A NOVEL EXTRACELLULAR MEMBRANE ELABORATED BY A MOUSE EMBRYONAL CARCINOMA-DERIVED CELL LINE

Albert E. Chung*, Ian L. Freeman+, and Janina E. Braginski*

*Department of Life Sciences, Faculty of Arts and Sciences, and +Department of Ophthalmology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261

Received October 25, 1977

An extracellular membranous structure is synthesized by an embryonal carcinoma-derived cell line, M1536-B3, in suspension cultures. Analysis of the solubilized membranous structure on polyacrylamide gels in sodium dodecyl sulfate yielded two major classes of glycoproteins with molecular weights of approximately 230,000 and 320,000 respectively. The amino acid composition of the purified membranous structures revealed the absence of both hydroxyproline and hydroxylysine. Carbohydrate analysis demonstrated the presence of fucose, xylose, mannose, galactose, glucose, N-acetylglucosamine, N-acetylgalactosamine, and N-acetylneuraminic acid. These carbohydrates represented approximately 9% of the weight of the membrane. A comparison of the electrophoretic patterns of cells grown on monolayers and in suspension revealed a marked accumulation of the glycoproteins under the latter growth conditions. D-[^{3}H]-glucosamine was incorporated into these two and a third major glycoprotein by cells in suspension culture.

Glycoproteins play important roles in cell recognition (1,2), transformation (1,2,3,4), cell adhesion (5), membrane structure (6), and other vital cell functions. Recently, cell lines have been derived from mouse embryonal carcinoma cells (7,8) which synthesize, in cell culture, an extracellular carbohydrate containing membranous structure. These cell lines provide a potentially useful system for the study of glycoprotein synthesis, secretion, and assembly of supramolecular structures. The usefulness of these cell lines depends, in part, on the isolation and characterization of these membranes. This communication describes the isolation and preliminary characterization of an extracellular membrane from the cell line M1536-B3, which was derived from mouse embryonal carcinoma.

MATERIALS AND METHODS

Cells and Cell Culture: The cell line used in these studies were derived from the mouse embryonal carcinoma cell line, PCC4-F, as described by Chung et

*This research was supported by Grants GM-19948 from the NIH and Grant BMS74-05262 from the National Science Foundation.

ISSN 0006-291X

al. (8). Cells were grown on Dulbecco's modified Eagle's medium (9) containing 15% fetal calf serum (Grand Island Biological Company, Grand Island, NY) in 100 mm Falcon plastic dishes. The cells were incubated in humidified 10% CO_2-air at 37°. Cells were routinely transferred after detachment by trypsinization on a 2- to 3-day schedule. For suspension cultures, cells were seeded in 10 ml of medium at a cell density of 1×10^5 cells/ml in 100 mm bacterial culture dishes. Hollow spherical aggregates developed within 3 days and increased in size up to approximately 0.5 mm in 10 days. These aggregates were used for membrane isolation.

Polyacrylamide Gel Electrophoresis: Gel electrophoresis was carried out in a slab gel electrophoresis apparatus (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). The methods of Laemmli (10) were employed. The running gel consisted of 7.5% acrylamide cross-linked with diallytartardiamide. The stacking gel contained 5% acrylamide. Gels, 1.5 x 100 mm, were run at a constant current of 20 mA/slab. Samples were solubilized by heating for 5 min at 100° in a sample buffer containing 10% v/v, glycerol; 2% w/v, sodium dodecyl sulfate (SDS); and 5% v/v, 2-mercaptoethanol. Protein was detected with Coomassie Blue R250 (11). Gels analyzed for radioactivity were sliced longitudinally and then cut manually into 2 mm transverse sections, these were solubilized by incubating overnight at room temperature in 0.5 ml 2% w/v periodic acid. The samples were then mixed with 8 ml Biofluor (New England Nuclear, Boston, MA) and counted in a Packard Liquid Scintillation Spectrometer.

Protein Determination: Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as standard. Cells were digested at room temperature in 0.1 NaOH overnight prior to protein determination. Samples in sample buffer were precipitated twice with 9 volumes of acetone before digestion and protein determination. This treatment removed both SDS and mercaptoethanol.

Amino Acid Analysis: Samples were hydrolyzed as described by Liu and Chang (13). The hydrolyzate was analyzed by the accelerated single column method on a Beckman 121 M amino acid analyzer as described in the application manual. The Beckman standard amino acid mixture with the addition of 3-hydroxyproline, taurine (Calbiochem, Los Angeles, CA), and hydroxylysine (Sigma, St. Louis, MO) was employed to identify and determine the amino acid content of the hydrolyzate. No corrections were applied for hydrolytic loss.

Carbohydrate Analysis: The procedures of Clamp et al. (14) were used. Methanolysis, re-N-acetylation and trimethylsilylation were carried out as follows: Samples (750 µg) of membrane were dissolved in 0.5 ml of methanolic 1M-HCl in an ampoule. The ampoule was sealed under nitrogen and methanolyzed for 24 hr at 85°. The acid solution was neutralized with solid silver carbonate. Re-N-acetylation was carried out by the addition of 0.1 ml of acetic anhydride. This mixture was kept at room temperature for 24 hr. The precipitate was triturated thoroughly and the supernatant solution collected by centrifugation at 2,000 x g for 5 min. The residue was washed twice with 0.5 ml of dry methanol. The pooled supernatant solutions were evaporated under reduced pressure at 35°. The residue was dried for 12 hr in a vacuum desiccator over P_2O_5. The sample was then trimethylsilylated with 0.1 ml silylating agent containing pyridine:hexamethyldisilazane:trichlorosilane, 5:1:1, v/v/v, for 30 min at room temperature. The samples were finally analyzed on a Packard Becker model 412 gas chromatograph under the conditions described by Kamerling et al. (15).

Isolation of Extracellular Membranes: Cell aggregates grown in bacterial culture dishes for 10-12 days were collected by centrifugation in a Sorvall GLC-1 centrifuge at 400 rpm for 2 min. The aggregates from 4-5 dishes were pooled and washed twice by centrifugation as above after resuspension in 5 ml

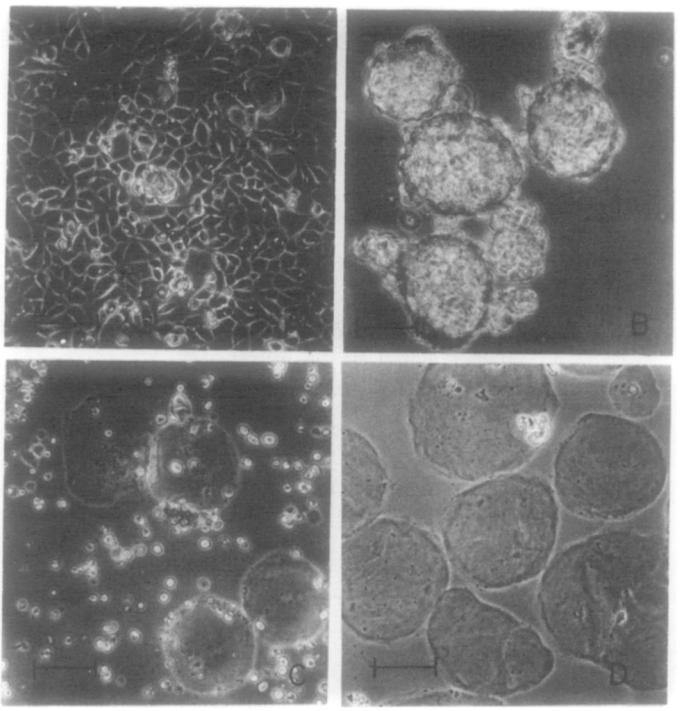

Figure 1. Phase-contrast photomicrographs of M1536-B3 cells and extracellular membranes. The bar is equivalent to 100 μm.

of phosphate buffered saline. The washed aggregates were suspended in 5 ml of phosphate buffered saline to which was added cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) at a concentration of 20 μg/ml. The suspension in a plastic tube was incubated for at least 6 hr at 37°. At the end of the incubation, the suspension was agitated vigorously on a Vortex mixer for 30 sec. The cells were dislodged from the membranous sacs by this procedure. The suspension of cells and membranes was centrifuged at 400 rpm for 1 min. The majority of the detached cells and cell debris remained suspended in the supernatant solution and the membranes formed a loose pellet. The supernatant fluid was gently removed and the loose pellet resuspended in 5 ml buffer. The centrifugation-resuspension cycle was repeated until an almost pure preparation of membrane was obtained after 10 such cycles. The progress of the purification was monitored by phase contrast microscopy. An alternate purification scheme yielded almost identical results. This scheme utilized the adsorption of the membranes to concanavalin A covalently attached to Sepharose 4B beads (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). The detached cells did not readily adsorb to the gel particles. The beads, with the membranous bags attached, were separated from the detached cells by differential centrifugation. The attached membranes were readily solubilized by heating for 5 min at 100° in sample buffer containing 2% w/v sodium dodecyl sulfate and 5% v/v 2-mercaptoethanol. Alternatively, partial release of the membranes could be achieved by treatment of the loaded beads with 0.2M α-methylmannoside in phosphate buffered saline.

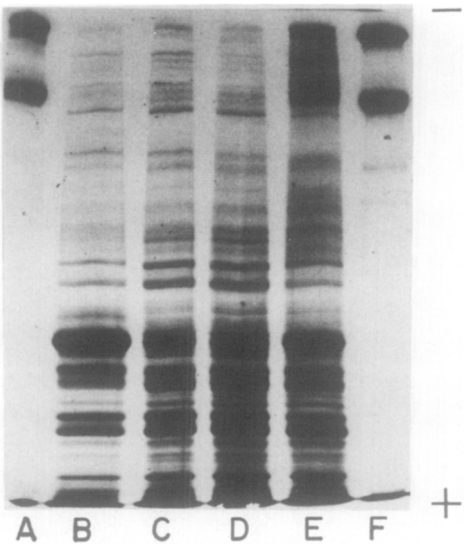

Figure 2. SDS gel electrophoretic patterns of purified membranes and solubilized M1536-B3 cells grown on monolayers and in suspension. See text for further description.

RESULTS

In Figures 1A and 1B the typical appearance of M1536-B3 cells grown on plastic tissue culture dishes and in bacterial culture dishes are compared. On the monolayers, the cells have a polygonal shape with small foci where the cells form rosettes. These rosettes are held together by a glycoprotein matrix (8). In suspension the cells initially form small aggregates, and as the culture ages, these aggregates enlarge and assume a hollow spherical shape with a layer on the exterior one or two cells thick. The cells are detached from the exterior of the hollow sphere by cytochalasin B treatment as shown in Figure 1C. The purified membranous structure is shown in Figure 1D. These structures, when examined closely, have a disc-like appearance. The membranes adhere avidly to plastic and glass surfaces. Their glycoprotein nature is readily confirmed by the strong positive reaction elicited by the periodic acid-Schiff test (16).

In Figure 2, a comparison is made of the electrophoretic patterns of the proteins found in cells grown in monolayers for 1, 2, and 3 days, channels B,

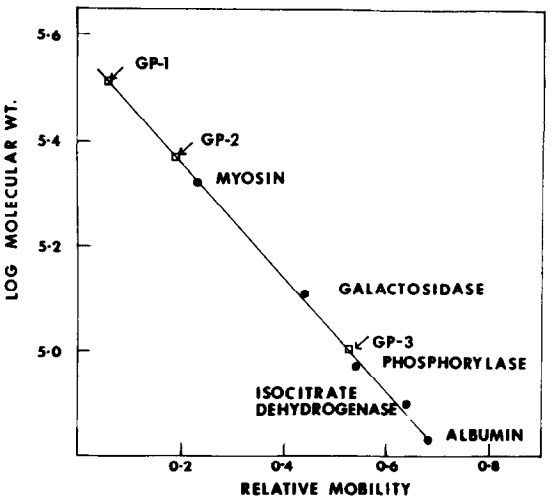

Figure 3. Molecular weight determination of glycoproteins by SDS gel electrophoresis. Standards and their molecular weights were: bovine serum albumin, 68,000; Azotobacter isocitrate dehydrogenase, 80,000; phosphorylase, 93,000; β-galactosidase, 130,000; myosin, 210,000.

C, and D; spheres grown in suspension for 10 days, channel E; membrane sacs purified by repeated centrifugation, channel A; and membrane sacs purified by concanavalin A-Sepharose, channel F. The results indicate that there is a marked increase in two size classes of proteins in the spherical aggregates and that these are present as the almost exclusive components of the membrane preparations. The two major proteins gave positive reactions with the periodic acid-Schiff reaction, thus indicating their glycoprotein nature. The apparent molecular weights of these two major glycoproteins were determined by a comparison of their electrophoretic mobility with known standards in SDS gels. The results shown in Figure 3 indicate that the apparent molecular weights of these are approximately 320,000 for the larger glycoprotein, GP-1, and approximately 230,000 for the second glycoprotein, GP-2. It is not assumed that these glycoproteins are single protein species since there are no substantiating criteria.

The amino acid composition of the membrane was determined and the results are shown in Table 1. The amino acid composition reveals a high content of the

TABLE 1

Amino Acid Composition of Isolated Extracellular Membrane

Amino Acid	Residues/1000 Residues
	Extracellular Membrane (GP-1 + GP-2)
Hydroxyproline	0
Aspartic Acid	109
Threonine	52
Serine	62
Glutamic Acid	119
Proline	50
Glycine	80
Alanine	72
Half-cystine	31
Valine	56
Methionine	24
Isoleucine	53
Leucine	106
Tyrosine	27
Phenylalanine	27
Histidine	27
Hydroxylysine	0
Lysine	51
Tryptophan	--
Arginine	53

TABLE II

Carbohydrate Composition of Extracellular Membrane

Carbohydrate	g carbohydrate/100 g protein
Fucose	0.97
Xylose	0.25
Mannose	3.24
Galactose	2.24
Glucose	0.30
N-Acetylgalactosamine	0.62
N-Acetylglucosamine	1.05
N-Acetylneuraminic acid	0.48

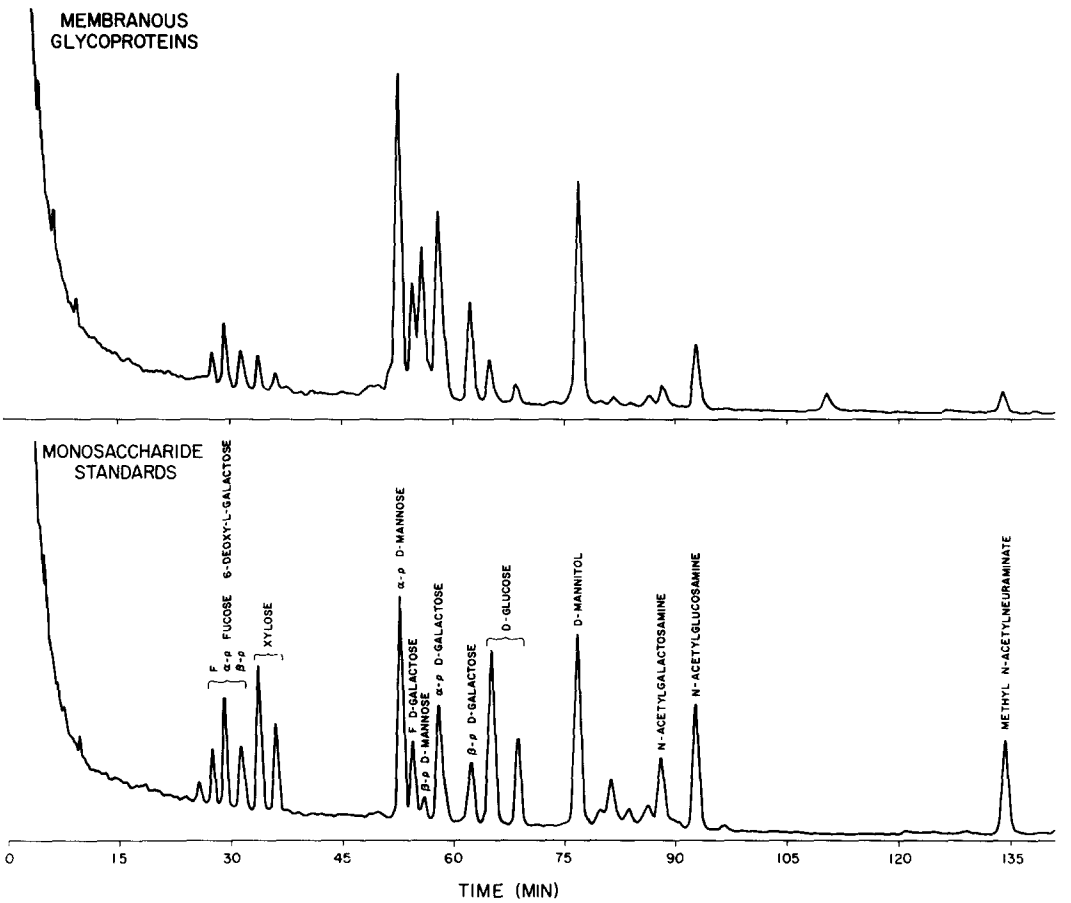

Figure 4. Gas-liquid chromatograms of monosaccharide standards, bottom panel, and monosaccharides derived from the purified membranes, upper panel. The methods were as described in the text. The reference compound, D-mannitol, was included in the membrane sample.

acidic amino acids aspartic and glutamic acids, a high content of half cysteine residues, and the absence of the hydroxylated amino acids, hydroxyproline and hydroxylysine. The absence of the latter two amino acids indicate that the isolated membranes are not related to collagen. The carbohydrate composition of the purified membranes was determined. The gas-liquid chromatographic tracing of the constituent monosaccharides are compared with authentic standards in Figure 4. The relative quantities of the monosaccharides are presented in Table II. The composition indicates that the membranes consist of approximately 9%

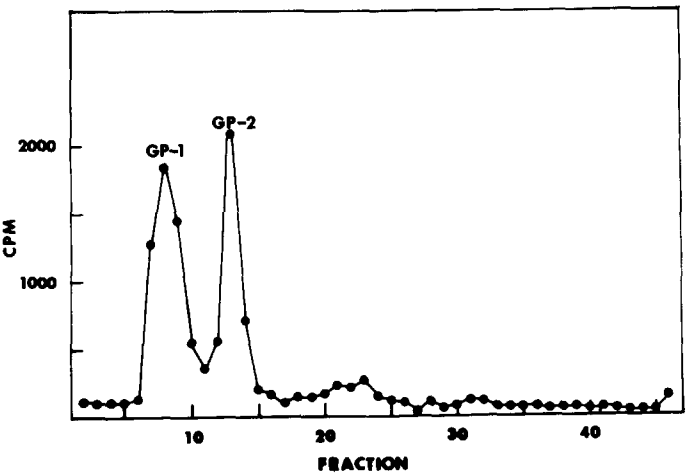

Figure 5. Sodium dodecyl sulfate gel electrophoretic pattern of D-[6-^{3}H]-glucosamine-labeled purified extracellular membrane. Solubilized membrane containing 14,000 cpm was applied to the gel, total recovery of counts from the gel was approximately 80%. The relative mobilities of the radioactive peaks were identical to those of GP-1 and GP-2 obtained by staining with Coomassie Blue.

carbohydrate with the most abundant species being mannose and galactose, with small quantities of xylose and glucose.

Experiments were carried out to determine if the spherical aggregates were capable of incorporating D-[^{3}H]-glucosamine into the glycoproteins found in the membranes. Suspension cultures were, therefore, exposed to 10 μcuries of D-[6-^{3}H]-glucosamine (specific activity 10.3 Ci/mmole)/100 mm dish on day 4 for 4 days. The membranes were purified from the cell aggregates and analyzed by electrophoresis on SDS gels. As shown in Figure 5, the label was confined to two major peaks which corresponded to GP-1 and GP-2. Upon examination of intact cell aggregates labeled for 24 hr with D-[6-^{3}H]-glucosamine in cultures ranging in ages from 1 to 11 days, it was found that the label was incorporated into GP-1 and GP-2 throughout the experiment. Furthermore, a third major glycoprotein peak, GP-3, with an apparent molecular weight of 100,000 was found in these samples. The relative magnitude of these peaks varied with the age of the culture, however, no conclusions could be drawn on the existence of a

precursor-product relationship. It might be noted that the cell number in the suspension cultures reached a steady state level of 4 x 10^6 cells/dish after 3 to 4 days. However, the protein content of the cultures increased throughout the experiment from a value of 0.3 mg protein/dish on the first day to 3.8 mg protein/dish on the eleventh day. This suggests that a significant portion of the protein synthesized beyond day 4 is incorporated into the membrane structure.

DISCUSSION

The arrangement of the cells in the cell aggregates suggests that the membranous structure is elaborated to enhance the survival of the cells by providing an anchor for attachment, and to facilitate exchange of nutrients with the environment. This membrane, in contrast to other extracellular membranes, such as basement membranes, is not collagenous in nature. This is suggested by the absence of hydroxyproline and hydroxylysine, and the resistance to digestion by protease-free collagenase. Low, but significant, quantites of xylose in the membrane indicates the presence of an unusual glycoprotein. Xylose is normally found in the carbohydrate-peptide linkage of glycosaminoglycans. The absence of significant quantities of hexuronic acids, which have retention times shorter than mannitol, and the relatively low content of carbohydrate, 9% by weight, in the membrane, however, tend to rule out the presence of a typical glycosaminoglycan. The membrane consists of only two major glycoproteins, thus, the isolation and further characterization of each component should be feasible.

ACKNOWLEDGEMENTS

We wish to thank Dr. Elijah Adams, Department of Biological Chemistry, University of Maryland School of Medicine for his generous gift of 3-hydroxyproline, Dr. Maurice Vaughan and Mr. John Malloy, Department of Life Sciences, University of Pittsburgh for samples of β-galactosidase and chick muscle myosin respectively, and Mr. J.P. Vergnes for the amino acid and carbohydrate analyses.

REFERENCES

1. Burger, M.M. (1973) Fed. Proc. 32, 91-101.
2. Sharon, N., and Lis, H. (1972) Science 177, 949-959.

3. Buck, C.A., Glick, M.C., and Warren, L. (1970) Biochemistry 9, 4567-4576.
4. Hynes, R.O. (1976) Biochim. Biophys. Acta 458, 73-107.
5. Roseman, S. (1970) Chem. Phys. Lipids 5,270-297.
6. Marchesi, V.T. (1975) Cell Membranes, Biochemistry, Cell Biology and Pathology, pp. 45-53, H.P. Publishing Co., New York.
7. Lehman, J.M.,Speers, W.C., Swartzendruber, D.E., and Pierce, G.B. (1974) J. Cell Physiol. 84, 13-28.
8. Chung, A.E., Estes, L.E., Shinozuka, H., Braginski, J., Lorz, C., and Chung, C.A. (1977) Cancer Res. 37, 2072-2081
9. Dulbecco, R. and Freeman G. (1959) Virology 8, 396-397
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. Fairbanks, G., Steck, T.L., and Wallach, D.F.H., (1971) Biochemistry 10, 2607-2717.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
13. Liu, T. -Y., and Chang, Y.H. (1971) J. Biol. Chem. 246,2842-2848.
14. Clamp, J. R., Bhatti, T., and Chambers, R.E. (1971) Methods in Biochemical Analysis, pp. 229-334, John Wiley & Sons, New York.
15. Kamerling, J.P., Gerwig, G.J., Vliegenthart, J.F.G., and Clamp, J.R. (1975) Biochem. J. 151, 491-495.
16. Humason, G.L. (1972) Animal Tissue Techniques, pp. 325-330, W.H. Freeman Co., San Francisco.