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Surface Proteins of a Transitional Carcinoma Cell Line (KS-31E)

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Summary. Biosynthetic and post-synthetic labeling procedures have been used to examine the cell surface proteins of a cell line (KS-31) originating from a human transitional cell carcinoma. One isolate of this cell line (KS-31E) is epithelial in appearance and has retained the characteristic of the original tumour with regard to synthesis of a viscous mucin-like material. A 220-250, 000 (250K) dalton large external transformation-sensitive (LETS) glycoprotein, and often a 95, 000 (95K) dalton glycoprotein, is released into the medium of KS-31E in association with the mucin-like material produced by the cell line. The finding of LETS and a 95K glycoprotein expressed on the cell surface and extruded into the growth medium of epithelial cells originating from a spontaneous human carcinoma is of interest in relation to the role of cell surface glycoproteins in normal cellular association and the antisocial behaviour exhibited by cancer cells.

Key words: LETS - Protein association - Transitional cell carcinoma

INTRODUCTION

Probably the most extensively examined cell surface protein is the large (220-250K dalton) external transformation-sensitive protein. This glycoprotein is also known as fibronectin (13) cell surface protein (27), large external transformation-sensitive protein (10) and cold insoluble globulin (17) and has been exhaustively investigated in avian and rodent fibroblast systems in relation to its loss following viral transformation (11). However, studies on the occurrence of LETS on epithelial cells (3) have been scarce and investigations concerning the expression and role of LETS per se and the relation of LETS to other cell surface proteins on human cancers of epithelial origin are essentially non-existent (19).

The present study demonstrates that LETS and a 95K protein occur on the cell surface of KS-31E. LETS constitutes over 50% of total labeled protein released into the culture medium of KS-31E following metabolic labeling. A 95K glycoprotein also is often excreted into the medium and represents approximately 25% of total labeled medium protein. Evidence, although indirect, indicates that the 95K glycoprotein is released into the medium in association with LETS. LETS and the 95K protein are most likely released from the cell surface in a supramolecular association with the mucin-like material characteristic of this cell line.

MATERIALS AND METHODS

Tissue Culture

Tumour tissue from a large transitional cell carcinoma was mechanically dispersed into single cells and small clumps which were seeded into a single 60 mm dish by the "spillout" method (15) and were subsequently transferred into collagen coated petri dishes (8). The growth medium used was medium 199 (Gibco) supplemented with 20% fetal bovine serum (Gibco), 62.5 units/ml penicillin G (Squibb), 50 µg/ml streptomycin sulphate (Eli Lilly.) and 0.5 µg/ml fungizone (Squibb). Subculture was accomplished by 7-10 min incubation with 0.025% trypsin-1 mM ethylene diamine tetra acetate (EDTA) in calcium- and magnesium-free phosphate buffered saline (PBS), pH 7.4. Cells were subcultured at 1:5 to 1:20 dilutions depending on the growth rate at the time of subculture.

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Karyotypic Anaysis

For chromosome studies, cells in logarithmic growth were treated with colchicine at a final concentration of 0.5 μg/ml for two hours. After harvesting with trypsin, the cells were treated with hypotonic culture medium (growth medium diluted with three volumes of water) and fixed in methanol-acetic acid (3:1); the cell suspension was dropped on ice-chilled slides which were then air-dryed. G-banding was obtained by the trypsin-Giemsa procedure (24).

Labeling Procedures, Membrane Isolation

Metabolic Labeling. Four to eight days after subcultivation, confluent cultures were washed twice with phosphate buffered saline (PBS) and then re-incubated with medium containing ^{14}C-glucosamine (5 μC/ml; 10 m C/mmole), or ^{3}H-leucine (5 μC/ml; 40-60 C/mmole). Following incubation at 37°C for 5 days, the medium and cell layer were separated. Labeled proteins in the medium were collected by dialysis against Earle's phosphate buffered saline followed by dialysis against distilled water at 4°C; the resulting precipitate was pelleted by low speed centrifugation and then solubilized for analyses by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) or by isoelectric focusing as described below. The cells were washed with PBS, scraped from the growth vessel, and collected by centrifugation (3500 x g/15 min). Membranes were isolated by a two-phase polymer system (25) modified from Brunette and Till (2).

Lactoperoxidase Iodination. KS-31E cell surface proteins were labeled with ^{125}I (carrier free; 1 mC per 100 x 20 mm culture dish) as described by Hynes and Bye (12). After labeling, the cells were washed with 0.1 M sodium phosphate buffer, pH 7.4 and the dish overlaid with chilled phosphate buffer containing the protease inhibitor Trasylol (Mobay Chemical Corp.) at a concentration of 1000 KIU/ml or buffer. The cells were collected by scraping with a rubber policeman and centrifugation (3500 x g/15 min). The cell pellet was resuspended in 50-100 μl of distilled water and stored at -20°C until solubilization for electrophoretic analysis.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide (30% acrylamide, 0.8% bisacrylamide stock solution) slab gel electrophoresis was modified from Laemmli (14) and carried out as previously described (26). Unless otherwise stipulated, a 3-20% gradient gel was used for separation of labeled proteins; a linear separation of proteins over a 260,000 to 16,000 dalton molecular weight range was routinely achieved (Fig. 1). Molecular weight references included BDH oligimers (56K-280K; Gallard-Schlesinger), cross-linked BSA (66K-264K; Sigma), cross-linked haemoglobin 16K-64K; Sigma), B-galactosidase (135K), phophorylase a (95K), pepsin (34K), and trypsinogen (24K); a wide variety of marker proteins were used so that any discontinuties in the gradient gel could be readily detected. Labeled cells, isolated cell membranes, or precipitates resulting from dialysis of the culture supernatant were prepared for electrophoresis by mixing the sample and solubilization buffer in a v/v ratio of 1:2 to the following final concentrations: 0.5-5 mg protein/ml, 6.6% glycerol (v/v), 3.3% 2-mercaptoethanol (v/v), 2% SDS (w/v), 0.0625M Tris-HCl, pH 6.8, 0.015% bromphenol blue. The sample-solubilization buffer mixtures were heated at 100°C for 2-3 min. Following electrophoresis, the slab gels were stained and destained as previously described (26). Gel lanes containing ^{125}I-labeled proteins were sliced into 1 mm sections before counting. One mm slices from lanes containing ^{14}C- or ^{3}H-labeled proteins were solubilized at 37°C for 12 hours in a Protosol-Econofluor (NEN) mixture prior to counting.

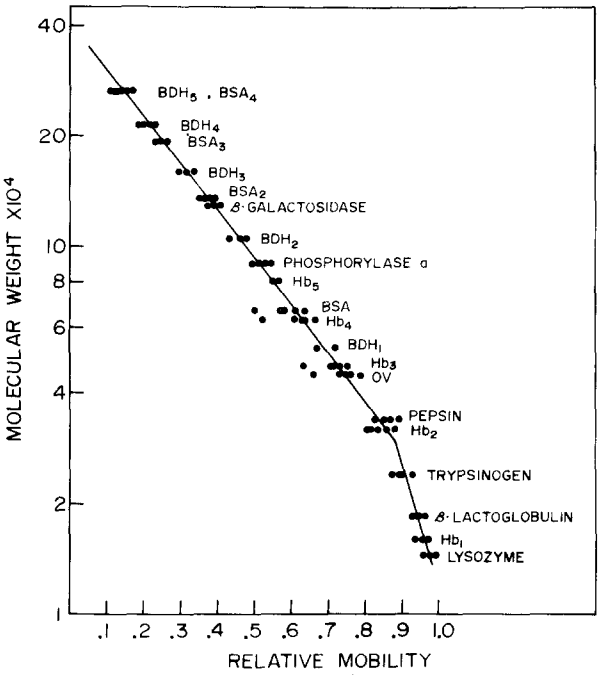

Fig. 1. Relative mobility of marker proteins on 3-20% gradient gels run on five different dates over a three month period. The gradient is reproducibly linear with a break at 30,000 daltons. Some scatter is apparent for marker proteins in the 70-40K MW range

Isoelectric Focusing

Focusing was done at 4°C in 0.5 x 10 cm cylindrical gels. The electrofocusing gel was 3.75% acrylamide (30% acrylamide, 0.5% bisacrylamide, stock solution), 8M urea, 0.075% ammonium persulfate, 2% (w/v) Ampholine (LKB) carrier ampholytes. Cathodal and anodal electrode solutions were 0.4% ethanolamine and 0.4% sulfuric acid, respectively. Hemoglobin marker protein was run on companion gels to monitor the progress of focusing. The cold insoluble globulin (CIG) column eluate was prepared for focusing by mixing 10 µl (0.5 µg/µl) CIG and 10 µl (0.2 µg/µl) ^{125}I labeled CIG (2.5 x 10^4 cpm/µg) in a buffer 8 M in urea, 5% 2-mercaptoenthanol, and 10% glycerol. Samples were heated at 100°C for 2 min and, after cooling to room temperature, the sample buffer was made 2% in ampholytes. Samples were applied after the gels had been pre-focused at 100V for 30 min. Voltage was increased at 50V increments to a maximum of 50V/cm gel length until the conductivity drop stabilized. Since the hemoglobin marker routinely focused prior to a plateau in conductivity, the later parameter was used as a criterion that focusing was complete. After focusing, the haemoglobin marker gels were sliced into 2 mm sections and placed into 1 ml distilled water which had been previously boiled to remove CO_2 which interfered with pH measurements; the ampholytes were allowed to elute at 4°C overnight and the pH gradient was measured the following morning. Gels containing labeled proteins were sliced into 2 mm sections and counted.

Immunoelectrophoresis

Immunoelectrophoresis was carried out at pH 8.6 in a barbital buffer system (8.25 g sodium barbital, 1.6 g barbital per liter) or at pH 5 using a TEMED-acetate buffer (0.01 mole, N, N, N, N'-tetramethylenthylene-diamine, 0.029 mole acetic acid per liter). One percent Seakem agarose (Marine Colloids, Inc.) was used.

CIG Isolation

Cold insoluble globulin was isolated from normal human plasma by a modified method of Engvall and Rouslahti (5). Gelatin (Sigma) was coupled to CM-BioGel A (Biorad) by means of the water-soluble carbodiimide, 1-ethyl-3 (3-diethylaminopropyl) carbodiimide-HCl (EDAC) (Biorad). Twenty mg of gelatin was coupled to 1 ml of BioGel using an EDAC concentration of 12 mg/ml bed volume of gel. The amount of gelatin coupled to the column was determined by a Coomassie blue dye binding assay (Biorad).

Affinity chromatography of human plasma was carried out at room temperature on a 1.5 x 10 cm column of gelatin - BioGel equilibrated with PBS, pH 7.2 Adsorbed material (CIG) was eluted with PBS/6M urea. The elution profile was monitored at 280 nm by a Gilson UV recorder. Adsorbed material was shown to be immunologically and electrophoretically pure. In those cases where the column eluate was radiolabeled for electrophoretic analysis, the chloramine-T method of Greenwood et al. (6) was used.

Antisera

Antiserum to human CIG was produced in rabbits by subcutaneous multiple site injections of CIG (0.25-0.5 mg) in incomplete adjuvant twice monthly. Rabbits were bled, 5, 7, and 9 days after each boost.

RESULTS

The cell line KS-31E was derived from a single grade IV transitional cell carcinoma in an 81 year old female. As in the tumour itself, the resulting cultured cells showed evidence of active mucin production. The original tumour stained significantly for acid mucopolysaccharide in histological section (Fig. 2a). KS-31E is epithelial in appearance with large surface-type cells (Fig. 2b) and has undergone over 65 passages at present. Cell line KS-31 started as a very pleomorphic group of cells. At about 35 transfers, one isolate of these cells (KS-31E) began to appear more epithelial and uniform than the other cells (KS-31P). Results of experiments with KS-31E are reported here.

KS-31E is a very stable cell line with regard to its karyotype. The cell line possessed a constant near triploid modal chromosome number of 71 (range 60-84) when examined at passages 20, 37, and 43 (16); a constant hypotriploid number of chromosomes (66-68), with a small reduction in modal number, was observed at passage 63. Detailed karyotypic analysis by G-banding (24) revealed some marker chromosomes. A representative karyotype at passage 63 is shown in Figure 3. The morphology and growth characteristics of cell line KS-31E are described elsewhere (16).

Evidence that the 250K and 95K proteins are produced in association with the mucin-like material characteristic of the cell line is indirect. Metabolic labeling of KS-31E cultures with amino acids followed by prolonged dialysis of the culture medium against distilled water resulted in the formation of a significant precipitate with a concomitant decrease in the viscosity of the medium. Solubilization of the precipitate

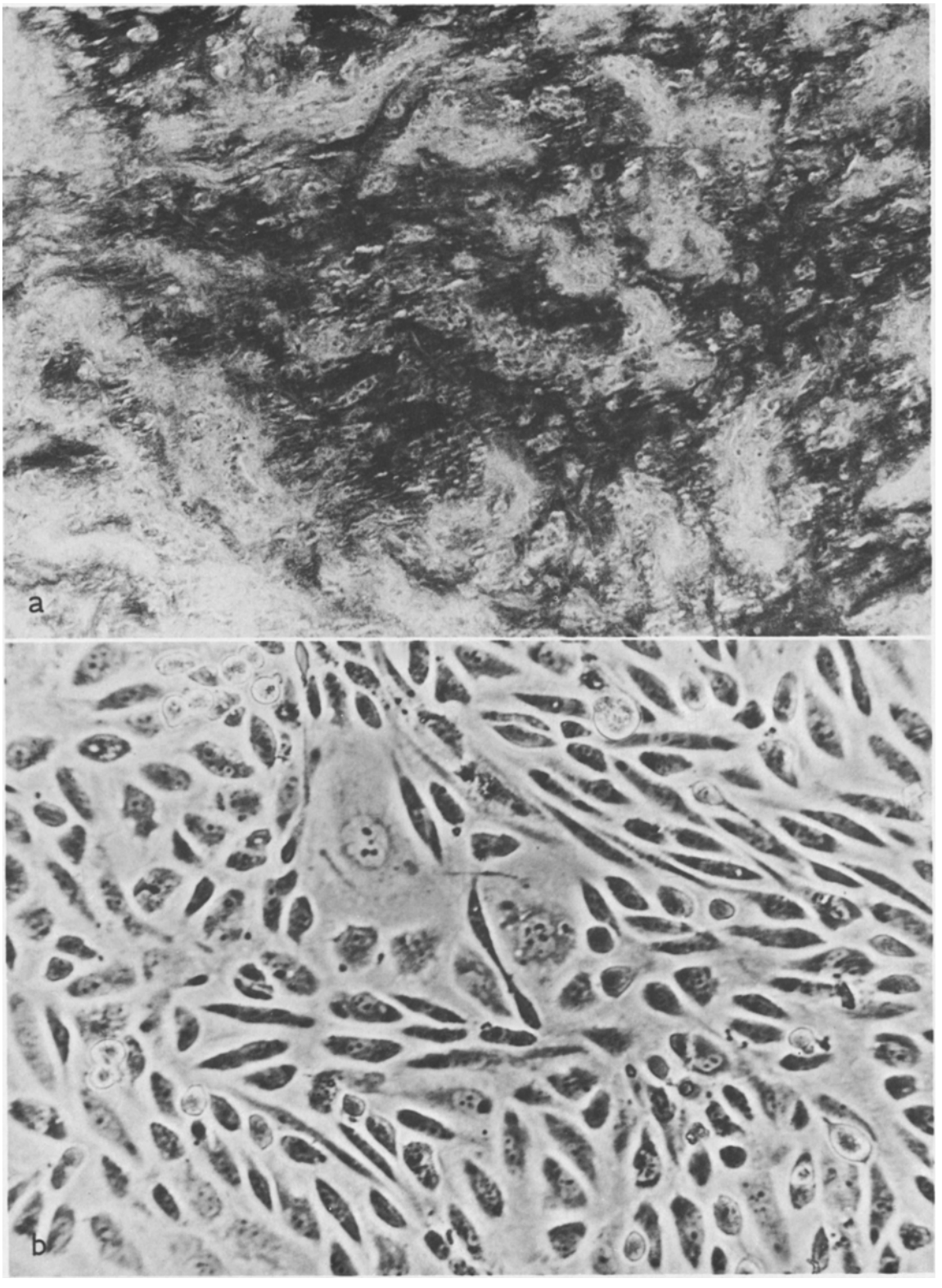

Fig. 2. a (TOP). Original tumour stained with H&E and counterstained with colloidal iron. Colloidal iron staining is prominent in interstitial areas. x 235. b (BOTTOM). Phase contrast micrography of KS-31E at passage 67. x 150

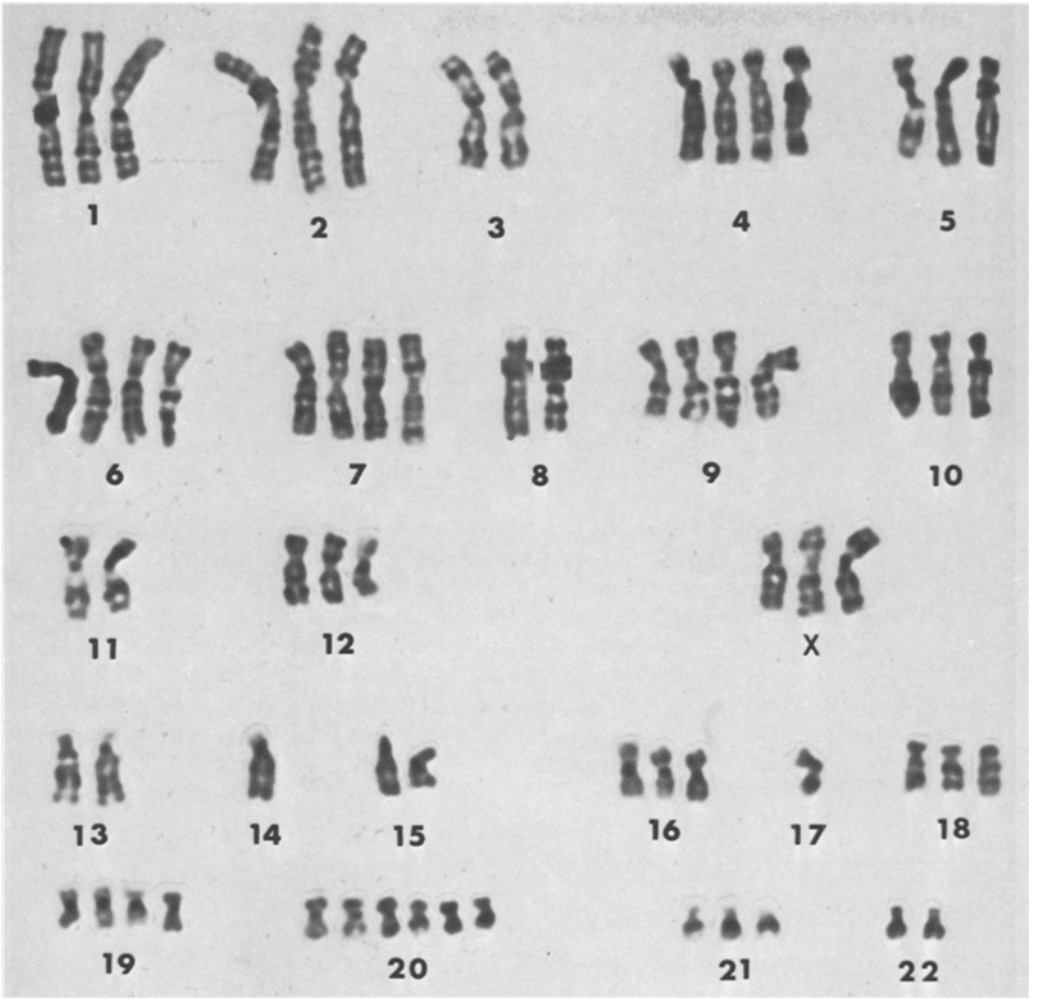

Fig. 3. G-banded karyotype of KS-31E at passage 63 showing abnormal marker chromosomes

and subsequent SDS-PAGE analysis revealed two labeled peaks of 250K daltons and 95K daltons (Fig. 4); the 250K and 95K peaks represent 58% and 27%, respectively, of the total gel counts.

The glycoprotein nature of the 250K and 95K proteins was ascertained by labeling of cultures with ^{14}C-glucosamine followed by harvesting of cells and membrane isolation. The labeled membrane proteins were analysed by SDS gradient gel electrophoresis; the resulting protein profile is shown in Figure 5. Labeled protein of molecular weight exceeding 220K is considered to be disulphide cross-linked LETS. Yamada et al. (28) have reported LETS to migrate as a multimeric complex; migration as the 220K monomer is contingent upon reducing and alkylating conditions employed. The 95K glycoprotein could invariably be detected on the cell surface of KS-31E by metabolic labeling with leucine (Fig. 6) or with amino acid hydrolysate, but could not always be detected with glucosamine metabolic labeling nor with lactoperoxidase catalysed iodiniation of cultured cells (Table 1).

Several criteria were used to identify the 220-250K dalton protein as cell surface LETS. The molecular weight of the protein as determined by SDS gel analysis is consistent with that of LETS and the protein co-electrophoresed with chick embryo fibroblast LETS (data not shown). The protein was accessible to lactoperoxidase-catalyzed iodiniation, a procedure capable of tagging cell surface proteins without penetrating the plasma membrane (10). The quantity of the 220-250K dalton protein was dependent on culture density. The expression of LETS on the cell surface of fibroblasts is generally density dependent (12). When KS-31E cultures at 35% confluency were lactoperoxidase iodinated, the LETS peak comprised 10% of the total gel counts. Lactoperoxidase labeling of a subculture at 95% confluency showed an approximate two-fold increase (18%) in the amount of LETS to total labeled membrane protein. The 95K protein remained constant in relation to total labeled protein at both levels of confluency. Cell surface LETS of KS-31E does not exhibit a density dependence of the same mag-

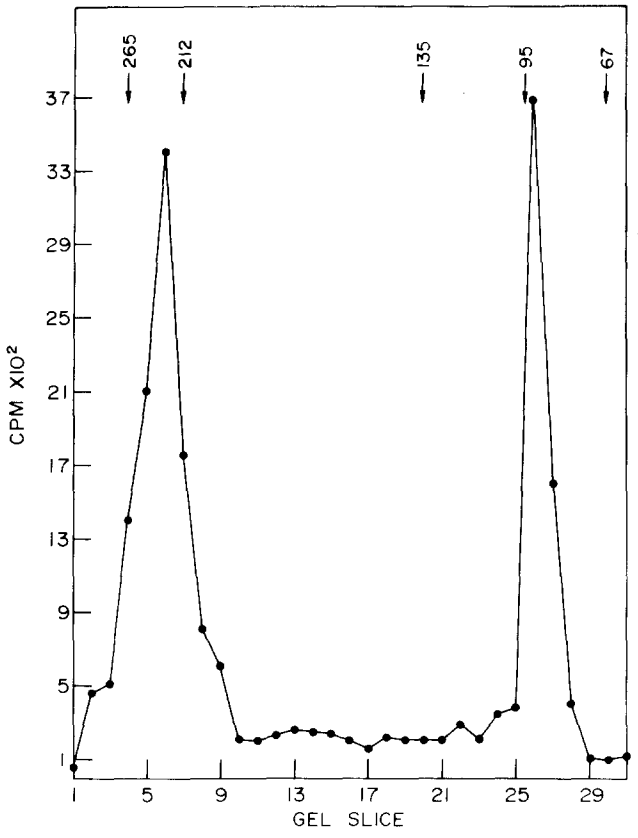

Fig. 4. KS-31E (T 37) cultures were labeled with ^{14}C amino acid hydrolysate (5 μC/ml medium) for 5 days. The precipitate was obtained by dialysing the cell culture against distilled water and then solubilized and analysed on a 7.5% SDS polyacrylamide gel. Molecular weight markers shown are BDH pentamer (262K), B-galactosidase subunit (135K), phosphorylase a (95K), and bovine serum albumin (67K). Gel slices were 2 mm

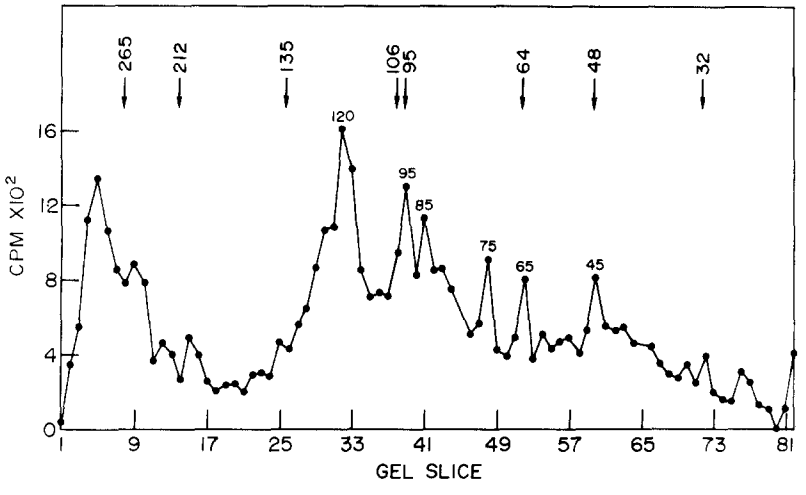

Fig. 5. KS-31E (T 40) cultures were labeled with ^{14}C-glucosamine (5 μC/ml medium) and the isolated cell membranes were analysed on a 3-20% gradient gel. Molecular weight markers (x 10^3) are as indicated. Gel slices were 1 mm

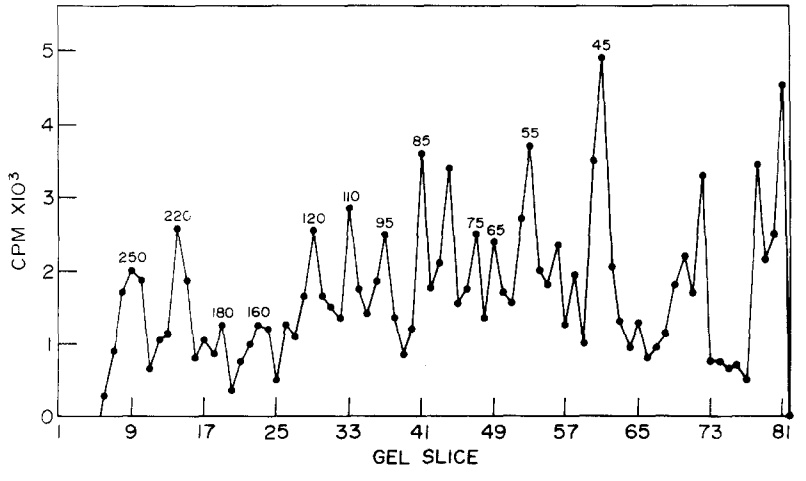

Fig. 6. KS-31E (T 41) cultures were labeled with ^{3}H-leucine (5 μC/ml medium) and the isolated membranes analysed on a 3-20% gradient gel. Gel slices were 1 mm

Table 1. Differential behavior of LETS and 95K glycoprotein

	LETS	95K
Lactoperoxidase iodination	+	+ or -
Glucosamine metabolic labeling	+	+ or -
Leucine or amino acid hydrolysate metabolic labeling	+	+
Release into culture medium	+	

nitude of that reported for fibroblast cells. Baum et al. (1) reported a fivefold increase of cell surface LETS on human fibroblasts between the first and fifth days after subculture. A massive fibril-like LETS protein network is characteristic of confluent fibroblast cultures; epithelial cells, however, appear to be unable to build up such a structure (3).

Examination of the binding properties of LETS show that this protein readily binds to fibrinogen (22), to collagen (18), and to gelatin, or random-coil collagen (5). Cold insoluble globulin, the plasma form of LETS (17, 22) was isolated from normal human plasma by preparative affinity chromatography on carboxymethylated Bio-Gel to which gelatin had been coupled. The specificity of column binding was established by SDS-PAGE analysis and by isoelectric focusing of the column eluate. SDS-PAGE of radiolabeled CIG showed a single peak with a molecular weight of 220-250K daltons (Fig. 7a); electrofocusing of the column urea eluate also revealed a single peak with an isoelectric point of 5.5-6.0 (Fig. 7b).

Non-ionic detergent extracts of KS-31E cells and the conditioned culture medium were tested immunoelectrophoretically (pH 8.6) with an antiserum raised against chromatographically isolated plasma CIG. A single precipitin band in the beta region of the immunoelectropherogram was recognized in KS-31E extracts and conditioned culture medium; a beta mobility is characteristic of LETS (9). When KS-31E extracts or culture supernatant were immunoelectrophoresed at pH 5, the LETS precipitin band remained at the origin (gamma region) of the agarose gel; this result is consistent with the pH 5.5-6.0 isoelectric point obtained for chromatographically purified CIG. The antiserum raised against CIG isolated from human plasma did not recognize CIG in fetal bovine serum, thus precluding the possibility that the precipitin band obtained upon immunoelec-

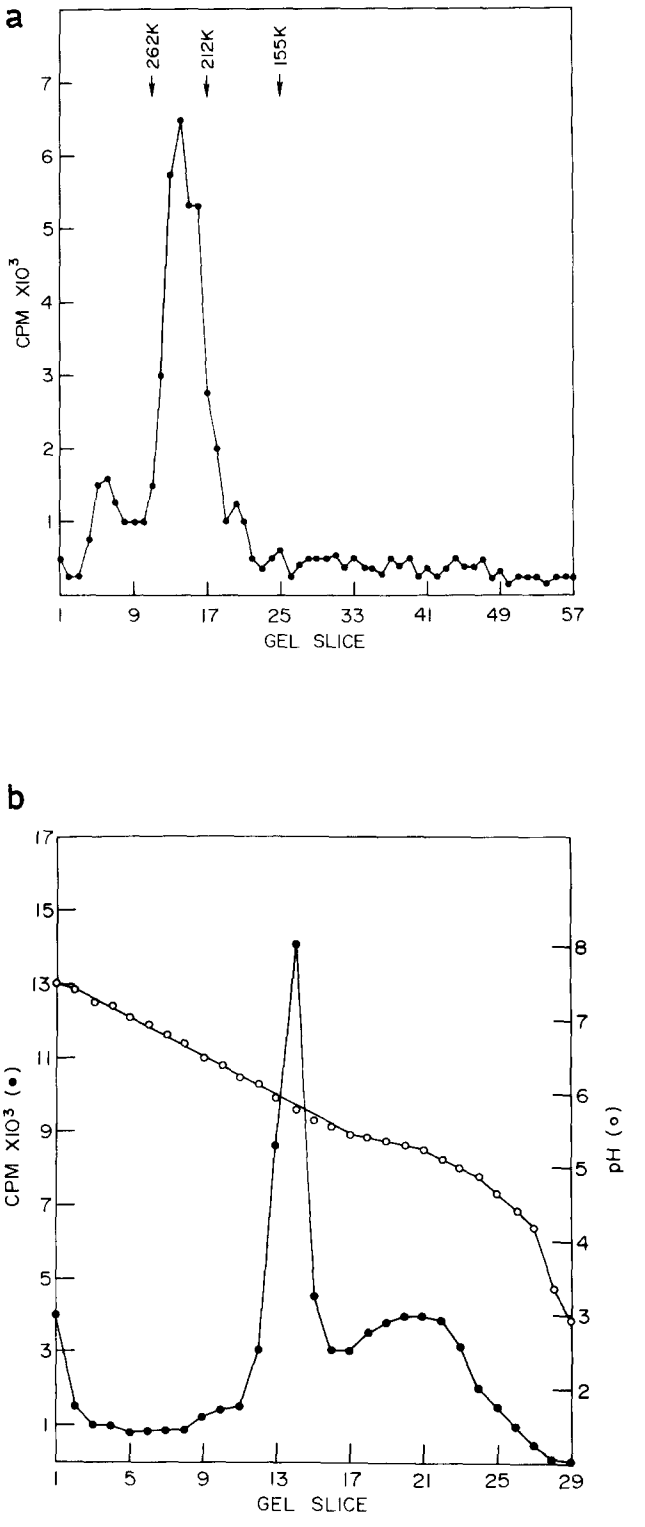

Fig. 7. a (TOP) 3-20% gradient gel analysis of of gelatin affinity column eluate. Gel slices were 1.5 mm. b (BOTTOM) Isoelectrofocusing profile of affinity column urea eluate. The focusing gel used an ampholyte range of 3.5-10, supplemented with pH 4-6 narrow range ampholytes. The skewed peak at the anodal end of the gel represents charge heterogeneity of CIG induced by the chloramine-T labeling procedure. Gel slices were 2 mm

trophoretic analysis of KS-31E cell extracts or conditioned medium was a serum component. Phillips and Perdue (20) have reported on cell surface antigenic proteins derived from the fetal bovine serum used in the culture medium. Interestingly, antiserum to human CIG did recognize CIG in adult bovine serum. CIG present in fetal bovine serum, although not recognized by antiserum to human CIG, did bind to the gelatin affinity column and thereby complicated attempts to isolate LETS from the KS-31E culture supernatant by affinity chromatography.

DISCUSSION

The presence of LETS and a 95K glycoprotein has been demonstrated on the cell surface of a transitional bladder carcinoma cell line and it has been shown that these proteins are extruded into the culture medium, possibly in a supramolecular association with the viscous mucin-like material produced by the cell line. The original patient tumour produced large amounts of viscous material most likely acid mucopolysaccharide in nature, as evidenced by the prominent colloidal iron staining in histological sections. Dermer and Kern (4) used colloidal iron to stain for sialic acid residues at the surfaces of transitional bladder epithelium and observed that higher grade invasive bladder carcinomas exhibit a marked reduction in affinity for colloidal iron, as compared to non-invasive grade I transitional cell carcinomas. The tumour from which KS-31E was derived was a grade IV transitional cell carcinoma and yet stained prominently for colloidal iron.

By far the majority of experiments on LETS protein have been carried out on fibroblastic cells and whether or not such research can be extrapolated to the understanding of human cancer, which is mostly of epithelial origin, is questionable. Pearlstein et al. (19) surveyed a variety of normal and transformed cells in an attempt to correlate the amount of cell surface LETS with the extent of fibrinolytic activity exhibited by the cells; four spontaneous human carcinoma cell lines, including bladder cell lines RT4, J82, and T24, were examined and all were negative for LETS. Pearlstein et al. did find high levels of LETS, consituting 10-20% of total radioactivity, on primary explants of human prostate. During the course of studies reported here, similarly high levels of LETS were found on primary explants of prostate tissue; however, none of these cells were able to be maintained beyond 10-15 subcultures (Webb et al., unpublished observations). The level of LETS on the surface of cell line KS-31E constitutes 10-20% of total proteins labeled, the absolute amount being dependent on culture confluency.

Chen et al. (3) have stressed the importance of establishing whether or not LETS protein is present on the surface of normal epithelial cells and of determining whether or not this protein is quantitatively reduced in tumourigenic epithelial cells. Unfortunately, the normal counterpart of malignant surgical specims initiated in culture is not always available, or , if available, can only be maintained in culture for a few passages. It is pertinent, however, to investigate possible mechanisms mediating the release of LETS from the surface of neoplastic cells, particularly in regard to its loss as a result of specific interaction with other cell surface proteins.

The significance of the 95K glycoprotein on the cell surface of KS-31E and its relation with LETS extruded into the medium is conjectural. Grinell et al. (7) have reported on a high molecular weight glycoprotein - cell adhesion and spreading factor - in fetal bovine serum. This factor contains two active globular glycoprotein components; the large component has a molecular weight of approximately 215K daltons and the small component is a mixture of 94K, 80K, and 70K dalton proteins. The 250K and 95K proteins of KS-31E are not fetal bovine serum components since both proteins can invariably be metabolically labeled with amino acids. That the 95K protein reported here is not a LETS subunit cannot be entirely dismissed but it does not seem likely since the 95K protein did not show the density dependence exhibited by LETS; also, antiserum to LETS did not recognise a 95K protein in either KS-31E extracts or in culture medium.

The 95K protein of KS-31E seems similar in its behaviour to the glucose-regulated fibroblast cell surface glycoprotein described by Pouyssegur and Yamada (21). This 97-95K glycoprotein is of interest because it can exist in the plasma membrane in two molecular forms and its synthesis can be regulated either by the extracellular glucose concentration or by the capacity of cells to glycosylate proteins. The nonglycosylated form remains accessible to antibodies but its membrane conformation precludes lactoperoxidase catalyzed iodination, presumably by conformation-induced masking of tyrosine residues. Further experiments will be necessary to determine if the glycosylation state of the 95K protein affects its release from KS-31E membranes.

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Note added in proof:

During the time elapsed between submission of this manuscript and its publication, other investigators (Smith, H. et al: Production of fibronectin by human epithelial cells in culture. Cancer Research 39, 4179, 1979) have reported on LETS expression on human epithelial cell lines derived from both normal and malignant tissue.