Occurrence of α-D-Galactosyl-Containing Glycoproteins on Ehrlich Tumor Cell Membranes†

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ABSTRACT: Ehrlich ascites tumor cells were strongly agglutinated by 0.4 μg/mL *Griffonia simplicifolia* I-B_4 isolectin (GS I-B_4), indicating the presence of nonreducing terminal α-D-galactopyranose (α-D-Gal*p*) residues on the cell surface. Incubation of the cells with GS I-B_4 labeled with either fluorescein isothiocyanate (FITC–B_4) or ferritin followed by examination with the light and electron microscope revealed a random distribution of α-D-Gal*p* residues over the entire cell surface. Cell-binding studies with [^{3}H]propionate-labeled GS I-B_4 demonstrated a minimum of 18.1×10^6 α-D-Gal*p* sites per Ehrlich cell. An enriched Ehrlich cell plasma membrane preparation subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and stained with FITC–B_4 revealed a number of α-D-galactosyl-containing glycoproteins ranging in molecular weight from 50 000 to over 200 000. The major plasma membrane glycoprotein of the Ehrlich cell (GP 130) was isolated in near homogeneous form by using nonionic detergent extraction, affinity chromatography over GS I–Sepharose 4B, and preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Injection of Ehrlich cells into the mouse peritoneal cavity stimulated the appearance of low levels of α-D-galactosyl-containing glycoproteins in the ascites fluid ranging in molecular weight from 34 000 to 260 000. These glycoproteins differed in molecular weight when compared to the α-D-galactosyl-containing glycoproteins observed in either ascites fluid induced with Freund's complete adjuvant or the glycoproteins in the Ehrlich cell plasma membrane.

The α-D-galactopyranosyl-specific isolectin isolated from *Griffonia simplicifolia* seeds has been used as a probe for the detection of α-D-galactosyl-containing glycoconjugates on the surface of several murine cell lines and tissues. For example, the TA3-St mammary adenocarcinoma ascites cell subline was observed to be agglutinated by a saline extract of *G. simplicifolia* seeds (Friberg, 1972), and a fluorescein isothiocyanate labeled GS I-B_4[1] isolectin and a ^{125}I-labeled GS I lectin mixture bound to the plasma membranes of murine C-1300 neuroblastoma cells and 3T3 cells, respectively (Basu et al., 1976; Stanley et al., 1979). Various mouse tissues such as capillaries, glomeruli and basement membrane of the kidney, lens capsule and epithelium, skin basal epithelial cells, Bowman's membrane, Descement's membrane, and the epithelium of the cornea all have α-D-galactopyranosyl-containing glycoconjugates, detectable by fluorescein isothiocyanate labeled GS I-B_4 (Peters & Goldstein, 1979). Murine liver and human biopsy kidney sections were negative.

Although common constituents of animal glycolipids (Eto et al., 1968; Hakomori et al., 1971; Hakomori & Strycharz, 168; Li et al., 1972; Naiki & Marcus, 1974; Slomiany & Slomiany, 1978), α-D-Gal*p* end groups have been reported in only a limited number of glycoproteins. These include blood group B substances (Kabat, 1956; Watkins, 1966; Newman & Kabat, 1976), earthworm cuticle collagen (Muir & Lee, 1969), bonnet monkey cervical mucin (Hatcher et al., 1977), bovine thyroid (Okada & Spiro, 1980), glycopeptides released by alkaline elimination from rat, rabbit, and chicken brain (Finne, 1975; Finne & Krusius, 1976), and cell-surface glycoprotein from stimulated murine macrophages (Maddox et al., 1982).

We report in this paper the detection of α-D-galactopyranosyl-containing glycoconjugates on the plasma membrane of the Ehrlich ascites tumor cell line, a spontaneous murine mammary adenocarcinoma adapted to ascites form. α-D-Gal*p* end groups were also demonstrated to be components of a number of plasma membrane glycoproteins that may be shed into the ascites fluid. The major plasma membrane glycoprotein of the Ehrlich cell was isolated in a highly enriched form, and its chemical properties are described.

Experimental Procedures

Materials

The following materials were obtained commercially: Tris–ATP and 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) (Sigma Chemical Co.); Omnifluor and CDP–[*methyl*-^{14}C]choline (New England Nuclear Corp.); *N*-succinimidyl [2,3-$^3H_2$]propionate (Amersham Corp.); fluorescein isothiocyanate (Nutritional Biochemicals Corp.); horse spleen ferritin (Miles Laboratories, Inc.); dansylhydrazine and constant boiling HCl, sequanal grade (Pierce Chemical Co.); cyanogen bromide activated Sepharose 4B (Pharmacia, Inc.); Freund's complete adjuvant and *Phaseolus vulgaris* lectin (Difco Laboratories); SDS–PAGE molecular weight protein standards (Bio-Rad Laboratories); Pronase (B grade) and *Triticum vulgaris* lectin (Calbiochem-Behring Corp.). *Griffonia simplicifolia* I lectin mixture and the GS I-A_4 and GS I-B_4 isolectins were purified as described by

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[1] Abbreviations: $DC_{10}PO$, dimethyldecylphosphine oxide; FITC–B_4, fluorescein isothiocyanate labeled *Griffonia simplicifolia* I-B_4 isolectin; ferritin–B_4, ferritin-labeled *Griffonia simplicifolia* I-B_4 isolectin; [^{3}H]-propionate–B_4, [2,3-$^3H_2$]propionate-labeled *Griffonia simplicifolia* I-B_4 isolectin; GS, *Griffonia simplicifolia*; GS I-B_4, *Griffonia simplicifolia* I-B_4 isolectin; INT, 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride; PAGE, polyacrylamide gel electrophoresis; PBS–BSA buffer, 0.01 M sodium phosphate, 0.15 M sodium chloride, and 1 mg/mL bovine serum albumin, pH 7.0; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Me-α-D-Gal*p*, methyl α-D-galactopyranoside; Me-α-D-Glc*p*, methyl α-D-glucopyranoside; EDTA, ethylenediaminetetraacetic acid; Con A, concanavalin A.

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Hayes & Goldstein (1974) and Murphy & Goldstein (1977). Concanavalin A was purified as described by Agrawal & Goldstein (1967). *Lotus tetragonolobus* lectin was purified as described by Blumberg et al. (1972). *G. simplicifolia* II lectin was purified as decribed by Shankar-Iyer et al. (1976). The following materials were obtained as gifts: *Helix pomatia* lectin from Dr. Sten Hammarstrom (Wenner Gren Institute, Stockholm, Sweden); *Dolichos biflorus* and *Ricinus communis* lectins from Dr. Marilynn Etzler (University of California, Davis, Davis, CA); antihuman blood group A, B, and P_1 antisera from John Judd (University of Michigan Blood Bank, Ann Arbor, MI); dimethyldecylphosphine oxide (Hays, 1968) from Dr. H. R. Hays (Procter and Gamble Co.).

Methods

Maintenance of Ehrlich Cells. Ehrlich ascites tumor cells were maintained by weekly intraperitoneal injection of outbred male Swiss ICR mice with 0.4 mL of ascites fluid (approximately 5×10^7 cells). For experimentation, cells were harvested 8 or 9 days after transfer and washed free of erythrocytes by repeated centrifugation at 900*g* for 30 s in 0.15 M sodium chloride at 2 °C.

Agglutination Assay. Serial dilutions of various lectins and antisera in PBS buffer (25-μL final volume) were performed in a microtiter V-plate (Cooke Engineering Co.). To each well was added 1.7×10^5 Ehrlich cells suspended in 25 μL of PBS. The plate was gently agitated and allowed to incubate at room temperature for 30 min. Results, determined microscopically, were expressed as the minimum concentration of lectin required for formation of small aggregates of cells.

Assay Procedures. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard. Neutral sugar was determined by the phenol–sulfuric acid colorimetric assay of Dubois et al. (1956) with Me-α-D-Gal*p* as the standard. The sialic acid content of glycoconjugates was determined by the method of Warren (1959). *N*-Acetylneuramic acid was used as the standard. Carbohydrate composition was determined by the method of Porter (1975). Amino acid composition was determined as described by Spackman et al. (1958) after hydrolysis in constant boiling hydrochloric acid at 107 °C for 18 h. No correction was made for the loss of serine and threonine during hydrolysis.

Preparation of Fluorescein Isothiocyanate Conjugated GS I-B_4 Isolectin. FITC-B_4, prepared according to the method of Peters and Goldstein (1979), contained 2.5 mol of fluorescein/mol of lectin. The hemagglutination activity of FITC–B_4 toward human type B erythrocytes was identical with that of native GS I-B_4.

Preparation of Ferritin-Conjugated GS I-B_4 Isolectin. Horse spleen ferritin was coupled to GS I-B_4 isolectin by the procedure of Nicolson & Singer (1974). After conjugation, the mixture was made 15% saturated in ammonium sulfate, stirred for 2 h at 2 °C, and clarified by centrifugation at 15000*g* for 30 min. The reaction mixture was applied to a Bio-Gel P-300 column (1.5 × 100 cm) equilibrated with PBS buffer at 2 °C. The high molecular weight fractions containing free and polymerized ferritin and ferritin–B_4 conjugates were pooled and applied to a column (7 × 75 mm) of epichlorohydrin-cross-linked guaran (Lonngren et al., 1976) equilibrated with PBS buffer. The guaran column was washed with PBS buffer followed by 0.1 M Me-α-D-Gal*p* in PBS buffer to elute ferritin–B_4. The ferritin–B_4 conjugate was dialyzed against PBS buffer and contained 1 mol of GS I-B_4/mol of ferritin.

Preparation of [2,3-$^3H_2$]Propionate–GS I-B_4 Isolectin. A 1.25-mL aliquot of *N*-succinimidyl [2,3-$^3H_2$]propionate (sp act. 50 Ci/mmol; dissolved in benzene at 2 mCi/mL) was evaporated to dryness under a stream of nitrogen. Two milliliters of GS I-B_4, 5 mg/mL, in coupling buffer (0.1 M sodium bicarbonate and 0.15 M sodium chloride buffer, pH 8.5) containing 0.1 M Me-α-D-Gal*p* was added to the *N*-succinimidyl [2,3-$^3H_2$]propionate and mixed vigorously at 2 °C. After 4 h, the reaction was terminated by the addition of 8 mL of coupling buffer containing 0.25 M glycine, followed by stirring for 5 min. The radiolabeled lectin was dialyzed exhaustively at 2 °C against PBS buffer until the dialyzate showed less than 200 cpm/mL. The [^{3}H]propionate–B_4 had a specific activity of 56 800 cpm/μg of protein and a hemagglutination activity against human type B erythrocytes identical with that of native GS I-B_4.

Preparation of GS I–Sepharose. Coupling of GS I to CNBr-activated Sepharose 4B was performed according to the directions of the manufacturer to give a final concentration of 3.3 mg of GS I/mL of Sepharose 4B.

FITC–B_4 Labeling of Ehrlich Cells. To 1 mL of a 5% suspension (v/v) of washed Ehrlich cells in PBS buffer was added FITC–B_4 (final concentration 25 μg/mL), and the suspension was stirred gently for 15 min at 2 °C. The incubation mixture included either 10 mM Me-α-D-Glc*p*, a noninhibitor of GS I-B_4, or 10 mM Me-α-D-Gal*p*, an inhibitory hapten. The cells were washed 3 times with 10 mL of PBS buffer at 2 °C, resuspended in 0.5 mL of PBS buffer, and examined.

Ferritin–B_4 Labeling of Ehrlich Cells. Ferritin–B_4 (final lectin concentration 50 μg/mL) was added to 1 mL of a 5% suspension (v/v) of washed Ehrlich cells in PBS buffer containing either 10 mM Me-α-D-Glc*p* or 10 mM Me-α-D-Gal*p* and the suspension gently stirred for 15 min at 2 °C. The cells were washed 3 times with 10 mL of PBS buffer at 2 °C, fixed by the addition of 1.0 mL of PBS buffer with 2% glutaraldehyde, processed for electron microscopy by standard techniques, and examined.

[^{3}H]Propionate–GS I-B_4 Binding to Ehrlich Cells. Washed Ehrlich cells [(6–7) × 10^6 cells] were added to tubes containing 1.4–10.6 μg/mL [^{3}H]propionate–B_4 and 10 mM Me-α-D-Glc*p* or Me-α-D-Glc*p* in 2.0 mL of PBS–BSA buffer and incubated for 90 min at 2 °C with intermittent mixing. The cells were centrifuged at 250*g* for 10 min at 2 °C; the supernatant solution was removed and counted to determine the concentration of unbound lectin. The unwashed cell pellets were dissolved by the addition of 0.2 N sodium hydroxide (2.0 mL), followed by vigorous mixing and incubation in a boiling water bath for 10 min. A 0.95-mL aliquot of solubilized cells was added to 50 μL of 5.0 N HCl and the solution counted to determine the amount of lectin bound to the cells. Binding determinations for each lectin concentration were performed in duplicate. The binding of [^{3}H]propionate–B_4 to Ehrlich cells in the presence of 10 mM Me-α-D-Gal*p* was never greater than 1% of lectin binding in the presence of 10 mM Me-α-D-Glc*p*.

Preparation of Ehrlich Cell Plasma Membranes and Criteria of Purity. An enriched plasma membrane fraction from Ehrlich cells was prepared according to Im et al. (1976) except 15 mM Tris was used to replace the sodium phosphate in all buffers. The activity of several enzymes characteristic of specific subcellular organelles, as well as the DNA and RNA content, was determined in both the whole-cell homogenate and the enriched plasma membrane fraction in order to demonstrate the degree of enrichment of the plasma membrane fraction.

The Na^+,K^+-dependent ATPase activity, a plasma membrane marker, was determined as described by Kilberg &

Christensen (1979). The succinate-INT reductase activity, a mitochondrial enzyme marker, was determined as described by Morre (1971). The CDP-choline transferase activity, an endoplasmic reticulum marker, was determined as described by Schneider (1963) and Schneider & Behki (1963). α-Galactosidase activity, a marker for lysosomes, was determined in the usual fashion by using as substrate 30 mM *p*-nitrophenyl α-D-galactopyranoside–50 mM sodium citrate buffer, pH 4.0.

The deoxyribonucleic acid (DNA) content, a nuclei marker, was determined by injecting 10 μCi of [*methyl*-^{3}H]thymidine into the peritoneal cavity of a mouse inoculated with Ehrlich cells 8 days previously. After 1 h, the mouse was sacrificed, and the Ehrlich cells were harvested and washed with 0.15 M sodium chloride until 1.0 mL of wash supernatant showed less than 30 cpm. These labeled cells were pooled with washed unlabeled cells, and plasma membranes were prepared as described previously. DNA and ribonucleic acid (RNA) were extracted with perchloric acid from the whole-cell homogenate and the plasma membrane fraction as described by Burton (1956). Aliquots of the extracts were counted to determine the amount of [*methyl*-^{3}H]thymidine in the extracted DNA. The specific acitivity, in units of cpm/mg of protein, of the homogenate extract was compared to the specific activity of the plasma membrane extract as an indicator of nuclei contamination. The RNA content of the homogenate and the plasma membrane fraction was determined by the method of Burton (1956).

Electrophoresis. Discontinuous polyacrylamide gel electrophoresis of protein and glycoprotein in the presence of SDS was performed according to Laemmli (1970) on either slab or tube gels. Fixed gels were stained for protein with Coomassie blue or stained for carbohydrate with dansylhydrazine after periodate oxidation according to Eckhardt et al. (1976). Polyacrylamide gels were also stained with FITC–B_4 to detect α-D-galactosyl-containing glycoproteins. Two identical slab gels were soaked in 500 mL of PBS buffer for 30 min at room temperature with two changes. The gels were then immersed in 100 μg/mL FITC–B_4 in PBS–azide buffer containing either 10 mM Me-α-D-Glc*p* or 10 mM Me-α-D-Gal*p* and incubated at 2 °C for 4 days. The gels were destained for 4 h in PBS buffer containing either 10 mM Me-α-D-Glc*p* or 10 mM Me-α-D-Gal*p*. Glycoproteins containing terminal α-D-Gal*p* residues were visualized with a long-wavelength ultraviolet transilluminator with a peak output of 366 nm (Ultraviolet Products, Inc., San Gabriel, CA).

Solubilization of Ehrlich Cell Plasma Membrane. To 2 mg of plasma membrane protein in 1.0 mL of water was added 1.0 mL of the solubilizing agent. The suspensions were stirred for 16 h at 2 °C and centrifuged at 100000*g* for 1 h. The supernatant solutions were removed, and 10 μL of 2% SDS was added to each. The extracts were dialyzed against 2 L of 0.01% SDS–0.04% NaN_3 at room temperature with several changes, assayed for protein, neutral sugar, and reaction with GS I-B_4 in hematocrit tubes, and subjected to SDS–PAGE. The solubilizing agents examined were as follows: (1) water; (2) 4 M sodium chloride; (3) 1 mM EDTA, 5 mM β-mercaptoethanol, 50 mM glycine–HCl buffer, pH 9.0; (4) 8 M urea; (5) 4 M potassium thiocyanate; (6) 2% Triton X-100 in PBS buffer; (7) 2% dimethyldecylphosphine oxide (DC_{10}PO) in PBS buffer; (8) 2% sodium deoxycholate in 0.01 M Tris-HCl, 0.15 M sodium chloride buffer, pH 7.9; (9) 2% SDS. A tenth sample was initially extracted with acetone at 2 °C for 10 min and centrifuged at 27000*g* for 15 min. The plasma membranes were washed once with water and suspended in 1% DC_{10}PO in PBS buffer with stirring for 16 h at 2 °C. The acetone–DC_{10}PO extract was processed as described above.

Isolation of GP 130. An enriched Ehrlich cell plasma membrane preparation (85 mg of protein) was suspended in 42.5 mL of water and forced through a 25-gauge needle into 170 mL of acetone at 2 °C. The suspension was stirred for 10 min at 2 °C and centrifuged at 27000*g* for 15 min. The supernatant was removed and the pellet was reextracted with 170 mL of acetone at 2 °C for 10 min. The suspension was centrifuged at 27000*g* for 15 min and the supernatant removed. The pellet was washed for 5 min with water (170 mL) at 2 °C and centrifuged at 27000*g* for 15 min. The supernatant solution was removed and the pellet suspended in 85 mL of DC_{10}PO (10 mg/mL), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and PBS–azide buffer and stirred at 2 °C for 16 h. The suspension was centrifuged at 100000*g* for 1 h at 2 °C and the supernatant solution removed and designated first DC_{10}PO extract. The pellet was resuspended in the same extraction medium (43 mL) and reextracted for 16 h at 2 °C. The suspension was centrifuged at 100000*g* for 1 h and the supernatant solution designated second DC_{10}PO extract.

The first DC_{10}PO extract was chromatographed over a GS I–Sepharose 4B column (1.5 × 15 cm) equilibrated with 1 mg/mL DC_{10}PO in PBS–azide buffer (flow rate 10 mL/h). The GS I–Sepharose 4B column was washed with 1 mg/mL DC_{10}PO in PBS–azide buffer until the absorbance at 280 nm was zero, and the bound material eluted with 0.1 M Me-α-D-Gal*p* in the same buffer. The eluted material designated GS I bound glycoprotein mixture was dialyzed against several changes of 0.04% sodium azide.

A small portion of the GS I bound glycoprotein mixture was dansylated to serve as a marker during preparative SDS–PAGE. Three milliliters of GS I bound glycoprotein mixture (0.175 mg/mL) was dialyzed against 0.1 M Tris-HCl buffer, pH 8.2. To the glycoprotein mixture was added 0.75 mL of 10% (w/v) SDS and 75 μL of 10% (w/v) dansyl chloride in acetone. The mixture was immersed in a boiling water bath for 5 min followed by dialysis against 0.1% SDS–0.04% sodium azide with several changes. The mobilities of the dansylated glycoproteins during SDS–PAGE were identical with those of the native mixture.

The GS I bound glycoprotein mixture was fractionated by preparative SDS–PAGE as described by Koziarz et al. (1978) except EDTA was omitted from all buffers. The 5% polyacrylamide gel (5.9 × 10 cm) was preelectrophoresed with 3 mL of sample buffer containing 1 M thioglycollic acid titrated to pH 7.4 with sodium hydroxide. A total of 5–10 mg of the GS I bound glycoprotein mixture and 90 μg of dansylated glycoprotein mixture were pooled, lyophilized, and redissolved in 5 mL of sample buffer. The glycoprotein mixture was applied to the polyacrylamide gel and electrophoresed until the tracking dye was 1 cm from the bottom of the gel. The gel was removed, and the glycoprotein bands were visualized by illuminating the gel with a UV transilluminator (360-nm output). The major glycoprotein, GP 130, in a 6 mm wide band was excised from the gel. The gel was minced and the glycoprotein extracted for 36 h with 50 mL of 0.01% SDS–0.04% sodium azide in water at room temperature with vigorous stirring. The suspension was centrifuged at 27000*g* for 30 min and the supernatant filtered through a 0.45-μm Millipore filter and lyophilized. The purified GP 130 was redissolved in 20 mL of water and dialyzed against 0.04% sodium azide in water or PBS–azide buffer with 0.1 mM $CaCl_2$.

Table I: Agglutinability of Ehrlich Cells with Various Lectins

lectin	minimum concn required for agglutination [μg of lectin $(3.4 \times 10^6$ cells$)^{-1}$ mL^{-1}]
Griffonia simplicifolia I-B_4	0.4
Griffonia simplicifolia I-A_4	0.55
Ricinus communis	1.23
Phaseolus vulgaris (PHA)	2.66
Triticum vulgaris (WGA)	18
Helix pomatia	102
Canavalia ensiformis (Con A)	135
Lotus tetragonolobus	>182
Griffonia simplicifolia II	>275
Dolichos biflorus	>675

To obtain glycopeptides, GP 130 (0.62 mg of protein in 7.5 mL of PBS–azide buffer with 0.1 mM $CaCl_2$) was digested with 3 mg of Pronase and incubated at 37 °C. Additional Pronase (3 mg) was added at 24 and 48 h and the digestion terminated after 72 h by immersion in a boiling water bath. The Pronase digest was chromatographed over a GS I–Sepharose 4B column (1 × 8 cm) equilibrated with PBS–azide buffer. The column was washed with buffer until the absorbance at 280 nm was zero and eluted with 10 mL of 0.1 M Me-α-D-Gal*p* in PBS–azide buffer. All fractions containing Me-α-D-Gal*p* were pooled, lyophilized, redissolved in 0.75 mL of water, and chromatographed at a rate of 12 mL/h over a Bio-Gel P-2 column (0.9 × 25 cm) to separate the α-D-galactosyl-containing glycopeptide(s) from Me-α-D-Gal*p*.

Detection of α-D-Galactosyl-Containing Glycoproteins in Ehrlich Cell Induced Ascites Fluid. Ehrlich cells and ascites fluid, collected from seven mice 8 days after tumor cell inoculation, were centrifuged at 900*g* for 5 min at 2 °C without additional 0.15 M sodium chloride. The supernatant (49 mL) was centrifuged at 27000*g* for 30 min at 2 °C, after which a small pellet of erythrocytes was observed. The cell-free supernatant solution was centrifuged in an SW 27 rotor at 100000*g* for 2 h at 2 °C. Ten milliliters of the 100000*g* supernatant solution, designated Ehrlich cell ascites fluid, was chromatographed over a GS I–Sepharose 4B column (1 × 2.5 cm) equilibrated with PBS–azide buffer. After the column was washed with PBS–azide buffer until the absorbance at 280 nm was zero, the bound material was eluted with 0.1 M Me-α-D-Gal*p* in the same buffer. The eluted material was dialyzed against several changes of an aqueous solution of sodium azide (0.04% w/v). Ehrlich cell ascites fluid and the GS I bound material were subjected to SDS–PAGE and stained for protein with Coomassie blue or FITC–GS I-B_4 to detect α-D-galactosyl-containing glycoproteins.

Detection of α-D-Galactosyl-Containing Glycoproteins in Ascites Fluid Induced by Freund's Complete Adjuvant. Three male Swiss ICR mice were injected intraperitoneally with Freund's complete adjuvant (FCA). Each mouse was given a total of six 0.2-mL injections of FCA with 4 days between injections. Two weeks after the last injection, the mice were sacrificed, and the FCA-induced ascites fluid was collected and centrifuged at 27000*g* for 30 min at 2 °C. The pooled ascites fluid (40 mL) was centrifuged at 100000*g* for 2 h at 2 °C. Ten milliliters of supernatant FCA-induced ascites fluid was chromatographed over a GS I–Sepharose 4B column (1 × 2.5 cm) as above to obtain a GS I bound fraction. FCA-induced ascites fluid and the GS I bound fluid were subjected to SDS–PAGE and stained with Coomassie blue or FITC–GS I-B_4.

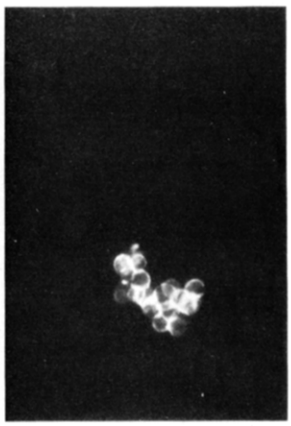

FIGURE 1: FITC–B_4 labeling of Ehrlich ascites tumor cells. Labeling was performed at 2 °C at a lectin concentration of 25 μg/mL with 10 mM Me-α-D-Glc*p* (125×).

Results

Detection of Terminal α-D-Galactopyranosyl Residues on the Ehrlich Cell Surface. The agglutinability of Ehrlich ascites tumor cells by a number of lectins and antisera was examined. The results of these experiments are summarized in Table I. Those lectins capable of reacting with terminal α-D-Gal*p* residues, GS I-B_4, GS I-A_4, and *R. communis*, exhibited the strongest agglutinating activity toward Ehrlich cells. The lectin from *P. vulgaris* also strongly agglutinated Ehrlich cells, while moderate agglutinating activity was exhibited by concanavalin A, wheat germ agglutinin, and *H. pomatia* agglutinin. No agglutination of Ehrlich cells was observed with the lectins from *L. tetragonolgus*, *G. simplicifolia* II, and *D. biflorus* at the maximum concentrations tested. Although GS I-A_4 is capable of reacting with terminal α-D-GalNAc*p* residues, this appears unlikely with the Ehrlich cell inasmuch as the *D. biflorus* lectin, which is highly specific for terminal α-D-GalNAc*p* residues, failed to agglutinate Ehrlich cells. Antisera raised against blood group B and blood group P_1 substances, both of which terminate in α-D-Gal*p* residues, and antisera to blood group A substance failed to agglutinate Ehrlich cells at the maximum concentrations tested.

The GS I-B_4 isolectin was used to probe further the distribution and number of α-D-Gal*p* sites on the Ehrlich cell surface. Fluorescence microscopy of Ehrlich cells incubated with FITC–B_4 at 2 °C in the presence of 10 mM Me-α-D-Glc*p* revealed an intense fluorescence over the entire cell surface (Figure 1). Cells allowed to warm up to 25 °C were observed to give rise to some patch formation. The binding of FITC–B_4 to the Ehrlich cell surface in the presence of 10 mM Me-α-D-Gal*p* was completely abolished, demonstrating the specificity of lectin binding.

Incubation of Ehrlich cells with ferritin–B_4 at 2 °C in the presence of 10 mM Me-α-D-Glc*p* followed by fixation with glutaraldehyde and examination with the electron microscope revealed a random distribution of ferritin–B_4 bound to the plasma membrane (Figure 2A). No areas of clustering were observed. In the presence of 10 mM Me-α-D-Gal*p*, the binding of ferritin–B_4 to the Ehrlich cell surface was completely abolished (Figure 2B).

The binding of [^{3}H]propionate–GS I-B_4 to Ehrlich cells plotted according to Scatchard (1949) indicated the lectin binding sites to be independent (noninteracting) and homogeneous with respect to association constant (data not shown). Making the assumption that at saturation each lectin molecule binds to only one α-D-Gal*p* residue allows one to calculate the presence of 18.1 × 10^6 α-D-Gal*p* sites per Ehrlich cell.

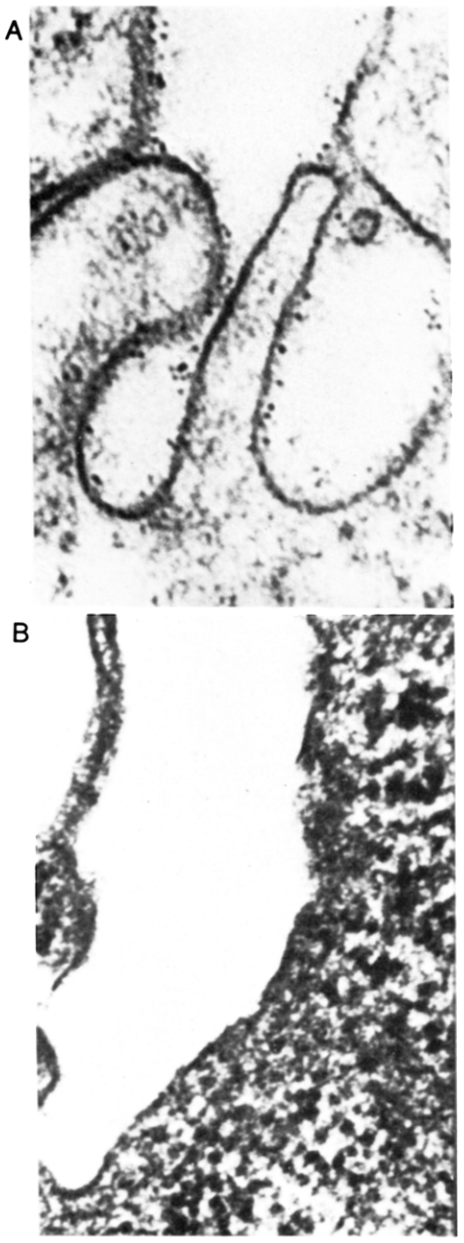

FIGURE 2: Ferritin–B_4 labeling of Ehrlich ascites tumor cells. Labeling was performed at 2 °C at a lectin concentration of 50 μg/mL with (A) 10 mM Me-α-D-Glc*p* or (B) 10 mM Me-α-D-Gal*p* (47650×).

However, this assumption is probably not valid because the apparent association constant for the binding of [^{3}H]-propionate–GS I-B_4 to the Ehrlich cell was found to be 1.13×10^8 M^{-1}. This is 1000-fold higher than the intrinsic association constant of GS I for Me-α-D-Gal*p*, which was determined to be 8.6×10^4 M^{-1} at 2 °C (Hayes & Goldstein, 1975). This increase in the association constant may be due either to nonspecific, noncovalent interactions between the lectin and the cell surface and/or to, more probably, binding of tetravalent GS I-B_4 to Ehrlich cells via multiple binding sites. Hornick & Karush (1972) and Hammarstrom (1973) examining the multivalent binding of antibodies and *H. pomatia* lectin, respectively, observed increases in the apparent association constants of 3–6 orders of magnitude compared to the intrinsic association constants of monovalent carbohydrate ligands. If multivalent binding does occur between GS I-B_4 and Ehrlich cells, then 18.1×10^6 α-D-Gal*p* sites per cell would be a lower limit.

Characterization of Ehrlich Cell Plasma Membrane Fraction. The yield of enriched plasma membrane prepared from 40 mL of washed Ehrlich cells (approximately 1.2×10^{10} cells) ranged from 15 to 25 mg of protein. The degree of enrichment of the plasma membrane fraction was established by comparing the level of several subcellular organelle markers in the whole-cell homogenate and the plasma membrane fraction. The data are shown in Table II. The relative specific activity was obtained by dividing the specific activity in the plasma membrane fraction by that found in the whole-cell homogenate.

Table II: Subcellular Markers Present in the Homogenate and Enriched Plasma Membrane of the Ehrlich Cell[a]

subcellular marker	homogenate	plasma membrane	RSA[b]
(Na^+,K^+)-ATPase (plasma membrane)	370	9900	26.7
succinate-INT reductase (mitochondria)	2270	1250	0.55
CDPcholine transferase (endoplasmic reticulum)	484	135	0.28
α-galactosidase (lysosomes)	7.5	3.25	0.43
RNA (endoplasmic reticulum)	149.4	58	0.388
DNA (nuclei)	451	432	0.957

[a] The activities of (Na^+,K^+)-ATPase, succinate-INT reductase, CDPcholine transferase, and α-galactosidase are reported as nmol h^{-1} (mg of protein)$^{-1}$. RNA is reported as μg of RNA/mg of protein. DNA is reported as cpm [*methyl*-^{3}H]thymidine incorporated/mg of protein. [b] Relative specific activity.

(Na^+,K^+)-ATPase, a plasma membrane marker, was highly enriched in the plasma membrane fraction with a relative specific activity of 26.7 and a specific activity of 9.9 μmol h^{-1} (mg of protein)$^{-1}$. These values were comparable to those obtained by Im et al. (1976) for an Ehrlich cell plasma membrane fraction [(Na^+,K^+)-ATPase specific activity 9.59 μmol h^{-1} (mg of protein)$^{-1}$ with a relative specific activity of 30.9] and indicated a high degree of enrichment for the plasma membrane preparation.

Enzymatic activities associated with other subcellular membrane fractions, succinate-INT reductase (mitochondria), CDPcholine transferase (endoplasmic reticulum), and α-galactosidase (lysosomes), were found at lower specific activities in the enriched plasma membrane fractions compared to those in the whole-cell homogenate. The relative specific activity values for these three enzyme activities respectively were 0.55, 0.28, and 0.43 (Table II). RNA, an endoplasmic reticulum marker, was found in significant amounts in the plasma membrane fractions, 58 μg of RNA/mg of protein, although the amount was reduced compared to that in the whole-cell homogenate, which contained 149.4 μg of RNA/mg of protein. This was consistent with the findings of others (Kilberg & Christensen, 1979; Rittenhouse et al., 1978; Columbini & Johnstone, 1973) and suggests the possibility that RNA may be an integral part of the Ehrlich cell plasma membrane, as well as the endoplasmic reticulum.

DNA, a nuclei marker, was found in fairly high amounts in the plasma membrane fraction. The relative specific activity value was 0.957, making nuclei the major contaminating organelle of the plasma membrane preparation. This high level of DNA in the plasma membrane fraction was inconsistent with the observations of others. Relative specific activity values for DNA in the Ehrlich cell plasma membrane preparations of Rittenhouse et al. (1978) and Colombini & Johnstone (1973) were calculated from the data presented and determined to be, respectively, 0.10 and 0.15. In summary, the subcellular marker data demonstrated a highly enriched plasma membrane fraction that contained low levels of mi-

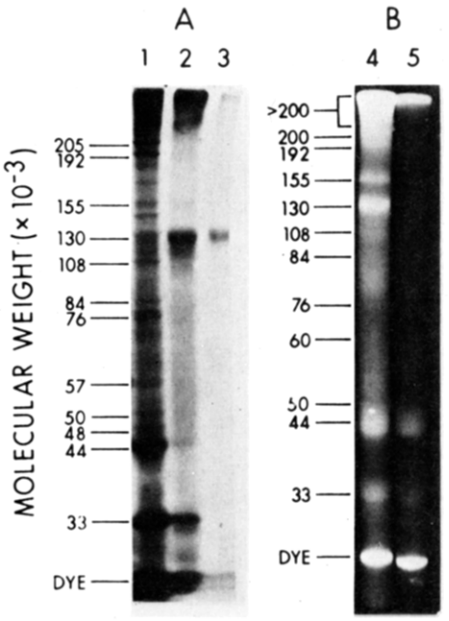

FIGURE 3: (A) SDS–PAGE of enriched Ehrlich cell plasma membrane solubilized with SDS, GS I bound plasma membrane glycoprotein mixture, and GP 130 stained for protein with Coomassie blue. (Gel 1) 100 μg of enriched Ehrlich cell plasma membrane protein; (gel 2) 50 μg of GS I bound glycoprotein mixture; (gel 3) 10 μg of GP 130. (B) SDS–PAGE of enriched Ehrlich cell plasma membrane solubilized with SDS and stained for carbohydrate with dansylhydrazine. Both gels 4 and 5 contained 200 μg of protein and were treated identically except the periodate oxidation step was omitted for gel 5.

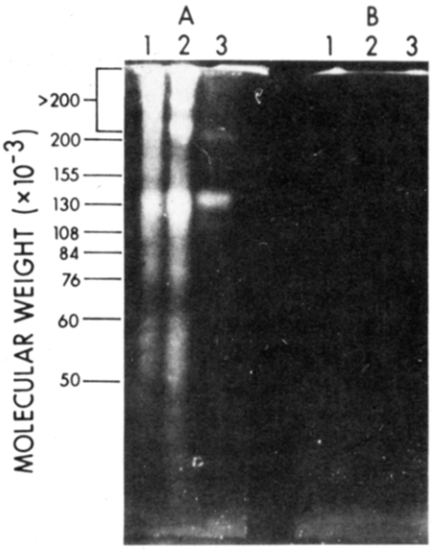

FIGURE 4: SDS–PAGE of enriched Ehrlich cell plasma membrane, GS I bound plasma membrane glycoprotein mixture, and GP 130 stained for α-D-galactosyl units with FITC–B_4. Both (A) and (B) were treated identically except (A) was stained in the presence of 10 mM Me-α-D-Glc*p* and (B) was stained in the presence of Me-α-D-Gal*p*. (Gel 1) 100 μg of enriched Ehrlich cell plasma membrane protein; (gel 2) 50 μg of GS I bound glycoprotein mixture; (gel 3) 10 μg of GP 130.

tochondria, lysosomes, and endoplasmic reticulum and moderately high levels of nuclei.

Polyacrylamide gel electrophoresis of the Ehrlich cell plasma membrane (SDS solubilized) in the presence of sodium dodecyl sulfate (SDS–PAGE) by the procedure of Laemmli (1970) revealed a large number of protein bands as seen in Figure 3A. In order to assay for glycoprotein components, the SDS-solubilized Ehrlich cell plasma membrane was subjected to SDS–PAGE and stained for carbohydrate with dansylhydrazine (Figure 3B). The glycoproteins reacting most strongly with dansyl hydrazine had molecular weights of 130 000 and 155 000. Glycoproteins with molecular weights of 192 000 and 200 000 also stained with dansylhydrazine as did the high molecular weight aggregate near the origin of the gel. Weak and diffuse dansylhydrazine staining also was observed for the 108 000 molecular weight glycoprotein and in the 76 000–84 000 and 50 000–60 000 molecular weight regions. The apparent dansylhydrazine reaction with the proteins of molecular weight 44 000 and 33 000 in gel 4 probably represents artifacts inasmuch as these components in control gel 5 (not treated with periodate) also exhibited intense fluorescence (Figure 3B). The results of Figure 3 indicated that a large number of the prominent proteins in the Ehrlich cell plasma membrane were also glycoproteins.

The presence of terminal nonreducing α-D-Gal*p* groups on the glycoproteins was detected by subjecting the Ehrlich cell plasma membrane fraction to SDS–PAGE followed by staining with FITC–B_4 (Figure 4). Two gels, each with the same amounts of plasma membrane protein, were treated identically except one gel was stained with FITC–B_4 containing 10 mM Me-α-D-Glc*p* whereas the second gel was stained with FITC–B_4 containing 10 mM Me-α-D-Gal*p*. The presence of haptenic 10 mM Me-α-D-Gal*p* during the staining served as a control for nonspecific binding of FITC–B_4. The 130 000 molecular weight glycoprotein was found to react strongly with FITC–B_4. Weak reactivity with FITC–B_4 was observed for the 155 000 and 108 000 molecular weight glycoproteins, for the high molecular weight aggregate, and for the 76 000–84 000 and 50 000–60 000 molecular weight regions. The gels incubated with FITC–B_4 in the presence of 10 mM Me-α-D-Gal*p* were not stained (Figure 4). These results suggested that the majority of the glycoproteins present in the Ehrlich cell plasma membrane probably contain terminal α-D-Gal*p* residues. The 130 000 molecular weight glycoprotein that stained most intensely with dansylhydrazine also reacted most strongly with FITC–B_4.

The α-D-galactosyl-containing glycoproteins in the Ehrlich cell plasma membrane are probably integral membrane components strongly associated with the lipid bilayer. Surfactants such as dimethyldecylphosphine oxide, Triton X-100, sodium deoxycholate, and sodium dodecyl sulfate solubilized respectively 40, 34, 50, and 75% of the plasma membrane protein and 56, 58, 62, and 82% of the plasma membrane hexose. Each of these extracts gave a strong precipitin reaction with GS I-B_4 in the hematocrit tube test, indicative of solubilized α-D-galactosyl-containing glycoproteins.

Agents that dissociate peripheral or extrinsic proteins and glycoproteins in weak association with the membrane lipid were ineffective in solubilizing plasma membrane hexose and α-D-galactosyl-containing glycoproteins. Two molar sodium chloride, 4 M urea, and 0.5 M EDTA, 2.5 mM β-mercaptoethanol, and 25 mM glycine-HCl, pH 9.0, solubilized respectively 7, 7, and 12% of the total hexose. The chaotropic salt potassium thiocyanate solubilized 20% of the plasma membrane protein and 18% of the hexose. This solubilized material gave a moderate reaction with GS I-G_4 in the hematocrit tube test. Suprisingly, SDS–PAGE revealed only minor differences in the protein profiles for the plasma membrane extracts solubilized with potassium thiocyanate, Triton X-100, $DC_{10}PO$, sodium deoxycholate, or SDS.

Extraction of the plasma membrane with cold acetone followed by 1% $DC_{10}PO$ produced an extract that reacted vigorously with GS I-G_4 in the hematocrit tube test. Examination of this extract by SDS–PAGE revealed a highly se-

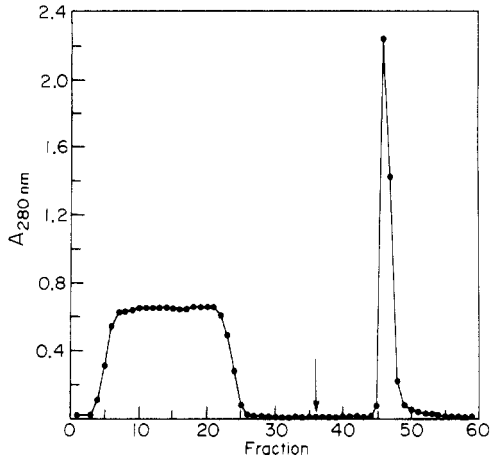

FIGURE 5: Elution profile of 1% $DC_{10}PO$-solubilized Ehrlich cell plasma membrane chromatographed over a GS I–Sepharose 4B column. Solubilized plasma membrane (16.4 mg of protein, 80.5 mL) was layered onto the column (1.5 × 15 cm) equilibrated with 1 mg/mL $DC_{10}PO$ in PBS-azide buffer at a flow rate of 10 mL/h. The column was washed free of unbound protein. Addition of 0.1 M Me-α-D-Gal*p* (indicated by the arrow) resulted in the displacement of α-D-galactosyl-containing glycoproteins. Volume of fractions 1–35 was 4.4 mL and 36–60 was 1.9 mL.

lective enrichment for the major glycoprotein of the Ehrlich cell plasma membrane, the 130 000 molecular weight α-D-galactosyl-containing glycoprotein designated GP 130. Although the yield of solubilized protein and hexose was rather poor, because of the selective enrichment in GP 130, the acetone–$DC_{10}PO$ extraction procedure was chosen for the initial step in the purification of GP 130.

Purification of GP 130. Incubation of Ehrlich cell plasma membrane (85 mg of protein, 7.5 mg of hexose) with 1% $DC_{10}PO$ in PBS buffer for 16 h, following cold acetone extraction, solubilized 17 mg (20%) of the plasma membrane protein and 1.2 mg (16.3%) of the plasma membrane hexose. A second extraction with 1% $DC_{10}PO$ in PBS buffer was unproductive, solubilizing only 0.7 mg of protein and no hexose. The solubilized material was subjected to affinity chromatography over a GS I–Sepharose 4B column. α-D-Galactosyl-containing glycoproteins were eluted from the column with 0.1 M Me-α-D-Gal*p* as a sharp peak (Figure 5). This GS I bound glycoprotein mixture contained 5.6 mg of protein, which represented 32.9% of the solubilized protein and 6.6% of the total plasma membrane protein. Over 65% of the solubilized hexose was eluted from the GS I–Sepharose 4B column with haptenic sugar.

SDS–PAGE of the GS I reactive glycoprotein mixture revealed an enrichment of GP 130 compared to the whole plasma membrane (Figure 3). The GS I bound glycoprotein mixture contained a diffuse, weakly staining zone of unresolved proteins ranging in molecular weight from 44 000 to 125 000, as well as a high molecular weight aggregate. Staining of the GS I bound glycoprotein mixture with FITC–B_4 after SDS–PAGE revealed a staining pattern similar to the pattern obtained for whole plasma membrane (Figure 4). The high molecular weight aggregate and GP 130 were the most intensely stained bands while diffuse FITC–B_4 staining was observed in the 50 000–60 000 and 76 000–165 000 molecular weight regions. These results indicated that the entire spectrum of α-D-galactosyl-containing glycoproteins present in the plasma membrane was partially solubilized by the acetone–1% $DC_{10}PO$ extraction procedure. Me-α-D-Gal*p* (10 mM) abolished completely staining of the GS I bound glycoprotein mixture with FITC–B_4.

Table III: Composition of GP 130

residue	residues per 1000 amino acid residues	residue	residues per 1000 amino acid residues
lysine	51.3	valine	69.4
histidine	14.7	methionine	3.2
arginine	39.7	isoleucine	46.0
aspartic acid	106.5	leucine	102.7
threonine	61.9	tyrosine	25.2
serine	82.2	phenylalanine	42.0
glutamic acid	127.0	tryptophan	nd
proline	47.3	fucose	24
glycine	104.0	*N*-acetylglucosamine	44
alanine	76.8	mannose	60
cysteine	0.0	galactose	149

[a] nd, not determined.

GP 130 was obtained in a highly enriched form by fractionating the GS I bound glycoprotein mixture by preparative SDS–PAGE. The yield of GP 130 from 85 mg of plasma membrane protein was 0.36 mg of protein. Analytical SDS–PAGE showed only one protein band for the GP 130 preparation when stained with Coomassie blue (Figure 3). However, staining of the preparation with FITC–B_4 revealed a heavily stained GP 130 band and, in addition, two glycoproteins with molecular weights of 200 000 and 108 000, which were not detected with Coomassie blue (Figure 4). Although absolutely pure GP 130 was not obtained, the highly enriched GP 130 preparation was subjected to further chemical analysis. The molecular weight of GP 130 was determined to be 130 000 by SDS–PAGE on 7.5% polyacrylamide gels. The presence of β-mercaptoethanol did not affect the electrophoretic mobility of GP 130.

The reactivity of GP 130 (0.14 mg/mL) with lectins (1.0 mg/mL) was found to parallel lectin agglutinability of Ehrlich cells. GP 130 formed a precipitate with GS I-B_4, *R. communis*, *T. vulgaris*, and *P. vulgaris* lectins, the same four lectins that strongly agglutinated Ehrlich cells (Table I). GP 130 did not form a precipitate with *G. simplicifolia* II or the lectins from *Canavalia ensiformis*, *L. tetragonolobus*, *H. pomatia*, *Phaseolus lunatus*, and *D. biflorus*; these six lectins either weakly agglutinated or failed to agglutinate Ehrlich cells. The amino acid composition of GP 130 is shown in Table III. Glutamic acid, aspartic acid, leucine, and glycine were present in highest concentration, accounting for 44% of the total amino acid composition. The sulfur-containing amino acids were present in very small amounts with only 3.2 residues of methionine present per 1000 amino acid residues. No cysteine residues were detected. Although the solubilization experiments indicated the α-D-galactosyl-containing glycoproteins were membrane bound, GP 130 did not contain a high proportion of hydrophobic residues. Except for its low content of methionine and cysteine, GP 130 has an amino acid composition very similar to that found for a large number of proteins (Dayhoff et al., 1978). This result could indicate that only a small hydrophobic portion of GP 130 is embedded in the plasma membrane. On a weight basis, GP 130 was found to contain 27% carbohydrate. Galactose was the major monosaccharide, 14% by weight, followed by mannose, *N*-acetylglucosamine, and fucose at 5.7, 5.2, and 2.1%, by weight, respectively. No sialic acid was detected.

Glycopeptides of GP 130. GP 130 (0.62 mg of protein, 140 μg of hexose) was digested with Pronase and chromatographed over a GS I–Sepharose 4B column and a Bio-Gel P-2 column to obtain an α-D-galactosyl-containing glycopeptide (72.2 μg of hexose recovered). The GP 130 glycopeptide formed a

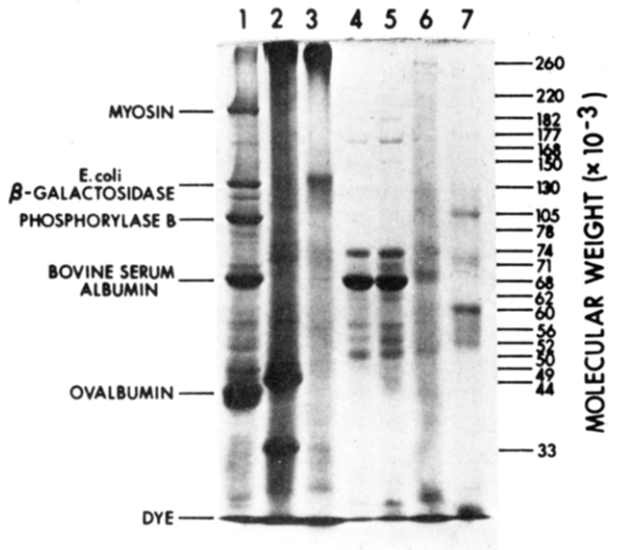

FIGURE 6: SDS–PAGE of unfractionated and GS I bound Ehrlich cell induced and Freund's complete adjuvant induced ascites fluids stained for protein with Coomassie blue. (Gel 1) Molecular weight standards; (gel 2) 100 μg of enriched Ehrlich cell plasma membrane protein; (gel 3) 50 μg of GS I bound glycoprotein mixture; (gel 4) 0.25 μL of Ehrlich cell induced ascites fluid; (gel 5) 0.25 μL of Freund's complete adjuvant induced ascites fluid; (gel 6) 20 μg of GS I bound Ehrlich cell induced ascites fluid protein; (gel 7) 20 μg of GS I bound Freund's complete adjuvant induced ascites fluid protein.

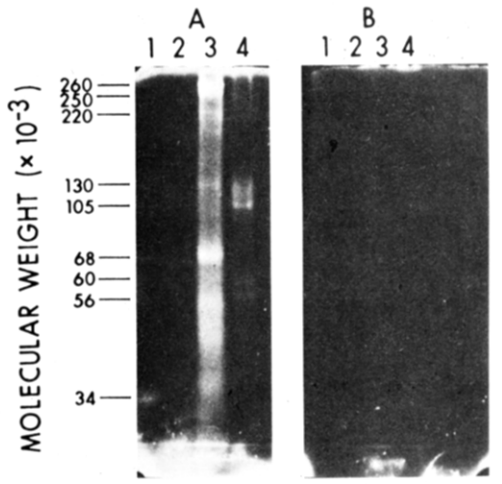

FIGURE 7: SDS–PAGE of unfractionated and GS I bound Ehrlich cell induced and Freund's complete adjuvant induced ascites fluids stained for α-D-galactosyl groups with FITC–B_4. Both (A) and (B) were treated identically except (A) was stained in the presence of 10 mM Me-α-D-Gal*p*. (Gel 1) 0.25 μL of Ehrlich cell induced ascites fluid; (gel 2) 0.25 μL of Freund's complete adjuvant induced ascites fluid; (gel 3) 20 μg of GS I bound Ehrlich cell induced ascites fluid protein; (gel 4) 20 μg of GS I bound Freund's complete adjuvant induced ascites fluid protein.

precipitate with GS I-B_4, *T. vulgaris*, and *R. communis* lectins. The glycopeptide did not form a precipitate with the lectins from *P. vulgaris*, *C. ensiformis*, *L. tetragonolobus*, *D. biflorus*, or *G. simplicifolia* II. The carbohydrate composition of the GP 130 α-D-galactosyl-containing glycopeptide normalized to three mannose residues was found to be galactose–*N*-acetylglucosamine–mannose–fucose in the molar ratio of 9.4:11.2:3:0.5. The carbohydrate portion of the GP 130 glycopeptide would have a calculated molecular weight of 4364. There was insufficient glycopeptide for further chemical characterization.

α-D-Galactosyl-Containing Glycoproteins in Ehrlich Cell Induced and Adjuvant-Induced Ascites Fluid. In order to determine whether α-D-galactosyl-containing glycoproteins were shed from the Ehrlich cell into the surrounding fluid, cell-free ascites fluid was subjected to SDS–PAGE followed by staining with Coomassie blue and FITC–B_4. The Freund's complete adjuvant induced ascites fluid served as a control for the presence of α-D-galactosyl-containing glycoproteins in Ehrlich cell free ascites fluid.

SDS–PAGE of whole Ehrlich cell ascites fluid and whole Freund's complete adjuvant induced ascites fluid indicated proteins with similar apparent molecular weights (Figure 6). Staining of both samples with FITC–B_4 after SDS–PAGE revealed the absence of α-D-galactosyl-containing glycoproteins (Figure 7).

Chromatography of Ehrlich cell induced ascites fluid (10 mL) and Freund's complete adjuvant induced ascites fluid (10 mL) on a GS I–Sepharose 4B column resulted in the elution of 0.6 and 0.94 mg of protein, respectively, with 0.1 M Me-α-D-Gal*p*. Examination of the two GS I bound ascites samples after SDS–PAGE by staining with Coomassie blue and FITC–B_4 revealed very dissimilar compositions. The GS I bound Ehrlich cell ascites sample contained protein throughout the gel with prominent bands having molecular weights of 260 000, 250 000, 220 000, 182 000, 168 000, 105 000, 78 000, 68 000, 56 000, 52 000, and 50 000 (Figure 6). FITC–B_4 also stained the GS I bound Ehrlich cell ascites sample throughout the entire gel, with prominently labeled glycoproteins having molecular weights of 260 000, 250 000, 220 000, and 68 000, as well as a 56 000–34 000 region that was heavily stained (Figure 7). Only a trace of fluorescence was detected at the position at which GP 130 would be found. Addition of 10 mM Me-α-D-Gal*p* totally abolished lectin binding.

The FITC–B_4 staining profile for GS I bound Ehrlich ascites fluid was very different from the FITC–B_4-stained profile for GS I bound Ehrlich cell plasma membrane glycoproteins in which GP 130 was the heaviest staining glycoprotein (Figure 4). Qualitatively, the GS I bound Ehrlich cell ascites fluid appeared to have a higher density of lower molecular weight (<68 000) FITC–B_4-staining glycoproteins compared to that of the GS I bound Ehrlich cell plasma membrane glycoproteins. The origin of the Ehrlich cell ascites fluid α-D-galactosyl-containing glycoproteins is not know but may include shedding or secretion from the Ehrlich cell plasma membrane, release from the cell surface by proteolytic activity, or synthesis by the host in response to the tumor.

The GS I bound FCA-induced ascites fluid when stained for protein after SDS–PAGE had prominent protein bands with molecular weights of 177 000, 105 000, 74 000, 60 000, 56 000, and 52 000. The FITC–B_4 staining of GS I bound FCA-induced ascites fluid was very limited. Two bands of molecular weight 56 000 and 60 000 were weakly stained as was the 250 000–220 000 region (Figure 7). Heavy FITC–B_4 staining was observed for two glycoproteins of molecular weight 310 000 and 105 000. Me-α-D-Gal*p* (10 mM) totally abolished lectin binding. Whether these α-D-galactosyl-containing glycoproteins are normal components of mouse plasma or are synthesized by the mouse in response to Freund's complete adjuvant remains to be tested. In any event, it is clear the Ehrlich tumor resulted in the appearance in the ascites fluid of low concentrations of α-D-galactosyl-containing glycoproteins, which appear to be different than the glycoproteins in ascites fluid induced with Freund's complete adjuvant. The Ehrlich ascites fluid glycoproteins cannot be attributed to fragments of Ehrlich cell plasma membrane. The FITC–B_4 reactive glycoproteins found in Ehrlich ascites fluid are very different than the glycoproteins found in the Ehrlich cell plasma membrane. However, shedding of a very spe-

cialized portion of the Ehrlich cell plasma membrane cannot be excluded.

Discussion

The data presented herein support the presence of glycoproteins containing nonreducing terminal α-D-Gal*p* residues on the Ehrlich ascites tumor cell plasma membrane. The distribution and quantitation of this carbohydrate residue on the cell surface was determined by using the α-D-galactopyranosyl-specific isolectin *G. simplicifolia* I-B_4 labeled with fluorescein isothiocyanate, ferritin, or [^{3}H]propionate. The minimum number of α-D-Gal*p* residues on the Ehrlich cell surface was found to be 18.1×10^6 per cell. For comparison, Nachbar et al. (1976) determined that the Ehrlich cell had 6.4×10^6 sites for concanavalin A, 10.0×10^6 sites for *R. communis* lectin, and 1.4×10^6 sites per cell for soybean agglutinin. The most prevalent carbohydrate moiety detected to date on the Ehrlich cell is the receptor for *P. vulgaris* [β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→2)-α-D-Man*p*] with 66×10^6 sites per cell (Steck & Wallach, 1965). Other mammalian cells for which the number of α-D-Gal*p* residues on the cell surface has been determined by GS I binding studies include human type B erythrocytes (Hayes & Goldstein, 1975) and Swiss 3T3 mouse fibroblast (Stanley et al., 1979). Type B erythrocytes were shown to have a minimum of $(0.72–1.34) \times 10^5$ GS I sites per cell and Swiss 3T3 cells a minimum of 3.9×10^5 sites per cell. These values are 46–250-fold lower than the number of GS I sites observed for the Ehrlich cell.

Antisera specific for blood group B or blood group P_1 substance, both of which terminate in α-D-Gal*p* residues, did not agglutinate Ehrlich cells. Although a terminal α-D-Gal*p* residue is a necessary part of the determinant for these two antigens, this sugar residue alone is insufficient to bring about reaction with the antisera. Antibody recognition of these two antigens is also dependent on both the nature and linkage of the sugar residue penultimate to the terminal α-D-Gal*p* group (Schiffman & Marcus, 1964). These observations suggest structural differences between the nonreducing terminal portion of blood group B and P_1 substances and the Ehrlich cell-surface α-D-Gal*p*-containing glycoproteins.

A major proportion of Ehrlich cell plasma membrane glycoproteins appear to contain terminal α-D-galactosyl residues, and these glycoproteins are probably integral membrane components inasmuch as they require detergent for solubilization. It is not known if other subcellular organelles of the Ehrlich cell have α-D-galactosyl-containing glycoproteins as membrane components.

Pronase digest of GP 130 generated a large glycopeptide with $M_r \sim 4300$, calculated from the carbohydrate composition. This glycopeptide had a high galactose and *N*-acetylglucosamine content and formed a precipitate with GS I-B_4 and wheat germ agglutinin. This lectin reactivity indicated the glycopeptide possesses multiple terminal α-D-Gal*p* residues and either multiple terminal *N*-acetyl-β-D-glucosaminyl residues or more likely internal *N*-acetyl-β-D-glucosaminyl residues linked at the O-4 position. The glycopeptide derived from GP 130 can be compared to the high molecular weight megaloglycopeptides with keratan-like oligosaccharide chains recently isolated from human erythrocyte membranes (Krusius et al., 1978; Finne et al., 1978; Jarnefelt et al., 1978) and Chinese hamster ovary cells grown in suspension culture (Li et al., 1980). These asparagine-linked glycopeptides had molecular weights ranging from 4000 to 13 000 and a high content of galactose and *N*-acetylglucosamine residues. Fukuda et al. (1979) has shown the megaloglycopeptide isolated from the erythrocyte is located on band 3, the major intrinsic membrane glycoprotein. Structural analysis of the glycopeptides isolated from the Ehrlich cell plasma membrane will be reported in the following paper.

Two murine leukemic cell lines, P-388 and L-1210 (Chen et al., 1978), and the murine sarcoma 180 ascites tumor cell line (Shin & Carraway, 1973) all contain a plasma membrane glycoprotein that may be related to GP 130 of the Ehrlich cell. The molecular weights for the major glycoproteins of the leukemic cell lines and the sarcoma cell line are extremely close to the molecular weight of GP 130. It was not determined if any of the three cell lines contained α-D-galactosyl residues, and no attempt was made to isolate the glycoproteins. These experiments raise the possibility that GP 130 may be present on the surface of a number of murine cells.

Examination of the peritoneal ascites fluid induced in the mouse by Ehrlich cells revealed the presence of a number of α-D-galactosyl-containing glycoproteins at a total protein concentration of 6 μg/mL. The number and molecular weight of the Ehrlich cell induced α-galactosyl-containing glycoproteins differed considerably when compared to the α-D-galactosyl-containing glycoproteins induced by Freund's complete adjuvant.

The function of the Ehrlich cell induced α-D-galactosyl-containing glycoproteins is at the present time unknown, although one possibility may be that the glycoproteins released by the tumor cell interfere with the humoral and cell-mediated immune response of the host against the tumor. For example, Cooper et al. (1974) demonstrated the shedding of a glycoprotein from the TA3-Ha cell surface into the ascites fluid at concentrations up to 0.5 mg/mL. This cell was able to grow in H-2-incompatible mice. The TA3-St subline, which can grow only in H-2-compatible mice, did not shed any of this cell-surface glycoprotein into the ascites fluid. The authors postulated the released glycoproteins blocked an immune response toward TA3-Ha cells in H-2-incompatible hosts that otherwise would have eliminated the tumor.

The spontaneous release of the cell coat (glycocalyx) by Ehrlich cells in vitro was demonstrated by Rittenhouse et al. (1978). Released glycocalyx, when subjected to SDS–PAGE followed by staining with ^{125}I-labeled concanavalin A and autoradiography, gave one heavily concanavalin A reacting glycoprotein of molecular weight 130 000 and many lightly reacting glycoproteins. A comparison of an enriched plasma membrane fraction with glycocalyx revealed no glycoproteins unique to one or the other. It is not known if the 130 000-dalton glycoprotein described by Rittenhouse is the same as GP 130. Although GP 130 did not form a precipitate with Con A, it is possible Con A could still bind to GP 130.

Although the identity of the α-D-Gal*p*-containing glycoprotein(s) is unknown at this time, we suggest that it may be related to the basement-membrane glycoprotein laminin (Timpl et al., 1979). This is based on our recent discovery that laminin contains α-D-Gal*p* end groups (Shibata et al., 1982) and the observation that rabbit antibodies raised against laminin interact strongly with Ehrlich ascites tumor cells, as well as with thioglycollate-stimulated murine peritoneal macrophages (Huard et al., 1983).

Registry No. α-D-Galactopyranose, 3646-73-9.

References

Agrawal, B. B. L., & Goldstein, I. J. (1967) *Biochim. Biophys. Acta 147*, 262–271.

Basu, S., Moskal, J. R., & Gardner, D. A. (1976) in *Ganglioside Function: Biochemical and Pharmacological Implications* (Porcellati, G., Ceccarelli, B., & Tettamanti, G.,

Eds.) pp 45–63, Plenum Press, New York.
Blumberg, M., Hildesheim, J., Yariv, J., & Wilson, K. (1972) *Biochim. Biophys. Acta 264*, 171–176.
Burton, K. (1956) *Biochem. J. 62*, 315–323.
Chen, K. Y., Kramer, R. H., & Canellakis, E. S. (1978) *Biochim. Biophys. Acta 507*, 107–118.
Columbini, M., & Johnstone, R. M. (1973) *Biochim. Biophys. Acta 323*, 69–86.
Copper, A. G., Codington, J. F., & Brown, M. D. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1224–1228.
Dayhoff, M. O., Hunt, L. T., & Hurst-Calderone, S. (1978) in *Atlas of Protein Sequence and Structure*, Vol. 5, p 363, National Biomedical Research Council Foundation, Silverspring, MD.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28*, 350–356.
Eckhardt, A. E., Hayes, C. E., & Goldstein, I. J. (1976) *Anal. Biochem 73*, 192–197.
Eto, T., Ichikawa, Y., Nishimura, K., Ando, S., & Yamakawa, T. (1968) *J. Biochem. (Tokyo) 64*, 205–213.
Finne, J. (1975) *Biochim. Biophys. Acta 412*, 317–325.
Finne, J., & Krusius, T. (1976) *FEBS Lett. 66*, 94–97.
Finne, J., Krusius, T., & Rauvala, H. (1978) *FEBS Lett. 89*, 111–115.
Friberg, S. (1972) *J. Natl. Cancer Inst. (U.S.) 48*, 1477–1489.
Fukuda, M., Fukuda, M. N., & Hakomori, S. (1979) *J. Biol. Chem. 254*, 3700–3703.
Hakomori, S., & Strycharz, G. D. (1968) *Biochemistry 7*, 1279–1286.
Hakomori, S., Siddiqui, B., Li, Y. T., Li, S. C., & Hellqvist, C. G. (1971) *J. Biol. Chem. 246*, 2271–2277.
Hammarstrom, S. (1973) *Scand. J. Immunol. 2*, 53–66.
Hatcher, V. B., Schwarzmann, G. O. H., Jeanloz, R. W., & McArthur, J. W. (1977) *Biochemistry 16*, 1518–1524.
Hayes, C. E., & Goldstein, I. J. (1974) *J. Biol. Chem. 246*, 1904–1914.
Hayes, C. E., & Goldstein, I. J. (1975) *J. Biol. Chem. 250*, 6837–6840.
Hays, H. R. (1968) *J. Org. Chem. 33*, 3690–3694.
Hornick, C. L., & Karush, F. (1972) *Immunochemistry 9*, 325–340.
Huard, T. K., Goldstein, I. J., Malinoff, H., & Wicha, M. S. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 1077, Abstr.
Im, W. B., Christensen, H. N., & Sportes, B. (1976) *Biochim. Biophys. Acta 436*, 424–437.
Jarnefelt, J., Rush, J., Li, Y. T., & Laine, R. A. (1978) *J. Biol. Chem. 253*, 8006–8009.
Kabat, E. A. (1956) *Blood Group Substances*, Academic Press, New York.
Kilberg, M. S., & Christensen, N. H. (1979) *Biochemistry 18*, 1525–1530.
Koziarz, J. J., Kohler, H., & Steck, T. L. (1978) *Anal. Biochem. 86*, 78–89.
Krusius, T., Finne, J., & Rauvala, H. (1978) *Eur. J. Biochem. 92*, 289–300.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Li, E., Gibson, R., & Kornfeld, S. (1980) *Arch. Biochem. Biophys. 199*, 393–399.
Li, Y. T., Li, S. C., & Dawson, G. (1972) *Biochim. Biophys. Acta 260*, 88–92.
Lonngren, J., Goldstein, I. J., & Bywater, R. (1976) *FEBS Lett. 68*, 31–34.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Maddox, D. E., Shibata, S., & Goldstein, I. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 166–170.
Morre, D. J. (1971) *Methods Enzymol. 22*, 130–148.
Muir, L., & Lee, Y. G. (1969) *J. Biol. Chem. 244*, 2343–2349.
Murphy, L. A., & Goldstein, I. J. (1977) *J. Biol. Chem. 252*, 4739–4742.
Nachbar, M. S., Oppenheim, J. D., & Aull, F. (1976) *Biochim. Biophys. Acta 419*, 512–529.
Naiki, M., & Marcus, D. M. (1974) *Biochem. Biophys. Res. Commun. 60*, 1105–1111.
Newman, W., & Kabat, E. A. (1976) *Arch. Biochem. Biophys. 172*, 535–550.
Nicolson, G. L., & Singer, S. J. (1974) *J. Cell Biol. 60*, 236–248.
Okada, Y., & Spiro, R. G. (1980) *J. Biol. Chem. 255*, 8865–8872.
Peters, B. P., & Goldstein, I. J. (1979) *Exp. Cell Res. 120*, 321–334.
Porter, W. H. (1975) *Anal. Biochem. 63*, 27–43.
Rittenhouse, H. G., Rittenhouse, J. W., & Takemoto, L. (1978) *Biochemistry 17*, 829–837.
Schiffman, G., & Marcus, D. M. (1964) *Prog. Hematol. 4*, 97–116.
Schneider, W. C. (1963) *J. Biol. Chem. 238*, 3572–3578.
Schneider, W. C., & Behki, R. M. (1963) *J. Biol. Chem. 238*, 3565–3571.
Shankar-Iyer, P. N. S., Wilkinson, K. D., & Goldstein, I. J. (1976) *Arch. Biochem. Biophys. 177*, 330–333.
Shibata, S., Peters, B., Roberts, D., Goldstein, I. J., & Liotta, L. A. (1982) *FEBS Lett. 142*, 194–198.
Shin, B. C., & Carraway, K. L. (1973) *Biochim. Biophys. Acta 330*, 254–268.
Slomiany, B., & Slomiany, A. (1978) *Eur. J. Biochem. 83*, 105–111.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Stanley, W. S., Peters, B. P., Blake, D. A., Yep, D., Chu, E. H. Y., & Goldstein, I. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 303–307.
Steck, T. L., & Wallach, D. F. H. (1965) *Biochim. Biophys. Acta 97*, 510–522.
Timpl, R., Rohde, H., Robey, P., Rennard, S., Fordart, J.-M., & Martin, G. R. (1979) *J. Biol. Chem. 254*, 9933–9937.
Warren, L. (1959) *J. Biol. Chem. 234*, 1971–1975.
Watkins, W. M. (1966) *Science (Washington, D.C.) 152*, 172–181.