Purification and partial characterization of a tumour-metastasis-associated high- M_r glycoprotein from rat 13762NF mammary adenocarcinoma cells

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The expression of a high- M_r sialogalactoprotein (gp580) on rat 13762NF mammary adenocarcinoma cells was identified and correlated with spontaneous metastatic potential to colonize lung [Steck & Nicolson (1983) *Exp. Cell Res.* **147**, 255–267]. Using a highly metastatic tumour-cell clone, MTLn3, we isolated and characterized gp580 from cells growing *in vitro* and *in vivo* in the mammary fat-pads of Fischer 344 rats. The glycoprotein was extracted with 4 M-guanidinium chloride/4% Zwittergent 3-12 solution in the presence of proteinase inhibitors. The extracts were then subjected to dissociative CsCl-density-gradient centrifugation, gel filtration on Sepharose CL-2B columns and ion-exchange chromatography on DEAE-Sephacel. The isolated glycoprotein possessed low electrophoretic mobility in SDS/polyacrylamide gels, and after desialylation bound ^{125}I -labelled peanut agglutinin. Electrophoresis of gp580 in polyacrylamide-gradient gels resulted in a diffuse but homogeneous migrating band of M_r approx. 550 000. After removal of carbohydrate, gp580 was demonstrated to have a protein core of M_r approx. 150 000. The gp580 had a high density (1.430 g/ml) on isopycnic centrifugation in 4 M-guanidinium chloride and was resistant to most proteinases and other degradative enzymes, suggesting a mucin-like structure. Amino acid and carbohydrate analyses revealed that gp580 has high contents of serine, threonine, glutamic acid, aspartic acid, glucosamine and galactosamine; several acidic and neutral oligosaccharides were obtained from alkaline-borohydride digests. Cellular localization studies suggested that gp580 is associated mainly with the cell-surface and extracellular-matrix fractions of MTLn3 cells.

INTRODUCTION

Breast cancer is a leading cause of female deaths in North America and northern Europe (Kelsey, 1979). Although recent advances have significantly improved the rates of detection and eradication of primary breast cancers, a large percentage of breast-cancer patients will develop metastatic lesions and eventually die (Denoix & Mather, 1979).

The process of tumour metastasis occurs by means of a number of sequential and highly selective steps (Sugarbaker, 1979; Nicolson & Poste, 1983). Many of these steps require a number of complex interactions between the tumour cells and the host environment, and the majority of these interactions appear to be mediated by cell-surface components (Nicolson, 1982, 1984). To investigate the possible functional roles of certain cell-surface components in the metastatic process, various researchers have correlated the expression or enzymic activities of these components with the metastatic potential of tumour-cell sublines or clones (Chatterjee & Kim, 1978; Liotta *et al.*, 1980; Raz *et al.*, 1980; Sloane *et al.*, 1981, 1982; Nakajima *et al.*, 1983). Alternatively, the surfaces of tumour cells have been modified metabolically or enzymically and the metastatic properties of the modified cells examined (Fidler, 1978; Poste & Nicolson, 1980; Irimura *et al.*, 1981).

We have begun investigating cell-surface components

of the spontaneously metastasizing rat 13762NF mammary adenocarcinoma to examine possible biochemical markers associated with breast-cancer metastasis (Steck & Nicolson, 1983). These cells share several important characteristics with human breast cancer, including similar pathological development of metastases (Neri *et al.*, 1982). We have obtained various cell clones and lines from the 13762NF system that differ in their abilities to metastasize spontaneously to regional lymph nodes and distant organs. We have been especially interested in the expression of two glycoproteins that correlate with the metastatic potential of the 13762NF tumour cells. One of these, a sialoglycoprotein of M_r approx. 80 000 (gp80), was decreased in expression in more metastatic cells; another, a high- M_r (approx. 580 000) glycoprotein (gp580), was increased in expression in highly metastatic cells (Steck & Nicolson, 1983). We now describe here the purification and partial characterization of the metastasis-associated glycoprotein gp580 from tumour cells grown *in vitro* and *in vivo*.

EXPERIMENTAL

Materials

D-[6- ^3H]Glucosamine (20–30 Ci/mmol), D-[1- ^3H]galactose (1–5 Ci/mmol), L-[6- ^3H]fucose (20–35 Ci/mmol), L-[4,5- ^3H]leucine (30–50 Ci/mmol), L-[6- ^3H]serine

Abbreviation used: PAGE, polyacrylamide-gel electrophoresis.

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(5–10 Ci/mmol), L-[U- 14 C]serine (135–165 mCi/mmol) and $\text{Na}_2^{35}\text{SO}_4$ were purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.); D-[2- ^3H]mannose (10–20 Ci/mmol) and Na^{125}I were from New England Nuclear (Boston, MA, U.S.A.); *Streptomyces* hyaluronidase and chondroitinase ABC were from ICN Biochemicals (Miles Laboratories, Elkhart, IN, U.S.A.); collagenase type VII was from Sigma Chemical Co. (St. Louis, MO, U.S.A.); trypsin (crystallized three times) was from Worthington Corp. (Freehold, NJ, U.S.A.); Pronase was from Calbiochem (San Diego, CA, U.S.A.); α -modified minimum essential medium was from GIBCO (Grand Island, NY, U.S.A.); foetal bovine serum was from Sterile Systems (Logan, UT, U.S.A.); CsCl was from Bethesda Research Laboratories (Rockville, MD, U.S.A.). All other reagents were of the highest commercial grade obtainable.

Cells and culture conditions

The rat 13762NF tumour-cell clone MTLn3 of high metastatic potential was used in these studies. MTLn3 cells were grown at 37 °C in an atmosphere of 5% CO_2 in humidified air in α -modified minimum essential medium containing 10% (v/v) foetal bovine serum and no antibiotics in 100 mm-diam. tissue-culture plates (Corning). The cells used in this study were in exponential growth phase and were from passages T14 to T20 *in vitro*. Cells were routinely screened and found to be free of mycoplasma and viral contamination (Chen, 1976).

Mammary tumours were obtained by subcutaneous injection of 1×10^6 viable MTLn3 cells into the inguinal mammary fat-pad of lightly anaesthetized syngeneic female Fischer 344 rats (Charles River Breeding Laboratories, Portage, MI, U.S.A.) (Neri *et al.*, 1982). This procedure resulted in tumours with a diameter of 11.7 ± 4.6 mm 30 days after injection, with 100% and 50% of tumour-bearing animals having lymph-node and pulmonary metastatic lesions respectively. Because the centre of the locally growing tumour was necrotic at 30 days, the majority of such tumours were harvested at 14 days.

Metabolic radiolabelling of cells grown *in vitro* was accomplished in complete medium (α -modified minimum essential medium containing 10% foetal bovine serum; 5 ml in a 100 mm-diam. culture plate) containing 10 μCi of [^3H]glucosamine, [^3H]galactose or [^3H]fucose/ml for 24 h to label the carbohydrate moieties. In the experiments in which the cells were labelled with [^3H]mannose, 200 μCi /ml was added to complete medium in which the α -modified minimum essential medium contained one-half of the normal concentration of D-glucose (1 g/l). To prepare cells labelled with [^3H]leucine, [^{14}C]serine or [^3H]serine, the medium contained 10 μCi of the radioactive precursor/ml and one-tenth the usual concentration of the amino acid (5.25 mg/l and 2.5 mg/l respectively). The MTLn3 cells were labelled with 50 μCi of $\text{Na}_2^{35}\text{SO}_4$ /ml in α -modified minimum essential medium in which the MgSO_4 (80.9 μmol) was replaced with MgCl_2 . After the 24 h labelling period, the medium was collected, centrifuged at 4 °C for 10 min at 2000 *g* to remove cellular debris, and frozen at –80 °C. The culture plates were washed twice with phosphate-buffered saline (0.15 M- NaCl /8 mM-sodium phosphate buffer, pH 7.2) and the cells extracted as described below.

Isolation of gp580

The extraction of gp580 was accomplished by using procedures described by Hascall & Kimura (1982) for proteoglycans and outlined in Scheme 1. The material eluted at the excluded volumes of the Sephadex G-50 columns was prepared for isopycnic dissociative density-gradient centrifugation by the addition of CsCl (0.55 g of CsCl/g of extract; Hascall & Kimura, 1982). The samples were centrifuged at 35000 rev./min in a Beckman SW 50.1 or Ti 50.2 rotor for 48–72 h at 9 °C. The gradients were divided into eight equal fractions, and the density and radioactivity, if any, of each fraction were determined. The fractions were then pooled to give four equal-volume fractions, D1–D4 [the nomenclature indicates the fractions recovered from the bottom (densest) to the top of dissociative CsCl-density-gradient ultracentrifugation in 4 M-guanidinium chloride (Hascall & Kimura, 1982)], and each fraction was then analysed by gel-filtration chromatography. Certain radiolabelled samples of purified gp580 were subjected to isopycnic centrifugation by mixing [^{14}C]serine-labelled gp580

Scheme 1. Schematic diagram of the procedures used in the isolation procedure of gp580

The cells were solubilized (approx. 5×10^6 cells per dish) by adding 1 ml of extraction buffer (containing 4% Zwittergent 3-12, 4 M-guanidinium chloride, 0.1 M-6-aminohexanoic acid, 10 mM- Na_2EDTA , 5 mM-phenylmethanesulphonyl fluoride, 5 mM-benzamidine hydrochloride, 2 mM-N-ethylmaleimide, 10 μg of leupeptin/ml, 10 munits of aprotinin/ml and 0.1 M-sodium acetate buffer, pH 6.0) per five dishes. The plates were scraped, extracts were combined and the solubilization was continued at 4 °C for 18 h with stirring. Isolation of gp580 from tumours was accomplished by finely mincing the tissues and then extracting with 5 vol. of extraction buffer per g wet wt. of tissue. The extracts were frozen at –80 °C or immediately used after filtration through Whatman no. 1 paper. The filtrate was then subjected to gel filtration on Sephadex G-150 columns (either 10 cm $\times$ 1 cm or 50 cm $\times$ 4 cm) and equilibrated with extraction buffer minus the Zwittergent.

(1×10^4 c.p.m.) with 4 M-guanidinium chloride and 4.3 M-CsCl (or 0.5 M-guanidinium chloride and 6.5 M-CsCl) in 10 mM-Tris/HCl buffer, pH 7.2. The samples were centrifuged at 35000 rev./min in a Beckman SW50.1 rotor for 60 h at 4 °C and then separated as described by Sherblom *et al.* (1980) into 20 equal fractions.

Column chromatography

Portions of the D1–D4 fractions were applied to calibrated Sepharose CL-2B columns (115 cm $\times$ 0.8 cm) equilibrated with 4 M-guanidinium chloride in 0.1 M-sodium acetate buffer, pH 5.8. The fractions were pooled, dialysed extensively against deionized water and freeze-dried. Certain pooled fractions were redissolved in 8 M-urea and 0.2% CHAPS {3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonate} in 50 mM-Tris/HCl buffer, pH 7.2, and applied to a DEAE-Sepacel column (5 cm $\times$ 1 cm) in the same buffer. The column was washed and eluted with a linear gradient of 0–1 M-NaCl. After completion of the gradient, the column was washed with 8 M-urea containing 3 M-NaCl. The recovery of radioactivity was greater than 90%. The fractions were pooled according to their radioactivity, dialysed against water and freeze-dried. Alternatively some fractions were loaded on a Sepharose CL-2B column (115 cm $\times$ 0.8 cm) which was equilibrated and eluted with 1% SDS and 5 mM-2-mercaptoethanol in 10 mM-Tris/HCl buffer, pH 7.2. Samples used for carbohydrate analysis were applied to a Bio-Gel P6 or a Bio-Gel P4 column (115 cm $\times$ 0.8 cm) equilibrated with 50 mM-pyridine/acetate buffer, pH 5.3.

Gel electrophoresis

SDS/PAGE was performed according to the method of Laemmli (1970) with a 7.5% polyacrylamide running gel and a 3% polyacrylamide stacking gel. For glycoprotein detection the gels were overlaid with 125 I-labelled peanut agglutinin (Burrige, 1976) with or without prior mild acid treatment to remove sialic acid residues (Irimura & Nicolson, 1983). Alternatively, SDS/PAGE was performed with the same system except that a linear 2.0–17.5% polyacrylamide gradient gel with *NN'*-diallyltartardiamide instead of *NN'*-methylene-bisacrylamide as the cross-linking agent (Baumann & Chramback, 1976) and a stacking gel of 3% *NN'*-diallyltartardiamide-cross-linked polyacrylamide was used. The gel was constructed from a 37.8% acrylamide/1.6% *NN'*-diallyltartardiamide stock solution. This increased the porosity of the gel and permitted migration of the gp580 into the running-gel matrix. Gels containing 125 I-labelled material were washed, dried and subjected to autoradiography. For gels containing 3 H- or 14 C-labelled samples, gels were first processed for fluorography by treatment with En 3 Hance (New England Nuclear). Densitometric scans of the exposed X-ray film were performed with a Beckman DU-8 spectrophotometer with gel-scan accessory. Isoelectric focusing in a sucrose density gradient was performed as described by Behnke *et al.* (1975), except that a 1:1 (v/v) mixture of Ampholines (pH 2.5–5.0 and pH 3.0–10.0; Pharmacia, Uppsala, Sweden) was used.

Enzymic treatments

The sensitivity of gp580 to various degradative enzymes was determined by incubation of [3 H]glucosamine-, [3 H]serine- or periodate/boro[3 H]hydride-

labelled gp580 in a volume of 100 μ l of Dulbecco's phosphate-buffered saline (GIBCO) for 1 h at 37 °C with purified trypsin, Pronase, papain, α -chymotrypsin, subtilopectidase A (1 or 10 mg/ml), collagenase type VI (1000 units/ml), chondroitinase ABC (1 unit/ml), *Streptomyces* hyaluronidase (100 turbidity-reducing units/ml), *Vibrio cholerae* neuraminidase (100 munits/ml) or β -galactosidase (1000 units/ml). The samples and untreated control were analysed by SDS/PAGE on a 2.0–17.5% *NN'*-diallyltartardiamide-cross-linked polyacrylamide gradient gel followed by densitometric quantification.

Core-protein determination

Unlabelled gp580 or gp580 metabolically labelled with [14 C]serine was added to 100 μ g of bovine serum albumin used as carrier protein; the mixture was treated with trifluoromethanesulphonic acid in anisole (2:1, w/w) in a Reacti-vial for 10 or 30 min at 4 °C in an N_2 -purged environment (Edge *et al.*, 1981). The samples were extracted with diethyl ether, dialysed and freeze-dried. The unlabelled treated material was chemically labelled afterwards with 125 I by using the chloramine-T method (Hunter & Greenwood, 1962). Samples were analysed by SDS/PAGE with 2.0–17.5% *NN'*-diallyltartardiamide-cross-linked polyacrylamide gels.

Samples of gp580 for amino acid analysis were freeze-dried in acid-washed tubes and hydrolysed with 6 M- or 4 M-HCl for 4, 10 or 24 h at 110 °C. Analyses were performed by Dr. D. Ward of University of Texas M. D. Anderson Hospital with an LKB model 401 amino acid analyser, with norleucine as an added internal standard.

Alkaline-borohydride treatment

Freeze-dried samples of isolated gp580 with radioactively labelled carbohydrate were treated with 50 mM-NaOH/1.0 M-NaBH $_4$ at 45 °C for 24 h. The samples were neutralized with acetic acid, passed through a Dowex 50 (H $^+$ form) column and eluted with distilled water, and the fractions were dried repeatedly in the presence of methanol. The oligosaccharitol samples were redissolved in 5 mM-Tris/HCl buffer, pH 7.5, and applied to a QAE- (*NN'*-diethyl-*N*-2-hydroxypropyl-aminoethyl-)Sephadex column (5 cm $\times$ 1 cm) in the same buffer. The column was washed with 25 ml of 5 mM-Tris/HCl buffer, pH 7.5, followed by a linear gradient elution with 120 ml of 0.2 M-Tris/HCl buffer, pH 7.5. The radioactive peaks were pooled and then applied to a Bio-Gel P6 column (Carlson, 1968; Hull *et al.*, 1984).

Cell localization

The distribution of gp580 in the cells, cell-surface, matrix and medium compartments was determined after cell fractionation as described previously for glycosaminoglycans (Kraemer, 1971). After MTLn3 cells were metabolically radiolabelled with [3 H]serine for 24 h, the medium was collected, the cells were washed with phosphate-buffered saline and the wash fraction was combined with the medium fraction. Cells were removed from the culture dish by a 30 min treatment with 5 mM-EDTA at 37 °C with gentle shaking. The matrix fraction (the insoluble material remaining on the tissue-culture dish) was washed four times with phosphate-buffered saline and observed microscopically to assure removal of all cells. Then 2 ml of phosphate-buffered saline was added to each plate. The cell-surface

fraction was obtained by treatment of the cell suspension with trypsin (100 $\mu\text{g/ml}$) for 15 min at 37 °C with gentle shaking. The cell fraction (intact trypsin-treated cells) was collected by centrifugation and then resuspended in 5 ml of phosphate-buffered saline. To each fraction was added Triton X-100 and trypsin to final concentrations of 0.1% and 1 mg/ml respectively. The samples were redissolved, after being freeze-dried, in 1% SDS and 5 mM-2-mercaptoethanol in 10 mM-Tris/HCl buffer, pH 7.5, and subjected to gel-filtration chromatography on a Sepharose CL-2B column.

RESULTS

Isolation of gp580

Cultured MTLn3 cells were fractionated according to the procedure outlined in Scheme 1, resulting in four density fractions (D1–D4). The gp580 was detected by binding of peanut agglutinin, mainly in the D2 fraction ($\rho = 1.49 \pm 0.03 \text{ g/ml}$) with some small amounts in the D1 and D2 fractions. Approx. 50% of the [^3H]glucosamine-labelled macromolecules but less than 8% of the [^3H]serine-labelled macromolecules were found in fractions D1–D3. Density-gradient gp580 fractions derived from MTLn3 cells metabolically labelled with different precursors were subjected to gel filtration on Sepharose CL-2B columns (Fig. 1). Cells that were labelled with [^3H]galactose, [^3H]glucosamine or [^3H]serine yielded elution profiles containing two radioactive peaks (K_{av} , approx. 0.22 and 0.60). The first peak eluted contained 18.6% of the [^3H]glucosamine, 0.45% of the [^3H]galactose and 0.1% of the [^3H]serine incorporated into the total macromolecular material. The MTLn3 cells metabolically labelled with [^3H]leucine, [^3H]mannose or [^3H]fucose resulted in a similar profile of two peaks, but the void-volume fractions contained less than 0.002–0.05% of incorporated radioactivity. Only one radioactive peak (K_{av} , approx. 0.60; Fig. 1) was observed if the cells were incubated with $\text{Na}_2^{35}\text{SO}_4$. Analysis of the D2 peaks revealed that the void-volume fractions contained most (at least 90%) of the gp580, as detected by binding of ^{125}I -labelled peanut agglutinin after desialylation and SDS/PAGE.

The Sepharose CL-2B void-volume fractions from density fractions D1–D3 were pooled, and then subjected to ion-exchange chromatography on Sephacel DEAE-cellulose columns with an NaCl elution gradient. Cells labelled with [^3H]glucosamine or [^3H]galactose resulted in column profiles containing four peaks (Fig. 2a, peaks I–IV). The radioactivity associated with peaks I, II, III and IV corresponded to 2.0%, 7.8%, 8.1% and 0.9% of the total [^3H]glucosamine incorporated respectively. In contrast, only three radioactive peaks were observed when MTLn3 cells were labelled with [^3H]mannose or [^3H]fucose (Fig. 2b, peaks I, III and IV). Only the last two peaks (III and IV) were found with [^{14}C]serine-labelled material (Fig. 2c).

MTLn3 tumours were harvested, and the extracts were subjected to dissociative density-gradient centrifugation. Density fractions corresponding approximately to fraction D2 ($\rho = 1.48 \pm 0.09 \text{ g/ml}$) were pooled, and applied to a calibrated Sepharose CL-2B column. As previously determined for the MTLn3 cells grown *in vitro*, most of a high- M_r glycoprotein that bound ^{125}I -labelled peanut agglutinin after desialylation and had a low migration in 7.5% SDS/PAGE gels was

Fig. 1. Gel filtration of radioactively labelled macromolecules synthesized by MTLn3 cells after dissociative CsCl -density-gradient ultracentrifugation

[^3H]Glucosamine-labelled (—) and [^{35}S]sulphate-labelled (·····) cellular extracts from the various density-gradient fractions were subjected to Sepharose CL-2B column chromatography in 4 M-guanidinium chloride/0.1 mM-sodium acetate buffer, pH 5.8. The average density of each of the fractions is denoted (ρ values), and the arrows represent the void-volume (V_0) and total-volume (V_t) elution fractions of the columns. The columns were standardized by dextrans (M_r 2000000, 500000, 150000 and 70000).

found in the Sepharose CL-2B void-volume fractions (results not shown). Portions of the pooled, dialysed and freeze-dried void-volume fractions were labelled with ^{125}I by using the chloramine-T method for proteinaceous material or periodate/ $^{3\text{H}}$ borohydride for sialic acid moieties (Van Lenten & Ashwell, 1971). Portions of the unlabelled and labelled extracts were combined and then applied to a DEAE-Sephacel column. The ^3H -labelled material was eluted in four radioactive peaks, similarly to the [^3H]glucosamine labelled extract, except that the second peak contained less radioactivity. The ^{125}I -labelled extract was eluted with a profile most similar to that of the [^3H]serine-labelled material (cf. Figs. 2 and 3a), except for an increased peak I, which contained low- M_r -migrating material including non-covalently bound ^{125}I (Table 1). In the remainder of this paper, the sources of gp580 fractions are differentiated as chemically labelled for tumour-derived material and metabolically labelled for material prepared from cultured cells.

Fig. 2. Ion-exchange chromatography on DEAE-Sephacel columns of metabolically radiolabelled pooled void-volume fractions from the Sepharose CL-2B elution

Elution profiles of (a) [^3H]glucosamine-labelled, (b) [^3H]fucose-labelled and (c) [^{14}C]serine-labelled samples from cultured MTLn3 cells are shown. The continuous line at the top of each panel represents the concentration of NaCl elution. The various fractions I, II, III and IV were pooled for further analysis. The radioactive peaks were designated by their elution position as compared with the [^3H]glucosamine-labelled sample.

Fig. 3. Analysis of rat tumour Sepharose CL-2B void-volume fractions by ion-exchange chromatography

(a) DEAE-Sephacel elution profile of rat tumour-extract density-gradient fractions. The continuous line at the top of the panel represents the concentration of NaCl used in the elution of the column. The various peaks (I–IV) were pooled individually, and then samples were subjected to SDS/PAGE. (b) Autoradiograph of binding of ^{125}I -labelled peanut agglutinin to the purified DEAE-Sephacel pooled peaks after SDS/PAGE on a 7.5% polyacrylamide gel. The lanes A–D correspond to peaks I–IV from the DEAE-Sephacel column. The binding of peanut agglutinin was shown to be specific by a control gel in which lactose was included in the incubation. The protein standards were myosin, β -galactosidase, phosphorylase *b*, bovine serum albumin and ovalbumin, with M_r 200000, 130000, 92000, 68000 and 45000 respectively.

To characterize the various DEAE-Sephacel peaks, portions were incubated with various degradative enzymes and subsequently analysed by SDS/PAGE. The [^3H]glucosamine-labelled material in peak II appeared to be insensitive to Pronase and trypsin, but it could be degraded by *Streptomyces* hyaluronidase (Table 1), suggesting that this component is hyaluronic acid. Components from peaks III and IV had identical sensitivities to various enzymes and were degraded only by Pronase (Table 1). Furthermore, materials in peaks III and IV bound ^{125}I -labelled peanut agglutinin to high- M_r components after mild acid treatment, suggesting that these peaks contained gp580 (Fig. 3b and Table 1). Analysis of DEAE-Sephacel fractions from chemically labelled MTLn3 tumour tissue showed identical characteristics.

SDS/PAGE analysis

Examination of the four [^3H]glucosamine-labelled peaks from DEAE-Sephacel chromatography after electrophoresis and fluorography showed that peak I contained only low- M_r material ($M_r < 30000$). Peaks II–IV were observed to contain only high- M_r material that migrated as diffuse but single bands ($M_r \sim 350000$ – 800000 ; Table 1 and Fig. 4). No other low- M_r bands were detected in either metabolically or chemically labelled carbohydrate fractions, even with overexposure of the X-ray films, suggesting that essentially all of the components were detected with these techniques. When the [^3H]serine-labelled or ^{125}I -labelled peaks were analysed, similar results were obtained, except that peak II material was essentially unlabelled. Estimation of the average M_r of gp580 by densitometric analysis of the fluorogram and extrapolation from the standard proteins yielded an M_r value of approx. 550000

Table 1. SDS/PAGE analysis of DEAE-Sephacel fractions from MTLn3 cells

Samples of DEAE-Sephacel fractions from MTLn3 cells grown either *in vivo* or *in vitro* displayed identical results in response to the various treatments. The M_r is the relative migration of [^3H]glucosamine- or periodate/[^3H]borohydride-labelled fractions on NN' -diallyltartardiamide-cross-linked polyacrylamide-gradient gels in SDS. The binding of ^{125}I -labelled peanut agglutinin and the enzyme treatments are described in the Experimental section.

Treatment	Peak I	Peak II	Peak III	Peak IV
M_r	<30000	>350000	>350000	>350000
Peanut agglutinin binding	—	—	+	+
Sensitivity to:				
Trypsin	—	—	—	—
Pronase	—	—	+	+
Hyaluronidase	—	+	—	—

(Fig. 4, lane A), which agrees with the gel-filtration estimation (Steck & Nicolson, 1983). In addition, the migration of gp580 on gel filtration or SDS/PAGE was not altered with or without reducing conditions. After purification, gp580 was found to be homogeneous but diffuse by SDS/PAGE analysis and to contain approx. 0.08% of the total [^{14}C]serine incorporated into acid-precipitable radioactivity (Fig. 4).

Chemical composition of gp580

The amino acid composition of purified gp580 is

Fig. 4. SDS/PAGE analysis of purified and radiolabelled gp580 fraction

[^{14}C]Serine-labelled purified gp580 (lane A) and trifluoromethanesulphonic acid-treated gp580 (lane B) are shown. Electrophoresis was performed on a 2–17.5% NN' -diallyltartardiamide-cross-linked polyacrylamide gel followed by fluorography. The protein standards were myosin, phosphorylase α and bovine serum albumin, with M_r 200000, 94000 and 68000 respectively.

shown in Table 2. Glutamate, aspartate and serine were the major amino acids present, and with threonine, glycine and lysine represented more than 50% of the amino acid composition of gp580. A further 20% was made up of the amino sugars glucosamine and galactosamine (Table 2).

The gp580 purified from MTLn3 cells was deglycosylated with trifluoromethanesulphonic acid to produce the protein core (Edge *et al.*, 1981) and then analysed by SDS/PAGE. The protein core migrated as a single band with an estimated M_r of approx. 150000 (Fig. 4 lane B). No other labelled bands were detected. The protein core of gp580 isolated from tumour tissue and subsequently radiolabelled with ^{125}I was of identical size by SDS/PAGE.

The apparent density of gp580 was determined by isopycnic centrifugation in the presence of 4 M-guanidinium chloride and revealed a density of around 1.432 g/ml (> 90% between 1.475 and 1.398 g/ml). However, in the presence of an associative concentration of guanidinium chloride (0.5 M) the apparent density was 1.615 g/ml (> 90% between 1.671 and 1.563 g/ml). An estimate of the content of sialic acid on gp580 was obtained by treatment of [^3H]glucosamine-labelled gp580 with mild acid (50 mM- H_2SO_4 for 1 h at 80 °C), followed by chromatography on a Bio-Gel P4 column. Approx. 25.5% of the radioactivity incorporated into gp580 was released by this treatment, suggesting that sialic acid is a major component of gp580. Furthermore gp580 migrated as a diffuse band on sucrose-density-gradient isoelectric focusing with a pI of 3.2 ± 0.5 , thus confirming its acidic composition.

Table 2. Amino acid analysis of gp580 synthesized by rat mammary-adenocarcinoma cells

Values represent averages of duplicate analyses for gp580 purified from cultured MTLn3 cells and tumour tissues (peak III, Figs. 2 and 3). The purity of gp580 was analysed by SDS/PAGE. The carbohydrate values were determined by extrapolation of the values at different hydrolysis times to zero time of hydrolysis. Tryptophan was not detected.

Amino acid or amino sugar	Composition (residues/1000 amino acid residues)	
	Cultured MTLn3 cells	MTLn3 tumour
Aspartic acid	105	103
Threonine	92	95
Serine	143	148
Glutamic acid	139	137
Proline	29	33
Glycine	101	98
Alanine	33	31
Cysteine	5	6
Valine	65	59
Methionine	7	8
Leucine	62	60
Isoleucine	37	38
Tyrosine	26	24
Phenylalanine	37	35
Histidine	13	16
Lysine	84	82
Arginine	22	25
Glucosamine	114	125
Galactosamine	90	102

Table 3. Sensitivity of gp580 to degradative enzymes

The enzymic treatment of [^3H]serine-labelled gp580 is described in the Experimental section. For determination of M_r , the various samples after enzyme incubation were subjected to PAGE on 2–17.5% *NN'*-diallyltartardiamide-cross-linked polyacrylamide gels and fluorography. The relative density was normalized to the area under the densitometric scans of the fluorograms from the SDS/PAGE gels by taking the untreated lane as 1. Areas were derived on a Beckman DU-8 spectrophotometer by using the lowest-valley program. Abbreviation: N.D., not detected.

Enzyme	Amount (mg/ml or units/ml)	$10^{-3} \times M_r$	Relative density (area)
Control	–	> 400	1
Trypsin	1	> 400	1
	10	> 400	1
Pronase	1	30–200	0.35
	10	N.D.	0
Subtilopeptidase A	1	50–300	0.43
	10	N.D.	0
Papain	1	> 400	1
	10	> 400	1
Pepsin	1	> 400	1
	10	> 400	1
α -Chymotrypsinogen	1	> 400	1
	10	> 400	1
Collagenase	1	> 400	1
	10	> 400	1
Chondroitinase ABC	1	> 400	1
Hyaluronidase	100	> 400	1
Neuraminidase	100	> 400	1
β -Galactosidase	100	> 400	1
Neuraminidase + β -galactosidase	100 + 100	> 350	0.9

Previous data suggested that gp580 possessed sialomucin-like characteristics, and this finding was confirmed by incubation of [^3H]serine-labelled gp580 with various degradative enzymes. Purified gp580 was resistant to a variety of proteinases, chondroitinase ABC and hyaluronidase (Table 3). Digestion was observed only with Pronase, subtilopeptidase A and a combination of neuraminidase and β -galactosidase. MTLn3-cell extracts were capable of degrading gp580, and therefore the isolation was performed in the presence of proteinase inhibitors.

Oligosaccharides of gp580

A number of oligosaccharides were released by alkaline-borohydride treatment of gp580 that was radiolabelled with [^3H]glucosamine, [^3H]galactose or [^3H]fucose. [^3H]Glucosamine-labelled oligosaccharides were applied to a QAE-Sephadex column and separated into neutral (pass-through fractions representing 23.7% of total radioactivity recovered) and acidic (retained fractions, 76.3% of the radioactivity) components. The acidic fractions were eluted under conditions where mono- and di-sialylated oligosaccharitols are obtained (10–40 mM-Tris/HCl buffer, pH 7.5). Further fractiona-

Fig. 5. Gel filtration on a Bio-Gel P6 column of [^3H]glucosamine-labelled oligosaccharides from gp580 after alkaline-borohydride treatment

The elution profiles of the neutral oligosaccharides (a), which constitute 23% of the labelled material, and of the acidic oligosaccharides (b), representing 76% of labelled carbohydrate, are shown. The total recovery of the column was in the range 85–95%. Arrows represent the elution positions (left to right) of Dextran Blue (void volume), NeuAc2-3Gal β -1-3(NeuAc2-6)-GalNAcol, Gal β 1-3GalNAcol, N-acetylneuraminic acid and fucose.

tion was performed by gel-filtration chromatography on Bio-Gel P6 columns. Most of the neutral oligosaccharides were found to be associated with one radioactive peak (Fig. 5a). Several relatively large radioactive peaks were observed for the acid oligosaccharitols (Fig. 5b). Oligosaccharides released from gp580 isolated from tumour tissue and whose sialic acid moieties were chemically labelled showed a similar profile to that shown in Fig. 5(b).

Localization of gp580

Analysis of the relative amounts of gp580 in various cellular compartments was made because of the relative resistance of gp580 to proteolytic degradation and its high M_r . The relative amounts of high- M_r [^3H]serine-labelled gp580 released into the medium (approx. 3%) and contained intracellularly (approx. 11%) by cultured MTLn3 cells were determined by Sepharose CL-2B chromatography. The matrix-associated gp580 constituted approx. 25% and the cell-surface fraction contained 61% of the total [^3H]serine-labelled gp580 (results not shown). The void-volume fractions were subsequently analysed by SDS/PAGE, and only high- M_r labelled material was observed.

DISCUSSION

Few biochemical properties or markers are associated with the metastatic behaviour of tumour cells (Nicolson, 1982, 1984). Exceptions are the differential expression of certain glycoproteins (Chatterjee & Kim, 1978; Irimura & Nicolson, 1984) and degradative enzymes (Liotta *et al.*, 1980; Nakajima *et al.*, 1983; Sloane *et al.*, 1981) in highly metastatic tumour cells. With the possible exception of the laminin receptor (Terranova *et al.*, 1983) and gp580 (Steck & Nicolson, 1983, 1984), biochemical markers associated with malignancy have not been identified on mammary carcinoma cells.

Several isolation procedures have been described for sialomucins similar to gp580; however, most of these methods have used suspension cells (Sherblom *et al.*, 1980) or released cellular products (Bhavanandan *et al.*, 1977; Herzburg *et al.*, 1979; Shimizu & Yamauchi, 1982) as the starting materials. We used an extraction procedure for gp580 that is used for proteoglycans (Hascall & Kimura, 1982) and is more suitable for adherent cultured cells and solid tumours. Although the highly metastatic MTLn3 cells synthesize a heparan sulphate proteoglycan that is extracted and shows density migration similar to that of gp580, the proteoglycan is eluted from the Sepharose CL-2B column with K_{av} approx. 0.60 and does not interfere with the purification of gp580 (P. A. Steck, M. Nakajima & G. L. Nicolson, unpublished work). Dissociative CsCl-density-gradient centrifugation also resulted in the separation of gp580 from most of the proteinaceous material (over 90% of [3 H]leucine-labelled material) of MTLn3 cells.

By using electrophoresis in an *NN'*-diallyltartardiamide-cross-linked polyacrylamide-gradient gel and densitometric analysis, we showed that gp580 migrates as a diffuse single band centred around M_r approx. 550 000. The diffuse band formed by gp580 on SDS/PAGE is probably due to differences in net charge and the presence of carbohydrate heterogeneity. However, the possibility cannot be excluded that gp580 may exist as a series of oligomers. No major biochemical or compositional differences have been observed between gp580 isolated from cultured cells or from tumours, indicating that the expression of gp580 is not an artifact of cell growth *in vitro*. However, gp580 isolated from tumour extracts bound more 125 I-labelled peanut agglutinin without prior desialylation than gp580 from cells cultured *in vitro*, suggesting decreased sialylation of this molecule *in vivo*.

The relatively high density of gp580 in CsCl gradients containing 4 M-guanidinium chloride indicated the presence of a relatively large quantity of acidic carbohydrate chains as compared with protein. The pI of gp580 was demonstrated to be 3.2. Mild acid treatment of [3 H]glucosamine-labelled gp580 revealed that 25% of the incorporated radioactivity was released, and that 76% of the oligosaccharitols were retained in a QAE-Sephadex ion-exchange column, suggesting a high percentage of sialic acid moieties. A high carbohydrate/protein ratio was also demonstrated by removal of *O*-linked oligosaccharides, resulting in a protein core of M_r approx. 150 000 by SDS/PAGE analysis. These findings suggest that gp580 is a mucin-type glycoprotein, a notion further supported by the resistance of gp580 to several degradative enzymes.

In other mammary-tumour systems high- M_r molecules have been described, such as epiglycan from mouse (Cooper *et al.*, 1974; Miller *et al.*, 1977) and ASGP-1 from rat (Sherblom *et al.*, 1980; Howard *et al.*, 1982) ascites-tumour cells. ASGP-1 and gp580 probably represent similar high- M_r sialomucin-like glycoproteins, although there are several distinct differences between these two molecules. First, amino acid analyses of ASGP-1 and gp580 differ in the quantities of aspartic acid and threonine. Secondly, sulphated oligosaccharides appear on ASGP-1 (Hull *et al.*, 1984) but not on gp580. Thirdly, peanut agglutinin binds to ASGP-1s without removal of sialic acid moieties. Fourthly, AGP-1 is the major cell-surface component labelled by lactoperoxidase-catalysed iodination or metabolically labelled with [3 H]glucosamine on the ascites variants of 13762 cells, and it is not apparently synthesized and deposited in an extracellular form.

We found that gp580 is present in a variety of cellular compartments. Most of the gp580 is found in the cell membrane (approx. 60%) or in the extracellular matrix (approx. 25%) synthesized by the mammary-tumour cells. This finding is in agreement with information on localization obtained by using a monoclonal antibody against gp580 (S. M. North, P. A. Steck & G. L. Nicolson, unpublished work).

High- M_r mucins have also been described on normal mammary epithelial cells or on their secreted products (Imam *et al.*, 1981; Shimizu & Yamauchi, 1982; Johnson *et al.*, 1986). A high- M_r glycoprotein (PAS-0) has been identified and characterized on the human milk-fat-globule membrane (Shimizu & Yamauchi, 1982). Although PAS-0 is mucin-like in its characteristics, such as resistance to proteinase, binding of peanut agglutinin, wheat-germ agglutinin and *Ricinus communis* agglutinin and relatively high M_r on SDS/PAGE, gp580 does not appear to be the same molecule. Amino acid analysis of PAS-0 indicates that it does not contain a high percentage of acidic amino acids, and its M_r appears to be lower than that of gp580. In addition, a high- M_r tumour-associated glycoprotein (TAG-72) has recently been defined by the monoclonal antibody B72.3, which reacts with colon- and breast-carcinoma cells (Johnson *et al.*, 1986). This monoclonal antibody did not react with gp580 (S. M. North, P. A. Steck & G. L. Nicolson, unpublished work). It is possible that PAS-0, TAG-72 and gp580 may represent similar types of glycoproteins, but they appear to express different antigenic determinants.

The presence of sialomucins on a number of epithelial tumours has been reported, but their functional roles have not been ascertained. It has been proposed that mucins and similar molecules act as cell-protective agents and lubricants. For example, such mucins may mask tumour-cell antigens or block host anti-tumour responses (Cooper *et al.*, 1974; Alexander, 1974; Kim *et al.*, 1975). Mucins may also act in cellular adhesive and migratory processes during metastasis (Chernoff *et al.*, 1983). We have generated several monoclonal antibodies directed against gp580 (S. M. North, P. A. Steck & G. L. Nicolson, unpublished work) that should allow us to investigate the possible functional roles of gp580 and to compare this molecule with other mammary-carcinoma antigens.

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