Multiple Classes of Heparan Sulfate Proteoglycans from Fibroblast Substratum Adhesion Sites

AFFINITY FRACTIONATION ON COLUMNS OF PLATELET FACTOR 4, PLASMA FIBRONECTIN, AND OCTYL-SEPHAROSE*

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Both newly formed and long-term culture-generated substratum adhesion sites, generated by EGTA-mediated detachment of BALB/c SVT2 cells, were extracted with an n-octyl-β-D-glucopyranoside buffer containing salt and several protease inhibitors under conditions which result in maximal solubilization of the sulfate-radiolabeled proteoglycans. Because of the functional importance of heparan sulfate proteoglycans in the fibronectin-dependent cell-substratum adhesion processes of these cells, these proteoglycans were fractionated on affinity columns of octyl-Sepharose or of the heparan sulfate-binding protein platelet factor 4 or plasma fibronectin. These affinity matrices resolved a number of both binding and nonbinding classes of heparan sulfate proteoglycan from both types of adhesion sites. In particular, the platelet factor 4 column could resolve several proteoglycans with differing binding affinities. Approximately twice as much heparan sulfate proteoglycan from newly formed sites bound to all three matrices as proteoglycan from long-term sites. The proteoglycan which bound to one matrix was then tested for binding to a second matrix; this approach resolved a number of biochemically distinct species. For example, one-half of the fibronectin-Sepharose-binding fraction from the long-term sites could also bind to platelet factor 4-Sepharose; however, over 90% of the fibronectin-binding fraction from newly formed sites could bind to platelet factor 4. A major portion of the octyl-Sepharose-binding fractions of the original extracts could bind to fibronectin-Sepharose. These studies indicate that some of these proteoglycans have overlapping affinities for fibronectin, platelet factor 4, and octyl-Sepharose and that a portion of the heparan sulfate proteoglycan from these adhesion sites cannot bind to any of these affinity matrices. These results are discussed with regard to the functional significance of these various heparan sulfate proteoglycans in mediating adhesion to extracellular matrices containing fibronectin or platelet factor 4.

The molecular mechanisms through which fibroblast-like cells adhere to extracellular matrix components are slowly being elucidated. A simplified model system in which fibroblasts attach to a serum-adsorbed tissue culture substratum has been used to delineate the molecules involved in these interactions and to assign some functional significance. These cells attach to the fibronectin-containing serum layer through focal footpad adhesion sites (1, 2). These sites can be isolated as substratum-attached material away from the remainder of the cell body upon EGTA-mediated detachment of cells (3) and are biochemically similar to the sites left at the posterior of the cell during active cell movement (4). When cells initially attach to the fibronectin-coated substratum, they do so through footpad adhesion sites composed primarily of close adhesive contacts as determined by interference reflection microscopy. With time, tight focal contacts appear within the close contact adhesions at the end of the microfilamentous stress fibers (5–7). Biochemical characterization of these newly formed sites, which are principally close contacts isolated as R-SAM from cells allowed to attach to the substratum for only 1 h, indicates that these sites are enriched in heparan sulfate proteoglycan (8). By comparison, L-SAM isolated from cells actively moving in culture contain both footpad adhesion sites as well as "footprints" (8, 10, 11) which are abandoned at the posterior of the cell during movement; this material is also enriched in tight focal contacts (7). Compositionally, L-SAM contains less heparan sulfate proteoglycan and a greater quantity of chondroitin sulfate proteoglycan and hyaluronic acid (8). The chondroitin sulfate proteoglycan from R-SAM is extractable using low concentrations of GdnHCl while this proteoglycan in L-SAM resists solubilization with this treatment (9, 12). This indicates that there is not only a quantitative change in this proteoglycan with time but there is also a change in the association of this molecule with other molecules within the adhesion site. A portion of the heparan sulfate proteoglycan found in R-SAM is also catabolized into smaller single chain glycosaminoglycans (9, 12, 13).

The interaction of cell surface heparan sulfate and substratum-bound fibronectin appears necessary to mediate physiologically compatible attachment and spreading of cells to the substratum (4). It has recently been shown that cells attach to the heparan sulfate-binding protein platelet factor 4 (14) through only close contact adhesion sites (7); however, these

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1 The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N' ‑tetraacetic acid; GdnHCl, guanidine hydrochloride; L-SAM, long-term metabolically radiolabeled substratum-attached material; PBS, phosphate-buffered saline; R-SAM, reattaching substratum-attached material; SDS, sodium dodecyl sulfate.
potentially was isolated as previously described (21). Briefly, 7.5 ng/mL of platelet factor 4 also resulted in the reorganization of pseudopod bundles in the cytoplasm of these cells, resulting in a transmembranal linkage of heparan sulfate proteoglycan to the cell surface to cytoskeletal networks in the cytoplasm (15, 16).

Since attachment and spreading of fibroblasts to both platelet factor 4 and fibronectin-coated substrate shared many facets in common, it was necessary to characterize the heparan sulfate proteoglycans in the adhesion sites which could bind to these matrix molecules and which may play a role in the various morphological responses observed. Since a number of modifications have been observed in the proteoglycans between L-SAM and R-SAM, it was important to identify any differences in binding affinities for these matrix molecules which may somehow affect cellular adhesiveness and/or changes in contact formation (e.g., close to tight focal). Therefore, the heparan sulfate proteoglycans from both L-SAM and R-SAM were fractionated on affinity matrices of platelet factor 4 or fibronectin. A hydrophobic binding matrix was also used in an initial attempt to identify molecules which may have a hydrophobic core protein sequence and could potentially be membrane-intercalated proteoglycans (17, 18, 40). A molecule such as this could directly transfer signals from the extracellular matrix to cytoskeletal responses resulting in altered cell morphology (19). Using these three affinity matrices, a number of overlapping subclasses of heparan sulfate proteoglycan were identified. These various subclasses of heparan sulfate proteoglycans are discussed as to the presumed role they may play in the different morphological responses observed as cells attach to various extracellular matrix components.

**EXPERIMENTAL PROCEDURES**

**Cell Growth**—Simian virus 40-transformed Balb/c 3T3 cells (clone SVT2) shown to be Mycoplasma-free (20) were used between their 15th and 25th passages and grown in Eagle's minimal essential medium supplemented with four times the requirements of these cells during the radiolabeling protocol; (a) 50 pCi/ml of Na2[35S]O4 (6774 Bq/mL) which is an excess of the requirements of these cells for 30 min. To quantitatively detach the cells, the EGTA-released cell monolayer was rinsed twice in PBS, twice in glass-distilled water, and any material resistant to extraction was then rinsed twice in PBS, twice in glass-distilled water. Any material resistant to extraction with octyl glucoside-resistant buffer was chromatographed on a Sephadex G-75 (1.5 x 100 cm) column eluted with 1 M NaCl in 0.5 M Tris/HCl, pH 7.2. The more included peak of material was identified as factor 4 plus some contaminating high molecular weight protein. These fractions were pooled, concentrated at 4 °C, dialyzed at 4 °C prior to covalent attachment to 3 ml of the Sepharose 4B resin. The Sepharose 4B was activated using 0.01 volume of cyanogen bromide in acetone (2 mg/ml) for 1 min at room temperature and subsequently mixed with 1 mg/ml of protein at 4 °C for 4 h. Block any unoccupied and activated sites on the resin, the reaction mixture was brought to 1 M urea in 0.5 M Tris/HCl, pH 7.2 (referred to as Tris buffer), (b) 1 M urea, pH 7.5, (c) 2 M NaCl in Tris buffer, and finally (d) Tris buffer alone and stored at 4 °C prior to covalent attachment to 3 ml of the Sepharose 4B resin. The Sepharose 4B was activated using 0.01 volume of cyanogen bromide in acetone (2 mg/ml) for 1 min at room temperature and subsequently mixed with 1 mg/ml of protein at 4 °C for 4 h. To block any unoccupied and activated sites on the resin, the reaction mixture was brought to 1 M Tris/HCl, pH 7.2 (referred to as Tris buffer), (b) 1 M urea, pH 7.5, (c) 2 M NaCl in Tris buffer, and finally (d) Tris buffer alone and stored at 4 °C. To purify platelet factor 4 binding molecules, extracts of both L-SAM or R-SAM solubilized with octyl glucoside buffer (above) were dialyzed against glass-distilled water for 12 h and then against three changes of Tris buffer for 48 h at 4 °C. Samples were affinity-adsorbed on a 3-ml platelet factor 4-Sepharose column. The column was rinsed with Tris buffer and eluted with a linear gradient of 0 to 0.6 M NaCl in 5 ml of Tris buffer. Alternatively, 0.6 M NaCl in Tris, and finally 2 M NaCl in Tris. Refractive indices of column fractions were taken on a Bausch and Lomb refractometer to determine salt concentrations.

**Adhesion Site Heparan Sulfate Proteoglycans**

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Octyl-Sepharose Affinity Chromatography—To isolate putative hydrophobic binding molecules (17, 18), either L-SAM or R-SAM extracts solubilized with octyl glucoside buffer were dialyzed against glass-distilled water for 12 h at 4 °C and then against 3 M NaCl, 0.5 M GdnHCl, 0.2 M Tris, pH 7.3, for 48 h at 4 °C. The samples were then batch-adsorbed to the hydrophobic binding matrix, octyl-Sepharose CL-4B, overnight at room temperature. The resin containing partially eluted with (a) 0.1 M Tris, pH 7.3, (b) 3 M NaCl in 0.2 M Tris, pH 7.3, and (c) 1% octyl glucoside, 3 M NaCl in 0.2 M Tris, pH 7.3. Material resistant to elution with these buffers was eluted with 0.2% SDS in glass-distilled water for 12 h and then against Tris buffer for 48 h at 4 °C and chromatographed over columns of Sepharose 4B which had been covalently linked to 1 mg/ml of human fibronectin as previously described (29). The adhesion site extracts were applied to the column in Tris buffer and eluted with a linear gradient of 0–0.4 M NaCl in Tris, 0.4 M NaCl in Tris, and finally 2 M NaCl in Tris. All fractions were assayed for radioactivity by scintillation counting and refractive indices determined using a Bausch and Lomb refractometer to calculate salt concentrations.

Materials—Materials were purchased from the following sources: [3H]-glucosamine hydrochloride, L-[4,5,3H]-leucine, and Na2[35S]SO4 from Amer sham Corp.; N.E.F. Nues s aqueous scintillation cocktail from New England Nuclear; guanidine hydrochloride from Bethesda Research Laboratories; sodium dodecyl sulfate from Bio-Rad; [3H]-octyl-β-D-glucopyranoside from Sigma; Sepharose CL-2B, CL-6B, Sephindex G-75, and DEAE-Sephadex and octyl-Sepharose CL-4B from Pharmacia Fine Chemicals; EGTA from Eastman; minimal essential medium 4X from Grand Island Biological Co.; donor calf serum from K.C. Biologicals, Inc.; plastic tissue culture dishes from Falcon; CX-10 ultrafiltration units from Millipore Corp. A sample of [3H]-radioabeled chick limb cartilage chondroitin-keratan sulfate proteoglycan was kindly provided by Dr. Arnold Caplan of the Department of Biology at Case Western Reserve University.

RESULTS

Extractability of Substratum Adhesion Sites with Octyl Glucoside-containing Buffers—To characterize the subclasses of heparan sulfate proteoglycans from fibroblast substratum adhesion sites, solubilization conditions were designed to maximally extract the sulfated proteoglycans from these phospholipid-enclosed structures (2, 30). The nonionic detergent, octyl glucoside, was chosen since it has been used to isolate membrane-bound proteins without their undergoing extensive denaturation (31). This detergent can also be easily removed from protein extracts by dialysis (32), permitting functional analyses to be completed on these molecules. A concentration of 1% (w/v) octyl glucoside was found to give optimal extraction of radiolabeled L-SAM; however, this treatment resulted in solubilization of only a portion of the radiolabeled material. In contrast, SDS solubilizes all of the radiolabeled species from these adhesion sites (22). The addition of 1 M NaCl and 0.5 M GdnHCl, which are also easily removed by dialysis, to the 1% octyl glucoside solution results in extraction of the majority of radiolabeled glycoconjugates from the substratum-attached material (Table I). Over 90% of both the [3H] glucosamine-radioabeled glycoconjugate and [35S]O−-radiolabeled glycosaminoglycan-containing molecules are extractable from both L-SAM and R-SAM under these conditions, compared to 80% of the [3H]-leucine-radio labeled protein. L-SAM contained approximately 1640 cpm of 35S-radio labeled material per 106 cells while R-SAM contained 390 cpm of 35S material per 106 cells. Therefore, this combination of 1% octyl glucoside, 1 M NaCl, and 0.5 M GdnHCl (designated octyl glucoside-containing buffer) was used to extract [35S]O−-radio labeled proteoglycans for the remainder of studies reported here.

Extractability of macromolecules from adhesion sites with octyl glucoside-containing buffer

<table>
<thead>
<tr>
<th>Type of SAM</th>
<th>Radioactivity (percentage of total)</th>
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<tbody>
<tr>
<td></td>
<td>[3H]-Glucosamine</td>
</tr>
<tr>
<td>L-SAM*</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>R-SAM*</td>
<td>95 ± 1</td>
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* L-SAM was isolated and extracted with 1% octyl glucoside, 1 M NaCl, 0.5 M GdnHCl in buffer I as described under “Experimental Procedures.” The material resistant to this treatment was subsequently solubilized with 0.2% SDS in buffer II. The percentage of extractable material is expressed as the percentage of total radiolabeled L-SAM extractable with SDS (±S.D. of four separate L-SAM preparations), since SDS quantitatively solubilizes all radiolabeled L-SAM (22).

Heparin Sulfate and Proteoglycan Content of L-SAM and R-SAM Extracts—To initially characterize the [35S]O−-radio labeled L-SAM material extractable with the octyl glucoside-containing buffer, the solubilized material was chromatographed over a Sepharose CL-6B column eluted with an SDS-containing buffer. As observed in the control profile of Fig. 1, the majority of this material elutes in the Vo region of this column profile. When this same extract was chromatographed over a Sepharose CL-2B column eluted with an SDS-containing buffer, it eluted as a relatively homogeneous peak of radiolabeled material with a Kav = 0.55 (data not shown). To identify the amount of N-sulfated heparan sulfate in the extract, the [35S]O−-radio labeled material was treated with nitrous acid (Fig. 1A). Over 70% of this material (approximately 1200 cpm/106 cells) is sensitive to nitrous acid deamination and shifts to an included position on a Sepharose CL-6B column. This same material was also treated with alkaline-sodium borohydride (Fig. 1B) under conditions which lead to breakdown of protein-polysaccharide linkages (23, 24). After such a treatment, the [35S]O−-radio labeled material eluting in the Vo region of the control profile shifts to an included position on a Sepharose CL-6B column (Kav = 0.3), indicating that it is proteoglycan. The shoulder of radiolabeled material in the undigested experiment of Fig. 1B at fractions 27–36 is probably single chain heparan sulfate, as described previously (13), and not proteoglycan. Taken together, these results indicate that over 70% of the [35S]O−-radio labeled L-SAM material extractable with the octyl glucoside-containing buffer is heparan sulfate, and the vast majority of it is proteoglycan.

Similar analytical procedures were used to characterize the octyl glucoside-soluble R-SAM material. The majority of this material also elutes in the Vo region (Fig. 2) of a Sepharose CL-6B column indicating that it is large. Treatment of the [35S]O−-radio labeled R-SAM extract with nitrous acid (Fig. 2A) results in a shift of approximately 85% of the radiolabeled material (i.e. 330 cpm/106 cells) toward the Vf region of the same column, indicating again that the majority of this extract is heparan sulfate. Upon alkaline-borohydride reduction (Fig. 2B), the majority of this [35S]O−-radio labeled R-SAM extract shifts from the Vf region to an included position on a Sepharose CL-6B column, indicating that this sulfated material is also proteoglycan. Comparison of the R-SAM and L-SAM
extracts indicate that they both contain a sizable amount of large heparan sulfate proteoglycan (K_w = 0.55 on Sepharose CL-2B columns); however, a greater amount of the [35S]-radiolabeled material in the R-SAM extract is heparan sulfite than in the L-SAM extract.

**Ion Exchange Chromatography**—Ion exchange resins have proven successful in the fractionation of proteoglycans from other systems (33-35); therefore, an anion exchange resin was also used in an attempt to fractionate proteoglycans from these adhesion site extracts. When an octyl glucoside extract of L-SAM was chromatographed over a DEAE-Sephadex column (data not shown), the material eluted as a broad peak of material at 0.6 M NaCl with a small amount of heterodisperse material eluting between 0.6 M NaCl and 1 M NaCl. These results indicate that columns of DEAE-Sephadex have limited usefulness in the fractionation of proteoglycans in these adhesion site preparations.

**Fractionation on Platelet Factor 4 Affinity Columns**—It has been shown previously that cells can attach, spread, and reorganize microfilament bundles on substrata containing the heparan sulfate-binding protein platelet factor 4 via a cell surface heparan sulfate proteoglycan-dependent mechanism (7, 15). Therefore, affinity matrices of platelet factor 4 were used to fractionate and classify the proteoglycans from both L-SAM and R-SAM which may play a role in these adhesion processes. To characterize the binding affinity of the platelet factor 4-Sepharose, [35S][O]-radiolabeled chick cartilage chondroitin sulfate proteoglycan and [3H]heparin were separately eluted through this affinity resin. As observed in Fig. 3, the affinity column can very effectively resolve the chondroitin sulfate proteoglycan eluting at 0.1 M NaCl from the heparin which elutes at 0.55 M NaCl.

The [35S][O]-radiolabeled L-SAM extract was eluted through the identical platelet factor 4 column, resulting in resolution of four peaks of radioactivity (Fig. 4A). Chromatography of an identical extract over a bovine serum albumin-Sepharose column resulted in binding of none of the L-SAM extract to the column (data not shown). When the platelet factor 4-binding and -nonbinding fractions were pooled separately and rechromatographed over the same column (data not shown), each of the fractions chromatographed in the identical position as observed initially. Also, batch adsorption for up to 16 h (data not shown) did not increase the amount of material binding to the affinity resin. These results indicate that the platelet factor 4 can be used to fractionate L-SAM into three binding and one nonbinding fractions and that the nonbinding fraction is not the result of saturation of the affinity resin. Each fraction eluted from the platelet factor 4 column was pooled separately and treated with nitrous acid.
and alkaline borohydride (data not shown) to identify heparan sulfite and proteoglycan, respectively. Results from this assay indicate that the nonbinding fraction contains heparan sulfate proteoglycan and a large "single chain" heparan sulfate; however, approximately 50% of this nonbinding fraction was resistant to nitrous acid and was identified as chondroitin sulfate, since L-SAM contains no keratan sulfate and very little dermatan sulfate (8). The three binding fractions were all found to contain heparan sulfate proteoglycan; however, the major binding fraction eluting at approximately 0.2 M NaCl does contain a small amount of chondroitin sulfate as identified by sensitivity to chondroitinase ABC digestion (data not shown). These results indicate that platelet factor 4 can be used to fractionate the heparan sulfate proteoglycans from L-SAM into subclasses with different binding affinities.

Octyl glucoside-extracted R-SAM was also chromatographed over an identical affinity column (Fig. 4B). This material eluted as two major pools of radioactivity, one binding and one nonbinding. There is also a third very small high affinity fraction eluting at 1–2 M NaCl similar to that observed from the L-SAM extract (compare Fig. 4, A and B). Comparison of Fig. 4, A and B, also indicates that approximately 45% of the [35S]O:--radiolabeled L-SAM extract does not bind to the affinity resin while only 20% of the R-SAM extract does not bind to this matrix. Biochemical analyses of the binding and nonbinding fractions of the R-SAM extract (data not shown) indicate approximately 60% of the nonbinding R-SAM is sensitive to chondroitinase ABC digestion, indicating that it is a chondroitin sulfate moiety. The remainder of this fraction is sensitive to nitrous acid deamination and eluted in the V4 region of a Sepharose CL-6B column, indicating that it is a heparan sulfate proteoglycan. Over 85% of the binding fraction is sensitive to both nitrous acid deamination and alkaline borohydride reduction and is, therefore, a heparan sulfate proteoglycan. Taken together, these results establish that the majority of the proteoglycan from both L-SAM and R-SAM can bind to platelet factor 4; however, a portion of the heparan sulfate proteoglycan in both of these extracts does not bind to this affinity matrix.

**Hydrophobic Affinity Chromatography on Octyl-Sepharose Columns**—Hydrophobic affinity chromatography has been used in several systems to identify proteins (36) and proteoglycans (17, 18, 40) which have a hydrophobic binding domain in their protein sequence. To identify any proteoglycans from adhesion site extracts which might have a hydrophobic core protein sequence and could potentially by a membrane-bound form of the proteoglycan, the extracts were adsorbed to octyl-Sepharose CL-4B using modifications of the procedure of Kjellen et al. (17). After overnight batch adsorption, the material was eluted with high ionic strength buffer to elute any molecules binding to the matrix through ionic interactions. The material binding to the column through a hydrophobic detergent-sensitive interaction was subsequently eluted with an octyl glucoside-containing buffer. When [3H]heparin was chromatographed over this affinity matrix (data not shown), none of the material bound, indicating that the glycosaminoglycan sequences in the heparan sulfate proteoglycans will not contribute to binding to the hydrophobic matrix.

When [35S]O:--radiolabeled L-SAM is adsorbed to octyl-Sepharose (Fig. 5A), approximately 20% of the material binds to the resin and is eluted with an octyl glucoside-containing buffer. This material is completely sensitive to both nitrous acid and alkaline borohydride (data not shown), indicating that it is heparan sulfate proteoglycan. Approximately 50% of the nonbinding fraction from this column has been iden-
Affinity Chromatography of Octyl-Sepharose-binding Fractions on Columns of Platelet Factor 4-Sepharose—Since both L-SAM and R-SAM contain proteoglycans which bind to octyl-Sepharose and to platelet factor 4, it was necessary to identify any overlapping subsets between these affinity-fractionated classes. The octyl-Sepharose-binding fractions from both L-SAM and R-SAM (see Fig. 5) were pooled separately and dialyzed against Tris buffer to remove detergent prior to affinity chromatography over platelet factor 4-Sepharose. The majority of the L-SAM proteoglycan elutable from octyl-Sepharose with octyl glucoside binds to platelet factor 4 (Fig. 7A). This material elutes from the affinity column as essentially one peak of material at 0.2 M NaCl, but again with a small amount of high affinity proteoglycan eluting at 1.0 M NaCl. Comparing this profile to the profile of the total extract chromatographed over fibronectin-Sepharose (see Fig. 4A) indicates that the major factor 4-binding fraction is enriched in heparan sulfate proteoglycan which also binds to octyl-Sepharose (see Table II).

As described earlier, the [35S]0^-radiolabeled R-SAM material which binds to octyl-Sepharose is heparan sulfate proteoglycan (see Fig. 6). The proportion of this "hydrophobic" proteoglycan which could also bind to platelet factor 4 was then determined. As observed in Fig. 7B, a large amount of this octyl-Sepharose-binding material from R-SAM has the ability to bind to platelet factor 4, most with a modest affinity (0.2-0.4 M NaCl) but also a sizable fraction with high affinity (1-2 M NaCl). These data indicate that both L-SAM and R-SAM contain proteoglycans which bind to a hydrophobic binding matrix, as well as to factor 4, even though there appears to be less of this octyl-Sepharose-binding fraction in the L-SAM extract than in the R-SAM extract. There are also sizable amounts of octyl-Sepharose-binding proteoglycans from both extracts which do not bind to factor 4.

Affinity Fractionation on Columns of Fibronectin-Sepharose—Laterra et al. (29) have shown that heparan sulfate chains isolated upon proteolytic digestion of adhesion site extracts have the ability to bind to plasma fibronectin-Sepharose affinity columns. Since the majority of the heparan sulfate isolated from adhesion sites is proteoglycan, it was necessary to identify if all, or only a portion, of this proteoglycan could bind to fibronectin-Sepharose. It was also important to determine if any proteoglycan had overlapping affinities for octyl-Sepharose, fibronectin, and platelet factor 4. In order to achieve this objective, an affinity chromatography experiment was performed using aliquots of the isolated proteoglycan fractions from both L-SAM and R-SAM. The results of this experiment are illustrated in Fig. 8A and B. The data indicate that the proteoglycans from both L-SAM and R-SAM have overlapping affinities for fibronectin-Sepharose, platelet factor 4-Sepharose, and octyl-Sepharose. However, there are also differences in the relative affinities of the proteoglycans from L-SAM and R-SAM for these three affinity columns. For example, the proteoglycans from L-SAM have a higher affinity for fibronectin-Sepharose than for platelet factor 4-Sepharose or octyl-Sepharose. In contrast, the proteoglycans from R-SAM have a higher affinity for platelet factor 4-Sepharose than for fibronectin-Sepharose or octyl-Sepharose.
When \[^3H\]heparin was chromatographed through a fibronectin-Sepharose column, 95% of the glycosaminoglycan bound and was eluted at a rather low salt concentration (0.25 M) (data not shown). By comparison, none of the \[^35S\]O\(^-\)-radiolabeled chick chondroitin sulfate proteoglycan binds to this column under identical conditions (data not shown).

When \[^35S\]O\(^-\)-radiolabeled L-SAM material was chromatographed over a fibronectin column (Fig. 8A), approximately 30% of the material bound to the column and was eluted as a fairly broad peak of material at approximately 0.1-0.2 M NaCl; however, a small portion of this material binds to the column with high affinity (at 0.4 M NaCl). When \[^35S\]O\(^-\)-radiolabeled R-SAM is chromatographed over the same column (Fig. 8B), approximately 50% of this material binds with weak affinity (0.1-0.2 M NaCl) and a small portion with high affinity (0.4 M NaCl). These results indicate that a greater proportion of the extractable R-SAM material binds to fibronectin-Sepharose than the material extracted from L-SAM.

To identify overlapping subsets of heparan sulfate proteoglycans in L-SAM and R-SAM, the octyl-Sepharose-binding fractions (see Fig. 5) from each of these extracts were pooled, extensively dialyzed against Tris buffer, and chromatographed on fibronectin-Sepharose (data not shown). In both cases, approximately 70% of the octyl-Sepharose-binding material, previously identified as heparan sulfate proteoglycan, also bound to fibronectin (data not shown). Since twice as much of the R-SAM heparan sulfate proteoglycan as L-SAM proteoglycan binds to octyl-Sepharose, these results indicate that R-SAM also contains approximately twice as much proteoglycan which can bind to both ligands.

Since the precise heparan sulfate sequences required for binding to fibronectin or to platelet factor 4 have not been determined, it is important to identify the overlapping affinities of the heparan sulfate proteoglycans for fibronectin and platelet factor 4. In an attempt to identify subfractions of the fibronectin-binding material which also has the ability to bind to platelet factor 4, fibronectin-binding fractions (see Fig. 8) were eluted, pooled, concentrated, and dialyzed against Tris buffer prior to chromatography on a platelet factor 4 column. When the R-SAM fraction is chromatographed (Fig. 9A), 90% of the material binds at 0.15-0.35 M NaCl. By comparison, when the fibronectin-binding fraction of the L-SAM extract is chromatographed over the same factor 4 column (Fig. 9B), approximately 60% of the material binds to the affinity resin and is eluted at 0.15-0.25 M NaCl. These results indicate that both the L-SAM and R-SAM extracts contain heparan sulfate proteoglycans which can bind to both fibronectin and platelet factor 4; however, L-SAM contains much less material with these overlapping affinities than R-SAM. Also, a portion of these proteoglycans also has the capacity to bind to hydrophobic binding matrices (see above).
The proteoglycans from both newly formed sites and long-term culture-generated adhesion sites were extracted and fractionated on three different affinity matrices. Overlapping subsets of proteoglycans were identified which may play some role in the various physiological responses observed when cells attach to extracellular matrices containing these specific binding proteins. In this regard, cells form only close contact adhesions when attaching to platelet factor 4-coated substrata (8); however, they form both close contacts and tight focal (at later times) contacts when attaching to fibronectin (5, 7). Thus, the attachment responses observed as cells attach to these ligands are quite different even though both attachment events are at least partially mediated by cell surface heparan sulfate proteoglycan. Therefore, these proteins were used as affinity ligands to identify functionally important proteoglycans.

The proteoglycans from both newly formed sites and long-term culture-generated adhesion sites were extracted and compared in an effort to identify differences which may affect the adhesion process as cells attach and move across the fibronectin-coated substratum. A number of biochemical differences have previously been observed between R-SAM and L-SAM (8). The latter has recently increased amounts of chondroitin sulfate proteoglycan and hyaluronic acid as compared to R-SAM, which contains principally heparan sulfate proteoglycan. Also, there is a change in the association of the chondroitin sulfate proteoglycan with other molecules within these sites with time resulting in its resistance to solubilization with the chaotropic agent 0.5 M GdnHCl (9). A number of changes in the heparan sulfate proteoglycan have also been observed. R-SAM contains a high density heparan sulfate proteoglycan which sediments to the bottom of an isopycnic cesium chloride density gradient; this moiety is depleted in L-SAM (21, 37). Pulse-chase analysis has also shown that a portion of the sulfate-radiolabeled heparan sulfate proteoglycans in R-SAM is catabolized into single chain glycosaminoglycans with time (13). The formation of both tight focal and close contacts observed when cells attach to fibronectin-coated substrata appears to result from the binding of fibronectin's heparan sulfate-binding domain to cell surface heparan sulfate proteoglycan concurrent with binding of fibronectin's cell-binding domain to its unknown cell surface receptor (15, 16). The exact role which these changes in the adhesion site proteoglycans play in the formation and dissolution of both close contact adhesions and tight focal contacts remains to be determined.

A number of different heparan sulfate proteoglycans have now been identified in this study. Two major and one minor platelet factor 4 binding fractions and one nonbinding fraction were identified in the L-SAM extract. R-SAM contained one major and one minor binding fraction, as well as a nonbinding fraction. However, a higher percentage of the L-SAM did not bind to the matrix as compared to R-SAM material (Table II). Approximately one-half of this nonbinding material in both cases is chondroitin sulfate proteoglycan with the remainder being heparan sulfate proteoglycan. It appears that L-SAM contains more heterodisperse classes of heparan sulfate proteoglycans, as compared to R-SAM, which are resolved on the platelet factor 4-binding matrix. It is possible that this heterodispersity and the fairly large amount of nonbinding material observed in the L-SAM extract may result from catabolism of the proteoglycans during aging processes of fibroblast adhesion sites (9, 13). The molecular basis for the nonbinding proteoglycans remains to be determined.
resolving properties of the platelet factor 4 is presently unknown; however, fractionation of adhesion site proteoglycans on this resin cannot be completely based on charge differences since an ion exchange resin cannot resolve proteoglycans from these same extracts.

When the L-SAM and R-SAM extracts were affinity-fractionated on fibronectin, they both partitioned into binding and nonbinding fractions. Interestingly, twice as much of the R-SAM extract can bind to this ligand as compared to the L-SAM extract (Table II). Overlapping affinities were observed between the heparan sulfate proteoglycan which binds to fibronectin and to platelet factor 4 (Table II). Over 90% of the fibronectin-binding R-SAM-derived proteoglycan also binds to factor 4. Since R-SAM is enriched in close contacts and since cells attach to platelet factor 4 through only close contact adhesions (7), it is possible that the proteoglycan isolated from R-SAM which binds to both platelet factor 4 and to fibronectin is the principal molecule responsible for close contact formation. It has been shown, using epithelial cells, that the extracellular matrix component to which cells attach affects the catabolism of the proteoglycans synthesized by these particular cells (38, 39). Therefore, the formation of close contacts may be the initial event in cell adhesion requiring multivalent heparan sulfate proteoglycans. When cells attach to fibronectin-containing substrata, catabolism of a portion of their proteoglycan may normally occur coincident with the formation of tight focal contacts at later times. On the other hand when cells attach to platelet factor 4, this catabolic event may not take place, for some unknown reason, and the cells may thereby be inhibited from forming tight focal contacts. Only about one-half of the fibronectin-binding L-SAM extract also binds to platelet factor 4. It is possible that the proteoglycan from L-SAM which binds to both proteins is important in maintaining the close contact structure; however, the material which does not bind to platelet factor 4 but which does bind to fibronectin may be involved in the formation of tight focal contacts, since both structures are observed when cells are grown on the fibronectin-containing substratum.

The original L-SAM and R-SAM proteoglycan extracts were also affinity-adsorbed onto octyl-Sepharose to identify molecules which may have a hydrophobic core protein sequence (17, 18, 40) and may, therefore, be membrane-intercalated proteoglycans. Both L-SAM and R-SAM contained octyl-Sepharose-binding heparan sulfate proteoglycans; however, twice as much of R-SAM as L-SAM bound to this material. Over 70% of the octyl-Sepharose-binding proteoglycan from L-SAM or R-SAM can also bind to either fibronectin or platelet factor 4 (Table II). A portion of the octyl-Sepharose-binding material does not bind to the heparan sulfate-binding matrix. There is also some proteoglycan which has a relatively high affinity for platelet factor 4 but does not bind to octyl-Sepharose. The proteoglycan which did bind to octyl-Sepharose and to both fibronectin and platelet factor 4 may be an intercalated membrane proteoglycan which can interact with extracellular matrix molecules and internal cytoskeletal networks simultaneously (15, 16, 19, 40). Further studies, such as liposome reconstruction experiments (40), must be completed to help resolve this issue. Previous cell biology studies have suggested that heparan sulfate proteoglycan may be involved in cytoskeletal reorganization (15, 16). When cells attach on a platelet factor 4-containing substratum, they form broad lamellae and they develop lengthy actin microfilament bundles within these lamellae (15). This cytoskeletal reorganization is the first evidence for a transmembrane linkage of heparan sulfate proteoglycan to cytoplasmic microfilament networks in the fibroblast substratum adhesion site.

A number of differences were observed in the binding affinity for platelet factor 4 and fibronectin between the proteoglycans isolated from R-SAM or L-SAM. These differences may reflect metabolic processing within those sites which may be controlled somewhat by the matrix component to which the cells attach (38, 39). A significantly smaller proportion of heparan sulfate proteoglycan from L-SAM bound to all three matrices. This may reflect two different catabolic events occurring with time. First, there may be metabolism of the carbohydrate chain via endoglycosidase action (41-43) which may disrupt the ability of the proteoglycan to associate properly with extracellular matrix components. Secondly, there may be a proteolytic cleavage of the putative hydrophobic core protein sequence which would lead to the formation of a proteoglycan which would bind to the membrane only through a receptor (17) or would be completely released from the cell membrane into the extracellular matrix. It is also possible that the cells synthesize a number of different proteoglycans separately and that there is no catabolic processing of one proteoglycan into another. Further studies must be undertaken to resolve these two important possibilities.

In conclusion, these experiments have identified a number of different subclasses of heparan sulfate proteoglycan from fibroblast adhesion sites which may be responsible for specific adhesive responses observed as cells grow on different extracellular matrix components. Also, differences in these proteoglycans have been identified which occur during maturation or aging of the fibroblast adhesion sites. Determination of the functional role that these different classes of proteoglycan play should help clarify the molecular mechanisms through which cells interact with extracellular matrices.

REFERENCES
Adhesion Site Heparan Sulfate Proteoglycans