phenol extraction has been used to selectively isolate the major glycoprotein, glycoprotein A, from human erythro-
ocyte membranes [14]. This method, with certain modifications, has since been applied to several other cell lines [23,32,33]. In their pioneering work with L-1210 cells, Burger and his coworkers [33] used phenol, guanidine, pyri-
dine and lithium diiodosalicylate to solubilize the glycoproteins and reported that the lithium diiodosalicylate-extracted fractions showed four bands on SDS-polyacrylamide gel with apparent molecular weights ranging from 40,000
to 50,000. Hourani et al. [34] also used lithium diiodosalicylate to solubilize the glycophorins from the L-1219 cell surface and reported that six proteins were extracted, among them four were glycophorins as defined by periodic acid-Schiff base staining. These glycophorins had apparent molecular weights of 24,000, 60,000, 44,000 and 33,000 on 6% SDS-polyacrylamide gel. The yield was approx 4-7 mg protein/10^8 cells.

With the availability of more recent methodology we first labeled the cells with ^14C and demonstrated that most, if not all, of the extracted polypeptides are exposed on the cell surface. Furthermore, we have used a number of carbohydrate labeling methods, both surface labeling (galactose oxidase-reductive tritiation) and metabolic labeling, to show that indeed most of the extracted proteins are glycophorins and they are synthesized by the cell. We have also found that the lithium diiodosalicylate-phenol extraction method will preferentially isolate cell surface glycophorins, even from the post-nuclear cell homogenate (Note the similarity in pattern obtained in lane a and lane b of Fig 1 which are lithium diiodosalicylate-phenol extracts of post-nuclear supernatant and of plasma membranes from L-1210 cells, respectively). These additional methodological refinements permit us to avoid a dependence on the Coomassie Blue stain, which does not detect all the extracted glycophorins, or on the periodic acid-Schiff base staining, which also has limitations. For example, the substitution of an acetyl group on C1 of saccharic acid virtually inhibits oxidation of saccharic acid by periodate and renders it undetectable by periodic acid-Schiff base staining method [35]. In addition, Gahmberg recently showed that, with the human erythrocites, the periodic acid-Schiff base stains detects only three bands while the galactose oxidase plus ^14C/NADH reveals more than 20 glycophorins [36]. In our early experiments we provided evidence that the 1 mM PMSF should be present during homogenization and membrane isolation to prevent the degradative activities of endogenous proteolytic enzymes [9,10]; we have also been careful to use intact viable cells rather than frozen cells [34]. These additional modifications have permitted us to increase the yields of glycophorins to approx. 4-6% of the membrane protein [38]. In addition, the use of high pressure gas chromatography [39] has permitted us to determine that about 80% of the saccharic acid in the mouse leukemic cells is in a glycolyzed form and that the total saccharic acid content is only 0.5-0.6% by weight of the glycophorin extracts. This low content of saccharic acid in the glycophorin extracts derived from mouse leukemic cells probably explains their relative lack of sensitivity to periodic acid-Schiff staining; the latter appears to be greatly dependent on the saccharic acid content of glycophorins [37]. On the other hand, it is known that the plasma membranes of HeLa cells have high saccharic acid content [40].

Gahmberg and his coworkers have recently [41] applied a reductive tritiation method to study the glycophorin patterns of different mouse lymphoid cells. They found that most of the tritiated glycophorins of mouse lymphoid cells are of high molecular weights (>100,000). Since it has been suggested that the L-1210 cell is derived from a "B" lymphocyte precursor [42], we compared the glycophorin patterns of the B lymphoblasts obtained by Gahmberg with the glycophorin pattern of lithium diiodosalicylate-phenol extracts from L-1219 cells. The tritiated glycophorins in mouse B lymphoblasts have appar-
ent molecular weights of 210,000, 180,000, 125,000, 86,000 and 77,000, which may be compared to our values from both L-1210 and P-388 cells of 220,000, 180,000, 135,000, 120,000, 96,000, 88,000 and 60,000. The correspondence of many of these bands is extremely close considering the acceptable ±10% error range in the estimation of the apparent molecular weight on the SDS-polyacrylamide gel. On the other hand, the mouse T lymphoblasts lack the 220,000 band [41].

The Coomassie Blue staining pattern in the lithium diiodosalicylate-phenol extracts from leukemic cells and HeLa cells appears very similar. Differences in the extracts from the two cell types become apparent only when other detection procedures are used, such as iodination or carbohydrate labeling methods.

Acknowledgments

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