Carbohydrate Research, 151 (1986) 157–171
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

FURTHER CHARACTERIZATION OF THE GLYCOPROTEINS AND GLYCOSAMINOGLYCANS RELEASED FROM TA3 MURINE ADENOCARCINOMA CELLS IN CULTURE*

DOUGLAS K. MILLER
Department of Biochemistry, Merck Institute of Therapeutic Research, Rahway, NJ, 07065 (U.S.A.)

AND AMIEL G. COOPER
Department of Pathology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA, 02111 (U.S.A.)

(Received September 3rd, 1985; accepted for publication, December 12th, 1985)

ABSTRACT

TA3 murine ascites adenocarcinoma cells were compared for their ability to release radioactive glucosamine and $^{35}SO_4$-labeled glycoproteins and glycosaminoglycans into the culture medium. Both TA3-Ha and TA3-St cells contained cell-surface heparan sulfate that was released into culture, but not chondroitin sulfate. Both cells released a membranous aggregate of labeled components from the cell surface and hyaluronic acid from inside the cells that fractionated in the void volume of Sepharose CL-4B. This void-volume fraction from the TA3-Ha cells contained glucosamine-labeled epiglycanin at a higher concentration relative to other glucosamine-labeled components than that found on plasma membranes. Glycoproteins associated with epiglycanin found on the cell surface, as well as released into culture medium, contained sulfate that could not be removed by chondroitinase ABC, heparinase, or keratinase. Kinetic analysis of the glucosamine-labeled material released from TA3-Ha cells indicated that hyaluronic acid was released rapidly with a 45-min half-life, whereas the other membranous components were released much more slowly.

INTRODUCTION

The allogeneically transplantable, TA3-Ha ascites mouse adenocarcinoma cell-line[1] has been found to release a variety of glycoproteins *in vivo* into ascites fluid and serum[2] and *in vitro* into tissue-culture medium[3,4]. The predominant glycoprotein released is an unusual, rod-shaped glycoprotein that has a molecular weight >500,000 and is composed of 80% carbohydrate arranged in a series of short chains bearing the common attachment structure of GalNAc-Ser(Thr)[5,6]. This

*Dedicated to Roger W. Jeanloz.

0008-6215/86/$ 03.50

glycoprotein, termed "epiglycanin", is absent from a sister ascites subline, the TA3-St line[1], which has only syngeneic transplantability[7]. The presence of this glycoprotein on the cell surface (epiglycanin comprises 0.5% of the dry weight of the TA3-Ha cell) and as the soluble form released outside the cell during normal cellular growth has been suggested as a key event in permitting the cell's allo- and xeno-transplantability[8].

In earlier reports[4,6,7,9] we have indicated that the [^{3}H]glucosamine-labeled glycoproteins found in the TA3-Ha cell were released *in vitro* from the cell surface in 2 high-molecular-weight forms: one found in the void volume of Sepharose 4B (termed Peak A) and a second with a $K_{av} = 0.25$ (Peak B). Both components were soluble in perchloric acid. Peak B was essentially pure epiglycanin, similar to that isolated earlier from ascites fluid, whereas Peak A contained both epiglycanin and hyaluronic acid. These 2 epiglycanin fractions were released with different kinetics: Peak A was released biphasically with half-lives of 0.75 and >70 h, whereas Peak B was released with a half-life of >70 h. The TA3-St cells, which lacked epiglycanin, released no Peak B material and only a small amount of glycoproteins in the void-volume region.

Because of the presence of hyaluronic acid in Peak A, we looked for the presence of other glycosaminoglycans by labeling TA3 cells in $^{35}SO_4$ as well as [^{3}H]glucosamine. We now report that each TA3 subline both contains cell-surface glycosaminoglycans containing sulfate, particularly heparin sulfate, and also releases them to the culture medium. In addition, in the TA_3-Ha cells, sulfate is found attached to cell-surface carbohydrate chains of epiglycanin or of coeluting glycoproteins and is released into culture. The hyaluronic acid in Peak A is the component released with the rapid kinetics whereas much of the remainder of the material in Peak A is slowly released membraneous material.

EXPERIMENTAL

Cell growth and labelling conditions. — Strain A/J mice (Jackson Labs, Bar Harbor, ME) were inoculated i.p. with 10^5 or 10^6 TA3-Ha and TA3-St cells respectively in 0.2 mL of Dulbecco's phosphate-buffered saline [balanced salt solution (BSS); Whittaker M.A. Bioproducts, Walkersville, MD] and harvested from the ascites fluid on day 7. Heparin was not employed in the cell harvesting. TA3 cells were grown in suspension culture at 0.5–1.5 × 10^6 cells/mL MEM medium modified for suspension culture and supplemented with 10% heat-inactivated fetal calf serum and 50 μg/mL of gentamicin.

TA3 cells were labeled *in vivo* by i.p. inoculation of each mouse with 5 μCi of D-[^{3}H]glucosamine (10 Ci/mmol) or 20 μCi of D-[^{14}D]glucosamine (55 mCi/mmol) which had been evaporated to dryness under nitrogen and reconstituted in 0.2 mL BSS. TA3 cells were labeled *in vitro* for 24 h in suspension culture with D-[^{3}H]- or D-[^{14}C]-glucosamine (0.3 μCi/10^6 cells/mL), $^{35}SO_4$ (carrier free; 60 μCi/10^6 cells/mL), or [^{3}H]leucine (84 Ci/mmol; 1 μCi/10^6 cells/mL). All

radioactive isotopes were obtained from New England Nuclear (Boston, MA). In experiments in which TA3 cells were labeled with radioactive glucosamine for <1 h, 10 times as much label was used as for the 24-h labeling period to ensure adequate labeling. In experiments with [^{3}H]leucine, MEM lacking leucine was used. The culture medium from these cells was concentrated with PM-10 ultra filters (Amicon Corp., Lexington, MA), centrifuged at 37,000*g* for 10 min, and fractionated on Sepharose columns (see later). All isolated glycoprotein fractions were concentrated with PM-10 filters.

Removal of cell-surface glycopeptides and sialic acid. — Cell-surface glycopeptides were cleaved from intact TA3 cells with trypsin (18 μg/mL BSS; TPCK-trypsin, Worthington Biochemicals, Freehold, NJ) as described earlier[4]. Sialic acid was removed from glycoproteins by neuraminidase [25 U/mL BSS; (1U releases 1 μmol per min of NeuNAc from human acid α_1-glycoprotein at 37°); Behring Diagnostics, Somerville, NJ] for 3 h at 37°.

Identification of glycosaminoglycans. — Glucosamine-labeled glycoproteins released from TA3 cells were analyzed for the presence of hyaluronic acid by treatment with purified testicular hyaluronidase (Leo AB, Helsingborg, Sweden; 50 μg/mL sodium acetate buffer, 0.05M in 0.08M NaCl, pH 5.1) or *Streptomyces* hyaluronidase (Calbiochem; 0.2 U/mL of the same sodium acetate buffer) for 3 h at 37° in the presence of 2.2 mg/mL of unlabeled hyaluronic acid (Sigma Chemicals, St. Louis , MO) included as an internal control. The hyaluronidase digests were chromatographed on Sepharose CL-4B, and fractions were counted for radioactivity and analyzed for uronic acids (derived from the unlabeled hyaluronic acid) by a modifiation of the AutoAnalyzer continuous-flow method of Heinegard[10] in which samples were mixed with 18 volumes of carbazole reagent, incubated in boiling water for 13 min, and monitored for absorbance at 550 nm in a Technicon Colorimeter II (Technicon Corp., Tarrytown, NY).

The presence of chondroitin sulfate was determined by incubation of labeled glycoproteins with chondroitinase ABC (Miles Labs., Elkhardt, IN; 0.5 U in 0.05M Tris HCl, pH 8.0) for 2 h at 37° together with 2.6 mg of chondroitin 4-sulfate (Miles Labs.) used as an internal control. After Sepharose CL-4B chromatography, uronic acids were measured as before. Aliquots of the chondroitinase ABC digest of D-[^{14}C]glucosamine-labeled glycoproteins were separated by paper chromatography by the method of Saito *et al.*[11] and compared to known oligosaccharide digestion-products of chondroitin sulfate (available from Miles Labs.).

The presence of heparin and heparan sulfate were determined by incubation of the radioactive glycoproteins with heparinase[12]. Heparinase (in 0.2 mL of 0.5M sodium acetate[13]) was added to 2.5 mL of sample in phosphate-buffered saline (PBS; 0.15M NaCl with 0.05M sodium phosphate, pH 7.0) containing 2mM calcium acetate and 4 mg of unlabeled heparin. The tubes were incubated first for 2.5 h at 30°, and then for 2.5 h at 43°. Monitoring of the absorbance at 232 nm indicated the completeness of the hydrolysis of the heparin. The mixture was chromatographed

on Sepharose CL-4B, and aliquots were counted for radioactivity and monitored for uronic acid as already described.

Gel chromatography. — All Sepharose CL-4B chromatography (2.5 × 94 cm column) was performed at 5° and eluted with PBS at an upward flow-rate of 20 mL/h. A column (2.5 × 94 cm) of Sepharose CL-2B was eluted with 1% sodium dodecyl sulfate in 0.5M sodium phosphate, pH 8.1 (SDS) at 25° at an upward flow-rate of 9 mL/h. The fractions collected were 6.5 mL for both columns. All gels, the chromatography columns, and the P-3 peristaltic pumps were obtained from Pharmacia Fine Chemicals (Piscataway, NJ).

SDS–Polyacrylamide gel electrophoresis. — Samples were reduced with 5% (v/v) 2-mercaptoethanol and heated in 2% SDS for 5 min at 100°, loaded onto 1.5-mm thick slab-gels containing a 5–20% linear gradient of acrylamide [2:75 bis(acrylamide):acrylamide, w/w] and subjected to electrophoresis for 280 mA.h after the method of Maizel[14]. Each gel track was sliced in 3-mm (or 5-mm) sections, incubated for 2 days at 37° with mixing in glass scintillation-vials containing 10 mL of a scintillation cocktail (911:39:50:6 toluene–Liquifluor–Protosol–water, New England Nuclear), and counted at 25°. Sections of gel containing protein standards were fixed and stained overnight in 50:50:7 (v/v) methanol–water–acetic acid containing 0.2 g of R-250 Coomassie Blue/100 mL (Bio-Rad Labs, Richmond, CA) and destained for 1 day in 25:68:7 ethanol–water–acetic acid containing AG 501-X8 resin (Bio-Rad).

Preparation of plasma membrane. — TA3 plasma membranes were prepared by nitrogen cavitation, differential centrifugation, and linear sucrose-gradient centrifugation as previously described[4].

Hemagglutination-inhibition assay. — Samples were analyzed for the presence of epiglycanin by a continuous-flow hemagglutination-inhibition assay in which the agglutination of human blood-group N specific erythrocytes by a PBS extract of *Vicia graminea* seeds was specifically inhibited by epiglycanin[2,15]. This technique is uniquely sensitive to the presence of epiglycanin. Because a soluble glycoprotein competes for the lectin that would otherwise bind tightly to erythrocytes containing many adjacent MN glycoproteins, only those soluble glycoproteins having extremely high affinity show inhibition. Indeed, no other TA3 cell component was such an inhibitor, nor was any other Gal-GalNAc-Ser(Thr)-containing glycoprotein that was tested[3].

RESULTS

Cell-surface and released epiglycanin contains SO_4. — As is shown in Fig. 1 (top), the TA3-Ha cell released both Sepharose CL-4B void-volume (Peak A) and slightly retained (Peak B) D-[^{3}H]glucosamine-labeled peaks that contained both *Vicia graminea* inhibitory activity (epiglycanin) and $^{35}SO_4$. In contrast, from the TA3-St cell (Fig. 1, bottom), just as there was no epiglycanin in the whole cell or in the medium, there was also no discrete Peak B D-[^{3}H]glucosamine or $^{35}SO_4$ peak.

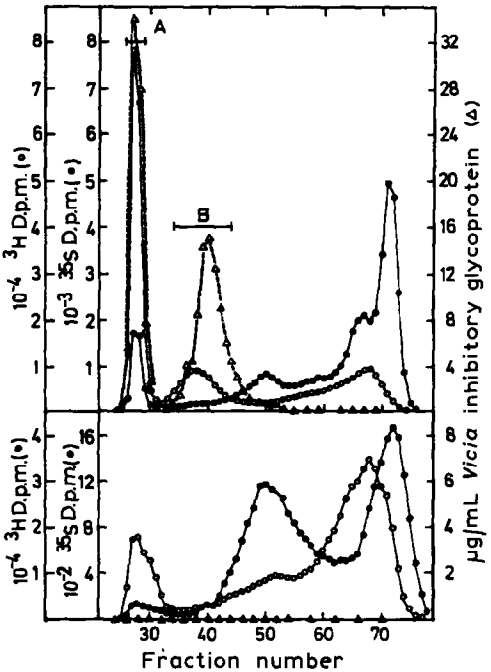

Fig. 1. Sepharose CL-4B fractionation of media released from TA3-Ha (top) and TA3-St cells (bottom). TA3 ascites cells were incubated for 25 h in medium with D-[^{3}H]glucosamine and $^{35}SO_4$, washed, and incubated for 16 h in serum-free medium. This medium was concentrated and fractionated as described under Experimental. Peak A and B described in the text are indicated by the bars. D-[^{3}H]Glucosamine, ○; $^{35}SO_4$, ●; *Vicia graminea* inhibitory glycoprotein, △.

When plasma membranes were prepared of each cell type, solubilized in 1% SDS, and chromatographed over Sepharose CL-2B, only the TA3-Ha cell contained a [^{3}H]glucosamine-labeled peak that eluted in the position of epiglycanin (Fig. 2). This peak also contained small amounts of $^{35}SO_4$.

Intact TA3-Ha and TA3-St cells doubly labeled for 1 day with D-[^{3}H]glucosamine and $^{35}SO_4$ were treated with trypsin and the resultant glycopeptides were fractionated over Sepharose CL-4B (Fig. 3). In the TA3-Ha cells (Fig. 3A), most of the D-[^{3}H]glucosamine radioactivity was in the form of large glycopeptides that eluted with epiglycanin. In contrast, the TA3-St cell produced no such large glycopeptides (Fig. 3B). Both cells contained glycopeptides labeled with $^{35}SO_4$, but the glycopeptides of the TA3-Ha cell contained a trailing of $^{35}SO_4$ at the high-molecular-weight end, coincident with D-[^{3}H]glucoamine and epiglycanin activity (Fractions 35–40, Fig. 3A). The majority of the $^{35}SO_4$ label, however, chromatographed similarly in both cells, despite the variability in D-[^{3}H]glucosamine label, indicating the presence in both cells of other glycosaminoglycans having less glucosamine content.

Glycosidase treatment of Peak B. — To better study the association of sulfate

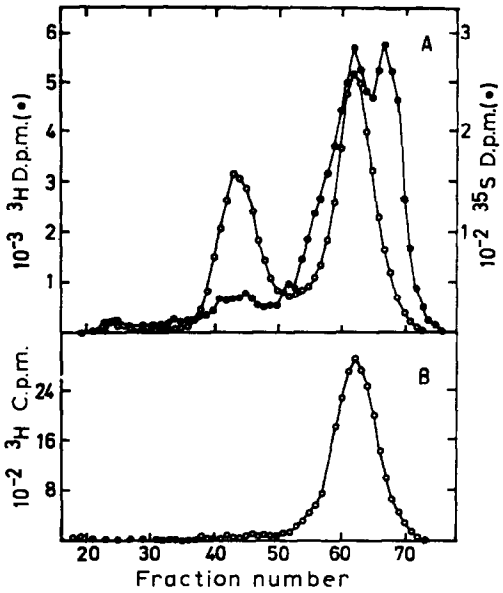

Fig. 2. Sepharose CL-2B fractionation in 1% SDS of plasma membrane from TA3-Ha (A) and TA3-St (B) cells. Cells were labeled for 24 h with D-[^{3}H]glucosamine alone (TA3-St cells) or D-[^{3}H]glucosamine plus $^{35}SO_4$ as described in Experimental. D-[^{3}H]Glucosamine, ○; $^{35}SO_4$, ●.

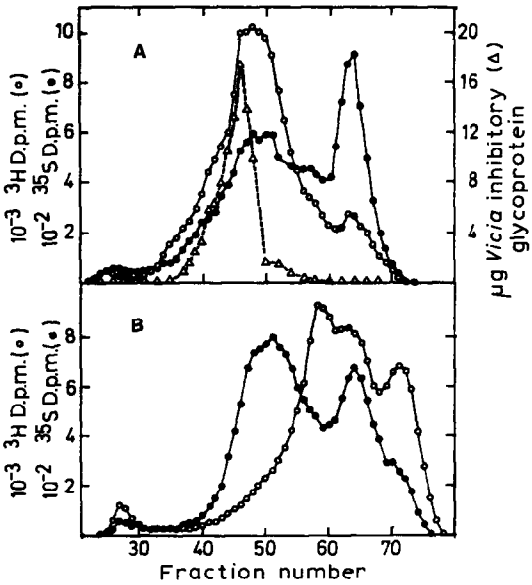

Fig. 3. Sepharose CL-4B fractionation of TA3-Ha (A) and TA3-St (B) cell-surface tryptic glycopeptide from cells labeled for 24 h with D-[^{3}H]glucosamine (○) and $^{35}SO_4$ (●). Tryptic fragments were prepared as described in Experimental.

with epiglycanin, both released epiglycanin (Peak B of Fig. 1, top) and the glycopeptides of the TA3-Ha cells (Fig. 3A) were fractionated by anion-exchange chromatography on DEAE-cellulose (Fig. 4). Peak B produced one major D-[^{3}H]glucosamine peak (Fig. 4A). The leading edge (fraction 31) contained less label as sialic acid (21%) than the trailing edge (25%), as determined by neuraminidase release and BioGel fractionation (not shown), as would be anticipated from a fractionation technique highly dependent on negatively charge groups. The epiglycanin (as monitored by *Vicia graminia* inhibition) was also associated with this D-[^{3}H]glucosamine-labeled peak, with the maximum activity in fraction 33 (arrow, Vg) while a small amount was found in the column void-volume (Fraction 8). The $^{35}SO_4$ was localized in 2 peaks: one with the D-[^{3}H]glucosamine and a second, larger peak eluting at higher salt concentrations between marker hyaluronic acid and chondroitin sulfate. Neither $^{35}SO_4$ peak was affected by digestion with testicular hyaluronidase or chondroditinase ABC, but the second of the two $^{35}SO_4$ peaks could be completely digested by heparinase treatment (not shown). Similar heparinase treatment of the combined fractions of the D-[^{3}H]glucosamine peak (indicated by the bar in Fig. 4A) released no further $^{35}SO_4$ (not shown). This heparinase-resistant material was incubated with keratanase[16], which is known to digest keratan sulfate. Such treatment did not release any $^{35}SO_4$ nor change the position of this peak on Sepharose CL-4B (not shown).

When the D-[^{3}H]glucosamine- and $^{35}SO_4$-labeled TA3-Ha glycopeptides containing epiglycanin (fractions 45–55 of Fig. 3A) were separated on DEAE-cellulose, an elution pattern similar to that of Peak B appeared (Fig. 4B): a small amount of $^{35}SO_4$ associated with the D-[^{3}H]glucosamine peak and a second, larger $^{35}SO_4$ peak. Heparinase treatment of the glycopeptides removed the second, larger $^{35}SO_4$ peak but left $^{35}SO_4$ associated with the D-[^{3}H]glucosamine peak (fractions 27–50, Fig. 4B).

Fractionation of peak A. — Fractions of Peak A that eluted from the Sepharose CL-4B column appeared opalescent, especially after concentration. In contrast, Peak B appeared clear and transparent at similar protein concentrations. As the TA3-Ha plasma membrane was previously shown to contain essentially all of the cellular epiglucanin[4], it seemed likely that the high-molecular-weight form of released epiglycanin might contain membrane fragments probably derived from the plasma membrane. If Peak A did contain membraneous components, then they should be sedimented by ultracentrifugation. When Peak A was sedimented at 300,000*g* for 2 h, most of the glucosamine radioactivity (Table I) and all of the *Vicia graminea* inhibitory activity (not shown) were pelleted. When Peak A material was first treated with testicular hyaluronidase and refractionated on Sepharose CL-4B to remove labeled breakdown products, the void-volume material sedimented completely (Table I). This result indicated that the soluble component of Peak A was largely hyaluronic acid. In a separate experiment, when D-[^{3}H]glucosamine-labeled TA3-Ha Peak A was bottom loaded below a 15-50% linear sucrose gradient and centrifuged for 16 h at 30,000 r.p.m. in an SW25.5 rotor, none of the material

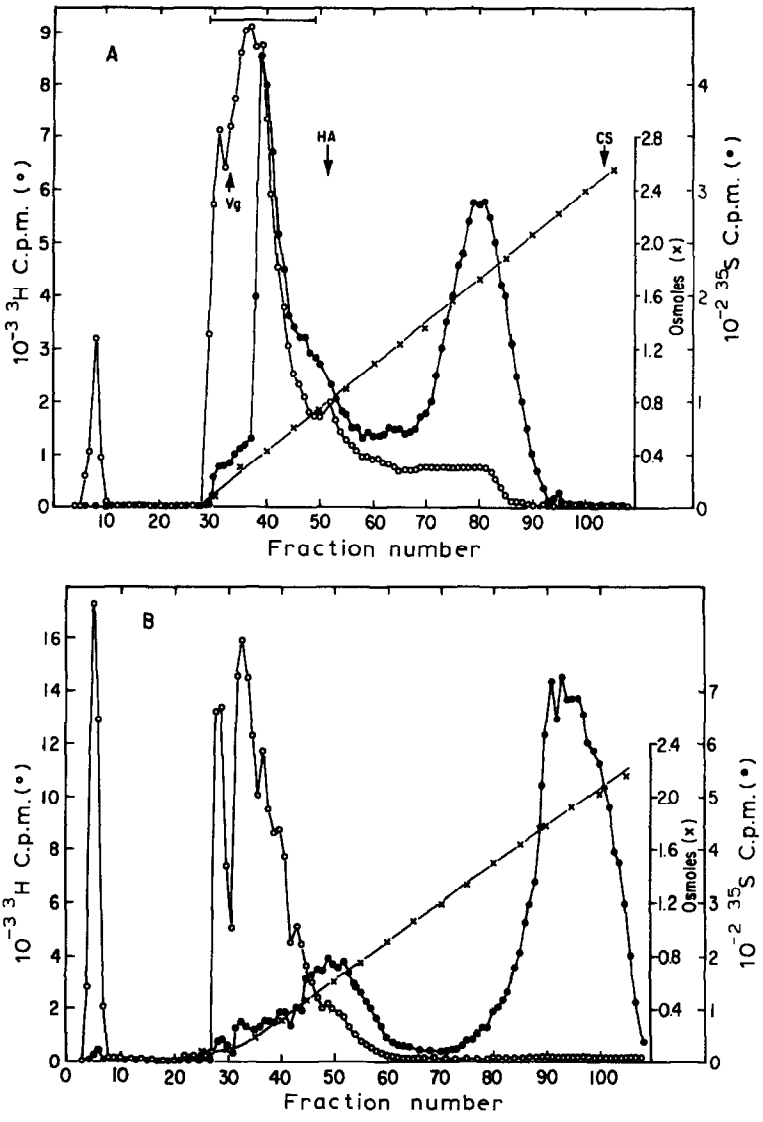

Fig. 4. DEAE-cellulose chromatography of Sepharose CL-4B Peak B (*A*) and cell-surface tryptic glycopeptides (*B*) of TA3-Ha cells labeled with D-[^{3}H]glucosamine (○) and $^{35}SO_4$ (●). Preswollen DEAE-cellulose (Whatman DE52) was precycled, preequilibrated with 0.01M ammonium acetate, and eluted with a linear gradient of ammonium acetate as described by Kraemer[26]. The column was 1.8 cm × 34 cm; ~3-mL fractions were taken in (*A*) and 2.5 mL in (*B*). Osmolarity (x) was measured on an Advanced Instruments osmometer. Arrows indicate the positions marking the peaks of *Vicia graminea* inhibitory activity (Vg), unlabeled hyaluronic acid, and chondroitin-4-SO_4.

floated, whereas a sample of D-[^{14}C]glucosamine-labeled TA3-Ha plasma membrane floated to a level corresponding to 39% sucrose (not shown). Thus while Peak A could be sedimented in a similar fashion as membranes, it was clearly much denser than normal membrane components.

TABLE I

ULTRACENTRIFUGATION OF SEPHAROSE CL-4B PEAK A AND PEAK B[a]

Fraction	*Hyaluronidase treatment*	*D.p.m. in supernatant*		*Percent soluble*
		Before spin	*After spin*	
Peak A	–	6680	1570	24
Peak A	+	2890	120	4
Peak B	–	1650	1190	72

[a]Aliquots of D-[^{14}C]glucosamine-labeled Peak A or D-[^{3}H]glucosamine-labeled Peak B were centrifuged for 2 h at 50,000 r.p.m. in a Beckman SW 50.1 rotor, and the radioactivity in the supernatant solution was determined. Prior to ultracentrifugation, the aliquot of Peak A had been treated with testicular hyaluronidase followed by gel filtration on Sepharose CL-4B to remove degraded products.

The TA3-Ha Peak A and Peak B was compared to TA3-Ha plasma membranes by SDS–polyacrylamide gel electrophoresis. When the released Peak A and plasma membrane prepared from leucine-labeled cells (Fig. 5A and B) or glucosamine-labeled cells (Fig. 5D and E) were compared by electrophoresis on 5–20% gradient gels, a large number of labeled components were found producing radioactivity profiles of similar complexity. In contrast, the corresponding leucine and glucosamine labeling patterns from Peak B were much simpler, indicating the presence of primarily one high-molecular-weight glycoprotein (Fig. 5C and F). The broad labeled peak in the 20% gel from fractions 59–66 was probably glycolipid, as little leucine radioactivity was found in this area, observations supporting the conclusion that the Peak A material was membrane-associated. Electrophoresis was also performed on each of the 2 glucosamine-labeled Sepharose CL-2B/SDS peaks isolated from purified TA3-Ha plasma membranes (see Fig. 2A). Electrophoresis of the first Sepharose 2B peak (epiglycanin) resulted in only a large peak at the origin of the gel, whereas the second Sepharose 2B peak contained the rest of the peaks found in Fig. 5E (data not shown). Electrophoresis of glucosamine-labeled TA3-St plasma membranes showed a profile similar to that of the TA3-Ha plasma membrane (Fig. 5E), but without the major peak at the origin (not shown).

When attempts were made to solubilize and fractionate Peak A, only those treatments which included detergents were successful. When Peak A was treated with 4M guanidine HCl (adequate to solubilize proteoglycans), all of the radioactivity remained in the void volume of Sepharose CL-4B or CL-2B. With chromatography on Sepharose CL-2B in 1% SDS, however, D-[^{3}H]glucosamine-labeled TA3-Ha Peak A could be separated into a void-volume peak, a second peak in a position slightly larger than TA3-Ha Peak B, and a third peak of smaller molecular-weight material (Fig. 6A). When D-[^{3}H]glucosamine-labeled Peak A from TA3-St cells was similarly fractionated, the Peak B material was absent (Fig. 6C).

Glycosidase treatment of Peak A. — Testicular hyaluronidase treatment of

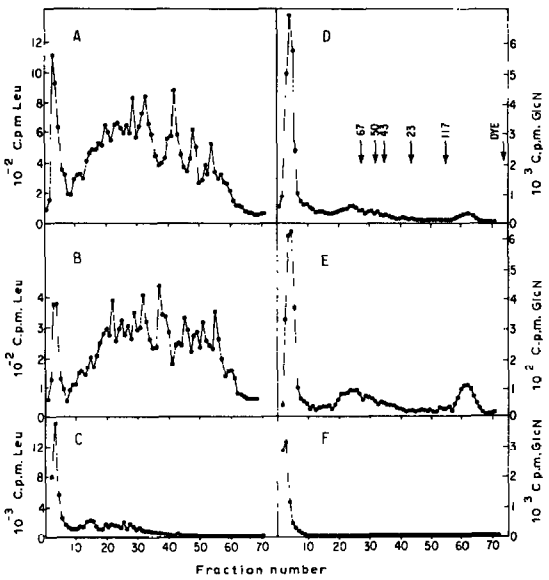

Fig. 5. SDS-gel electrophoresis of [^{3}H]leucine (*A–C*) and D-[^{3}H]glucosamine- (*D–F*) labeled TA3-Ha samples of Sepharose CL-4B Peak A (*A* and *D*), plasma membrane (*B* and *E*), and Peak B (*C* and *F*). Samples were handled as described in Experimental. Gel slices were 3 mm (fractions 1–56) and 5 mm (fractions 57–72). Arrows indicate migration of protein markers of bovine serum albumin (67,000), immunoglobulin G (50,000 and 23,000), ovalbumin (43,000), and cytochrome C (11,700).

D-[^{3}H]glucosamine- and $^{35}SO_4$-labeled Peak A from TA3-Ha or TA3-St cells resulted in the degradation of the Sepharose CL-2B void-volume, D-[^{3}H]glucosamine-labeled component. Thus the high-molecular-weight, glucosamine-labeled material in Peak A is hyaluronic acid. The elution of the Peak B-sized material in the solubilized TA3-Ha Peak A (Fig. 6B) was unaffected by either testicular or *Streptomyces* hyaluronidase.

Just as the TA3-Ha Peak B material earlier was shown to contain $^{35}SO_4$ so also was the Peak B-sized material in Peak A found to contain $^{35}SO_4$ (Fig. 6B). To determine if this sulfate were associated with chondroitin sulfate, the TA3-Ha Peak A was incubated with chondroitinase ABC. No [^{3}H]glucosamine or $^{35}SO_4$ was released, despite the complete degradation of marker unlabeled chondroitin-4-SO_4 (not shown). When the chondroitinase ABC-resistant material was treated with heparinase and fractionated on Sepharose CL-4B, 40% of the $^{35}SO_4$, <5% of the D-[^{3}H]glucosamine, and all of an unlabeled heparin marker were degraded (data not shown). When the heparinase-resistant Peak A material was rechromatographed on Sepharose CL-2B in SDS, there was a profile similar to that shown for the epiglycanin fraction in Fig. 6B; that is, a substantial amount of $^{35}SO_4$ remained with the D-[^{3}H]glucosamine. Treatment with nitrous acid by the method of Roblin *et al.*[17] did not release any further ^{3}H or ^{35}S radioactivity, also indicating the absence of any remaining heparin in the sample. Throughout all of these incubations, the epiglycanin of the TA3-Ha Peak A, as measured by *Vicia graminea* inhibition,

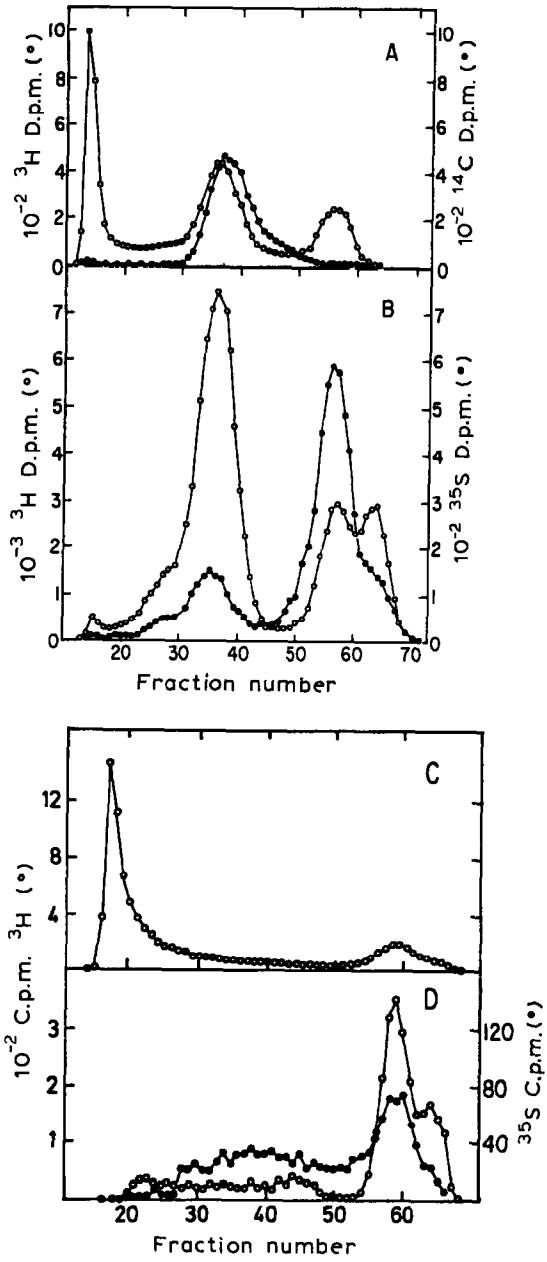

Fig. 6. Sepharose CL-2B fractionation in 1% SDS of Sepharose CL-4B Peak A from TA3-Ha cells (*A* and *B*) and TA3-St cells (*C* and *D*). *A*. D-[^{3}H]Glucosamine labeled TA3-Ha Peak A (○). D-[^{14}C]Glucosamine TA3-Ha Peak B (●) has been added as a marker. *B*. D-[^{3}H]Glucosamine (○) and $^{35}SO_4$ (●) labeled TA3-Ha Peak A after treatment with testicular hyaluronidase. *C*. D-[^{3}H]Glucosamine-labeled TA3-St Peak A. *D*. D-[^{3}H]Glucosamine (○) and $^{35}SO_4$ (●) labeled TA3-St Peak A, after treatment with *Streptomyces* hyaluronidase.

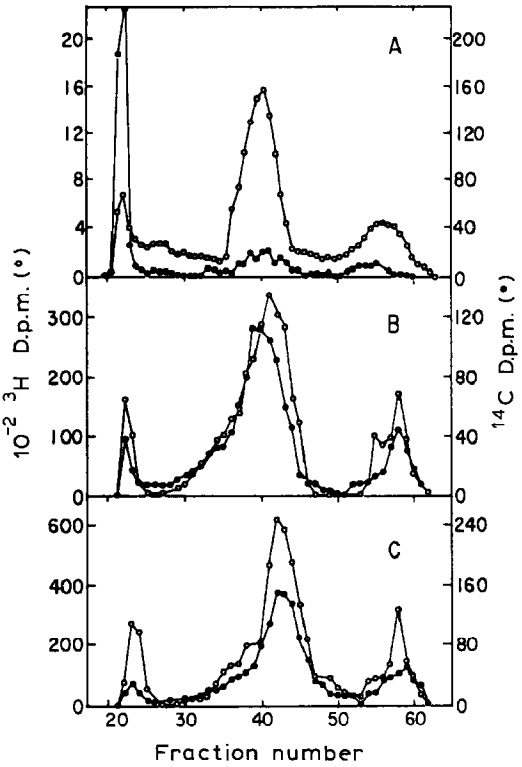

Fig. 7. Sepharose CL-4B fractionation in PBS of TA3-Ha Peak A doubly labeled with D-[^{3}H]glucosamine and D-[^{14}C]glucosamine before (*A*) and after (*B*) testicular hyaluronidase and after hyaluronidase and neuraminidase treatments (*C*) as described in the text. *A*. D-[^{3}H]Glucosamine (old label), ○; D-[^{14}C]glucosamine (new label), ●. *B* and *C*, D-[^{14}C]glucosamine (old label), ●; D-[^{3}H]glucosamine (new label), ○.

remained in the void volume of Sepharose CL-4B, largely resistant to degradation, although small amounts consistently appeared in the position of Sepharose CL-4B Peak B epiglycanin.

Kinetics of release of Peak A components. — To determine which of the components of Peak A were released rapidly with a half-life of 0.75 h and which had the very long half-life previously reported[3], TA3-Ha cells previously labeled for 24 h in D-[^{3}H]glucosamine and grown for 16 h in nonradioactive medium were labeled for 0.75 h in D-[^{14}C]glucosamine and incubated for 2.5 h in nonradioactive medium. When this second chase-medium was fractionated on Sepharose CL-4B and the resultant Peak A was chromatographed on Sepharose in SDS, most of the newly labeled, high-molecular-weight radioactivity was found in the void-volume fraction containing hyaluronic acid (Fig. 7A).

In a similar double glucosamine-label experiment [in which cells were labeled for 24 h with D-[^{14}C]glucosamine and pulse-labeled for 30 min with D-[^{3}H]glucosamine], Peak A material released from TA3-Ha cells after 1 h incubation in non-

radioactive medium was treated with hyaluronidase, refractionated over Sepharose CL-4B, and then chromatographed over Sepharose CL-2B in SDS. After hyaluronidase treatment, the newly labeled void-volume peak largely disappeared (Fig. 7B). The major difference between the old, long-term label (^{3}H) and the new pulse-label (^{14}C) radioactivity profiles was that the epiglycanin components of newly labeled Peak A released *in vitro* migrated on Sepharose CL-2B at an apparent lower molecular weight than the older, labeled material (Fig. 7B). When this hyaluronidase-treated Peak A was treated with neuraminidase prior to Sepharose CL-2B/SDS fractionation, the difference in the elution profiles of the radioactivity in the epiglycanin peak was eliminated, indicating that the migration difference was due to the decreased amount of labeled sialic acid on the newly synthesized glycoproteins (Fig. 7C). After removal of the hyaluronic acid, the remaining glucosamine-labeled components contained similar proportions of both the [^{3}H] pulse and [^{14}C] long-term glucosamine labels, suggesting that these newly labeled Peak A components were released in a concomitant fashion as the older labeled components. Thus the rapid phase of the biphasic release of Peak A was due to hyaluronic acid, whereas the low rate was attributed to the other components.

DISCUSSION

TA3-Ha and TA3-St cells have been found to contain, on their plasma membranes, heparan sulfate but not chondroitin sulfate or hyaluronic acid. Both cell lines synthesize and release heparan sulfate and hyaluronic acid into culture medium. The absence of cell-surface-bound hyaluronate in the TA3 cells contrasts with the binding of hyaluronate to normal and transformed fibroblasts reported earlier by Toole and colleagues[18,19]. In the MAT-B cell line, another ascites mammary adenocarcinoma also studied for the role of its sialoglycoproteins in transplantation and metastasis[20], hyaluronic acid was also not found in culture medium bound to the cell surface[21]. The absence of hyaluronate binding on ascites tumor cells may be responsible for their ability to grow and remain unattached to the substratum.

The major difference that was seen between the two TA3 cell types was the presence, on the TA3-Ha cell-surface and in the medium, of the glycoprotein epiglycanin which is not present in the TA3-St cells as was previously documented[2–8]. This epiglycanin is found in a totally soluble form (Peak B, Fig. 1A) and in a membraneous aggregate (Peak A). Both of the 2 released forms, as well as the plasma membrane-bound form contain sulfate associated with the glycoprotein in an unknown fashion. The sulfate appears to be primarily associated with those forms of epiglycanin containing the least amount of sialic acid, but all of the glycopeptides contained some sulfate (Fig. 4). These results are analogous to those reported earlier by Sherblom and Carraway[21] in the MAT-B1 cells, in which most of the glycopeptides of the major sialoglycoprotein were found to contain

sulfate. Later work indicated that the sulfate was found on GlcNAc residues attached in the 6 position of GalNAc[22].

The origin of the membraneous released glycoproteins (Peak A) is undoubtedly plasma membrane. In the first place, we have previously shown that the plasma membrane contains essentially all of the epiglycanin[4], and the >70 h half-life of shedding indicates that this is not newly synthetic material. In addition, SDS gel electrophoresis of Peak A isolated from TA3-Ha cells that had been labeled externally with [^{3}H]$NaBH_4$ after treatment by galactose oxidase indicated[3] that those same components labeled endogenously by glucosamine were also labeled by the [^{3}H]$NaBH_4$. A major difference between Peak A material and plasma membrane is the different proportions of components. Peak A contains, for example, a higher proportion of epiglycanin than does the plasma membrane, as measured by glucosamine labeling. In Fig. 5A and B, the glucosamine-labeled epiglycanin (fractions 32–42) was found in greater amount than the smaller sized, glucosamine-labeled material (fractions 51–61). However, in comparative experiments where the plasma membrane (Fig. 2A) was isolated from the same group of cells that produced labeled Peak A, the epiglycanin (fractions 39–49) was found in a lesser amount relative to the smaller-sized, glucosamine-labeled material (fractions 58–68). This occurrence may be due to the postulated microvillar origin of membrane fragments[23] released; indeed, thin-section electron micrographs have indicated that, in the TA3-Ha cell, epiglycanin was found preferentially on microvilli[24]. A similar membraneous glycoprotein-complex was also found to be released from MAT-C1 and MAT-B1 rat adenocarcinoma cells[25].

Our studies show that the glucosamine-labeled material released rapidly from TA3 cells is hyaluronic acid, that it is this high-molecular-weight glycosaminoglycan that is the component of Peak A with the half-life of synthesis and release of 45 min. The epiglycanin and other components of Peak A are released much more slowly. It is clear, however, that some of the epiglycanin in Peak A can be released very rapidly. In our earlier report[3], we found that newly released Peak B as labeled by a glucosamine pulse eluted on Sepharose at a smaller effective size than those molecules released with a long half-life. This difference between rapidly released and slowly released epiglycanin was solely related to the presence of sialic acid, which was more highly labeled in the slowly released epiglycanin. An identical phenomenon occurs with the epiglycanin contained in Peak A (Fig. 7). Thus both the membraneous and soluble forms of epiglycanin contain molecules, relatively poor in sialic acid labeling, that are released quickly to culture medium. Both forms of epiglycanin contain molecules relatively enriched in sialic acid that are released much more slowly to the medium.

ACKNOWLEDGMENTS

We thank the following for their generous gifts: Dr. Jeramiah Silbert for heparinase, Dr. S. Suzuki for keratinase, and Dr. H. C. de Witt for the *Vicia graminea* seeds.

REFERENCES

1 T. S. HAUSCHKA, L. WEISS, B. A. HOLDRIDGE, T. L. CUDNEY, M. ZUMPFT, AND J. A. PLANINSEK, *J. Natl. Cancer Inst.*, 47 (1971) 343–359.
2 A. G. COOPER, J. F. CODINGTON, AND M. C. BROWN, *Proc. Natl. Acad. Sci. U.S.A.*, 71 (1974) 1224–1228.
3 D. K. MILLER AND A. G. COOPER, *J. Biol. Chem.*, 253 (1978) 8798–8803.
4 D. K. MILLER, A. G. COOPER, AND M. C. BROWN, *J. Biol. Chem.*, 253 (1978) 8804–8811.
5 H. S. SLAYTER AND J. F. CODINGTON, *J. Biol. Chem.*, 248 (1973) 3405–3410.
6 J. F. CODINGTON, K. B. LINSLEY, R. W. JEANLOZ, R. IRIMURA, AND T. OSAWA, *Carbohydr. Res.*, 40 (1975) 171–182.
7 J. F. CODINGTON, B. H. SANFORD, AND R. W. JEANLOZ, *J. Natl. Cancer Inst.*, 51 (1973) 585–591.
8 J. F. CODINGTON, in *Cellular Membranes and Tumor Cell Behavior*, William and Wilkins, Baltimore, 1975, pp. 399–419.
9 D. K. MILLER AND A. G. COOPER, in J. GREGORY AND R. W. JEANLOZ (Eds.), *Glycoconjugates*, Vol. 1, Academic Press, New York, 1979, pp. 221–227.
10 D. HEINEGARD, *Chem. Scr.*, 4 (1973) 199–201.
11 H. SAITO, T. YAMAGATA, AND S. SUZUKI, *J. Biol. Chem.*, 243 (1968) 1536–1542.
12 A. LINKER AND P. HOVINGH, *Methods Enzymol., Part B*, 28 (1972) 902–911.
13 B. Y. J. T. YUE, J. L. BAUM, AND J. E. SILBERT, *Biochem. J.*, 158 (1976) 567–573.
14 J. V. MAIZEL, *Methods Virol.*, 5 (1971) 179–246.
15 A. G. COOPER AND M. C. BROWN, in R. F. RICHIE (Ed.), *Automated Immunoanalysis*, Part 2, Marcel Dekker, New York, pp. 471–506.
16 K. NAKAZAWA AND S. SUZUKI, *J. Biol. Chem.*, 25 (1975) 912–917.
17 R. ROBLIN, S. O. ALBERT, N. A. GELB, AND P. H. BLACK, *Biochemistry*, 14 (1975) 347–357.
18 C. B. UNDERHILL AND B. P. TOOLE, *J. Cell Physiol.*, 110 (1982) 123–128.
19 R. L. GOLDBERG, J. D. SEIDMAN, G. CHI-ROSSO, AND B. P. TOOLE, *J. Biol. Chem.*, 259 (1984) 9440–9446.
20 K. L. CARRAWAY, J. W. HUGGINS, A. P. SHERBLOM, R. W. CHESNUT, R. L. BUCK, S. P. HOWARD, C. L. OWNBY, AND C. A. C. CARRAWAY, in E. F. WALBORG, JR. (Ed.), *Glycoproteins and Glycolipids in Disease Processes*, ACS Symp. Ser. No. 80, 1978, pp. 432–445.
21 A. P. SHERBLOM AND K. L. CARRAWAY, *Biochemistry*, 19 (1980) 1213–1219.
22 S. R. HULL, R. A. LAINE, T. KAIZU, I. RODRIGUEZ, AND K. L. CARRAWAY, *J. Biol. Chem.*, 259 (1984) 4866–4877.
23 G. J. STEWART, G. G. GASIC, T. GASIC, AND J. L. CATALFALMO, *J. Supramol. Struct.*, Suppl. 3 (1979) 187.
24 S. C. MILLER, E. D. HAY, AND J. F. CODINGTON, *J. Cell Biol.*, 72 (1977) 511–529.
25 S. C. HOWARD, A. P. SHERBLOM, J. W. HUGGINS, C. A. C. CARRAWAY, AND K. L. CARRAWAY, *Arch. Biochem. Biophys.*, 207 (1981) 40–50.
26 P. M. KRAEMER, *Biochemistry*, 10 (1971) 1437–1445.