Purification and partial characterization of a tumor-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-Sephasel. The isolated glycoprotein possessed low electrophoretic mobility in SDS/polyacrylamide gels, and after desialylation bound $^{131}$I-labelled peanut agglutinin. Electrophoresis of gp580 in polyacrylamide-gradient gels resulted in a diffuse but homogeneous migrating band of $M_r$ approx. 550000. After removal of carbohydrate, gp580 was demonstrated to have a protein core of $M_r$ approx. 150000. 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. 80000 (gp80), was decreased in expression in more metastatic cells; another, a high-$M_r$ (approx. 580000) 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

$\text{d-[6-}^3\text{H]}$Glucosamine (20-30 Ci/mmol), $\text{d-[1-}^3\text{H]}$galactose (1-5 Ci/mmol), $\text{l-[6-}^3\text{H]}$fucose (20-35 Ci/mmol), $\text{l-[4,5-}^3\text{H]}$leucine (30-50 Ci/mmol), $\text{l-[6-}^3\text{H]}$serine
(5–10 Ci/mmol), L-[U-14C]serine (135–165 mCi/mmol) and Na235SO4 were purchased from ICN Pharmaceuticals (Irvine, CA, U.S.A.); D-[2-3H]mannose (10–20 Ci/mmol) and Na181I 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 minimal 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% CO2 in humidified air in α-modified minimal 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 x 106 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 minimal 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 minimal essential medium contained one-half of the normal concentration of D-glucose (1 g/l). To prepare cells labelled with [3H]leucine, [14C]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 Na235SO4/ml in α-modified minimal essential medium in which the MgSO4 (80.9 μmol) was replaced with MgCl2. 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 [14C]serine-labelled gp580.

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

The cells were solubilized (approx. 5 x 106 cells per dish) by adding 1 ml of extraction buffer (containing 4% Zwittergent 3-12, 4 M-guanidinium chloride, 0.1 M-6-amino-hexanoic acid, 10 mM-Na2EDTA, 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 x 1 cm or 50 cm x 4 cm) and equilibrated with extraction buffer minus the Zwittergent.
(1×10⁴ 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 cen-
trifuged 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 × 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]-di-
methylammoniopropyl)-propyl-l-sulphonate) in 50 mm-Tris/
HCl buffer, pH 7.2, and applied to a DEAE-Sephadex
column (5 cm × 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 × 0.8 cm) which was equilibrated and
eluted with 1% SDS and 5 mm-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 × 0.8 cm) equilibrated with
50 mm-pyridine/acetic 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
125I-labelled peanut agglutinin (Burridge, 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 125I-labelled material were washed, dried and
subjected to autoradiography. For gels containing 3H or
14C-labelled samples, gels were first processed for
fluorography by treatment with En3Hance (New England
Nuclear). Densitometric scans of the exposed X-ray film
were performed with a Beckman DU-5 spectrophoto-
meter 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 [3H]glucos-
amine, [3H]serine or periodate/boro[3H]hydride-
labelled gp580 in a volume of 100 µl of Dulbecco’s
phosphate-buffered saline (GIBCO) for 1 h at 37 °C with
purified trypsin, Pronase, papain, α-chymotrypsin,
subtilopeptidase 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
[14C]serine was added to 100 µg 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 N₂-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 125I 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 radio-
actively labelled carbohydrate were treated with 50 mm-
NaOH/1.0 m-NaBH₄ 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 oligosaccharidate 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 × 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 glycos-
aminoglycans (Kraemer, 1971). After MTLn3 cells were
metabolically radiolabelled with [3H]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 μg/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 ($\rho = 1.49 \pm 0.03$ 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 Na$_2$35SO$_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 125I-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, II and IV). Only the last two peaks (II and IV) were found with $[^4C]$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 ($\rho = 1.48 \pm 0.09$ 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 125I-labelled peanut agglutinin after desialylation and had a low migration in 7.5% SDS/PAGE gels was 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 125I by using the chloramine-T method for proteinaceous material or periodate/$[^3H]$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 $^4$H-labelled material was eluted in four radioactive peaks, similarly to the $[^3H]$glucosamine labelled extract, except that the second peak contained less radioactivity. The 125I-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 125I (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.
To characterize the various DEAE-Sephasel peaks, portions were incubated with various degradative enzymes and subsequently analysed by SDS/PAGE. The [H]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 125I-labelled peanut agglutinin to high-Mr components after mild acid treatment, suggesting that these peaks contained gp580 (Fig. 3b and Table 1). Analysis of DEAE-Sephasel fractions from chemically labelled MTLn3 tumour tissue showed identical characteristics.

**SDS/PAGE analysis**

Examination of the four [H]glucosamine-labelled peaks from DEAE-Sephasel chromatography after electrophoresis and fluorography showed that peak I contained only low-Mr material (M_r < 30000). Peaks II–IV were observed to contain only high-Mr material that migrated as diffuse but single bands (M_r ~ 350000–800000; Table 1 and Fig. 4). No other low-Mr 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 [H]serine-labelled or 125I-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

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

Elution profiles of (a) [H]glucosamine-labelled, (b) [H]fucose-labelled and (c) [14C]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 [H]glucosamine-labelled sample.

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

(a) DEAE-Sephasel 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 125I-labelled peanut agglutinin to the purified DEAE-Sephasel pooled peaks after SDS/PAGE on a 7.5% polyacrylamide gel. The lanes A–D correspond to peaks I–IV from the DEAE-Sephasel 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.
Samples of DEAE-Sepharose 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 $[^{3}H]$glucosamine- or periodate/$[^{3}H]$bromo-
hydride-labelled fractions on $NN'$-diallyltartardiamide-
cross-linked polyacrylamide-gradient gels in SDS. The binding of $^{131}I$-labelled peanut agglutinin and the enzyme treatments are described in the Experimental section.

### Chemical composition of gp580

The amino acid composition of purified gp580 is 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 deglyco-
sylated with trifluoromethanesulfonic 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. 150,000 (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 $[^{3}H]$glucosamine-labelled gp580 with mild acid (50 mM-H$_2$SO$_4$ 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.

<table>
<thead>
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<th>Amino acid or amino sugar</th>
<th>Cultured MTLn3 cells</th>
<th>MTLn3 tumour</th>
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Table 3. Sensitivity of gp580 to degradative enzymes

The enzymatic 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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount (mg/ml or units/ml)</th>
<th>$10^{-9}\times M_r$</th>
<th>Relative density (area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>–</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Trypsin</td>
<td>1</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Pronase</td>
<td>1</td>
<td>30–200</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td>Subtilopeptidase A</td>
<td>1</td>
<td>50–300</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>N.D.</td>
<td>0</td>
</tr>
<tr>
<td>Papain</td>
<td>1</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Pepsin</td>
<td>1</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>$\alpha$-Chymotrypsinog</td>
<td>1</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Collagenase</td>
<td>1</td>
<td>&gt; 400</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>1</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>100</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>100</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>$\beta$-Galactosidase</td>
<td>100</td>
<td>&gt; 400</td>
<td>1</td>
</tr>
<tr>
<td>Neuraminidase $+$ $\beta$-galactosidase</td>
<td>100 + 100</td>
<td>&gt; 350</td>
<td>0.9</td>
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</table>

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 $\beta$-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 fractionation 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 (Hascal & 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 Kᵥ. 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 [3H]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 Mr, approx. 550000. 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 125I-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 [3H]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 Mr, approx. 150000 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-Mr 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-Mr 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 [3H]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-Mr 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-Mr 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 Mr, 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 Mr appears to be lower than that of gp580. In addition, a high-Mr 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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