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Aggregation Competence of Proteoglycans from the Substratum Adhesion Sites of Murine Fibroblasts†

Judy A. Garner‡ and Lloyd A. Culp*

ABSTRACT: Supramolecular, proteoglycan-containing complexes from the serum-coated tissue culture substratum adhesion sites of Balb/c and Swiss 3T3 murine fibroblasts have been characterized after [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid mediated detachment of cells, extraction of the substratum-bound sites with 4 M guanidine hydrochloride (Gdn·HCl), and reassociation of proteoglycan-containing complexes by dialysis against 0.4 M Gdn·HCl. Sepharose CL2B gel filtration of extracts under associative conditions demonstrates a large proportion of aggregated proteoglycan material, some of which resists dissociation with 4 M Gdn·HCl and requires a detergent for complete dissociation. Isopycnic CsCl density gradient centrifugation of long-term-radiolabeled adhesive material under associative conditions reveals radiolabeled bands at three densities. A small fraction of two bands can be shown to be aggregates by exclusion from Sepharose CL2B columns and their dissociation with detergent. Digestion of the gradient bands with selective glycosaminoglycan-degrading enzymes documents that (a) the highest buoyant density band contains primarily heparan sulfate and chondroitin sulfate, with possibly a small amount of hyaluronic acid; (b) the mid-density band is comprised only of hyaluronate; and (c) the lightest band is a complex mixture of hyaluronate, chondroitin sulfate, and glycoprotein. The proteoglycan nature of some of the material in the gradient bands is shown by its sensitivity to Pronase and its lability to alkaline sodium borohydride reduction. Isopycnic density gradient analyses of Gdn·HCl extracts of newly formed footpad adhesion sites under associative conditions are qualitatively similar to those described above for long-term-generated adhesive material (enriched in "footprint" material as a result of natural cell movement across the substratum). However, there are significant quantitative and density gradient behavioral differences. These studies indicate that (a) some of the proteoglycan material from fibroblast cell–substratum adhesion sites is competent to form supramolecular aggregates, (b) there appear to be multiple types of aggregates which differ in several properties from cartilage-like hyaluronate/chondroitin proteoglycan aggregates, (c) the adhesion sites of both cell types have proteoglycans with similar properties, and (d) the proteoglycan material in newly formed adhesion sites is different from that in long-term-generated adhesion sites.

The survival of most mammalian cells in culture depends upon their ability to attach and adhere to a suitable tissue culture substratum, much the way cells in vivo attach to an extracellular matrix. Fibroblasts attach to a serum-coated substratum with both very tightly apposed focal regions and broad "close" contact regions (Izzard & Lochner, 1976, 1980; Heath & Dunn, 1978; Couchman & Rees, 1979). The tightly apposed focal regions have been called "footpads" upon visualization with the scanning electron microscope (Revel et al., 1974; Rosen & Culp, 1977; Vogel, 1978; Britch & Allen, 1980). Fibroblasts that are motile in culture appear to break off from footpads at their posterior or trailing edge and make new ones directly behind the ruffling anterior membrane. The footpad adhesion sites thus left behind are referred to as "footprint" material (Culp, 1976, 1978; Chen, 1977).

Exposure of fibroblasts to the calcium-specific chelator [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA)[1]

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‡ Present address: Department of Chemistry, Indiana University, Bloomington, IN 47405.

[1] Abbreviations used: C0S, unsulfated chondroitin; C6S, chondroitin 6-sulfate; C4S, chondroitin 4-sulfate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; GAG, glycosaminoglycan; Gdn·HCl, guanidine hydrochloride; HA, hyaluronic acid; HS, heparan sulfate; L-SAM, long-term metabolically radiolabeled substratum-attached material containing both footpad and footprint adhesive material; MEM × 4, Eagle's minimal essential medium supplemented with a 4-fold concentration of vitamins and essential amino acids; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline without divalent cations; PMSF, phenylmethanesulfonyl fluoride; R-SAM, reattaching substratum-attached material containing newly formed footpad adhesion sites; ΔDi-6S, ΔDi-4S, and ΔDi-0S, $\Delta^{4,5}$-unsaturated disaccharides liberated by chondroitinase digestion of chondroitin 6-sulfate, chondroitin 4-sulfate, and unsulfated chondroitin, respectively; DS, dermatan sulfate.

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leads to cytoskeletal disorganization within the cells resulting in cell body rounding and retraction away from their substratum adhesion sites (Rosen & Culp, 1977). Eventually, the cell bodies break away, and the footpad adhesion sites are left behind as substratum-attached material enriched in microfilaments, filaments, fibronectin, and glycosaminoglycan-containing proteoglycans (Culp, 1978; Rollins & Culp, 1979a,b; Culp et al., 1979). Preparations containing both footpad and footprint adhesion macromolecules are obtained after long-term metabolic radiolabeling of cultures. Preparations enriched in newly formed adhesion sites containing primarily footpads may be obtained by allowing EGTA-detached, long-term-radiolabeled cells to reattach to a fresh serum-coated substratum for 1 h before EGTA-mediated detachment.

A considerable body of evidence suggests that hyaluronate and the proteoglycans play an active role in the adhesion and detachment of cells by their binding to cell-surface and/or substratum-bound fibronectins (Yamada et al., 1980; Ruoslahti & Engvall, 1980; Laterra et al., 1980; Rollins et al., 1981). In newly formed adhesion sites, the most prominent GAG is heparan sulfate; as cells spread and initiate motility, hyaluronate and the chondroitins accumulate in a coordinate manner in these sites. Rollins & Culp (1979b) have also shown that these GAG's, with the single exception of hyaluronate, exist as proteoglycans. In addition, preliminary results using approaches originally designed to analyze aggregation of hyaluronate/chondroitin proteoglycan complexes from cartilage (Hascall, 1977) indicate that adhesion site proteoglycans may also aggregate into large complexes (Rollins & Culp, 1979b).

The present work characterizes the competence of adhesion site proteoglycans from Balb/c and Swiss 3T3 cells in forming supramolecular aggregates after dissociative extraction from these sites. We have compared the properties of these extracts during isopycnic density gradient centrifugation or gel filtration chromatography under associative or dissociative conditions. It has been found that the adhesion site proteoglycan complexes are significantly different from the cartilage-derived hyaluronate/chondroitin proteoglycan aggregates (Hascall, 1977). Because of the increasing evidence for the importance of multivalent binding in promoting adhesion of cells to extracellular matrixes (Carter et al., 1981; Rollins et al., 1981), the characterization of adhesion site proteoglycans and their potential aggregates should provide greater insight into the mechanism(s) of cell–substratum adhesion.

Materials and Methods

Radiolabeling and Preparation of Substratum-Attached Material. *Mycoplasma*-free Swiss 3T3 (C1-1) or Balb/c 3T3 (clone A31) cells were grown as described previously (Rollins & Culp, 1979a,b). For long-term radiolabeling of adhesion sites (Culp, 1976), 0.25×10^6 3T3 cells were inoculated into 64×100 mm plastic tissue culture dishes containing 10 mL of medium. After 24 h, the medium was replaced with 10 mL of one of the following radiolabeling media: (a) complete (containing 10% calf serum) Eagle's minimal essential medium (with 4× concentration of vitamins and essential amino acids) plus 5.0 μCi/mL D-[6-^{3}H]glucosamine hydrochloride; (b) complete medium (without streptomycin sulfate) plus 50 μCi/mL $Na_2{}^{35}SO_4$; or (c) 90% leucine-depleted complete medium plus 10 μCi/mL L-[4,5-$^3H_2$]leucine. Long-term-radiolabeled substratum-attached material (L-SAM, containing both footprint and footpad adhesive material) was harvested after labeling for 72 h, at which time cells had covered 70–80% of the dish surface. The radiolabeling medium was removed, and the cell layer was rinsed twice with PBS. Cells were incubated for 30 min in PBS containing 0.5 mM EGTA on a gyratory shaker at 37 °C. The rounded cells were detached from the substratum by gently pipetting the suspension over the plate surface and were retained for reattaching experiments (see below). Dishes containing substratum adhesion sites were rinsed 3 times with PBS and once with glass-distilled water. L-SAM was extracted with 5 mL of 4 M Gdn·HCl in buffer A (0.05 M sodium acetate containing the following protease inhibitors: 0.05 M benzamidine hydrochloride, 0.1 M 6-aminohexanoic acid, and 0.01 M EGTA at pH 5.8) for 18–24 h on a rocking platform at 4 °C. All buffers contained this mixture of protease inhibitors. The solubilized material was concentrated at 4 °C by vacuum dialysis, then dialyzed against large volumes of 0.4 M Gdn·HCl in buffer A to allow proteoglycan material to reassociate into large complexes (Hascall, 1977), and stored at 4 °C. By the criteria described in this study, samples were stable upon storage for several weeks. In some cases, bovine albumin (100 μg/mL) was added as a competitive inhibitor of any proteolysis that may occur during sample preparation; however, results identical with those reported here were observed. Similarly, addition of trace amounts of cartilage chondroitin proteoglycan to these samples failed to detect any degradation of proteoglycan material.

For the isolation of newly formed adhesion sites as reattaching substratum-attached material (R-SAM), 1.5×10^8 3T3 cells that had been radiolabeled previously for 72 h and gently detached from the substratum by EGTA treatment (see above) were inoculated into 32×100 mm plastic tissue culture dishes containing 5 mL of complete medium. Cells were allowed to attach for 1 h during which new footpad adhesion sites form (Rosen & Culp, 1977). R-SAM was then harvested as described above after EGTA-mediated detachment of cells. Rollins & Culp (1979a) have previously shown that the distribution and specific radioactivity of the various cellular GAG pools remain constant during the reattachment process. Glycosaminoglycan analyses of glucosamine-radiolabeled Balb/c or Swiss 3T3 cells and preparations of substratum-attached material were performed as described by Cohn et al. (1976) as modified by Rollins & Culp (1979a).

Isopycnic Density Gradient Centrifugation. Preparations of L-SAM or R-SAM that had been 4.0 M Gdn·HCl extracted in buffer A were dialyzed against 0.4 M Gdn·HCl in buffer A to permit re-formation of proteoglycan aggregates (termed "associative" conditions). Samples were brought to a density of 1.63 g/mL through addition of solid CsCl and were centrifuged in polyallomer tubes in a Beckman type 65 rotor at 100000*g* for 72 h at 18 °C. Fractions (0.5 mL) were collected from the bottoms of the tubes, and 50-μL aliquots were assayed for radioactivity. In order to determine the quantity of radioactive material adhering to the polyallomer tubes (up to 40% of the total radioactivity in some instances), they were cut into seven pieces and were fitted into scintillation vials for direct radioactivity determination; radioactive material was distributed equivalently along the sides of the tube [see Rollins & Culp (1979b)].

Gel Filtration Chromatography. Associative L-SAM or R-SAM samples were applied to columns of cross-linked Sepharose without further treatment. Samples were also chromatographed under the following dissociative conditions: with (a) 0.2% $NaDodSO_4$ (w/v) in buffer B (0.15 M sodium acetate, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM PMSF, pH 5.8) or with (b) 4.0 M Gdn·HCl in buffer A. Samples to be chromatographed in an $NaDodSO_4$-containing buffer were

generated by dialyzing associative extracts extensively against buffer B and then against 0.2% $NaDodSO_4$ in buffer B. Samples to be chromatographed in 4.0 M Gdn·HCl-containing buffer A were obtained either by (a) extracting plates with 4.0 M Gdn·HCl in buffer A and then vacuum dialyzing the extract to low volume or by (b) increasing the Gdn·HCl concentration of associative extracts. All samples were applied to Sepharose CL2B (0.8 × 60 cm) or CL6B (1 × 120 cm) columns that had previously been equilibrated with the appropriate buffers. Samples were eluted with buffer at flow rates of 6–10 mL/h, and fractions of 0.6 or 2.2 mL, respectively, were collected. Aliquots of each fraction were analyzed for radioactivity in a liquid scintillation counter. Recoveries were 85–95% for $NaDodSO_4$-eluted columns and 50–60% for columns eluted with Gdn·HCl-containing buffers. Prerunning associative columns with large quantities of unlabeled proteoglycan material substantially increased the yield and did not appear to affect the quality of material (as monitored by various analytical procedures to be described under Results).

Enzyme Digestion. For Pronase treatment, gradient or column fractions were initially dialyzed against buffer B without protease inhibitors followed by dialysis against 0.2% $NaDodSO_4$ in buffer B. They were then concentrated with molecular separators (Millipore Corp.) and divided into two equal parts. Pronase (2 mg) in buffer B was added to the experimental half of each sample, while the other half had an equivalent amount of buffer B without Pronase added as a control. Samples were incubated at 56 °C for 18 h at which time an additional 2 mg of Pronase was added for an additional 24-h incubation. For testicular hyaluronidase treatment (to degrade hyaluronate and the various chondroitins), gradient fractions were dialyzed against buffer B at 4 °C overnight and divided into experimental (10 μg/mL enzyme) and control halves for digestion at 37 °C for 4 h. For *Streptomyces* hyaluronidase treatment (to specifically degrade hyaluronate), gradient fractions were dialyzed against buffer C (0.1 M sodium acetate, 150 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$, pH 6.0) at 4 °C overnight and split into control and experimental halves (10 TRU/mL) for incubation at 37 °C for 18 h. $NaDodSO_4$ was then added to all samples to a final concentration of 0.2% for chromatography on Sepharose CL6B columns eluted with 0.2% $NaDodSO_4$ in buffer B.

Alkaline Sodium Borohydride Reduction. To test for O-glycosidic linkages of carbohydrate chains to protein (Carlson, 1968), we divided gradient or column samples into experimental and control halves. An equal volume of 7.5% $NaBH_4$ in 0.1 N NaOH was added to each experimental sample, and all samples were incubated at 45 °C for 48 h. Samples were then neutralized with acetic acid, and $NaDodSO_4$ was added to a final concentration of 0.2% for chromatography on columns of Sepharose CL6B as described above.

Materials were purchased from the following sources: D-[6-^{3}H]glucosamine hydrochloride, $Na_2{}^{35}SO_4$, and L-[4,5-$^3H_2$]leucine from New England Nuclear Corp.; Pronase and *Streptomyces* hyaluronidase from Calbiochem Corp.; testicular hyaluronidase from Worthington Biochemical Corp.; cesium chloride and guanidine hydrochloride from Bethesda Research Laboratories; Sepharose CL2B, CL4B, and CL6B from Pharmacia Fine Chemicals, Inc.; EGTA from Eastman Organic Chemicals; chondroitinase ABC, chondroitinase AC, ΔDi-6S, ΔDi-4S, and ΔDi-0S from Miles Laboratories, Inc.; MEM × 4 from Grand Island Biologicals Co.; donor calf serum from K. C. Biological, Inc.; plastic tissue culture dishes from Lux Scientific Co.; reference standard GAG's kindly provided by Drs. Cifonelli and Mathews of the University of Chicago; and bovine nasal septum hyaluronate/chondroitin proteoglycan aggregates kindly provided by Dr. Arnold Caplan of the Biology Department at Case Western Reserve University.

Table I: Glycosaminoglycan Composition of Swiss 3T3 Cell Adhesion Sites[a]

	radioactivity (%)[c]		
polysaccharide[d]	cell associated	L-SAM[a]	R-SAM[b]
glycopeptide	68.1 (73.6)	11.1 (27.9)	40.9 (33.4)
GAG	31.9 (26.4)	88.9 (72.1)	59.1 (66.6)
total	100.0	100.0	100.0
HS	52.7 (48.8)	15.4 (26.3)	41.0 (80.2)
HA	25.0 (17.6)	16.2 (22.7)	10.5 (4.5)
C6S	(0.8)	2.8 (2.3)	3.2 (1.6)
C4S	21.2 (25.7)	49.5 (22.4)	33.2 (8.0)
COS	1.0 (3.1)	16.1 (23.6)	11.5 (4.8)
DS	(4.0)	(2.7)	(0.9)
total	100.0	100.0	100.0

[a] Cells were metabolically radiolabeled for 72 h in the presence of [^{3}H]glucosamine after which L-SAM was quantitatively solubilized with 0.2% $NaDodSO_4$. The cell or adhesion site fractions were fractionated as described by Rollins & Culp (1979a) to obtain protein-free polysaccharide preparations. The polysaccharides were digested with chondroitinase ABC or AC, and the digestion products were separated either by paper chromatography or by Sepharose CL6B chromatography before and after nitrous acid treatment to identify specific GAG's or glycoprotein-derived glycopeptide as described previously (Rollins & Culp, 1979a). [b] Cells were grown for 72 h in the presence of [^{3}H]-glucosamine, detached from the dishes by treatment with EGTA, washed, and allowed to reattach to dishes for 1 h. After reattachment, substratum-attached material (R-SAM) was harvested as described under Materials and Methods, and the polysaccharide content was determined as described in footnote *a* above. [c] Glycopeptide and GAG (upper columns) are presented as the percentage of total polysaccharide radioactivity in the total extract, while the individual GAG's are presented as the percentage of total GAG radioactivity. The numbers in parentheses represent values obtained previously for Balb/c 3T3 preparations by Rollins & Culp (1979a). When these analyses are performed on three separate preparations, accuracy in measuring each GAG class is generally better than ±10%. [d] HA, C6S, C4S, COS, and DS quantities were determined by measuring the radioactivity eluted from the areas of paper chromatograms corresponding to the known disaccharide digestion products after chondroitinase ABC or AC treatments. HS was determined as the percentage of total radioactivity in a chondroitinase ABC digest labile to nitrous acid treatment. Glycopeptide was determined during elution from Sepharose CL6B columns of chondroitinase digests [see Rollins & Culp (1979a)].

Results

Polysaccharide Composition in Adhesion Sites. The GAG compositions of Swiss 3T3 cells, L-SAM's, and R-SAM's are presented in Table I for comparison with similar data generated previously for Balb/c 3T3 cells by Rollins & Culp (1979a). Both L-SAM and R-SAM are enriched in GAG relative to glycoprotein when compared with the cell-associated material. This is particularly notable in the very low glycoprotein content of the Swiss 3T3 L-SAM. When the individual species of GAG are analyzed, it can be seen that the L-SAM and R-SAM preparations are enriched in C4S and COS in comparison to the cellular GAG's. Newly formed adhesion sites (R-SAM) for both cell types are appreciably enriched in HS when compared with "mature" adhesion sites (L-SAM). The ratio of the hyaluronate plus chondroitin classes of polysaccharide to the heparan sulfate plus glycopeptide class of

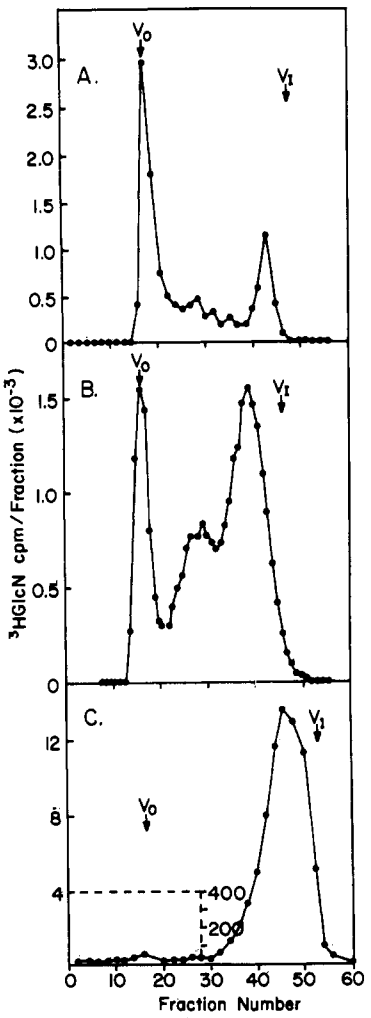

FIGURE 1: Aggregation competence of polysaccharide-containing components assayed by Sepharose chromatography. [^{3}H]Glucosamine-radiolabeled Swiss 3T3 L-SAM was extracted with 4 M Gdn·HCl as described under Materials and Methods, dialyzed to associative conditions (0.4 M Gdn·HCl in buffer A), and split into three parts. Sample A was chromatographed over Sepharose CL2B under associative conditions (0.4 M Gdn·HCl in buffer A). Sample B was restored to dissociative conditions (4 M Gdn·HCl in buffer A) through addition of solid Gdn·HCl and then chromatographed over a Sepharose CL2B column eluted with 4 M Gdn·HCl in buffer A. Sample C was dialyzed against buffer B overnight to remove the Gdn·HCl and then against 0.2% NaDodSO$_4$ in buffer B overnight; it was then chromatographed on a Sepharose CL2B column previously equilibrated with 0.2% NaDodSO$_4$ in buffer B. Markers were blue dextran (V_0) and dinitrophenylated glycine (V_I).

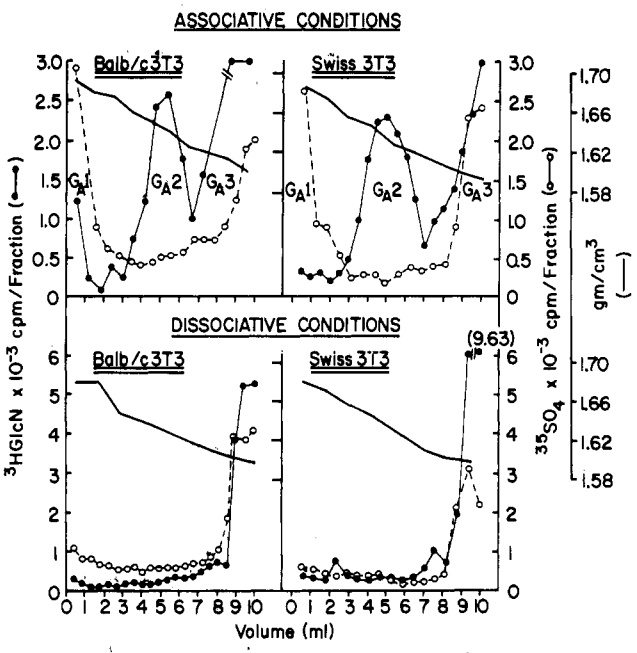

FIGURE 2: Isopycnic density gradient analyses of long-term metabolically radiolabeled adhesion site extracts. For samples centrifuged under associative conditions (upper profiles), [^{3}H]glucosamine- or $^{35}SO_4^{2-}$-radiolabeled Swiss or Balb/c 3T3 L-SAM's were harvested with 4 M Gdn·HCl in buffer A, concentrated, and dialyzed to associative conditions. For samples centrifuged under dissociative conditions (lower profiles), the reassociated L-SAM's described above were restored to 4 M Gdn·HCl through addition of solid Gdn·HCl. Centrifugation was performed as described under Materials and Methods. Fractions were analyzed for their content of tritium radioactivity (●), $^{35}SO_4^{2-}$ radioactivity (○), and density (—) as described under Materials and Methods, correcting for quenching if required.

polysaccharide is much higher for Swiss 3T3 L-SAM than for Balb/c 3T3 L-SAM. This correlates with the observation that the Swiss 3T3 cells are considerably more motile on their substratum than the Balb/c 3T3 cells (Gail & Boone, 1972), a correlation which applies to a large number of cultured cell types (Rollins et al., 1981).

Guanidine Hydrochloride Extraction of Adhesion Sites. Sodium dodecyl sulfate has previously been shown to quantitatively solubilize murine fibroblast adhesion site macromolecules from serum-coated plastic substrata (Cathcart & Culp, 1979). The relative efficiency of 4 M Gdn·HCl dissociative extraction was determined for both L-SAM and R-SAM from the two cell types by comparison with NaDodSO$_4$ extractions. 4 M Gdn·HCl is much less efficient in extracting protein material (24% and 13%, respectively) from Balb/c 3T3 L-SAM and R-SAM than in extracting polysaccharide material (46% and 21%, respectively) from these preparations. On the other hand, 4 M Gdn·HCl extracts approximately twice as much protein and polysaccharide from Swiss 3T3 L-SAM and R-SAM as from the comparable Balb/c 3T3 fractions. Increasing the concentration of Gdn·HCl or the length of the extraction time did not affect the efficiency of extraction.

Reassociation of Polysaccharide-Containing Components from the Gdn·HCl Extract. When glucosamine-radiolabeled extracts of Swiss or Balb/c 3T3 L-SAM are analyzed by gel filtration on Sepharose CL2B columns under associative conditions, there are two populations of glucosamine-radiolabeled macromolecules distinguished (Figure 1A)—a predominant class at the exclusion region of the elution profile (V_0) and a smaller class which elutes just before the inclusion region of the profile (V_I) which is heterodisperse as demonstrated on Sepharose CL6B columns (see below). When [Gdn·HCl] is raised to 4 M (Figure 1B), only a portion of the material at V_0 is disaggregated. However, treatment of the L-SAM extract with 0.2% NaDodSO$_4$ before chromatography almost completely dissociates the aggregated material (Figure 1C). Enzyme susceptibility studies demonstrate that the excluded material (V_0 of Figure 1A) is primarily composed of HA with some chondroitin sulfate and heparan sulfate (data not shown). These data demonstrate that there are noncovalent interactions between macromolecules from adhesion sites resulting in a significant proportion of aggregation.

Figure 2 illustrates the behaviors of $^{35}SO_4^{2-}$- or [^{3}H]-glucosamine-radiolabeled L-SAM extracts from both Balb/c and Swiss 3T3 cells when subjected to isopycnic CsCl density gradient centrifugation under associative and dissociative conditions. In the associative gradients, three bands of [^{3}H]glucosamine-radiolabeled material are revealed: One at a density of 1.68–1.71 g/cm^3 (referred to as associative gradient band 1 or G_A1), one at 1.62–1.66 g/cm^3 (G_A2), and one at 1.58–1.61 g/cm^3 (G_A3). The proportion of [^{3}H]glucos-

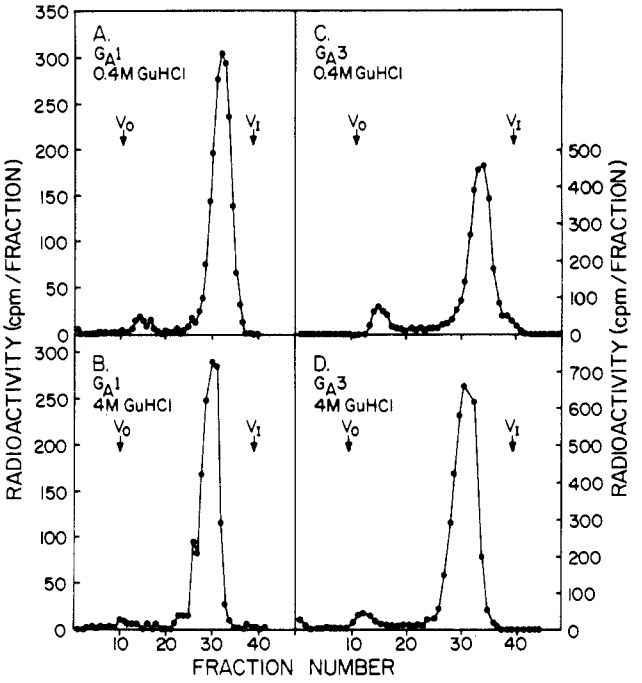

FIGURE 3: Sepharose CL2B chromatography of density gradient fractions. G_A1 or G_A3 density gradient fractions of [^{3}H]glucosamine-radiolabeled Balb/c 3T3 L-SAM were pooled (see Figure 2), dialyzed against 0.4 M Gdn·HCl in buffer A overnight at 4 °C, and divided into two portions. One portion was chromatographed on Sepharose CL2B under associative conditions, and the other portion was brought to 4 M Gdn·HCl concentration and chromatographed on Sepharose CL2B under dissociative conditions: (A) G_A1 chromatographed under associative conditions (0.4 M Gdn·HCl in buffer A); (B) G_A1 chromatographed under dissociative conditions (4 M Gdn·HCl in buffer A); (C) G_A3 chromatographed under associative conditions as in (A); and (D) G_A3 chromatographed under dissociative conditions as in (B).

amine-derived radioactivity for Balb/c or Swiss 3T3 L-SAM in each of these bands is slightly different. Only G_A1 and G_A3 contain sulfated material. Under dissociative conditions, all the radioactive material is shifted to the top of the gradients. This suggests that the capacity of the material at the bottom and the middle of the gradient to bind cesium ions is significantly reduced by the increased guanidinium ion concentration, consistent with these components being GAG or proteoglycan (see below).

When leucine-radiolabeled L-SAM (or R-SAM) from Balb/c or Swiss 3T3 cells is analyzed on CsCl gradients under associative conditions (data not shown), 95% of the radioactivity is found in the G_A3 peak, with 3% (or less) of the radiolabeled material associated with material in the G_A1 or G_A2 peaks (data not shown). Under dissociative conditions, all (~99%) of the leucine-radiolabeled material is shifted to the top of the gradients. These data indicate that the majority of the protein from adhesion sites has a fairly low charge density, as would be expected by considering previous evidence (Culp, 1976; Culp et al., 1979) that the major portion of the protein in these adhesion site preparations is cytoskeletal.

Figure 3 presents the Sepharose CL2B elution profiles of the Balb/c 3T3 L-SAM G_A1 and G_A3 gradient bands chromatographed under associative or dissociative conditions. While the G_A1 band exhibits high buoyant density in the isopycnic density gradients, it can be seen that relatively little of the G_A1 radioactivity elutes in the exclusion volume during gel filtration (Figure 3A). The material within the void volume of the associative column, however, is moderately sensitive to 4 M Gdn·HCl (Figure 3B) and is completely dissociated in the presence of $NaDodSO_4$ (not shown). This indicates that a small amount of gradient band G_A1 is truly aggregated material. Similar properties are observed for band G_A3 (Figure 3C,D). The G_A2 gradient band, on the other hand, is excluded from Sepharose CL2B columns in all buffers and is completely sensitive to *Streptomyces* hyaluronidase (data not shown), consistent with this material being high molecular weight hyaluronate.

There are three possible explanations for the low yield of aggregate from the isopycnic gradients: (a) the gradient conditions may dissociate a sizable fraction of the aggregates; (b) the aggregated macromolecules are lost by selective binding to the walls of centrifuge tubes (we have been unable to extract this material under sufficiently gentle conditions to analyze aggregation; all attempts to coat the walls of the tube to prevent binding have also been unsuccessful); or (c) formation of aggregates is dependent on the presence of materials found in more than one of the gradient bands. Addition of carrier quantities of bovine nasal septum hyaluronate/chondroitin proteoglycan aggregates to these gradients failed to prevent binding of radiolabeled adhesion site material to the walls of the tube.

Enzyme Digestions of Gradient Bands G_A1, G_A2, and G_A3. Rollins & Culp (1979b) have previously shown that bands G_A1 and G_A2 contain small amounts of glycoprotein, although the great majority of glycoprotein is recovered in the G_A3 band (see below). Because of the paucity of the material in the isopycnic gradient fractions, it was not possible to determine their GAG content by conventional methods (Cohn et al., 1976; Rollins & Culp, 1979a). We therefore tested their sensitivity to specific GAG-degrading enzymes in order to obtain information about which GAG's were present. Figure 4 illustrates the Sepharose CL6B chromatographic behavior in $NaDodSO_4$-containing buffer of control glucosamine-radiolabeled G_A1 and after prior treatment with bovine testicular hyaluronidase (Figure 4A) or *Streptomyces* hyaluronidase (Figure 4B). It should be noted that the [^{3}H]glucosamine-radiolabeled control profiles in Figure 4A,B are different; the profile in Figure 4B displays high molecular weight material sensitive to *Streptomyces* hyaluronidase digestion. These were different experiments, and it is unknown at this time whether the high molecular weight material (probably HA) is a significant portion of the G_A1 band or is a result of contamination of other HA-containing fractions. Figure 4C demonstrates the change in chromatographic behavior after treatment of $^{35}SO_4^{2-}$-radiolabeled G_A1 with the two enzymes. It should be noted that the testicular hyaluronidase digestion products (Figure 4A,C) do not elute at the inclusion volume, suggesting the possibility of the chondroitin sulfate chains being linked to higher molecular weight macromolecules. These data establish that a sizable portion of the polysaccharide is chondroitin sulfate and that there may also be some HA present. The resistance of the majority of the $^{35}SO_4^{2-}$-radiolabeled material to either enzyme indicates the presence of a large amount of heparan sulfate in G_A1, as also indicated by the high charge density and high $^{35}SO_4^{2-}$/glucosamine labeling ratio in the G_A1 gradient band (Figure 2). The presence of HS in G_A1 is also indicated by its ability to bind to affinity columns of plasma fibronectin linked to Sepharose 4B (J. A. Garner, J. Laterra, and L. A. Culp, unpublished experiments), since heparan sulfate is the only polysaccharide from adhesion sites that binds to such a column (Laterra et al., 1980).

Glucosamine-radiolabeled Swiss (Figure 5) or Balb/c (not shown) 3T3 G_A3 has a complex profile when chromatographed on a Sepharose CL6B column in $NaDodSO_4$-containing buffer. Some of the material with the highest molecular weight

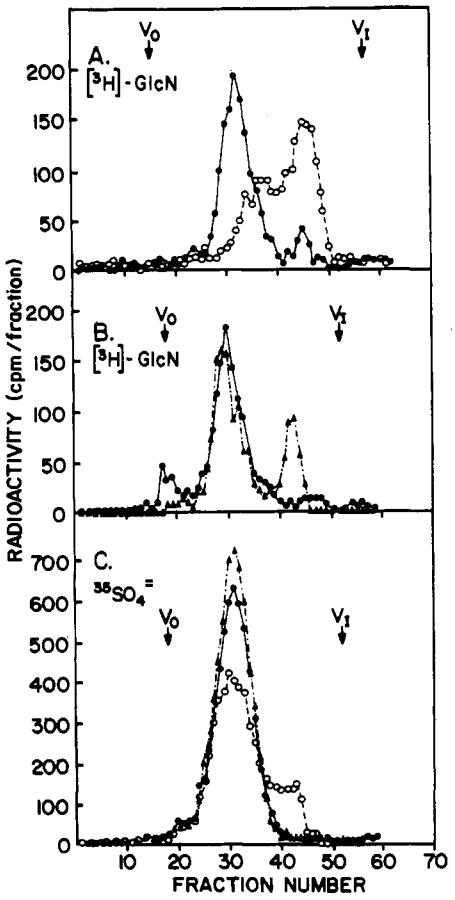

FIGURE 4: Sensitivity of density gradient fraction G_A1 to digestion with hyaluronidases. G_A1 fractions from [^{3}H]glucosamine- (A and B) or $^{35}SO_4^{2-}$-radiolabeled (C) Balb/c or Swiss 3T3 L-SAM associative density gradients (see Figure 2) were pooled and digested with *Streptomyces* (▲) or testicular (○) hyaluronidase as described under Materials and Methods for comparison of Sepharose CL6B elution characteristics in NaDodSO$_4$-containing buffer B to control (●) samples: (A) [^{3}H]glucosamine-radiolabeled Swiss 3T3 L-SAM G_A1 digested with testicular hyaluronidase; (B) [^{3}H]glucosamine-radiolabeled Swiss 3T3 L-SAM G_A1 digested with *Streptomyces* hyaluronidase; (C) $^{35}SO_4^{2-}$-radiolabeled Balb/c 3T3 L-SAM G_A1 digested with testicular or *Streptomyces* hyaluronidase.

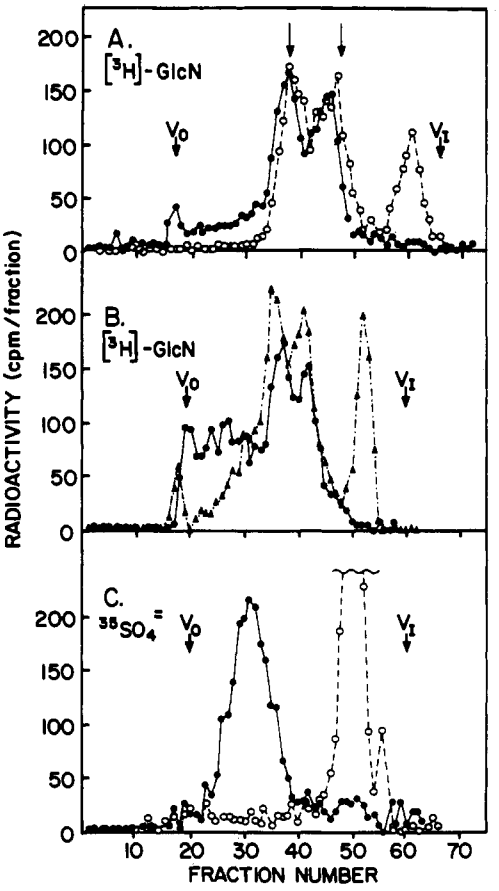

FIGURE 5: Sensitivity of density gradient fraction G_A3 to digestion with hyaluronidases. G_A3 fractions were pooled from [^{3}H]glucosamine- or $^{35}SO_4^{2-}$-radiolabeled Swiss 3T3 L-SAM associative density gradients (see Figure 2) and were enzyme digested as described under Materials and Methods and as follows: (A) [^{3}H]glucosamine-radiolabeled G_A3 treated with control buffer (●) or testicular hyaluronidase (○); (B) [^{3}H]glucosamine-radiolabeled G_A3 digested with control buffer (●) or *Streptomyces* hyaluronidase (▲); (C) $^{35}SO_4^{2-}$-radiolabeled G_A3 treated with control buffer (●) or testicular hyaluronidase (○). All samples were then made 0.2% in NaDodSO$_4$ and chromatographed on identical Sepharose CL6B columns equilibrated with 0.2% NaDodSO$_4$ in buffer B. The two arrows at fractions 35 and 40 in (A) indicate the elution of two characteristic glycoprotein peaks (see text).

(fractions 16–26) disappears from the elution profile after digestion with either hyaluronidase (Figure 5A,B), indicating that it is HA. The two peaks at lower molecular weights in the control profile of Figure 5 (arrows) appear to retain their elution characteristics after enzyme treatment, suggesting their glycoprotein nature (see below). The $^{35}SO_4$-radiolabeled G_A3 elution profile is much less complex, consisting of a single broad peak between fractions 24 and 35 (Figure 5C). This peak is resistant to *Streptomyces* hyaluronidase (not shown) and entirely sensitive to testicular hyaluronidase, verifying that the $^{35}SO_4$-radiolabeled material in G_A3 is primarily chondroitin sulfate.

Proteoglycan Nature of Gradient-Fractionated Material. In order to determine if any of the associative gradient bands have covalently bound polypeptide, they were subjected to Pronase digestion. If one compares the $^{35}SO_4^{2-}$-radiolabeled G_A1 elution profile after digestion with either hyaluronidase (Figure 4C) with that of the G_A1 elution profile after additional Pronase digestion (Figure 6A), it can be seen that no further digestion products are generated (beyond those normally seen by using only hyaluronidase). The G_A2 band (Figure 6B) appears to be entirely resistant to treatment with Pronase. These data indicate that if the G_A1 and G_A2 GAG's are associated with protein, the protein is not available to Pronase for digestion. The glucosamine-radiolabeled G_A3 material (Figure 6C), however, is entirely sensitive to Pronase digestion, after predigestion with testicular hyaluronidase. This indicates that the glucosamine-radiolabeled GAG-containing material in G_A3 is proteoglycan in nature. In addition, the two characteristic glycoprotein peaks have been digested by the Pronase enzyme, verifying their protein nature. Identical results were obtained from Balb/c or Swiss 3T3 density gradient fractions.

In order to determine if carbohydrate chains were protecting a protein core from degradation by Pronase in the G_A1 and G_A3 material, both SO_4^{2-}-radiolabeled and glucosamine-radiolabeled G_A1 and G_A3 were subjected to alkaline sodium borohydride reduction, a hydrolysis which will cleave serine- or threonine-linked polysaccharide chains but not asparagine-linked chains. Some of the $^{35}SO_4^{2-}$-radiolabeled G_A1 (Figure 7A) or glucosamine-radiolabeled (not shown) material elutes near the inclusion volume as a result of the alkaline borohydride reduction. This suggests the presence of a significant amount of polysaccharide linkage to protein via O-glycosidic serine or threonine bands in the G_A1 gradient band. Since predigestion of chondroitin chains in G_A1 with testicular hyaluronidase (Figure 6A) is insufficient in allowing penetration of Pronase to the protein core, much of the material

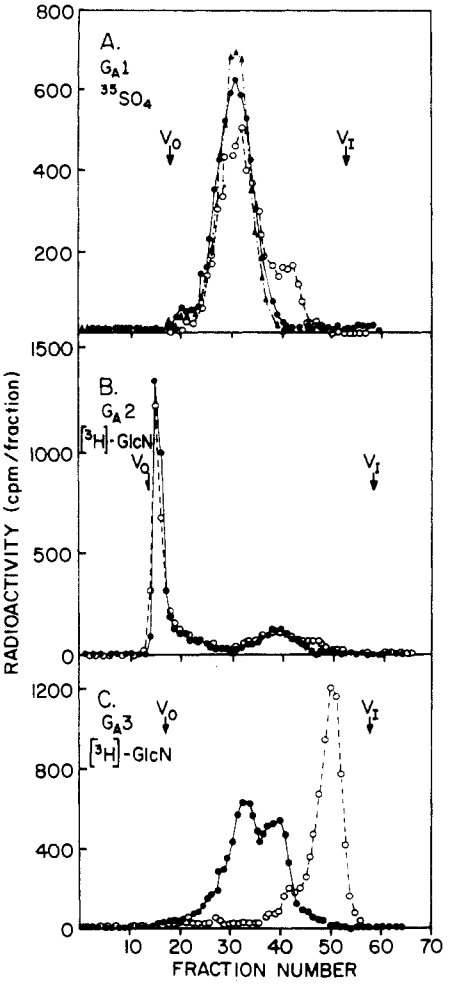

FIGURE 6: Sensitivity of density gradient fractions to digestion with Pronase. The associative density gradient G_A1, G_A2, and G_A3 bands from $^{35}SO_4^{2-}$-radiolabeled Balb/c L-SAM or [^{3}H]glucosamine-radiolabeled Swiss 3T3 L-SAM were dialyzed against buffer B overnight at 4 °C and subjected to control (●) or enzyme digestion as follows: (A) $^{35}SO_4^{2-}$-radiolabeled Balb/c 3T3 L-SAM G_A1 treated with either *Streptomyces* hyaluronidase (▲) or testicular hyaluronidase (○), then made 0.2% in $NaDodSO_4$, and digested with Pronase as described under Materials and Methods; (B) [^{3}H]glucosamine-radiolabeled Swiss 3T3 L-SAM G_A2 made 0.2% in $NaDodSO_4$ and digested with Pronase (○) without prior digestion with hyaluronidase; (C) [^{3}H]-glucosamine-radiolabeled Swiss 3T3 L-SAM G_A3 initially digested with testicular hyaluronidase (○), then made 0.2% in $NaDodSO_4$, and subjected to Pronase digestion. All samples were chromatographed on Sepharose CL6B columns eluted with $NaDodSO_4$ containing buffer B.

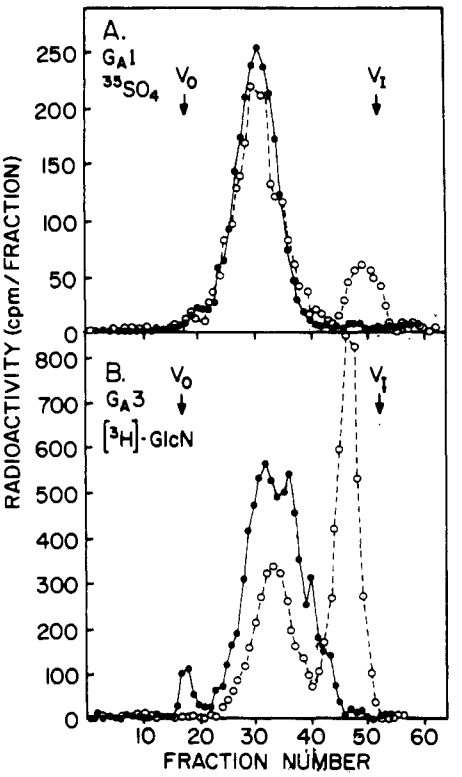

FIGURE 7: Sensitivity of density gradient fractions to alkaline borohydride reduction. $^{35}SO_4^{2-}$-radiolabeled Balb/c 3T3 L-SAM G_A1 (A) or [^{3}H]glucosamine-radiolabeled G_A3 (B) was pooled and treated with control buffer (●) or with an equal volume of alkaline borohydride solution (○). Samples were then brought to a slightly acidic pH with glacial acetic acid and made 0.2% in $NaDodSO_4$ for chromatography on Sepharose CL6B columns as described under Materials and Methods.

in G_A1 that is sensitive to alkaline borohydride is probably heparan sulfate proteoglycan. The alkali-stable glucosamine- or $^{35}SO_4^{2-}$-radiolabeled material that elutes with the control peak may represent either very large polysaccharide chains or proteoglycans in which the polysaccharide–protein linkage is N-glycosidic to an asparagine residue. In Figure 7B, it can be seen that a high proportion of the glucosamine-radiolabeled G_A3 material is shifted to the low molecular weight region of the profile after alkaline borohydride reduction. This indicates that most of the GAG species in fractions 16–24 are proteoglycan in nature, and much, but not all, of the glycoprotein in fractions 24–40 contains serine- and/or threonine-linked oligosaccharide.

Density Gradient Analysis of R-SAM. In Figure 8, the density gradient profiles of radiolabeled Swiss and Balb/c 3T3 R-SAM's are presented. The glucosamine-radiolabeled material in Balb/c 3T3 R-SAM has a banding distribution similar to that seen in Balb/c 3T3 L-SAM in Figure 2. The Swiss 3T3 R-SAM, on the other hand, contains a much larger proportion of glucosamine-derived radioactivity in G_A1 than Swiss 3T3 L-SAM. The $^{35}SO_4^{2-}$-radiolabeled R-SAM's also behave differently than the $^{35}SO_4^{2-}$-radiolabeled L-SAM's in that a much larger proportion of sulfated material appears in the G_A1 peak. In addition, under dissociative conditions, only a small portion of the $^{35}SO_4^{2-}$-radiolabeled material in the G_A1 peak is shifted to lower buoyant density even though most of the glucosamine-radiolabeled G_A1 material is shifted to the gradient top. This significantly different behavior under dissociative gradient conditions between the $^{35}SO_4^{2-}$-radiolabeled R-SAM and L-SAM proteoglycans from either cell type establishes what must be their intrinsic difference in overall macromolecular structure or their intermolecular complexing.

Composition of R-SAM Gradient Bands. The glucosamine-radiolabeled associative gradient bands G_A1, G_A2, and G_A3 of R-SAM's from either Balb/c or Swiss 3T3 cells are all qualitatively similar in elution characteristics and in enzyme digestion susceptibility to the G_A1, G_A2, and G_A3 bands of the respective L-SAM's (data not shown). There is, however, a notable quantitative difference in that a higher proportion of R-SAM GAG is heparan sulfate. Figure 9A demonstrates the insensitivity of the R-SAM G_D1 peak to *Streptomyces* hyaluronidase digestion and its slight sensitivity to the testicular enzyme. This suggests that the G_D1 gradient band is qualitatively similar to the G_A1 band of both L-SAM and R-SAM. However, when the G_D1 band is subjected to alkaline borohydride reduction (Figure 9B), a much higher proportion of $^{35}SO_4^{2-}$-radiolabeled material is shifted to the inclusion volume, indicating that more of this material is proteoglycan in nature than the material found in G_A1 of L-SAM (compare Figure

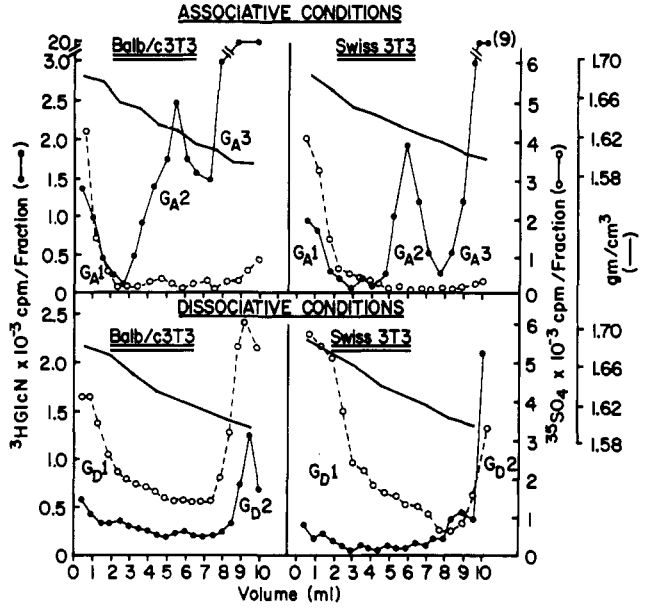

FIGURE 8: Isopycnic density gradient analyses of reattaching adhesion site extracts. [^{3}H]Glucosamine- or $^{35}SO_4^{2-}$-radiolabeled Swiss 3T3 or Balb/c 3T3 R-SAM's (see Materials and Methods) were extracted with 4 M Gdn·HCl in buffer A, concentrated, and brought to associative conditions. For centrifugation under associative conditions (upper profiles), samples were subjected to no further treatment; for centrifugation under dissociative conditions (lower profiles), samples received solid Gdn·HCl to a final concentration of 4 M. All samples were brought to an initial density of 1.63 gm/cm^3 through addition of solid CsCl and analyzed as described under Materials and Methods for content of tritium radioactivity (●), $^{35}SO_4$ radioactivity (○), and density (—).

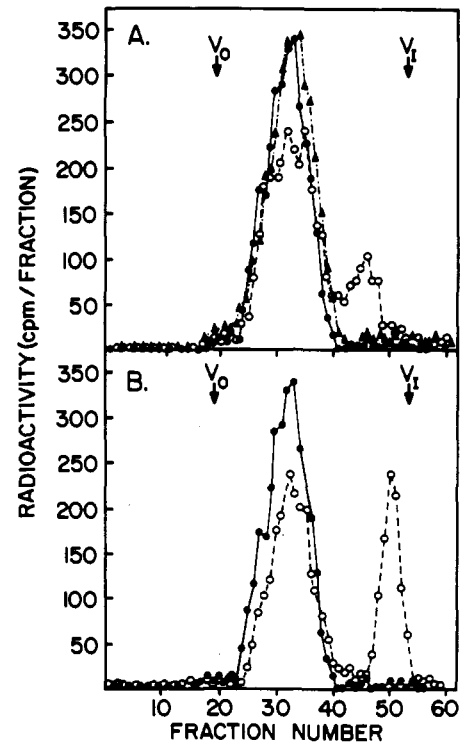

FIGURE 9: Sensitivity of R-SAM density gradient fraction G_D1 to various treatments. The $^{35}SO_4$-radiolabeled dissociative density gradient band G_D1 from Balb/c 3T3 R-SAM (see Figure 8) was pooled and divided into five equal portions for treatment as follows: (A) digested with control buffer (●), *Streptomyces* hyaluronidase (▲), or testicular hyaluronidase (○); (B) treated with control buffer (●) or alkaline borohydride (○) as described under Materials and Methods. All samples were made 0.2% in $NaDodSO_4$ and chromatographed on Sepharose CL6B columns equilibrated with 0.2% $NaDodSO_4$ in buffer B.

9B with Figure 7A). On the basis of the resistance of a large proportion of the G_D1 band material to digestion with testicular hyaluronidase, its high $^{35}SO_4^{2-}$/[^{3}H]glucosamine content, its binding affinity for plasma fibronectin–Sepharose columns (J. Laterra, J. A. Garner, and L. A. Culp, unpublished experiments), and its lability to alkaline sodium borohydride reduction, a large proportion of the G_D1 material is heparan sulfate proteoglycan which differs significantly from the heparan proteoglycan of L-SAM.

Discussion

Previous studies (Culp, 1976; Rollins & Culp, 1979a,b) have demonstrated that all of the SO_4^{2-}-radiolabeled and most of the [^{3}H]glucosamine-radiolabeled material in newly formed and long-term-generated substratum adhesion sites from Balb/c 3T3 murine fibroblasts is GAG and, with the single exception of hyaluronate, is covalently linked to protein as proteoglycan. Similar results have now been shown in this study for the substratum-attached adhesion sites of Swiss 3T3 fibroblasts. The present study utilized methodologies developed in the model bovine cartilage system (Hascall, 1977) to examine the *capability* of adhesion site HA and various proteoglycans to interact with each other and with glycoprotein. Sepharose CL2B gel filtration indicates that a major portion of the 4 M Gdn·HCl-extractable polysaccharide forms large aggregates. Some of the aggregate was sensitive to dissociation with 4 M Gdn·HCl, while much of it required a detergent to achieve complete dissociation. This latter result, along with other data to be presented separately, suggests that some aggregation is dependent upon hydrophobic protein interaction. Therefore, at least two different classes of aggregates are demonstrated by this approach, one that is sensitive to increased guanidinium ion concentration and one to detergent treatment. This system appears to be much more complex than cartilage HA/proteoglycan aggregation, while some similarities to the cartilage system have been observed. Human fibroblasts contain chondroitin proteoglycan which reversibly associates with HA in a Gdn·HCl-sensitive manner (Cöster et al., 1979). Similarly, Rollins & Culp (1979b) have shown that some Balb/c 3T3 adhesion site proteoglycan aggregation is sensitive to addition of HA_{80}, and 80-sugar oligomer of HA which acts as a monovalent hapten inhibitor of HA-dependent aggregation.

Examination of radiolabeled L-SAM and R-SAM extracts by isopycnic CsCl density gradient centrifugation has revealed that adhesion site material can be fractionated into three major density bands (G_A1, G_A2, and G_A3). However, only small fractions of G_A1 and G_A3 are demonstrated to be true aggregates by their exclusion from Sepharose CL2B columns and their dissociation upon detergent treatment. The sizable amount of only HA in G_A2 indicates that this component is not stoichiometrically limiting in formation of aggregates as assayed with the associative density gradient system. The "stickiness" of as much as 40% of the polysaccharide to the walls of the gradient tubes may reflect selective loss of aggregated material in this system. The inability to prevent this binding to the tube walls by adding carrier bovine nasal septum HA/chondroitin proteoglycan complexes or by pretreating the walls with protein or siliconizing agents limits the usefulness of associative density gradients for measuring aggregation competence of these adhesion site extracts.

Isopycnic density gradient analyses did, however, reveal a significant difference between R-SAM and L-SAM extracts. Whereas most of the L-SAM polysaccharide banded at the top of *dissociative* gradients, only a portion of the highly

sulfated proteoglycan from R-SAM shifted to lighter buoyant densities in dissociative gradients. It has previously been shown that R-SAM is considerably enriched in heparan sulfate proteoglycans (Rollins & Culp, 1979a) (see also Table I). G_D1 (the material in R-SAM which resists a shift to lighter buoyant densities) is composed principally of heparan sulfate polysaccharide with *some* "associated" chondroitin sulfate. It is not known whether the high buoyant density of G_D1 material results from proteoglycan monomers containing a very high density of polysaccharide chains arranged on a core protein or results from aggregation of heparan proteoglycans into dense structures which resist dissociation with 4 M Gdn·HCl. In this regard, Fransson et al. (1980) have demonstated a propensity for some heparan sulfates to aggregate by polysaccharide chain–chain interaction. In any case, the heparan proteoglycan deposited in newly formed adhesion sites differs significantly from the heparan proteoglycans isolated from "mature" sites.

The fractionation of the radiolabeled adhesion site material on gradients made it possible to identify and further characterize some of the macromolecular constituents of the adhesion site. Digestion of gradient bands of L-SAM with selective enzymes revealed that G_A1 contains heparan sulfate, chondroitin sulfate, small amounts of protein, and possibly some HA. The *Streptomyces* hyaluronidase sensitivity of all of the polysaccharide in G_A2 indicates that it is only HA. G_A3, on the other hand, is composed principally of glycoprotein and chondroitin sulfate, with smaller amounts of HA. The sensitivity of some of the GAG polysaccharide in G_A1 and G_A3 to Pronase digestion and particularly alkaline borohydride reduction confirmed earlier indications of their proteoglycan nature (Rollins & Culp, 1979b). Most of the chondroitin sulfate and HS isolated from a variety of systems exists as proteoglycan and not as free polysaccharide chains (Lindahl & Höök, 1978). Heparan sulfate proteoglycans have previously been demonstrated on the surface of cells (Kraemer, 1971a,b, 1977; Horner, 1971; Kjellen et al., 1977; Oldberg et al., 1979; Mutoh et al., 1980) or associated with the basement membranes of various tissues (Kanwar & Farquhar, 1979; Hassell et al., 1980; Letourneau et al., 1980). The size of the HS proteoglycans described in the present study is comparable to that seen for HS proteoglycans in mast cells (Yurt et al., 1977) or rat hepatoma cells (Hassell et al., 1980). The findings that (a) heparin or heparan sulfate bind to plasma fibronectin (Stathakis & Mosesson, 1977; Ruoslahti & Engvall, 1980; Laterra et al., 1980) and cell-surface fibronectin (Yamada et al., 1980), (b) adhesion sites of fibroblasts are enriched in fibronectin-binding HS sequences (Laterra et al., 1980), and (c) HS is the principal GAG deposited in newly formed adhesion sites (Rollins & Culp, 1979a) indicate that heparan sulfate proteoglycans may be important mediators of the adhesive bond between cell-surface fibronectin and plasma fibronectin adsorbed to the tissue culture substratum [Culp, 1978; Grinnell, 1978; this and other evidence are reviewed in greater detail in Rollins et al. (1981)]. The aggregation competence of heparan sulfate proteoglycans on the cell surface may be a critical factor in the adhesion mechanism. It may allow patching on the cell surface of these large highly charged macromolecules resulting in localized concentration of multivalent receptors able to bind to substratum-bound plasma fibronectin (Rollins et al., 1981). The studies reported here begin to characterize the aggregation competence of these proteoglycans with methodologies found productive in analyses of cartilage proteoglycan aggregation (Hascall, 1977), but clearly other approaches will be required because of the complexity of the adhesion site system.

Acknowledgments

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Direct Determination by Raman Scattering of the Conformation of the Choline Group in Phospholipid Bilayers†

Hideo Akutsu*

ABSTRACT: For clarification of the assignments of the vibrational modes of the choline group, Raman spectra of choline iodides selectively deuterated at three different positions were investigated. The isotope shifts of the C–N stretching vibrations suggested that they are conformation sensitive. When the Raman spectra of choline chloride, carbamoylcholine iodide, carbamoylcholine chloride, and methoxycarbonylcholine iodide are compared with the crystal structures of these compounds, a correlation between the vibrational frequency and the conformation of the O–C–C–N^+ backbone could be established. The Raman bands attributed to the "totally" symmetric stretching (ν_1) and symmetric stretching vibrations (ν_2) of the C–N bonds of the quaternary ammonium group appeared at about 720 cm^{-1} and about 870 cm^{-1}, respectively, for the gauche conformation of the O–C–C–N^+ backbone, and in the trans conformation, they shifted to about 770 cm^{-1} (ν_1) and about 910 cm^{-1} (ν_2), respectively. On the basis of this correlation and from measurements of phosphatidylcholine and sphingomyelin bilayers, it was concluded that most of the choline groups in both bilayers take the gauche conformation not only in the solid state but also in the gel and liquid-crystalline states. These data represent the first direct evidence that a gauche conformation for the O–C–C–N^+ bond is preferred in the gel and liquid-crystalline states. These key bands, especially the ν_1 band, are a powerful tool to study the conformation of the choline group in situ not only in the membrane field but also in the neuroscience in connection with acetylcholine.

The choline group is an important chemical substituent in biological systems. It is found in lipids as polar head groups of phosphatidylcholine and sphingomyelin as well as in the neurotransmitter as a part of acetylcholine. The crystal structures of both acetylcholine (Jagner & Jensen, 1977, and related references in it) and the phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (Pearson & Pascher, 1979) are now known. It is relevant to physical studies on lipid membranes to know if these structures are retained in other states. For example, lipid bilayers of biomembranes are in the liquid-crystalline state under many biological conditions. It is difficult, however, to determine the molecular structures of the lipids in the liquid-crystalline state.

Several techniques such as neutron scattering, X-ray scattering, and deuterium and phosphorus NMR[1] can yield information on the positions or order parameters of various segments, but they cannot give direct information on bond conformations. In contrast, vibrational spectroscopy, especially Raman scattering, is a powerful method for such an investigation. Raman bands attributed to skeletal vibrations are relatively strong and correlate closely with the conformation. Furthermore, Raman scattering can be observed in the solid, gel, and liquid-crystalline states. A good example is the hydrocarbon chain region of lipids where Raman bands at 1066, 1130, and 1089 cm^{-1} are observed. The former two bands were assigned to the C–C stretching vibration in the all-trans conformation, and the last one was assigned to the C–C stretching vibration in the presence of the gauche conformations (Lippert & Peticolas, 1971). These assignments have been useful for the investigation of the phase transitions of lipid bilayers. In spite of its potential usefulness, the contribution of Raman spectroscopy to structural studies of the polar head groups of phospholipids has been limited due to the lack of a Raman band sensitive to the conformation. In this work, it will be shown that the Raman bands assigned to the C–N stretching vibrations are sensitive to the conformation of the choline group and that because of its strong intensity, one of these bands is appropriate for the study of the structure of the choline group in situ. Although applications are presented only for phosphatidylcholine and sphingomyelin bilayers, this method has general significance for any investigation into a molecule containing a choline group.

Materials and Methods

Selectively deuterated choline iodides and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine were kindly provided by Dr. J. Seelig. They were synthesized according to reported

† From the Department of Biophysical Chemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland. *Received June 9, 1981.* Part of this work was performed at the Institute for Protein Research, Osaka University, Suita, Osaka, Japan. This work was partly supported by Swiss National Foundation Grant 3.346.78. The author was a long-term fellow of the European Molecular Biology Organization, 1979.
* Address correspondence to the Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan.

[1] Abbreviations used: ChCl, choline chloride; CarChI, carbamoylcholine iodide; CarChCl, carbamoylcholine chloride; MetChI, methoxycarbonylcholine iodide; ^1H NMR, proton magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; NMR, nuclear magnetic resonance.

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