Biosynthesis of Heparan Sulfate Proteoglycan by Human Colon Carcinoma Cells and Its Localization at the Cell Surface

RENATO V. IOZZO
Department of Pathology and Laboratory Medicine, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

ABSTRACT After 24 h of continuous labeling with radioactive precursors, a high molecular weight heparan sulfate proteoglycan (HS-PG) was isolated from both the medium and cell layer of human colon carcinoma cells (WiDr) in culture. The medium HS-PG eluted from a diethylaminoethyl anion exchange column with 0.45–0.50 M NaCl, had an average density of 1.46–1.49 g/ml on dissociative CsCl density-gradient ultracentrifugation, and eluted from Sepharose CL-2B with a $K_{av} = 0.57$. This proteoglycan had an estimated M_r of $\simeq 8.5 \times 10^5$, with glycosaminoglycan chains of $M_r = 3 \times 10^4$ which were all susceptible to HNO_2 deaminative cleavage. Deglycosylation of the HS-PG with polyhydrogen fluoride resulted in a ^{3}H-core protein with $M_r \simeq 2.4 \times 10^5$. The cell layer contained a population of HS-PG with characteristics almost identical to that released into the medium but with a larger $M_r = 9.5 \times 10^5$. Furthermore, an intracellular pool contained smaller heparan sulfate chains ($M_r \simeq 1 \times 10^4$) which were mostly devoid of protein core. In pulse chase experiments, only the large cell-associated HS-PG was released (~58%) into the medium as intact proteoglycan and/or internalized and degraded (~42%), with a $t_{1/2} = 6$ h. However, the small intracellular component was never released into the medium and was degraded at a much slower rate. When the cells were subjected to mild proteolytic treatment, only the large cell-associated HS-PG, but none of the small component, was displaced. Addition of exogenous heparin did not displace any HS-PG into the medium. Both light and electron microscopic immunocytochemistry revealed that the cell surface reacted with antibody against an HS-PG isolated from a basement membrane-producing tumor. Electron microscopic histochemistry using ruthenium red and/or cuprolinic blue revealed numerous 10–50-nm diam granules and 70–220-nm-long electron-dense filaments, respectively, on the surface of the tumor cells. The results indicate that colon carcinoma cells synthesize HS-PGs with distinct structural and metabolic characteristics: a large secretory pool with high turnover, which appears to be synthesized as an integral membrane component and localized primarily at the cell surface, and a small nonsecretory pool with low turnover localized predominantly within the cell interior. This culture system offers an opportunity to investigate in detail the mechanisms involved in the regulation of proteoglycan metabolism, and in the establishment of the neoplastic phenotype.

Proteoglycans are major constituents of the extracellular matrix and cell surface. They are complex macromolecules in which glycosaminoglycan side chains are covalently linked to a protein core forming polydisperse populations of proteoglycan monomers (1). Although significant progress has been made in elucidating the structure of cartilage proteoglycans (1–8), relatively little is known about the noncartilaginous proteoglycans, but their ubiquitous distribution and strategic location among tissues suggest that they play important physiological roles (9, 10).

Since the discovery of cell-associated heparan sulfate (11), it has been evident that this macromolecule is intimately

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involved with fundamental cellular processes such as cell growth, receptor binding, and transformation (9, 12). The cell surface heparan sulfate can self-interact via its side chains (13) and is a critical mediator of cell adhesion by specific interactions with fibronectin (14), laminin (15), and collagen (16). Recent studies (17) indicate that these macromolecules are complexed at the cell surface, and that heparan sulfate proteoglycan plays an important role in the assembly of such supramolecular complexes.

Heparan sulfate has also been shown to be qualitatively different in transformed cells. In particular, it has been found that the degree of sulfation is markedly reduced upon transformation with SV40 (18, 19), and that this undersulfation occurs predominantly in *O*-sulfate groups of the heparan sulfate chains (20). It is noteworthy that in vivo studies of human hepatomas have also identified a class of heparan sulfate with a lower degree of sulfation as compared with normal human liver (21), and this difference is specific for heparan sulfate (21). These studies have proposed that changes in heparan sulfate may be related to the altered cell interaction and growth behavior of transformed cells.

We have previously demonstrated (22) that normal human colon tissue maintained in organ culture synthesizes two major classes of proteoglycans: a large monomer containing heparan sulfate, and a smaller monomer containing dermatan sulfate. Colon carcinoma tissue, on the other hand, synthesizes predominantly a small chondroitin sulfate-rich proteoglycan and lesser amounts of heparan sulfate proteoglycan. We have also shown, using both cytochemistry and autoradiography (23), that the mesenchymal cells surrounding the tumor are active in the synthesis of sulfated proteoglycans. These previous findings have raised the possibility that the heparan sulfate proteoglycan is synthesized primarily by the neoplastic epithelial cells, whereas the small chondroitin sulfate proteoglycan is synthesized by the connective tissue stroma surrounding the invasive tumor cells.

In the present study, we have investigated in detail the biosynthesis, secretion, and topographical distribution of proteoglycans in cultured carcinoma cells derived from a spontaneous human colon carcinoma. The results demonstrate that cultured colon carcinoma cells synthesize exclusively heparan sulfate proteoglycans with distinct location and metabolism: a "secretory" pool, which is located primarily at the cell surface and is partly released by limited proteolysis and partly internalized and degraded, and a "nonsecretory" pool, which is mostly intracellular, never released, and slowly degraded.

MATERIALS AND METHODS

Materials: All the chemicals used were of analytical grade unless otherwise stated. Guanidine HCl (grade I), phenylmethylsulfonyl fluoride, and anisole were purchased from Sigma Chemical Co. (St. Louis, MO); cesium chloride from Matheson, Coleman and Bell (Elk Grove Village, IL); benzamidine HCl, 6-aminohexanoic acid, and ruthenium red from Eastman Kodak Co. (Rochester, NY); Zwittergent 3-12 was from Calbiochem-Behring Co. (San Diego, CA); and pyridinium polyhydrogen fluoride from PCR Inc. (Gainesville, FL); Sepharose CL-2B, CL-6B, Sephacryl-300, Sephadex G-25 (PD-10), and DEAE-Sephacel from Pharmacia Fine Chemicals (Piscataway, NJ); papain from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium, fetal calf serum, and trypsin were purchased from GIBCO Laboratories, Grand Island Biological Co. (Grand Island, NY). Carrier-free $^{35}SO_4^{2-}$ as sodium salt (900 mCi/mmol), L-[G-^{3}H]serine (19 Ci/mmol), L-[3,4,5-^{3}H (*N*)]leucine (141 Ci/mmol), and D-[6-^{3}H (*N*)]glucosamine HCl (19.7 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Chondroitinase ABC (*Proteus vulgaris*), AC II (*Arthrobacter aurescens*), and glycosaminoglycan standards were from Miles Laboratories Inc. (Elkhart, IN). Heparitinase from *Flavobacterium heparinum* was a generous gift of Dr. A. Linker, University of Utah (Salt Lake City). Affinity-purified antibodies raised against the protein core of the heparan sulfate proteoglycan from the Engelbreth-Holm-Swarm tumor were a generous gift of Dr. J. Hassell, National Institute of Dental Research, NIH. Proteoglycan monomer (A_1-D_1)[1] and aggregates (A_1) from bovine nasal cartilage were generous gifts of Dr. L. Rosenberg, Montefiore Hospital (Bronx, NY). Proteoglycan monomers (D_1)[1] from Swarm rat chondrosarcoma were generous gifts of Dr. T. N. Wight, University of Washington (Seattle). Protein standards for calibration of the columns and for SDS PAGE were from Bio-Rad (Richmond, CA). The Aquapore columns for the high-performance liquid chromatography (HPLC)[2] were bought from Brownlee Laboratory (Santa Clara, CA). Cuprolinic blue was from Gallard Schlesinger Chemical Mfg. Corp. (Carle Place, NY).

Cell Culture and Metabolic Labeling: Human colon carcinoma cells (WiDr) were purchased from the American Type Culture Collection (Baltimore, MD). By both scanning and transmission electron microscopy, these cells revealed a rich microvillar surface and well-developed junctional complexes (not shown), which are characteristically found in neoplastic cells of epithelial origin. The cells were cultured in 75-cm^2 Falcon flasks (Becton, Dickinson & Co., Sunnyvale, CA) in Dulbecco's modified Eagle's medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, and 10% fetal calf serum (Gibco Laboratories). After reaching confluency in a humidified atmosphere of 5% CO_2 and 95% air at 37°C, the cells were washed twice with Hanks' balanced salt solution (HBSS) and metabolically labeled in the presence or absence of serum. Radioactive precursors included 40 μCi/ml of carrier-free [^{35}S]sulfate alone or in combination with 10 μCi/ml of L-[^{3}H]serine, L-[^{3}H]leucine, or D-[^{3}H]glucosamine. Steady state was reached between 20 and 24 h (see Fig. 1), and the cells were metabolically labeled for at least 24 h in all the experiments described in this paper.

Isolations of Proteoglycans: After labeling, the incubation medium was removed, the cell layer (~10^7 cells/flask) was washed twice with HBSS and the washes were combined with the medium. Solid guanidine HCl (0.5 g/ml) was added and the solutions were either processed immediately or frozen at −70°C until subsequent analysis. Proteoglycans in the cell layer were solubilized at 4°C using the sequential addition of 8% Zwittergent 3-12, 30 min, and 8 M guanidine HCl, 50 mM sodium acetate, pH 6.0, 30 min (24) in the presence of the following protease inhibitors (25): 20 mM sodium EDTA, 5 mM benzamidine HCl, 0.1 M 6-aminohexanoic acid, and 2 mM phenylmethylsulfonylfluoride. The combined extracts (~8 ml) were removed and the cell layers were extracted for an additional 30 min with 4% Zwittergent 3-12, and 4 M guanidine HCl (~6 ml). The solutions were centrifuged at 1,500 *g* to remove cellular debris, and the unincorporated radioisotopes were removed from all samples by gel chromatography on prepacked Sephadex G-25 (PD-10) columns, equilibrated, and eluted with 4 M guanidine HCl, 0.1 M sodium sulfate, pH 7.0, containing 0.2% Triton X-100.

Purification of Proteoglycans: The material eluted at the excluded volumes of PD-10 columns was dialyzed against distilled water at 4°C and lyophylized. The samples were dissolved in 1-2 ml of 50 mM Tris-HCl buffer, pH 8.0, 0.1 M NaCl in 8 M urea, and 0.2% Triton X-100, and applied to an 8-ml DEAE-Sephacel column equilibrated with the same buffer (Yanagishita, M., and V. C. Hascall; personal communication). The DEAE column was washed with ~30 ml of equilibration buffer and then eluted with a linear gradient (30+30 ml) from 0.1 to 0.8 M NaCl, under a constant flow rate of 5 ml/h. Linearity of the gradient was confirmed by measuring the conductivity of various fractions and converting the obtained values to NaCl concentrations by use of a standard curve. Fractions eluting at high salt concentration (0.4-0.5 M) were pooled, concentrated by dialysis and lyophylization, and resuspended in 4 M guanidine HCl buffer in the presence of protease inhibitors. Isopycnic CsCl density-gradient ultracentrifugation (starting $\rho = 1.41$ g/ml) was performed for 48-60 h at 10°C, 37,000 rpm in a Beckman SW-50.1 rotor (Beckman Instruments, Inc., Fullerton, CA). Gradients were divided into 10 equal fractions (500 μl each) using an Auto Densi-Flow II (Buchler Instruments Inc., Ft. Lee, NJ) and the distribution of radioactivity and density in 100-μl aliquots of each fraction was determined. Portions of the CsCl density-gradient fractions were applied to an analytical Sepharose CL-2B column (0.6 × 90 cm) eluted with 4 M guanidine HCl, 50 mM sodium sulfate buffer, pH 7.0, with 0.2% Triton X-100. Fractions of 0.5 ml were mixed with 10 ml of Redi-Solv™EP (Beckman Instruments, Inc.) and analyzed for radioactivity in a

[1] The nomenclature D_1–D_{10} indicates fractions recovered from the bottom to top of dissociative (4 M guanidine HCl) CsCl density-gradient ultracentrifugation.

[2] *Abbreviations used in this paper:* HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography.

Beckman LS-6800 scintillation counter (Beckman Instruments, Inc.). Appropriate quenching curves for the various buffers were determined; efficiency was calculated with H number, and corrections for the shift of ^{35}S radioactivity into ^{3}H channel were made.

Proteoglycan-enriched samples were resuspended in 0.1 M Tris–sulfate buffer, pH 7.0, and subjected to HPLC as previously described (26). This system utilized two Aquapore columns OH-500 and OH-1000 (4.6 × 250 mm each) in tandem, with nominal exclusion limits in H_2O as determined with dextrans of 2 and 20 × 10^6, respectively. Samples of 50–100 μl (1–2 × 10^4 cpm, ^{35}S) were subjected to HPLC under a constant pressure of ~500 psi and a flow rate of 0.5 ml/min. The HPLC columns were calibrated using proteoglycans from bovine nasal cartilage and rat chondrosarcoma as well as proteins of known M_r as described previously (26). The recovery of radiolabeled macromolecules exceeded 95%.

Characterization of Glycosaminoglycans: Glycosaminoglycans were released from proteoglycans by alkaline borohydride treatment (27) and/or papain digestion (28). The released glycosaminoglycans were chromatographed on an analytical Sepharose CL-6B column eluted with 0.5 M sodium acetate buffer, pH 7.0, and their relative average molecular weight was estimated as described (29). The ^{35}S-labeled glycosaminoglycans were subjected to HNO_2 degradation (30), and/or chondroitinase ABC and AC-II treatments (31). The degree of sulfation was determined by electrophoresis in 0.1 N HCl, pH 1.0, where only the sulfate groups are ionized (32), and by elution from anion-exchange chromatography.

Analysis of Core Protein: Proteoglycan monomers derived from the bottom fifth of CsCl density gradients (D_1+D_2) were deglycosylated with a modified polyhydrogen fluoride treatment (33). Briefly, 100 μl of anisole scavenger and 1 ml of 70% polyhydrogen fluoride in pyridine buffer were added to a lyophylized aliquot of purified proteoglycans doubly labeled with L-[^{3}H]leucine and [^{35}S]sulfate. The mixture was stirred for 20–30 min and then dried under vacuum. In preliminary experiments, several incubation times (up to 8 h) were tested. However, significant degradation of the core peptide was found if incubation was protracted for more than 30 min. Other procedures to release the heparan sulfate chains from the core protein, such as heparitinase treatment, resulted in a marked degradation of the core peptide even when 0.1% bovine serum albumine (wt/vol) was present to minimize proteolysis of the radiolabeled peptide core.[3] The dried samples were resuspended in a large (~20 ml) volume of H_2O, dialyzed and lyophylized, and then subjected to chromatography on Sephacryl S-300 in 0.2 M NaCl, 0.1 M Tris–HCl buffer, pH 7.0 (33). SDS PAGE before and after deglycosylation was performed according to Laemmli (34) on 10% polyacrylamide slab gels 1.5 mm thick. After electrophoresis the gels were placed in 10% trichloroacetic acid and 20% methanol at 4°C for 30 min, and were stained with Coomassie Brilliant Blue R-250 to detect the protein standards or incubated with Enlightening (New England Nuclear, Boston, MA) for 30 min, washed, and dried under vacuum at 80°C. The dried gels were then prepared for autoradiography using Kodak X-OMAT AR film (Eastman Kodak Co.). Amino acids were analyzed as previously described (25).

Release of Surface Proteoglycans by Trypsin or Heparin: Confluent monolayers were incubated with radioactive precursors for 24–48 h in 2 ml of Dulbecco's modified Eagle's medium supplemented with 5% calf serum. The cells were then washed with 3 × 1 ml of Dulbecco's modified Eagle's medium and then incubated for 15 min in isotope-free medium in the presence or absence of 0.1% trypsin (35). This treatment did not cause any significant cell detachment or damage. Proteolysis was stopped with soybean trypsin inhibitor, and the medium was removed. The cell layer was washed with 2 ml of Dulbecco's modified Eagle's medium and the remaining material was extracted with Zwittergent 3–12 and 4 M guanidine HCl as above. The material was studied by gel chromatography on Sepharose CL-2B and by isopycnic CsCl density-gradient ultracentrifugation as above (starting ρ = 1.45 g/ml). To determine the relative amounts of proteoglycans that were bound to the cell surface through ionic interactions, various concentrations (10–500 μg/ml) of exogenous heparin were added to confluent monolayers which have been labeled for 24 h with the same concentrations of isotopes (36). After 30 min of incubation, the amount of labeled proteoglycans in the medium and cell layer was determined as above.

Pulse Chase Experiments: To determine the fate of the cell associated proteoglycans, pulse chase experiments were performed. Confluent monolayers were labeled with L-[^{3}H] leucine (10 μCi/ml) and [^{35}S]sulfate (40 μCi/ml) for 24 h. The medium was removed and the cell layers were washed three times with HBSS and chased in isotope-free medium for various periods of time. Macromolecular as well as free [^{35}S]sulfate radioactivity from the medium and cell layer at various chase times was quantitated by PD-10 chromatography. Sepharose CL-2B chromatography was also performed to determine whether the macromolelcular radioactivity excluded by PD-10 was present as intact proteoglycan.

Light and Electron Microscopic Immunocytochemistry: Colon carcinoma cells were grown to confluency and, after several washes in HBSS, were fixed for 30 min at 4°C in either 95% ethanol or Zamboni's fixative (37). The cells were washed three times in phosphate-buffered saline, incubated with various dilutions of rabbit anti-heparan sulfate proteoglycan antibodies (38), and then incubated with fluorescein isothiocynate-conjugated goat anti-rabbit antibodies. Slides were examined with a Leitz fluorescent photomicroscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epillumination. For immunoelectron microscopy, monolayers were fixed in either 1% glutaraldehyde in phosphate-buffered saline or Zamboni's fixative and were processed as previously described (39) with minor modifications. After fixation, the cells were washed several times in phosphate-buffered saline and then treated with 3% H_2O_2 in H_2O for 30 min to inhibit endogenous peroxidase activity. The cells were rinsed in phosphate-buffered saline and then sequentially incubated with normal goat serum (1:5), primary rabbit antibodies (1:100), goat anti-rabbit IgG, and then rabbit peroxidase antiperoxidase. The peroxidase reaction was developed by incubating in diaminobenzidine solution with H_2O_2. After washing in phosphate-buffered saline for 30 min, the cells were photographed, and then reacted with 1% OsO_4 for 30 min, dehydrated in graded ethanols, and embedded in Epon. Unstained 40–60-nm-thin sections were observed with a Hitachi-600C electron microscope, at 50 kV (Hitachi America, Ltd., Allendale, NJ).

Electron Microscopic Cytochemistry of Surface Proteoglycans: Monolayers grown in the absence of serum were fixed in the presence of ruthenium red (10, 23), a cationic dye capable of retaining and binding to proteoglycans (40). Thin sections were sequentially stained with uranyl acetate and lead citrate (41) and examined by electron microscopy. Polycarboxylated and polysulfated surface macromolecules were distinguished by processing monolayers in the presence of cuprolinic blue in either 0.1 M or 0.3 M $MgCl_2$, according to the critical electrolite concentration method (42, 43). As further control, adult rat tail tendons were processed at the same time in the presence or absence of the dye in 0.1 M and/or 0.3 M $MgCl_2$ as above. Parallel monolayers were incubated with chondroitinase ABC (31) for 30 min or trypsin (0.1 mg/ml) at 37°C for 15 min before processing in the presence of the cationic dyes.

Stereologic Analysis of Cuprolinic Blue-stained Proteoglycans: Because the cuprolinic blue-stained filaments are interpreted to represent extended proteoglycans (42, 43), quantitative studies of the length of the Cuprolinic Blue filaments were performed. Randomly selected pictures were analyzed quantitatively at a final magnification of 60,000–90,000. Magnification was calibrated with a carbon-grating replica carrying 21,600 lines cm^{-1} (Ernest F. Fullam, Inc., Schenectady, NY). An appropriate sample size was determined statistically (44), and section thickness was estimated using the "fold" method (45). Because the cuprolinic blue proteoglycans appeared as electron-dense linear structures of variable length, random orientation, and constant thickness (see Results), it was possible to estimate the true length from the mean "observed" projected length (46): $L = \Pi/2 \times \bar{L}'$; where L is the true length of a straight segment randomly oriented in a volume, and $\bar{L}'$ is the mean projected length. This approach is feasible, because in a system of projected lines in a space or volume, overlap is a highly improbable event (46) if the section thickness (T) is smaller than the average length ($\bar{L}$) and, in particular, if $T/\bar{L} < 2$. In the present study, we found $T/\bar{L} = 0.56 \pm 0.06$.

RESULTS

Synthesis and Secretion of Proteoglycans

After a lag period of ~4 h, the secretion of ^{35}S-labeled macromolelcules into the medium increased rapidly and almost linearly for up to 68 h (Fig. 1). In the cell layer, the accumulation of the ^{35}S-labeled material increased rapidly between 4 and 18 h, and then more slowly, reaching a plateau after 24 h and staying fairly constant up to 68 h. In most experiments, cultures were labeled for 24 h at which time the cells had reached a steady state with ~70% of the ^{35}S-macromolecule present in the culture medium and ~30% in the cell layer (Fig. 1). The kinetics of accumulation and secretion of D-[^{3}H]glucosamine-labeled macromolelcules were similar to

[3] Treatment with heparitinase (0.1–1.0 mg/ml) for 15 min also resulted in severe damage to the cells, as determined by electron microscopy, indicating that this enzyme preparation was contaminated with proteases. This enzyme, therefore, was not further used.

those of the ^{35}S-labeled macromolecules, although in the former case, the radioactivity was equally divided between the medium and cell layer after 24 h incubation (not shown). In accordance with previous reports (24, 35), sequential extraction with Zwittergent 3–12 and guanidine HCl solubilized nearly 100% of the labeled macromolelcules from the cell layer.

Purification of Proteoglycans

The labeled macromolelcules recovered from both medium and cell layer were concentrated and subjected to anion-exchange chromatography on DEAE–Sephacel, in the presence of 8 M urea and 0.2% Triton X-100 and a linear gradient from 0.1 to 0.8 M NaCl. The polyanionic macromolecules (peak III, Fig. 2) were clearly separated from the bulk of the glycoprotein (peaks I and II), which did not bind to the DEAE. The majority (~95%) of the ^{35}S radioactivity was present as a sharp peak in both the medium (M-III) and cell layer (C-III) eluting at the same salt concentration (0.5 M). In the cell extract, a small shoulder of ^{35}S-labeled material was also present in C-III eluting with 0.45 M NaCl (Fig. 2*B*). Furthermore, the radiolabeled macromolecules in C-III had a much higher $^{3}H/^{35}S$ ratio than those present in M-III in preparations labeled with [^{35}S]sulfate and either L-[^{3}H]leucine or L-[^{3}H] serine.

The M-III and C-III fractions were pooled separately, concentrated, and further purified by ultracentrifugation in a CsCl density gradient (Fig. 3). In the medium the majority of ^{35}S radioactivity (~60%) was recovered in the bottom three fractions (D_1–D_3)[1] with densities higher than 1.38 g/ml, and the remainder was distributed in the fractions with intermediate and low densities (Fig. 3*A*). In the cell layer a large proportion of ^{35}S radioactivity was also recovered in the higher-density fractions (Fig. 3*B*). However, significant amounts of ^{35}S activity were found in the fractions of lower buoyant density, and up to 54% of [^{3}H]leucine radioactivity sedimented in the top three fractions. To determine the composition and relative hydrodynamic size of the proteoglycans from medium and cell layer, we pooled the original fractions from the CsCl density-gradient ultracentrifugation to yield five approximately equal fractions. For M-III, the fractions of highest buoyant density (D_1 + D_2, 1.46 g/ml) contained a single population of proteoglycans that eluted from Sepharose CL-2B with a K_{av} = 0.57 and [^{3}H]Leu/^{35}S ratio of 1.1 (Fig. 4*A*). This proteoglycan was distributed

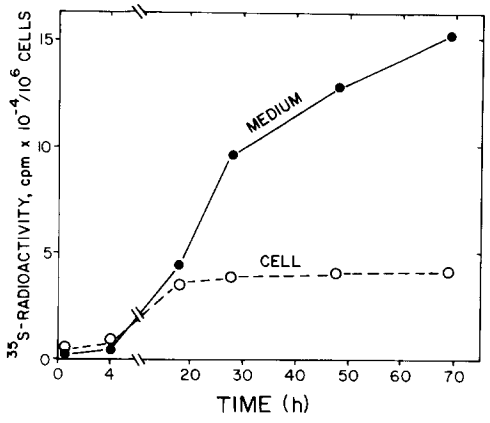

FIGURE 1 Accumulation of ^{35}S-labeled macromolecules in culture medium (●) and cell layer (○) of human colon carcinoma cells. Aliquots (0.5 ml) of medium and Zwittergent-guanidine HCl extracts of the cell layer were chromatographed separately on PD-10 columns in the presence of proteoglycan monomer carrier (250 μg) from rat chondrosarcoma. Each value represents the average of triplicate cultures with SD ≤ 10% of the mean.

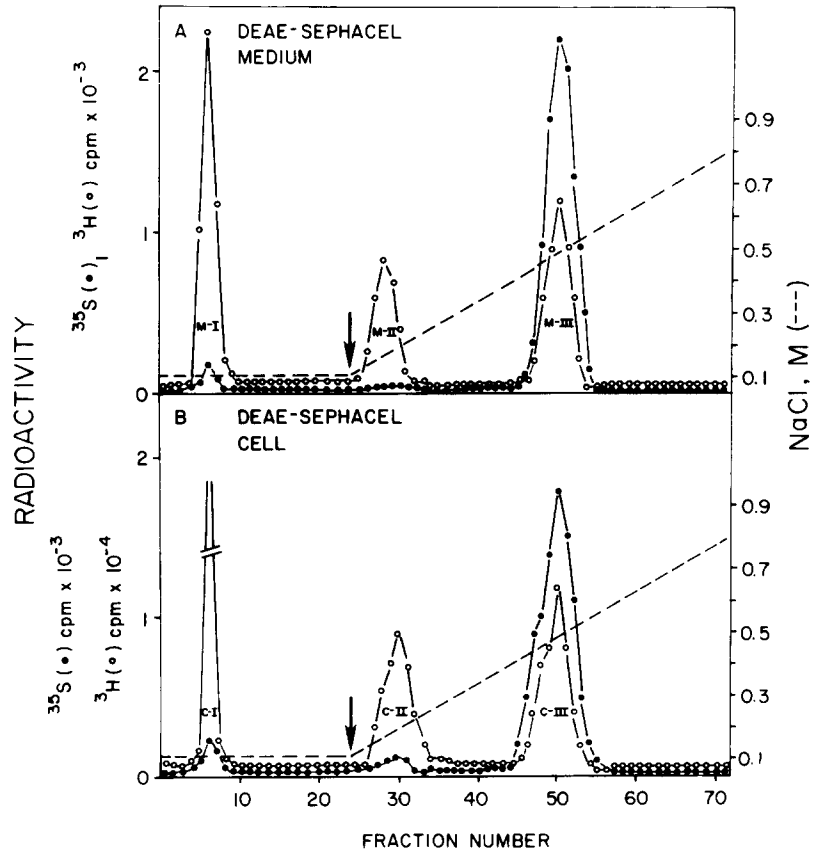

FIGURE 2 Ion exchange chromatography on DEAE-Sephacel of [^{35}S]sulfate- (●) and [^{3}H]leucine-labeled (○) medium (*A*) and cell layer (*B*) from human colon carcinoma cells. The arrow indicates the starting of the gradient. Polyanionic fractions from medium (*M-III*) and cell layer (*C-III*) were pooled for further analysis.

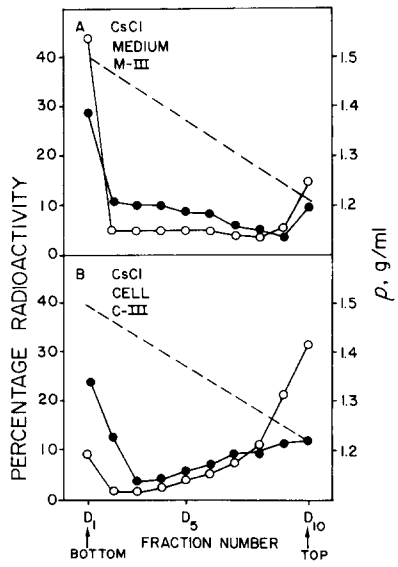

FIGURE 3 CsCl density-gradient ultracentrifugation of DEAE-Sephacel-purified proteoglycans from medium (*A*) and cell layer (*B*). Isopycnic CsCl density-gradient ultracentrifugation (starting $\rho = 1.41$ g/ml) was performed for 48 h at 10°C, 37,000 rpm in a Beckman SW-50.1 rotor. Fractions of 500 μl were collected and 100-μl aliquots were analyzed for [^{35}S]- (●) and [^{3}H]leucine radioactivity (○) and for density (- - -).

throughout all fractions of the gradient in decreasing amounts from bottom to top. The [^{3}H]Leu/^{35}S ratio in this peak increased from 1.1 in the fraction of highest density to 2.5 in the fraction of lowest density (Fig. 4*A*), suggesting a higher proportion of glycosaminoglycan to protein in the bottom fractions. Furthermore, no contaminant ^{3}H-labeled proteins were found in the proteoglycan-enriched fractions, whereas significant amounts of ^{3}H-labeled proteins were found in the fractions of lower density eluting before and after the major proteoglycan peaks. In the cell layer, however, two distinct populations of macromolecules were found: a large population (peak A, Fig. 4*B*) which eluted slightly earlier (K_{av} = 0.55) than the large proteoglycans of the medium, and a smaller population (peak B, Fig. 4*B*) which eluted near the V_t of the column. The large proteoglycan population was found in all the fractions of the gradient in decreasing concentration from bottom to top, and the [^{3}H]Leu/^{35}S ratio for this peak increased from 1.5 in the fraction of highest density to 21.3 in the fractions of lowest density (Fig. 4*B*). The results indicate that the CsCl density-gradient ultracentrifugation effectively purified most of the proteoglycan from lower buoyant-density glycoproteins, and that the larger hydrodynamic size of the cell-associated proteoglycan may be due in part to a higher protein content, ^{3}H/^{35}S ratio of 1.5 vs. 1.1.

The average molecular size of the proteoglycan monomers in the bottom fraction was also studied by HPLC (26). The retention time of the medium proteoglycan, $T_r = 10$ min (Fig. 5*A*), differed from that of the cell layer, $T_r = 9$ min (Fig. 5*B*). As expected, in cell layer, the minor component (peak B of Fig. 4*B*) eluted near the V_t of the HPLC system with a T_r = 14 min (Fig. 5*B*). It was estimated, based on the elution position of proteoglycan monomers from bovine nasal cartilage, rat chondrosarcoma, and proteins of known M_r (see legend to Fig. 5), that the medium proteoglycan had an average $M_r = 8.5 \times 10^5$ and the large cell-associated proteoglycan had an $M_r \simeq 9.5 \times 10^5$.

Characterization of the Glycosaminoglycans

The ^{35}S-labeled glycosaminoglycans derived from the large proteoglycan population in the medium (Fig. 6*A*) and cell layer (peak A, Fig. 6*B*) were included in the Sepharose CL-6B column after alkaline borohydride treatment with a K_{av} = 0.42 ($M_r \simeq 3 \times 10^4$), whereas the smaller cell-associated component (peak B, Fig. 6*C*) had a K_{av} of 0.63 ($M_r \simeq 1 \times 10^4$). All the ^{35}S-labeled polysaccharides in both medium and cell layers were susceptible to deaminative cleavage with HNO_2 (Fig. 6, ○), but were all resistant to chondroitinase ABC and AC-II. Identical results were obtained with the fractions of lower density derived from CsCl ultracentrifugation indicating that human colon carcinoma cells synthesize and secrete only one species of sulfated glycosaminoglycan, heparan sulfate. The ^{35}S-glycosaminoglycans from both medium and cell layer co-migrated on electrophoresis in 0.1 M HCl and eluted at the same ionic strength on DEAE-Sephacel indicating that they had the same degree of sulfation (not shown). Furthermore, the smaller cell-associated component eluted as a broad polydisperse peak ($K_{av} = 0.51$) on Sepharose CL-6B before treatment with papain or alkaline/borohydride in contrast with the larger proteoglycans which were excluded (not shown). These results suggest that this material is composed primarily of single heparan sulfate chains of small molelcular weight.

Analysis of Core Protein

After deglycosylation with polyhydrogen fluoride, ~90% of the ^{35}S radioactivity was removed and the core protein eluted as a single sharp peak on Sephacryl S-300 in both the medium (Fig. 7*A*) and the large cell layer (Fig. 7*B*) proteoglycan preparations. The deglycosylated core protein from the cell layer, however, eluted from Sephacryl S-300 slightly earlier (K_{av} = 0.355, Fig. 7*A*) than the medium form (K_{av} = 0.387, Fig. 7*B*). The deglycosylated protein cores of both cell and medium preparations migrated as a single band on SDS PAGE as detected by autoradiography (Fig. 7*C*), with an estimated $M_r \simeq 2.4 \times 10^5$. The fact that the small difference in elution behavior on Sephacryl S-300 between medium and cell preparations could not be clearly detected by SDS PAGE suggests that the difference between the protein core of medium and cell layer is small.

The amino acid analysis of both medium and cell-associated heparan sulfate proteoglycan derived from the $D_1 + D_2$ fractions was similar but not identical (Table I). The major amino acids were glutamic acid, glycine, alanine, aspartic acid, valine, leucine, and lysine. No detectable cysteine[4] or methionine was found in both proteoglycan preparations and, in addition, no proline was detected in the medium proteoglycan. The medium proteoglycan contained ~15% less hydrophobic amino acids as compared to the cellular form (319 vs. 374 residues).

Release of Surface Proteoglycans by Trypsin or Heparin

Colon carcinoma cells were labeled for 48 h with [^{35}S] sulfate and either L-[^{3}H]leucine or D-[^{3}H]glucosamine and subjected to mild trypsinization (34). This treatment removed up to 26% and 17.5% of the ^{35}S- and D-[^{3}H]glucosamine-

[4] Lack of detection of this amino acid may be in part artifactual due to destruction during acid hydrolysis.

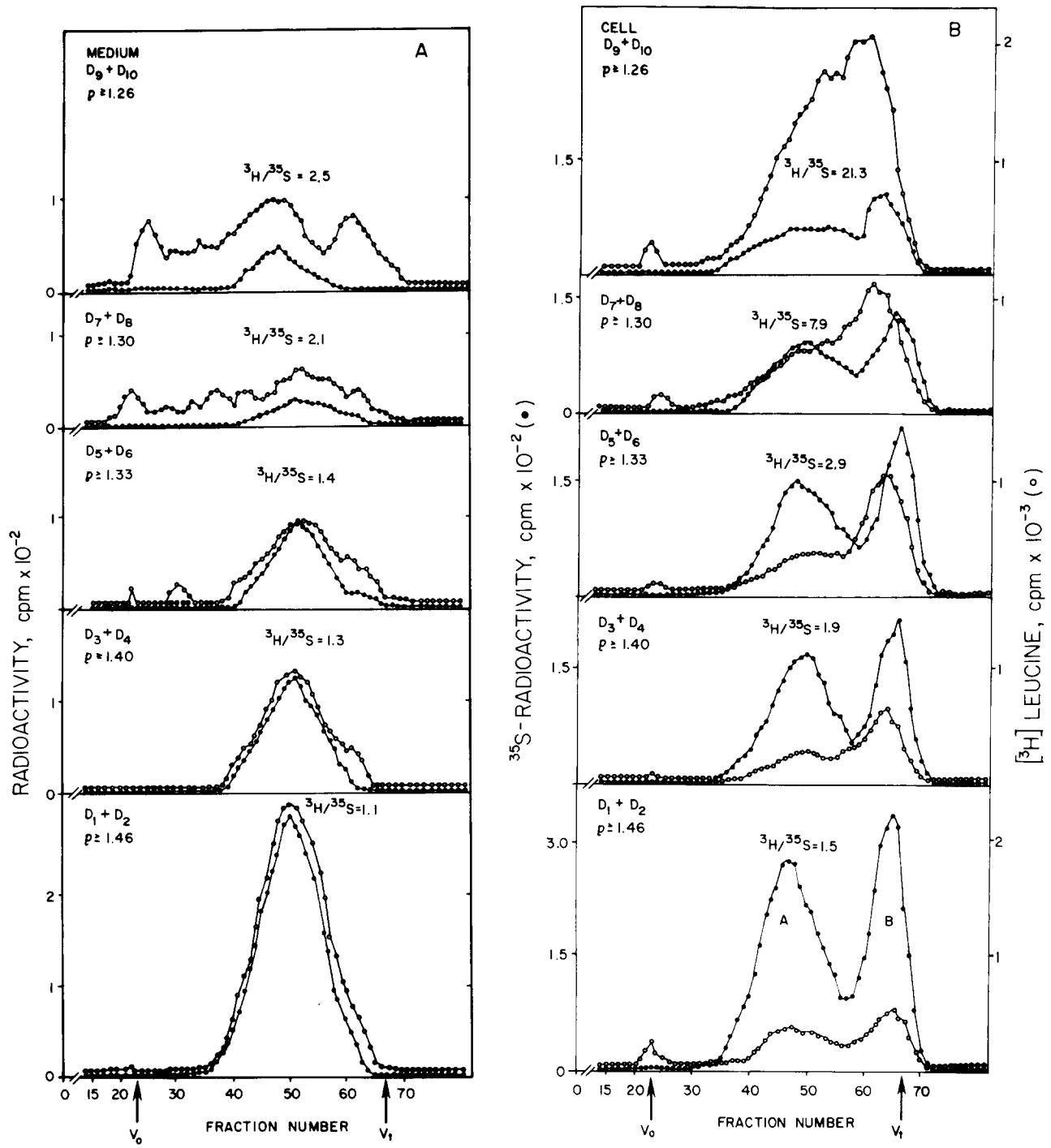

FIGURE 4 Sepharose CL-2B elution profiles of ^3H-^{35}S-labeled macromolecules synthesized by colon carcinoma cells and purified by anion exchange chromatography and CsCl density-gradient ultracentrifugation. The [^3H]leucine/^{35}S ratio of the major proteoglycan peaks in medium (*A*) and cell layer (*B*) and the average density for combined fractions are shown. (●) ^{35}S; (○) [^3H] leucine.

labeled macromolelcules, respectively, but only 8% of L-[^3H] leucine-labeled macromolelcules (Table II). To determine what proportion of the proteoglycans was bound to the cell surface through ionic interactions, we added increasing concentrations of exogenous heparin (10–500 μg/ml) to confluent monolayers, which had been incubated for at least 24 h in the same concentration of isotopes as above. After 30 min, there was no significant increase in the release of labeled proteoglycans in the presence of heparin when compared to controls (Table II).

The $^3H/^{35}S$-labeled macromolecules released during 15 min of chase in the presence or absence of trypsin were further studied by gel chromatography on Sepharose CL-2B and CsCl density-gradient ultracentrifugation. The control sample released into the medium after 15 min of chase eluted as a single peak from the Sepharose CL-2B column (Fig. 8*A*) with

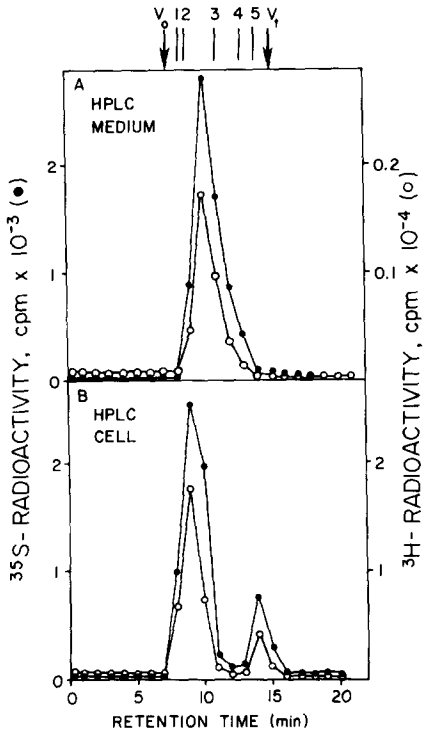

FIGURE 5 HPLC elution profiles of proteoglycan-enriched fractions (D_1 + D_2) purified by DEAE-Sephacel and CsCl density-gradient ultracentrifugation. Aliquots from medium (*A*) and cell layer (*B*) were subjected to HPLC using two Aquapore columns in tandem, OH-500 and OH-1000. The samples were run under constant pressure of ~500 psi, and a flow rate of 0.5 ml/min. Fractions of 0.25 ml were analyzed for radioactivity. The V_0 and V_t were determined by using aggregate proteoglycan (A_1) from bovine nasal cartilage and free $^{35}SO_4^{2-}$, respectively. The numbers represent the elution position detected by ultraviolet at 280 nm of (*1*) proteoglycan monomer from bovine nasal cartilage, (2) proteoglycan monomer from rat chondrosarcoma, (*3*) thyroglobulin, (*4*) ferritin, and (*5*) catalase. ^{35}S (●); [^{3}H]leucine (○).

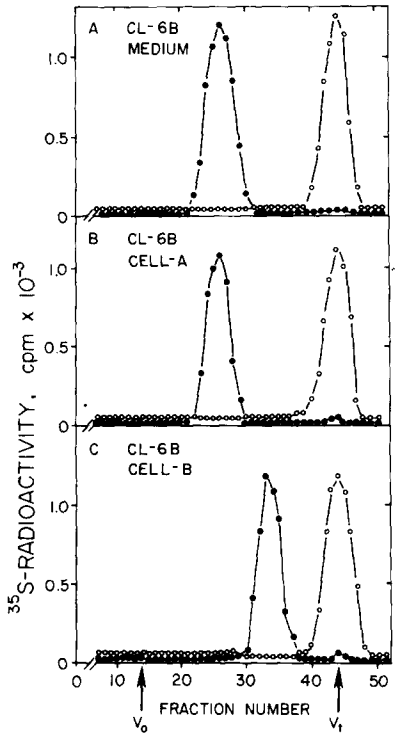

FIGURE 6 Sepharose CL-6B elution profiles of glycosaminoglycans released by alkaline borohydride treatment from proteoglycans of medium (*A*) and cell layer peak A (*B*) and peak B (C). Radiolabeled macromolecules isolated from the proteoglycan-enriched fractions (D_1 + D_2) were purified by Sepharose CL-2B. The included peaks were treated with alkaline borohydride and analyzed by Sepharose CL-6B chromatography before (●) and after HNO_2 (○) treatment. Similar elution profiles were found in the samples treated with papain.

identical characteristics (K_{av} = 0.57) to that isolated from the medium after 24 h of continuous labeling (see Fig. 4*A*). On the other hand, the trypsin-released proteoglycan (Fig. 8*C*) was slightly reduced in hydrodynamic size (K_{av} = 0.61), indicating that trypsin treatment had cleaved a portion of the peptide core. Furthermore, only the large cell-associated proteoglycan, but none of the small component was accessible to trypsin. In a CsCl density-gradient ultracentrifugation, the distribution of the spontaneously released proteoglycan (Fig. 8*B*) was similar to that of the trypsin-released proteoglycan (Fig. 8*D*). These results indicate that the heparan sulfate proteoglycan exists as a cell-surface macromolecule and that it is not bound by electrostatic interactions between the glycosaminoglycan side chains and membrane protein(s).

Turnover of Heparan Sulfate Proteoglycan

The fate of the cell-associated heparan sulfate proteoglycan was determined by studying the turnover of the ^{35}S-labeled macromolecules after chase for various periods of time in isotope-free medium. The total ^{35}S-macromolecules in the cell layer decreased rapidly and then more slowly with a $t_{1/2} \simeq 6$ h (Fig. 9*A*). The nonlinearity of the disappearance curve, even when plotted on logarithmic scale (not shown), indicates that the turnover of the proteoglycans did not follow simple first-order kinetics, which suggests that the fractions contain two or more components with different turnover rates (47). In fact, when the ^{35}S-macromolelcules present in the medium and cell layer were analyzed by Sepharose CL-2B chromatography, it was found that after 12 h only 10–20% of the large heparan sulfate proteoglycan remained associated with the cell layer and 58% was found intact in the medium (not shown), and this proportion remained constant for up to 24 h (Fig. 9*B*). On the contrary, the small cell-associated component was never found in the medium and was degraded at a much slower rate, because after 24 h of chase, ~30% of it was still found in the cell layer, whereas the larger proteoglycan was completely metabolized. Free [^{35}S]sulfate was constantly released into the medium with a maximum of 52% after 24 h of chase (Fig. 9*C*). In the cell layer there was ~5% of free ^{35}S radioactivity and this did not change with time (Fig. 9*C*).

Taken together these results indicate that (*a*) the larger heparan sulfate proteoglycan localized at the cell surface is the precursor of the medium proteoglycan; (*b*) this proteoglycan is in part (58%) released into the medium as intact proteoglycan and in part (42%) internalized and degraded; (*c*) the cell-associated pool of smaller heparan sulfate is never released into the medium, does not represent a precursor pool for the large heparan sulfate proteoglycan, and is degraded at a much slower rate than the large proteoglycan.

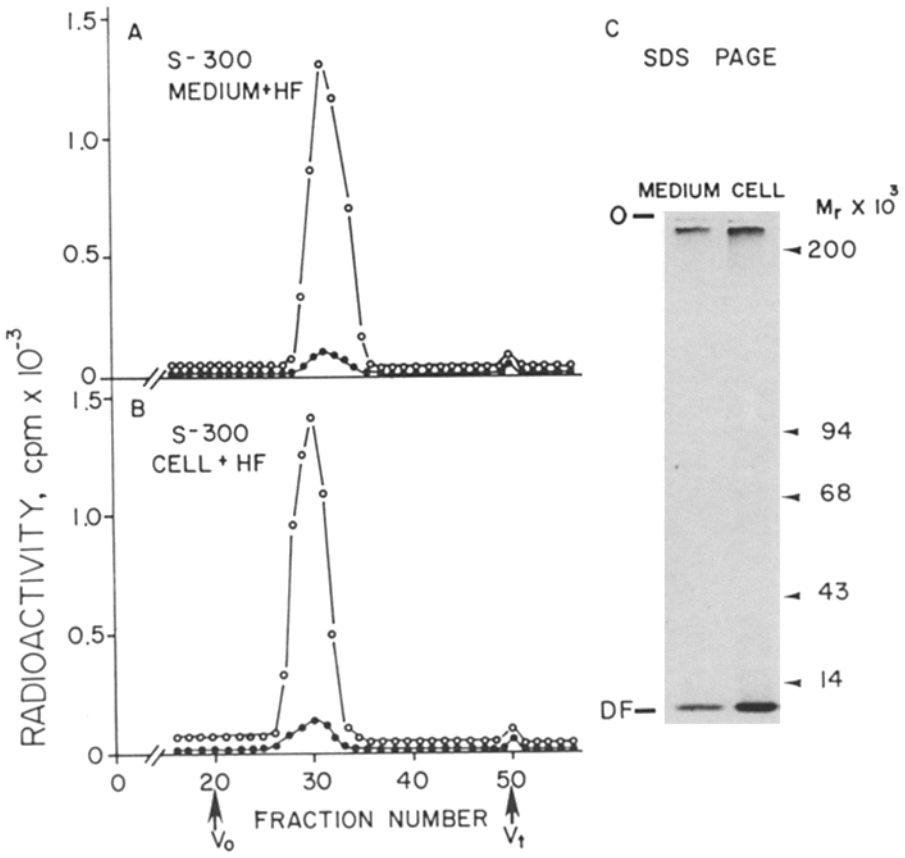

FIGURE 7 Sephacryl S-300 elution profiles (*A* and *B*) and SDS PAGE autoradiographs (*C*) of polyhydrogen-fluoride-treated proteoglycan monomers isolated from the medium and cell layer (peak A of Fig. 4*b*) of colon carcinoma cells. The deglycosylated proteoglycans, double-labeled with [^{35}S]sulfate (●) and [^{3}H] leucine (○), were subjected to Sephacryl S-300 chromatography in 0.3 M NaCl, 0.1 M Tris–HCl, pH 7.0. SDS PAGE was performed in slab polyacrylamide gels containing 0.1% SDS with 5% polyacrylamide stacking gel and a 10% resolving gel. Protein standards include myosin, phosphorylase b, albumin, ovalbumin, and lactalbumin with M_r of 200, 94, 68, 43, and 14 × 10^3, respectively. *O*, Origin; *DF*, dye front.

TABLE I

*Amino Acid Analysis of Proteoglycans Synthesized by Human Colon Carcinoma Cells**

Amino acid	Medium	Cell layer‡
	residues/1,000 amino acids	
Hydroxyproline	nd§	nd
Aspartic acid	90.6	88.6
Threonine	20.7	38.4
Serine	59.5	60.5
Glutamic acid	190.8	135.5
Proline	nd	37.6
Glycine	135.7	111.8
Alanine	108.9	103.8
Valine	87.2	82.6
Cysteine‖	nd	nd
Methionine	nd	nd
Isoleucine	25.1	36.0
Leucine	87.9	84.0
Tyrosine	12.4	10.7
Phenylalanine	10.0	28.6
Lysine	80.6	89.0
Histidine	18.4	16.7
Arginine	72.2	76.2

* Values represent the average of two analyses on two different preparations of purified proteoglycans (D_1 + D_2) with variability <10%.
‡ Values represent amino acid analysis of peak A (see Fig. 4 *B*, *bottom* panel).
§ *nd*, not detected.

TABLE II

*Release of Cell-surface Proteoglycans by Trypsin and Heparin**

	Incubation time	Released macromolecular radioactivity		
		[^{35}S]-Sulfate	D-[^{3}H]-Glucosamine	L-[^{3}H]-Leucine
	min	%	%	%
Control	15	2.4	0.8	0.4
+ Trypsin (100 μg/ml)	15	26.1	17.5	8.3
Control	30	4.7	1.9	0.7
+ Heparin (10 μg/ml)	30	5.8	1.6	0.7
+ Heparin (100 μg/ml)	30	5.6	1.7	0.7
+ Heparin (500 μg/ml)	30	4.5	1.4	0.6

* Triplicate confluent cultures were incubated for 24 h with radioactive precursors. The cells were washed three times with HBSS and incubated for 15 and/or 30 min at 37°C with trypsin or increasing concentrations of heparin, respectively. The radioactivity in the medium and cell layer was calculated as described under Materials and Methods. The values are expressed as percentage of macromolecular radioactivity released from the cell layer. None of the radioactivity released by heparin is statistically different from the control values.

Light and Electron Microscopic Immunocytochemistry

Human colon carcinoma cells reacted with affinity-purified antibodies raised against the protein core of Engelbreth–Holm–Swarm tumor heparan sulfate proteoglycan. Fluorescence was observed in the form of punctuate or linear deposits especially along the cellular contours, whereas control sections incubated with normal rabbit serum remained totally unlabeled (not shown). Immunofluorescence deposits were completely removed by pretreating the cells with trypsin, but no immunoreactivity could be displaced by chondroitinase ABC treatment. Light microscopy (*inset*, Fig. 10*A*) of tumor cells reacted with anti-heparan sulfate and labeled with peroxidase antiperoxidase revealed intense deposits around the tumor

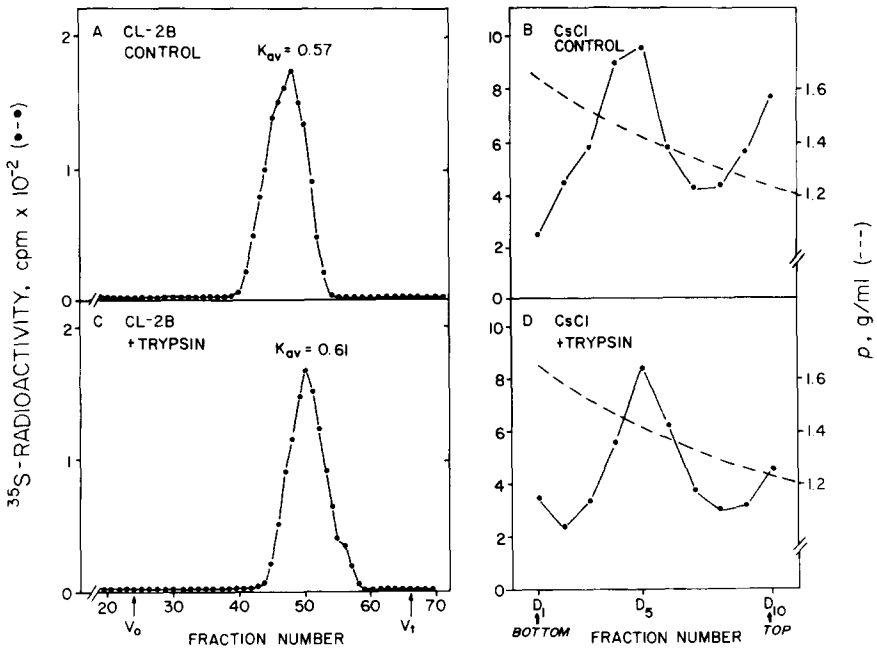

FIGURE 8 Sepharose CL-2B elution profiles (A and C) and CsCl density-gradient ultracentrifugation (B and D) of proteoglycans released from colon carcinoma cells with or without mild proteolytic treatment. The cells were incubated with isotopes for 24 h, washed with HBSS (×3) and then chased for 15 min in isotope-free medium in the absence or presence of trypsin (0.1 mg/ml, 37°C). The released material was desalted through PD-10 columns and subjected to chromatography on Sepharose CL-2B and isopycnic CsCl density-gradient ultracentrifugation (starting ρ = 1.45 g/ml) in the presence of 4 M guanidine HCl and protease inhibitors.

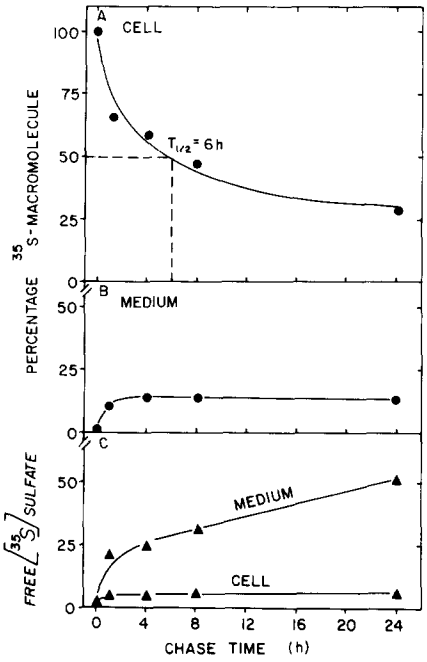

FIGURE 9 Turnover of ^{35}S-labeled proteoglycan synthesized by human colon carcinoma cells. The ^{35}S-macromolecules (●) as well as the free [^{35}S]sulfate radioactivity (▲) were monitored in both the medium and cell layer as described under Materials and Methods.

cells with a distribution similar to that obtained with immunofluorescence. Immunoelectron microscopy (Fig. 10*A*) of the same cells revealed electron-dense deposits of antigen-antibody complexes localized exclusively at the cell surface, around microvilli, and along the avillous membrane. The electron-dense products were seen as nearly continuous deposits in close apposition to the plasma membrane (Fig. 10*A*). Normal rabbit serum failed to react with any components of colon carcinoma cells (Fig. 10*B*). When the cells were permeabilized with the freezing–thawing method (48), no significant immunoreactivity was noted in the cytoplasmic components of colon carcinoma cells. The results indicate that the heparan sulfate proteoglycan synthesized by human colon carcinoma cells is immunologically related to that synthesized by Engelbreth-Holm-Swarm tumor and that it is localized predominantly at the cell surface, in agreement with the biochemical data.

Electron Microscopic Cytochemistry of Surface Proteoglycans

Numerous ruthenium red positive granules, 20–50 nm in diameter, were observed on the outer plasmalemmal surface of the tumor cells (Fig. 11*A*). These granules, which are thought to represent collapsed proteoglycan monomers (10, 23, 40, 49), were present in both the microvillar surface and along the avillous portion of the plasma membrane. No granules were observed in cells processed without the cationic dye (Fig. 11*B*), nor were they seen in cells that were subjected to mild proteolysis. Chondroitinase ABC also did not remove any significant amount of ruthenium red-positive material.

Polycarboxylated and polysulfated surface glycoconjugates were distinguished by processing the cells in the presence of cuprolinic blue using the critical electrolyte concentration approach (42, 43). With high $MgCl_2$ concentration (0.3 M), the uptake of the dye is relatively specific for sulfate-containing polyanions, whereas polycarboxylated and polyester phosphates remain unstained (42, 43). Colon carcinoma cells contained numerous cuprolinic blue-positive structures on the cell surface (Fig. 12). No difference was noted whether the cells were incubated with the dye in the presence of 0.1 M (Fig. 12*A*) or 0.3 M $MgCl_2$ (Fig. 12*B*), indicating that these cuprolinic blue filaments were polysulfated. These structures were filamentous and unbranched and often projected from the surface of the microvilli as well as from the avillous portion of the plasma membrane. Occasionally, cuprolinic blue filaments were seen intracellularly and were exclusively localized in the cisternae of Golgi complexes. After mild trypsinization,

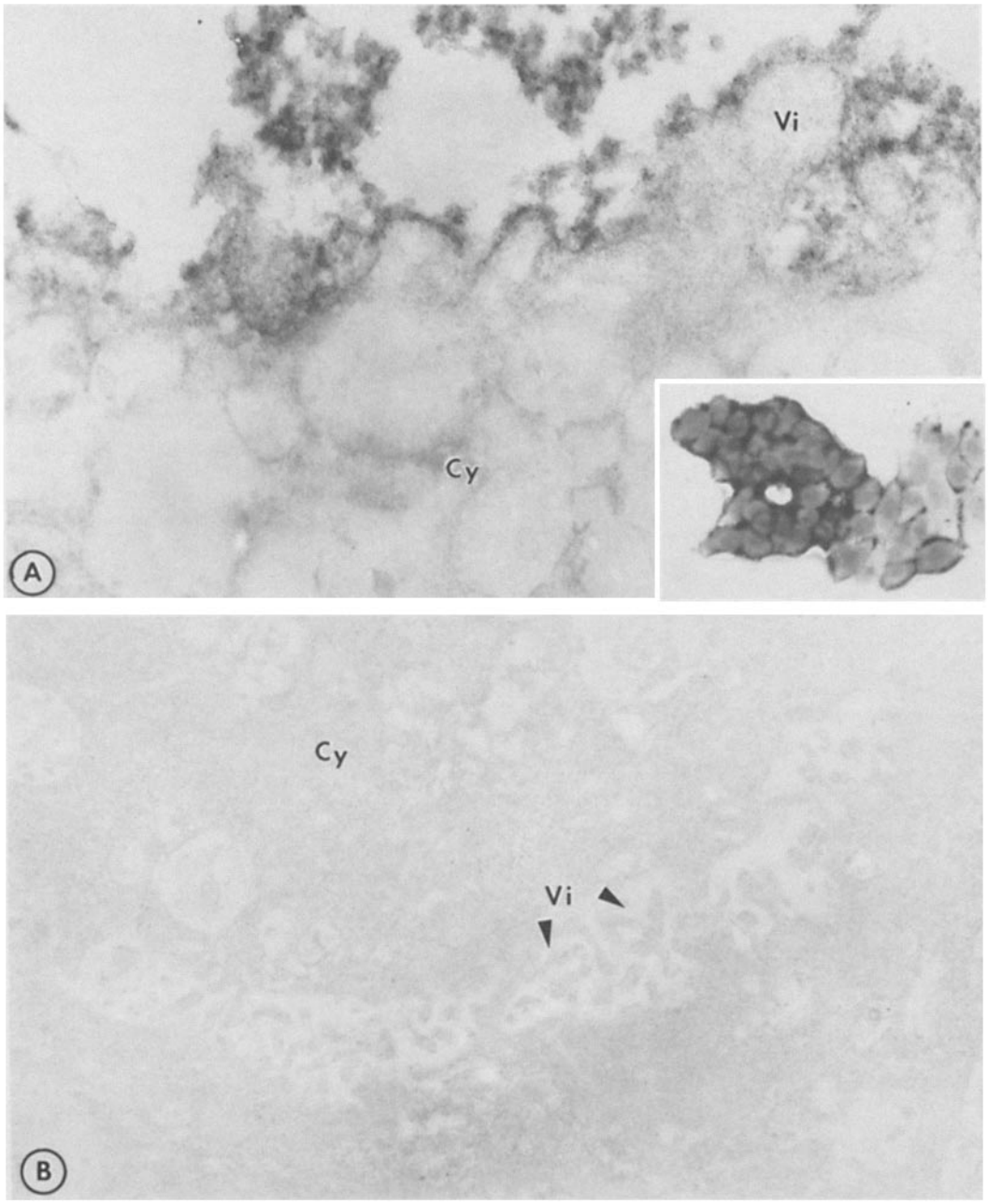

FIGURE 10 Electron microscopic immunocytochemistry of human carcinoma cells reacted with anti-heparan sulfate antibodies (*A*) and normal rabbit serum (*B*). The inset represents a light micrograph of same cells with strong surface positivity before reaction with OsO_4 and further processing for immunoelectron microscopy. The peroxidase reaction is localized exclusively at the cell surface (*A*) where large amounts of electron-dense material are in close apposition to the plasma membrane. No reaction is seen over the cytoplasm (*Cy*) or in the control (*B*). (*Vi*) microvilli. (*A*) × 96,000. (*B*) × 75,000. (*Inset*) × 120.

the majority of the cuprolinic blue material was removed from the cell surface, and only a few, short dotlike structures remained (not shown). However, these structures were unaffected by treatment with chondroitinase ABC. The dimension of these cuprolinic blue filaments varied widely from 20 to 220 nm, and quantitative studies revealed that the mean corrected length (L) was 124 ± 6 nm ($n = 574$). Ultrastructural examination of rat tail tendon stained in parallel as a control revealed electron-dense filaments interacting with collagen with characteristics and measurements ($L = 74 \pm 8$ nm) identical to those described by Scott (42, 43).

These results indicate that colon carcinoma cells express a surface heparan sulfate proteoglycan which appears as a collapsed granule or as an extended electron-dense linear structure when ruthenium red or cuprolinic blue is used, respectively.

DISCUSSION

The present study demonstrates that cultured human colon carcinoma cells synthesize unique species of heparan sulfate proteoglycans with distinct structural characteristics and met-

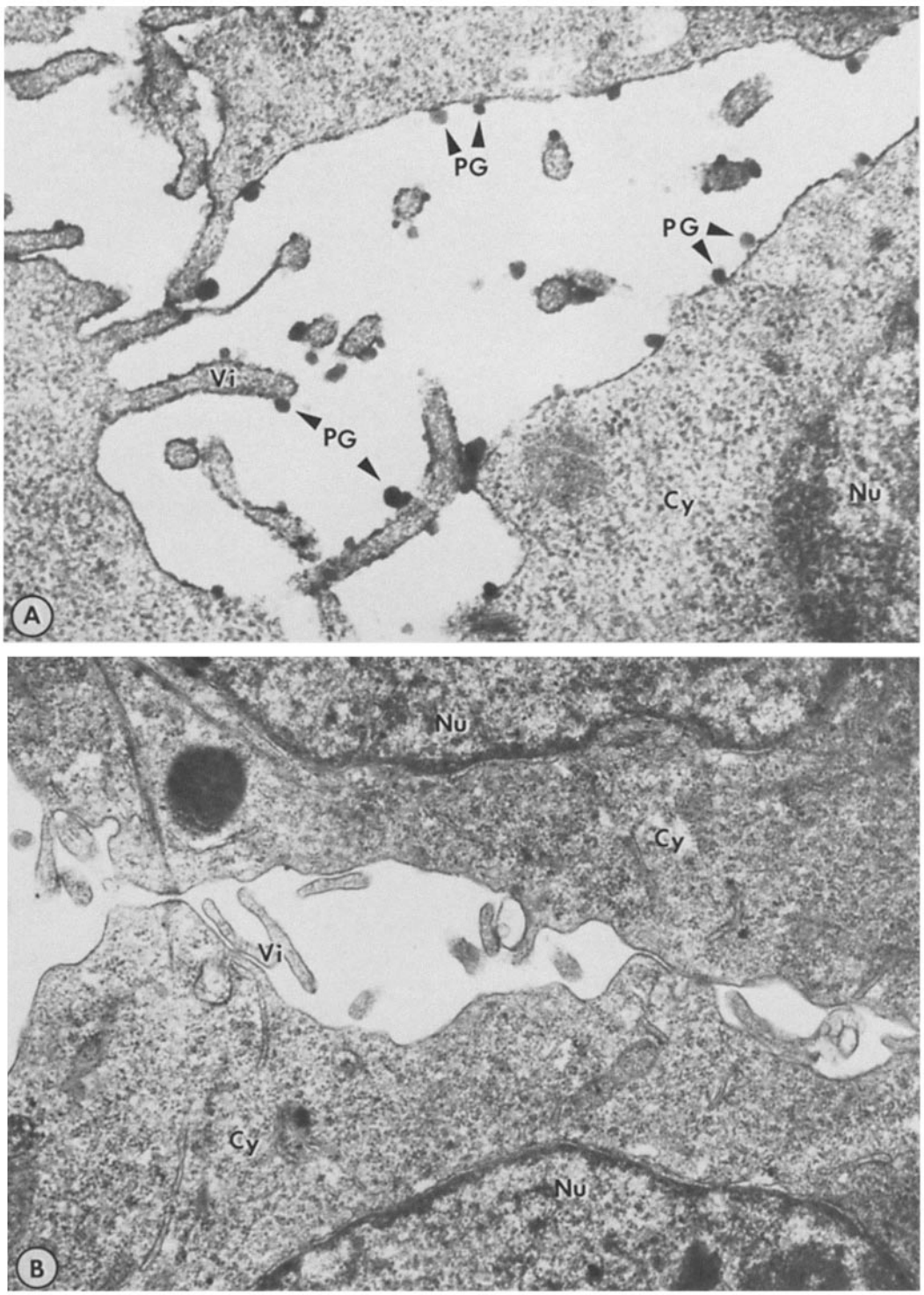

FIGURE 11 Transmission electron micrographs of ruthenium red-stained colon carcinoma cells. Notice the presence of several ruthenium red positive granules (*PG*) on the outer plasma membrane of the tumor cells (*A*), whereas control tissue processed in the absence of dye (*B*) shows no electron-dense granules. (*Nu*) Nucleus. (*Cy*) Cytoplasm. (*Vi*) Microvilli. (A) × 68,000. (B) × 32,000.

abolic fates. A large cell-associated proteoglycan with an estimated overall $M_r \simeq 9.5 \times 10^5$, a protein core with $M_r \simeq 2.4 \times 10^5$, and heparan sulfate side chains with $M_r \simeq 3 \times 10^4$, appears to be localized primarily at the surface plasma membrane. A slightly smaller ($M_r \sim 8.5 \times 10^5$) proteoglycan with identical heparan sulfate side chains is released continuously into the culture medium. These proteoglycans, therefore, could contain as many as 20 heparan sulfate chains per core peptide,[5] and share significant structural similarities with those synthe-

[5] Preliminary studies have demonstrated that the heparan sulfate proteoglycan contains a small fraction of *O*-linked oligosaccharide. (Iozzo, R. V., M. Yanagishita, and V. C. Hascall; manuscript in preparation.)

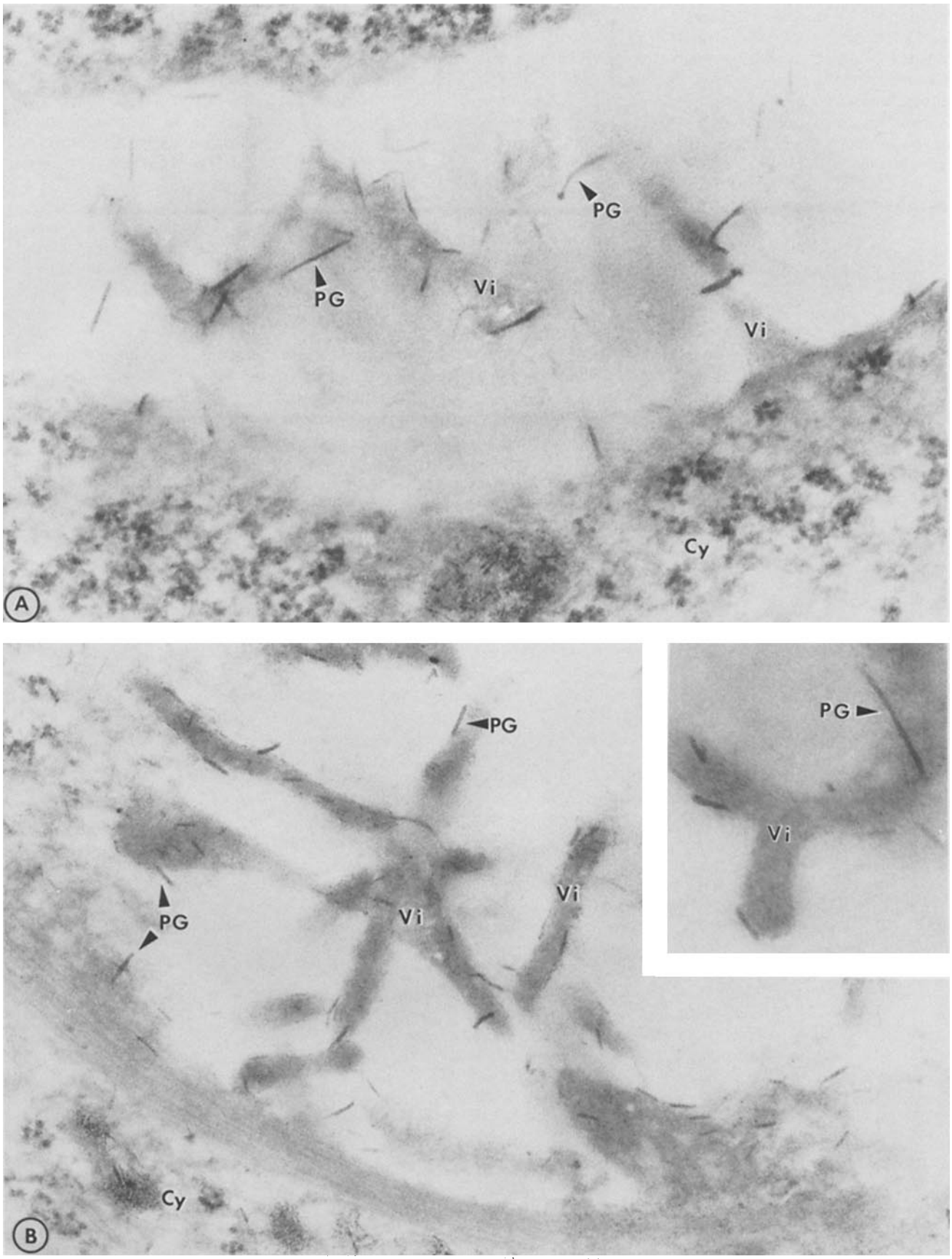

FIGURE 12 Transmission electron micrographs of cuprolinic blue–stained colon carcinoma cells in the presence of 0.1 M (*A*) and 0.3 M (*B*) $MgCl_2$. Notice the presence of numerous electron-dense filamentous structures (*PG*) on the surface of microvilli (*Vi*) in close association with the plasma membrane. The inset in *B* shows at a higher magnification the linear nature of the cuprolinic blue-positive structures, which can be >200 nm in length. (*Cy*) Cytoplasm. (*A*) × 73,000. (*B*) × 72,000. (*Inset*) × 105,000.

sized by normal human colon tissue in organ culture as we have previously reported (22). The latter proteoglycans differ from the colon carcinoma proteoglycans described here in that they have larger hydrodynamic size, higher buoyant density, but shorter glycosaminoglycan side chains (22). However, the amino acid composition of their respective protein cores is similar, particularly in their high content of aspartic acid, glutamic acid, alanine, and glycine suggesting that they may be genetically related. Comparison with other heparan sulfate proteoglycans indicates that the proteoglycan synthesized by colon carcinoma cells is considerably larger than that of liver plasma membrane (50), fibroblasts (51), or parietal yolk sac-2 cells (52), but is similar in overall size to that synthesized by vascular endothelial cells (53) and Engelbreth-Holm-Swarm tumor (38).

In addition to these two high-M_r proteoglycans, the colon carcinoma cells contain a cell-associated pool of small heparan sulfate chains ($M_r = 1 \times 10^4$). These components are admixed with small oligopeptides, are never found in the medium, and are not trypsin accessible suggesting that they may be located in an intracellular compartment. At least in part, this material could represent degraded products from the large cell-associated proteoglycan, with intermediate sized species composed of truncated proteoglycans. Intracellular pools of heparan sulfate of small size have been previously described in a variety of cells (35, 53–55), including embryonal carcinoma (56) and ovarian granulosa cells (57).

There is also evidence indicating that the large cellular proteoglycan is a precursor for the medium proteoglycan. In chase experiments, only the larger heparan sulfate proteoglycan is released into the medium as intact macromolecule, is accessible to trypsin, and exhibits nearly identical structural properties to that released during continuous labeling. Several lines of evidence presented here indicate that the release of the surface heparan sulfate proteoglycan occurs via a "clipping off" mechanism of its protein core. First of all, the medium proteoglycan has a smaller hydrodynamic size than the cell-associated form. Secondly, the deglycosylated protein core of the medium proteoglycan is slightly smaller, and thirdly, it contains ~15% less hydrophobic amino acids and a [^{3}H]Leu/^{35}S ratio significantly lower than the cellular form. The latter must therefore be either rich in protein or have heparan sulfate side chains that are sparsely sulfated (51). However, the ^{35}S-labeled glycosaminoglycans from the medium and cell layer co-migrate in 0.1 N HCl electrophoresis and elute from DEAE-Sephacel at the same NaCl molarity, indicating that they are equally sulfated. Taken together, these results suggest that the heparan sulfate proteoglycan may follow a biosynthetic pathway similar to that of integral membrane glycoproteins, in which the core protein could be inserted into the membrane bilayer or bound to a membrane receptor (58). After its arrival at the plasma membrane, it may then be released by limited cleavage of the core protein. Support for this model derives from recent studies showing that heparan sulfate proteoglycans from a variety of sources contain lipophilic domains and can be solubilized by detergents (59–62). Furthermore, in the present study, no heparan sulfate could be displaced by addition of exogenous heparin, thus indicating that none of the proteoglycan is bound through ionic interactions of its side chains with plasma membrane components (36). However, lack of heparin-mediated displacement does not exclude the possibility that electrostatic interactions involving the core protein could be responsible for anchoring the proteoglycan at the plasma membrane.

Previous studies using skin fibroblasts (55) have demonstrated that, during chase experiments, nearly all the cell-associated dermatan sulfate is not secreted as macromolecule but is completely degraded to dialyzable fragments, oligosaccharides, and/or inorganic sulfate. In the present study, we have found that after a 24-h chase, a large proportion of the cell-associated heparan sulfate is secreted into the medium as intact proteoglycan, whereas the rest is degraded after internalization. This discrepancy may be due to (*a*) a difference in cell types; (*b*) the fact that fibroblasts do not express a large amount of cell surface heparan sulfate; and/or (*c*) that the metabolism of heparan sulfate differs from that of other secretory products such as dermatan sulfate-rich proteoglycans. In agreement with recent investigations (63), the colon carcinoma cells metabolize membrane heparan sulfate proteoglycan through intracellular transport and catabolic pathways as well as by release into the medium. It has been speculated that the shedding of heparan sulfate is an event correlated to cell division and turnover rather than to secretion (64).

The immunocytochemical studies have revealed heavy deposits along the surface of colon carcinoma cells after reaction with an antiserum specific for the heparan sulfate proteoglycan core protein (38). These results have been confirmed at the ultrastructural level demonstrating coarse electron-dense deposits on the surface of microvilli and the free plasma membrane. Ruthenium red staining has revealed numerous 20–50-nm diameter electron-dense granules on the surface of the tumor cells. These granules are similar to those found in cartilage and in other soft tissues (10, 23, 40) and are thought to represent collapsed proteoglycan monomers (49). It has been shown that when cultured cells are stained with ruthenium red in the presence of serum, they exhibit a thick fuzzy coat on the surface membrane, whereas the lack of serum nearly eliminates it (65, 66). In the present study we have used serum-free medium to culture the tumor cells, and a rich, fuzzy coat has not been found, indicating that the electron-dense granules observed on the surface of colon carcinoma cells are not artifactual but represent polyanionic macromolecules newly synthesized by the cells. This has been confirmed by cuprolinic blue staining using the critical electrolite concentration method (42, 43). This approach has a major advantage over ruthenium red staining because it allows visualization of proteoglycan monomers in an extended configuration. Similar extended structures have been recently demonstrated in arterial smooth muscle cells using Safranin O (67). The evidence indicating that the cuprolinic blue-stained filaments are indeed heparan sulfate proteoglycans is based upon the following observations; (*a*) identical structures are seen in either 0.1 or 0.3 M $MgCl_2$ indicating that the molecules contain polyester sulfate groups; (*b*) nearly 100% of the cell surface ^{35}S-labeled macromolecules are present as heparan sulfate proteoglycan; (*c*) the cuprolinic blue material is sensitive to mild trypsinization, which also removes the majority of the cell-surface heparan sulfate, but is insensitive to chondroitinase ABC; (*d*) electron-dense filaments with similar characteristics are observed in the rat tail tendon processed at the same time, with results identical to those of Scott et al. (42, 43); (*e*) staining of hyaluronic acid and glycoprotein could be excluded in that the $MgCl_2$ concentrations used in these experiments prevent their staining (42, 43). Thus, the data indicate that these electron-dense filaments

visualized by cuprolinic blue represent proteoglycan monomers and their length may reflect the length of the core protein. We have found cuprolinic blue filaments that measure up to 220 nm in length, surprisingly close to those obtained with purified cartilage proteoglycans spread and rotary shadowed with platinum (49). Indeed, the protein core of this proteoheparan sulfate ($M_r = 2.4 \times 10^5$) is similar in overall size to that synthesized by cartilage cells (1).

In conclusion, this study has demonstrated that human colon carcinoma cells produce unique heparan sulfate proteoglycans, with distinct structural and metabolic characteristics. The strategic localization at the cell surface together with the large hydrodynamic size and charge suggest that these macromolecules may contribute to the altered surface properties of neoplastic cells. The availability of a well-controlled and characterized cell culture system and the expression of aberrantly high amounts of a single gene product, such as the heparan sulfate proteoglycan described here, provides an opportunity to investigate in detail the mechanisms that regulate proteoglycan metabolism and that concur with the establishment of the neoplastic phenotype.

The author thanks S. Finazzo, N. Pillsbury, and J. DiRienzi for their excellent technical assistance, and J. Woodruff for her skillful help in the preparation of the manuscript. The author is deeply indebted to Dr. G. G. Pietra for his continuous support and Dr. T. N. Wight, Dr. V. C. Hascall, Dr. M. Yanagishita, and Dr. M. Pacifici for critical review of the manuscript.

This work was presented in part (68) at the 23rd Annual Meeting of the American Society for Cell Biology, December 1983, San Antonio, Texas, and was supported in part by American Cancer Society Grant (IN-135C) and Junior Faculty Research Award (JFRA-95) and by a Biomedical Research Support Grant from the University of Pennsylvania.

Received for publication 21 December 1983, and in revised form 6 April 1984.

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