ISOLATION AND PARTIAL CHARACTERIZATION OF "GALACTOPROTEIN a" (LETS) AND "GALACTOPROTEIN b" FROM HAMSTER EMBRYO FIBROBLASTS*

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Summary- The cell surface glycoproteins of hamster NIL cells, labeled with galactose oxidase and NaB^3H_4, were selectively solubilized by sequential extraction with Tris buffer containing 1) sucrose-ATP-EDTA, 2) zwitterionic detergent (Empigen BB), and 3) 8 M urea. The previously reported "galactoprotein b" (Gap b) and "galactoprotein a" (Gap a or LETS) were isolated by affinity chromatography on insoluble *Ricinus communis* lectin columns (RCA column) from extracts 2) and 3), respectively. The affinity-purified Gap a contained an actin-like protein, whereas the other affinity-purified galactoproteins did not contain the actin-like protein. The isolated Gap b was heterogeneous, and an additional glycoprotein, specific for NILpy cells was co-purified on RCA-column with Gap b.

The presence of a unique glycoprotein in NIL cells, but deleted in NILpy cells, was revealed by cell surface-labeling with galactose oxidase-NaB^3H_4 and termed "galactoprotein a" (Gap a) (1,2). Increased label was found in one glycoprotein of NILpy as compared to NIL cells and termed "galactoprotein b" (Gap b) (1). Gap a increased greatly on cell contact and increased at G_1 phase of the cell cycle (3) and interacted with Con A and RCA (2). A similar glycoprotein present in various non-transformed fibroblasts and deleted in various transformed cells has been described and variously termed LETS (4), Zeta (5), SFA (6), CSP (7), etc (for a review, see 8). Yamada and Weston (7) isolated Gap a or LETS by selective solubilization in hypotonic 0.25 M urea from chick embryo fibroblasts,

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Abbreviations: ATP, adenosine triphosphate; buffer saline, 10 mM sodium phosphate buffer pH 7.2 containing 0.9% NaCl, 1 mM $CaCl_2$; CON A, *Canavalia ensiformis* lectin; EDTA, ethylenediamine tetraacetic acid (disodium salt); Gap a, galactoprotein a; Gap b, galactoprotein b; NIL, hamster embryo fibroblasts; NILpy, NIL cells transformed by polyoma virus; PAHS, polyacrylhydrazido-Sepharose; PHA, *Phaseolus vulgaris* lectin, PNA, *Arachis hypogaea* lectin; RCA, *Ricinus communis* lectin; SBA, *Glycine max* lectin; SDS, sodium dodecyl sulfate; TKS, 50 mM Tris-HCl buffer pH 7.6 containing 25 mM KCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$; TLCK, N-α-p-tosyl-L-lysine chloromethyl ketone HCl; Tris, Tris (hydroxymethyl) amino methane; WGA, *Triticum vulgaris* lectin.

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the method was however not applicable to NIL cells, and only a very small portion of Gap a was released in hypotonic urea. With sequential solubilization followed by affinity chromatography on RCA-PAHS-column, the Gap a and Gap b were extensively solubilized and isolated in good yield. This paper also describes a preliminary note on a possible complex between Gap a and an actin-like protein and the presence of a unique glycoprotein, co-purified with Gap b fraction, specific for NILpy cells. A possible association of LETS and actin-like material was previously suggested by Kuusela *et al* (9).

MATERIAL AND METHODS

Cells, cell culture and surface labeling with galactose oxidase and NaB^3H_4 are essentially the same as previously reported (1,2).
Isolation of Lectins and Hamster Muscle Actin: Lectins, PNA, SBA, WGA, from *Dolichos biflorus* and *Lotus tetragonolobus* were purified by published procedures in Dr. Nathan Sharon's laboratory by one of the authors. RCA, (RCA_I and RCA_{II}) were purified as described by Nicolson *et al* (10). PHA and Con A were obtained from Sigma Chemical Co. (St. Louis, Mo.). Hamster muscle actin was purified as described by Mommaert (11).
Lectin Affinity Column: RCA was coupled to a glutaraldehyde derivative of PAHS (12) followed by $NaBH_4$ reduction (13). In preliminary experiments, neither 1% Empigen BB (a zwitterionic detergent from Marchon Division of Albright and Wilson, Ltd., England (13,14) nor 2 M urea were found to inhibit the precipitation of RCA with [^{14}C]-asialofetuin in a precipitin test previously described (13).
Polyacrylamide Gel Electrophoresis: Linear polyacrylamide gradient (5-14%) slab gels containing 0.1% SDS were prepared following the basic stacking SDS gel technique of Laemmli (15). Cell samples and isolated glycoproteins (25-50 µg) were dissolved in sample buffer containing 2% SDS and 5% 2-mercaptoethanol and heated in a boiling water bath for 5 min. Non-reduced samples were treated as above in sample buffer without 2-mercaptoethanol. Slab gels were stained with Coomassie blue R250 (16). Fluorography of slab gels followed the procedure of Bonner and Laskey (17).
Preparation of Antisera: Gap a (ca 200 µg) was emulsified with 5 mg of attenuated *Mycobact. tuberculosis* in 1.5 ml of Freund's adjuvant and injected into 8 sites along the back and hind legs of a New Zealand white rabbit. After 3 weeks identical booster injections were given. Two weeks later blood was taken from the ear vein and the serum collected.
Sequential Extraction and Affinity Chromatographic Isolation of Labeled Glycoprotein: See legend of Fig. 1.

RESULTS

Isolation and properties of "Gap a".

The fluorography of surface-labeled glycoproteins of intact and trypsin-treated NIL cells as compared to the total protein pattern is shown in Fig. 2A gel 2, 3 and 4. The Gap a band (marked by "a" Fig. 2A) shows an acute sensitivity to trypsin cleavage of the intact cells (Fig. 2A gel 4). Whereas most of

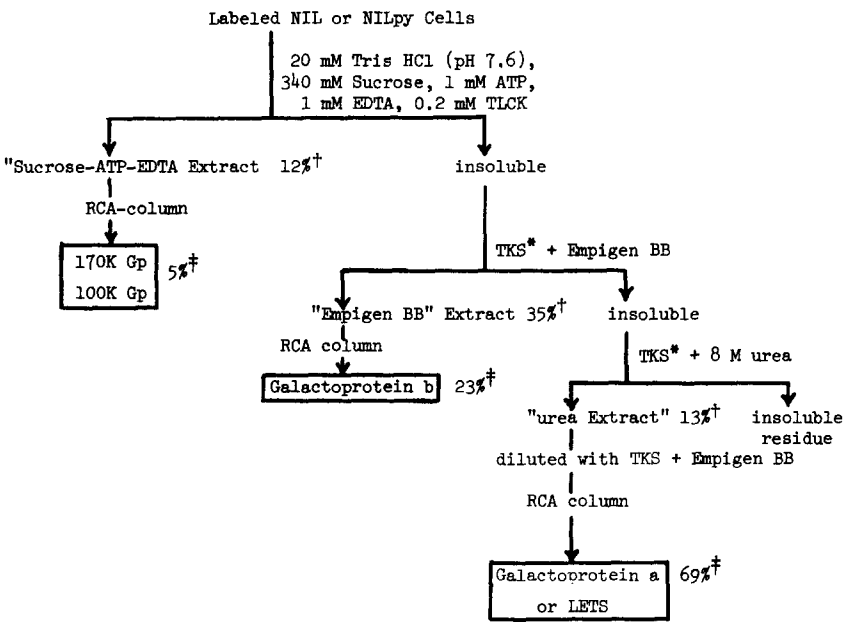

Fig. 1. Sequential Extraction of Surface-Labeled Galactoproteins of NIL and NILpy Cells.

Galactose oxidase-NaB^3H_4 labeled NIL cells (1.0 ml packed) were mixed with 6.5 ml of packed unlabeled NIL cells and suspended in 37.5 ml of ice-cold 20 mM Tris-HCl buffer pH 7.6 containing 0.34 M sucrose, 1 mM ATP, 1 mM EDTA and 0.2 mM TLCK. After 60 min on ice the cell suspension was centrifuged at 800 xg for 10 min (low speed) and the supernatant removed and recentrifuged at 35,000 xg for 20 min (high speed). The high speed pellet was added back to the low speed pellet and reextracted as above. The first and second high speed supernatants were pooled and labeled sucrose-ATP-EDTA extract. The above procedure was repeated on the cell pellet utilizing two 60 min extraction cycles of ice-cold TKS, containing 1.0% Empigen BB and 0.2 mM TLCK. The two combined high speed supernatants were labeled Empigen BB extract. The remaining cell pellet was suspended in 37.5 ml of TKS buffer containing 8 M urea and 0.2 mM TLCK. The suspension was heated in a boiling water bath for 5 min and then centrifuged at 35,000 xg for 20 min. Lower urea concentrations or extraction at 4° C resulted in reduced solubilization of radioactivity. The supernatant was removed and labeled Urea-extract.

The sucrose-ATP-EDTA extract was applied to a RCA-PAHS column (25 ml bed volume, 3 mg RCA/ml resin) which had been equilibrated with 20 mM Tris-HCl buffer pH 7.6, washed with the same buffer until no further radioactivity was eluted and then eluted with the same buffer containing 0.5 M galactose. The peak of radioactivity eluted with galactose was extensively dialyzed against H_2O and lyophilized. The Empigen BB extract was chromatographed on RCA-PAHS as above except all buffers contained TKS-0.5% Empigen BB. The urea extract was diluted with 3 volumes of TKS buffer containing 1.3% Empigen BB then recentrifuged at 35,000 xg for 20 min and the supernatant chromatographed on RCA-PAHS as above except all buffers contained TKS-0.5% Empigen BB and 2 M urea. The Empigen BB and urea extract RCA receptors eluted with galactose were extensively dialyzed against H_2O, 80% acetone, H_2O (22), lyophilized and weighed.

*TKS, see Abbreviation (footnote of title page).
‡% activity of each extract to the total label on cells.
†% activity of glycoprotein isolated by RCA-affinity column to the total activity of each extract.

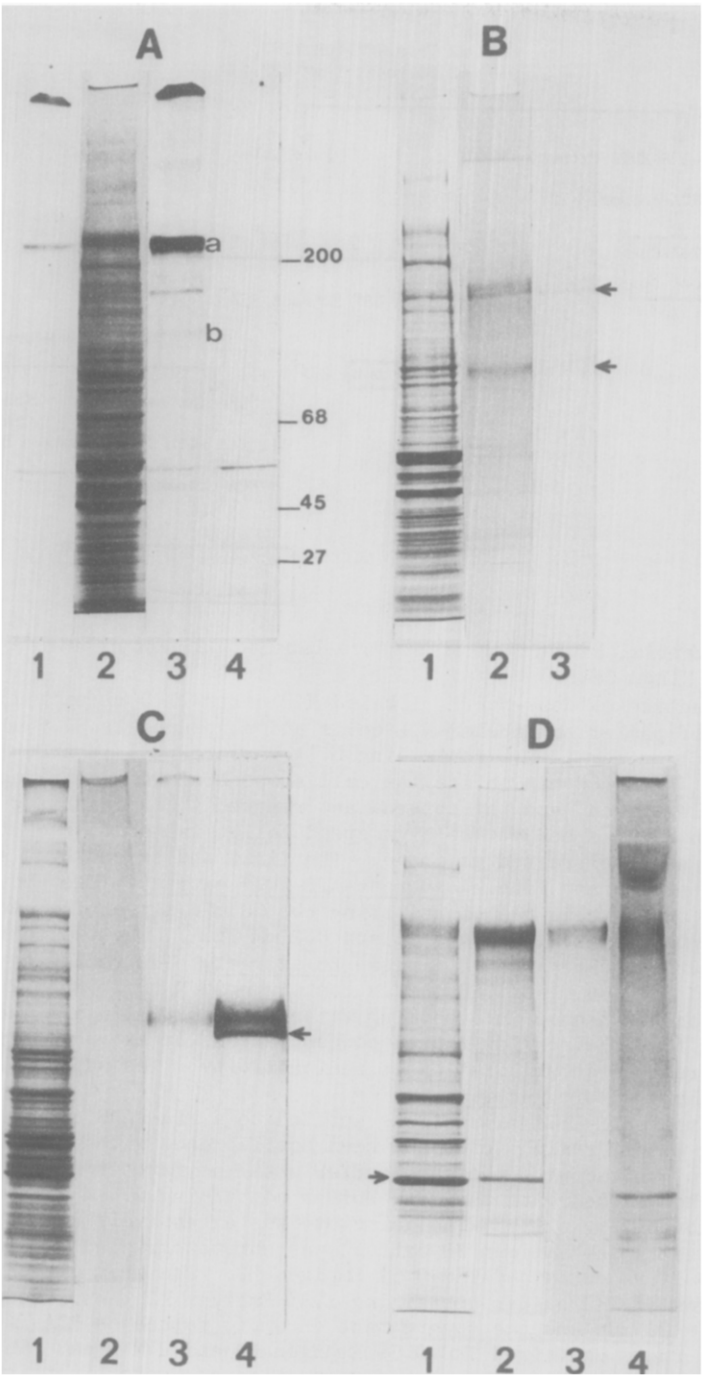

Fig. 2. Electrophoretic patterns of isolated RCA receptors from galactose-oxidase-NaB^3H_4 labeled NIL and NILpy cells on pH 7.6 polyacrylamide gradient (5-14%) slab gels in the presence of 0.1% SDS.
(A) Pattern of Total Cells: (1) Fluorograph of NIL cells reduced with NaB^3H_4 (no galactose oxidase); (2) Protein stain of NIL cells; (3) Fluorograph of gel 2;

the membrane proteins and glycoproteins were solubilized by successive extraction with sucrose-ATP-EDTA and with Empigen BB, Gap a remained unextracted under these conditions. The Gap a was finally extracted by 8 M urea from the insoluble residue (see Fig. 1), and was purified from the diluted urea extract by affinity chromatography on RCA-PAHS-column. The isolated glycoprotein (yield, 3.0 mg from 7.5 ml of packed NIL cells) co-migrated on SDS-polyacrylamide gels with trypsin-sensitive Gap a with an apparent molecular weight of 230,000 (see Fig. 2D gel 2 and 3 compared to Fig. 2A gel 2 and 3) when detected by protein staining (Fig. 2D gel 2) and by fluorography (Fig. 2D gel 3). Without prior reduction with 2-mercaptoethanol the isolated glycoprotein contained significant quantities of material that would not enter the 3.0% stacking gel or migrated with an apparent molecular weight of >300,000 (see Fig. 2D gel 4). The appearance of a reduction dependent subunit interaction was supported by amino acid analysis (Table I) which indicated the presence of cystine/2 in the isolated glycoprotein. The isolated Gap a showed precipitin reaction with RCA, Con A, and WGA (see Fig. 3). PNA, SBA, PHA, *Lotus tetragonolobus* and *Dolichos biflorus* lectins (results not shown) did not form precipitin bands.

Presence of an Actin-Like Protein Associated with "Gap a".

As seen in Fig. 2D gel 2, the Gap a fraction contains a small quantity of a 45,000 molecular weight non-labeled protein which co-migrates with purified hamster and rabbit muscle actin (marked with arrow Fig. 2D gel 1). The anti-

(4) Fluorograph of NIL cells labeled with galactose oxidase-NaB^3H_4 followed by trypsin digestion (10 µg trypsin/ml) for 10 min at 37°; 200, 68, etc. indicate mobilities of proteins with known molecular weights; rabbit muscle myosin 200,000, bovine serum albumin 68,000, rabbit muscle actin 45,000 *Dolichos biflorus* lectin subunit 27,000; a and b indicate Gap a and Gap b, respectively. (B) Pattern of Sucrose-ATP-EDTA Extract (see Fig. 1). (1) Protein stain of sucrose-ATP-EDTA extract of labeled NIL cells; (2) Protein stain of RCA receptors isolated from 1; (3) Fluorograph of gel 2; arrows indicate light bands visible on original fluorograph.
(C) Pattern of Empigen BB Extract (see Fig. 1). (1) Protein stain of Empigen BB extract of labeled NIL cells; (2) Protein stain of RCA receptor isolated from 1 (Gap b); (3) Fluorograph of gel 2; (4) Fluorograph of RCA receptor isolated from Empigen BB extract of labeled NILpy cells. Arrow indicates extra band present in NILpy cells.
(D) Pattern of Urea Extract (see Fig. 1). (1) Protein stain of urea extract of labeled NIL cells; (2) Protein stain of RCA receptor isolated from 1 (Gap a); (3) Fluorograph of gel 2; (4) Protein stain of RCA receptor isolated from 1 (sample not reduced with 2-mercaptoethanol); arrow indicates mobility of isolated hamster muscle actin.

TABLE I. Amino Acid Composition of Galactoprotein a and Galactoprotein b Isolated From NIL Cells* (expressed in mole percent).

	Gap a	Gap b
Alanine	7.8	7.1
Arginine	3.2	5.3
Aspartic acid	7.8	11.1
Cystine/2†	3.2	3.5
Glutamic acid	7.7	11.1
Glycine	13.9	8.5
Histidine	3.0	2.3
Isoleucine	3.7	4.3
Leucine	5.4	8.3
Lysine	3.3	4.5
Methionine‡	1.2	1.9
Phenylalanine	2.1	4.4
Proline	5.1	5.2
Serine	17.7	7.7
Threonine	7.3	6.0
Tryptophan	N.D	N.D
Tyrosine	2.3	3.0
Valine	5.4	5.9
	100.1	100.1

*24-hour 6N-HCl hydrolysis at 110° C; serine increased by 10% and threonine increased by 5% to compensate for destruction by acid. Analysis performed by AAA Laboratory, Seattle, WA.
†Performic acid oxidized prior to acid hydrolysis. Calculated from cystic acid/alanine ratio.
‡Methionine plus methionine sulfoxide.

Gap a rabbit antisera was found to precipitate with conditioned NIL growth media and isolated Gap a but not with purified hamster muscle actin on agarose double diffusion plates (results not shown). The same antisera co-precipitated the isolated Gap a and actin-like protein suggesting that the Gap a and actin-like material exist as a complex (data not shown).

Isolation and properties of "Gap b" fraction.

SDS polyacrylamide gel electrophoresis of the RCA receptor isolated from the Empigen BB extract (Fig. 1) (4.2 mg of glycoprotein isolated per 7.5 ml of

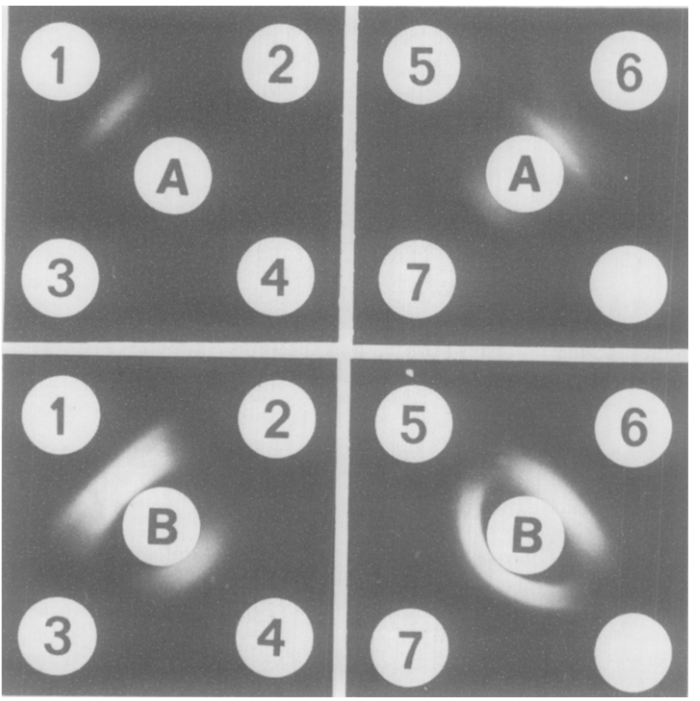

Fig. 3. Reactivity of Gap a and Gap b with various lectins in Ouchterlony double diffusion in agarose.
Center wells: (A) Gap a, 2.0 mg/ml; (B) Gap b, 3.0 mg/ml.
Outer wells: (1) RCA; (2) *Lotus tetragonolobus* lectin; (3) SBA, (4) PHA; (5) PNA; (6) CON A; (7) WGA. All lectins were dissolved in buffered saline at a concentration of approximately 3 mg/ml. Glycoproteins were dissolved in 2% Triton X-100 in 50 mM barbital buffer pH 8.6. Gels contained 0.5% agarose in 50 mM Tris-HCl buffer pH 7.6, 0.5% Triton X-100. Each well contained 10 µl and were developed for 2 days at room temperature.

starting packed cells) failed to detect any significant protein staining material although over 30 µg of material was applied to the gel (Fig. 2C gel 2). Fluorography (Fig. 2C gel 3) detected a labeled band with apparent molecular weight of 125,000 probably corresponding to the previously reported Gap b (marked "b" Fig. 2A). The sample with or without reduction in 2-mercaptoethanol had the same mobility. Fig. 3 indicated that the isolated Gap b possess polyvalent receptor sites for RCA, Con A, PHA, and WGA but not *Lotus tetragonolobus*, SBA or PNA. *Dolichos biflorus* lectin also gave negative precipitin results (data not shown). Table I gives the amino acid composition of the isolated Gap b.

Appearance of a New Glycoprotein after Transformation of NIL cells by Polyoma Virus.

Previously reported results (1) indicate that after polyoma viral transformation of NIL (NILpy) cells, the Gap b possess an increased reactivity with lectins. As can be seen in Fig. 2C gel 4, the RCA receptor isolated from Empigen extract of galactose oxidase-NaB^3H_4 labeled NILpy cells contains a glycoprotein receptor co-migrating with the Gap b isolated from NIL cells (Fig. 2C gel 3) plus an additional higher mobility labeled glycoprotein band (marked with an arrow).

Sucrose-ATP-EDTA Extract-RCA Receptor(s).

Two labeled glycoproteins (apparent molecular weights of 170,000 and 100,000) were purified on RCA-PAHS-column from the sucrose-ATP-EDTA extract, and detected both by protein staining and fluorography (Fig. 2B gel 2 and 3). The sucrose-ATP-EDTA extraction is a modification of a procedure originally designed for extraction of actin complexes from homogenates of alveolar macrophages (18). No detectable quantities of [^{3}H]-labeled Gap a or Gap b was present in the isolated receptors (Fig. 2B gel 2 and 3).

DISCUSSION

The affinity-purified Gap a fraction from the urea extract contained quantities of a 45,000 molecular weight actin-like protein. Reduction with 2-mercaptoethanol (Fig. 2D gel 2 and 4) had no effect on the mobility of the actin-like protein on SDS polyacrylamide gels indicating that it was not disulfide linked to Gap a. A large quantity of actin-like protein is present in both sucrose-ATP-EDTA extract and in Empigen extract but it was not co-purified with glycoproteins 170K, 100K or Gap b on RCA-PAHS-column. Moreover, precipitation of Gap a with rabbit anti-Gap a from the urea extract co-precipitated the actin-like material, indicating a possible specific interaction of Gap a and an actin-like protein. However, the proportion of Gap a and actin-like protein varied and was not stoichiometric. Further study on identification of actin-like protein and its interaction with Gap a is in progress. Kuusela *et al* (9) have previously observed the presence of an actin-like material in

immune precipitates of SF antigen complex from chicken fibroblasts, while Moore *et al* (19) have described the presence of cytoskeletal proteins associated with cell surface envelopes of ascites tumor cells. The possible involvement of cytoskeletal-membrane associations in cell shape or motility changes (20,21) could be supported by isolation of membrane receptor-actin-like complex(es).

Isolated Gap b (apparent molecular weight of 125,000) was not detectable with protein staining on SDS polyacrylamide gels, and approximately 55% of its weight was recovered as amino acid residues by amino acid analysis thus indicating extensive glycosylation of the protein backbone. Both Gap a and Gap b precipitated with Con A, RCA and WGA but Gap b showed an additional reaction with PHA under these conditions. The carbohydrate composition, structure and variations of Gap a and b are not within the scope of this preliminary note and will be described elsewhere. NILpy cells labeled with galactose oxidase-NaB^3H_4 and run on SDS gels have been previously reported to possess increased label in the Gap b region (1). This may, in part, be explained by the appearance of an additional labeled band (Fig. 2C gel 4) in the Gap b region isolated from NILpy cells. This additional band was not detected in Gap b from NIL cells.

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