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ISOLATION AND CHARACTERIZATION OF AN EPITHELIAL BASEMENT MEMBRANE GLYCOPROTEIN FROM MURINE KIDNEY AND FURTHER CHARACTERIZATION OF AN EPITHELIAL BASEMENT MEMBRANE GLYCOPROTEIN SECRETED BY MURINE TERATOCARCINOMA CELLS IN VITRO

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SUMMARY

A glycoprotein component of epithelial basement membranes (EBM) has been isolated from murine kidney homogenates by extraction with 0.05 M phosphate buffer, pH 7.2, precipitation with $(NH_4)_2SO_4$ and chromatography on controlled pore glass. Antiserum produced against this glycoprotein reacts specifically with the basement membranes of renal glomeruli and tubules. The EBM glycoprotein of renal origin is antigenically identical with a glycoprotein component of epithelial basement membrane secreted by a murine teratocarcinoma grown in vitro, and the amino acid composition of the two EBM glycoproteins is markedly similar. Both glycoproteins were isolated as high molecular weight aggregates. Disaggregation with sodium dodecyl sulfate and 2-mercaptoethanol resulted in release of monomers of 32 000 and 34 000 daltons for kidney EBM glycoprotein and teratocarcinoma EBM glycoprotein, respectively. The difference in molecular weight is apparently due to increased amounts of fucose, mannose, *N*-acetylglucosamine and sialic acid in the glycoprotein secreted by the teratocarcinoma. In addition, both EBM glycoproteins contain galactose, glucose and *N*-acetylgalactosamine.

INTRODUCTION

In 1972, a method for the isolation of a murine glycoprotein component of epithelial basement membranes (EBM) was reported [1]. This glycoprotein was isolated as a high molecular weight aggregate from tissue culture medium in which a murine teratocarcinoma called parietal yolk sac carcinoma was grown. This glycoprotein of neoplastic cell origin was antigenically similar or identical to a component of normal epithelial basement membranes as demonstrated by specific reactivity of

Abbreviations: NEBM, basement membrane glycoprotein of neoplastic epithelial origin; KEBM, void volume protein from molecular sieve chromatography of S_3.

antibodies to glomerular and tubular basement membranes of murine kidney. Biochemical characterization indicated that the glycoprotein was not contaminated by basement membrane collagen.

Comparison of this neoplastic EBM glycoprotein with epithelial basement membrane components extracted from several normal mammalian tissues such as the lens capsule [2–6] and renal tubules [7–9] was unsuccessful. In nearly all these cases and one other report of the characterization of neoplastic epithelial basement membranes [10], techniques employed in the isolation and separation of epithelial basement membrane components utilized several washings and physical disruption of the tissues by sonication in order to obtain morphologically identifiable basement membrane. Solubilization has been achieved by the use of denaturants, strong alkaline solutions or proteolytic enzymes. Although the collagenous and non-collagenous glycoprotein components can be solubilized by such treatment, there are no assurances that degradation has not resulted. For example, amino acid analyses of glycoproteins isolated from basement membranes almost invariably include small amounts of hydroxyproline and hydroxylysine. Comparative studies were therefore dependent on isolating epithelial basement membrane components from normal tissue by methods which insured against physical or chemical modification. Establishment of such a method would also facilitate further characterization of epithelial basement membrane components.

The studies reported in this paper were initiated to determine whether a similar EBM glycoprotein could be isolated from normal tissue, simply by extraction with aqueous buffers. In addition, further characterization of the neoplastic EBM glycoprotein was dependent on successful disaggregation and isolation of monomeric species. Although the propensity of the neoplastic EBM glycoprotein to aggregate was an indispensible property with regard to its isolation, it severely restricted further characterization of the molecule.

The present study reports (1) the isolation of an aqueous soluble EBM glycoprotein from murine kidney by methods essentially the same as those previously reported [1], (2) the amino acid and carbohydrate composition of the kidney glycoprotein and (3) comparison of the biochemical, immunologic and physical characteristics of monomeric EBM glycoproteins of neoplastic and normal epithelial cell origin.

METHODS

Isolation of kidney glycoprotein. Adult Swiss Webster mice of either sex were sacrificed by decapitation. The kidneys were removed from 50 to 100 mice and immediately placed in ice-cold saline. After excising the capsule and pelvic structures, the remaining renal tissue was transferred to ice-cold 0.05 M phosphate buffer, pH 7.2 (five volumes buffer per volume of wet tissue). The kidneys were homogenized in a Waring blender and extracted for 24–48 h at 4 °C. Cell debris was removed by centrifugation at 13 000 $\times g$ for 30 min at 4 °C. The supernatant (S_1) was decanted and solid $(NH_4)_2SO_4$ was added slowly to 50 % of saturation at 0 °C. The protein was allowed to precipitate for 24 h at 4 °C and was collected by centrifugation at 13 000 $\times g$ for 30 min at 4 °C. The supernatant (S_2) was decanted and the precipitate (P_1) was dissolved in 100–200 ml of 0.05 M phosphate buffer, pH 7.2. Undissolved or denatured

protein (P_2) was removed by centrifugation at 13 000 $\times g$ for 30 min at 4 °C and was discarded. The final supernatant (S_3) was chromatographed on a column of CPG-10-350 (Electro-Nucleonics, Inc), 90 $\times$ 2.5 cm, eluted with 0.05 M phosphate buffer, pH 7.2. The column eluates were monitored at 280 nm and collected in 5-ml fractions.

In some instances, the protein peak which eluted with the void volume (Peak I in Fig. 1) was collected, concentrated and rechromatographed on a column of *P*-cellulose which was eluted with a linear phosphate buffer gradient from 0.05 to 1.0 M at pH 7.2.

Preparation of rabbit antisera to EBM glycoproteins. Antisera were produced against the basement membrane glycoprotein of neoplastic epithelial cell origin [1], designated NEBM and the void volume protein from molecular sieve chromatography of S_3, designated KEBM. New Zealand white rabbits were immunized by intramuscular injection of 10 mg glycoprotein dissolved in 0.5 ml phosphate-buffered saline, pH 7.2, which was emulsified with 0.5 ml complete Freund's adjuvant. The rabbits were innoculated and bled from the marginal vein of the ear on days 0 (prebleed serum for controls), 4 and 10. Sera collected from these bleedings and subsequent bleedings on days 21 and 30 were heated at 56 °C for 30 min to inactivate complement and tested for anti-epithelial basement membrane activity.

Immunoassays. The glycoprotein isolated from murine kidney was identified as a component of the epithelial basement membrane by the indirect immunofluorescent method [11]. Sections of frozen murine kidney and spleen were cut at a thickness of 6 μm. Sections were reacted with either antiserum against KEBM or normal rabbit serum (prebleed serum). The sections were then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit γ-globulin (Cappel Laboratories, Inc., Downingtown, Pa. 19335). Stained sections were examined with a Leitz fluorescent microscope.

KEBM and NEBM were reacted against anti-NEBM according to the immunodiffusion procedure described previously [1]. Further immunologic characterization of NEBM and KEBM was obtained by passive hemagglutination of appropriately sensitized sheep erythrocytes [12].

Molecular weight estimations of EBM glycoproteins. NEBM and KEBM were concentrated and dialyzed extensively against 0.1 M Tris buffer, pH 8.2, containing 1 % sodium dodecyl sulfate (SDS). After adding 2-mercaptoethanol to a concentration of 5 %, aliquots of the glycoproteins were incubated for 1 min at 100 °C followed by incubation at 37 °C for 2 h. Alternatively, the incubation time at 100 °C was increased to 5 min. The reduced, denatured proteins were chromatographed on a column of Bio-gel A-5m (90 $\times$ 2.5 cm) eluted with 0.1 M Tris buffer, pH 8.2, containing 1 % SDS. Eluates were monitored at 280 nm and collected in 5-ml fractions. The elution positions of the EBM glycoproteins were compared with the elution positions of reduced, denatured transferrin (90 000 mol. wt.), bovine serum albumin 68 000 mol. wt.), ovalbumin (45 000 mol. wt.) and cytochrome *c* (12 400 mol. wt.). Reduced, denatured EBM glycoproteins were also examined by SDS-acrylamide gel electrophoresis at pH 8.2 [13]. Gels were either 5 or 7.5 % with respect to acrylamide concentration. Proteins were detected by staining gels with Coomassie blue, and sugars were detected by staining gels with periodic acid-Schiff reagent [14]. Molecular weight estimations were obtained by comparison of the EBM glycoproteins to the migration distances of the calibration proteins (indicated previously) which were electrophoresed simultaneously.

Chemical determinations. The protein content of column fractions were routinely determined by the method of Lowry et al. [15]. Amino acid analyses of NEBM, KEBM and peaks 2 and 3 obtained by chromatography of these glycoproteins after reduction and denaturation (Fig. 4) were performed on a Jeolco model JLC-6AH amino acid analyzer. Aliquots of the glycoproteins were dialyzed extensively against distilled water, lyophilized and hydrolyzed with 6 M HCl for 24 h at 110 °C in vacuo. Analyses were performed according to the method of Miller and Peiz [16] to resolve hydroxyproline and according to the method of Moore et al. [17] for all other amino acids. When methionine sulfoxide was found, it was added to methionine.

The sugar compositions of NEBM and KEBM were determined by gas chromatography. The heterosaccharides were cleaved and converted to monosaccharides by the method of Reinhold et al. [18]. The trimethylsilyl derivatives of the monosaccharides were separated on a glass column, 6 ft in length and 3 mm in diammeter, packed with Chromosorb HP coated with 2 % Lopol (Beckman Instruments, Inc, Fullerton, Calif.) in a Hewlett Packard Model 7610A gas chromatograph equipped with dual flame ionization detectors, automatic sample injector and a model 3352D Lab Data System. Sugar derivatives dissolved in heptane were injected onto the column at an initial temperature of 130 °C, the column was maintained at 130 °C for 4 min, increased at the rate of 4 °C per min to a final isothermal temperature of 172 °C which was maintained for 10 min. A standard mixture composed of the sugars commonly present in glycoproteins was chromatographed to determine the molar response of each sugar. Sialic acids were also determined by the thiobarbituric acid assay [19] and glucose was determined by digestion with glucose oxidase (Glucostat, Worthington Biochemical Corp, Freehold, N.J.).

Because of the possibility of renal cell membrane contaminants co-chromatographing with EBM glycoprotein, aliquots of the fractions obtained at each step in the isolation procedure were assayed for ATPase activity according to the method of Potter [20].

RESULTS

Assay of the fractions obtained during the isolation procedure indicated significant amounts of ATPase in all fractions. Of the activity detected in P_1, approx. 90 % appeared in P_2 and only 10 % in S_3. The elution pattern of Fraction S_3 following chromatography on CPG-10-350 is shown in Fig. 1. The protein in Fractions 30–50 eluted with the void volume, which was determined by chromatographing tobacco mosaic virus. The bulk of the protein was retarded and eluted considerably later in the run. No ATPase activity was detected in the void volume peak.

The chromatography of Peak I glycoprotein on *P*-cellulose (Fig. 2) resulted in the elution of only one peak. The increasing baseline rise was due to absorbance of the phosphate buffer as the concentration of the gradient increased.

The results of immunofluorescent microscopy are shown in Fig. 3. Specific linear fluorescence was present in the glomerular tufts, Bowman's capsule and around renal tubules when kidney sections were incubated with anti-KEBM. Kidney sections reacted with normal rabbit serum were unstained. Sections of spleen were unstained after incubation with either anti-KEBM or normal rabbit serum.

The results of immunodiffusion are shown in Fig. 4. When antiserum directed

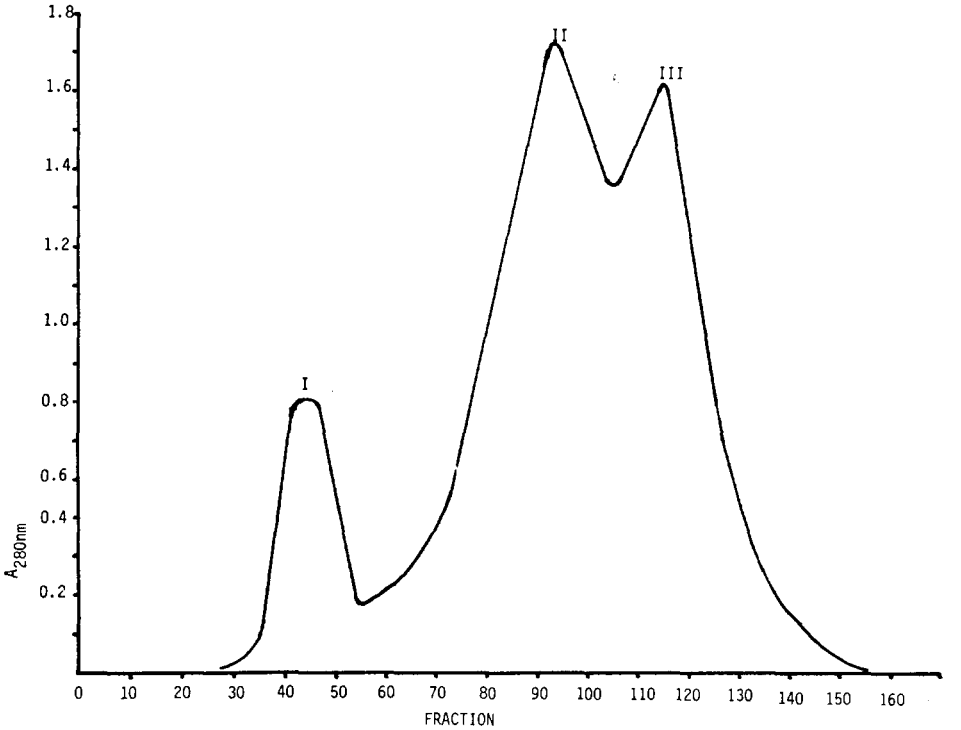

Fig. 1. Elution pattern of (S_3) on CPG-10-350. The column (90 × 2.5 cm) was eluted with 0.05 M phosphate buffer, pH 7.2. Peak I eluted with the void volume.

against NEBM glycoprotein was incubated with NEBM or KEBM, a single precipitin line developed against each antigen and antigenic identity was demonstrated. Identical results were obtained when the antigens were diffused against anti-KEBM.

The results of passive hemagglutination are shown in Table I and confirm those obtained by immunodiffusion. Antisera to NEBM- and $KEBM_2$-agglutinated sheep erythrocytes sensitized with either of the glycoproteins. The values stated are the reciprocals of the greatest antiserum dilution which resulted in hemagglutination. When each of the antisera was absorbed with either of the antigens, sensitized sheep erythrocytes failed to agglutinate. Both antisera failed to agglutinate unsensitized sheep erythrocytes, and normal rabbit sera (obtained when rabbits were prebled prior to immunization) failed to agglutinate sensitized sheep erythrocytes.

The results of chromatography of reduced, denatured KEBM are shown in Fig. 5. Chromatography of Peak I glycoproteins on Bio-gel A-5m in the presence of 1 % SDS resulted in a minor peak in the void volume and larger peaks with apparent molecular weights of 60 000 and 30 000 when compared with the elution positions of transferrin (90 000), bovine serum albumin (68 000), ovalbumin (45 000) and cytochrome *c* (12 400). These results were obtained if initial incubation was at 100 °C for 1 min. Increasing the initial incubation time to 5 min resulted in elimination of the void volume peak and the 60 000 dalton peak. All glycoprotein eluted with a molecular weight of 30 000. This peak was collected, concentrated and dialyzed against 0.1 M

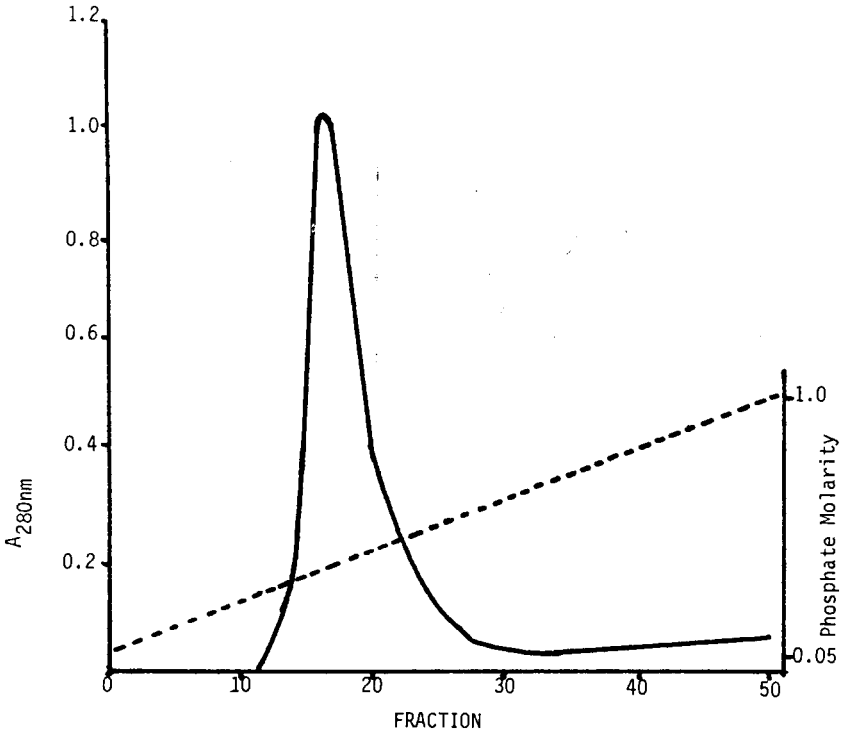

Fig. 2. Elution pattern of Peak I protein on *P*-cellulose. Peak I protein obtained by molecular sieve chromatography was rechromatographed on *P*-cellulose, eluted with a phosphate buffer gradient from 0.05 to 1.0 M, pH 7.2. The slope of the phosphate gradient is indicated by the broken line.

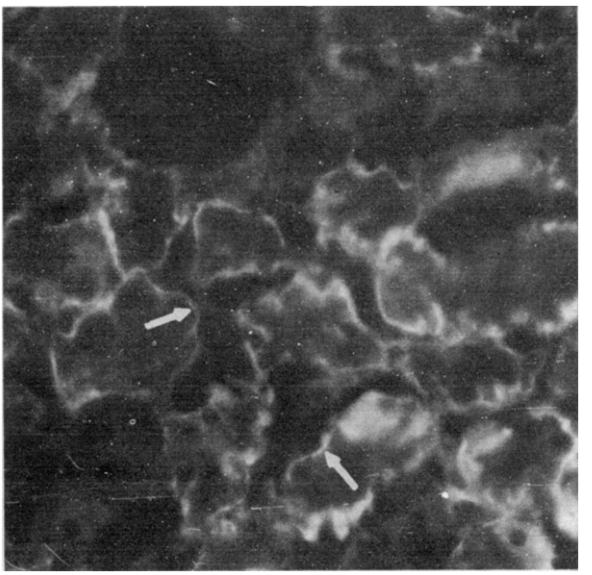

Fig. 3. Reaction of anti-KEBM with mouse kidney. Linear fluorescence is present specifically over the basement membrane (arrows).

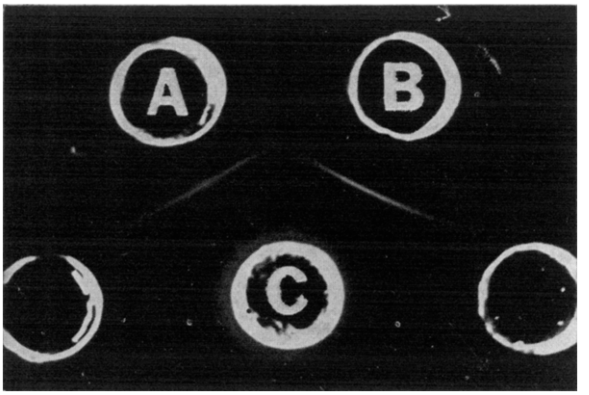

Fig. 4. Immunodiffusion of NEBM and KEBM. Well C contained rabbit antiserum directed against NEBM. Wells A and B contained NEBM and KEBM, respectively. Identical results were obtained when antiserum against KEBM was placed in the center well.

Tris buffer, pH 8.2, containing 1 % SDS to remove 2-mercaptoethanol. Rechromatography resulted in elution of all glycoprotein with an apparent molecular weight of 60 000. Chromatography of NEBM resulted in an identical elution profile.

The results of SDS-acrylamide gel electrophoresis are shown in Fig. 6. Electrophoresis of NEBM resulted in a narrow band at the origin and bands with apparent molecular weight of 68 000 and 34 000. Electrophoresis of KEBM resulted in essentially the same pattern, however, the molecular weights of the two major glycoprotein bands were estimated at 64 000 and 32 000.

The amino acid composition of KEBM is shown in Table II. The values are expressed in residues of amino acid per 1000 residues of total amino acid. There were no significant differences when analysis of the aggregated KEBM glycoprotein was compared with analyses of the dimer (60 000 daltons) and monomer (30 000 daltons) obtained by reduction and denaturation. The final column in Table II is the amino acid

TABLE I

HEMAGGLUTINATION OF EBM GLYCOPROTEIN-SENSITIZED SHEEP ERYTHROCYTES

Sheep erythrocytes were sensitized with either NEBM or KEBM, designated antigens, and were incubated with antisera directed against each of these glycoproteins. The numbers are the reciprocal of the final antiserum dilution which resulted in hemagglutination. Antisera absorbed with each antigen are designated (Abs.). The final column indicates reactivity of antisera against washed, non-sensitized sheep erythrocytes.

Antiserum	Antigen		
	NEBM	KEBM	None
Anti-NEBM	256	256	0
Anti-KEBM	128	256	0
Anti-NEBM (Abs.)	0	0	0
Anti-KEBM (Abs.)	0	0	0

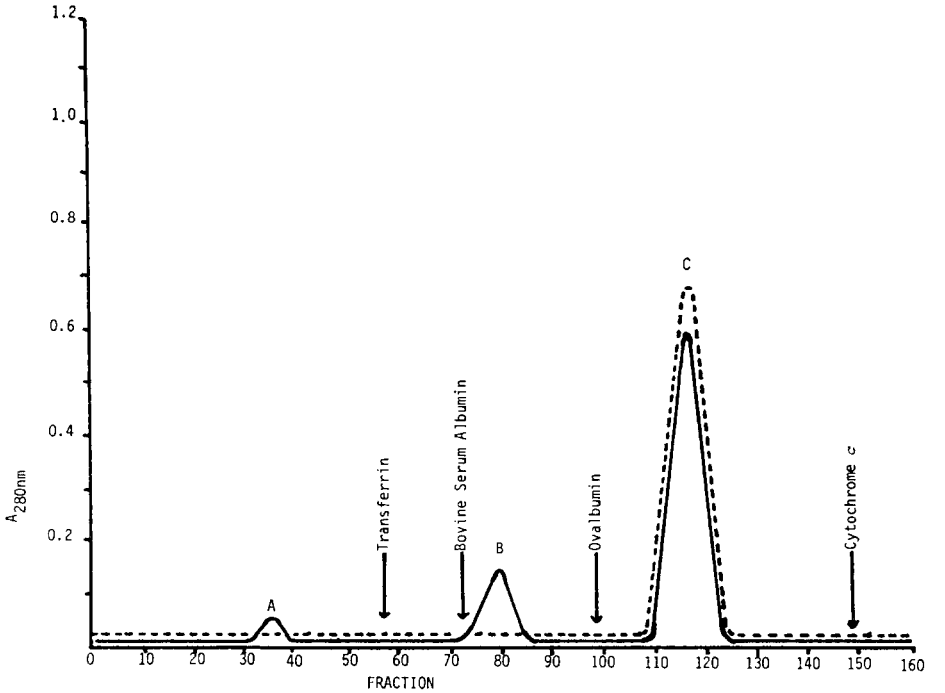

Fig. 5. Elution pattern of reduced, denatured KEBM on Bio-gel A-5m. Peak I glycoprotein from CPG-10-350 chromatography of kidney protein, reduced and denatured with 5 % 2-mercaptoethanol (2-ME) and 1 % sodium dodecyl sulfate (SDS) was eluted with 0.1 M Tris buffer, pH 8.2, containing 1 % SDS. The solid curve indicates the elution profile when the incubation with 2-mercaptoethanol and SDS at 100 °C was for 1 min. The elution profile indicated by the broken curve occurred when incubation at 100 °C was increased to 5 min. Peak A eluted with the void volume, Peaks B and C with apparent molecular weights of 30 000 and 60 000, respectively. The elution positions of transferrin (90 000 daltons), bovine serum albumin (68 000 daltons), ovalbumin (45 000 daltons) and cytochrome *c* (12 400 daltons) are indicated.

composition of aggregated NEBM. The amino acid compositions of NEBM dimeric and monomeric forms obtained by reduction and denaturation were virtually identical.

The sugar compositions of NEBM and KEBM are shown in Table III. The quantity of each sugar is reported in μg per mg of lyophilized glycoprotein. The total sugar content of the NEBM glycoprotein was approx. 13 %, as reported previously [1], however, the exact composition differs in that sialic acid is present. The presence of sialic acid was confirmed colorimetrically.

The total sugar content of the kidney EBM glycoprotein is lower than that of the glycoprotein secreted by teratocarcinoma cells, 10.6 % as compared with 12.9 %. The differences are due to statistically significant increases in the amounts of mannose, sialic acid, fucose and *N*-acetylglucosamine in NEBM glycoprotein (see Table III).

DISCUSSION

The techniques previously applied to the isolation of an epithelial basement glycoprotein from an in vitro system have been successfully modified for the isolation

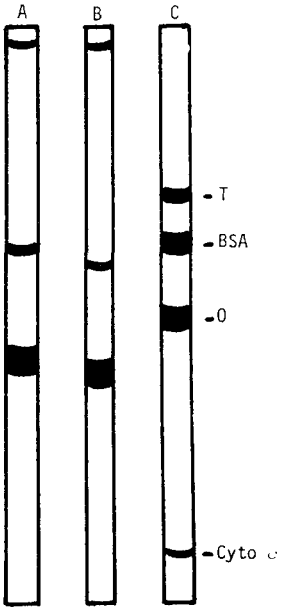

Fig. 6. SDS-acrylamide gel electrophoresis of EBM glycoproteins. NEBM (A) and KEBM (B) electrophoresed on either 5 or 7.5 % SDS-acrylamide gels resolved into three bands; band 1 near the origin, bands 2 and 3 with apparent molecular weights of 68 000 and 34 000, respectively for NEBM; 64 000 and 32 000 for KEBM. C is the pattern obtained when transferrin (T), bovine serum albumin (BSA), ovalbumin (O) and cytochrome *c* (cyto *c*) were electrophoresed.

TABLE II

AMINO ACID COMPOSITION OF EBM GLYCOPROTEINS

The content of each amino acid is reported as residues per 1000 residues of total amino acid. Analyses of KEBM glycoprotein aggregates (KEBM), Peaks B and C from SDS-agarose chromatography of KEBM V_0 protein (dimer and monomer, respectively) and NEBM aggregates (NEBM) are shown. Analyses of NEBM dimeric and monomeric forms were virtually identical.

Amino acid	KEBM	Monomer	Dimer	NEBM
Lysine	68.4	65.0	62.8	70.5
Histidine	26.3	24.9	22.9	21.3
Arginine	46.1	45.9	43.4	44.0
Aspartic acid	99.1	101.9	101.2	102.5
Threonine	63.9	63.1	60.7	62.5
Serine	76.9	84.7	79.1	73.3
Glutamic acid	90.1	87.7	90.5	89.8
Glycine	80.8	83.5	79.4	81.5
Alanine	79.0	80.7	79.4	77.6
Valine	56.9	52.8	56.6	57.5
Methionine	7.7	8.0	8.3	8.8
Isoleucine	40.4	38.5	41.9	47.5
Leucine	101.8	102.6	106.4	99.6
Tyrosine	38.3	36.4	37.6	36.3
Phenylalanine	46.9	47.7	50.9	51.1
Proline	57.0	58.6	58.8	56.7
Half-cystine	20.5	18.7	21.1	20.0

TABLE III

SUGAR COMPOSITION OF EBM GLYCOPROTEINS

The quantity of each sugar is expressed in μg/mg of lyophilized glycoprotein as determined by gas chromatography.

Sugar	NEBM	KEBM
Fucose*	17.40	15.00
Mannose**	18.00	12.30
Galactose	20.60	22.20
Glucose	10.00	10.40
N-Acetylgalactosamine	20.90	21.50
N-Acetylglucosamine**	40.30	23.60
Sialic acid**	1.84	1.36
	129.04	106.36

* Paired *t*-test between NEBM and KEBM gives $P < 0.01$.
** Paired *t*-test between NEBM and KEBM gives $P < 0.001$.

of in vivo EBM glycoprotein. The extraction of murine kidney homogenates with aqueous buffer is sufficient to release substantial amounts of a glycoprotein which is antigenically similar, if not identical to the EBM glycoprotein synthesized by murine neoplastic cells [1]. In contrast to other procedures for the isolation of basement membrane components, extraction with buffer in all probability does not modify the glycoprotein. The isolated glycoproteins eluted from CPG-10-350 appear to be relatively pure since only one peak was demonstrated by chromatography on *P*-cellulose and only a single precipitin band was identified by immunodiffusion. Although considerable contamination by cell membranes was present prior to chromatography on CPG-10-350, as indicated by ATPase activity, no activity was detectable in the column effluent. Mizutani and Mizutani [21] recently reported absorption of protein to controlled-pore glass, and subsequent elution with phosphate buffers failed to remove the absorbed protein. We noted continual accumulation of material on columns of CPG-10-350, which elutes with amino acid buffers and contains considerable ATPase activity.

Comparison of the normal and neoplastic EBM glycoproteins reveals several similarities. In addition to antigenic identity, the amino acid compositions of both glycoproteins are markedly similar. The molecular weights are slightly different, 34 000 for neoplastic EBM glycoprotein and 32 000 for normal EBM glycoprotein, the increased size of the neoplastic glycoprotein apparently due to an increased sugar content.

The acidic nature of these glycoproteins is in agreement with other reports of the amino acid composition of non-collagenous basement membrane glycoproteins isolated from several tissues [5, 22, 23]. Both glycoproteins contain approx. 20 residues of half-cystine per 1000 residues total amino acid. Based on a molecular weight of 32 000 for the kidney glycoprotein monomer, there are 3–4 residues of half-cystine per molecule.

The difference in composition between the normal and neoplastic EBM glycoproteins occurs in their content of mannose, fucose, *N*-acetylglucosamine and

sialic acid. These differences could reflect isolation of glycoproteins from non-identical tissues or alterations accompanying neoplastic change. The literature is non-contributory in assessing the possibility that epithelial cells in various organs synthesize basement membrane glycoproteins which differ in sugar composition. As our studies have shown, the EBM glycoproteins from two murine tissues are markedly similar, tending toward the conclusion that the difference reflects the neoplastic process.

Numerous studies have indicated differences in the carbohydrate content of cell membrane or surface glycoproteins isolated from normal cells and their neoplastic counterpart. Typically, the cell membrane glycoproteins from malignantly transformed cells have less sialic acid and appreciably less neutral and amino sugars than glycoproteins isolated from normal cell membranes as first demonstrated by Wu et al. [24] in 3T3 fibroblasts and virally transformed 3T3 fibroblasts. Failure to complete the oligosaccharide chain is not due to a decrease in the pool of nucleotide sugars [24] nor is it due to a decrease in the activity of glycosyltransferases [25].

There is at present no data comparing the content of sugars in glycoproteins secreted by normal or neoplastic cells. The presence of glucose is somewhat puzzling as it is not commonly found in glycoproteins other than collagen. However, a glycopeptide containing glucose and fucose has been isolated from normal human urine [26]. Common sources of contamination have been excluded. The glycoproteins were not chromatographed on beaded dextrans and samples chromatographed on *P*-cellulose were not used for sugar analysis. Therefore, glucose appears to be a component of EBM glycoproteins.

The functional significance of increased sugar in neoplastic EBM glycoprotein is dependent on further elucidation of the function of normal EBM glycoprotein. The changes observed could possibly interfere with normal interaction between basement membrane collagen and glycoprotein or alter the function of the basement membrane as a filter.

In summary, at least one glycoprotein component of epithelial basement membranes can be obtained from normal mammalian tissues simply by extraction with aqueous buffer. The normal EBM glycoprotein is markedly similar to neoplastic EBM glycoprotein in most respects, but significant differences exist in the sugar composition of the two glycoproteins.

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