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# EPIGLYCANIN AS A MEMBRANE GLYCOPROTEIN. ISOLATION OF PLASMA MEMBRANE FROM THE TA3-HA TUMOR CELL*,†

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ABSTRACT

A plasma membrane fraction (M) was isolated from ascites cells of mouse TA3-Ha mammary carcinoma by the procedure of Brunette and Till [*J. Membr. Biol.*, 5 (1971) 215], involving homogenization, slow-speed centrifugation, and finally, differential centrifugation in a two-phase system. Marker enzyme activities indicated only minimal contamination of M by the endoplasmic reticulum, a result confirmed by transmission electron microscopy. To monitor the presence of epiglycanin (a large cell-surface glycoprotein), each fraction was tested in a radioimmunoassay for epiglycanin content, by gas chromatography for carbohydrate and amino acid compositions, and by scintillation spectrometry for radioactivity. Cells had been treated with galactose oxidase, followed by reduction with sodium borotritide, prior to homogenization. Of the total recovered epiglycanin, 15% was present in M, but, as indicated by g.l.c., M also contained other glycoproteins in high concentration. A direct correlation was found between epiglycanin concentration, GalNAc content, and radioactivity. Electron microscopy of fraction M by shadow casting showed multiple filaments emanating from some of the particles. The dimensions of these filaments corresponded to those of isolated epiglycanin molecules.

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## INTRODUCTION

The presence at the cell surface of epiglycanin, the major glycoprotein of the allotransplantable TA3-Ha mammary carcinoma ascites cell of the strain A mouse, has been demonstrated by its capacity, in intact cells, to bind both antibodies[1] and lectins[2,3]; by its removal from viable cells by proteases, such as TPCK-trypsin[4,5] or papain[6]; and by its visualization at the cell surface with transmission electron microscopy[7,8]. Nevertheless, little has been reported concerning the characteristics of its binding to the plasma membrane of the cell or of methods to dislodge it from isolated cell-membranes. Miller *et al.*[9], however, were able to obtain a membrane fraction from glucosamine-labeled TA3-Ha cells. After treatment with sodium dodecylsufate, a labeled component from the membrane was eluted from a column of Sepharose 4B in the effluent volume expected for epiglycanin. In this manuscript, we describe the isolation of membrane fragments from TA3-Ha cells by the method of Brunette and Till[10]. The concentration of epiglycanin in isolated membranes was determined, and the binding of epiglycanin to isolated membrane fragments was shown by electron microscopy.

## EXPERIMENTAL

*Isolation of TA3-Ha cells.* — One week following the i.p. inoculation of male A/WySn mice (8–12 weeks of age) with $10^5$ ascites cells, cells were harvested ($2 \times 10^8$ cells per mouse) and washed several times in the cold with phosphate-buffered saline[11], as previously described[5], and finally with isotonic saline (0.9%).

*Preparation of membranes.* — The procedure was essentially that of Brunette and Till[10]. The washed cells were suspended in mM $ZnCl_2$, at a concentration of $5 \times 10^7$ cells/mL. After 15 min at 23°, the suspension was cooled to 0–5°. Approximately 5 mL of the suspension was placed in a 15-mL Dounce homogenizer with a tight-fitting (Type B) pestle (Kontes, Vineland, NJ). Homogenization, as monitored with a Zeiss phase-contrast microscope, was usually complete (>90% of the cells ruptured) after 120–150 strokes. The homogenate (H) was centrifuged in the cold for 15 min at ~500*g*, and the supernatant solution (S) was withdrawn. The pellet was suspended in 7 mL of the upper phase of a two-phase aqueous dextran–polyethylene glycol system[10], consisting of dextran (T-500, Pharmacia, Piscataway, NJ) and polyethylene glycol (Eastman Kodak, Rochester, NY). After adding an equal volume of the lower phase and shaking vigorously, the mixture was transferred to three 5-mL polyallomer tubes and centrifuged in a Beckman–Spinco centrifuge, equipped with an SW-65L rotor, at 10,000 r.p.m. for 10 min at 3°. The membrane particles were removed from the interface between the two layers and stored at 0–5° until the remaining pellet had been similarly treated. The pooled membrane-fractions were again shaken in the two-phase system as before and centrifuged similarly. After again repeating this centrifugation, the membrane fraction was washed with 10 vol. of cold water. Usually two

washes ($M_1$ and $M_2$) were collected, but with labeled cells, three additional washes ($M_b$, $M_{b1}$, and $M_{b2}$) were obtained. The resulting fraction (M) was used for all determinations. For electron microscopy, however, washes were performed with 0.1M ammonium acetate. The pellet at the bottom of the polyallomer tubes was washed twice with cold water (washes $P_1$ and $P_2$), and the resulting pellet was designated P. Material remaining in the three two-phase layer mixtures was designated $2PL_1$, $2PL_2$, and $2\ PL_3$.

*Enzyme assays.* — $Na^+$, $K^+$-activated ATPase, used as a marker for the plasma membrane, was assayed by the method of Wallach and Kamat[12], but with the solution concentrations employed by Brunette and Till[10]. The phosphate released from ATP by ATPase was determined by the method of Lowry and Lopez[13]. β-NADH-dehydrogenase, used as a marker for smooth endoplasmic reticulum, was determined by the procedure of Wallach and Kamat[12]. Cytochrome C oxidase as a marker for mitochondria was determined by the method of Wharton and Tzagoloff[14]. Protein concentrations prior to enzyme assays were determined by the method of Lowry *et al.*[15], with bovine serum albumin as standard.

*Tritium labeling.* — Terminal galactose and *N*-acetylgalactosamine residues at the cell surface were labeled by a modification of the method of Morell and Ashwell[16]. To ~5 mL of a suspension of TA3-Ha ascites cells ($1.1 \times 10^9$ cells, 97% viable) was added 0.5 mL of galactose oxidase (110 units, Worthington Biochemicals, Freehold, NJ), and the mixture was incubated, with shaking, for 1.5 h at 37°. After twice washing in phosphate-buffered saline[11], the cells were washed twice with Tris buffer (0.2M, pH 7.4). At this stage the viability of the cells was 94% (Trypan Blue exclusion test). Five portions of $NaB^3H_4$ (1.0 mCi each), (New England Nuclear, Boston, 100 mCi/mmol, stored at 1 mCi/100 μL, 0.05M NaOH, −80°), each in 400 μL of Tris buffer (0.2M, pH 7.4), were added, in turn, to the cell suspension at 22°. After mixing, the suspension was kept for 1.5 h at 22°. The reduction was completed by the addition of 1.7 mg of $NaBH_4$ in 500 μL of Tris buffer. After mixing, the suspension was kept for 30 min at 22°, and then centrifuged. The cells were washed several times with Tris buffer, and then twice with 0.9% NaCl. The cells ($8.1 \times 10^8$, 74% recovery) were 50% viable. Radioactivity of the fractions was determined in Hydrofluor (National Diagnostics, Somerville, NJ) after digesting the samples with 300 μL of 0.50M NaOH for 40 h at 80°, and then adding 0.5M HCl until neutral. All samples were adjusted to the same volume with water, and 200-μL portions were withdrawn for counting.

*Radioimmunoassays.* — TPCK-trypsin (0.05 μg/μL, 50 μL) was added to 50 μL of each fraction. After mixing, these were incubated for 2 h at 22° before adding 25 μg of soybean-trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). The samples were allowed to remain for 16 h at 4°. Aliquots of 50 μL in 1.2 mL conical tubes, at appropriate dilutions, were then incubated with 100 μL of anti-epiglycanin antiserum[1] (dilution 1:800 in 0.1M ammonium acetate) for 60 min at 37°, followed by 60 min at 4°. To each tube was added 50 μL of $^{125}$I-epiglycanin (1000 c.p.m.), prepared by the Bolton–Hunter procedure[17]. After incubation for 16–20 h at 4°,

150 μL of goat anti-rabbit IgG antiserum and 200 μL of normal rabbit serum (1:50) were added, and the incubation continued at 4° for an additional 16–20 h. After vigorous mixing, the tubes were centrifuged at 500*g* for 55 min. The radioactivities of both the residues and the supernatant solutions were determined. As standards, solutions of epiglycanin at seven concentrations were run simultaneously. Values for solutions of unknown concentrations were calculated from a straight-line standard curve plotted from the log of the epiglycanin concentration *versus* the percentage of radioactivity in the precipitate.

*Electron microscopy. Thin sectioning.* — Cells were first fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 1 h, washed three times in Sabatini's solution[18], buffered with 0.1M cacodylate buffer (pH 7.4), cut into small blocks, and then post-fixed in 2% osmium tetraoxide containing 1% potassium ferrocyanide, buffered with 0.1M cacodylate. Following three further washes in Sabatini's solution, as before, the tissue was dehydrated with ethanol and propylene oxide, and finally infiltrated with Epon.

Following infiltration with the pure resin, the tissue blocks were placed in resin in embedding trays and cured for 3 h at 37° and for 16–20 h at 60°. Polymerization was completed by a short (1.0 h) period at 100°.

Blocks were sectioned and positively stained with uranyl acetate, followed by lead citrate, according to Echlin[19]. Magnifications were calibrated by recording the 25-Å spacing from crystals of Indanthrene Olive[20]. Sections were examined in a Phillips EM 300 microscope at 60 kV.

*Shadowed preparations.* — As it was considered that the epiglycanin coat on membrane fragments of TA3-Ha cells would be more readily made visible by using high-contrast metal shadowing, a suspension of the membrane fraction at an appropriate dilution in 50% glycerol (0.15M $NH_4OAc$, pH 7.0) was placed on a carbon-filmed parlodion grid and the excess liquid withdrawn. Grids were placed in a high-vacuum shadow caster and pumped for 24 h to remove glycerol, water, and the volatile salt. Rotary shadowing[21] was then carried out using $10^{-6}$ g/cm$^2$ of platinum followed by about a 2.5-nm layer of carbon. Specimens were then examined and photographed at a magnification of 20,000×.

*Gas chromatography.* — For carbohydrate and amino acid analyses, 0.5-mL aliquots of each fraction were dialyzed against water at 4° to remove salts. Carbohydrates were analysed as their trimethylsilyl derivatives, as previously described[22], and amino acids by the method of Roach and Gehrke[23].

## RESULTS

*Enzyme assays.* — The results from five separate experiments to determine the specific activities of three marker enzymes are presented in Table I. The $Na^+$, $K^+$-activated ATPase activity was measured by the increase in inorganic phosphate ($P_i$) released from ATP when $K^+$ is present in addition to $Na^+$ and $Mg^{2+}$. Controls contained $Na^+$ and $Mg^{2+}$, but no $K^+$, in the incubation mixture. The specific activity

TABLE I

SPECIFIC ACTIVITIES OF ENZYMES USED AS MARKERS. NUMBERS DENOTE AVERAGE VALUES FOR FIVE SEPARATE EXPERIMENTS PERFORMED WITH NON-TRITIUM-LABELED CELLS

| *Fraction*[a] | *$Na^+$, $K^+$-ATPase* *Increase in $P_i$ μmol/h/mg protein* | *Ferrocytochrome c oxidase* *μmol oxidized/-min/mg protein* *× $10^3$* | *β-NADH dehydrogenase* *μmol oxidized/-min/mg protein* *× $10^2$* |
|---|---|---|---|
| H | 0.41 (±0.06)[c] | 10.5 (±2.7) | 2.0 (±0.4) |
| S | 0.31 (±0.15) | nda[b] | 1.6 (±0.6) |
| M | 4.33 (±0.76) | 1.9 (±1.5) | 3.6 (±0.6) |
| P | 0.38 (±0.11) | 14.0 (±2.5) | 9.3 (±4.4) |

[a]The identity of fractions is described in the Experimental Section. [b]nda, no detectable activity. There was an increase, rather than a decrease in absorbance. This suggests the presence of an inhibitory factor. [c]Numbers in parentheses represent the standard deviation of the mean, ($s_m$).

if this enzyme marker for the cell-surface membrane, M (Table I), was, on the average, 11.4 times higher than for P, 10.6 times higher than for H, and 14 times higher than for S. These results provide evidence that fraction M consists of a high proportion of plasma-membrane particles. The ferrocytochrome c oxidase assay, a test for the presence of mitochondria, showed no activity in fraction M in three experiments and only minor activity in two other experiments. These results suggest that only minimal amounts of mitochondria were present in M. Brunette and Till[10] reported that, for L cells, by far the highest concentration of mitochondria was present in the supernatant fraction. In these studies, only one experiment resulted in the detection of ferrocytochrome c oxidase activity in S. No evidence for the presence of this enzyme in S was found in the other four experiments. There was, however, evidence to support the presence of an inhibitor of this enzymic activity in the S fraction, namely the observation that the absorbance at 550 nm increased, rather than decreased, in the assay.

The activity of β-NADH dehydrogenase, as a marker for the presence of smooth endoplasmic reticulum, was, on the average, 2.5 times greater in the pellet P, than in M, 4.7 times greater in P than in the homogenate H, and 5.8 times greater in P than in S. These results suggest that there was some contamination of the M fraction with fragments of the endoplasmic reticulum, which were not removed under the conditions of the fractionation. This suggestion was supported by electron micrographs of M (see Fig. 1).

In a separate experiment, one in which cells were tritium labeled, cell-surface galactose and *N*-acetylgalactosamine residues were the primary objects of the reduction procedure. The results of the assays for enzymic activities (Table II) were consistent with those presented in Table I. The presence of ATPase activity was several fold greater in fraction M than in either the P or H fractions. The results also suggest that endoplasmic reticulum fragments may be present in fraction M.

*Distribution of radioactivity in fractions.* — Table III lists the relative propor-

tions of tritium in the isolated fractions. The extraordinarily high value found in the S fraction could not be attributed solely to the presence of labeled macromolecules but was probably due mainly to the presence of products of small molecular weight resulting from the borohydride reduction. Fraction M contained a significant amount of labeled material (20,000 c.p.m.), as would be expected if it contained epiglycanin, of which Gal and GalNAc residues together constitute ~60% of the mass. High levels of radioactivity were also found in the pellet and two-phase layer fractions, but these could not be accounted for solely by the epiglycanin present (Table III), nor, in the case of P, where a carbohydrate analysis could be obtained, by the concentration of Gal and GalNAc (Table IV).

*The presence of epiglycanin in the fractions.* — Concentration of epiglycanin was determined in two ways. The radioimmunoassay[1] was employed for all fractions (Table III). Because of the composition of the two-phase system used, g.l.c. analysis for carbohydrate composition was meaningful for only five of the fractions (Table IV). As we have shown previously that epiglycanin is the major source of GalNAc in the TA3-Ha ascites cell, and that GalNAc constitutes ~30% of the mass of epiglycanin, it was possible to calculate the approximate concentration of epiglycanin in the five fractions. Four of the five values agree reasonably well with those determined by the radioimmunoassay (r.i.a.). There is no ready explanation for the low GalNAc value found for fraction P. The sum of all values, as determined by the r.i.a., was 0.8 mg, ~40% of the 2 mg estimated to be present at the surface of $10^9$ TA3-Ha ascites cells[4]. Epiglycanin appeared to be present in all fractions, and only 15% of the total was detected in fraction M. Slightly more (18%) was detected in the supernatant solution (S), probably due to the loss of free glycoprotein from the particulate material. High concentrations, 18 and 14%, respectively, were also found in P and the $2PL_1$. No major differences in amino acid compositions were found in the four fractions analyzed (Table V), but the

TABLE II

SPECIFIC ACTIVITIES OF THE ENZYMES USED AS MARKERS FOR MEMBRANES IN EXPERIMENT WITH TRITIUM-LABELED TA3-Ha CELLS

| *Fraction*[a] | *$Na^+$, $K^+$-ATPase* *Increase in $P_i$ µmol/h/mg protein* | *Ferrocytochrome c oxidase* *µmol oxidized/-min/mg protein (× $10^3$)* | *NADH dehydrogenase* *µmol oxidized/-min/mg protein (× $10^2$)* |
|---|---|---|---|
| H | 0.30[c] | 7.1 | 1.6 |
| S | 0.00 | nda[b] | 0.9 |
| M | 1.10 | 0.6 | 5.5 |
| P | 0.20 | 11.0 | 6.3 |

[a]The identity of the fractions is described in the Experimental Section. [b]nda, no detectable activity. [c]This experiment was performed similarly to those reported in Table I. The variability in results would be expected to be about the same.

TABLE III

AMOUNTS OF EPIGLYCANIN, AS DETERMINED BY A RADIOIMMUNOASSAY, AND RADIOACTIVITY IN FRACTIONS FROM $1.8 \times 10^8$ TRITIUM-LABELED TA3-Ha CELLS

| *Fraction* | *Epiglycanin ($\mu g/10^9$ cells)* | | *Radioactivity[c] (c.p.m. $\times 10^{-4}$)* |
|---|---|---|---|
| | *by r.i.a.[a]* | *by g.l.c.[b]* | |
| H[d] | 580 | | |
| S | 144 | 109 | 53 |
| M | 120 | 105 | 2 |
| $M_1$ | 17 | | 0.1 |
| $M_2$ | 56 | 36 | 0.3 |
| $M_b$ | 48 | | |
| P | 140 | 35 | 4.7 |
| $P_1$ | 25 | | 0.3 |
| $P_2$ | 33 | 38 | 9.4 |
| $2PL_1$ | 110 | | 5.9 |
| $2PL_2$ | 41 | | 1.1 |
| $2PL_3$ | 33 | | 1.1 |

[a]Average of three independent assays. The average variation in r.i.a. values was ~7%. [b]Epiglycanin value calculated from GalNAc value (Table IV), as 30% of epiglycanin. Values by g.l.c. (Table IV) considered to be accurate within ±5%. [c]Radioactivity determined on neutralized NaOH digests, with Hydrofluor. Values were consistent within ±10%. [d]For a description of symbols, see text.

TABLE IV

CARBOHYDRATE ANALYSIS BY GAS CHROMATOGRAPHY OF FRACTIONS FROM $8.1 \times 10^8$ TRITIUM-LABELED TA3-Ha CELLS[a]

| *Carbohydrate residue* | *S* | *M* | *M-2* | *P* | *P-2* |
|---|---|---|---|---|---|
| GalNAc | 32.7 | 31.5 | 10.7 | 10.6 | 11.4 |
| | (1.0)[b] | (1.0) | (1.0) | (1.0) | (1.0) |
| Gal | 36.5 | 52.8 | 26.9 | 41.1 | 18.8 |
| | (1.4) | (2.1) | (3.2) | (11.5) | (2.1) |
| GlcNAc | 18.1 | 36.5 | 5.4 | 19.4 | 45.8 |
| | (0.6) | (1.2) | (0.5) | (0.4) | (4.0) |
| Man | 14.2 | 17.6 | 7.0 | 47.0 | 6.8 |
| | (0.7) | (0.7) | (0.8) | (13.2) | (0.7) |
| NeuAc | 18.4 | 44.8 | 11.2 | 22.8 | 10.2 |
| | (0.4) | (1.1) | (0.8) | (4.0) | (1.5) |
| Glc | 1179 | 151 | 1386 | 1992 | 1380 |
| | (45) | (6.0) | (167) | (570) | (156) |
| Rib | nd[c] | 88.2 | 7.4 | 131 | 14.8 |
| | | (4.3) | (1.1) | (45) | (2.0) |
| Xyl | 25.7 | 10.2 | 3.7 | 17.7 | 2.6 |
| | (1.2) | (0.5) | (0.5) | (6.1) | (0.3) |

[a]Values represent $\mu g/10^9$ cells. Accuracy by this procedure has been found to be within ±5%. [b]Values in parentheses represents mol relative to mol of GalNAc. Ratios are consistent within ±2%. [c]nd = not detectable.

differences in the protein concentrations, as determined by g.l.c., were significant. Fraction S contained relatively little protein compared to carbohydrate (2.2:1) and, if glycoprotein, the percent carbohydrate would be 43%. For M and P the percent carbohydrate would be, respectively, 8.3 and 2.2%. Thus, although fraction P contained an equivalent amount of epiglycanin, by the r.i.a., as compared to fraction M, it contained 3.1 times as much protein.

The detection of ribose and xylose in Fraction M (Table IV) was not surprising. A small amount of RNA has been consistently found in isolated membrane-components from the TA3-Ha ascites cell. The xylose probably resulted from contamination by proteoglycans that had become adsorbed by membrane components. The glucose probably resulted from the dextran used in the membrane preparation.

*Electron microscopy of the plasma membrane fraction M.* — Fig. 1 shows a transmission electron micrograph of fraction M. Some fields contain what are identified as endoplasmic reticulum fragments, and a few examples of mitochondrial membranes have been found. Nevertheless, a large proportion of the material possesses morphologic characteristics consistent with those of plasma membrane fragments.

*Visualization by shadow casting.* — Fraction M produced striking results, as shown in Fig. 2. Long filaments may be observed to emanate from some particles. The range of most-frequent lengths of the strands is 400–500 nm, and with widths of about 2 nm. These dimensions are consistent with those previously found for

TABLE V

AMINO ACID ANALYSIS BY G.L.C. OF FRACTIONS OBTAINED FROM $8.1 \times 10^8$ TA3-Ha ASCITES CELLS. VALUES ARE GIVEN AS $\mu g/10^9$ CELLS[a]

| *Amino acid residue* | *S* | *M* | *P* | *2PL$_I$* |
|---|---|---|---|---|
| Ala | 7 | 156 | 465 | 128 |
| Asn/Asp | 28 | 232 | 686 | 353 |
| Gln/Glu | 54 | 337 | 1090 | 642 |
| Gly | 6 | 63 | 271 | trace |
| Ile | 17 | 131 | 377 | 173 |
| Leu | 25 | 228 | 695 | 276 |
| Lys | 39 | 238 | 886 | 507 |
| Phe | 16 | 125 | 344 | 372 |
| Pro | 11 | 102 | 340 | 64 |
| Ser | 18 | 131 | 397 | 270 |
| Thr | 16 | 127 | 389 | 148 |
| Val | 23 | 158 | 472 | 353 |
| Total | 260 | 2028 | 6412 | 3286 |

[a]Values by this procedure are considered to be accurate within ±7%. Repeated results on the same sample are consistent within ±4%.

Fig. 1. Transmission electron micrograph of membrane fraction M (130,000×).

isolated epiglycanin molecules[4]. In order to examine the possibility that these filaments might be strands of DNA, fraction M was first incubated extensively with DNAase, then investigated by electron microscopy, with the same shadow-casting procedure. No change in either the occurrence or the length of the filaments was observed.

## DISCUSSION

The decision to undertake this investigation was strongly influenced by the report of Sherblom *et al.*[24]. These investigators were able to isolate in pure form a

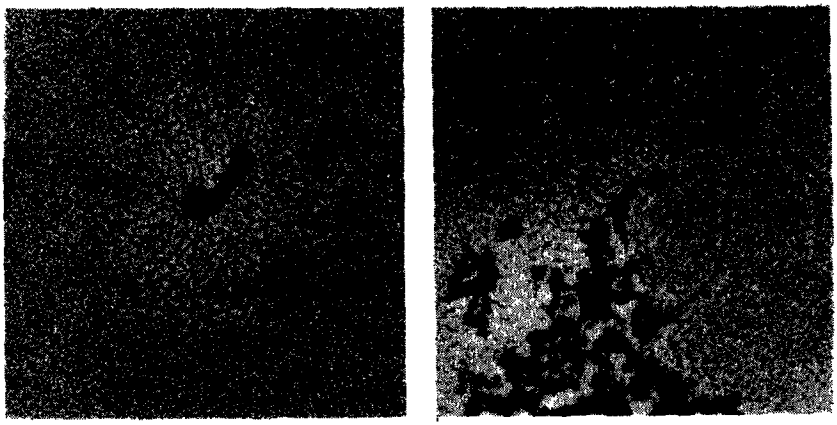

Fig. 2. Membrane fraction M shown in macromolecular replicas, shadow-cast with $10^{-6}$ g/cm$^2$ of Pt (64,000×). *Left:* small membrane particle surrounded by attached macromolecular epiglycanin; *right:* large membrane fragment with attached epiglycanin.

large glycoprotein, ASGP-1, from the isolated plasma membrane fraction of each of two rat mammary carcinoma ascites cells. The two ASGP-1 glycoproteins differed in oligosaccharide-chain structure, but each differed more significantly in both carbohydrate and amino acid compositions, from epiglycanin[25]. Nevertheless, epiglycanin was similar in several respects to the rat glycoproteins, as each consisted of >65% carbohydrate, most of which was present in *O*-linked chains, and each possessed a molecular weight of the same order of magnitude, 500–600 K. After homogenization, Sherblom *et al.*[24] employed, in the final centrifugation, a cesium chloride gradient with 4M guanidinium chloride. Our decision to use the two-phase dextran–polyethylene glycol system[10] was based upon its simplicity and the availability of enzyme markers for evaluating the success of the fractionation. Indeed, no major problems were encountered in performing the fractionation. Clearly, however, the loss of epiglycanin from the isolated membrane fraction was much greater than that experienced in the rat system. This may be due, however, to the different characteristics of the rat and mouse (TA3-Ha) cells. For example, in previous studies we were completely unable to isolate microvilli from the TA3-Ha cell by the use of a procedure that had been successful for the rat cells[26].

The results obtained with marker enzyme activities (Tables I and II) suggest that the marker for the plasma membrane, namely $Na^+$, $K^+$-ATPase, was present mainly in fraction M. The activity in M was 5–10 times greater than that found in either S or P. The observation that the concentrations of epiglycanin in fractions S and P, as well as $2PL_1$, are equivalent to that found in M does not suggest, however, that epiglycanin is not a membrane glycoprotein. That the behavior in the fractionation procedure (Table III) of this large ($M_r$ 500,000), rod-like glycoprotein of 75–80% carbohydrate[4] is not similar to that of the marker enzyme, a glycoprotein of $M_r$ 180,000–350,000 and with no more than 15% carbohydrate[27], is not surprising. It was expected that some epiglycanin would be found in the S fraction, as it has been demonstrated that epiglycanin is slowly "shed" from intact TA3-Ha cells into the surrounding medium when the cells are grown either *in vivo* or *in vitro*. The composition of S (Tables IV and V) suggests the presence, not only of epiglycanin, but of other glycoproteins, as the relative proportions of Man, GlcNAc, and aspartic acid (asparagine) are far greater than those found in epiglycanin. The significant concentrations of epiglycanin detected in fraction P, as well as in the 2PL fractions, may reflect a relatively weak binding of epiglycanin to membrane particles, an unusual effect of the high carbohydrate concentration upon the buoyant density of membrane bound epiglycanin, and/or a loss of the membrane structure itself, despite the presence of $Zn^{2+}$.

Despite the low concentration of epiglycanin in M, the quality of the membranes appears to be fairly good, as attested to by the high marker-enzyme activity. There was, however, some contamination by endoplasmic reticulum particles (Tables I and II). Transmission electron micrographs of fraction M (Fig. 1) are consistent with these results. There appears to be a high concentration of

plasma membrane particles, but also evidence of some contaminating particulate material.

The successful use of shadowcasting electron microscopy for viewing membrane particles is illustrated in Fig. 2. Fraction M, suspended in 0.1M $NH_4OAc$, had been sprayed onto mica sheets and shadowed to permit visualization of epiglycanin. By this technique, the fields examined appeared to be grossly heterogeneous with particles of various shapes and sizes. However, the presence of fragments of material with large numbers of macromolecular-sized filaments emanating from them, as shown in Fig. 2, was not uncommon. The identity of these filamentous strands as epiglycanin molecules has not been established. However, their unusual characteristics, their similarity in length and width to similarly observed molecules, previously shown to be epiglycanin[4], and the absence of a more likely explanation suggests this identification. An alternative suggestion that these filaments might be DNA molecules was shown to be improbable by the observance of similar filaments following extensive incubation of fraction M with DNAase. Thus, the results shown in Fig. 2 may constitute the first example of the direct visualization by electron microscopy of the binding of a cell-surface glycoprotein to a membrane fragment.

The total recovery of epiglycanin in all fractions, as determined by the r.i.a. (Table III) was only 40% of that expected. However, this decreased yield is thought to be related in part to the difficulty of solubilizing the epiglycanin from the particulate matter without destroying its inhibitory activity. In the interest of consistency, all fractions, whether in solution or in suspension, were treated uniformly with 50 $\mu$g of TPCK-trypsin/mL for 2 h at 22° before adding soybean trypsin inhibitor. Although these conditions were probably capable of releasing all antigen into solution, it is possible that they may have resulted in a significant loss of activity in some fractions. Indeed, we have shown that more extensive proteolysis by this enzyme may destroy >80% of the inhibitory activity in epiglycanin.

One of the objectives of this investigation was to examine the possibility of obtaining epiglycanin from isolated membrane fragments, as a method of producing this glycoprotein. The results reported here have discouraged us from taking this route as a means of obtaining this glycoprotein in its native form. With only ~15% of the detected epiglycanin present in the membrane (M) fraction, and with other contaminating glycoproteins also present (Tables III, IV, and V), this approach does not seem promising. Nevertheless, this study may prove valuable in furthering our understanding of the physicochemical characteristics of tumor-specific, cell-surface glycoproteins, such as epiglycanin, particularly as they function as cell-membrane components, and should help us to understand their role(s) as tumor antigens.

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