

Cell Surface Heparan Sulfate Mediates Some Adhesive Responses to Glycosaminoglycan-binding Matrices, Including Fibronectin

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ABSTRACT Proteins with affinities for specific glycosaminoglycans (GAG's) were used as probes for testing the potential of cell surface GAG's to mediate cell adhesive responses to extracellular matrices (ECM). Plasma fibronectin (FN) and proteins that bind hyaluronate (cartilage proteoglycan core and link proteins) or heparan sulfate (platelet factor 4 [PF4]) were adsorbed to inert substrata to evaluate attachment and spreading of several 3T3 cell lines. Cells failed to attach to hyaluronate-binding substrata. The rates of attachment on PF4 were identical to those on FN; however, PF4 stimulated formation of broad convex lamellae but not tapered cell processes fibers during the spreading response. PF4-mediated responses were blocked by treating the PF4-adsorbed substratum with heparin (but not chondroitin sulfate), or alternatively the cells with *Flavobacter heparinum* heparinase (but not chondroitinase ABC). Heparinase treatment did not inhibit cell attachment to FN but did inhibit spreading. Cells spread on PF4 or FN contained similar Ca^{2+} -independent cell-substratum adhesions, as revealed by EGTA-mediated retraction of their substratum-bound processes. Microtubular networks reorganized in cells on PF4 but failed to extend into the broadly spread lamellae, where fine microfilament bundles had developed. Stress fibers, common on FN, failed to develop on PF4. These experiments indicate that (a) heparan sulfate proteoglycans are critical mediators of cell adhesion and heparan sulfate-dependent adhesion via PF4 is comparable in some, but not all, ways to FN-mediated adhesion, (b) the uncharacterized and heparan sulfate-independent "cell surface" receptor for FN permits some but not all aspects of adhesion, and (c) physiologically compatible and complete adhesion of fibroblasts requires binding of extracellular matrix FN to both the unidentified "cell surface" receptor and heparan sulfate proteoglycans.

Fibronectin is an extracellular matrix-associated glycoprotein found in vivo that mediates fibroblast attachment, spreading, motility, and longterm survival in vitro when adsorbed to tissue culture substrata. Fibronectin consists of dimeric subunits (54) and has the potential to interact with several cell surface-associated macromolecules including collagen (13), certain glycosaminoglycans (24, 56), and possibly gangliosides (21), and another unidentified cell surface receptor (37, 43, 45, 56). Accumulating evidence indicates that cell surface glycosaminoglycans and proteoglycans mediate fibroblast attachment and possibly more complex adhesive responses on fibronectin-containing extracellular matrices (10, 41, 47). Hyaluronic acid and heparan sulfate are two particular classes of cell surface

glycosaminoglycan that have received considerable attention as potential determinants of the state of cellular adhesiveness and motility in numerous experimental systems (2, 11, 25, 34, 44). It has yet to be determined, however, if direct interactions between cell surface glycosaminoglycans and the extracellular matrix determine properties of cellular adhesiveness and motility.

Fibronectin "receptors" on the surface of the cell might be expected to initiate certain aspects of the cellular adhesive response when interacting with substrata coated with ligands other than fibronectin that bind the "receptor" molecule. Recent in vitro studies by Carter et al. (7) have shown that tissue culture substrata coated with certain lectins or enzymes, which

interact with cell surfaces but are not necessarily associated with physiological processes of cellular adhesion, are capable of mediating simple cell attachment and in special cases cytoplasmic spreading. These studies demonstrate that cells can respond with attachment and spreading to matrices containing proteins of vastly different biochemical binding properties. The cell surface components which bind directly to the artificial substrata used by Carter et al. (7) may either contain, in the form of macromolecular complexes, or be identical to the cell surface components which mediate similar spreading responses on more physiological fibronectin-containing extracellular matrices.

We have therefore developed experimental approaches for testing the potential of specific cell surface glycosaminoglycans to mediate simple cell attachment and the more complex phenomena associated with cytoplasmic spreading. Murine fibroblasts are plated on tissue culture substrata adsorbed with proteins that bind to either hyaluronic acid or specific sulfated glycosaminoglycans. Cell attachment kinetics, detailed cellular morphologies, and cytoskeletal reorganization in response to these novel substrata are directly compared to the fibronectin-mediated phenomena. The similarities and differences between the observed responses are discussed in relation to previous reports and developing theories concerning the specific biochemical determinants of cell-extracellular matrix interactions.

MATERIALS AND METHODS

Materials: Plasma-derived fibronectin (FN) was isolated from outdated blood bank human plasma or from bovine calf serum by affinity chromatography on gelatin-Sepharose columns (13). Human platelet factor-4 (PF4), which binds very well to heparin or fibroblast heparan sulfate and weakly to chondroitin sulfate (15), was purified essentially according to Levine and Wohl (27). Briefly, platelets were collected from outdated human platelet concentrates by centrifugation, resuspended in phosphate-buffered saline (PBS), frozen-thawed three times, and centrifuged at 40,000 *g* for 20 min. Saturated ammonium sulfate was added dropwise to the supernatant to a final concentration of 50%, the mixture stirred for 15 min at room temperature, and then centrifuged at 40,000 *g* for 20 min. The supernatant was dialyzed extensively against PBS and passed through a heparin-Sepharose affinity column which was washed sequentially with PBS, 0.5 M NaCl (in 50 mM Tris, pH 7.4), which elutes quantitatively any contaminating FN (24), 1 M NaCl (in 50 mM Tris), and finally 2 M NaCl (in 50 mM Tris) to elute the bound PF4. The identity and purity of the recovered protein was verified by amino acid analysis (12) on a Durrum 500 amino acid analyzer and by polyacrylamide gel electrophoresis. The PF4 preparations were >99% pure and free of contaminating FN. Bovine nasal septum cartilage proteoglycan core protein was isolated according to Hascall and Heinegard (16). This proteoglycan as an A₁D₁ fraction from a cesium chloride gradient (graciously provided by Dr. Lawrence C. Rosenberg, Montefiore Hospital and Medical Center, Bronx, NY) was exhaustively digested with chondroitinase ABC to remove most of the chondroitin sulfate. The remaining intact proteoglycan core protein was isolated by Sepharose CL-4B chromatography in 0.1 M sodium acetate-0.1 M Tris (pH 7.3) while monitoring O.D.₂₈₀. Bovine nasal septum cartilage proteoglycan link protein was purified according to the method of Tang et al. (49) and was also kindly provided by Dr. Rosenberg. Concentrations of purified proteins were determined by the method of Lowry (31).

To obtain heparinase activity (14), lyophilized *F. heparinum* bacteria were suspended in 0.5 M sodium acetate (pH 7.0), sonicated for 60 s, and centrifuged at 30,000 *g* for 20 min. The supernatant was recovered and stored at -20°C. This crude enzyme preparation has been shown to contain chondroitinase, heparinase, and heparinase activities (29) but to lack detectable protease, sialidase, or collagenase activities (14). Each enzyme preparation was assayed for activity by incubating an aliquot of crude enzyme in 500 µg/ml of either highly purified heparin or chondroitin-6-sulfate in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂ (PBS-II) at 37°C while monitoring O.D.₂₃₂ (29, 57).

Reference standard glycosaminoglycans were kindly provided by Drs. M. B. Mathews and J. A. Cifonelli of the University of Chicago, Chicago, IL. Porcine mucosal [³H]heparin (0.624 mCi/mg; 1 Ci = 3.7 × 10¹⁰ becquerels) was purchased from New England Nuclear Corp., Boston, MA; methyl[³H]thymidine (72 Ci/mmol) from ICN, Chemical and Radioisotope Division; ethylene diamine tetraacetic acid, bovine serum albumin (BSA) and swine skin gelatin (type I) from

Sigma Chemical Co., St. Louis, MO; round 13-mm diameter Thermanox plastic coverslips from Flow Laboratories, Inc., Rockville, MD; round glass coverslips from Clay Adams Co., Parsippany, NJ; glutaraldehyde (50% wt/wt), paraformaldehyde, and formaldehyde (37% wt/wt) from Fischer Scientific Co., Pittsburgh, PA; sheep antibody to bovine brain 6S tubulin from CAABCO, Inc., Houston, TX; rhodamine-conjugated rabbit anti-sheep IgG from Cappel Laboratories Inc., Cochranville, PA; nitrobenzoxadiazole-conjugated phalloidin from Molecular Probes Inc., Plano, TX.

Determination of Glycosaminoglycan Binding to Protein-coated Coverslips: Thermanox plastic coverslips were incubated at room temperature for 1 h with 20 µg/ml of binding proteins in PBS. The coverslips were rinsed three times with PBS and then incubated for 30 min in 1 mg/ml BSA in PBS. After three more rinses in PBS-II, each type of coated coverslip (triplicate samples) was incubated for 2 to 24 h, with either metabolically radiolabeled [³H]hyaluronic acid (25) (9,000 cpm/µg) or commercial [³H]heparin (5 × 10⁶ cpm/µg). Equilibrium binding was achieved at incubation times of 1–2 h. Each coverslip was rinsed three times in PBS-II and directly assayed for bound radioactivity by scintillation counting. Uncoated coverslips or coverslips treated with only 1 mg/ml BSA were used as negative controls and consistently failed to bind glycosaminoglycan.

Cell Culture: Stock cultures of mouse BALB/c3T3 (clone A31), BALB/c3T3 cells transformed by Simian Virus 40 (clone SVT2), and Swiss 3T3 cells were grown *Mycoplasma*-free in supplemented Eagle's Minimal Essential Medium as described previously (40). For radiolabeling of cells to be used in attachment assays, cells were freshly passaged by trypsinization; 24 h later, the medium was replaced with one containing 0.1 µCi/ml of methyl-³H]thymidine (72 Ci/mmol). After 24 h, the radiolabeling medium was replaced with unlabeled medium for an additional 24 h.

Cell Adhesion Assays: Protein-coated coverslips were placed in the wells of Costar tissue culture clusters. In some instances, the coverslips were posttreated for 1 h with reagents being tested to inhibit cell adhesion and further rinsed three times with PBS before cell inoculation.

[³H]thymidine-radiolabeled cells (80% confluent) in stock cultures were rinsed three times in PBS and treated for 30 min at 37°C with 0.5 mM EGTA in PBS on a rotary shaker to detach cells. After rinsing twice in PBS, cells were resuspended by gentle pipetting in serumless MEM×4 containing 2 mg/ml BSA. Cell concentrations were determined and adjusted to 1.5 × 10⁶ cells/ml. Attachment assays were initiated by adding 100 µl of suspended cells to each well containing a coverslip and 400 µl MEM×4 (preequilibrated for temperature and pH), 2 mg/ml BSA, and in particular cases GAG's or enzymes being tested to inhibit attachment responses. At appropriate times, coverslips containing radiolabeled cells were removed from the wells, gently rinsed three times in PBS-II to remove nonadherent cells, and placed into scintillation vials for determination of bound radioactivity. In assays using *F. heparinum* enzyme or chondroitinase ABC enzyme, PBS-II containing 2 mg/ml BSA was substituted for serumless medium. After the addition of cells to wells containing enzymes, both the enzyme-containing samples and controls were shaken for 5 min at 37°C to permit enzyme digestion of cell surface polysaccharides before initiating the attachment of cells by terminating shaking.

To visualize the morphology of cell-substratum adhesion sites during EGTA-mediated cell detachment (42), cells were incubated on protein-coated coverslips for 1 h to allow cell attachment and spreading, rinsed three times in PBS, and then incubated for 15 to 30 min at 37°C in 0.5 mM EGTA in PBS. The coverslips, which contained rounded but still adherent cells, were then gently rinsed twice in PBS and prepared for scanning electron microscopy as described below by procedures which are optimized to retain the integrity of fragile cell processes.

Preparation of Samples for Microscopy: For phase contrast microscopy, substrata and cells were prepared as described above with the exception that, instead of coverslips, Costar 16-mm tissue culture wells were used directly as substrata. Adherent cells were rinsed twice with PBS-II, fixed for 30 min at room temperature with filtered (0.45-µm pore size Millipore membrane) 3.7% formaldehyde in PBS-II buffered to pH 7.4, and rinsed three times with PBS. Phase contrast micrographs were taken on a Nikon Diaphot inverted phase contrast microscope.

For scanning electron microscopy, cells on Thermanox coverslips were rinsed twice in PBS-II, fixed for 2 h at room temperature in filtered 2% (vol/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in Hanks's balanced salt solution buffered to pH 7.4, rinsed twice in PBS and three times in glass-distilled water, and dehydrated with sequential 10-min incubations at room temperature in increasing concentrations of ETOH in glass-distilled water. The ethanol dehydration was completed with three 10-min incubations in absolute ETOH at room temperature. Samples were then gently rinsed with liquid CO₂ in the critical point drying apparatus (Technics Critical Point Apparatus; Technics EMS, Inc., Springfield, VA), sputter-coated with gold-palladium (Technics Hummer V), and stored desiccated until examined using an ETEC Omniscan scanning electron microscope.

Fluorescent Staining of Cytoskeletons: Cells on PF4 or FN were fixed for 20 min with 3.7% formaldehyde in PBS-II, rinsed three times in PBS, and permeabilized by incubation at room temperature for 1 min in PBS containing 0.2% Triton X-100, followed by three rinses in PBS. Immunolabeling procedures were based on those of Lazarides and Weber (26). Cells were incubated for 60 min at 37°C with 200 µg/ml sheep antiovine brain 6S tubulin IgG in PBS, rinsed in PBS, and then incubated for 30 min at 37°C with rhodamine isothiocyanate-conjugated rabbit antisheep immunoglobulin (1:40 dilution). Coverslips were either directly mounted on glass microscope slides over PBS containing 50% glycerol (pH 7.4) or stained further for F-actin before mounting. To stain specifically for F-actin, fixed and permeabilized cells prepared as above were incubated for 20 min at room temperature in PBS containing 900 ng/ml nitrobenzoxadiazole (NBD)-conjugated phalloidin (50). Cells were then rinsed in PBS and mounted on microscope slides as described above. Stained preparations were viewed in a Leitz Dialux microscope with phase contrast optics and epifluorescence illumination and photographed using Kodak 2475 Recording Film.

RESULTS

Glycosaminoglycan Binding to Protein-coated Substrata

Protein-coated coverslips were tested for GAG binding by incubation in PBS with either [³H]heparin or [³H]hyaluronate. Human plasma fibronectin continued to bind heparin when adsorbed (Table I). Hyaluronate, as expected, did not interact with plasma FN-coated coverslips, since this form of fibronectin has been shown to lack hyaluronate binding activity in a number of *in vitro* assays (25). PF4, which has been demonstrated to have a single GAG-binding site with specificity heparin > heparan sulfate >> chondroitin sulfates (15), bound significant levels of heparin or heparan sulfate but undetectable amounts of hyaluronate (Table I). The relatively high level of heparin binding to PF4-coated coverslips in comparison to FN is consistent with PF4's higher univalent affinity for this glycosaminoglycan (24, 27) and the much larger concentration of binding sites of this small protein per area of substratum. The core protein of bovine nasal septum cartilage proteoglycan and link protein, which have been shown to interact specifically with hyaluronate (17, 51), also retain this activity when adsorbed to plastic coverslips. Incubations of protein-coated coverslips with radiolabeled GAG for up to 10 times longer than those used to obtain the data in Table I failed to increase the level of radioactivity bound indicating that equilibrium had

TABLE I
GAG Binding Activity of Protein-coated Substrata

Protein coat	GAG bound per coverslip	
	Hyaluronate	Heparin
	<i>ng</i>	
Plasma fibronectin	<1.0	0.5 ± 0.1
Platelet factor-4	<1.0	1.2 ± 0.2
Proteoglycan core protein	5 ± 0.5	<0.05
Link protein	13.5 ± 0.4	<0.05
Bovine serum albumin	<1.0	<0.05

Thermanox plastic coverslips were incubated for 1 h at room temperature with 20 µg/ml in PBS of either plasma fibronectin, platelet factor-4, bovine nasal septum cartilage proteoglycan core protein, bovine nasal septum cartilage link protein, or BSA. The treated coverslips were then rinsed three times in PBS-II and incubated in triplicate for 2 h in either [³H]heparin (5 × 10⁵ cpm/µg) or [³H]hyaluronate (9 × 10³ cpm/µg). Each coverslip was rinsed three times in PBS and assayed for bound radioactivity by scintillation counting. Nanograms of polysaccharide bound per coverslip were determined directly from the amount of radioactive material bound. Data represent the mean of triplicate determinations ± standard error. The different limits for the minimal detectable amounts of bound GAG (<1.0 ng for hyaluronate and <0.05 ng for heparin) result from differences in specific activity of the radiolabeled glycosaminoglycans.

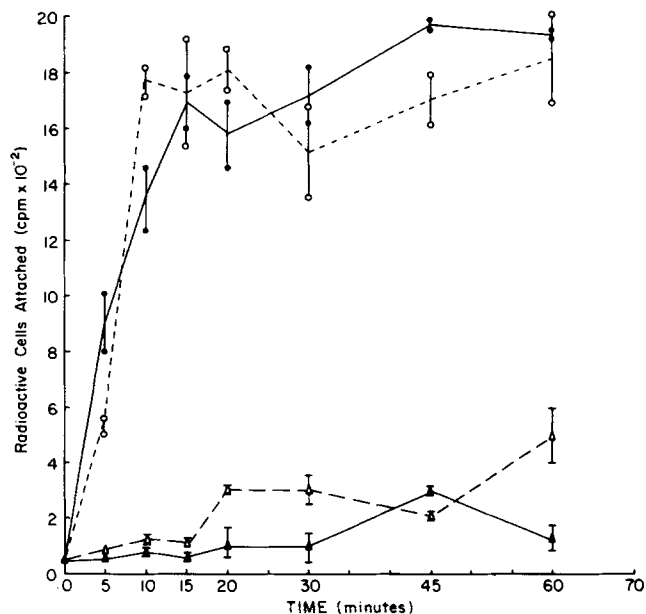


FIGURE 1 Cell attachment to GAG-binding substrata. SVT2 cells radiolabeled with [³H]thymidine (Materials and Methods) were detached from stock cultures with 0.5 mM EGTA, rinsed twice in PBS, and resuspended in serumless medium containing 2 mg/ml BSA (attachment medium) at 1.5 × 10⁶ cells/ml. At 0 min, 100 µl of suspended cells were added to Costar tissue culture wells containing 400 µl of attachment medium and a coverslip coated with either plasma FN (●—●), PF-4 (○—○), bovine cartilage proteoglycan core protein (▲—▲), or bovine cartilage link protein (△—△) which had been equilibrated at 37°C with 10% CO₂. At indicated times, duplicate coverslips were removed, gently rinsed three times in PBS-II, and placed in scintillation vials for determination of bound radiolabeled cells. Total radioactivity associated with 100 µl of suspended cells was also determined to permit calculation of percent-attached cells which attached. Similar data were obtained using either BALB/c3T3 or Swiss 3T3 cells.

been reached under the binding conditions used. These data indicate that these proteins retain their ability to interact with specific GAG's when adsorbed to tissue culture plastic; their GAG binding sites are thus available to interact with GAG's present on cell surfaces in the following studies.

Attachment of Cells to Protein-coated Substrata

EGTA-detached fibroblasts preradiolabeled in their DNA with [³H]thymidine incorporation were tested for their ability to form stable attachments to coated coverslips. The kinetics of cell attachment were directly compared to that on FN-coated coverslips. The presence of BSA in the serumless medium during these and other experiments described below served two functions. (a) To compete with the substratum-bound GAG-binding proteins for nonspecific proteolysis, and (b) to compete with released cellular proteins for binding to areas of uncoated plastic made available by spontaneous release of the preadsorbed GAG-binding proteins. The results for SVT2 cells are shown in Fig. 1. Cell attachment to FN or PF4-coated surfaces was initiated within 5 min of addition of cells with a maximal level achieved at 10–15 min. Plating efficiencies were identical on PF4- and FN-coated substrata and ranged from 50 to 70% during these and other experiments described below served two functions. In contrast to cell responses to FN or PF4, cells failed to attach to the hyaluronate-binding cartilage proteoglycan core or link proteins (Fig. 1). These results indicate that cell surface hyaluronate, although abun-

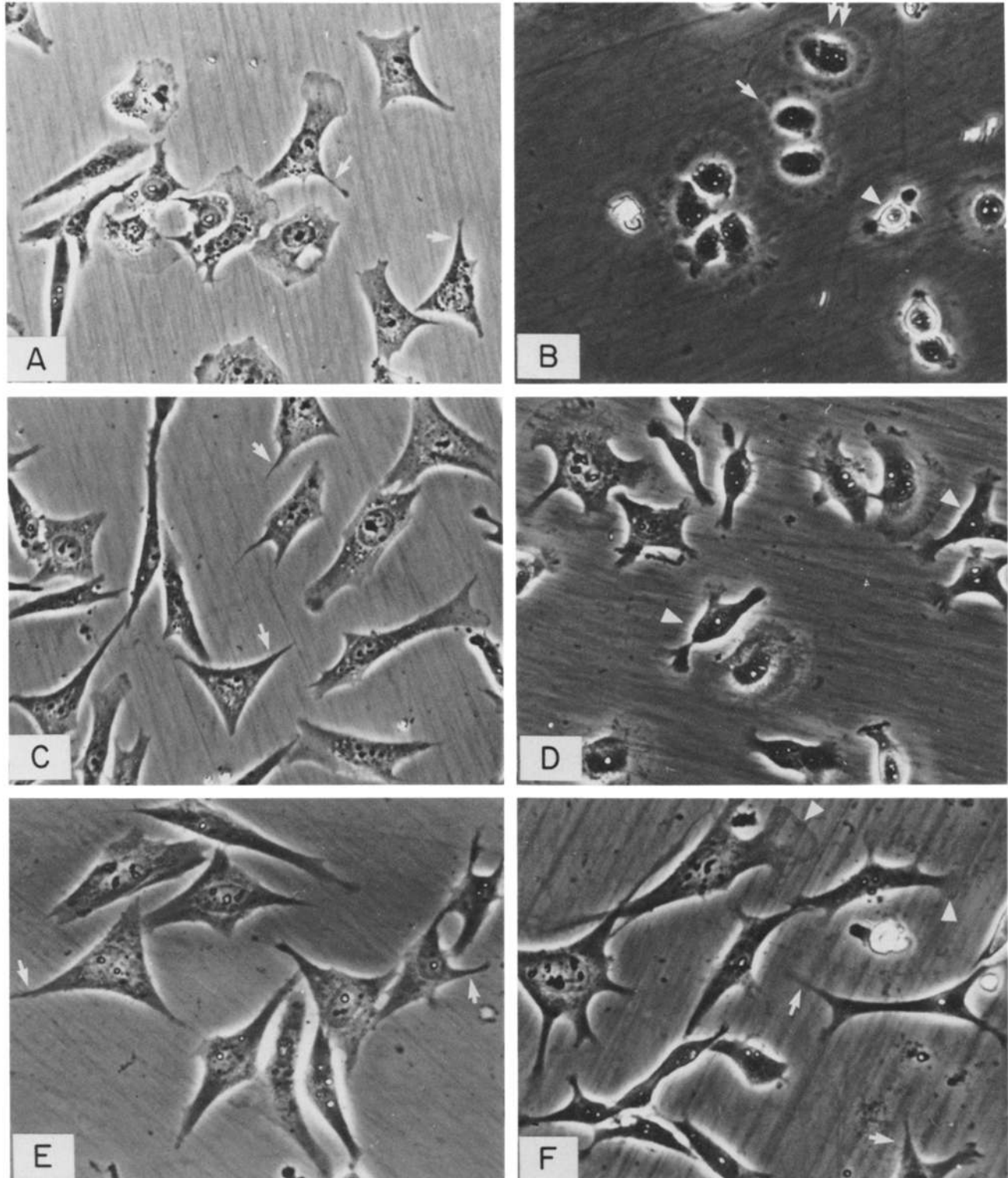


FIGURE 2 Morphology of cells adhering to FN-coated or PF4-coated substrata. SVT2 cells, detached from stock cultures with EGTA, were prepared and inoculated on FN-coated (A, C, and E) or PF4-coated (B, D, and F) tissue culture wells as described in the legend to Fig. 1. After incubation for various times at 37°C, samples were rinsed twice with PBS-II and fixed for 30 min with 3.7% formaldehyde in PBS-II. (A, C, and E) 1, 4, and 24 h of attachment on FN-coated substrata, respectively. Typical SVT2 polygonal morphologies contain tapered linear cell processes (arrows) uncommon in cells on PF4. (B) At 1 h on PF4, cells have spread centrifugally with either one broad lamella encircling the cell (arrow) or multiple lamellae each partially encircling the cell (double arrow). Note the radial folds on broad lamellae. Cells which remain relatively rounded contain multiple cytoplasmic processes which end in well-spread lamellae (arrowhead). (D) At 4 h on PF4, cells have increased their degree of spreading and elongation relative to cells at 1 h. Single or multiple broad lamellae are present on well-spread cells. Less spread cells contain smaller lamellae at ends of extended cytoplasmic processes (arrow heads). (F) By 24 h on PF4, cytoplasmic lamellae contain fewer radial folds (arrow heads) common at earlier attachment times. Linear cytoplasmic processes commonly taper to discrete distal endings (arrows) unlike cells on PF4 at earlier times. $\times 400$.

dant on the cells used in these studies (40), is unable to mediate stable cell attachment to surfaces with demonstrated hyaluronate-binding activity. Attachment kinetics of BALB/c3T3 or Swiss 3T3 cells were identical to those described for SVT2 cells (data not shown).

Morphology of Cell Spreading in Response to FN or PF4

EGTA-detached SVT2 cells were allowed to attach and spread on surfaces coated with FN or PF4. At appropriate times from 1 to 24 h, cultures were rinsed, fixed, and then photographed using phase contrast optics as described in Materials and Methods. Fig. 2 represents typical cell morphologies. Cellular morphologies on FN (Fig. 2A, C, and E) were similar to those on serum-coated surfaces (not shown), since FN is the major attachment and spreading-promoting factor in serum (55). Adherence for 1–4 h on PF4-coated substrata (Fig. 2B and D) resulted in morphologies somewhat distinct from those seen on FN. At 1 h on PF4, oval-shaped cells with broad lamellae were most common (Fig. 2B). These lamellae were

either extended completely around the periphery of the cell (Fig. 2B, single arrow) or terminated after $<180^\circ$ abutting another broad lamella (Fig. 2B, double arrow). The other common morphologic type at 1–4 h on PF4 was a relatively rounded cell containing two or more cytoplasmic extensions ending in smaller lamellae (Fig. 2B and D, arrowhead). Both the broad and smaller lamellae on PF4 contained radial folds, as indicated by phase dark lines (Fig. 2B and D). These radial folds were gently reduced in number on cells spreading for 24 h on PF4 (Fig. 2F, arrow head) and were consistently absent on FN (Fig. 2A, C, and E).

At all times studied, FN-mediated spreading resulted in the formation of retractionlike fibers—linear cytoplasmic processes which tapered to distal areas of discrete attachment (Fig. 2A, C, and E; arrows). The distal cytoplasmic extensions of cells spreading on PF4 more often terminated in relatively broad well-spread lamellae. Only at longer times of attachment were tapered cell processes formed in response to PF4 (Fig. 2F, arrows). This change, together with the absence of the radial lamellar folds at 24 h on PF4, resulted in cell morphologies more similar to those observed on FN. Identical morphologic

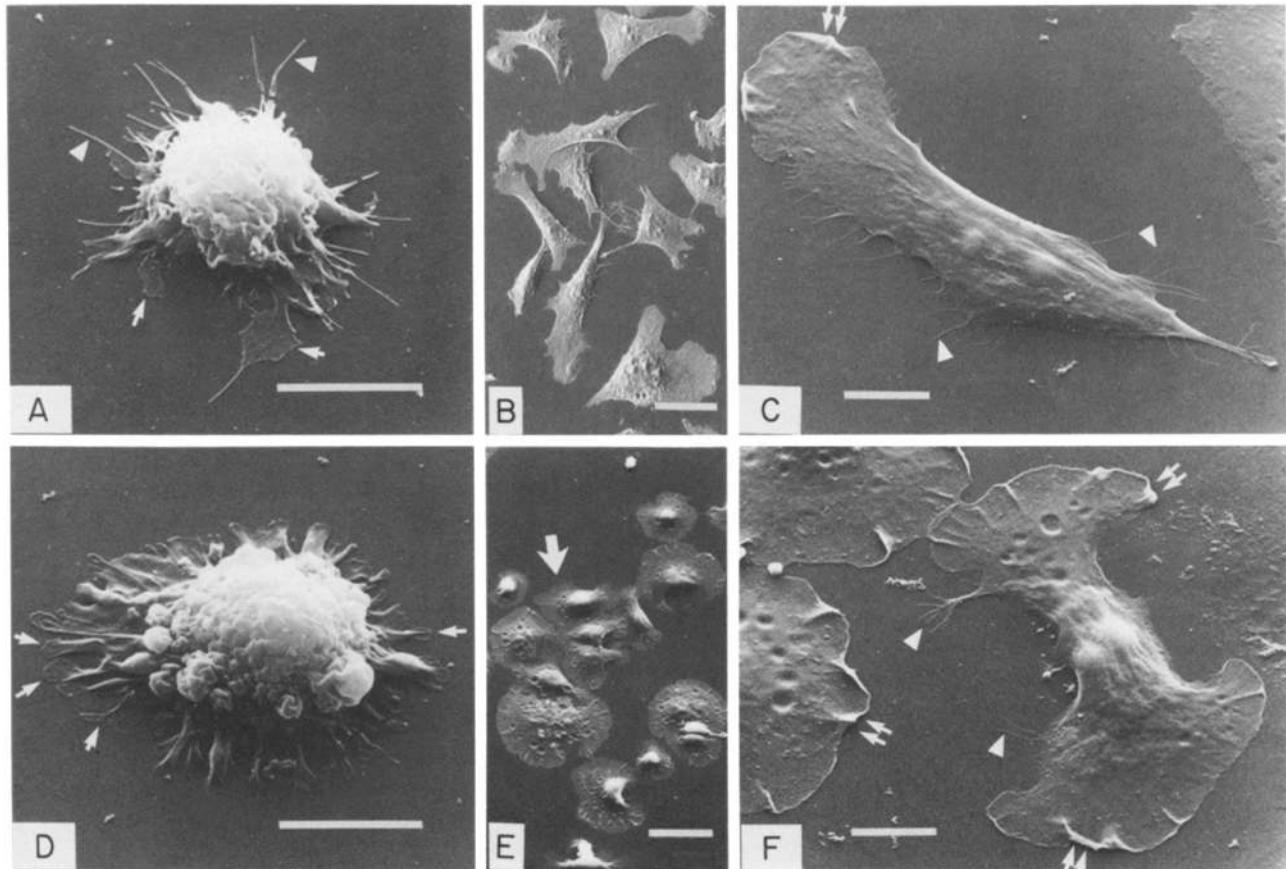


FIGURE 3 Fine structure of cells on FN-coated and PF4-coated substrata. BALB/c3T3 cells, detached from stock cultures with EGTA, were prepared and inoculated onto FN-coated (A–C) or PF4-coated (D–F) Thermanox cover slips as described in the legend to Fig. 1. After incubations for various times at 37°C , coverslips were rinsed and processed for scanning electron microscopy as described in Materials and Methods. Cells at 5 min on FN indicate the initiation of spreading by extension of numerous filopodia (A, arrowheads), some of which evolve into footpads (A, arrows) (42). Cells on PF4 (D) display a similar degree of spreading at 5 min, but their adherent cytoplasmic processes form more numerous primitive footpads at this early time point (D, arrows). By 1 h on FN (B), cells have generated polygonal conformations and form typical leading lamellae and tapered processes in contrast to cells on PF4 (E), which spread centrifugally and possess broad lamellae which encircle most cells. Arrow in E indicates filopodial formation at the periphery of a lamella. A typical cell spread for 2 h on FN (C) exhibits ruffling leading lamellae (double arrow), a trailing cytoplasmic retraction fiber, and numerous long filopodia parallel and apparently adherent to the substratum (arrow head). Cells on PF4 at 2 h (F) also display ruffling lamellae (double arrow) and filopodia (arrow heads). Similar results were observed for SVT2 cells. Bars (A, C, D, and E), $10\ \mu\text{m}$ and (B and E) $40\ \mu\text{m}$.

responses were also observed for BALB/c or Swiss 3T3 cells.

To more effectively evaluate the fine-structure of spreading, we used scanning electron microscopy. EGTA-detached BALB/c3T3 cells were allowed to spread on PF4-coated or FN-coated coverslips. Between 5 min and 2 h, coverslips were rinsed and prepared for scanning electron microscopy as described in Materials and Methods. Fig. 3A and D demonstrate cells that were initiating cytoplasmic spreading after 5 min on FN-coated and PF4-coated substrata, respectively. Cell spreading on both substrata was initiated by binding, extension, and broadening of cytoplasmic processes which first contacted the substratum. The initial cytoplasmic extensions of cells on FN were most commonly filopodial (Fig. 3A, arrow head), which slowly evolved into footpads (Fig. 3A, arrows) as previously reported for serum-coated substrata (42). Although filopodia were also seen during initial cytoplasmic spreading on PF4, these broadened rapidly to form primitive footpad-like structures (Fig. 3D, arrows). Fig. 3B and E demonstrate larger fields of cells after 60 min of attachment on FN or PF4, respectively. While cytoplasmic extensions of cells on FN often ended by tapering to fine discrete distal attachments, these structures were not seen on cells responding to PF4. By 2 h (Fig. 3C and F), cells on both substrata displayed ruffling lamellae (double arrows) and numerous filopodia parallel to and apparently making contact with the substrata (arrow heads).

Inhibition of Attachment and Spreading on Substrata

COMPETITION BY GLYCOSAMINOGLYCANS: If interactions between the cell surface and PF4's glycosaminoglycan binding site are required for these cell responses, then heparin, which binds tightly to PF4, should compete effectively with cell surface GAG. Likewise, chondroitin-4-sulfate, which interacts much less effectively with PF4, should compete poorly (15). EGTA-detached SVT2 cells preradiolabeled with [³H]thymidine were inoculated onto PF4-coated coverslips in the presence of either 50 μg/ml heparin or chondroitin-4-sulfate. The rates of cell attachment under these conditions are presented in Fig. 4. Heparin decreased the cell attachment rate, and induced a sizable time lag of initial attachment and a 50% reduction in maximal attachment. Comparable levels of chondroitin-4-sulfate had no detectable effect (Fig. 4). Those cells attaching in the presence of heparin failed to spread as observed by phase contrast microscopy, and were easily detached from the substrata with minor increases in rinsing strength. Cell attachments in the absence of soluble competing GAG on PF4 that had been pretreated with saturating levels of heparin produced identical results (data not shown). In contrast to cell attachment on PF4 coatings, heparin concentrations of up to 1 mg/ml failed to inhibit cell attachment or spreading on FN.

Cell attachment and cytoplasmic spreading are often regarded as biochemically distinct processes, since the latter usually requires involvement of the cytoskeleton (39). We tested the ability of soluble GAG's to inhibit the spreading of SVT2 cells on PF4 independent of their effect on initial attachment. EGTA-detached cells were allowed to attach for 5 min on PF4 during which cytoplasmic extension and footpad formation have been shown to be initiated (Fig. 3D). The culture medium was then replaced with a medium containing 50 μg/ml heparin or chondroitin-4-sulfate. After further incubation for 90 min, the substrata were rinsed with PBS-II and the adherent cells fixed and photographed using phase contrast

optics. The results are shown in Fig. 5. The addition of chondroitin-4-sulfate after initial cell attachment produced no adverse effects on cellular morphologies observed at 90 min (Fig. 5A and B), whereas heparin completely inhibited cytoplasmic spreading and eventually resulted in detachment of some cells from the substratum (Fig. 5C).

INHIBITION BY GLYCOSAMINOGLYCAN LYASES: EGTA-detached BALB/c3T3 cells preradiolabeled with [³H]thymidine were inoculated onto PF4-coated coverslips in the presence of either 0.02 U/ml *F. heparinum* enzyme or 0.1 U/ml chondroitinase ABC. Attachment on PF4 (Fig. 6A) was completely inhibited with the *F. heparinum* heparinase enzyme under conditions in which biochemical analysis demonstrated that >85% of cell surface heparan sulfate, and <10% chondroitin sulfate and hyaluronate, were degraded (not shown); chondroitinase ABC at levels ten times higher than that present in the *F. heparinum* preparation was without effect (Fig. 6A) even though it resulted in removal of >60% of cell-associated chondroitin sulfate and hyaluronate (6). Pretreatment of the substrata alone or using enzyme preparations that had been heated to 100°C for 10 min failed to inhibit attachment.

The effect of *F. heparinum* enzyme on cell attachment to FN-coated substrata was also examined (Fig. 6B). In contrast to its effects on PF4-mediated cell attachment, the degradation of cell surface heparan sulfate under identical conditions had a minimal effect on FN-mediated attachment, consistent with previous work demonstrating that some other cell surface receptor for FN can mediate at least attachment without the involvement of cell surface heparan sulfate (37, 43, 45, 56).

We examined the effect of *F. heparinum* enzyme treatment on cellular morphology as evaluated by phase contrast microscopy. BALB/c3T3 cells were inoculated onto FN-coated substrata in the presence of chondroitinase ABC or *F. heparinum*

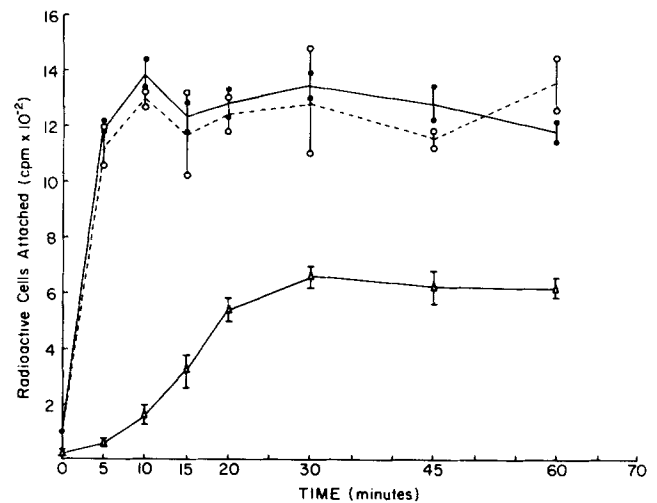


FIGURE 4 Inhibition of PF4-mediated cell attachment by soluble glycosaminoglycans. SVT2 cells radiolabeled with [³H]thymidine and detached from stock cultures with EGTA were inoculated onto PF4-coated coverslips as described in the legend to Fig. 1 in the presence of either 50 μg/ml reference standard heparin (Δ—Δ) or reference standard chondroitin-4-sulfate (○—○). Attachment in the absence of competing GAG served as control (●—●). At appropriate times, duplicate coverslips were gently rinsed three times in PBS-II and scintillation counted to detect bound radiolabeled cells. Inoculation of cells in the absence of soluble GAG on PF4-coated coverslips which had been pretreated with either heparin or chondroitin-4-sulfate produced similar results. Similar results were observed with Swiss and BALB/c3T3 cell lines.

enzyme. More than 90% of the attached cells were spreading by 15 min in the absence of enzyme or in the presence of chondroitinase (Fig. 7A and B); <10% of the attached cells were doing so in the presence of *F. heparinum* enzyme (Fig. 7C). By 60 min, substantial cell elongation and orientation had occurred for control or chondroitinase-treated cells (Fig. 7D and E), while the *F. heparinum* enzyme-treated cells were extending only very primitive cell processes (Fig. 7F). Although cell surface heparan sulfate is not required for simple attachment to FN-coated substrata, these data suggest that heparan sulfate-containing species on the cell surface are essential for the maturation of initial cellular attachments and the development of fully spread cytoplasmic morphologies.

Fine Structure of Adhesions as Revealed by EGTA-induced Retraction of Cells from the Substratum

Fibroblasts on serum-coated substrata respond to treatment with EGTA by rounding up, withdrawing their lamellae, and revealing the fine structure of adhesive contacts at the cell's undersurface (42). The responses of PF4-attached cells to EGTA treatment were compared with those of FN-attached cells. Fibroblasts were detached from stock cultures with EGTA and permitted to attach and spread for 2 h on either PF4 or FN. These cells were then treated with 0.5 mM EGTA in PBS as described in Materials and Methods. At various times during this treatment, the coverslips were rinsed and prepared for scanning electron microscopy. Cells on either substratum responded to 15 min of treatment by rounding of the cell body and initial retraction of peripheral membrane (Fig. 8A and D). Discrete areas of the peripheral membrane remained substratum-bound after 15 min of EGTA treatment of cells on FN and were generally fine and linear (Fig. 8A, arrows). In contrast, the peripheral structures of cells on PF4 remained somewhat broadly spread (Fig. 8D, arrowheads), even though they were contiguous with narrower retracted regions of more proximal cytoplasm (Fig. 8D, double arrowheads). The end-stage cellular responses to 30 min of Ca^{2+} chelation on both substrata were nearly identical (Fig. 8C and F).

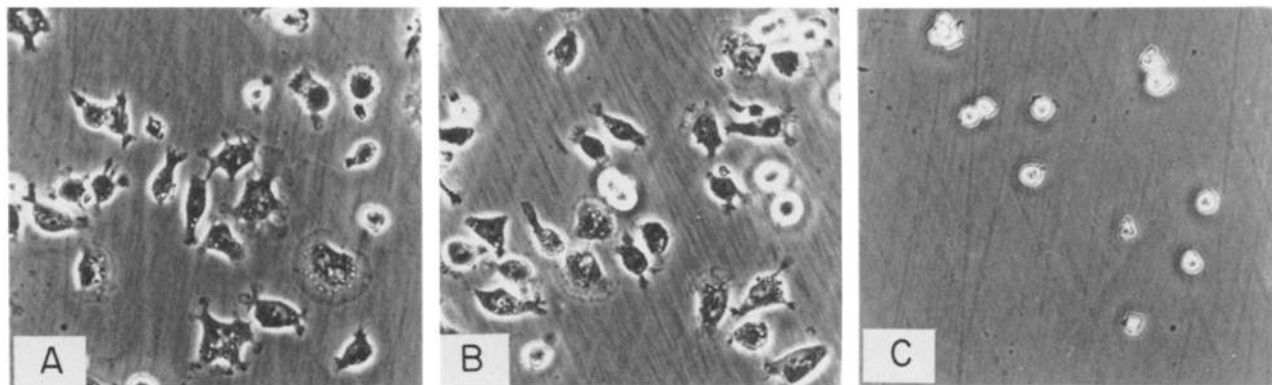


FIGURE 5 Inhibition of PF4-mediated cytoplasmic spreading by soluble glycosaminoglycan. SVT2 cells, detached from stock cultures with EGTA, were inoculated into PF4-coated tissue culture wells as described in the legend to Fig. 1 and incubated for 5 min at 37°C to allow initial cell attachment to occur. At 5 min, attachment medium was removed and replaced with either fresh attachment medium (A) or attachment medium containing 50 µg/ml chondroitin-4-sulfate (B), or heparin (C). Cells were then incubated at 37°C for 90 min more at which time wells were rinsed with PBS-II and the attached cells fixed as described in Fig. 2. Identical results were obtained with BALB/c3T3 and Swiss 3T3 cells. × 220.

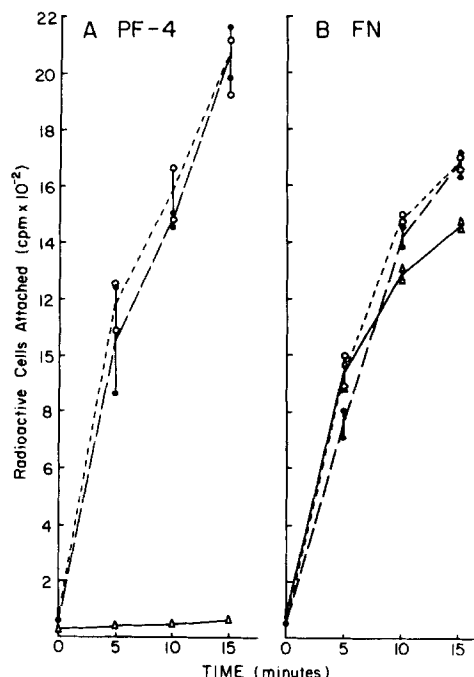


FIGURE 6 Effect of GAG lyases on cell attachment. BALB/c3T3 cells, radiolabeled with [3H]thymidine, were detached from stock cultures with EGTA and inoculated onto PF4-coated (A) or FN-coated (B) coverslips in PBS-II containing 2 mg/ml BSA plus either 0.02 U/ml heparinase as *F. heparinum* enzyme (Δ — Δ) or 0.1 U/ml chondroitinase ABC (\circ — \circ), which is ten times the level of chondroitinase activity found in the *F. heparinum* enzyme. The control (\bullet — \bullet) contained no enzyme. Cells were shaken for 5 min at 37°C to inhibit cell attachment and to allow cell surface polysaccharide digestion, after which shaking was stopped and the attachment assay started (0 min). Substituting untreated *F. heparinum* enzyme with enzyme that had been heated for 10 min at 100°C, or pretreating only the substrata with enzyme for 15 min at 37°C had no inhibitory effect. Similar results were obtained with SVT2 cells. The disparity in the levels of radioactivity remaining associated with coverslips in the controls (\bullet — \bullet) and chondroitinase containing samples (\circ — \circ) of A and B is due to different radioactive cell preparations used in A and B and not to significant differences in number of cells attached.

Cytoskeletal Reorganization in Spreading Cells

Cell spreading in cultures usually involves cytoskeletal reorganization (39). Therefore microtubules and microfilaments were examined to determine if cell surface heparan sulfate interactions with an extracellular matrix, in this case PF4, generates signals for the reorganization of specific cytoskeletal structures. BALB/c3T3 cells were inoculated onto glass coverslips coated with either FN or PF4. The adherent cells were fixed, permeabilized, and double stained using anti 6S tubulin IgG and NBD-phalloidin to visualize microtubules and microfilaments bundles, respectively (Fig. 9). The microtubular patterns of cells spread on FN (Fig. 9A) in serumless medium were similar to those typically seen in fibroblasts growing in serum-containing medium (not shown) (5). The microtubular network was visualized throughout the cytoplasm and approached the periphery. In contrast, cells on PF4 lacked detectable microtubules in their very adherent, broadly spread lamellae (Fig. 9C), even though networks were present in the center of the cells.

By 2 h of attachment, the majority of cells on FN had formed prominent longitudinal microfilament bundles or stress fibers which spanned throughout the cells (Fig. 9B). Stress fibers were rarely observed in cells on PF4 (Fig. 9D), but very common were fine linear accumulations of staining at the periphery of lamellae, which in particular instances extended more centrally toward the cell bodies. These fine microfilaments had formed in regions of cytoplasm completely devoid of detectable microtubules. Although bright regions of fluorescent staining at lamellae can result from membrane ruffling,

visualization with phase contrast optics indicated that the majority of the linear F-actin staining in cells on PF4 could not be attributable solely to membrane topography.

DISCUSSION

Molecular interactions between GAG's (and other components of the cell surface) and the extracellular matrix have been implicated in determining states of cellular adhesiveness and motile behavior in vitro and in vivo. Kraemer and co-workers have isolated CHO cell variants whose content of cell surface heparan sulfate and hyaluronate is directly and inversely related, respectively, with their strength of adhesion in vitro (1, 2). Consistent with a role for direct heparan sulfate-FN interactions in the formation of cell-substratum adhesions are the observations that the cellular material which remains substratum-bound after EGTA-mediated detachment of fibroblasts from FN-containing substrata is greatly enriched for heparan sulfate (40) and particularly for FN-binding heparan sulfate sequences (24, 25). Furthermore, Hedman et al. (18) have recently shown that FN and heparan sulfate proteoglycans codistribute in the pericellular matrix of cultured human fibroblasts. In addition, use of *F. heparinum* enzyme to release heparan sulfate from suspensions of skin fibroblasts results in removal of some FN from the cells (47). Numerous studies in vivo have correlated increases in hyaluronate content of tissue with cellular migration and increases in sulfated GAG's with the cessation of cell movement during tissue development and regeneration (3, 32, 38, 46, 52, 53). Because most of the data associating GAG's with varying states of cell adhesive behavior

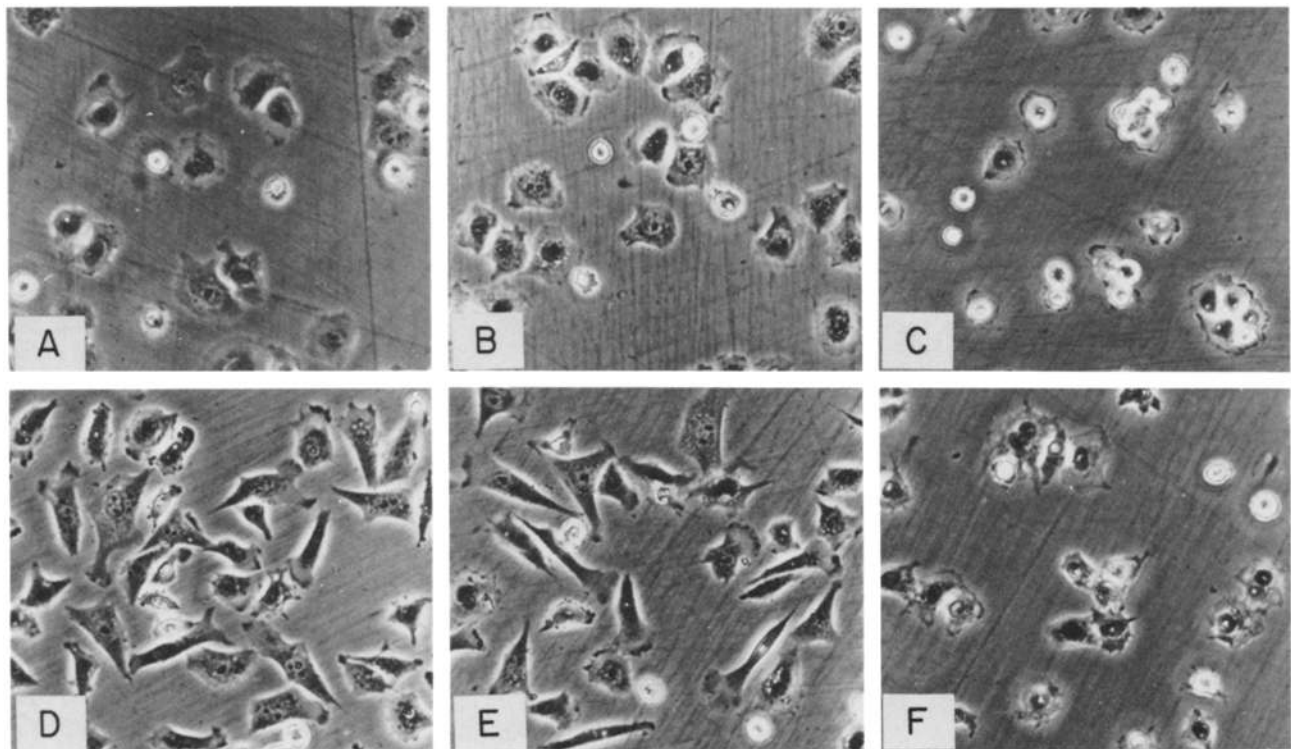


FIGURE 7 Inhibition of FN-mediated cell spreading by *F. Heparinum* enzyme. BALB/c3T3 cells, detached from stock cultures with EGTA, were inoculated into FN-coated tissue culture wells containing PBS-II, 2 mg/ml BSA plus either no enzyme (A, D), 0.02 U/ml heparinase activity as *Flavobacter heparinum* enzyme (C, F), or 0.1 U/ml chondroitinase ABC (B, E) and allowed to attach and spread as described in Fig. 6. After either 15 min (A-C) or 60 min (D-F) incubations at 37°C, wells were rinsed with PBS-II and the attached cells fixed for 30 min in 3.7% formaldehyde in PBS-II. Substituting untreated *F. heparinum* enzyme with enzyme that had been heated for 10 min at 100°C or pretreating the substrata with enzyme for 15 min at 37°C resulted in no inhibition of cytoplasmic spreading. Identical results were obtained with SVT2 cells. $\times 235$.

have been inferential in nature, we chose a direct test of fibroblast adhesive responses to specific GAG-binding proteins.

These studies demonstrate that normal and SV40-transformed murine fibroblasts attach and spread on tissue culture substrata coated with the heparan sulfate-binding glycoprotein PF4. That these cellular responses were mediated by direct binding of cell surface heparan sulfate to the substratum-bound PF4 was verified using two complementary approaches; (a) inhibition of cell attachment by specific removal of cell surface heparan sulfate and (b) competition for cell attachment and cytoplasmic spreading by addition of soluble heparin. Although PF4 has weak affinity for chondroitin sulfate, chondroitinase digestion of cells had no effect on either PF4-mediated cell attachment or cytoplasmic spreading. Cell surface chondroitin sulfate, therefore, does not mediate adhesion to this extracellular matrix. The similarity in the PF4-mediated adhesion responses of the BALB/c3T3 and SV40-transformed BALB/c3T3 cells is consistent with their similar cell surface heparan sulfate content (40).

For a cell surface receptor to bind to one or more components of the extracellular matrix and direct cell adhesion, it must be stably associated with the cell surface, accessible to interact

with the matrix, and sufficiently mobile to accumulate in focally high concentrations. A class of cell surface heparan sulfate has been shown to exist covalently linked to protein in a proteoglycan form (22, 36). Certain heparan sulfate proteoglycans insert into lipid bilayers via a hydrophobic core protein and may exist as integral membrane components on the surface of the cell (20, 30, 35). These plasma membrane forms of heparan proteoglycan should be so tightly bound to the cell surface that they would mediate at least simple cytoplasmic attachment responses to heparan sulfate-binding matrices such as that provided by PF4 or FN. Furthermore, the data in this report indicate that heparan sulfate is organized at the cell surface in sufficient quantity and density to also stimulate cytoplasmic spreading.

That fibroblasts were unable to demonstrate even simple attachment to substratum-bound proteins with specific affinity for hyaluronate reflects a fundamental difference between this GAG's and heparan sulfate's associations with the cell surface. Unlike heparan sulfate, the majority of cell surface hyaluronate has not been shown to be covalently associated with protein (28). Incubating the adhesion sites of murine fibroblasts, left bound to serum-coated substrata after EGTA-mediated detachment of cells, with either low concentrations of guanidine

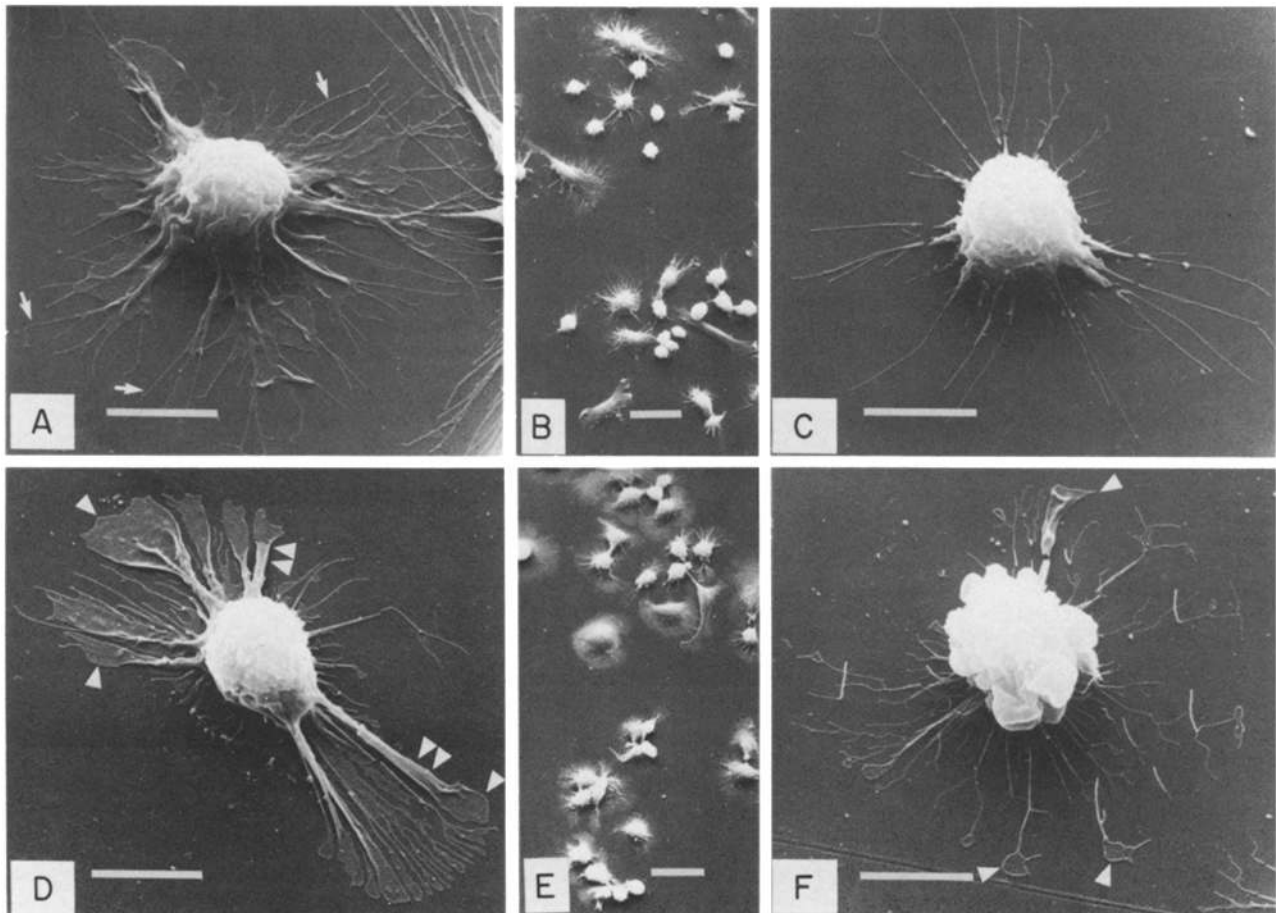


FIGURE 8 EGTA-induced retraction of spread cells. BALB/c3T3 cells were prepared and inoculated onto FN-coated (A–C) or PF4-coated (D–F) coverslips as described in the legend to Fig. 3. After 2 h of incubation at 37°C, the medium was removed and the cells rinsed three times with PBS. Attached cells were then incubated for 15 (A, D) or 30 min (B, C, E, and F) in 0.5 mM EGTA to reveal the fine structure of cell surface adhesions resistant to Ca^{2+} chelation, and prepared for scanning electron microscopy as described in Materials and Methods. Arrows (A) indicate that, after initial EGTA-induced cytoplasmic retraction, cells on FN form fine linear cytoplasmic processes which remain present on fully retracted cells (C). The comparable EGTA-resistant adhesions of PF4-adherent cells tend to remain well-spread at the cell peripheries (D and F, arrow heads), even though structures more proximal to the adhesions are collapsed and detached from the substratum (D, double arrows; F). Bars (A, C, D, and F), 10 μm and (B and E) 40 μm .

hydrochloride (23) or hyaluronidase (9) results in release of >80% of their hyaluronate content with little release of cell surface glycoproteins, cytoskeletal components, or heparan sulfate. These observations, along with the data presented here and elsewhere demonstrating that chondroitinase ABC digestion of cells has no effect on cell attachment or spreading on FN or serum (6), indicate that cell surface hyaluronate is not required for the formation or continued integrity of the adhesions which form between fibroblasts and FN-containing substrata in vitro.

A comparison of the cytoplasmic spreading sequence of cells on PF4 or FN revealed that they respond to both by initial extension and attachment of multiple cytoplasmic processes which then expand laterally to form primitive adhesive footpads (42). PF4 stimulated the formation of very broad cytoplasmic lamellae which indicates that heparan sulfate has the potential to mediate some aspects of the cytoplasmic spreading on FN (which also binds HS [25, 56]). Although digestion of cell surface heparan sulfate with *F. heparinum* enzyme failed to affect cell attachment on FN, it dramatically inhibited the rate of cytoplasmic spreading and development of fully spread morphologies. Since the three cell lines used in these studies lack collagen in their substratum adhesion sites (8), FN's previously described cell surface binding site, which is spatially

distinct from its collagen and heparan sulfate binding sites (43, 45, 56), probably mediates the attachment response of heparinase-treated cells.

A comparison of the fine structure of cell adhesions, as revealed by EGTA-mediated cytoplasmic retractions from substrata, revealed that the EGTA-resistant adhesions consist of a radial network of linear contacts with the substratum. Since PF4 and FN both bind heparan sulfate independent of the presence of Ca^{++} (24, 27), it is likely that these EGTA-resistant adhesions represent areas of highly stabilized interactions between focally high concentrations of heparan sulfate and the substrata. Consistent with this hypothesis is that the cellular material remaining on FN-containing substrata after EGTA-mediated cell release is significantly enriched relative to whole cells in cell surface heparan sulfate (33). EGTA may thus effect cell retraction from these substrata by destabilizing Ca^{++} -dependent cell surface or cytoplasmic interactions that are distinct from those which occur directly between cell surface heparan sulfate and the substratum.

Microtubule and microfilament organizations were examined to determine whether cell surface heparan sulfate binding to a substratum was sufficient to stimulate cytoskeletal reorganization. Although microtubules were polymerized to a significant extent on either PF4 or FN, the distribution of the

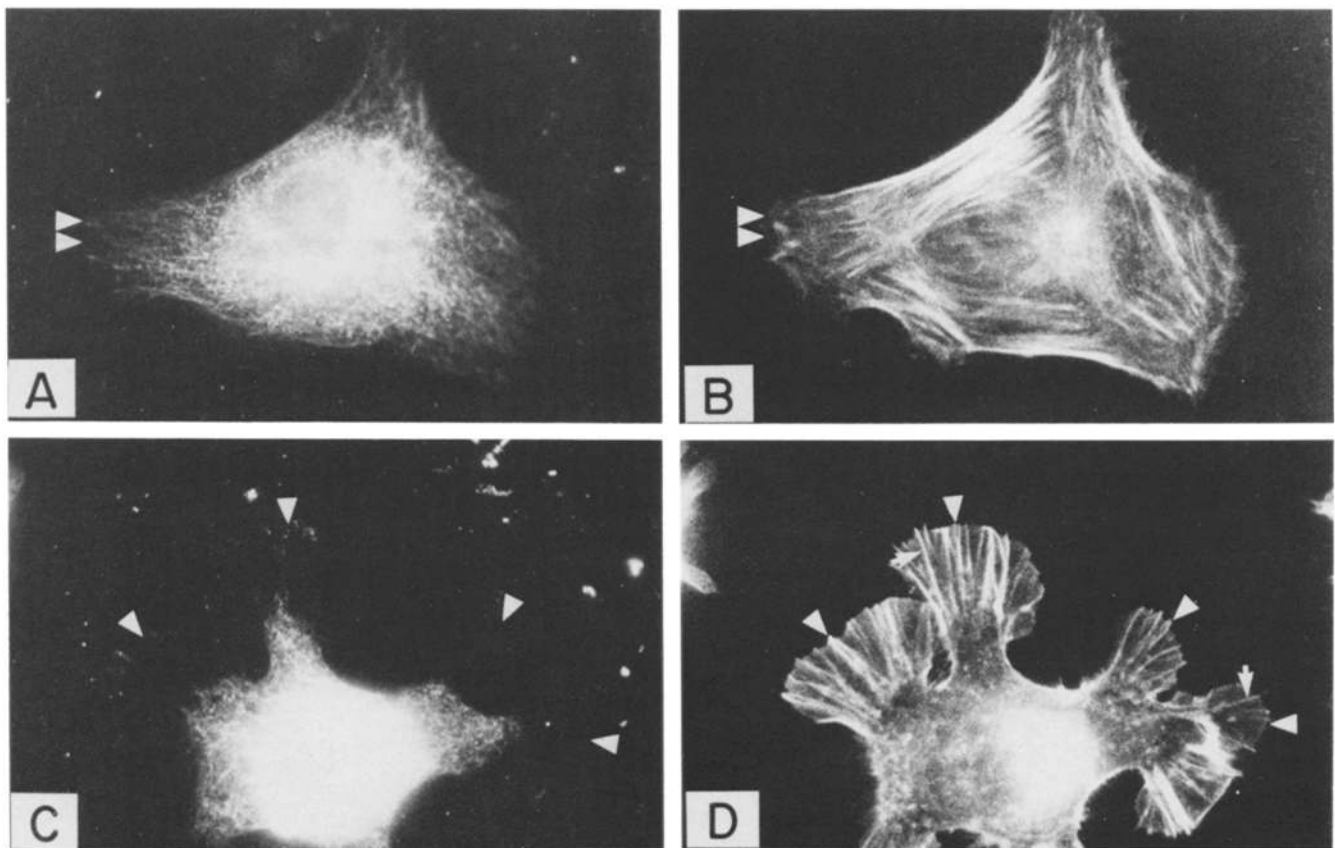


FIGURE 9 Reorganization of microtubules and microfilaments during spreading. BALB/c3T3 cells were detached from stock cultures with EGTA and inoculated onto FN-coated (A and B) or PF4-coated (C and D) glass coverslips as described in the legend to Fig. 1. After 2 h of attachment, the cells were fixed and double labeled as described in Materials and Methods to visualize both microtubules (A and C) and microfilaments (B and D). Double and single arrowheads indicate peripheral limits of cells on FN and PF4, respectively, as determined by phase contrast microscopy. Microtubules of cells on FN (A) are visualized throughout peripheral lamellae in contrast to the microtubules of cells on PF4 (C) which concentrate more proximal to the nucleus and are not present in well-spread lamellae. The majority of cells on FN contain well-defined longitudinal stress fibers (B) which are absent on PF4-attached cells (D). Cells on PF4 contain numerous fine filaments of F-actin (D, arrows) throughout lamellae that do not coincide with membrane folds or ruffles but may be associated with microspikes (see Discussion). $\times 750$.

microtubular networks differed. The absence of microtubules in the broad lamellae of cells on PF4 indicates that microtubules do not passively distribute throughout the cytoplasm during changes in fibroblast morphology. Furthermore, simple heparan sulfate directed cell spreading cannot in itself generate the proper biochemical signals required for the development of a microtubular network in newly spreading lamellae. On the other hand, microfilament reorganization has been more closely linked to the state of cell-substratum interactions, cellular morphology and motility than the organization of microtubules (4). Staining of cells with the F-actin-specific probe NBD-phalloidin revealed fine microfilaments in the absence of microtubules at the most adherent periphery of lamellae spreading on PF4, indicating spatially distinct processes of cytoskeletal reorganization. Cells on PF4 contain microspikes and associated core structures when observed with differential interference contrast optics (Lattera, Norton, Izzard, and Culp. Manuscript submitted for publication.). Since these structures have been demonstrated to contain fine linear bundles of microfilaments (19, 48), the linear filaments that we observed in this study could correspond to such structures. Cell surface heparan sulfate binding to an extracellular matrix can thus stimulate the reorganization of microfilaments; the development of more extensive microfilament networks in cells responding to FN-coated substrata possibly requires further interactions between substratum-bound FN and cell surface components other than heparan sulfate (such as the unidentified FN receptor on the cell surface).

In conclusion, these experiments provide direct evidence that cell surface heparan sulfate proteoglycans have the potential to direct significant cell adhesive behavior on extracellular matrices. Furthermore, cell surface heparan sulfate and the uncharacterized cell surface FN receptor appear to direct separate but complementary spreading functions on FN. Although the cell surface receptor for FN alone may direct simple attachment, cell surface heparan sulfate proteoglycans are required for a complete physiologic response. Cooperative interactions of multiple distinct cell surface receptors with adhesive components of the extracellular matrix are therefore likely to determine the adhesive state and migratory behavior of cells in simplified *in vitro* systems as well as *in vivo*.

The authors thank Drs. Pierluigi Gambetti and James Scillian of The Institute of Pathology, Case Western Reserve University School of Medicine for loan of their fluorescent microscopic equipment and Ms. Grace Maresh for her excellent technical assistance with the scanning electron microscopy.

This work was supported by National Institutes of Health Research Grants CA27755 and AM25646 (L. A. Culp) and the Medical Research Service of the Veterans Administration (J. E. Silbert). J. Lattera was supported by United States Public Health Service Training Grant GM07250.

Note Added in Proof: The evidence provided in this study, namely that adhesion to the PF4-coated substratum permits reorganization of membrane-associated microfilament bundles, indicating transmembrane linkage between heparan sulfate proteoglycan on the surface of the cell and elements of the microfilamentous network in the cytoplasm, is supported by an independent study. Alan C. Rapraeger and Merton Bernfield (in *Extracellular Matrix*, 1982, S. P. Hawkes and J. L. Wang, editors, Academic Press, NY, 265-269) have established that actin binds to the core protein of heparan sulfate proteoglycan on the internal side of the membrane. This binding would provide one possible mechanism for transmembrane linkage of these elements.

Received for publication 28 June 1982, and in revised form 13 September 1982.

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