Glycosaminoglycans in the Substrate Adhesion Sites of Normal and Virus-Transformed Murine Cells†

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ABSTRACT: The substrate-attached material of Balb/c 3T3, SV40-transformed, and concanavalin A selected revertant variant cells was examined for its glycosaminoglycan (GAG) distribution during various cellular growth and attachment conditions. This material, the components of which are part of the tissue culture substrate adhesion sites of these cells, is left adherent to the substrate after cells are detached by EGTA treatment. The GAG distribution in substrate-attached material was also compared to the distributions found in the EGTA-detached cells and in the material solubilized during EGTA treatment. These comparisons revealed the following: (1) heparan sulfate accounts for over 80% of the glucosamine–radiolabeled GAG in the adhesion sites of reattaching cells, in contrast to its accounting for only 25–50% of the radiolabeled GAG in the adhesion sites of long-term or pulse-radiolabeled cells; (2) substrate-attached material is relatively enriched for chondroitin 4-sulfate and unsulfated chondroitin and highly depleted of dermatan sulfate when compared to the other cell fractions after long-term or pulse radiolabeling; (3) EGTA-soluble material is enriched for hyaluronic acid and depleted of heparan sulfate; and (4) the ratio of the chondroitins to hyaluronic acid is much higher in substrate-attached material than in cell-associated or EGTA-soluble material and is constant in substrate-attached material for all cell types examined under each set of radiolabeling conditions used. The relative distribution of GAG in all three cell types was remarkably similar. A consideration of these and other observations suggests that cellular adhesion to a tissue culture substrate may be mediated by cell-surface heparan sulfate interacting with a serum receptor protein(s) adsorbed to the substrate. Subsequent cellular detachment, which occurs when the cell body separates from its footpad adhesion sites, may be effected by the accumulation of undersulfated chondroitin sulfate–hyaluronic acid proteoglycan complexes at the adhesion site.

After many years of intensive effort, the molecular basis of mammalian cellular adhesion remains elusive. Attention has recently been focused on the adhesion of cells to an artificial substrate. In such studies, the cell's adhesive apparatus can be well differentiated from the serum component(s) to which it is adhering. Specifically, when metabolically radiolabeled cells are detached from their substrate by incubation in the Ca^{2+}-specific chelator EGTA,[1] it is found that a significant pool of radiolabeled material remains bound to the substrate (Culp & Black, 1972a). Extraction of this substrate-attached material with subsequent analysis by Na$DodSO_4$ PAGE (Culp, 1976a,b) has revealed the enrichment in this material of several cytoskeletal components, as well as the LETS glycoprotein [Hynes, 1976; also commonly referred to as fibronectin (Keski-Oja et al., 1976)]. These studies, as well as morphological investigations (Rosen & Culp, 1977), have indicated that substrate-attached material consists, at least in part, of the footpads by which the cell adheres to the substrate (Revel et al., 1974; Rosen & Culp, 1977). These footpads remain attached to the substrate after the rest of the cell body is sheared away following cytoskeletal weakening associated with calcium chelation.

This approach to the study of cellular adhesion to an artificial substrate is complicated by the observation that cells in culture, rather than adhering to naked glass or plastic, appear to adhere to one or more of the serum proteins adsorbed to the tissue culture substrate (Revel & Wolken, 1973; Yaoi & Kanaseki, 1972; Grinnell, 1974; Culp & Buniel, 1976; Stamatoglou, 1977). In fact, the adhesive behavior of cells in culture differs greatly depending on the presence or absence of such a protein carpet (Grinnell, 1974; Culp & Buniel, 1976). Recent evidence suggests that cold insoluble globulin, as a substrate-bound serum component, may be an important mediator of cellular adhesion to collagenous or artificial tissue culture substrates (Grinnell, personal communication; Culp, 1978).

Early investigations of the carbohydrate components of the cell's adhesive footpad revealed that a large amount of hyaluronic acid and a smaller amount of sulfated glycosaminoglycan were present in substrate-attached material (Terry & Culp, 1974; Roblin et al., 1975). These cell-associated polyanionic carbohydrates could bind to one or more components of the serum protein layer and, in so doing, effect adhesion to the substrate. This possibility is suggested by the fact that heparin, another polyanionic connective tissue polysaccharide, is known to bind to CIg, the putative serum component responsible for cellular adhesion to a serum-coated substrate (Stathakis & Mosesson, 1977). With the documented synthesis of heparin-like macromolecules by cultured cells (Kraemer, 1971a,b), it seemed of interest to perform a systematic investigation of the glycosaminoglycan in substrate-attached material. Since only focal areas of the cell surface, namely footpads, are involved in adhesive interactions with the substrate, it was hoped that a functional role for GAG's in adhesion might be revealed by comparing their

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[1] Abbreviations used: CIg, cold-insoluble globulin which is very similar to cell surface LETS glycoprotein (Yamada et al., 1977); Con A, concanavalin A; ΔDi-6S, ΔDi-4S, ΔDi-OS, and ΔDi-OHA represent $\Delta^{4,5}$-unsaturated disaccharides liberated by chondroitinase digestion of chondroitin 6-sulfate, chondroitin 4-sulfate, nonsulfated chondroitin, and hyaluronic acid, respectively (Yamagata et al., 1968); EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; GAG, glycosaminoglycan; LETS, large external transformation-sensitive glycoprotein (Hynes, 1976); MEM × 4, Eagle's minimal essential medium supplemented with a fourfold concentration of vitamins and essential amino acids; Na$DodSO_4$, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline without divalent cations; SV40, simian virus 40.

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distribution in substrate-attached material to their distribution in the rest of the cell. This report describes the distribution of GAG's for various cell types under different attachment and growth conditions and demonstrates a selective enrichment in substrate-attached material for certain GAG's which have the potential to interact with other components to mediate cell-substrate adhesion.

Materials and Methods

Cell Growth. Mouse Balb/c 3T3 cells (clone A31), SV40-transformed 3T3 cells (clone SVT2), and Con A selected revertant variant cells of SVT2 (Culp & Black, 1972b) were grown as described previously (Terry & Culp, 1974). Cells were used between their 12th and 25th passages (approximately 48–100 generations), were grown in MEM × 4 supplemented with 10% donor calf serum, penicillin (250 units/mL), and streptomycin (0.25 mg/mL), and were incubated in humidified 5% CO_2–95% air at 37 °C. For experimental purposes, cells were routinely grown in 100-mm diameter plastic tissue culture dishes and were free of *Mycoplasma* according to the radiolabeling assay of Culp & Black (1972a).

Radiolabeling Procedures. For preparation of long-term radiolabeled fractions, cells (0.75×10^6 Balb/c 3T3 cells, 0.50×10^6 Con A revertant cells, or 2.0×10^6 SVT2 cells) were inoculated into each of 64 100-mm plastic tissue culture dishes containing 10 mL of medium. After overnight incubation, medium was removed and replaced with 10 mL of medium containing 5.0 μCi/mL of D-[1-^{3}H]glucosamine (specific activity, 7.3 Ci/mmol), 5.0 μCi/mL of D-[6-^{3}H]glucosamine (specific activity, 29.0 Ci/mmol), or 50 μCi/mL of $Na_2{}^{35}SO_4$ (specific activity, 800 mCi/mmol). Cells were radiolabeled by incubation in this medium for 72 h, after which 70–80% of the dish surface was covered by cells.

For preparation of short-term radiolabeled cells, the same number of cells was plated and, after overnight incubation, medium was changed to 10 mL of fresh medium without radioactive precursor. Cells were allowed to grow for 48 h (to 70–80% confluence). Medium was then removed and replaced with 4.0 mL of medium containing 20 μCi/mL of D-[1-^{3}H]glucosamine or 50 μCi/mL of $Na_2{}^{35}SO_4$. Cells were radiolabeled by incubation for 2 h.

To examine reattaching cells, long-term radiolabeled cells were EGTA-detached, pelleted by centrifugation, washed once in cold PBS, and resuspended in fresh medium. Cells (3.0×10^6 3T3, 1.5×10^6 Con A revertant, or 11.0×10^6 SVT2 cells) were inoculated into each of 32 100-mm dishes containing 5 mL of medium and allowed to attach for 1 h before fractionation. Plating efficiency of reattaching cells was routinely estimated to be between 40 and 60%. A minimal amount of chasing of radiolabeled material occurs under these conditions due to the extremely large endogenous pool of radiolabeled glucosamine and GAG's present in these cells which were prelabeled for 72 h (Kornfeld & Ginsburg, 1966). This is confirmed by the fact that the amount of radiolabeled cell-associated polysaccharide per reattaching cell is the same as the amount in the long-term radiolabeled cells from which the reattaching cells were taken (about 250 000 cpm/10^6 cells). None of the radiolabeling procedures used in these studies was deleterious to cell growth.

Isolation of Cellular Fractions. After radiolabeling, medium was decanted and the cell layer gently washed three times with PBS. Cells were removed from the substrate by incubation in 0.5 mM EGTA in PBS on a gyratory shaker at 37 °C for 30 min. The cell suspension was gently pipetted over the dish surface to ensure detachment of all cells, and the cell suspension was then removed from the dish and placed on ice. Dishes were washed three times with distilled water at 37 °C, and *substrate-attached material* was quantitatively removed by incubating dishes in 0.2% $NaDodSO_4$ (w/v in H_2O) with shaking at 37 °C for 30 min. This material was concentrated by vacuum dialysis at room temperature.

Cell-associated material was obtained by centrifugation of EGTA-suspended cells at 600*g* for 10 min at 4 °C. The supernatant was decanted and put on ice (*EGTA-soluble* fraction). Cells were gently resuspended in cold PBS and recentrifuged; the supernatant from this wash was combined with the first supernatant. EGTA-soluble material was concentrated by vacuum dialysis at 4 °C. One-half of the cell pellet was resuspended in cold PBS and the cell suspension was frozen–thawed three times. The cell suspension and the 0.1–0.3-mL concentrated samples of substrate-attached and EGTA-solubilized material were then dialyzed against distilled water for 48 h at 4 °C (for glucosamine-radiolabeled material) or against 0.2 mM Na_2SO_4 for 24 h and then distilled water for an additional 24 h at 4 °C (for $Na_2{}^{35}SO_4$-radiolabeled material) to remove any EGTA and unincorporated precursor. Retentates were lyophilized and stored at −20 °C.

Polysaccharide Preparation. Radiolabeled polysaccharides were isolated from cell-associated, EGTA-soluble, and substrate-attached materials using essentially the techniques described by Cohn et al. (1976) with some modifications. Briefly, after addition of carrier GAG, all cellular fractions were digested with Pronase. The Pronase-digested cell-associated material was then made 1.5 mM in $MgCl_2$, 0.04 mg/mL in DNase-I, and 0.04 mg/mL in RNase-A. Digestion proceeded for 8 h at 37 °C, after which equivalent amounts of DNase-I and RNase-A were added and digestion continued for an additional 16 h at 37 °C. These nuclease-digested samples, as well as the Pronase-digested EGTA-soluble material and substrate-attached material, were boiled for 10 min, cooled to 4 °C, and centrifuged at 20000*g* for 15 min. The clarified substrate-attached material was then dialyzed against distilled water for 48 h at room temperature to reduce the concentration of $NaDodSO_4$.

The fractions were then mixed with three volumes of cold 95% ethanol–1% potassium acetate. Samples were stored at −20 °C overnight and the polysaccharide precipitate was collected by centrifugation at 30000*g* for 20 min at −20 °C. The precipitate was dried under vacuum overnight and then resuspended in 0.5 mL of distilled water. Material was purified twice more by precipitation with three volumes of ethanol–potassium acetate and dried under vacuum.

Determination of Chondroitinase-Susceptible GAG. The techniques used were essentially those of Saito et al. (1968). For digestion with chondroitinase ABC, dried precipitates were resuspended in 25 μL of enriched Tris buffer (Saito et al., 1968) adjusted to pH 7.4. To this mixture was added 0.125 unit of chondroitinase ABC and digestion was allowed to proceed for 3 h at 37 °C. For digestion with chondroitinase AC, the precipitates were resuspended in 25 μL of the same buffer, except adjusted to pH 6.8. Again, 0.125 unit of chondroitinase AC was added and the sample digested for 3 h at 37 °C. Adequacy of digestion of various substrates with both enzymes under these conditions was established by the methods described by Yamagata et al. (1968) and Cohn et al. (1976). Digestion with as much as 0.25 unit of chondroitinase ABC and 0.45 unit of chondroitinase AC led to no alterations in the distributions of digestion products observed.

After digestion was complete, 25 μg each of carrier ΔDi-6S, ΔDi-4S, ΔDi-OS, and ΔDi-OHA was added to each digest.

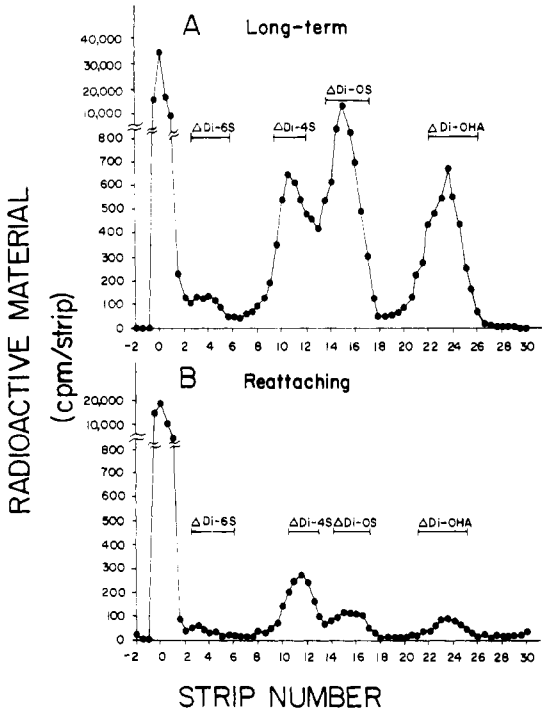

FIGURE 1: (A) SVT2 cells were grown for 72 h in the presence of 5 μCi/mL of [^{3}H]glucosamine, after which time the cells were harvested using EGTA and saved at 4 °C. The substrate-attached material was collected using $NaDodSO_4$, the polysaccharides from this material were isolated and digested with chondroitinase ABC, and the digestion products were separated by paper chromatography as described under Materials and Methods. The position at which the standard disaccharide digestion products chormatographed is shown by bars. (B) An aliquot of the long-term radiolabeled cells described in (A) was inoculated into dishes containing medium without radioactive precursor. The cells were allowed to reattach for 1 h, after which they were harvested using EGTA. Polysaccharides from the substrate-attached material of these reattaching cells were digested with chondroitinase ABC and the digest products were chromatographed as described in (A).

The mixture was desalted by paper chromatography for 48 h in *n*-BuOH–EtOH–H_2O (52:32:16) and then fractionated during 24 h in *n*-BuOH–HOAc–1 N NH_4OH (2:3:1). UV-absorbing spots were marked, and each tract was cut into strips. Radioactive material was eluted from the strips by adding 0.5 mL of 0.1 N HCl and determined by scintillation counting.

Determination of Nonchondroitinase-Digestible Material. Qualitative determinations of chondroitinase-resistant GAG's were performed by subjecting digests to two-dimensional cellulose acetate electrophoresis as described by Sato & Gyorkey (1974). After electrophoresis, the membranes were stained with Alcian Blue (Stefanovich & Gore, 1967) and the entire membrane was cut into sections. Each section was assayed for radioactive content by the direct addition of 5 mL of scintillation fluid.

Heparan sulfate was determined by nitrous acid deamination (Lindahl et al., 1973). An aliquot of a chondroitinase ABC digest was treated with 10 μL of 18% $NaNO_2$ in 1.8 M acetic acid in a total volume of 0.1 mL. The reaction proceeded for 80 min at room temperature and was stopped by the addition of 0.1 mL of 2 M ammonium sulfamate. Reaction products were separated by chromatography on columns of Sepharose CL-6B or Sephadex G-100 (both 1 × 50 or 1 × 120 cm) and eluted with 150 mM Tris-HCl (pH 7.4) with or without 0.2% $NaDodSO_4$ at a rate of 0.1–0.2 mL/min. An aliquot of each of the eluted fractions was used for determination of radioactive material by scintillation counting and the profile obtained was compared to the profile seen when another aliquot of the chondroitinase ABC digest was treated with 10 μL of distilled water instead of $NaNO_2$.

Since the adhesive footpad represents such a small percentage of total cellular material (Culp, 1978), the various preparations of substrate-attached material contained only a

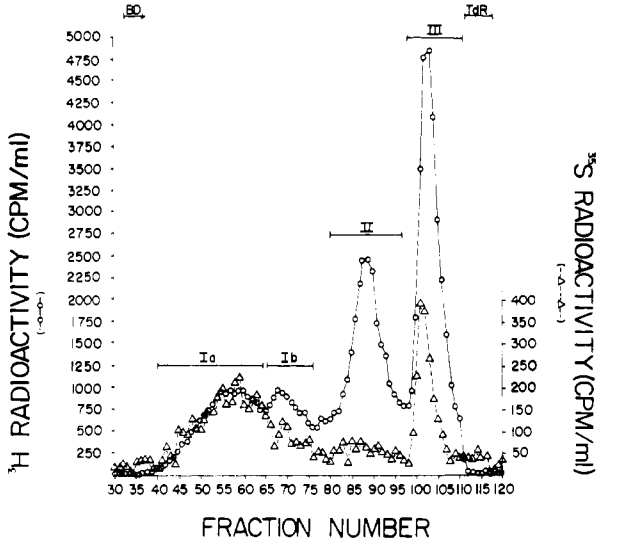

FIGURE 2: SVT2 cells were grown for 72 h in the presence of 5 μCi/mL of [^{3}H]glucosamine and 50 μCi/mL of $Na_2{}^{35}SO_4$. The cells were harvested using EGTA. Substrate-attached material was collected using $NaDodSO_4$; the polysaccharides from this material were isolated and digested with chondroitinase ABC and then chromatographed on a column of Sepharose CL-6B (1 × 120 cm; eluted with 150 mM Tris-HCl, pH 7.4, 0.2% $NaDodSO_4$). ^{3}H (O-O) and ^{35}S (Δ--Δ) radioactivity was determined for each sample using scintillation counter settings allowing less than 15% crossover. BD, blue dextran; TdR, [^{14}C]thymidine.

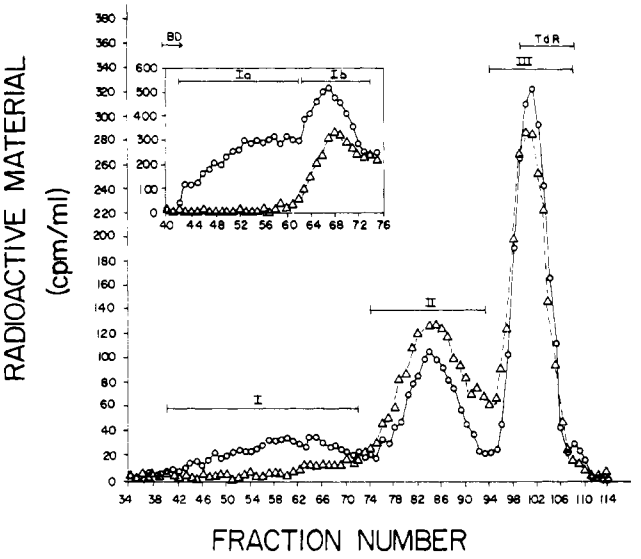

FIGURE 3: 3T3 cells were grown for 2 h in the presence of 20 μCi/mL of [^{3}H]glucosamine, after which the cells were harvested using EGTA. Substrate-attached material was collected using $NaDodSO_4$, the polysaccharides from this material were isolated and digested with chondroitinase ABC, and an aliquot of the digestion products was chromatographed on a column of Sepharose CL-6B (1 × 50 cm; eluted with 150 mM Tris-HCl, pH 7.4) (O-O). A second aliquot was treated with nitrous acid as described under Materials and Methods and chromatographed on the same column (Δ--Δ). *Insert*: A portion of a Sepharose CL-6B chromatography profile of the chondroitinase ABC digested polysaccharides from the substrate-attached material of long-term radiolabeled 3T3 cells before (Δ-Δ) and after (Δ--Δ) nitrous acid treatment. Fractions in areas Ia, Ib, II, and III were collected (see text). BD, blue dextran; TdR [^{14}C]thymidine.

Table I: Distribution of Long-Term Radiolabeled Polysaccharides[a]

cell line	polysaccharide[b]	% radioactivity[c] cell associated	EGTA soluble	cell + EGTA[d]	substrate attached
3T3	glycopeptide	73.6	36.0	72.7	27.9
	GAG	26.4	64.0	27.3	72.1
	total	100.0	100.0	100.0	100.0
	HS	48.8	22.2	48.2	26.3
	6S	0.8	1.1	0.8	2.3
	4S	5.7	8.3	5.8	22.4
	DS	24.0	11.7	23.7	2.7
	OS	3.1	18.0	3.5	23.6
	HA	17.6	38.7	18.0	22.7
	total	100.0	100.0	100.0	100.0
SVT2	glycopeptide	79.4	53.6	78.7	65.9
	GAG	20.6	46.4	21.3	34.1
	total	100.0	100.0	100.0	100.0
	HS	51.9	57.0	51.9	52.1
	6S	1.5	1.3	1.4	2.4
	4S	2.4	7.5	2.8	8.9
	DS	31.1	9.7	29.4	4.7
	OS	3.9	5.5	4.2	17.7
	HA	9.2	19.0	10.3	14.2
	total	100.0	100.0	100.0	100.0
rev	glycopeptide	69.3	9.7	65.5	22.9
	GAG	30.7	90.3	34.4	77.1
	total	100.0	100.0	100.0	100.0
	HS	46.2	25.7	42.8	36.0
	6S	1.7	0.8	1.5	1.6
	4S	11.8	4.3	10.6	13.9
	DS	12.1	3.2	10.8	1.2
	OS	5.9	2.5	5.3	11.9
	HA	22.3	63.5	29.0	35.4
	total	100.0	100.0	100.0	100.0

[a] Cells were grown in the presence of [^{3}H]glucosamine for 72 h, after which the various cellular fractions were collected, their polysaccharides were digested with chondroitinase ABC or AC, and the digestion products were separated either by paper chromatography or by chromatography on Sepharose CL-6B before and after nitrous acid treatment, as described under Materials and Methods. rev = revertant cells. [b] 6S (chondroitin 6-sulfate), 4S (chondroitin 4-sulfate), DS (dermatan sulfate), OS (unsulfated chondroitin), and HA (hyaluronic acid) were determined by measuring the amount of radioactivity eluted from the areas of paper chromatograms corresponding to the known disaccharide digestion products. HS (heparan sulfate) was determined as the percentage of total radioactivity in a chondroitinase ABC digest labile to nitrous acid (area I or areas Ia and Ib in Figure 3). Glycopeptide was determined as the percentage of total radioactivity remaining at the origin of a paper chromatogram of a chondroitinase ABC digest minus the percentage of total radioactivity identified as heparan sulfate. This amount of radioactivity was equivalent to that in area II of Figure 3 for each preparation. [c] Glycopeptide and GAG are shown as the percentage of total polysaccharide radioactivity, while the individual GAG's are shown as the percentage of total GAG radioactivity. [d] The percentage of radioactivity in the (cell + EGTA) fraction contained in a specific polysaccharide was determined as follows: % radioactivity = (cpm of specific polysaccharide in cell-associated and EGTA-soluble material)/(cpm of total polysaccharide in cell-associated and EGTA-soluble material) × 100.

Table II: Distribution of Short-Term Radiolabeled Polysaccharides[a]

cell line	polysaccharide[b]	% radioactivity[c] cell associated	EGTA soluble	cell + EGTA[d]	substrate attached
3T3	glycopeptide	56.3	21.1	50.5	8.8
	GAG	43.7	78.9	49.5	91.2
	total	100.0	100.0	100.0	100.0
	HS	46.5	5.2	34.2	15.0
	6S	0.9	1.2	1.0	2.5
	4S	3.7	10.2	5.5	21.7
	DS	16.4	16.2	16.6	0.0
	OS	8.8	14.7	10.6	34.5
	HA	23.7	52.5	32.1	26.3
	total	100.0	100.0	100.0	100.0
SVT2	glycopeptide	73.3	26.6	71.3	29.3
	GAG	26.7	73.4	28.7	70.7
	total	100.0	100.0	100.0	100.0
	HS	59.2	39.7	57.1	37.6
	6S	1.2	1.0	1.1	2.6
	4S	5.3	8.3	5.6	12.7
	DS	9.5	15.8	10.2	0.0
	OS	4.6	7.6	4.9	25.5
	HA	20.2	27.6	21.1	21.6
	total	100.0	100.0	100.0	100.0

[a] Cells were exposed to [^{3}H] glucosamine for 2 h, after which the various cellular fractions were collected and treated as described in Table I, footnote *a*. [b] See Table I, footnote *b*. HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid. [c] See Table I, footnote *c*. [d] See Table I, footnote *d*.

small amount of radioactivity (see Figures 1–3). Because of this, and because of minor variations in plating densities, duplicate experiments led to wide variations in total counts per minute and to differences of 0–50% in terms of percentages of GAG's in duplicate preparations. The qualitative relationships among the various polysaccharides were, however, always seen in duplicate experiments. Thus the data in Tables I–III are from representative experiments, and the trends and relationships discussed in the text and shown in Table IV were always seen from one preparation to the next.

Materials. Materials were purchased from the following sources: D-[1-^{3}H]glucosamine hydrochloride from Amersham/Searle Corp.; D-[6-^{3}H]glucosamine hydrochloride, $Na_2{}^{35}SO_4$, and NEF-963 aqueous counting cocktail from New England Nuclear Corp.; Pronase (grade B) from Calbiochem Corp.; bovine albumin (fraction V), hyaluronic acid (grade III), chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, DNase-I, and RNase-A (Type III-A) from Sigma Chemical Co.; sodium heparin (injectable) from Upjohn Co.; Sephadex G-100 and Sepharose CL-6B from Pharmacia Fine Chemicals, Inc.; EGTA from Eastman Organic Chemicals; sodium dodecyl sulfate from Bio-Rad Laboratories; cellulose acetate membranes (Micro Zone System) from Beckman; chondroitinase ABC, chondroitinase AC, ΔDi-6S, ΔDi-4S, and ΔDi-OS from Miles Laboratories, Inc.; MEM × 4 from Grand Island Biologicals Co.; donor calf serum from K. C. Biological, Inc.; and plastic tissue culture dishes from Lux Scientific Co. ΔDi-OHA was prepared by the methods of Cohn et al. (1976). Acid mucopolysaccharide reference standards were generously provided by Dr. M. B. Mathews, University of Chicago.

Results

Identification of Polysaccharides. When $NaDodSO_4$-extracted substrate-attached material from SVT2 cells, radiolabeled for 72 h with [^{3}H]glucosamine, is analyzed for its chondroitinase ABC digestion products, the chromatographic profile of Figure 1A is seen. It is apparent that substrate-attached material radiolabeled under these conditions contains significant amounts of chondroitin 6-sulfate, chondroitin 4-sulfate, and/or dermatan sulfate, and undersulfated varieties of these galactosaminoglycans, as well as hyaluronic acid. [Some of these species were not observed by Terry & Culp

Table III: Distribution of Radiolabeled Polysaccharides in Reattaching Cells[a]

cell line	polysaccharide[b]	% radioactivity[c] cell associated	EGTA soluble	cell + EGTA[d]	substrate attached
3T3	glycopeptide	59.7	53.1	59.4	66.6
	GAG	40.3	46.9	40.6	33.4
	total	100.0	100.0	100.0	100.0
	HS	56.8	54.8	56.6	80.2
	6S	2.5	2.4	2.5	1.6
	4S	4.3	5.0	4.3	8.0
	DS	17.7	22.4	18.1	0.9
	OS	2.5	2.4	2.5	4.8
	HA	16.2	13.0	16.0	4.5
	total	100.0	100.0	100.0	100.0
SVT2	glycopeptide	81.7	66.1	81.5	67.3
	GAG	18.3	33.9	18.5	32.7
	total	100.0	100.0	100.0	100.0
	HS	57.4	44.2	57.4	81.4
	6S	1.7	1.5	1.6	0.6
	4S	7.2	7.2	7.1	5.7
	DS	20.4	14.7	20.2	4.4
	OS	3.9	6.3	3.8	4.1
	HA	9.4	26.1	9.9	3.8
	total	100.0	100.0	100.0	100.0
rev	glycopeptide	60.8	29.9	56.7	51.8
	GAG	39.2	70.1	43.3	48.2
	total	100.0	100.0	100.0	100.0
	HS	49.6	43.3	48.4	70.4
	6S	2.6	1.9	2.3	2.5
	4S	11.4	8.6	10.7	8.2
	DS	15.0	11.5	14.3	4.6
	OS	5.7	3.6	5.1	5.7
	HA	15.7	31.1	19.2	8.6
	total	100.0	100.0	100.0	100.0

[a] Cells were grown in the presence of [^{3}H]glucosamine for 72 h, after which they were harvested with EGTA, washed, and allowed to reattach to dishes in fresh medium without radioactive precursor for 1 h. After this reattachment period, the various cellular fractions were collected and treated as described in Table I, footnote *a*. [b] See Table I, footnote *b*. HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid. [c] See Table I, footnote *c*. [d] See Table I, footnote *d*.

(1974) because they extracted substrate-attached material with alkali. Unlike NaDodSO$_4$, alkali does not extract this material quantitatively (Cathcart & Culp, unpublished data).] Figure 1B shows the profile of digestion products obtained by chondroitinase ABC treatment of the substrate-attached material from reattaching SVT2 cells. The same identifiable components can be seen, although in different relative amounts (see below).

It is apparent that a large percentage of the radiolabeled material does not migrate from the origin. This material does not represent incomplete digestion products since incubation of the same polysaccharide preparations with two to four times the amount of enzyme used to generate these chromatograms leads to no alteration in the profiles shown in Figure 1 (data not shown).

The nature of this nondigestible material was investigated by molecular sieve chromatography of [^{35}SO$_4$,^{3}H]glucosamine doubly radiolabeled polysaccharides after chondroitinase ABC digestion, as shown in Figure 2. Four areas of radioactivity can be discerned. When material from area Ia or Ib was electrophoresed as described under Materials and Methods, all of the ^{35}S and tritium radioactivity coelectrophoresed with standard heparan sulfate. No radioactive material coelectrophoresed with heparin or with bovine corneal or human skeletal keratan sulfates.

Table IV: Ratios of Specific Glycosaminoglycans from 3T3 Cells[a]

polysaccharides[b] compared	radiolabeling[c] conditions	ratio[d] cell associated	EGTA soluble	cell + EGTA	substrate attached
OS-HA	long term	0.18	0.46	0.19	1.04
	short term	0.37	0.28	0.33	1.31
	reattaching	0.15	0.18	0.16	1.07
(6S + 4S + OS)-HA	long term	0.54	0.71	0.56	2.13
	short term	0.56	0.50	0.53	2.23
	reattaching	0.57	0.75	0.58	3.20
(6S + 4S + DS)-OS	long term	10.17	1.17	8.66	1.16
	short term	2.95	1.89	2.18	0.70
	reattaching	9.80	12.42	9.96	2.19
HS-HA	long term	2.77	0.57	2.68	1.16
	short term	1.96	0.10	1.07	0.57
	reattaching	3.51	4.22	3.54	17.82

[a] The percentage of radioactivity associated with a specific polysaccharide from the indicated cellular fraction and radiolabeling condition was taken from Tables I-III. Similar ratios were seen for SVT2 and revertant cells. [b] HS, heparan sulfate; 6S, chondroitin 6-sulfate; 4S, chondroitin 4-sulfate; DS, dermatan sulfate; OS, unsulfated chondroitin; HA, hyaluronic acid. [c] Polysaccharides were isolated from cells radiolabeled for 72 (long term) and 2 h (short term) and from long-term radiolabeled cells allowed to reattach for 1 h as described under Materials and Methods. [d] Percentages from Tables I-III were added and/or divided directly as indicated.

The identification of this material as heparan sulfate was confirmed by its lability to nitrous acid (Cifonelli, 1968). Nitrous acid treatment of sulfate-radiolabeled polysaccharides from substrate-attached material leads to complete desulfation of the material in areas Ia,b of Figure 2 (data not shown). Similar treatment of polysaccharides from short-term [^{3}H]glucosamine radiolabeled cells is described in Figure 3. All of the material in area I is labile to nitrous acid, which would indicate that it is composed entirely of heparan sulfate. In some of the cellular fractions from long-term radiolabeled cells or reattaching cells, this area can be separated into two distinct areas (see Figure 2 and insert of Figure 3). Again, area Ia is completely nitrous acid labile and is thus composed of heparan sulfate. After nitrous acid treatment of area Ib all of the ^{35}S radioactivity disappears from this area (data not shown), and the remaining tritium radioactivity undergoes a shift in eluted position to one of lower molecular weight (insert, Figure 3). This suggests that area Ib may represent an under-N-sulfated, focally N-sulfated, or highly N-acetylated heparan sulfate, the degradation products of which would still be rather large (Linker & Hovingh, 1973, 1975).

The material in area II in Figures 2 and 3 is resistant to nitrous acid and has a fivefold lower ratio of ^{35}S to ^{3}H radioactivity than does the material in areas Ia,b. Furthermore, this material does not move from the origin during cellulose acetate electrophoresis, suggesting that it has a low charge density. These facts plus its relatively smaller size indicate that it probably represents glycopeptide derived from Pronase-digested glycoprotein.

Finally, the amount of radioactivity in area III in all preparations is equal to the percentage of radioactivity which migrates from the origin during paper chromatography. This fact plus the size of this material and its content of ^{35}S radioactivity indicate that it is composed of the disaccharide digestion products.

Thus in the subsequent analyses, heparan sulfate was determined as the amount of radioactivity in area I, or areas Ia

and Ib, and glycopeptide as the amount of radioactivity in area II of the Sepharose CL-6B profile of chondroitinase ABC digested polysaccharides.

Distribution of Long-Term Radiolabeled Polysaccharides. One of the results of cellular movement over the tissue culture substrate is that the cells leave their adhesion sites behind them (Culp, 1976b, 1978; Chen, 1977). These footpads remain firmly bound to the substrate after the cell body has pinched off the footpad and moved on. These remnants of the adhesion site have been termed "footprints" (Culp, 1976b, 1978). Thus a preparation of substrate-attached material derived from cells radiolabeled for 72 h consists of a mixture of both radiolabeled footpads and footprints. After exposure of cells to [^{3}H]-glucosamine for 72 h, radioactivity is incorporated into polysaccharides in the distribution shown in Table I. It is apparent that both EGTA-soluble and substrate-attached materials are relatively enriched for GAG.

The distribution of specific GAG's in these long-term radiolabeled preparations from 3T3 and SVT2 cells reveals several trends. For example, while the relative amount of chondroitin 4-sulfate rises almost fourfold in substrate-attached material as compared to cell-associated material, the amount of dermatan sulfate drops eightfold. In addition, the unsulfated content of the galactosaminoglycans, as determined by ΔDi-OS levels, rises four- to eightfold in substrate-attached material as compared to cell-associated or EGTA-soluble material. In fact, the ratio of sulfated to unsulfated galactosaminoglycan decreases from 9–10:1 in cell-associated material to 1:1 in substrate-attached material (Table IV), and the ratio of unsulfated chondroitin to hyaluronic acid rises from 0.2–0.4:1 in cell-associated and EGTA-soluble material to approximately 1:1 in substrate-attached material (Table IV).

Although revertant cells demonstrate the same characteristics as described for 3T3 cells, some of the trends are not quite so dramatic. Thus the amount of chondroitin 4-sulfate in substrate-attached material is only 30% greater than the amount in cell-associated material. Also, although substrate-attached material is enriched for unsulfated chondroitin, the ratio of chondroitin to hyaluronic acid is less than 1:1.

Distribution of Short-Term Radiolabeled Polysaccharides. In order to examine more specifically the newly synthesized components of footpads to the relative exclusion of footprints, cells were radiolabeled by means of a 2-h exposure to a high concentration of [^{3}H]glucosamine. Since these cells only move short distances during a 2-h period (Gail & Boone, 1972), the substrate-attached material prepared from such pulse-radiolabeled cells has radioactivity preferentially incorporated into footpads. The relative amounts of radioactive precursor incorporated into the various polysaccharides of these cells under these conditions are shown in Table II. As with long-term radiolabeling, substrate-attached material is considerably enriched for GAG.

Other trends seen in the long-term radiolabeled GAG distributions are retained in the pulse-radiolabeled profile. The levels of chondroitin 4-sulfate rise two to four times in substrate-attached material as compared to cell-associated material, while there are minimal amounts of dermatan sulfate in substrate-attached material. Also, the percentage of unsulfated galactosaminoglycan in each cellular fraction for both cell lines is nearly the same or slightly greater in pulse-radiolabeled than in long-term radiolabeled polysaccharide. This suggests that the high levels of undersulfated chondroitin sulfate in substrate-attached material from long-term radiolabeled cells are not due to simple accumulation of this GAG because of low turnover rate or endogenous sulfatase activity. The only striking difference between long-term and short-term radiolabeled GAG's in substrate-attached material is a significant diminution of radiolabeled heparan sulfate in the short-term radiolabeled material.

Distribution of Radiolabeled Polysaccharides in Reattaching Cells. In order to radiolabel all of the components of newly forming footpads, an alternative method of radiolabeling was investigated. In this method, cells radiolabeled for 72 h are detached with EGTA and then allowed to readhere to new substrates for 1 h. The distribution of radioactivity in the polysaccharides of the various fractions of reattaching cells is shown in Table III. The most striking aspect of the GAG distribution is the significant enrichment for heparan sulfate in substrate-attached material. It is anywhere from two to five times the amount seen in this fraction under any other set of conditions. This parallels, for 3T3 and revertant cells, the large rise in radiolabeled glycopeptide content in substrate-attached material from these reattaching cells. The amount of glycopeptide for SVT2 cells is very similar to that seen after 72 h of radiolabeling.

Other trends include (1) the same sharp decrease in dermatan sulfate when substrate-attached material is compared to cell-associated material, (2) a decrease of three- to fivefold in the ratio of sulfated to unsulfated galactosaminoglycans in substrate-attached material as compared to the other cellular fractions (Table IV), and (3) a rise in the ratio of unsulfated chondroitin to hyaluronic acid from much less than one in cell-associated and EGTA soluble material to 1:1 in substrate-attached material (Table IV).

All of these trends were seen in long- and short-term radiolabeled substrate-attached material (Tables I and II). In those cases, however, the last two ratios mentioned above (sulfated to unsulfated chondroitin and unsulfated chondroitin to hyaluronic acid) appeared to be generated by an enrichment for unsulfated chondroitin and, to a lesser extent, hyaluronic acid in substrate-attached material radiolabeled under those conditions. In this fraction from reattaching cells, however, there is no enrichment. Rather, a relative decrease in the amount of both the sulfated galactosaminoglycans and hyaluronic acid in the substrate-attached material of reattaching cells parallels the lack of enrichment for unsulfated chondroitin in this fraction, and so the same ratios are generated as were seen under the other radiolabeling conditions.

Discussion

The results described in this communication have demonstrated that the substrate adhesion sites of murine fibroblast cell lines, as represented by their substrate-attached material (Culp, 1978), have a GAG distribution strikingly different from the distribution found in the remainder of the cell. The most significant finding is that, when adhesion sites are newly formed, they are highly enriched for heparan sulfate and depleted of hyaluronic acid and the chondroitins (excluding dermatan sulfate) as compared to the rest of the cell. As the adhesion sites mature, i.e., as the cells begin to spread, there is a progressive accumulation of unsulfated chondroitin and hyaluronic acid in a fairly constant proportion (Table IV). This accumulation continues to such an extent that eventually the adhesion site becomes enriched for unsulfated chondroitin as compared to the rest of the cell. At all stages of development of adhesion sites, from the formation of the initial footpad to the formation of the pinched-off footprint, there is a relatively low level of dermatan sulfate in substrate-attached material. In contrast, both the cell-associated and EGTA soluble GAG's are highly enriched for dermatan sulfate. EGTA-soluble GAG's are also enriched for hyaluronic

acid. These results define the nature and distribution of the sulfated GAG's, their presence in substrate-attached material having been suggested by the preliminary experiments of Terry & Culp (1974) and Roblin et al. (1975).

As far as these distributions are concerned, there seems to be little difference between the normal and virus-transformed cells used in these studies, an observation also made by Cohn et al. (1976). The only significant differences between the cell lines seemed to involve chondroitin 4-sulfate levels. In each cellular fraction, 3T3 cells and revertant variants of SV40-transformed cells had more chondroitin 4-sulfate than the SV40 transformants. The significance of this difference is not clear, and it stands in contrast to the overall similarities in GAG distribution among the cell lines.

Heparan sulfate was identified in the various fractions by several criteria. Evidence that areas I, Ia, and Ib of Figures 2 and 3 represent heparan sulfate includes (1) elution near the exclusion volume of Sepharose CL-6B (Kleinman et al., 1975), (2) degradation by nitrous acid (Cifonelli, 1968), (3) high sulfate content, and (4) coelectrophoresis with standard heparan sulfate.

The material in area II of Figures 2 and 3 was identified as glycopeptide based on (1) an apparent molecular weight larger than disaccharides and smaller than heparan sulfate, (2) resistance to nitrous acid degradation, (3) low sulfate content, and (4) low charge density as judged by its lack of electrophoretic mobility. It is possible, however, that this material could be GAG fragments resistant to chondroitinase digestion. This is unlikely for two reasons. First, a reported undigestible GAG is galactosaminoglycan containing unsulfated *N*-acetylgalactosamine adjacent to sulfated iduronic acid residues (Fransson et al., 1974). Such fragments are sulfated while area II material shows very little sulfate content. (This does not, of course, exclude the possibility of an unsulfated, undigestible form of GAG.) Second, experiments separating whole components of substrate-attached material *before* Pronase or chondroitinase digestion have shown that one well-separated species alone accounts for over 98% of this area II material while other species account for all of the identifiable GAG (Rollins & Culp, manuscript in preparation). Therefore, unless there exists a proteoglycan composed of uniformly undigestible GAG, this area II material probably arises from Pronase-digested glycoprotein.

A more probable alternative is that area II material could be highly N-acetylated heparan sulfate (Linker & Hovingh, 1973, 1975). This possibility cannot be excluded at this time and would suggest that we may have underestimated heparan sulfate content.

The presence of large amounts of cell-associated heparan sulfate was not an unexpected finding (Kraemer, 1971a,b), and knowing the cellular origin of substrate-attached material (Terry & Culp, 1974; Culp 1976a,b; Rosen & Culp, 1977), a significant pool of heparan sulfate in this cellular fraction was also anticipated. The fact that heparan sulfate makes up at least 70–80% of the GAG in the adhesion site of reattaching cells, however, gives an indication of the possible importance of this polysaccharide in the initial adhesive interaction between the cell surface and the serum-coated substrate. It may be that this initial adhesive interaction involves cell-surface heparan sulfate acting as a cross-link between the cell-surface LETS glycoprotein (Culp, 1976a,b) and substrate-bound CIg. That such an interaction involving heparan sulfate could reasonably occur is suggested by the fact that heparin binds strongly and rather specifically to plasma CIg (Stathakis & Mosesson, 1977). Such a proposal also correlates well with all of the experimental indications to date that the cell-adhesion and spreading factor found in serum is CIg (F. Grinnell, 1976, personal communication; R. Haas, B. Murray, & L. Culp, unpublished results). Although interactions between CIg and collagen (Kleinman et al. 1976; Kleinman et al., 1978; Bornstein & Ash, 1977) and between proteoglycans and collagen (Toole, 1976) have been established, they are probably not relevant in this substrate adhesion system since the cells used in this study do not have any appreciable levels of collagen in their substrate-attached material (Culp & Bensusan, 1978).

Low heparan sulfate levels were found in the substrate-attached material from pulse-radiolabeled cells (Table II). Although initially considered an anomalous result, these levels could be explained by the fact that the half-life of at least one pool of heparan sulfate on the cell surface is on the order of several hours (Kraemer, 1977, personal communication), a period of time greater than that during which cells were exposed to radioactive precursor. Any new footpads formed during this pulse period will, with a high probability, be using heparan sulfate already present on the cell surface, that is, *nonradiolabeled* heparan sulfate. Thus the percentage of *radiolabeled* heparan sulfate in the adhesion sites of cells exposed to radioactive glucosamine for 2 h would be expected to be low.

During a full 72-h radiolabeling period undersulfated chondroitin and hyaluronic acid accumulate in the adhesion site (Table I). This accumulation is occurring *specifically* at the footpad since the GAG distribution in the rest of the 72-h radiolabeled cell is quite similar to that in the rest of the reattaching cell (Tables I and III). Furthermore, under all sets of conditions, these polysaccharides accumulate at the footpad in a fairly constant relationship (Table IV), suggesting that they may be somehow associated with each other. This situation is reminiscent of the hyaluronate–chondroitin sulfate proteoglycan complexes in cartilage (Hardingham & Muir, 1972; Hascall & Heinegard, 1974).

This GAG accumulation could be the chemical reflection of the physiological maturation which the footpad undergoes. This maturation takes the form of an apparently weakened connection between the cell body and its footpad since (1) well-spread cells are more easily detached with EGTA treatment than are attaching cells and (2) cells in culture move by detaching the cell body from the footpad, leaving footprints behind them (Chen, 1977; Culp, 1978). This suggests that some modification is occurring at the level of the footpad in order for it to become a footprint. The mechanism whereby such an accumulation of polysaccharide might weaken the footpad remains obscure, but it may be that as hyaluronic acid and chondroitin accumulate at the adhesion site, they can compete with heparan sulfate for binding to LETS. If LETS is connected in some way to the cell's cytoskeletal apparatus, such a competition could lead to localized destabilization of this apparatus and subsequent pinching off of the footpad.

There are several examples of systems in which stimuli for cell growth, movement, and detachment are correlated with hyaluronic acid accumulation (Tomida et al., 1975; Lembach, 1976). Conversely, cessation of growth and movement has been observed to be accompanied by a decrease in hyaluronic acid production (Morris, 1960; Tomida et al., 1974). More specifically, there is a CHO cell variant resistant to detachment from the substrate with trypsin or EGTA. This variant synthesizes extremely low levels of hyaluronate but normal levels of other GAG's (Atherly et al., 1977; Kraemer, personal communication). There is another variant which is more easily detached by these methods and synthesizes extremely high

levels of hyaluronic acid. (It also has a more highly rounded morphology than the parental CHO cells.) The correlation between hyaluronic acid production and ease of detachment from a substrate suggests, as proposed here and by Atherly et al. (1977), that hyaluronic acid may be one of the components necessary for efficient cellular release from the substrate. Interestingly, growth of the parental *or* detachment-resistant variant cells in spinner culture leads to diminution of hyaluronate content (Kraemer & Barnhart, 1978), suggesting that hyaluronate may be necessary only for growth-associated adhesion modifications on a substrate rather than for growth per se.

The mechanism for cellular adhesion to a substrate proposed here raises a large number of questions about GAG's and their interactions with other cell surface and serum materials. It will be interesting to further examine the individual components of the adhesion site described so far and to assess their potential for interaction. The investigation of interactions between the LETS glycoprotein and the various GAG's will be of special interest.

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