Adhesion of Glycosaminoglycan-deficient Chinese Hamster Ovary Cell Mutants to Fibronectin Substrata

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Abstract. We have examined the role of cell surface glycosaminoglycans in fibronectin-mediated cell adhesion by analyzing the adhesive properties of Chinese hamster ovary cell mutants deficient in glycosaminoglycans. The results of our study suggest that the absence of glycosaminoglycans does not affect the initial attachment and subsequent spreading of these cells on substrata composed of intact fibronectin or a fibronectin fragment containing the primary cell-binding domain. However, in contrast to wild-type cells, the glycosaminoglycan-deficient cells did not attach to substrata composed of a heparin-binding fibronectin fragment. Furthermore, the wild-type but not the glycosaminoglycan-deficient cells formed F-actin–containing stress fibers and focal adhesions on substrata composed of intact fibronectin. We propose, therefore, that cell surface proteoglycan(s) participate in the transmembrane linking of intracellular cytoskeletal components to extracellular matrix components which occurs in focal adhesions.

ADHESION to a supporting substrate is of vital importance for normal cell function. When a suspension of cells is seeded on a substratum, the cells will initially attach. Subsequently, a reorganization of cytoskeletal components occurs which results in a flattening and spreading of the cell body and eventually in the formation of specialized contacts (e.g., focal adhesions) between the cell surface and the extracellular matrix (for a review see Grinnell, 1978). Some cells, such as endothelial cells and fibroblasts, may recognize and adhere to several components in the extracellular matrix, e.g., fibronectin, laminin, vitronectin, and fibrinogen (for reviews see Yamada, 1983; Mosher, 1984; Hynes, 1986). Although the primary cell attachment sites in some of the adhesive proteins are structurally related (Ruoslahti and Pierschbacher, 1986), cellular receptors involved in attachment to one particular matrix component may be functionally specific (Johansson et al., 1981; Dedhar et al., 1987). Thus, a cell may have several mechanisms for substrate adhesion composed of distinct receptors that recognize different matrix proteins. However, in some cases, the later stages of cell spreading and focal adhesion formation appear to occur similarly on different matrix components (Couchman et al., 1983) and hence may involve additional components common to different adhesive mechanisms.

All adhesion proteins examined so far have been shown to bind sulfated glycosaminoglycans. This has led to the suggestion that cell-surface glycosaminoglycans are involved in cell-substrate adhesion (for reviews see Höök et al., 1984, 1986; Culp et al., 1986). Indirect evidence in support of this hypothesis has been obtained, although it has previously not been possible to directly compare the adhesive properties of related cell lines varying only in their proteoglycan content. With the development of cell mutants deficient in glycosaminoglycan biosynthesis (Esko et al., 1985, 1986, 1987), a new set of tools became available for functional studies of glycosaminoglycans. In the present study glycosaminoglycan-containing Chinese hamster ovary (CHO) cells were compared with those of glycosaminoglycan-deficient mutants with respect to their ability to attach, spread, and form focal adhesions and stress fibers on fibronectin substrata.

Materials and Methods

Materials

Ham's F-12 growth media and trypsin were obtained from Gibco, Grand Island, NY; fetal bovine serum from Flow Laboratories, Inc., McLean, VA or from HyClone Laboratories, Logan, UT; tissue culture flasks from NUNC, Irvine Scientific, Santa Ana, CA; and 24-well Linbro tissue culture plates from Flow Laboratories, Inc. [^{35}S]Methionine (0.1–0.5 Ci/mMol), [^{35}S]sulfate (25–40 Ci/mg), and Aqueous Counting Scintillant were purchased from Amersham Corp., Arlington Heights, IL; glass coverslips were from Fisher Scientific Co., Atlanta, GA; Whatman 3MM filter paper was from Whatman Inc., Clifton, NJ; DEAE-Sephacel and Sephadex G-50 were from Pharmacia, Piscataway, Inc., NJ. Pronase was from Calbiochem-Behring Co., San Diego, CA. Nitrobenzoxadiazole (NBD) phallacidin was purchased from Molecular Probes, Inc., Eugene, OR. All other reagents used were purchased from Sigma Chemical Co., St. Louis, MO.

Cell Culture

Wild-type CHO cells (CHO-K1) were obtained from the American Type Culture Collection (Rockville, MD) (CCL 61). Mutants deficient in xylosyl-

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transferase (745) and galactosyltransferase I (761 and 650) were isolated and characterized as previously described by Esko et al. (1985, 1986, 1987). Cells were maintained in Ham's F-12 medium (Ham, 1965) supplemented with 10% (vol/vol) FBS and used between the second and twelfth passages from their original isolation. The cells were routinely maintained in monolayer culture and detached by trypsin treatment for sequential passage every 3–4 d.

Preparation of Substrates

Fibronectin was purified from outdated human plasma (American Red Cross, Birmingham, AL) according to the method of Engvall and Ruoslahti (1977) as modified by Miekka et al. (1982). Fibronectin used to generate specific fragments was purified according to the method of Vuento and Vaheri (1979). Isolation and characterization of a 31-kD "heparin-binding" and a 105-kD "cell-binding" fragment generated by chymotrypsin digestion of fibronectin was previously described by Woods et al. (1986). 24-well tissue culture plates (2.0 cm^2 surface area per well) or glass coverslips (1.2 cm^2 surface area per coverslip) were coated with human fibronectin or equimolar amounts of 105-kD cell-binding fragments or 31-kD heparin-binding fragments. Nonadhesive substrates were made by coating the wells with 1% BSA previously heat treated at 56°C for 60 min and filtered. The coated wells or coverslips were processed similar to the method of Couchman et al. (1983). Briefly, they were air dried at room temperature; before use the substrate was rehydrated by incubation with PBS (8.0 g NaCl, 0.2 g KCl, 0.2 g KH_2PO_4, 1.73 g Na_2HPO_4, 0.13 g $CaCl_2 \cdot 2H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, H_2O to 1 liter, pH 7.3) two times for 15 min and nonspecific binding sites were blocked by incubation with 1% heat-treated BSA. The substrate was then rinsed with PBS and the appropriate assay buffer.

Cell Attachment Assay

Cells (2×10^5) were seeded into 80 cm^2 tissue culture flasks and incubated for 24 h. At this time, the medium was replaced with 20 ml of fresh growth medium containing 0.125 μCi/ml of [^{35}S]methionine. After further incubation for 48–72 h at 37°C, cells were detached with trypsin, centrifuged, resuspended in 30 ml of growth medium containing radiolabel at the same specific activity, and incubated in suspension culture on an orbital shaker at 170 rpm for 10–12 h before use. In all adhesion experiments 10 μg/ml of cycloheximide was added to culture media 2 h before harvest and included in subsequent washing and assay buffers. At harvest the cell suspensions were centrifuged, washed one to two times, and resuspended in assay buffer. Cell numbers were determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL).

[^{35}S]Methionine-labeled cells (5×10^4 in 0.5 ml of 8.0 g NaCl, 0.35 g KCl, 0.16 g $MgSO_4 \cdot 7\ H_2O$, 0.18 g $CaCl_2 \cdot {}^2H_2O$, 2.4 g Hepes, H_2O to 1 liter, pH 7.4 buffer 3[1] supplemented with 0.06 mg/ml of heat-treated BSA and 10 μg/ml of cycloheximide) were seeded in 24-well tissue culture plates coated with the appropriate substrate. The plates were incubated at 37°C for the indicated periods of time and the percent of cell attachment was determined essentially as described by Couchman et al. (1983). Briefly, unattached cells were collected, wells were washed once with 0.5 ml of PBS, and the wash was combined with the unattached cells. Attached cells were solubilized with 0.5 ml of 1% SDS in PBS for 3–6 h at 37°C and collected. The wells were washed with 2×0.5 ml of PBS and the wash combined with the solubilized cells. Radioactivity in the different fractions was quantified by scintillation spectrometry and the values obtained were expressed as percent of total radioactivity recovered. The percent of cells attached was calculated assuming that all radioactivity is associated with cells. Each value is the average of at least two determinations.

Analysis of Cell Spreading, Stress Fiber, and Focal Adhesion Formation

Cells (3.5×10^4 cells/well) were seeded onto substrate-coated glass coverslips in 24-well tissue culture plates as described above.

Cell spreading was analyzed as follows: After incubation for 3 h at 37°C, the cells were fixed with 3% glutaraldehyde in buffer 3 for 30 min at room temperature and washed three times for 15 min in PBS without calcium and magnesium salts (PBS^-). The coverslips were inverted and mounted onto a drop of PBS^- on glass microscope slides and sealed with clear nail varnish. Fixed cells were observed by phase-contrast microscopy using a Nikon Optiphot microscope. The spread cell area was measured using a Nikon drawing device in conjunction with a micro-plan II (Laboratory Computer Systems, Inc., Cambridge, MA).

For analysis of stress fibers, cells were incubated for 3 h at 37°C in buffer 3, fixed in 3.7% formaldehyde in PBS^- (5 min at room temperature), washed with PBS (15 min), 0.1 M ammonium chloride in PBS (15 min), rinsed 15 min in PBS, and permeabilized with 0.1% Triton X-100 in PBS (10 min). The cells were stained with NBD-phallacidin (diluted in PBS according to the recommendations of the manufacturer) for 30 min at room temperature and washed twice with PBS. The coverslips were inverted, mounted on a drop of mounting media on glass microscope slides, and sealed with clear nail varnish. The fluorescent image was photographed using a Nikon Optiphot microscope. When the same cell was examined for the presence of stress fibers and focal adhesions, the coverslips containing the fixed cells were mounted on a drop of PBS on the microscope slides. The fluorescent image was photographed before analysis by interference reflection microscopy. When cells were only examined for focal adhesions, glutaraldehyde-fixed cells (see above) were used. Photographs were taken on Ilford HP5 film.

Quantitation of Glycosaminoglycans in Monolayer and Suspension Cultures

Cells (2×10^5) were seeded into 80 cm^2 tissue culture flasks and incubated for 24 h. At this time, the medium was replaced with 20 ml of sulfate-free growth medium and [^{35}S]sulfate was added. Cells were incubated 48–72 h at 37°C and either used directly for analysis of glycosaminoglycan content or transferred to suspension culture in similar radiolabeled medium for an additional 10–12 h. Suspension cultured cells were harvested by centrifugation and the cell pellet was washed in PBS. The medium plus the wash was designated the "medium compartment." The medium and a PBS wash of monolayer cultured cells were similarly combined. The cell monolayer was detached with 0.25% trypsin (wt/vol) and the suspension-cultured cell pellet was resuspended in 1 ml of 0.25% trypsin. After 3 min at 37°C, the trypsin digestions were terminated by addition of soy bean trypsin inhibitor (0.25% [wt/vol] final concentration). The cell suspensions were centrifuged and the supernatants designated the "cell-surface compartments." The cell pellets were resuspended in PBS and the number of cells were counted followed by solubilization in 5% Triton X-100. These suspensions were designated the "intracellular compartments." All samples were stored at −20°C until analyzed.

Samples (0.5 ml) from each compartment were mixed with 0.5 ml of 0.64 M NaCl, 80 mM sodium acetate, pH 5.4, 4 mg/ml of pronase, 250 μg/ml of heparin and incubated for 24 h at 45°C. [^{35}S]sulfate-labeled glycosaminoglycans were quantified by spotting digested samples on Whatman 3MM paper (Whatman, Inc.) followed by precipitation with 1% cetylpyridinium chloride in 0.3 M NaCl as described by Wasteson et al. (1973). Radioactivity in the precipitates was quantified by liquid scintillation spectrometry and the data were normalized to equal cell numbers. Amounts of labeled glycosaminoglycans recovered from wild-type cells were set to 100%.

Characterization of Cell Surface Glycosaminoglycan

Pronase-liberated glycosaminoglycans were isolated by anion-exchange chromatography on a DEAE-Sephacel column (0.5 ml) equilibrated with DEAE buffer A (50 mM Tris, 4 M urea, 0.2 M NaCl, pH 8.0) containing 0.1% Triton X-100, 20 mM EDTA, 10 mM *N*-ethylmaleimide, 0.2 mM phenylmethylsulfonyl fluoride. The column was washed with 5 ml of DEAE buffer B (DEAE buffer A with 50 mM sodium acetate replacing Tris, pH 4.0) and radioactive glycosaminoglycans were eluted with DEAE buffer C (DEAE buffer B with 1.5 M NaCl). After dialysis against water, samples

Table I. Biochemical Characterization of CHO Cell Mutants

Strain	Defective enzyme
Wild type	–
Mutant	
745	Xylosyltransferase
761	Galactosyltransferase I
650	Galactosyltransferase I

1. *Abbreviations used in this paper*: buffer 3, 8.0 g NaCl, 0.35 gKCl, 0.16 g $MgSO_4 \cdot 7\ H_2O$, 0.18 g $CaCl_2 \cdot {}^2H_2O$, 2.4 g Hepes, H_2O to 1 liter, pH 7.4; NBD, nitrobenzoxadiazole; PBS^-, PBS without calcium and magnesium.

were mixed with an equal volume of 60 mM sodium acetate, 0.1 M Tris, pH 8.0, 2 mg/ml heat-treated BSA, 0.1 U of chondroitinase ABC, and incubated at 37°C for 15 h. Another portion of the sample was treated with nitrous acid according to the method of Shively and Conrad (1976). To analyze the extent of polysaccharide depolymerization by the different treatments, aliquots of each sample (5,000 cpm) were chromatographed on a column (1 × 45 cm) of Sephadex G-50 equilibrated and eluted with 0.5 M NaCl in PBS. 1-ml fractions were collected and analyzed for ^{35}S radioactivity.

Results

Characterization of Glycosaminoglycan-deficient CHO Cell Mutants

The CHO cell mutants used in this study are summarized in Table I. Mutant 745 is defective in the chain initiation xylosyltransferase whereas mutants 761 and 650 are defective in galactosyltransferase I (Esko et al., 1985, 1986, 1987). Because both enzymes are involved in the formation of the common glycosaminoglycan–protein linking region, the synthesis of both heparan sulfate and chondroitin sulfate is diminished in the mutants. Radiolabeled glycosaminoglycans recovered from the culture medium, the cell surface, and intracellular pools were quantified for the different strains as described in the experimental procedures. The results (Fig. 1, *a* and *b*) show that mutants 745 and 761 incorporated little, if any, of the labeled precursors into glycosaminoglycans. Mutant 650 is "leaky" and the levels of labeled glycosaminoglycans recovered from each of the different compartments from this mutant were consistently higher than those recorded for 745 or 761. However, the amount of radiolabeled glycosaminoglycans synthesized by mutant 650 was never >30% of that produced by wild-type cells. When cells in monolayer were incubated with [^{3}H]glucosamine and the amount of radiolabeled glycosaminoglycans in the different compartments was determined, qualitatively similar results were obtained: mutants 745 and 761 produced very low amounts of radiolabeled glycosaminoglycans whereas the amount of ^{3}H-labeled glycosaminoglycan recovered from mutant 650 was always higher than that from 745 or 761, but never exceeded 30% of that recovered from the wild type (LeBaron, R. G., and M. Höök, unpublished results). These data confirm the results of Esko et al. (1985, 1986, 1987) regarding the relative levels of overall glycosaminoglycan production by the different CHO strains. Furthermore, the amount of radiolabeled glycosaminoglycans recovered from the cell surface of the different mutants are shown to be proportional to their previously reported overall glycosaminoglycan synthesizing capacity. Hence, this set of mutants provides a valid experimental system for examining the role of cell-surface glycosaminoglycans in cell adhesion.

The cell adhesion assays used in this study required monolayer cultures to be detached by trypsin treatment followed by suspension culture for 10–12 h to allow replenishment of cell-surface components. The effects of these conditions on glycosaminoglycan biosynthesis were examined. Incubating cells in suspension culture did not affect the quantitative differences between total glycosaminoglycans produced by the different strains (Fig. 1 *b*). To exclude the possibility that secreted proteins may interfere with attachment of cells to the substratum during the adhesion assay, cycloheximide was added to the cultures 2 h before cell harvest. We therefore determined the distribution of glycosaminoglycans

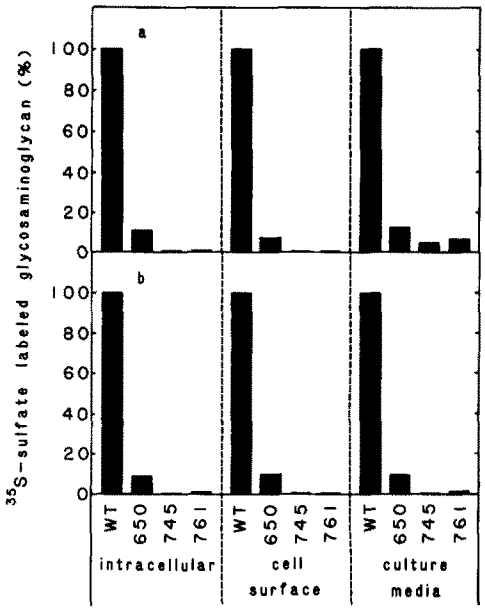

Figure 1. Radiolabeled glycosaminoglycans recovered from the intracellular, cell surface, and culture media compartments of wild-type and mutant CHO cells. (*a*) Monolayer cultures of wild-type (*WT*) and glycosaminoglycan-deficient mutant cells 650, 745, and 761. Cells were incubated in sulfate-deficient medium supplemented with 0.5 μCi/ml of [^{35}S]sulfate for 60–72 h. (*b*) Suspension cultures established from monolayers were incubated for 10–12 h in sulfate-deficient medium supplemented with 20 μCi/ml of [^{35}S]sulfate. The amount of [^{35}S]sulfate labeled glycosaminoglycan recovered in each compartment of the wild-type cells was taken as 100%. Values obtained for the mutants were normalized to wild-type values after correction for any variation in cell number.

on wild-type cells under these adhesion assay conditions, (suspension culture plus cycloheximide). Cells from monolayer cultures were prelabeled with [^{35}S]sulfate for 60 h, transferred to suspension culture in [^{35}S]sulfate-containing medium, and incubated for an additional 12 h. Cycloheximide (10 μg/ml) was added 2 h before harvest. The glycosaminoglycans were recovered from the culture medium, the cell surface, and the intracellular compartments. Quantitation of [^{35}S]sulfate-labeled glycosaminoglycans showed that 60% was recovered from the intracellular compartment whereas 30 and 10% was recovered in the media and cell-surface compartment, respectively. The distribution of ^{35}S-labeled glycosaminoglycans in suspension cultures grown in the absence of cycloheximide was similar. These data show that after cycloheximide treatment and growth in suspension cultures, cells still contain cell-surface associated glycosaminoglycans.

To quantitate the amount of [^{35}S]sulfate-labeled heparan sulfate and chondroitin sulfate recovered from the surface of wild-type cells labeled in spinner culture, trypsin-released material was treated with pronase, and chondroitinase ABC or nitrous acid. The products were subsequently analyzed by chromatography on a column of Sephadex G-50 (data not shown). Quantitation of the different peaks suggest that 25–30% of the labeled molecules were converted to disaccharides by treatment with chondroitinase ABC and 70–75% of the labeled macromolecules were degraded to oligosaccharides by treatment with nitrous acid. Similar results were

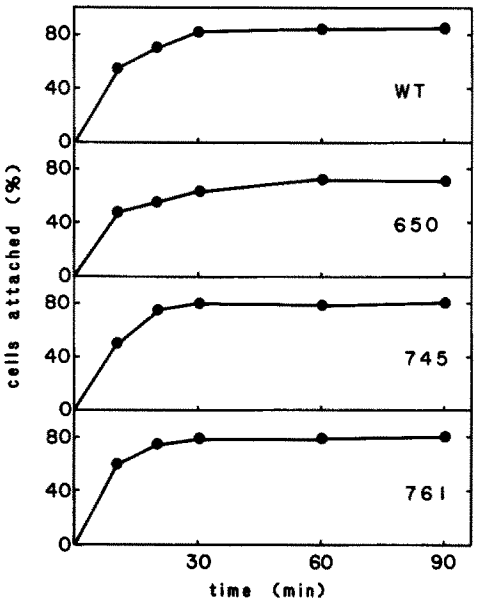

Figure 2. Attachment of cells to fibronectin substrata as a function of time. Cells (5×10^4 in 0.5 ml of buffer 3) were seeded in 24-well plates coated with fibronectin (6 μg/well) and incubated for the indicated periods of time at 37°C. The supernatant, containing nonadhering cells, was removed and cells attached to the substrate were quantified. Less than 5% of the seeded cells adhered to BSA-coated wells.

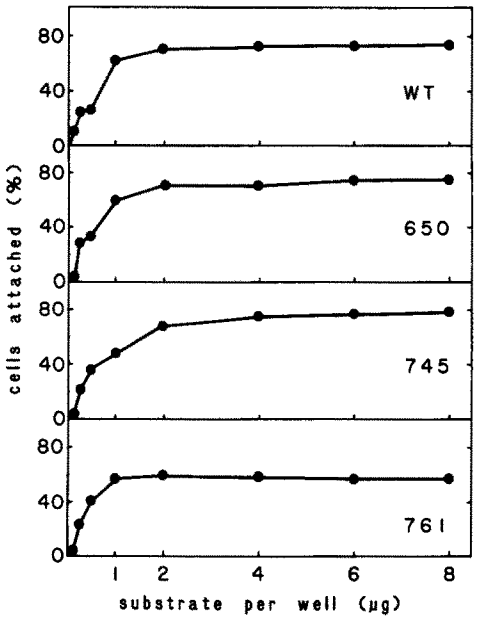

Figure 3. Attachment of cells to fibronectin substrata as a function of fibronectin concentration. Cells (5×10^4 in 0.5 ml of buffer 3) were seeded in 24-well plates coated with 0.1, 0.25, 0.5, 1, 2, 4, 6, and 8 μg/well of fibronectin and incubated for 90 min. See legend to Fig. 2 for further details.

obtained when cells were treated with cycloheximide 2 h before isolation of glycosaminoglycans. These findings demonstrate that heparan sulfate is the major glycosaminoglycan present at the cell surface, but that galactosaminoglycans are also present.

Attachment of Cells to Fibronectin Substrates

The attachment of cells to an adhesive substratum depends on the formation of a sufficient number of bonds between cell-surface receptors and corresponding ligands in the substratum to withstand the shear forces imposed when unattached cells are washed away. Cell attachment, therefore, is a reaction that depends on time of incubation as well as on the concentrations of cell-surface receptors and ligands. Since cell-surface proteoglycans have been proposed to participate in cell adhesion processes, we examined the attachment of wild-type and mutant cells to wells coated with fibronectin under conditions where the incubation time (Fig. 2) or concentration of fibronectin (Fig. 3) was varied. The different strains behaved almost identically; wild-type and mutant cells attached to a fixed amount of fibronectin in a time-dependent reaction that was completed in ~30 min (Fig. 2). The different cells also attached similarly on limiting amounts of fibronectin adsorbed in the wells (Fig. 3). Wells coated with <2 μg of fibronectin allowed only submaximal numbers of cells to attach.

To explore further the mechanism of cell attachment to fibronectin, wells were coated with isolated fibronectin fragments containing distinct binding domains and attachment of the wild-type and mutant cells to these substrates were analyzed (Fig. 4). On these substrates, wild-type cells behaved essentially like human embryo fibroblasts (Woods et al., 1986). Wild-type cells attached efficiently to substrates composed of fibronectin or a 105-kD fibronectin fragment containing the cell-binding RGD amino acid sequence. A significant number of wild-type cells also attached to a substrate composed of a 31-kD peptide containing the COOH-terminal heparin-binding domain.

Likewise, the different mutant cells attached as efficiently to the cell-binding fragment as to intact fibronectin. However, the glycosaminoglycan-deficient mutants 745 and 761 attached poorly to the heparin-binding fragment. Mutant

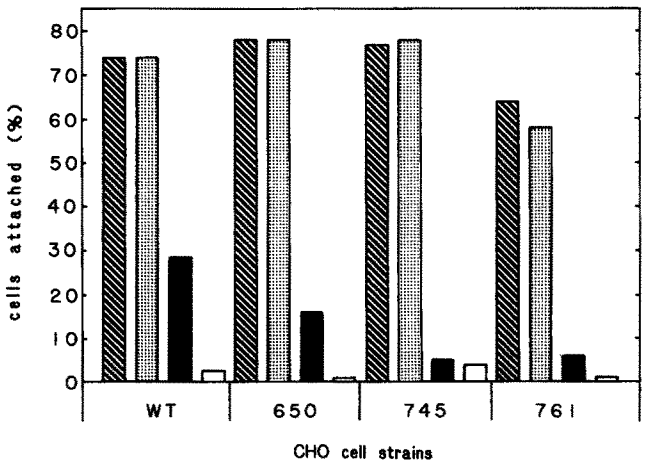

Figure 4. Attachment of cells to substrata containing fibronectin fragments. Cells (5×10^4 in 0.5 ml buffer 3) were seeded in 24-well plates coated with 6 μg/well of intact fibronectin (*hatched bars*), 2.86 μg/well of the 105-kD cell-binding fragment (*dotted bars*), 0.85 μg/well of the 31-kD heparin-binding fragment (*solid bars*), or 1% heat-treated BSA (*open bars*). The number of attached cells were quantified after a 90-min incubation at 37°C.

650, which has a low but significant level of glycosaminoglycans associated with its surface, showed an intermediate level of attachment to the heparin-binding fragment. Hence, the relative degree of attachment to the heparin-binding fibronectin peptide essentially paralleled the content of the cell-surface glycosaminoglycan. Neither the wild-type nor the different mutants attached to substrata containing only BSA (Fig. 4).

Spreading of Cells on Fibronectin

Cell spreading, which follows initial attachment to an adhesive substrate, was monitored by microscopic examination. Wild-type and mutant cells underwent cell spreading when seeded on a fibronectin substrata or a substrate composed of the 105-kD cell-binding fragment. The extent of cell spreading with time on each substrate was monitored for wild-type and mutant CHO cells by measuring the average area of 50 cells. As shown in Fig. 5, wild-type and mutant cells went through a cell spreading process that was essentially completed during the first hour of incubation. Furthermore, the extent of cell spreading was essentially identical for cells seeded on substrates containing intact fibronectin or the 105-kD cell-binding fragment. However, the area of a fully spread cell seems to vary somewhat among the different mutants. For example, cells of mutant 761 covered an average area of close to 600 μm^2 when fully spread, whereas the maximum area covered by fully spread cells of mutants 650 and 745 appeared to be somewhat smaller (400 μm^2). Fully spread cells of the wild type covered an intermediate size area. These diferences do not seem to be related to the presence or absence of cell-surface glycosaminoglycans.

Formation of Stress Fibers and Focal Adhesions

Later stages in the substrate-adhesion process of many cells involve the assembly of components of the microfilament system into stress fibers and the formation of focal adhesions. When the distributions of the major microfilament protein F-actin in well-spread cells of the different strains were compared, marked differences were noticed (Fig. 6). In the wild-type cells, F-actin was detected in stress fibers (Fig. 6 *a*), whereas in the glycosaminoglycan-deficient mutants it was found along the periphery of the cell and also in a patchy distribution throughout the cell body (Fig. 6, *b–d*). Occasionally, F-actin was seen in small fibrous structures in the mutant cells.

When analyzed by interference reflection microscopy, wild-type cells were found to contain substrate attachment sites resembling focal adhesions (Fig. 7 *b*). Most cells of the glycosaminoglycan-deficient mutants did not form these structures (Fig. 7 *d*). The size of focal adhesions formed by wild-type CHO cells was considerably smaller compared with those formed by fibroblasts (Woods et al., 1986), and hence they were more difficult to identify. To further examine the nature of these structures, the same cells were analyzed for focal adhesions by interference reflection microscopy and F-actin distribution by phallacidin staining. The structures tentatively identified as focal adhesions were found to represent termination points of stress fibers (*arrows* in Fig. 7, *a* and *b*). This observation strongly supports the idea that these attachment structures are in fact focal adhesions.

To reduce bias and error caused by the small size of focal

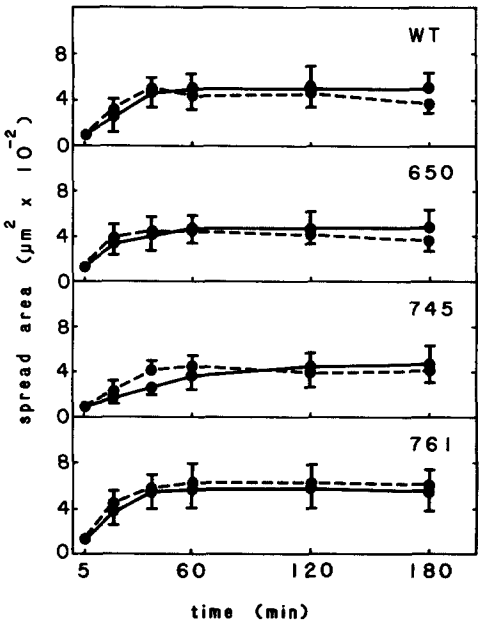

Figure 5. Cell spreading as a function of incubation time. Cells (3.5×10^4 in 0.5 ml of buffer 3) were seeded on glass coverslips coated with 4.52 μg/coverslip of fibronectin (*solid lines*) or 2.16 μg/coverslip of the 105-kD cell-binding fragment (*hatched lines*). Cells were incubated at 37°C for the indicated periods of time, fixed in glutaraldehyde, and the area of attached cells measured at each time point. Shown is the average area of 50 cells. Bars represent standard deviations.

adhesions in CHO cells, 100 cells of the wild type and each of the mutants were examined in double-blind experiments by two independent observers for the presence of focal adhesions. More than 60% of the wild-type cells were found to form focal adhesions, but <10% of cells of the different glycosaminoglycan-deficient mutants appeared to have these structures (Table II). Mutant 650 did not form more focal adhesions than did mutants 745 and 761. Possibly, the concentration of heparan sulfate on the cell surface of mutant 650 was too low to allow focal adhesions to form. Neither the wild-type nor the mutant cells formed significant numbers of focal adhesions on substrata composed of the 105-kD cell-binding fragment of fibronectin (Table II).

Discussion

A role for cell-surface proteoglycans in cell-substrate adhesion is supported by studies that show the following. (*a*) Heparan sulfate proteoglycans are present in structures connecting the cell to the substratum (Lark and Culp, 1984; Woods et al., 1984; Rapraeger et al., 1986). (*b*) Hydrophobic heparan sulfate proteoglycans are present in cell residues remaining after detergent solubilization of cell cultures. The residues contain cytoskeletal, extracellular matrix, and membrane components connecting the cytoskeleton and matrix (Woods et al., 1985; Culp et al., 1986). (*c*) Cells seeded on substrata containing different fibronectin fragments will form focal adhesions only if heparin-binding and cell-binding fragments are present (Woods et al., 1986). (*d*) Cells seeded on a substratum of intact fibronectin in the presence of antibodies to the heparin-binding domains will not de-

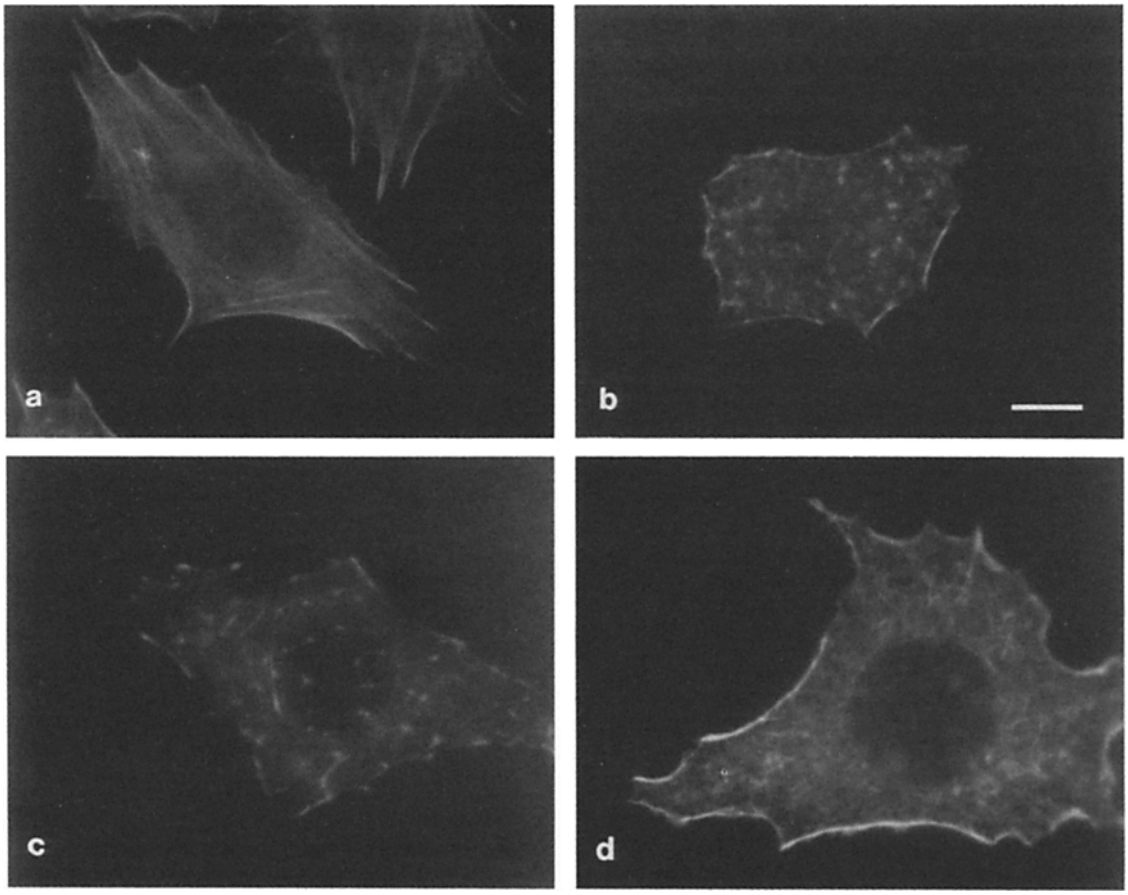

Figure 6. Actin staining of wild-type and mutant CHO cells. The cells were fixed and stained for F-actin using NBD-phallacidin as described in Materials and Methods. (*a*) Wild type; (*b*) mutant 745; (*c*) mutant 650; (*d*) mutant 761. Bar, 10 μm.

velop focal adhesions (Woods et al., 1986). (*e*) Digestion of cells with heparitinase may lead to impaired attachment to and spreading of cells on fibronectin substrata (Gill et al., 1986). (*f*) Addition of glycosaminoglycans or proteoglycans may interfere with the substrate attachment of cells (Brennan et al., 1983; Schwarz and Juliano, 1985; Rosenberg et al., 1986).

These reports all indicate that cell-surface proteoglycans are of importance for cell-substrate adhesion, however they contain only circumstantial evidence and each approach has some weaknesses. With the development of cell mutants deficient in glycosaminoglycan biosynthesis (Esko et al., 1985, 1986, 1987), a direct analysis of related cell lines differing in their content of proteoglycans became possible. In the present study, the role of cell-surface glycosaminoglycans in fibronectin-mediated cell adhesion was analyzed using glycosaminoglycan-deficient CHO cell mutants with identified biochemical defects. This approach also has potential weaknesses. Firstly, since the glycosaminoglycan-deficient CHO cell mutants were generated through the use of mutagenic compounds, the possibility of pleiotropic mutations must be considered. Secondly, it is possible that the glycosaminoglycan deficiency may have its origin in a biosynthetic step that also affects other glycoconjugates. However, to minimize these potential problems, we have used several independently isolated mutants that all have documented defects in enzymatic reactions forming part of the chain initiation processes that appear to be unique to glycosaminoglycan biosynthesis.

The results presented in this communication demonstrate that cell-surface glycosaminoglycans are not required for the attachment and spreading of cells on substrata composed of intact fibronectin molecules or fragments containing the primary cell-binding site. These data are consistent with our previous observation where fibroblasts were shown to attach and spread normally on substrata of fibronectin fragments containing the cell-binding domain but lacking a heparin-binding domain (Woods et al., 1986).

When a heparin-binding fragment was used as a substrate, an intermediate number of wild-type cells attached whereas attachment of the glycosaminoglycan-deficient mutants was largely reduced. This finding confirms our previous observation (Woods et al., 1986) showing that a heparin-binding domain of fibronectin can serve as a substrate ligand supporting cell attachment. Furthermore, the current report suggests that cell-surface proteoglycans serve as "receptors" in this cell attachment reaction. However, heparin-binding fragments only support an incomplete cell adhesion where spreading and focal adhesion formation are not observed.

Previous studies have indicated that for the cell types analyzed, a substratum needs to contain a heparin-binding domain in addition to a cell-binding site in order to support the formation of focal adhesions (Woods et al., 1986; Izzard et al., 1986). In contrast, recent studies by Singer et al. (1987)

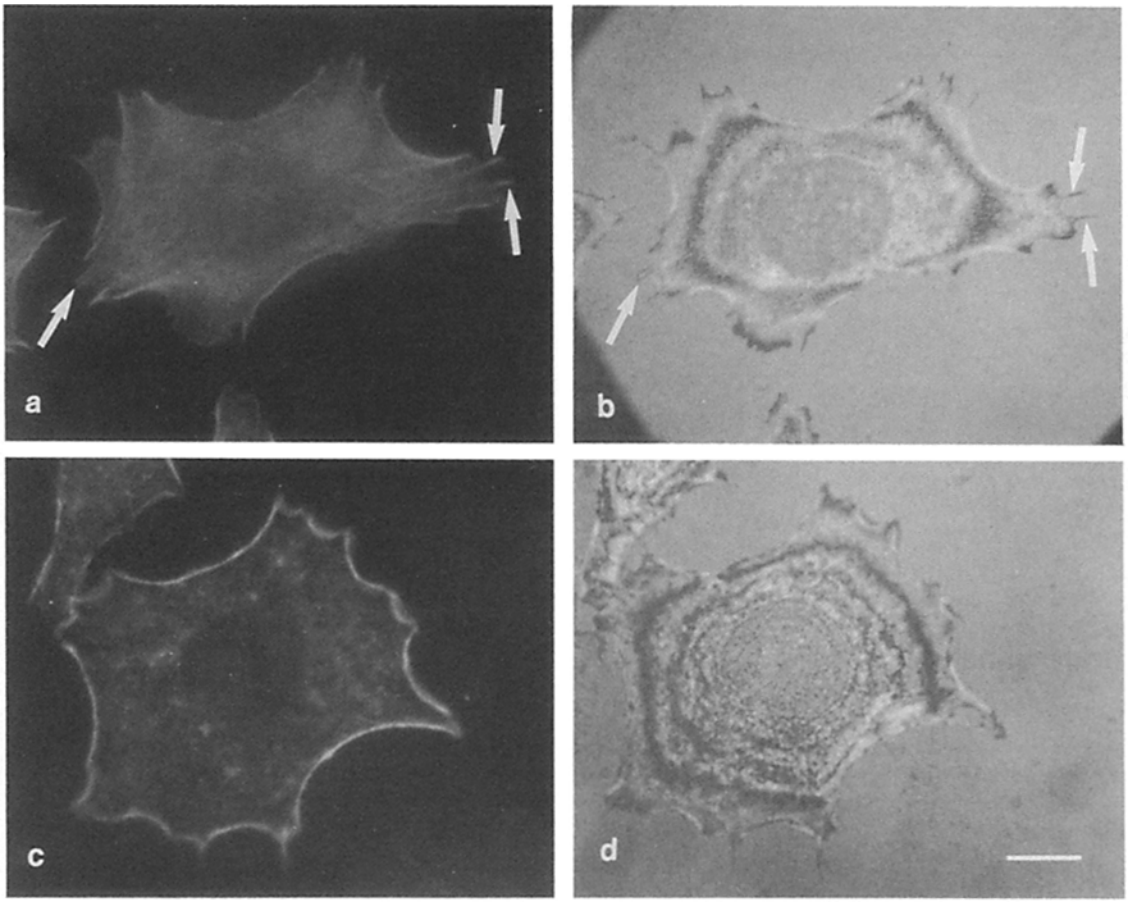

Figure 7. Actin staining and correlating interference reflection micrograph of wild-type and mutant CHO cells. (*a*) Wild-type cell stained with NBD-phallacidin. Arrows show ends of stress fibers. (*b*) The same wild-type cell examined by interference reflection microscopy. Arrows show focal adhesive plaques. (*c*) Actin staining of mutant 761. (*d*) The corresponding interference reflection micrograph. Bar, 10 μm.

and Streeter and Rees (1987) suggest that a substrate composed of RGD-containing cell binding domains might be sufficient to induce focal adhesion formation. However, the latter two studies were carried out under conditions allowing endogenous protein synthesis, and as pointed out by Streeter and Rees (1987), it is possible that the observed focal adhesions developed on an endogenous adhesion protein. In the present study, wild-type cells but not glycosaminoglycan-deficient mutants seeded on intact fibronectin formed focal adhesions. Neither cell formed focal adhesions on substrata containing the cell-binding site alone. These results strongly imply that heparin-binding domains of fibronectin play a role in stress fiber and focal adhesion formation and that a cytoskeletal organization may be mediated through an interaction with cell-surface proteoglycans. Thus, formation of focal adhesions presumably involves coordinated interactions at the cell surface whereby a complex in the membrane containing the fibronectin receptor (Pytela et al., 1985; Tamkun et al., 1986) and a heparan sulfate proteoglycan link extracellular matrix components to intracellular cytoskeletal components (Horwitz et al., 1986).

Table II. Focal Adhesions in Wild-type and Mutant Cells

Strain	Cells showing focal adhesions	
	FN	105
	%	%
Wild type*	62	2
Mutant		
650	9	2
745	4	0
761	10	6

Cells were treated as described in the legend to Fig. 7. The percent of cells that formed focal adhesion plaques was determined in a double-blind fashion for wild-type and mutant cells on a fibronectin substrate and a substrate containing only 105-kD cell-binding fragments of fibronectin.
* Since focal adhesions formed by wild-type CHO cells are small, the number shown may be underestimated.

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