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Chondroitin sulphate proteoglycan in the substratum adhesion sites of Balb/c 3T3 cells

Fractionation on various ion-exchange and affinity columns

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Proteoglycans on the cell surface play critical roles in the adhesion of fibroblasts to a fibronectin-containing extracellular matrix, including the model mouse cell line Balb/c 3T3. In order to evaluate the biochemistry of these processes, long-term [^{35}S]sulphate-labelled proteoglycans were extracted quantitatively from the adhesion sites of 3T3 cells, after their EGTA-mediated detachment from the substratum, by using an extractant containing 1% octyl glucoside, 1 M-NaCl and 0.5 M-guanidinium chloride (GdnHCl) in buffer with many proteinase inhibitors. Greater than 90% of the material was identified as a large chondroitin sulphate proteoglycan ($K_{av.}$ = 0.4 on a Sepharose CL2B column), and the remainder was identified as a smaller heparan sulphate proteoglycan; only small amounts of free chains of glycosaminoglycan were observed in these sites. These extracts were fractionated on DEAE-Sepharose columns under two different sets of elution conditions: with acetate buffer (termed DEAE-I) or with acetate buffer supplemented with 8 M-urea (termed DEAE-II). Under DEAE-I conditions about one-half of the material was eluted as a single peak and the remainder required 4 M-GdnHCl in order to recover it from the column; in contrast, greater than 90% of the material was eluted as a single peak from DEAE-II columns. Comparison of the elution of [^{35}S]sulphate-labelled proteoglycan with that of ^{3}H-labelled proteins from these two columns, as well as mixing experiments, indicated that the GdnHCl-sensitive proteoglycans were trapped at the top of columns, partially as a consequence of their association with proteins in these adhesion-site extracts. Affinity chromatography of these proteoglycans on columns of either immobilized platelet factor 4 or immobilized plasma fibronectin revealed that most of the chondroitin sulphate proteoglycan and the heparan sulphate proteoglycan bound to platelet factor 4 but that only the heparan sulphate proteoglycan bound to fibronectin, providing a ready means of separating the two proteoglycan classes. Affinity chromatography on octyl-Sepharose columns to test for hydrophobic domains in their core proteins demonstrated that a high proportion of the heparan sulphate proteoglycan but none of the chondroitin sulphate proteoglycan bound to the hydrophobic matrix. These results are discussed in light of the possible functional importance of the chondroitin sulphate proteoglycan in the detachment of cells from extracellular matrix and in light of previous affinity fractionations of proteoglycans from the substratum-adhesion sites of simian-virus-40-transformed 3T3 cells.

INTRODUCTION

When Balb/c 3T3 cells or their oncogenic-virus-transformed derivatives growing on a pFN-coated substratum are detached by treatment with the Ca^{2+} chelator EGTA, they retract away from the substratum and break their elastic retraction fibres, thereby liberating the cell into suspension and leaving their focal adhesion sites as substratum-attached material, i.e. cell-surface material that is very tightly bound to the substratum and that is highly enriched in membrane-associated cytoskeletal components and glycosaminoglycan-containing proteoglycans (Rollins *et al.*, 1982; Lark *et al.*, 1985; Lark & Culp, 1986). In the case of Balb/c 3T3 cells, this substratum-attached material has been shown by interference reflection microscopy to contain a subset of the so-called close contacts with the substratum (spacing distance of 25–30 nm) as well as tight-focal contacts (spacing distance of 10–15 nm) where microfilament stress fibres condense (Lattera *et al.*, 1983*b*; Lark *et al.*, 1985). Evidence that is too extensive to review here indicates that HS proteoglycan binds to substratum-bound pFN to mediate the close contacts of these cells and that substratum-bound pFN must bind to both HS proteoglycan and another receptor (Aplin *et al.*, 1981; Pierschbacher *et al.*, 1981; Yamada, 1983; Pytela *et al.*, 1985; Giancotti *et al.*, 1985) in order to generate tight-focal contacts (Laterra *et al.*, 1983*b*; Beyth & Culp, 1984; Lark *et al.*, 1985); interestingly, the intact pFN molecule appears to be much more effective at this latter response than are mixtures of the individual binding activities of the molecule (Beyth & Culp, 1984; Lark *et al.*, 1985; Lark & Culp, 1986). In a similar vein, evidence has been obtained that hyaluronate and/or CS proteoglycan (or a complex of the two) plays a central role in the detachment of cells from the pFN matrix. Consistent with this is the

Abbreviations used: SV40, simian virus 40; L-SAM, long-term-culture-generated substratum-attached material; pFN, plasma fibronectin; PF4, platelet factor 4; HS, heparan sulphate; CS, chondroitin sulphate; GdnHCl, guanidinium chloride.

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demonstration in three laboratories (Knox & Wells, 1979; Rich *et al.*, 1981; Brennan *et al.*, 1983) that purified CS proteoglycan can inhibit pFN-dependent adhesion processes.

In light of this evidence, our laboratory has undertaken the biochemical analyses of proteoglycans in the substratum adhesion sites of these cell types. Initial attention was focused on SV40-transformed Balb/c 3T3 cells (SVT2), which make only close contacts with the substratum. Their long-term-generated adhesion sites were shown to contain considerable HS proteoglycan and only small amounts of CS proteoglycan (Lark & Culp, 1982, 1983). In addition, the sites of these transformed cells from long-term cultures contain a sizable amount of free-chain HS, which can be selectively solubilized with Zwittergent 3-12, whereas the proteoglycan is completely resistant to such an extraction (Lark & Culp, 1982, 1983). Pulse–chase analyses of [^{35}S]sulphate-labelled cells demonstrated that there are two independent pathways of turnover of HS proteoglycan from SVT2 adhesion sites (Lark & Culp, 1984*a*): one involves the liberation of free chains of HS, and the other liberates proteoglycan directly back into the cell for catabolism, as shown now in two other systems (Bienkowski & Conrad, 1984; Yanagishita & Hascall, 1984*b*), or into the medium of these cultures. Affinity fractionation of HS proteoglycans from SVT2 adhesion sites (newly formed versus long-term-generated) on columns of octyl-Sepharose or the glycosaminoglycan-binding columns of PF4–Sepharose and pFN–Sepharose revealed that newly formed adhesion sites contained a high proportion of proteoglycan that was competent for binding to all three affinity matrices; in contrast, long-term-generated sites, in which catabolism of the proteoglycan had proceeded, contained overlapping subsets of proteoglycan that were or were not competent for binding to these matrices (Lark & Culp, 1984*b*).

In light of these results, we have undertaken the characterization of proteoglycans from the substratum adhesion sites of untransformed Balb/c 3T3 cells, using two different ion-exchange chromatography procedures and multiple affinity fractionations. The greater part of the proteoglycan in the long-term-culture-generated sites of these cells was found to be CS proteoglycan, which has specificities of binding to affinity matrices that are quite different from those of the HS proteoglycan either in 3T3 or in SVT2 adhesion sites. The significance of these results in the matrix adhesion of these cells is discussed.

MATERIALS AND METHODS

Materials

$Na_2{}^{35}SO_4$ and [4,5-^{3}H]leucine were purchased from Amersham Corp.; NEF-963 aqueous scintillation cocktail and [^{3}H]heparin (sp. radioactivity 0.3 mCi/mg) were from New England Nuclear Corp.; highly purified GdnHCl was from Bethesda Research Laboratories; SDS was from Bio-Rad Laboratories; n-octyl β-D-glucopyranoside was from Calbiochem–Behring and Sigma Chemical Co.; Sepharose CL6B, Sepharose CL4B, Sepharose CL2B, Sepharose 4B, DEAE-Sepharose and octyl-Sepharose CL4B were from Pharmacia Fine Chemicals; Dulbecco's modified Eagle's medium was from Grand Island Biological Co.; plastic tissue-culture dishes were from Becton Dickinson Labware; polypropylene mini-columns were from Isolab (Akron, OH, U.S.A.); CX-10 ultrafiltration units were from Millipore Corp.; dialysis tubing was from Spectrum Medical Instruments; *N*-ethymaleimide, EDTA, pepstatin A, phenylmethanesulphonyl fluoride, EGTA and bovine serum albumin from Sigma Chemical Co.; human platelets from the Cleveland Red Cross; outdated human plasma was from University Hospitals blood bank; CNBr was from Fisher Scientific; ethanolamine was from J. T. Baker Chemical Co.

Cell growth

Mycoplasma-free Balb/c 3T3 (clone A31) cells were used between their 15th and 35th passages. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) neonatal-calf serum, 250 units of penicillin/ml and 0.25 mg of streptomycin sulphate/ml, except where indicated (Lark & Culp, 1983, 1984*a*, *b*). The cells were maintained at 37 °C with CO_2/air (1:9).

Radiolabelling and harvesting of L-SAM

Cells (1.0×10^6) were inoculated into 100 mm-diameter tissue-culture dishes containing 10 ml of complete medium. After a 24 h period for attachment and spreading of cells, media were suctioned off and the cells were radiolabelled with either 10 μCi of [^{3}H]leucine/ml in complete medium or 50 μCi of $Na_2{}^{35}SO_4$/ml in Dulbecco's modified Eagle's medium containing serum, but with no penicillin and streptomycin sulphate. The final concentration of inorganic sulphate in this medium was not limiting in the sulphation of glycosaminoglycans (Lark & Culp, 1983, 1984*a*,*b*). The cells were then allowed to grow for 48 h at 37 °C to approx. 70–80% confluence.

The cells were harvested by first suctioning off the radioactive medium, rinsing them twice with phosphate-buffered saline (0.14 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4), and then treating them with 0.5 mM-EGTA in phosphate-buffered saline (5 ml per dish) for 30 min at 37 °C in a gyratory shaker–incubator. The cell suspension was gently pipetted in several washes over the tissue-culture dish to ensure quantitative detachment of the cells from the L-SAM. When the cells were detached completely, the plates bearing L-SAM were rinsed with two washes of phosphate-buffered saline and once with glass-distilled water. The L-SAM was then solubilized (see the Results section) with, per dish, 5 ml of 1% octyl glucoside, 1 M-NaCl, 0.5 M-GdnHCl and proteinase inhibitors (0.1 mM-*N*-ethylmaleimide, 10 mM-EDTA, 0.01 mM-pepstatin A, 0.1 M-6-aminohexanoic acid, 0.05 M-benzamidine hydrochloride and 0.5 mM-phenylmethane sulphonyl fluoride) in 50 mM-sodium acetate buffer, pH 5.8 (Lark & Culp, 1984*b*). The dishes were placed in the shaker incubator at 37 °C for 4 h.

In order to determine the effectiveness with which the octyl glucoside solution extracts L-SAM, the L-SAM-coated plates were agitated in phosphate-buffered saline overnight at 4 °C, and then rinsed with phosphate-buffered saline and glass-distilled water. The next day, 0.2% (w/v) SDS was applied and the plates were agitated in a shaker–incubator for 30 min at 37 °C. The octyl glucoside and phosphate-buffered-saline-containing extracts were concentrated down to 1.0–3.0 ml volumes by vacuum dialysis. The SDS-containing extract was concentrated down by use of a CX-10 submersible ultrafiltraiton unit. These fractions were compared with

plates treated with SDS only, which quantitatively solubilizes L-SAM (Cathcart & Culp, 1979).

Ion-exchange chromatography

A 3–5 ml batch of DEAE-Sepharose CL6B beads was allowed to swell for at least 24 h in 50 mM-sodium acetate buffer, pH 5.8, containing 0.02% NaN_3. The beads were then transferred to a polypropylene mini-column (0.5 cm diam. × 1.5 cm long) and rinsed extensively with acetate buffer to remove the azide. L-SAM samples containing octyl glucoside were diluted with 10 mM-octyl glucoside in 50 mM-acetate buffer at a ratio of 1:9 to decrease the salt concentration in the sample before application on the ion-exchange column (Lark & Culp, 1984*b*). Samples collected from previous preparative ion-exchange columns were dialysed for 48 h against 50 mM-acetate buffer and loaded straight on to the column without dilution. After the sample was loaded, the column was eluted with 10 ml of 50 mM-acetate buffer (flow rate 0.5 ml/min) to remove the non-binding material and the octyl glucoside detergent. A 100 ml salt gradient from 0 to 1.0 M-NaCl in 50 mM-acetate buffer was employed to elute some of the bound material. The gradient was followed with a 2.0 M-NaCl in 50 mM-acetate buffer wash, and, finally, with 4.0 M-GdnHCl in 50 mM-acetate buffer, which elutes a significant amount of material (see the Results section). These conditions are referred to as DEAE-I elutions. Fractions (0.5 ml) collected from these columns were assayed by scintillation counting of radioactivity.

Ion-exchange chromatography was also performed under a second set of conditions, which included 8.0 M-urea in the eluting buffers (DEAE-II). The procedure was exactly the same as that outlined above, except that the beads were swollen in 8.0 M-urea in 50 mM-acetate buffer, and all solutions included 8.0 M-urea. Samples loaded under these conditions were prepared either by adding solid urea to a diluted octyl glucoside sample or by dialysing a sample from a preparative ion-exchange column for 48 h against 8.0 M-urea in 50 mM-acetate buffer. Yields from either DEAE-I or DEAE-II columns were in excess of 85% of the material loaded.

Chemical treatments

Samples were treated with HNO_2 to test for the presence of HS (Lindahl *et al.*, 1973; Lark & Culp, 1982). $NaNO_2$ (18%, w/v) and acetic acid were added to the sample in the proportions of 1:1:8 (by vol.). The reaction was terminated after 80 min at room temperature with an equal volume of 2.0 M-ammonium sulphamate. Finally, the samples were adjusted to 0.2% SDS so that they would be compatible with gel-filtration-column buffers.

Samples were also treated with alkali and $NaBH_4$ under conditions of pH above 11 in order to test for proteoglycan content (Lark & Culp, 1983, 1984*a*, *b*). This treatment acts by cleaving protein–polysaccharide covalent bonds, while preventing 'peeling' of long polysaccharide chains (Carlson, 1968). Samples were treated with an equal volume of 2.0 M-$NaBH_4$ in 0.1 M-NaOH and then placed in a water bath at 45 °C for 19 h. At the end of the incubation period, the reaction was terminated by neutralizing the mixture with acetic acid. The samples were then adjusted to 0.2% SDS and frozen until chromatographed on gel-filtration columns.

Enzyme treatment

The NaCl-elutable material from a DEAE-Sepharose column was dialysed in enriched Tris buffer (0.25 M-NaCl/0.25 M-Tris/0.18 M-sodium acetate buffer, pH 6.8) for 24 h with two changes of buffer. Five units of freeze-dried chondroitinase AC or ABC were dissolved in 1.0 ml of enriched Tris buffer containing 2 mg of bovine serum albumin. This solution was kept on ice for 5–10 min to allow full solubilization of the enzyme. Then 0.25 unit of the enzyme was added to the sample, which was placed in a water bath at 37 °C for 2 h and shaken at intervals of 10 min to promote digestion. In some cases a second portion of enzyme was added at 1 h. The sample was adjusted to 0.2% SDS before gel-filtration chromatography.

Gel-filtration chromatography

All chemically and enzymically treated samples (0.2–0.5 ml) were chromatographed on Sepharose CL6B columns (1 cm × 100 cm) equilibrated in 0.2% SDS in 50 mM-sodium acetate buffer, pH 5.8, at a flow rate of 10–12 ml/h. Octyl glucoside L-SAM extracts were also characterized on a Sepharose CL2B column eluted with the same buffer at the same flow rate. Recoveries were greater than 92% of the material loaded on to the column. V_0 on Sepharose profiles represents the first fraction of detectable elution of high-M_r Blue Dextran from these columns (rather than the elution maximum); similarly, V_I marks the last fraction of detectable low-M_r dinitrophenyl-glycine (rather than its maximum elution).

Immobilization of PF4 and pFN on Sepharose

PF4 was isolated from human platelets as described by Lark & Culp (1984*b*). pFN was isolated from outdated blood-bank human plasma by the method of Engvall *et al.* (1978). The ligands were cross-linked to CNBr-activated Sepharose 4B beads by the method of March *et al.* (1974), with some modifications. The proteins at a concentration of 2 mg/ml in 0.1 M-$NaHCO_3$, pH 9.5, were added to an equal volume of activated Sepharose beads and mixed end-over-end at 4 °C for 4 h in the case of PF4, or overnight in the case of pFN. The solution was then adjusted to 1.0 M-monoethanolamine and mixed end-over-end for 2 h at room temperature. Efficiency of cross-linking varied in the range 35–47%, as described previously (Lark & Culp, 1984*b*; Maresh *et al.*, 1984). The beads were subsequently washed with 0.5M-NaCl in 0.1 M-$NaHCO_3$, pH 9.5, followed by 0.5 M-NaCl in 0.1 M-sodium acetate buffer, pH 4.0. These washes were each repeated five times. The pFN–Sepharose or PF4–Sepharose beads were stored at 4 °C in TMC buffer (50 mM-Tris/HCl buffer, pH 7.4, containing 1.0 mM-$MgCl_2$ and 1.0 mM-$CaCl_2$) containing 0.02% NaN_3 and tested with [^{3}H]heparin ($K_{av.} = 0.45$ on a Sepharose CL6B column; M_r 6000–20000; 0.3 mCi/mg) to determine the stoichiometry of binding; approx. 70% of the heparin binds to pFN–Sepharose columns and 80–85% to PF4–Sepharose columns. In all experiments, subsaturating conditions were used with all affinity columns.

Affinity chromatography

PF4–Sepharose 4B or pFN–Sepharose 4B columns (0.5 cm × 1.5 cm) were washed thoroughly with TMC buffer to remove the NaN_3. Various fractions of L-SAM samples (0.1–0.3 ml) were then loaded straight on to a

3 ml column and followed with 30 ml of TMC buffer wash at a flow rate of 0.3–0.5 ml/min. (Lark & Culp, 1984*b*; Maresh *et al.*, 1984). Batch adsorption of sample for 4 h gave identical results. A 40 ml salt gradient from 0 to 0.5 M-NaCl in TMC buffer was applied slowly to promote separation of binding material if more than one type were to bind, as observed in other systems (Lark & Culp, 1984*b*; Maresh *et al.*, 1984). Finally, a 2.0 M-NaCl in TMC buffer wash was applied to elute any material still remaining on the column. Recoveries were in excess of 87% of the radiolabelled material applied to the column.

Hydrophobic affinity chromatography

Approx. 3 ml of octyl-Sepharose CL4B beads was swollen in buffer I (1.0 M-NaCl/4.0 M-GdnHCl/50 mM-sodium acetate buffer, pH 5.8) at 4 °C. Detergent-free samples (final volume 9 ml) from a preparative ion-exchange column with proteinase inhibitors (1.0 mM-phenylmethanesulphonyl fluoride, 0.1 M-6-aminohexanoic acid, 0.05 M-benzamidine hydrochloride, 10 mM-EDTA) were dialysed against buffer I for 24 h at 4 °C. The high concentration of GdnHCl is critical to prevent trapping of aggregates in the column during the adsorption and initial elution steps (E. Vallen & L. A. Culp, unpublished work). Sample was then added to the beads at a ratio of 3:1 (v/v) in a plastic tube and rotated end-over-end overnight at room temperature. The column was then sequentially eluted at a flow rate of 6 ml/h with (*a*) buffer I, (*b*) 0.1 M-NaCl in 50 mM-acetate buffer, (*c*) 3.0 M-NaCl in 50 mM-acetate buffer, (*d*) 1.0% octyl glucoside and 3.0 M-NaCl in 50 mM-acetate buffer, and finally (*e*) 0.2% SDS. The fractions were assayed by scintillation counting of radioactivity.

RESULTS

Efficiency of extraction

Lark & Culp (1984*b*) have shown a very high recovery in solubilizaton of proteoglycan material from SV40-transformed Balb/c 3T3 adhesion sites with a mixture containing 1% octyl glucoside, 1 M-NaCl and 0.5 M-GdnHCl in acetate buffer containing many proteinase inhibitors. Such an extraction should only denature these molecules in a reversible manner, and the detergent, GdnHCl and salt can be readily removed by dialysis for subsequent assay of various biological activities. Therefore it was first important to evaluate the recovery of [^{35}S]sulphate-labelled proteoglycan from the L-SAM of untransformed Balb/c 3T3 cells. [^{35}S]Sulphate-labelling for 72 h leads to 13–15% of the total macromolecular radioactivity being recovered in the adhesion-site fraction. These extraction conditions were compared with SDS extraction, which quantitatively solubilizes L-SAM (see the Materials and methods section). The octyl glucoside mixture solubilized 98% of the radiolabelled proteoglycan material from Balb/c 3T3 adhesion sites with a 4 h incubation at 37 °C. Incubations at 4 °C, for shorter periods of time, with 4 M-GdnHCl alone or with detergent alone gave poorer recoveries of material (less than 60%). Therefore these conditions were chosen for routine isolation in order to maximize the recovery of sulphated macromolecules.

Proteoglycan content of octyl glucoside extract

The octyl glucoside L-SAM extracts were characterized before or after chemical treatment on a Sepharose CL6B column to determine whether the [^{35}S]sulphate-labelled material might be proteoglycan and whether any of it was of the HS class. The greater part of the [^{35}S]sulphate-labelled material was eluted at the exclusion volume (V_0) of a Sepharose CL6B column with SDS-containing buffer (Fig. 1), indicating reasonably high-M_r component(s). Reduction and blockage of disulphide linkages failed to change the gel-filtration properties of these molecules, demonstrating that they are not disulphide-cross-linked complexes (Cöster *et al.*, 1983; Lowe-Krentz & Keller, 1984). These extracts were tested by a second criterion for proteoglycan content by sensitivity to alkaline-borohydride treatment (Fig. 1). After alkaline-borohydride treatment, the radiolabelled polysaccharide shifted into the included region, again consistent with the radiolabelled material being proteoglycan. A small portion of this material (8–9%) is sensitive to HNO_2 degradation (Fig. 1), indicating that some of the presumed proteoglycan is of the HS class, whereas most is not. Chondroitinase AC

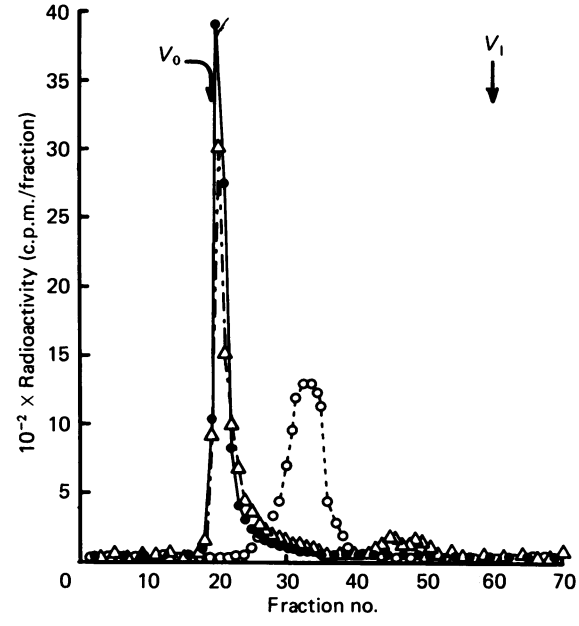

Fig. 1. Gel-filtration chromatography of Balb/c 3T3 octyl glucoside extracts with or without chemical treatment

[^{35}S]Sulphate-labelled cells were detached from the tissue-culture substratum with EGTA as described in the Materials and methods section. The substratum adhesion sites were then extracted with the octyl glucoside mixture, with the inclusion of proteinase inhibitors. After concentration, one portion of the extract was left untreated as a control (●); another was treated with alkaline borohydride to cleave glycosaminoglycan chains from the core protein (○); another was treated with HNO_2 to degrade *N*-sulphated HS chains (△). These samples were then chromatographed separately over the same Sepharose CL6B column eluted with 0.2% SDS in acetate buffer. V_0, the exclusion volume, and V_I, the inclusion volume, were determined with Blue Dextran and dinitrophenylglycine respectively for all gel-filtration columns, as described in the Materials and methods section. Fractions were assayed for radioactivity.

digestion (Fig. 2) revealed that the HNO_2-resistant material is of the CS class, with more than 95% of the treated sample shifting into the included region of the column. Chondroitinase ABC digestion gave the same results (not shown), indicating that fractions 28–38 contain core proteins with stubs of CS inaccessible to either enzyme; glycosaminoglycan chains liberated from alkaline-borohydride treatment are completely sensitive to chondroitinase AC. This is consistent with previous findings (Rollins & Culp, 1979) that there is very little dermatan sulphate in 3T3 adhesion sites. Therefore greater than 90% of the [^{35}S]sulphate-labelled material from the L-SAM of Balb/c 3T3 cells is CS proteoglycan with a smaller amount (approx. 8%) of HS proteoglycan.

The size distribution of these proteoglycans was examined on Sepharose CL2B columns eluted with an SDS-containing buffer (Fig. 3). Two components were resolved, with the larger proteoglycan (peak I) being the CS proteoglycan with $K_{av.} = 0.4$ (completely resistant to HNO_2); the smaller amount of lower-M_r proteoglycan ($K_{av.} = 0.7$) was completely HNO_2-sensitive and therefore of the HS class. The CS glycosaminoglycan chains display $K_{av.} = 0.35$ on Sepharose CL6B columns (Fig. 1).

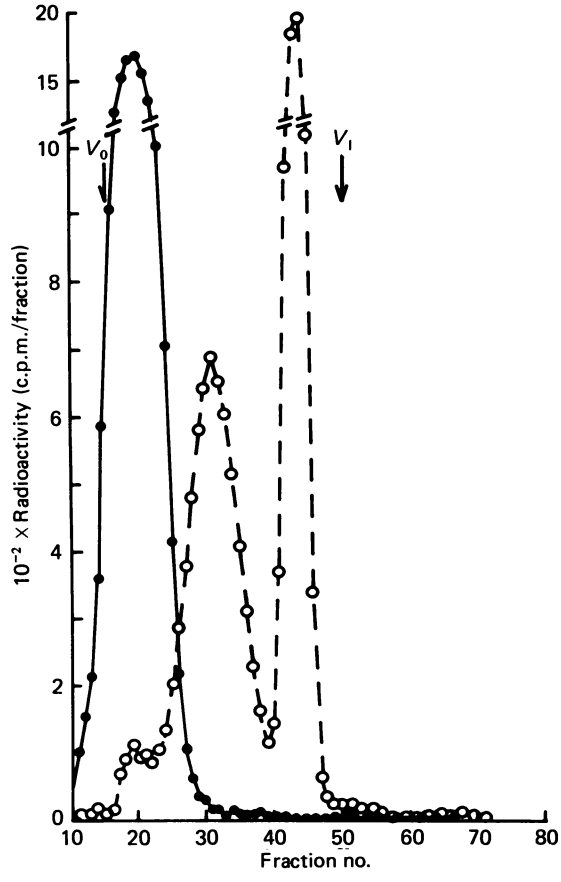

Fig. 2. Gel-filtration chromatography of chondroitinase AC-digested and non-digested octyl glucoside extract subfraction

Peak A from a DEAE-I column (see Fig. 4*a*) was dialysed against enriched Tris buffer and divided into two portions: (i) left untreated as a control (●); (ii) treated with chondroitinase AC as described in the Materials and methods section (○). The samples were then chromatographed over the same Sepharose CL6B column eluted with 0.2% SDS in acetate buffer.

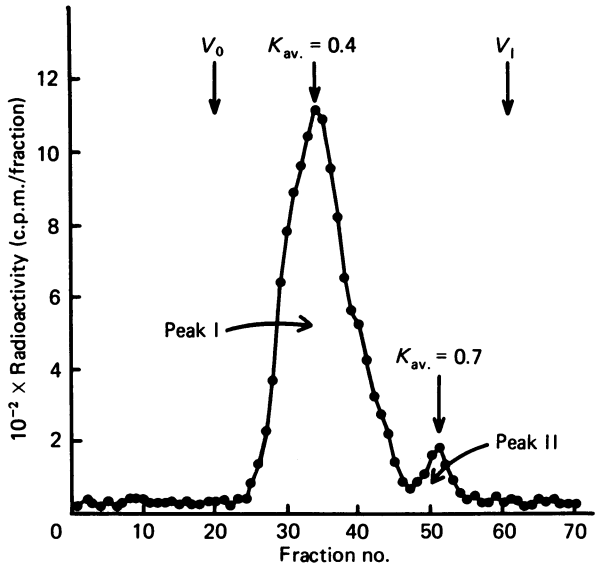

Fig. 3. Size distribution of proteoglycans in the octyl glucoside extract

The octyl glucoside extract of [^{35}S]sulphate-labelled L-SAM was dialysed overnight against 50 mM-sodium acetate buffer and then made 0.2% in SDS. It was chromatographed on a Sepharose CL2B column eluted with SDS/acetate buffer as described in the Materials and methods section, and samples of fractions were assayed for radioactivity.

Ion-exchange fractionation

Chromatography on mini-columns of DEAE-Sepharose was employed in an attempt to resolve different species of proteoglycans. As shown in Fig. 4 (*a*), L-SAM samples were loaded on ion-exchange columns in a sodium acetate buffer (DEAE-I conditions). A small fraction (less than 1%) of the radiolabelled material did not bind to the column. About half of the recovered proteoglycan was eluted as a broad peak during the salt gradient (peak A). The profile of peak A characteristically had a small shoulder eluted at approx. 0.6 M-NaCl and a major peak at 0.8 M-NaCl, followed by a very small peak at 1.0 M-salt. The remainder of the radiolabelled material (approx. 50%) was eluted as a sharp peak B only after application of 4.0 M-GdnHCl to the column. The original octyl glucoside extract, when chromatographed on non-dissociative Sepharose CL2B columns either as a concentrate or diluted with acetate buffer, gave poor yields, and most of the radioactivity was eluted from the V_0 region of the column. Peak B bound at the top of any Sepharose, Sephadex or Bio-Gel column to which it was applied. These and other pieces of evidence suggested that it is made up of large aggregates that are trapped at the top of these columns. Addition of a non-ionic or zwitterionic detergent to the eluting buffers of DEAE-I columns failed to change the elution characteristics of proteoglycan (results not shown). Peak A contained approx. 6.5% HS and peak B 9%; both peaks consisted of proteoglycan with little detectable protein-free glycosaminoglycan (results not shown).

Octyl glucoside extracts were also chromatographed on DEAE-Sepharose mini-columns under a second set of conditions (DEAE-II), which included 8.0 M-urea in all solutions, in order to promote disaggregation without interfering with the ion-exchange properties of the

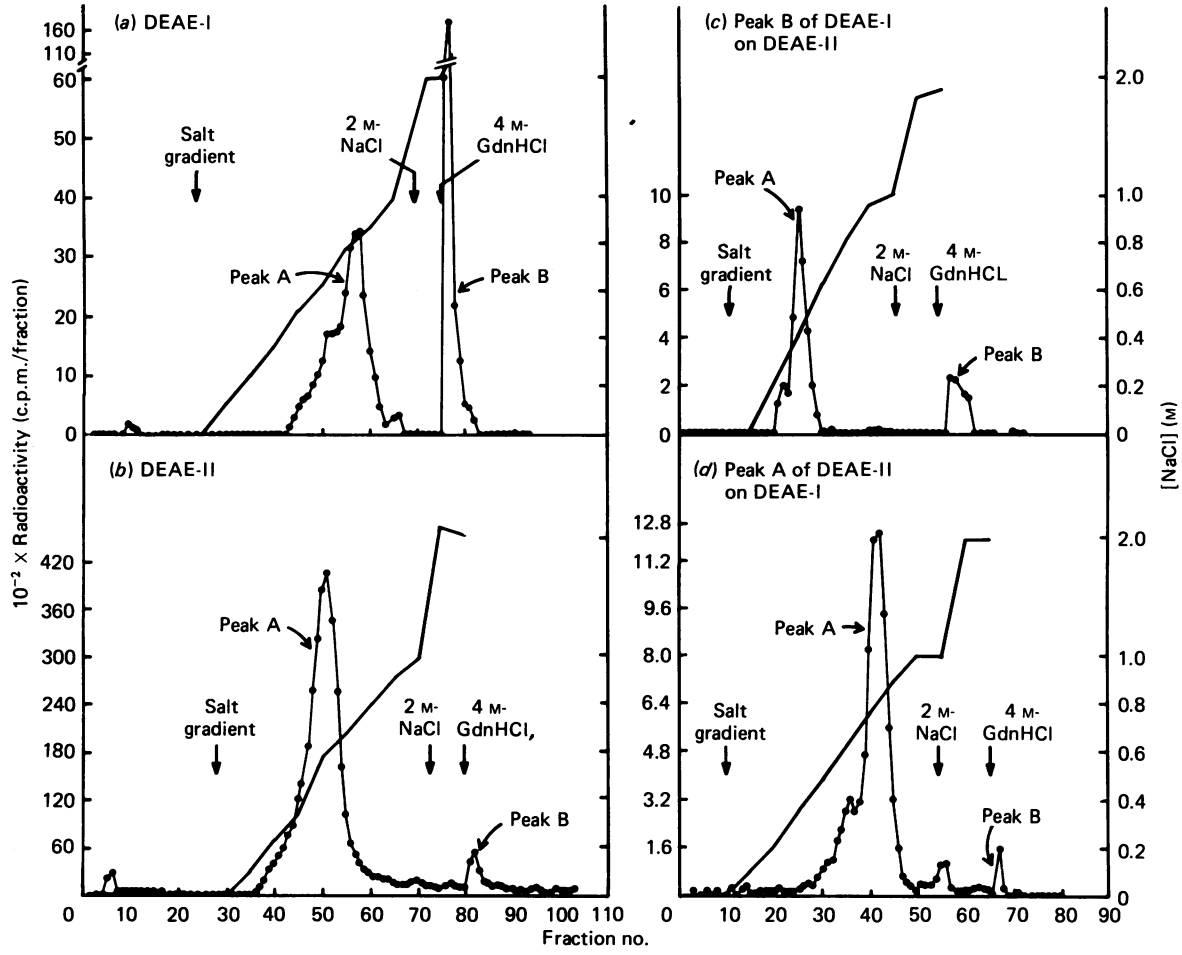

Fig. 4. Ion-exchange chromatography of [^{35}S]sulphate-labelled octyl glucoside extracts under two different elution conditions

Substratum adhesion sites metabolically radiolabelled with [^{35}S]sulphate were extracted with the octyl glucoside buffer as described in the Materials and methods section. The extract was diluted 10-fold with acetate buffer before passage on to DEAE-Sepharose mini-columns. In (*a*) the extract was chromatographed in acetate buffer (DEAE-I conditions). Elution proceeded with a 0–1.0 M-NaCl gradient, 2 M-NaCl and finally 4 M-GdnHCl, all in acetate buffer. In (*b*) the same extract was loaded on a column eluted with acetate buffer supplemented with 8.0 M-urea in each eluent (DEAE-II conditions). In (*c*) peak B material from (*a*) was dialysed against 8.0 M-urea/acetate buffer and chromatographed under DEAE-II conditions. In (*d*) peak A material from (*b*) was dialysed against acetate buffer and chromatographed under DEAE-I conditions. Samples of fractions were assayed for radioactivity (●) or refractive index (——) to determine salt concentration.

column (Yanagishita & Hascall, 1984*a*). A small fraction (3%) in Fig. 4 (*b*) does not bind to the column, a large and somewhat broad peak A is eluted in the salt gradient at 0.4–0.60 M-NaCl, and a much smaller peak B than that of DEAE-I columns requires GdnHCl for elution from the column. Peaks A and B represent 90% and 7% of the recovered radiolabelled material respectively. Again, inclusion of non-ionic or zwitterionic detergent in the eluting buffers failed to change these distributions.

Peaks A and B from DEAE-II columns were subsequently characterized by alkaline-borohydride and HNO_2 treatments (results not shown). Peak A contained only 1–1.5% HNO_2-sensitive HS and was entirely alkaline-borohydride-sensitive, indicating that it is made up principally of CS proteoglycan. Peak B was alkaline-borohydride-sensitive and 44% of the radiolabelled material was degraded by HNO_2, indicating that this fraction is approximately half HS proteoglycan and half CS proteoglycan.

The characteristics of peak B from a DEAE-I column were further investigated by loading peak B material on to a DEAE-Sepharose column under DEAE-II conditions (Fig. 4*c*). Under these dissociative conditions, 75% of the radiolabelled proteoglycan was eluted in the gradient at salt concentrations more narrowly defined (0.4–0.45 M-NaCl) than that of peak A from DEAE-II chromatography of the original extract (Fig. 4*b*).

Peak A from a DEAE-II column (Fig. 4*b*) was dialyzed against acetate buffer extensively and loaded on to a DEAE-I column in order to investigate further the reversibility of column trapping (Fig. 4*d*). Some 97% of the material was eluted as a peak with a profile similar to peak A under DEAE-I conditions (Fig. 4*a*) in the gradient at 0.6–0.8 M-NaCl, indicating that 8 M-urea treatment freed most of this proteoglycan from the aggregated or trapped site.

It was important in the purification of these proteoglycans from L-SAM to determine the fate of proteins (principally cytoskeletal; Rollins *et al.*, 1982) during ion-exchange fractionation and for future studies

to evaluate the contribution of these proteins in any aggregation processes of proteoglycans. Samples labelled with [^{3}H]leucine were applied to ion-exchange columns under DEAE-I or DEAE-II conditions. The recovery of [^{3}H]leucine-labelled material loaded on DEAE-I columns within the salt gradient (Fig. 5*a*) was extremely low (less than 10%), which is to be expected of proteoglycan with a low protein/carbohydrate ratio; loading of larger

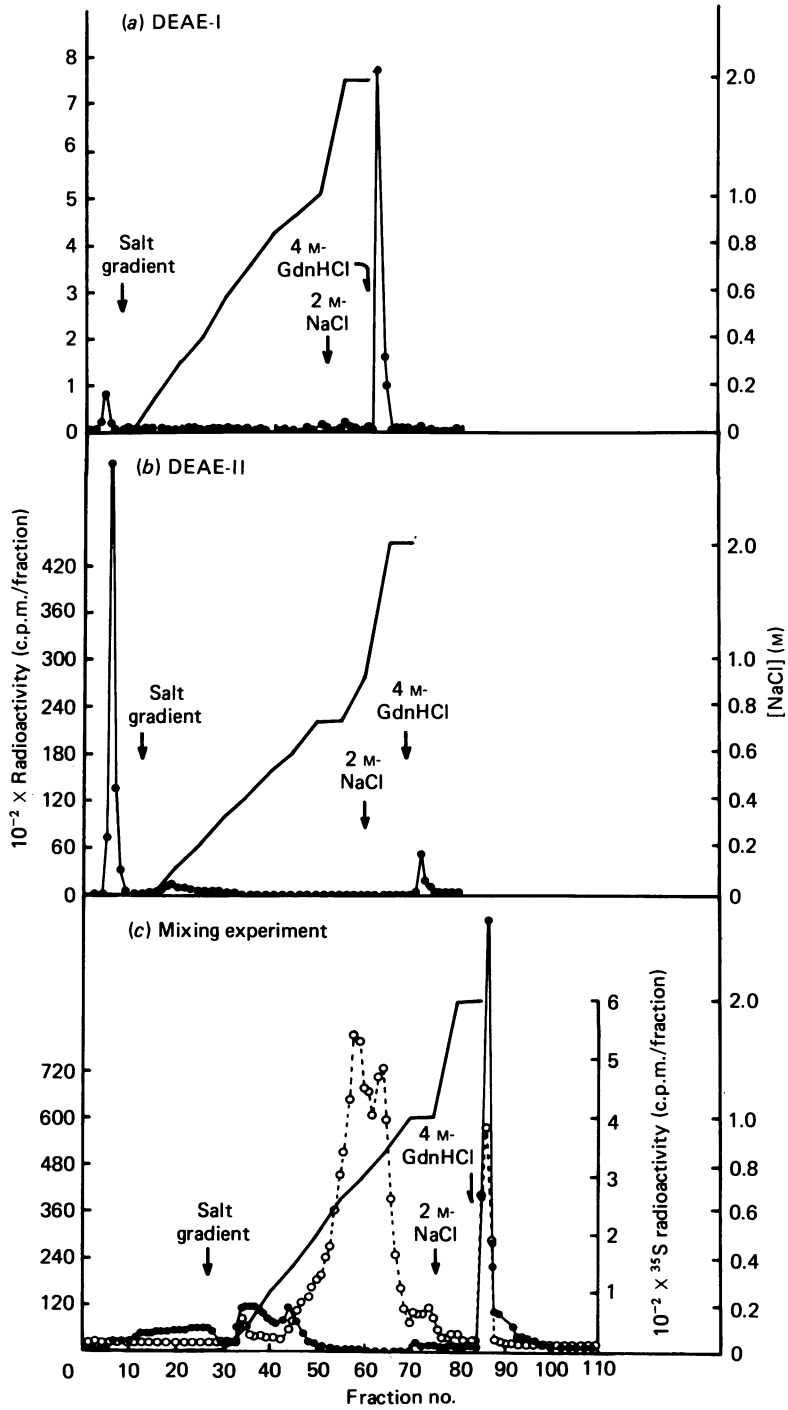

Fig. 5. Ion-exchange chromatography of [^{3}H]leucine-labelled extracts under DEAE-I or DEAE-II conditions

Substratum adhesion sites metabolically radiolabelled with [^{3}H]leucine were extracted with the octyl glucoside buffer as described in the Materials and methods section. The extract was diluted 10-fold and loaded on to a DEAE-Sepharose mini-column. In (*a*) the extract was chromatographed in acetate buffer (DEAE-I conditions) with a 0–1.0 M-NaCl gradient, 2 M-NaCl and finally 4.0 M-GdnHCl, all in acetate buffer. In (*b*) the same extract was chromatographed under DEAE-II conditions with 8.0 M-urea in eluting buffers (see Fig. 4). In (*c*) flow-through [^{3}H]leucine-labelled material from (*b*) was combined with peak A [^{35}S]sulphate-labelled material from a DEAE-II column (see Fig. 4*b*) and dialysed into acetate buffer without any urea. The mixed sample was loaded on to a DEAE-Sepharose column and chromatographed under DEAE-I conditions. The Figure shows profiles of ^{35}S radioactivity (○) and ^{3}H radioactivity (●), as well as the molarity of salt (——).

amounts of [^{3}H]leucine-labelled material established co-elution with [^{35}S]sulphate-labelled material (results not shown). Approx. 90% of the [^{3}H]leucine-labelled material required 4.0 M-GdnHCl for elution from the column. Under DEAE-II conditions, 75% (Fig. 5*b*) of the recovered material was eluted as a sharp flow-through peak before application of the salt gradient, indicating that the greater part of this protein is trapped at the top of DEAE-I columns, possibly with proteoglycan, and disaggregates in a high concentration of urea. A broad shallow peak in the early part of the salt gradient represented 15% of the material, and 10% required application of GdnHCl.

In order to explore the role of proteins in the trapping of proteoglycans on DEAE-Sepharose columns, a mixing experiment was performed (Fig. 5*c*). Flow-through [^{3}H]leucine-labelled material from a DEAE-II column (Fig. 5*b*) was mixed with peak A [^{35}S]sulphate-labelled material from a DEAE-II column (Fig. 4*b*). The volume of each fraction mixed represented the same number of dish equivalents in the original preparation in order to ensure the proper proportion of protein to proteoglycan material. The mixed sample was dialysed for 48 h into DEAE-I column buffer. The sample was then loaded on to an ion-exchange column under DEAE-I conditions. The profile in Fig. 5(*c*) represents a recovery of 45% of the loaded proteoglycan and 60% of the loaded protein. Approx. 20% of the recovered [^{3}H]leucine-labelled material was eluted before application of the salt gradient. Some 30% of the protein and 91% of the proteoglycan were eluted in the gradient, and 50% of the protein and 9% of the proteoglycan required elution with GdnHCl. Comparison with Fig. 4(*d*) indicates that re-addition of protein material results in a lowering of recovery of [^{35}S]sulphate-labelled material and a shift of the proteoglycan material from the gradient into the GdnHCl-elutable fraction, although the major portion of the proteoglycan has become refractile to aggregate formation.

Affinity chromatography

Sepharose columns with pFN or PF4 linked to them have been used to investigate the potential for cell-surface proteoglycans in adhesion sites to bind to these adhesion-promoting ligands (Lark & Culp, 1984*b*; Maresh *et al.*, 1984). pFN has little affinity for CS chains as compared with HS chains. PF4, on the other hand, allows both glycosaminoglycans to bind, with HS having a greater avidity.

Fig. 6(*a*) illustrates chromatography of peak A material from a DEAE-I (which contains both HS and CS proteoglycans) column on a PF4–Sepharose affinity column. (It was impossible to test peak A material from DEAE-II columns because of persistent aggregation and trapping at the top of the affinity columns, lowering column yields below 60%.) Of the recovered material, 80% bound to the column. HNO_2 and alkaline-borohydride treatment of the PF4-binding subset revealed that it is 94% CS proteoglycan and 6% HS proteoglycan (results not shown).

Peak A from a DEAE-I column was also loaded on a pFN–Sepharose column (Fig. 6*b*). Greater than 90% of the recovered material did not bind to the column and was completely HNO_2-resistant (results not shown), indicating that it is entirely CS proteoglycan. Approx. 8–9% of the recovered material bound to the pFN matrix and was

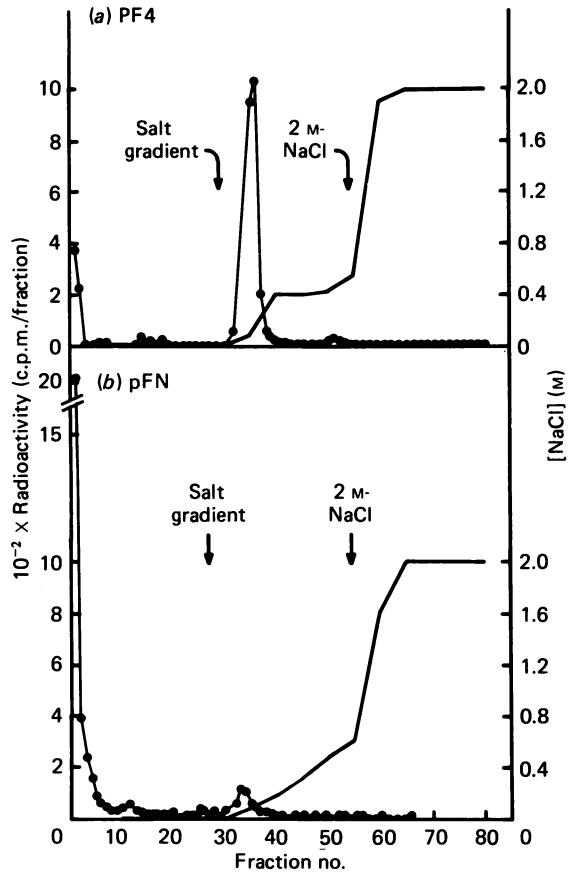

Fig. 6. Affinity chromatography on columns of PF4–Sepharose or pFN–Sepharose of peak A from a DEAE-I column

[^{35}S]Sulphate-labelled peak A material from a DEAE-I column (Fig. 4*a*) was dialysed against TMC buffer (see the Materials and methods section) and adsorbed on a PF4–Sepharose (*a*) or a pFN–Sepharose (*b*) column. An NaCl gradient was employed to elute material bound to the column, and finally the columns were washed with 2 M-NaCl in TMC buffer. Samples of fractions were assayed for radioactivity (●) and salt concentration (——).

completely HNO_2-sensitive (results not shown). These results demonstrate the effective discrimination of pFN–Sepharose columns for separating these two proteoglycan classes.

In order to determine if the L-SAM proteoglycans from 3T3 cells have hydrophobic regions that would allow them to be an integral part of the plasma membrane (Kjéllen *et al.*, 1981; Norling *et al.*, 1981; Rapraeger & Bernfield, 1983; Lark & Culp, 1984*b*; Maresh *et al.*, 1984), peaks A and B from a DEAE-II column were tested with octyl-Sepharose affinity columns (Fig. 7). These binding reactions were performed in buffer containing 4.0 M-GdnHCl to prevent aggregation and trapping of proteoglycan material on the column. Of the recovered peak A material (Fig. 7*a*), greater than 98% did not bind to the hydrophobic matrix and barely 1.0% was eluted with application of 1.0% octyl glucoside. In contrast, approx. 20% of the recovered peak B material (Fig. 7*b*) failed to bind to the column, and 50% was eluted with addition of octyl glucoside. The peak B material that did bind to the hydrophobic matrix in an octyl

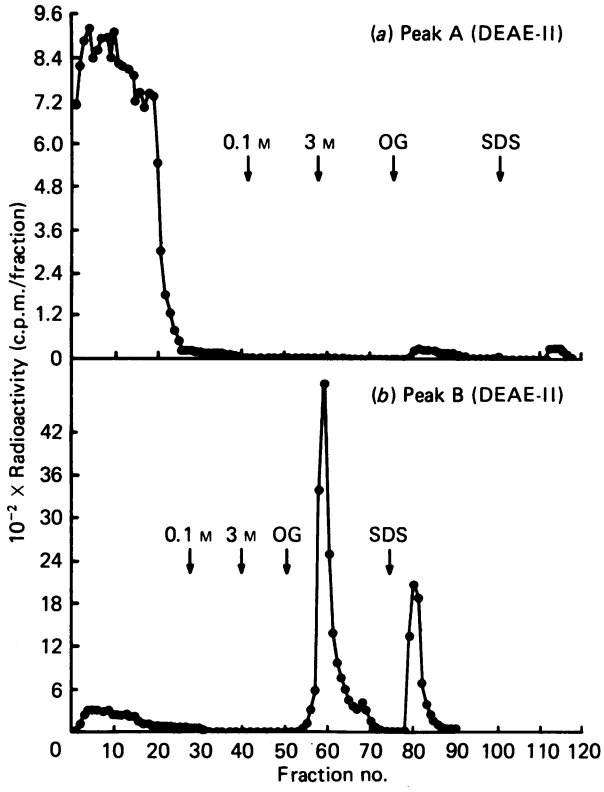

Fig. 7. Hydrophobic affinity chromatography of peaks A or B from a DEAE-II column

[^{35}S]Sulphate-labelled peaks A (*a*) or B (*b*) from DEAE-II columns (Fig. 4*b*) were dialysed against buffer I (see the Materials and methods section) and batch-adsorbed on octyl-Sepharose 4B beads overnight. The column was poured and eluted sequentially with 0.1 M-NaCl in acetate buffer (0.1 M), 3.0 M-NaCl in acetate buffer (3.0 M), 1.0% octyl glucoside and 3 M-NaCl in acetate buffer (OG) and 0.2% SDS (SDS). Samples of fractions were assayed for radioactivity.

glucoside-elutable manner (Fig. 7*b*) was entirely HNO_2-sensitive. Similarly, chromatography of peak A or B from DEAE-I columns demonstrated that virtually all of the HS proteoglycan in these fractions (6.5–8%) did bind to this hydrophobic matrix (results not shown). These data indicate that hydrophobic properties are limited to the HS class of proteoglycan in this adhesion-site extract.

DISCUSSION

Both the distribution and specific properties of the proteoglycans of Balb/c 3T3 substratum adhesion sites, as revealed in the studies here, are very different from those of proteoglycans from the SV40-transformed 3T3 derivative SVT2, as reported previously. In SVT2 L-SAM, most of the proteoglycan was of the HS class, with a smaller amount of CS (Lark & Culp, 1982, 1983), consistent with the preponderance of close contacts being formed in those transformed cells. In 3T3 L-SAM, greater than 90% of the proteoglycan was of the CS class, with the remainder being of the HS class; this correlates with a decreasing proportion of close contacts and an increasing proportion of tight-focal contacts in 3T3 cultures as they become dense (Izzard & Lochner, 1976, 1980). Close contacts are regions of adhesion at the undersurface for fibroblasts of 25–30 nm spacing distance between the plasma membrane and the substratum, and are principally, though not exclusively, associated with moving cells. In contrast, tight-focal contacts are smaller regions of adhesion with a spacing distance of 12–14 nm, where the microfilament stress fibres condense and are associated principally, although again not exclusively, with cells relatively immobile on the substratum. Evidence obtained in this laboratory has demonstrated the requirement for binding of pFN on the substratum to a non-HS-like molecule on the surface in order to generate tight-focal contacts with 3T3 cells (Laterra *et al.*, 1983*a*, *b*; Lark *et al.*, 1985), and two candidate glycoproteins have been reported to serve as possible 'receptors' in this capacity (Aplin *et al.*, 1981; Pytela *et al.*, 1985; Giancotti *et al.*, 1985). However, binding of pFN in the matrix to cell-surface HS proteoglycan appears to be a central mechanism for formation of close contacts by either untransformed or transformed cells (Laterra *et al.*, 1983*a*, *b*; Beyth & Culp, 1984; Lark *et al.*, 1985; Lark & Culp, 1986).

In addition to differences in the types of proteoglycans enriched in the adhesion sites of untransformed or transformed 3T3 cells, there are differences in the metabolism of these molecules. SVT2 cells accumulate a sizable proportion of HS chains in their L-SAM as a result of both proteolytic and endoglycosidic catabolism of HS proteoglycan (Lark & Culp, 1983). Pulse–chase analyses with [^{35}S]sulphate have revealed two differing mechanisms of turnover of HS proteoglycan molecules from SVT2 adhesion sites, only one of which involves the evolution of free glycosaminoglycan chains (Lark & Culp, 1984*a*). With the studies reported here, there were little detectable free chains of either HS or CS, indicating much less metabolism of proteoglycan in 3T3 adhesion sites, although this must be confirmed with pulse–chase-type analyses. Differences in metabolism are further supported by affinity-chromatography studies. Newly-formed adhesion sites of SVT2 cells contain HS proteoglycan with very high efficiencies for binding to three different affinity columns, namely PF4–Sepharose, pFN–Sepharose and octyl-Sepharose; in contrast, the L-SAM of SVT2 cells contain HS proteoglycans with diminished efficiencies for binding to all three of these columns and with overlapping subsets (Lark & Culp, 1984*b*). In contrast, these studies reveal that the HS proteoglycan in 3T3 L-SAM contains a very high proportion of molecules that bind to all three matrices, revealing much less 'processing' of glycosaminoglycan chains or core protein by enzymes in the adhesion sites of these cells as compared with the transformed counterpart. In fact, the high proportion of octyl-Sepharose-binding HS proteoglycan in 3T3 L-SAM argues for its essentiality in transmitting signals from the extracellular matrix across the plasma membrane to the cytoskeleton of these cells, as observed by other experiments (Laterra *et al.*, 1983*a*; Beyth & Culp, 1984; Lark *et al.*, 1985). Results similar to these with 3T3 cells have been observed with proteoglycans in the substratum adhesion sites of primary human dermal fibroblasts (Kent, *et al.*, 1986), whereas very active catabolism of HS proteoglycan has been observed in the adhesion sites of human neuroblastoma cells (E. Vallen & L. Culp, unpublished work). In this regard, more active catabolism of HS proteoglycan has been reported in highly

metastatic melanoma-cell variants than in low-metastatic selectants (Kramer *et al.*, 1982). Whether these marked differences in metabolism of proteoglycans in the substratum-bound fraction of the cell surface from untransformed or malignant cells provide a diagnostic property for their different phenotypes remains to be determined.

The experiments reported here on ion-exchange properties of proteoglycans reveal some complex intramolecular relationships that eventually must be sorted out, particularly if the specificity of aggregation of these molecules is to be resolved. Aggregates appear to form between the proteoglycans and the proteins (principally cytoskeletal) of these adhesion-site extracts, and these aggregates can be dissociated with high concentrations of urea or GdnHCl. Separation of the proteins from the proteoglycan on DEAE-II columns generates proteoglycans that still aggregate to some extent, making affinity-chromatography studies difficult with this class. However, re-addition of proteins to the proteoglycan fraction from DEAE-II columns, followed by DEAE-I chromatography, reveals that most of the proteoglycan is no longer susceptible to the aggregation process for some unapparent reason, although a small portion of proteoglycan continues to be susceptible. Aggregation of proteoglycans in extracts with proteins in the same extracts (i.e. proteins with which they should not form associations *in situ*) will form a significant obstacle for studying the specificity of formation of supramolecular complexes, as accomplished in the cartilage system (Hascall, 1977).

The CS proteoglycan being characterized from 3T3 adhesion sites shares some properties with CS proteoglycans isolated from other non-cartilage systems. Both the size of the intact proteoglycan as well as the size of CS chains are similar to values reported for a rat yolk-sac tumour CS proteoglycan (Oldberg *et al.*, 1981), a molecule that forms complexes with pFN and certain collagens (Oldberg & Ruoslahti, 1982). A somewhat smaller CS proteoglycan has been reported in bovine aortic endothelial cells (Oohira *et al.*, 1983). PF4 in platelets has been shown to be associated with a proteoglycan (Barber *et al.*, 1972), and extensive purification of this proteoglycan on affinity columns has identified a small chondroitin-4-sulphated proteoglycan that binds quantitatively to PF4 affinity columns (Huang *et al.*, 1982). In contrast, a very large CS proteoglycan enriched in 6-sulphated sequences has been isolated from 4 M-GdnHCl extracts of chick skeletal-muscle cultures (Carrino & Caplan, 1982). Rat ovarian granulosa cells are reported to contain three size classes of dermatan sulphate proteoglycan, which require both non-ionic detergent and GdnHCl for successful extraction (Yanagishita & Hascall, 1984*a*); some evidence was provided that one of the dermatan sulphate proteoglycan subsets may contain a hydrophobic sequence in its core protein. For the systems described here, it will now be interesting to determine whether any of these proteoglycans can bind specifically to hyaluronate sequences, the mechanisms of such binding, and other specific intermolecular binding relationships that might influence cellular physiological responses.

For the substratum adhesion sites of Balb/c 3T3 cells, a number of potentially important properties of CS proteoglycan have now been described that should lead to more detailed structure–function analyses. In addition, the simplicity of this system provides an ideal opportunity for studying cause-and-effect relationships between metabolism of this CS proteoglycan and the matrix adhesion properties of the cell.

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